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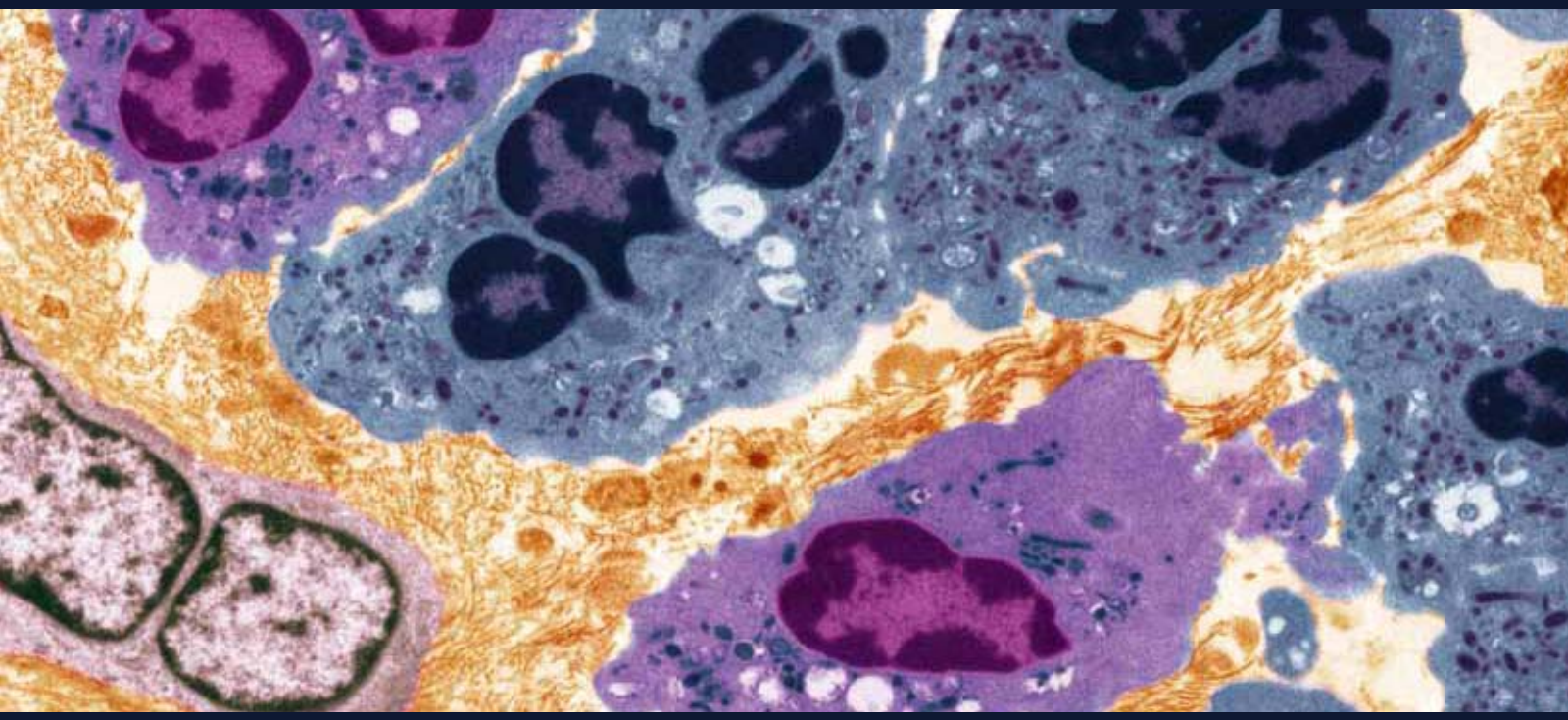
Cardiovascular Inflammation

Guest Editors: Masanori Aikawa, Ichiro Manabe, Adrian Chester, and Elena Aikawa



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Editorial

Cardiovascular Inflammation

Masanori Aikawa,[1] Ichiro Manabe,[2] Adrian Chester,[3] and Elena Aikawa[1]

[1] *Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*
[2] *Department of Cardiovascular Medicine, The University of Tokyo, Tokyo 113-8655, Japan*
[3] *Heart Science Centre, Imperial College London, London 4B9 64H, UK*

Correspondence should be addressed to Elena Aikawa, eaikawa@partners.org

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1. Introduction

Westernized countries face a growing burden of cardiovascular diseases including acute complications of coronary atherosclerosis. Cardiovascular diseases often associate with metabolic disorders such as insulin resistance and obesity. Emerging clinical and experimental studies suggest that inflammation is central to the development of these cardiometabolic disorders. A proinflammatory subset of monocytes/macrophages may importantly contribute to pathological processes in cardiometabolic organs. Crosstalks between cardiometabolic organs may enhance systemic and local inflammatory milieu. In addition, accumulating evidence supports a key role for inflammation in the pathogenesis of vascular mineral deposition and calcific aortic valve stenosis. The goal of this special issue is to highlight what is known concerning the inflammatory nature of cardiovascular diseases, such as atherosclerosis and aortic valve calcification, and their association with metabolic disorders. Armed with this understanding, it is hoped that novel therapeutic strategies will aid in the prevention and management of the cardiometabolic syndrome and its complications, and benefit the patients afflicted with calcific aortic valve disease.

2. Inflammation and Metabolic Diseases

Obesity, particularly visceral obesity, increases the clinical risk of metabolic and cardiovascular diseases. In addition to its function as a reservoir of lipids, adipose tissue is now known to be an active endocrine organ that produces a variety of "adipokines" and controls energy homeostasis. It has been suggested that the dysregulated production of proinflammatory mediators relative to the production of anti-inflammatory adipokines (e.g., adiponectin) is an important contributor to adverse metabolic and cardiovascular consequences. Recent studies have also demonstrated that the increased secretion of inflammatory mediators seen in obese visceral fat reflects the ongoing chronic inflammation of the adipose tissue, itself. The paper by V. Z. Rocha and E.J. Folco and M. Itoh et al. deal with the molecular mechanisms controlling adipose inflammation and the systemic effects of adipose inflammation on metabolic and cardiovascular diseases. The paper by T. R. Aprahamian and F. Sam focused on adiponectin. M. Furuhashi et al. reviewed the functional involvement of fatty acid-binding proteins in various cells in chronic diseases. These reviews provided molecular insights into development of inflammation in adipose tissue and its propagation to distant tissues that leads to the development of cardiovascular and metabolic diseases.

3. Inflammation in Atherosclerosis

Inflammation contributes critically to all stages of atherogenesis. Metabolic disorders such as dyslipidemia promote activation of circulating monocytes and endothelial cells and adhesion of these cell types, leading to accumulation of macrophages. Macrophages then undergo activation and produce proinflammatory cytokines and reactive oxygen species. Oxidative stress activates neighboring cells including endothelial cells and further promotes monocyte recruitment. Such an uncontrolled amplification mechanism represents inflammatory aspects of atherosclerosis. Naturally, many studies have thus addressed whether antioxidants can prevent development of atherosclerosis and its complications and provided unsatisfactory results. In this special issue, F. J. Pashkow overviewed this interesting controversy. Accelerated

atherogenesis in patients with autoimmune disease has long been recognized. E. Profumo et al. demonstrated that oxidative stress links these two disorders. Accumulating evidence suggested the causal role of endoplasmic reticulum (ER) stress in apoptosis. But ER stress may also cause various inflammatory disorders including atherosclerosis and metabolic syndrome. T. Gotoh et al. discussed biology of the ER stress pathway in atherogenesis and ischemic cardiac injury. Inflammation not only promotes atherogenesis but also may trigger acute onset of its clinical complication such as coronary thrombosis by reducing mechanical stability of the plaque. D. Segers et al. demonstrated the role of the chemokine CXCL10 in decreased plaque stability as gauged by collagen loss in mouse and human atherosclerotic lesions.

4. Inflammation in Cardiac Remodeling

Regardless of the origin, injury to the heart evokes a diverse and complex array of cellular responses involving both cardiomyocytes and nonmuscle cells that initiate and sustain a process of structural remodeling of the myocardium. Remodeling of the myocardium is a key determinant of the clinical course of heart failure. Many of the processes underlying cardiac remodeling have features in common with chronic inflammatory processes. In this issue, the paper by N. Takeda and I. Manabe and Y. Yoshimatsu and T. Watabe reviewed cellular and molecular processes underlying cardiac fibrosis and remodeling. The former paper focuses on the involvement of noncardiomyocytes, highlighting the dynamic cellular interplays in cardiac remodeling. The latter deals with endothelial-mesenchymal transition (EMT), suggesting the endothelial origin of a subset of cardiac fibroblasts. The paper by Y. Feng and W. Chao reviewed the involvement of Toll-like receptors (TLRs) in myocardial responses to infarction and sepsis. TLRs are the major pattern recognition receptors that detect not only the pathogen-associated molecular patterns (PAMPs), but also the damage-associated molecular patterns (DAMPs), including a variety of endogenous molecules. As reviewed in M. Itoh et al., TLRs have also been shown to be important for activation of inflammatory processes in metabolic tissues. These paper provide some of the key ideas in the emerging field of cardiac biology.

5. Cardiac Valve Inflammation

It is now widely accepted that inflammatory mechanisms also play a role in development of aortic stenosis. Aortic stenosis shares some risk factors and characteristics with those of atherosclerosis, but remains relatively less amenable to pharmacological intervention. Differences in the aetiology of the different disease process may be a reflection of the unique mechanical environment in which the aortic valve resides. The papers in this special issue that focus on heart valves illustrate the important role inflammatory mediators play in the development, tissue remodeling, and repair of the valve, as well as the initiation and progression of the disease process. The paper by G. J. Mahler and J. T. Butcher examined the role of inflammatory mediators from development to disease. Their paper illustrated how mediators such as TGF-β, TNF-α, and BMPs play an important role in the self-repair and tissue-remodeling properties that help maintain the durability and strength of the valve. The role of changes in the different types of mechanical force to which the aortic valve is exposed is discussed by K. Balachandran et al. and, more specifically, J. N. Warnock et al. presented new data on the influence of increased levels of pressure in inflammatory gene networks.

We will only then be able to identify targets that might be amenable to molecular or pharmacological modulation after we have gained a comprehensive understanding of the genes expressed, the molecules released, and the signaling pathways activated during the onset and progression of aortic stenosis. While work with animal and human cells and tissue is an important step in this process, development of robust animal models that recapitulates the human disease process will be fundamental to assaying the efficacy of new interventions. In this regard, K. L. Sider and colleagues provided a comprehensive summary of the currently available animal models of aortic stenosis.

6. Conclusion

The cardiometabolic syndrome represents a global health burden. An emerging concept suggests that inflammation participates in the pathogenesis of cardiometabolic disorders including atherosclerosis, obesity, insulin resistance, and aortic valve disease. Thus, controlling proinflammatory molecules or pathways may attenuate such diseases. The goal of this thematic series is to highlight what is known concerning the role inflammation plays in the cardiometabolic syndrome and what the therapeutic options are. We believe that this issue will offer updated concepts and help readers develop ideas leading to future investigations and new drug development.

Masanori Aikawa
Ichiro Manabe
Adrian Chester
Elena Aikawa

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Review Article

The Interface between Inflammation and Coagulation in Cardiovascular Disease

Gabriele Demetz and Ilka Ott

Deutsches Herzzentrum Technische Universität München, Lazarettstraße 36, 80636 München, Germany

Correspondence should be addressed to Ilka Ott, ott@dhm.mhn.de

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The intimate connection between coagulation and inflammation in the pathogenesis of vascular disease has moved more and more into focus of clinical research. This paper focuses on the essential components of this interplay in the settings of cardiovascular disease and acute coronary syndrome. Tissue factor, the main initiator of the extrinsic coagulation pathway, plays a central role via causing a proinflammatory response through activation of coagulation factors and thereby initiating coagulation and downstream cellular signalling pathways. Regarding activated clotting factors II, X, and VII, protease-activated receptors provide the molecular link between coagulation and inflammation. Hereby, PAR-1 displays deleterious as well as beneficial properties. Unravelling these interrelations may help developing new strategies to ameliorate the detrimental reciprocal aggravation of inflammation and coagulation.

1. Introduction

Systemic and local proinflammatory changes are in focus when investigating the pathophysiology of arteriosclerosis and acute coronary syndromes. In acute myocardial infarction (AMI), proinflammatory markers such as C-reactive protein (CRP), interleukins, or monocyte-chemoattractant protein (MCP)-1 are elevated [1–3] and their increase is of prognostic relevance for future cardiovascular events [4–6] and mortality [7–9]. Moreover, in healthy persons elevated proinflammatory markers are associated with an increase in cardiovascular risk [10–12]. Patients with increased circulating proinflammatory markers in AMI present with decreased myocardial salvage after coronary reperfusion therapy [13]. Similarly, in experimental studies, high levels of CRP deteriorate infarct size [14].

Sources of inflammatory response are vascular cells such as activated endothelial cells, which release proinflammatory cytokines such as interleukin (IL)-8 [15]. IL-8 is a CXC cytokine that acts as a chemoattractant and agonist for neutrophils, lymphocytes, and monocytes and is found in macrophage-rich atherosclerotic plaques [16]. Under flow conditions, IL-8 facilitates the arrest of monocytes on endothelium [16], which is necessary for migration into the intima in evolution of arteriosclerosis. Reperfusion injury after AMI as well as systemic inflammatory response syndrome can be associated to increased levels of IL-8 [17]. In experimental setting, murine IL-8 receptor knock-out mice display smaller arteriosclerotic lesions with less macrophages [18]. Apart from their contribution to arteriosclerosis, CXC cytokines are also produced by malignant cells and can promote tumor progression of a large variety of malignancies [19].

Besides IL-8, many other cytokines such as IL-6 take part in inflammatory responses by inducing B-cell differentiation, T-cell activation, and synthesis of acute phase proteins [20], but also contributing to proliferation of vascular smooth muscle cells (SMCs) [21]. Moreover TH1 activation was observed in acute coronary syndromes [22].

The pathogenesis of proinflammatory changes in acute coronary syndromes as well as the interplay between coagulation and inflammation is poorly understood and is subject to intense research. The mechanisms by which the coagulation system is altered by inflammatory interactions comprise enhanced synthesis and activation of coagulant

proteins, decreased synthesis of anticoagulants, and suppression of fibrinolysis [23]. However, not only inflammation activates coagulation but coagulation in turn perpetuates inflammatory response [24]. Accordingly, increased levels of prothrombin fragment F1+2, fibrinopeptide A and D-Dimer, reflecting activation of the coagulation cascade, are also associated with an unfavorable outcome in acute coronary syndromes [25–27].

In this paper we focus on possible mechanisms of the interplay between coagulation and inflammation in acute coronary syndromes.

2. Tissue Factor

A strict separation between the intrinsic and extrinsic coagulation cascade surely fails to reflect physiologic conditions. Nevertheless, in the setting of arteriosclerosis and acute coronary syndromes the extrinsic pathway of coagulation is of particular significance.

Tissue Factor (TF) is the most important initiator of the extrinsic coagulation cascade. TF, a 47 kDa transmembrane glycoprotein and member of the class II cytokine receptor family, is the cofactor for the activated plasma clotting factor VII (FVIIa). The TF-FVIIa complex catalyzes the activation of factor X and IX, which leads to the generation of thrombin and thus finally of a fibrin clot. Under physiologic conditions TF is abundantly expressed only in the adventitia and is induced by several inflammatory mediators such as IL-6, IL-8, and MCP-1 [28, 29]. After vascular injury, TF is rapidly augmented in SMC of the media and accumulates in the SMC of the developing neointima [30]. Consequently, TF is highly expressed within atherosclerotic lesions and displays high procoagulant activity suggesting a role in determining plaque thrombogenicity [30]. In atherosclerotic carotid lesions disruption of plaques exposes TF-positive cells within the plaque to plasma clotting factors and initiates local thrombosis with subsequent occlusion of the vessel [31].

Furthermore, increased TF expression can be noticed on circulating monocytes and microparticles in acute coronary syndromes and may, thereby, contribute to activation of coagulation [32–34]. A soluble form of TF within the circulating blood may also support coronary thrombosis [35]. It has been shown that cytokines can induce expression of soluble TF [36], which on the other hand has been shown to accumulate in developing thrombi [37]. However, the clinical significance and individual contributions of microparticle-derived and soluble TF remain a matter of debate. Several studies have demonstrated increased levels of circulating TF in patients with unstable Angina pectoris (uAP) and acute myocardial infarction (AMI) [32, 38–45]. Therefore, it has long been speculated that, in cases with no plaque rupture or only fractional superficial erosion, thrombus formation may mainly depend on circulating levels of TF. Consistent with this idea, several studies suggest that the levels of circulating TF and other haemostatic biomarkers may correlate to adverse cardiovascular events and mortality in patients with acute coronary syndrome [46–48].

Stimulation of the TF-thrombin pathway does not only occur at the site of the plaque but also within the ischemic myocardium where activated coagulation factors may enhance inflammatory responses and increase infarct size [49]. TF contributes to inflammation, cell migration, and remodelling after vascular injury [50]. Furthermore, TF expression has been reported in a number of cancers, such as glioma, pancreatic cancer, non-small-cell lung cancer, colorectal cancer, ovarian cancer, prostate cancer, hepatocellular cancer, and breast cancer [51]. TF expression in tumors not only correlates with the incidence of thrombosis [52] but also promotes metastasis [53], tumor progression, and tumor angiogenesis [54].

TF-mediated intracellular signal transduction has not been completely elucidated so far. On one hand TF allows docking and activation of FVII and, therefore, promotes the generation of downstream coagulation factors and activation of protease-activated receptors (PARs) which themselves possibly induce intracellular signal transduction. On the other hand there is evidence for direct signalling through the cytoplasmic domain of TF following TF-FVIIa complex formation [55, 56].

3. Tissue Factor Pathway Inhibitor

The endogenous Kunitz-type inhibitor Tissue Factor Pathway Inhibitor-1 (TFPI) inhibits initiation of TF-induced blood coagulation and is mainly expressed on vascular endothelial cells. TFPI binds and inactivates FXa. The TFPI-FXa complex then binds and inactivates FVIIa. Increased levels of the TFPI-FXa complex may reflect both increased FXa generation and increased TFPI concentrations [57]. In addition to the full length TFPI most of the plasma TFPI circulates in truncated forms that are bound to plasma lipoproteins. These truncated forms lack their C-terminal domains and exhibit reduced affinity for vascular wall proteolysis. Additionally, it has been shown that endogenous proteases [58] and elastase released by neutrophils degrade TFPI, resulting in enhanced local coagulation that contributes to prevent pathogen dissemination during infection [59]. Conversely, infusion of a mutant TFPI protein resistant to proteolysis by elastase strongly impaired host defence against systemic infection.

4. Protease-Activated Receptors

Important players in the interaction between coagulation and inflammation are protease-activated receptors (PARs). PARs are G-protein coupled receptors that mediate various cellular reactions as cytokine release, expression of adhesion molecules, cell migration, or proliferation. Unlike other receptors, PARs are not activated by a soluble, external ligand. Proteases, such as activated coagulation factors, detach a defined part of the NH2-terminal chain of the receptor, thereby inducing a conformational change of the receptor. This change causes a self-activation by a "tethered ligand." This activating sequence comprises only few amino acids. PARs can also be activated by synthetic peptides

consisting of the sequence of amino acids representing the tethered ligand.

In contrast to other receptors, the activation of PARs by enzymatic cleavage is irreversible. After proteolytic activation the receptor must be internalized, degraded, and resynthesized. PARs are mainly expressed in vascular cells, but also in many different other cell types such as gastrointestinal and bronchial epithelial cells. Four different PARs are known: PAR-1, -3, and -4 show responsible for thrombin signaling whereas PAR-2 is activated by trypsin-like serine proteases, FVIIa, and matriptase but not by thrombin. PAR-1 and PAR-2 are expressed on smooth muscle cells and endothelial cells, whereas mainly PAR-1 is expressed on monocytes.

PAR-1 agonists or thrombin induce IL-8 and IL-6 in SMC, EC, and mononuclear cells (MNCs) [60] therefore emphasizing the role of PAR-1 in inflammatory processes in vascular cells and confirming data about PAR-1-mediated cytokine release in EC and monocytes [61]. In smooth muscle cells PAR-1 and PAR-2 agonists induce cytokine release to a similar extent which underlines the relevance of both PARs [60]. In addition to coagulation factors other serine proteases, for example, matriptase secreted by monocytes stimulate proinflammatory cytokine release in endothelial cells via PAR-2 activation [62]. Increased PAR-2 expression in atherosclerotic lesions suggests a role for this proinflammatory pathway (Figure 1) [63].

In addition, PARs can also be cleaved downstream of the tethered ligand, resulting in receptor inactivation by preventing further proteolytic activation [64]. PAR-1 signalling not only induces inflammatory responses but also causes antiapoptotic and vasculoprotective reactions [65]. Since the anticoagulant protease-activated protein C can activate PAR1 when in complex with the endothelial cell protein C receptor (EPCR), which may account for much of the protective effects conferred by activated protein C (APC) in severe sepsis [66]. Different contributions of these two pathways may prevail. First, APC acts via PAR-1 when attached to EPCR [65], resulting in cellular responses distinct from thrombin signalling [64] by a mechanism dependent in trans-activation of the sphingosine 1 phosphate receptor 1. In mouse models with strongly reduced EPCR expression or PAR-1 deficiency, the loss of EPCR/APC signalling via PAR-1 resulted in increased endotoxemia-induced lethality [67]. Concordantly, APC mutants have been shown to contribute to protective effects during sepsis by pathways independent from anticoagulant properties [67, 68]. The second pathway described is independent from EPCR. In this case, the availability of the integrin CD11b/CD18 has been shown to be crucial for PAR-1 mediated APC signaling on macrophages, thereby exhibiting anti-inflammatory effects and reducing endotoxin-induced lethality [69]. Thus the strength of PAR1 and PAR2 activation by thrombin, factor Xa, and activated protein C can either promote or protect against changes in vascular permeability depending on the status of the endothelium.

Platelet activation with subsequent thrombus generation plays a major role in the development of acute coronary syndromes. At low concentrations thrombin activates PAR-1 on platelets through a hirudin-like site and at high concentrations additional PAR-4. This induces shape change, P-selectin, and CD40L mobilization to the platelet membrane and promotes the release of platelet agonists ADP, thromboxane A2, chemokines, and growth factors [70] and, thereby, enhances proinflammatory changes. Thus, inhibition of PARs by thrombin of FXa inhibitors may prove beneficial in reducing not only thrombotic but also proinflammatory responses.

5. FXa

Binding of the serine protease FVII to TF results in generation of the coagulation protease FXa (FXa) and subsequently thrombin both known to induce cell signaling. FXa shows dose-dependent induction of intracellular calcium transients in endothelial cells that is active-site-dependent, and independent of thrombin [71]. Potential pathophysiological responses to FXa include stimulation of proliferation, production of proinflammatory cytokines, and prothrombotic TF [72].

Elevated TFPI-FXa and prothrombin fragments F1+2 plasma levels indicate activation of the coagulation cascade in acute coronary syndromes. Under physiological conditions an inverse relationship between TFPI-FXa and F1+2 suggests that TFPI-FXa regulates prothrombinase activity in vivo [73]. Under conditions associated with activation of the coagulation cascade, however, increased TFPI-Xa plasma levels occur [57, 74]. Activation of coagulation as measured by TFPI-FXa but not by F1+2 is associated with plasma concentrations of the proinflammatory cytokine IL-8 in acute coronary syndromes [60]. Furthermore, subsequent elevated IL-6 levels in the course of acute coronary syndromes are associated with initial TFPI-FXa concentrations [60]. These results argue for a proinflammatory role of FXa in acute coronary syndromes that is independent of thrombin. Although thrombin provokes similar proinflammatory effects as FXa in vitro the effects of thrombin may be diminished after heparin treatment in vivo. Several trials of unfractionated heparin (UFH) [75], direct thrombin inhibitors [76], and enoxaparin [77] have thus far failed to demonstrate mortality reductions in acute coronary syndromes. Yet, the OASIS-6 trial suggests a reduction in reinfarction and mortality without excess bleeding in patients not undergoing PCI [78]. Therapeutic inhibition of the proinflammatory effects of Factor Xa may, therefore, prove additional benefits as compared to thrombin inhibition in the clinical course in acute coronary syndromes. This is currently investigated in the ATLAS-ACS 2 TIMI 51 trial that is testing the hypothesis that anticoagulation with the oral factor Xa inhibitor rivaroxaban reduces cardiovascular death, MI, and stroke among patients with ACS treated with guideline-based therapies for ACS [79].

In vitro experiments revealed that FXa stimulates IL-8 and MCP-1 transcription in endothelial cells and mononuclear leukocytes [60]. Genetic studies and receptor desensitization experiments indicate that signaling by FXa is mediated by PAR-1 and PAR-2 [80, 81].

According to the expression of PAR-1 and PAR-2, PAR-1 and PAR-2 agonists induce IL-8 and MCP-1 release in

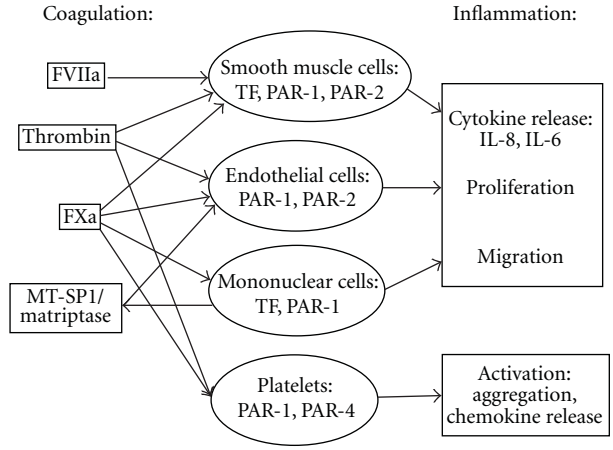

Figure 1

endothelial cells, whereas, only PAR-1 agonists stimulated cytokine release in mononuclear cells [60].

6. FVIIa

Several studies suggest a signaling mechanism of the TF-FVIIa complex via PAR-2 [82]. In this model, TF-bound FVIIa proteolytically activates PAR-2 and, to a lesser extent, PAR-1, and thereby evokes intracellular signaling cascades [83].

In contrast to FXa, FVIIa does not elicit a proinflammatory response in endothelial cells or mononuclear cells [82]. The TF-FVIIa-PAR-2 signalling was only observed in SMC [82] since they express both TF and PAR-2 and both of them seem to be a prerequisite for FVIIa action. EC expressing PAR-2 but lacking TF will not permit FVIIa docking, whereas MNC displaying TF but only low PAR-2 expression, probably allow FVIIa binding but do not express sufficient PAR-2 molecules being subsequently activated. However, PAR-2 and TF are induced by cytokine stimulation [28, 84]. Thus, FVIIa may still have an important impact in atherosclerotic vessels [31] and acute coronary syndromes [29].

In recent studies [85], it has been demonstrated that FVII is synthesized by different cancer cells (liver, ovary, prostate, lung, gastric, thyroid, and breast). Considering that these tumor cells also synthesize TF it is conceivable that supraphysiologic concentrations of FVIIa after binding of FVII to TF occur. On tumor cells, the TF-FVIIa binary complex mediates activation of PAR-2 [86]. Therefore TF-FVIIa-PAR-2 interaction with subsequent cytokine release may be relevant within a tumor environment. TF/FVIIa/PAR2 signalling has been shown to promote proliferation and metastasis of tumor cells [87, 88]. Consistently, TF/FVIIa-specific upregulation of IL-8 expression in breast cancer cells has been shown to be mediated by PAR-2 and to increase cell migration [83]. Whether TF-FVIIa-PAR-2 interaction may also contribute to local thrombus formation and progression of atherosclerotic disease remains to be elucidated.

7. Conclusion

The interplay between coagulation and inflammation is a matter of intense research. Proinflammatory changes in acute coronary syndromes may substantially influence prognosis [6]. Experimental evidence suggests that this interplay may contribute to the development of vascular remodeling or support plaque disruption of the artery. However transfer of these data to the clinical settings remains controversial since additional optimized medical and interventional treatments interfere. Therefore, understanding the causes of inflammation facilitate the development of new therapeutic strategies. To analyse whether these new therapies translate to improved clinical outcome needs to be studied in appropriate clinical trials.

While inhibiting proinflammatory cytokines such as Tumor-Necrosis Factor-α (TNF) has been shown to effectively improve survival in several animal models of sepsis [89–91], anti-TNF therapy in septic humans failed to ameliorate or even worsened clinical outcome [92–95]. In chronic inflammatory diseases however, anti-inflammatory treatment has become clinical routine. For example, inhibition of IL-6 by the first anti-IL-6 antibody, tocilizumab, has been shown to completely block TF-dependent thrombin generation in experimental endotoxemia [23, 96], and tocilizumab will be of special future interest as it has been approved for rheumatoid arthritis.

Inhibiting coagulation could depict a more promising mechanism in fighting overwhelming inflammatory response. Experimental studies that have shown that anticoagulant treatment not only diminishes activation of coagulation but also inhibits inflammation, underline the cross-talk between activation of coagulation and cytokine release in vivo [49, 97, 98]. In acute inflammatory disorders such as severe sepsis, administration of recombinant APC significantly improves survival and long-term outcome. In future, APC mutants that lack anticoagulant properties but still enable sphingosine 1 phosphate receptor 1 dependent activation of PAR-1 will be of special clinical interest as they have been shown to reduce sepsis-induced in mice but do not predispose to bleeding complications.

So far, anti-inflammatory treatments displayed no explicit benefit in patients with acute coronary syndromes [99]. However, there is evidence that FXa inhibitors prove to be superior to thrombin inhibitors [100]. Particularly, treatment with low molecular weight heparins, that include additional anti-FXa activity as compared to unfractionated heparin, has been shown to decrease inflammatory changes in vitro and in vivo [101, 102].

Yet the question remains if and what anticoagulant therapies will prove beneficial to alter systemic inflammatory responses.

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Review Article

Roles of TGF-β Signals in Endothelial-Mesenchymal Transition during Cardiac Fibrosis

Yasuhiro Yoshimatsu and Tetsuro Watabe

Department of Molecular Pathology, Graduate School of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Correspondence should be addressed to Tetsuro Watabe, t-watabe@umin.ac.jp

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Most cardiac diseases caused by inflammation are associated with fibrosis in the heart. Fibrosis is characterized by the accumulation of fibroblasts and excess deposition of extracellular matrix (ECM), which results in the distorted organ architecture and function. Recent studies revealed that cardiac fibroblasts are heterogeneous with multiple origins. Endothelial-mesenchymal transition (EndMT) plays important roles in the formation of cardiac fibroblasts during pathological settings. EndMT is regulated by signaling pathways mediated by cytokines including transforming growth factor (TGF)-β. Better understanding of the mechanisms of the formation of cardiac fibroblasts via EndMT may provide an opportunity to develop therapeutic strategies to cure heart diseases.

1. Introduction

Many of heart injuries end up in a common final pathway of pathologic tissue remodeling and fibrosis (defined by deposition of collagens, elastin, tenascin, and other matrix proteins), leading to the development of heart failure. Myocardial fibrosis induced by cardiac fibroblasts plays a dual role in cardiac remodeling after injury. While fibrosis plays important roles in wound healing, it also contributes to ventricular stiffening and heart failure progression. Recent reports have revealed that cardiac fibroblasts originate through the mesenchymal transition of endothelial cells (ECs) [1], which is termed "endothelial-mesenchymal transition (EndMT)." Here, after updating current views on the sources of cardiac fibroblasts, we will review epithelial mesenchymal transition (EMT), in which epithelial cells acquire mesenchymal phenotype, since this process has many similarities with EndMT and lays the groundwork for understanding EndMT. We will then review the roles of EndMT in physiological and pathological settings and address its potential mechanisms.

2. Source of Fibroblasts during Cardiac Fibrosis

Several lines of evidence suggest that cardiac fibroblasts are a heterogeneous population and derive from various distinct tissue niches in physiological and pathological conditions. During embryonic heart development, cardiac fibroblasts are differentiated from epicardium or endocardium of the heart [2–6]. In a healthy adult heart, cardiac fibroblasts reside in the interstitial tissue within the myocardium. Some reports have shown that heart-resident cardiac fibroblasts are the major source of tissue fibrosis associated with ischemic heart failure and hypertrophy [7, 8]. In addition, fibroblasts originated from bone marrow-derived cells including CD45-positive hematopoietic stem cells (HSCs) have also been shown to significantly contribute to remodeling of the injured heart [9–13]. Finally, emerging evidence suggests that a subset of cardiac fibroblasts is originated from ECs in a mouse model of pressure overload [1]. This endothelial mesenchymal transition has common features with epithelial mesenchymal transition. Taken together, cardiac fibroblasts are thought to be derived from resident fibroblasts, bone marrow-derived cells, and ECs.

3. Epithelial-Mesenchymal Transition (EMT)

EMT is a process in which epithelial cells lose their polarity and cell-to-cell contacts and undergo a dramatic remodeling of the cytoskeleton (Figure 1(a)) [14, 15]. During EMT, there is a marked decrease in the expression of epithelial

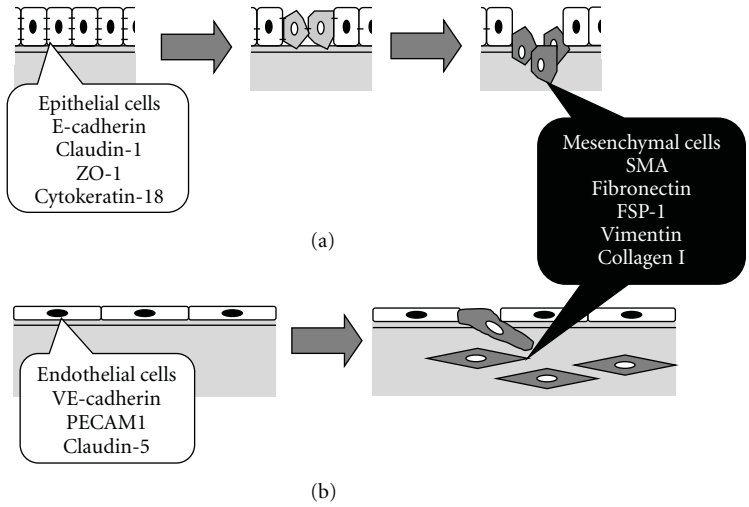

Figure 1: Schematic representation of mesenchymal transition of epithelial (a) and endothelial (b) cells.

markers including E-cadherin, claudin, zona occludens-1 (ZO-1), and cytokeratin-18, and concurrent increase in the expression of mesenchymal markers including smooth muscle α-actin (SMA), fibroblast-specific protein 1 (FSP1; also known as S100A4), fibronectin, and collagens. Furthermore, the mesenchymal cells manifest migratory and proliferative phenotypes. EMT has been implicated in many critical steps during embryonic development including gastrulation and formation of various tissues or cell clusters (neural crest, musculoskeletal system, cranial facial structures, and peripheral nervous system).

Emerging evidence suggests that EMT is also involved in tissue injury leading to tissue fibrosis [16]. For example, EMT is associated with progressive fibrosis in kidney disease. While fibroblasts are not particularly abundant in the normal kidney, there is a marked increase in the number of fibroblasts at the onset of fibrogenesis [17]. Furthermore, EMT also contributes to the fibrotic responses observed in several lung pathologies, such as rejecting lung allografts, silica-induced lung carcinogenesis, and in idiopathic pulmonary fibrosis [18].

4. Signaling Pathways Mediated by TGF-β Family Members

Although EMT has been implicated in many pathological processes described above, our knowledge of the molecular events that govern EMT remains relatively undefined. Transforming growth factor-β (TGF-β) is a multifunctional cytokine that plays many aspects of cell development, differentiation, and homeostasis, and suppresses their uncontrolled proliferation and transformation.

TGF-β belongs to the TGF-β superfamily, which includes 33 members in mammals; these include TGF-βs, bone morphogenetic proteins (BMPs), activins and inhibins, Nodal, myostatin, and Müllerian-inhibiting substance (MIS, also known as anti-Müllerian hormone) [19]. Members of the TGF-β family bind to two different types of serine/threonine kinase receptors (Figure 2). Upon ligand binding, the constitutively active type II receptor kinase phosphorylates the type I receptor which, in turn, activates the downstream signal transduction cascades, including Smad pathways. TGF-βs, activin, and Nodal signal through type I receptors are known as activin receptor-like kinase (ALK)-4, -5, and -7, respectively. The activated type I receptors phosphorylate receptor-regulated Smad proteins (R-Smads). Smad2 and 3 transduce signals for TGF-βs and activins, while Smad1, 5, and 8 are specific for signaling of BMPs [20]. As an exception, ALK-1, preferentially expressed in ECs, binds TGF-β and activates Smad1/5 pathways [21]. Recently, BMP-9 and BMP-10 were reported to bind to ALK-1 [22, 23]. Once activated, R-Smads complex with the common mediator Smad4 (co-Smad) and translocate to the nucleus, where Smad complexes regulate transcription of target genes through their interaction with various transcription factors.

In addition, TGF-β has been shown to activate diverse non-Smad parallel downstream pathways, such as extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAP kinase [24] (Figure 3). Furthermore, TGF-β activates the RhoA during EMT [25]. The Rho family of small GTPases is comprised of RhoA, Rac1, and Cdc42 and regulate the formation of stress fibers, lamellipodia, and filopodia, respectively [26]. Moreover, RhoA activation is also essential for TGF-β stimulation of SMA expression in renal epithelial cells undergoing EMT [27]. Taken together, these studies point to the overall importance of noncanonical TGF-β signaling as well as canonical TGF-β/Smad signaling to induce EMT in epithelial cells.

5. Other Signaling Pathways and Transcription Factors Involved in EMT

Several transcription factors, such as Snail, Slug, δEF1, SIP1, and Twist, have been implicated in EMT [28]. Snail, a zinc-finger containing transcription factor, represses E-cadherin

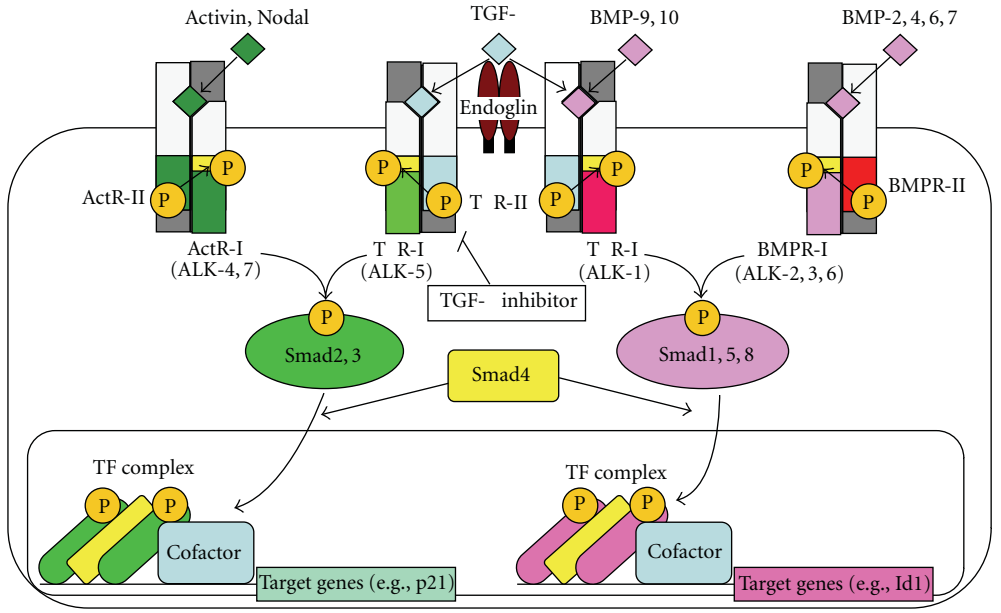

Figure 2: Smad signal transduction pathways mediated by TGF-β and BMP family members.

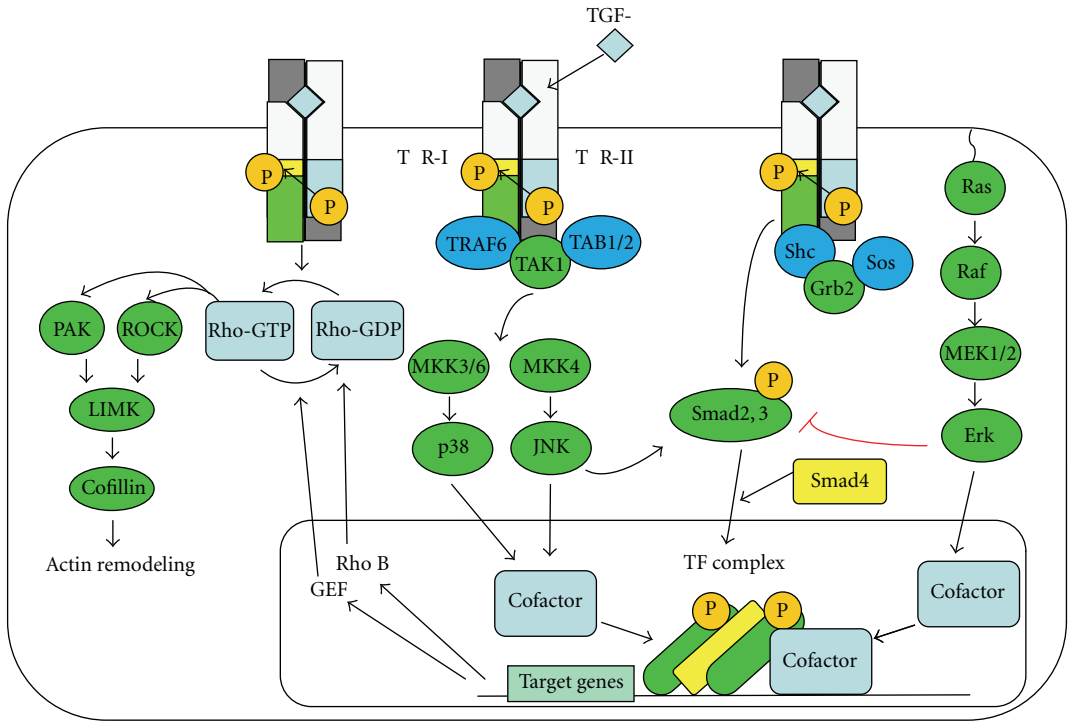

Figure 3: Non-Smad signal transduction pathways mediated by TGF-β family members.

expression and induces EMT when overexpressed in epithelial cells [29, 30]. Knockout mice deficient for Snail gene die at gastrulation as they fail to undergo a complete EMT process, forming an abnormal mesodermal layer that maintains E-cadherin expression [31]. While TGF-β signals have been shown to induce the expression of Snail, SIP1, and δEF1 during EMT of mammary epithelial cells [32, 33], the causal relationship between TGF-β-induced Snail expression and EMT has not yet been fully elucidated. In inflammatory conditions in which fibrosis is accelerated, the effect of inflammatory cytokines such as TNF-α cannot be disregarded. Stability of Snail protein was found to be increased by TNF-α in most cancer cell lines, which results in the elevated migration and invasion [34]. TNF-α also imparts breast cancer cells with a stem cell-like phenotype by inducing Slug expression via NFκB [35], a major transcription factor that functions downstream of TNF-α. Furthermore, several lines of evidence suggest that TGF-β and TNF-α synergistically induce the EMT

of lung carcinoma cells [36, 37], suggesting that TGF-β and other inflammatory signals collaborate to induce the mesenchymal transition of epithelial cells via regulation of EMT-related transcription factors. It is of our great interest that EMT-related transcription factors may also be involved in EndMT. Therefore, we will later discuss recent works on the involvement of EMT-related transcription factors in EndMT.

6. Endothelial-Mesenchymal Transition (EndMT)

Blood vessels are lined by ECs and, except for capillaries, surrounded by mural cells (pericytes or smooth muscle cells). ECs exhibit a wide range of phenotypic variability depending on local physiological requirement throughout the vascular network [38]. Furthermore, in pathologic conditions, the endothelium can be affected in a number of ways. One of the most remarkable ways is an extreme form of endothelial plasticity known as EndMT. During EndMT, resident ECs delaminate from a polarized cell layer and invade the underlying tissue (Figure 1(b)). This so-called mesenchymal phenotype can be characterised by loss of cell-cell junctions, acquisition of invasive and migratory properties, loss of EC markers, such as VE-cadherin and platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31), and gain of mesenchymal markers, such as SMA and FSP1 [1, 39–43]. Several lines of evidence have suggested that EndMT is involved not only in pathological [1, 43–45] but also in physiological conditions, such as the development of heart [46].

7. EndMT during Heart Development

During heart development, heart tube consists of two layers, an inner endocardium and an outer myocardium, which are separated by an acellular layer of extracellular matrix, so-called the cardiac jelly. Endocardial cells acquire endothelial cell markers, such as VE-cadherin and PECAM1. After the formation of cardiac cushion, signals from the outflow tract and atrioventricular (AV) myocardium stimulate transformation of the endocardial cells around the AV cushion and outflow tract into mesenchymal cells through EndMT to generate the primordia of the valves and membranous septa [47, 48]. This notion that valvular fibroblasts in the heart originate from endocardium was clearly confirmed by lineage analysis. In order to study cell fates in mice, Zeisberg and colleagues made use of the *R26Rosa-lox-STOP-lox-LacZ; Tie2-Cre* (*Rosa26-Tie2*) double-transgenic reporter mice [1]. In *Rosa26-Tie2* mice, EC-specific Cre recombinase removes the floxed STOP cassette, and the LacZ reporter gene is constitutively expressed under the control of ubiquitous R26R promoter. Therefore, all endothelial cells and their derivatives in this transgenic mouse will be irreversibly labeled with LacZ (β-galactosidase). Through this fate mapping studies, Zeisberg and colleagues found that EndMT occurs only in heart valves in physiological condition.

Slug, a well-known TGF-β target gene, especially, in the context of EMT, is a direct target of Notch and is required for initiation of cardiac EndMT [46]. Slug (−/−) embryos had significant fewer mesenchymal cells than wild-type control, indicating Slug is required for cardiac EndMT. Slug, but not Snail, is directly upregulated by Notch in ECs and is required for Notch-mediated repression of VE-cadherin expression. They also observed that CD31 expression is downregulated in ECs overexpressing Notch or Slug. These results suggest that Slug is involved in repressing EC markers although upregulation of mesenchymal markers depends on other transcription factors. Slug may be involved in only repressing EC markers during EndMT. Slug-deficient embryos increased Snail expression and knockdown of Snail expression in Slug (−/−) embryos significantly reduced AV canals, indicating that Snail expression can compensate for Slug deficiency and the redundant role of these Snail family members for cardiac cushion formation during cardiac EndMT.

8. Roles of TGF-β Family Signals in the EndMT during Heart Development

In vitro studies using tissue explants cultures in three-dimensional collagen gels have revealed the roles of TGF-β family members in cushion formation. More recently, analyses of knockout mice deficient in various signaling components have also shown that in addition to TGF-β family signals, multiple signaling pathways are involved in directing EndMT during cushion formation [49, 50].

Both *in vitro* and *in vivo* studies have shown that TGF-β2 plays central roles in endocardial EndMT. Among three members of TGF-β family (TGF-β1, 2, and 3), only TGF-β2 is expressed and required for endocardial cushion cell transformation in the mouse [51] since TGF-β2-deficient mice have multiple defects in the formation of AV cushion [52]. However, in the absence of endoglin, TGF-β2 was not able to induce AV cushion transformation, suggesting that this coreceptor plays an essential role in EndMT. Additionally, loss of the ALK-5, a type I TGF-β receptor, or the coreceptor endoglin decreased the number of mesenchymal cells in AV explant cultures [53]. To our interest, although endothelial-specific deletion of the TGF-β type II receptor (TβRII) prevented EMT in *in vitro* cultures, there were no EMT defects observed in the developing mouse embryo [54], suggesting a compensatory mechanism by other members of the TGF-β superfamily.

We previously reported that TGF-β plays important roles during mesenchymal differentiation of mouse embryonic stem cell-derived endothelial cells (MESECs). TGF-β2 induced the differentiation of MESECs into mural cells with decrease in expression of an endothelial marker, claudin-5, and increase in that of mural markers, SMA, SM22α, and calponin [55]. We also showed that TGF-β-induced Snail expression is necessary for the EndMT of MESEC, suggesting that EndMT and EMT share a common signal-transcription network to induce the changes in the expression of endothelial/epithelial and mesenchymal markers.

Multiple reports have shown that BMP2 released from the myocardium acts as an inductive signal initiating the

onset of EndMT [56, 57]. This myocardial signal leads to increased TGF-β synthesis in the endocardial cells, and this autocrine TGF-β induces EndMT [40]. While BMP signals induce EndMT in explant cultures [56], only limited genetic evidence has been obtained due to the early lethality of mice deficient in several BMP components. BMPs signal through specific type I receptors including ALK-2, 3, and 6. The ALK-2 deficiency in the endothelium resulted in the defective AV canal formation [58], and endothelial-specific deletion of ALK-3 [59] showed that ALK-3 is also required for EndMT of the heart. Additionally, cardiac muscle lacking ALK-3 expression is also deficient in TGF-β2 [60], supporting a two-step model for EndMT. Analyzing double-mutant embryos of the homeobox transcription factors Msx1 and Msx2, downstream effectors, and upstream regulators of BMP signaling revealed reduced number of SMA-positive cells in the AV cushions [61]. Smad4, a common downstream mediator of both TGF-β and BMP signals, is crucial for heart development. Smad4-deficient endocardial cells fail to proliferate and do not undergo EndMT, retaining morphological features of ventricular endocardium and decreased Id2 gene expression. Smad4 and GATA-4 interact in endocardium to induce Id2 expression cooperatively. In accordance with these findings, human GATA-4 mutations by which GATA-4 cannot bind to Smad4 cause atrioventricular septal defects due to loss of Id2 expression [62]. Taken together, TGF-β and BMP signals play essential roles in the EndMT-mediated formation of the valves and septa of the developing heart, providing a source of mesenchymal cells to form the valves and septa.

9. Roles of EndMT in Cardiac Fibrosis

Using the Rosa26-Tie2 reporter mouse described above, Zeisberg and colleagues performed fate mapping to trace the origin of the fibroblasts in cardiac fibrosis. Cardiac fibrosis was induced in these mice by exposing the heart to pressure overload for 5 days via aortic banding. Analysis of the fibrotic lesions revealed the presence of cells expressing both β-galactosidase (LacZ) and FSP1, a fibroblast marker. Analyzing fibrotic hearts of transgenic mice expressing green fluorescent protein (GFP) under the control of the FSP1 promoter showed the presence of GFP and PECAM-1 double-positive cells. These studies demonstrated that endothelial cells undergo EndMT and contributed to the total pool of cardiac fibroblasts, as observed during formation of the AV cushion in developing heart. Interestingly, in the fibrotic heart, approximately one-third of all fibroblasts were found to be originated from endothelial cells.

Two other groups have described the roles of EndMT in cardiac fibrosis. They used coimmunostaining method of endothelial and mesenchymal markers, instead of the irreversible fate mapping method to identify EndMT. Widyantoro and colleagues showed that endothelial cell-derived Endothelin-1(ET-1) promotes cardiac fibrosis via EndMT in streptozotocin-induced diabetic mouse model [44]. Cardiac function is decreased and perivascular fibrosis is enhanced in diabetic mice. Diabetes upregulated the production of ET-1 and TGF-β. Fibrosis markers, S100A4/FSP-1, Vimentin, and collagen 1α are regulated in CD31-positive cells, and an EC marker, VE-cadherin, is downregulated in diabetic mice. EC-specific deletion of ET-1 ameliorates these EndMT-related gene expression changes. They also addressed the mechanism by which ET-1 induces EndMT in cultured EC. They showed that ET-1 induces the TGF-β expression and claimed that TGF-β-Akt-Snail-axis is involved in this EndMT.

Ghosh and colleagues found deletion of plasminogen activator inhibitor-1 (PAI-1) results in constitutive active TGF-β signaling in aged mice [45]. Expression of Collagen1A1, TGF-β2, MMP-2, FGF-2, and its receptor, FGFR2, is also upregulated in aged mice. Importantly, in resemblance to EndMT, FGF signals play important roles in producing activated fibroblasts during EMT [63, 64]. The number of Mac-3-positive cells increased in the PAI-1 null heart, indicating that inflammation occurred and infiltrating macrophage may contribute to the pathogenesis and progression of cardiac fibrosis in PAI-1-deficient mice. Phosphorylation of Smad2 and ERK1/2 is also enhanced in the PAI-1 null heart, indicating upregulation of TGF-β signals. In their *in vitro* analysis, expression of CD31 decreases in concomitant with upregulation of FSP-1 and SMA in the PAI-1 null EC.

All of the works listed above suggested that TGF-β signal plays a critical role in cardiac fibrosis. It is of great interest whether EndMT-mediated cardiac fibrosis is regulated by other components of TGF-β signals and TGF-β-induced EMT-related transcription factors.

10. Roles of TGF-β Family Signals in the Cardiac Fibrosis That Does Not Involve EndMT

TGF-β is one of the critical regulators of cardiac fibrosis. Therefore, some groups have recently reported the roles of TGF-β family signals in cardiac fibrosis in which EndMT is not involved.

GDF15 (also known as MIC-1), a secreted member of the TGF-β superfamily, was identified as an antihypertrophic regulatory factor in the heart [65]. GDF15 is not expressed in the normal adult heart but is induced in response to conditions that promote hypertrophy and dilated cardiomyopathy. GDF15 transgenic mice were normal but were partially resistant to pressure overload-induced hypertrophy. Conversely, GDF15 knockout mice showed enhanced cardiac hypertrophic growth following pressure overload stimulation. GDF15 knockout mice also demonstrated a pronounced loss in ventricular performance. GDF15 stimulation promoted phosphorylation of Smad2/3 in an in vitro analysis. Overexpression of Smad2 attenuated cardiomyocyte hypertrophy similar to GDF15 treatment, whereas overexpression of the inhibitory Smad proteins, Smad6/7, reversed the antihypertrophic effects of GDF15. Therefore, GDF15 is an autocrine/endocrine factor that antagonizes the hypertrophic response and loss of ventricular performance and may be a candidate molecule for treating cardiac hypertrophy clinically.

TGF-β-ALK-5 signals are involved in GATA-6-mediated angiogenic function and survival in ECs [66]. Knockdown

of GATA-6 decreased proliferation, migration, and tube formation of ECs. This effect was partially rescued by TGF-β-neutralizing antibody or ALK-5 inhibitor. GATA-6 suppresses TGF-β expression in EC, which results in down-regulation of phosphorylation of Smad2 without affecting phosphorylation of Smad1, suggesting that GATA-6 suppresses only TGF-β-ALK-5 signals but not TGF-β-ALK-1 signals for exerting angiogenic function.

Sarcomere protein mutation (a-MHC R719W) causes hypertrophic cardiomyopathy, a disorder characterized by myocyte enlargement, fibrosis, and impaired ventricular relaxation. This mutation activates proliferative and profibrotic signals in nonmyocyte cells to produce TGF-β, and its responsive genes such as periostin. In treatment of TGF-β-neutralizing antibody or Losartan, an AT1 blocker abrogates nonmyocyte proliferation and fibrosis probably by blocking both TGF-β signals but through unknown precise mechanism [67]. Once again, TGF-β is one of the main causes of fibrosis here and can also be an efficient target for ameliorating fibrotic microenvironments in the heart.

11. Future Perspective and Concluding Remarks

The recent findings that EndMT is involved in different diseases suggest that modulating EndMT may serve a promising new therapeutic strategy for heart diseases and cancer. The endothelium is a promising target for drug delivery because it lies in direct contact with the bloodstream. Possible treatment strategies may target the TGF-β signaling pathways. We found that inhibition of endogenously activated TGF-β signals in ECs [68] by a small molecule that inhibits kinases of receptors for TGF-β led to a decrease in EndMT, suggesting that inhibition of TGF-β signals may suppress EndMT. Interestingly, Zeisberg and colleagues demonstrated that systemic administration of recombinant human BMP-7 (rhBMP-7) significantly reduced EndMT during cardiac fibrosis [1]. Additional studies are needed to identify the precise molecular mechanisms by which BMP-7 inhibits the EndMT.

Furthermore, accumulating evidence suggests that EndMT not only induces the formation of fibroblasts in cardiac fibrosis but also in other fibrotic disorders including intestinal fibrosis [69] and kidney fibrosis [70]. In conclusion, EndMT is expected to be a target for the development of new therapies for multiple fibrotic disorders.

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Research Article

Atherosclerotic Plaque Stability Is Affected by the Chemokine CXCL10 in Both Mice and Humans

Dolf Segers,[1] Jonathan A. Lipton,[1] Pieter J. M. Leenen,[2] Caroline Cheng,[1] Dennie Tempel,[1] Gerard Pasterkamp,[3] Frans L. Moll,[4] Rini de Crom,[5] and Rob Krams[6]

[1] *Department of Cardiology, Erasmus University Medical Center, 3522ZZ Rotterdam, The Netherlands*
[2] *Department of Immunology, Erasmus University Medical Center, 3522ZZ Rotterdam, The Netherlands*
[3] *Department of Cardiology, University Medical Center-Utrecht, 3584CX Utrecht, The Netherlands*
[4] *Department of Vascular Surgery, University Medical Center-Utrecht, 3584CX Utrecht, The Netherlands*
[5] *Department of Cell Biology and Genetics, Erasmus University Medical Center, 3522ZZ Rotterdam, The Netherlands*
[6] *Department of Bioengineering, Royal School of Mines, Imperial College, London SW7 2AZ, UK*

Correspondence should be addressed to Rob Krams, r.krams@imperial.ac.uk

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Background. The chemokine CXCL10 is specifically upregulated during experimental development of plaque with an unstable phenotype. In this study we evaluated the functional consequences of these findings in mice and humans. *Methods and Results*. In ApoE$^{-/-}$ mice, we induced unstable plaque with using a flow-altering device around the carotid artery. From week 1 to 4, mice were injected with a neutralizing CXCL10 antibody. After 9 weeks, CXCL10 inhibition resulted in a more stable plaque phenotype: collagen increased by 58% ($P = 0.002$), smooth muscle cell content increased 2-fold ($P = 0.03$), while macrophage MHC class II expression decreased by 50% ($P = 0.005$). Also, the size of necrotic cores decreased by 41% ($P = 0.01$). In 106 human carotid endarterectomy specimens we found that increasing concentrations of CXCL10 strongly associate with an increase in atheromatous plaque phenotype (ANOVA, $P = 0.003$), with high macrophage, low smooth muscle cell, and low collagen content. *Conclusions*. In the present study we showed that CXCL10 is associated with the development of vulnerable plaque in human and mice. We conclude that CXCL10 might provide a new lead towards plaque-stabilizing therapy.

1. Introduction

Atherosclerosis is a progressive inflammatory disease of the arterial vasculature with lesions that may turn unstable, which may lead to lesion rupture. The high mortality of these ruptures has been associated with atherosclerotic plaques that display a specific histological phenotype, characterized by a large pool of lipids and necrotic cell debris covered by a thin fibrous cap and the presence of inflammatory cells in the plaque shoulders [1].

Previously, we developed an experimental animal model in which plaques with stable and unstable characteristics are simultaneously induced in a single straight arterial segment [2]. We subsequently identified specific expression profiles of various chemokine genes in vulnerable plaque development [3]. In these, the chemokine CXCL10 was specifically upregulated during the early phase in the development of the vulnerable plaque segment.

CXCL10 is a member of the CXC family of chemokines. Upon stimulation with interferon-gamma (IFN-γ) it is expressed by many cell types, for example, monocytes/macrophages, endothelial cells, fibroblasts, and natural killer cells [4, 5]. Effects of CXCL10 are mediated by the G-protein coupled receptor CXCR3, which is known to be expressed by resting and activated T cells, NK cells, and a subset of peripheral blood monocytes [5–7]. Furthermore, CXCL10 plays a variety of roles in inflammatory diseases, like in the initiation and maintenance of T-helper-1- (Th1-) polarized immune responses [8, 9], migration of activated T cells [10], but also of natural killer cells [11], of monocytes [12, 13] and of smooth muscle cells (SMC) [14].

Despite these compelling findings described in the literature, in atherosclerosis CXCL10 has been less completely characterized. In human atherosclerosis, but not in healthy arteries, endothelial cells, smooth muscle cells, and

macrophages express CXCL10 [15]. Functional observations in CXCL10 gene knockout mice indicated that CXCL10 exerts pro-atherogenic effects, probably related to the specific recruitment and retention of activated Th1 cells and down-regulation of a regulatory T-cell response [16]. While these findings indicate that CXCL10 plays a role in atherogenesis, its effect on plaque composition is unclear. Based on our previous observations on gene expression in the mouse model, we hypothesized that CXCL10 mediates development of vulnerable atherosclerotic plaque. Hence, we assessed whether CXCL10 plays a critical role in unstable plaque development by evaluating the effect of antibody-mediated functional inhibition of CXCL10 on lesion phenotype in our vulnerable plaque mouse model. To provide evidence that the findings in the mouse bear relevance for human atherosclerosis, we also investigated the association between CXCL10 concentrations and plaque composition in human carotid endarterectomy lesions.

2. Materials and Methods

2.1. Animals and Surgical Procedures. Apolipoprotein-E deficient mice, 15–20 weeks of age, were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). They were fed a high fat, high cholesterol diet consisting of 15% (wt/wt) cocoa butter and 0.25% cholesterol (wt/wt) (Diet W, Hope Farms, Woerden, The Netherlands). Two weeks after this diet was initiated, the mice were instrumented with a shear stress-altering device around the right common carotid artery under 2% isoflurane anesthesia, as described previously [2]. Briefly, this device gradually narrows the vessel lumen to ~70% of its original diameter. As a consequence, shear stress is lowered upstream of the device, gradually increases inside the device, and is oscillating just downstream of the device [17]. After surgery, the mice were kept on the high fat, high cholesterol diet for the remainder of the experiment. The device remained in situ for 9 weeks. Intervention and control groups consisted of at least 10 animals per group. All animal experiments were approved by the institutional animal ethical committee and performed in compliance with institutional and national guidelines.

2.2. CXCL10 Inhibition. Mice were intravenously injected three times a week in week 2–4 after surgery with an anti-mouse bioactivity-neutralizing monoclonal CXCL10 antibody (MAB466, R&D Systems, Abingdon, United Kingdom) in a dose of 25 μg in 0.2 mL sterile PBS. Untreated cast-instrumented mice served as control.

2.3. Isolation and Analysis of Murine Tissues. Following sacrifice, blood was collected and mice were flushed systemically with PBS. Next, the common carotid artery was isolated, embedded in OCT compound (Tissue-Tek, Sakura, Japan), and snap-frozen in liquid nitrogen. For histology, 8 μm cryosections were cut and stained for routine histology (Hematoxylin-Eosin, HE), lipids (Oil Red O), collagen (Picrosirius Red), macrophages (anti-CD68, AbD Serotec, Oxford, UK), macrophage activation (anti-Major Histocompatibility Complex (MHC) class II, clone M5/114, ATCC TIB-120), and smooth muscle cells (anti-SMC α-actin, Sigma-Aldrich, The Netherlands). Subsequently, high-resolution images were taken using an Olympus BX-40 microscope or a Zeiss LSM5 Meta confocal microscope (Zeiss Jena, Germany). Collagen stainings were assessed using crossed circular polarization filters. To calculate lesion size and cellular content relative to the plaque area, all images were analyzed after digitalization by automated image analysis software (Clemex Technologies Inc, Canada) applying thresholding of the stained areas. Necrotic cores (defined as hypocellular plaque cavities devoid of collagen, containing cholesterol clefts) were assessed based on HE and Picrosirius Red stained sections and measured relative to the lesion area. Total cholesterol levels were measured in serum samples by an enzymatic colorimetric method (Cholesterol E, Wako Diagnostics, USA).

2.4. Endarterectomy Procedures. All patients in this study were participants of a prospective study aimed at investigating the predictive value of plaque characteristics for long-term outcome (Athero-Express), of which the study methods have been described extensively before [18]. In short, all patients underwent carotid endarterectomy, during which arterial wall tissue was obtained. Next, the culprit lesion, defined as the segment with the largest plaque burden, was processed for histological examination. In addition to HE staining, sections were stained for collagen (Picrosirius Red), SMC (α-actin), and macrophage (CD68) content. Histological assessment was performed in a semiquantitative fashion (none, minor, moderate, heavy), on basis of two independent observers. When results contradicted, a third independent observer was consulted. Subsequently, plaques were designated as fibrous (lipids constitute <10% of lesion surface area), fibroatheromatous (10–40% lipids), or atheromatous (>40% lipids) based on collagen and HE stainings. Besides, plaques were also designated as SMC dominant or macrophage dominant based on the prevailing cell type obtained from the specific stainings.

A 5 mm segment directly adjacent to the culprit lesion was crushed in liquid nitrogen, and subsequently total protein was extracted using 1 mL of TriPure Isolation Reagent (Boehringer Mannheim, Germany), according to the manufacturer's protocol. Then, CXCL10 concentration was measured by Enzyme-Linked Immuno Sorbent Assay (ELISA) according to manufacturers instructions (Quantikine human CXCL10 immunoassay, R&D Systems, Abingdon, United Kingdom) and blinded from histological and clinical data.

2.5. Statistical Analysis. SPSS (version 15.0, SPSS Inc., USA) was used for all analyses. To test the differences between the treated and the control mice at 9 weeks of cast placement, a student's t-test was used. When these data had a non-Gaussian distribution, a nonparametric t-test was used. For the endarterectomy samples, to compare CXCL10 levels between categorical baseline and plaque morphology variables, Kruskal-Wallis and Mann-Whitney tests for non-Gaussian distributed data were used. To investigate the differences between continuous baseline variables and CXCL10,

patients were categorized into quartiles, named 1 through 4 based on increasing CXCL10 concentration. Subsequently, these quartiles were used to sort all other variables. One-way ANOVA was used to compare continuous variables. The quartiles were also used to visualize the relationship between CXCL10 concentration and plaque morphology. Data are described as mean ± SD or median (Inter Quartile Range) as appropriate. A P value of <0.05 was considered statistically significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

3. Results

3.1. Suppression of CXCL10 Bioactivity Inhibits Experimental Vulnerable Plaque Formation. As we previously found that CXCL10 is expressed specifically in developing unstable lesions [3], we investigated if CXCL10 is also functionally involved in the development of plaque vulnerability using a mouse model that we developed before [2]. ApoE-deficient mice, in which a shear stress-altering device was applied, were injected with a bioactivity-neutralizing antibody during the onset of plaque formation. As expected, serum total cholesterol levels did not differ between treated and control mice (30.13 ± 4.6 versus 29.92 ± 6.7 mmol/L, $P = 0.92$). Short-term inhibition of CXCL10 did not influence the extent of plaque development, since we found no difference in lesion size between the treated and the control mice after 9 weeks of shear stress alteration. Because macrophage foam cells are characteristic of atherosclerosis, we measured both plaque lipid (31.3 ± 8.0% treated versus 29.5 ± 7.0% control) and macrophage content (31.7 ± 7.6% treated versus 27.8 ± 7.0 control; Figure 1(a)), where both remained unchanged upon CXCL10 inhibition.

To assess plaque vulnerability, we determined the amount of collagen in the lesions, which is the main stabilizing component of the plaque. Interestingly, we found a 57% increase in the relative amount of collagen in the plaques following CXCL10 suppression (17.8 ± 6.5% versus 11.3 ± 5.5%, $P = 0.002$; Figure 1(b)). The amount of plaque collagen is essentially the result of a balance between collagen deposition and breakdown. Therefore, the increase in collagen may be the result of decreased breakdown predominantly by proteinases secreted by activated macrophages. To determine the extent of immune activation, we measured MHC class II by immunohistochemistry. The cellular morphology, location in the plaque and spatial association of MHC class II staining with macrophage staining by CD68 antibodies in adjacent sections (Figures 1(a) and 1(c)) strongly suggests that MHCII-positive cells are the prime cells expressing this activation marker. We found a 50% reduction in the plaque MHC class II levels following CXCL10 inhibition (6.3 ± 3.3% versus 12.6 ± 7.4%, $P = 0.005$; Figure 1(c)). In addition, the amount of SMC, which is known to produce collagen, nearly doubled in the CXCL10-suppressed group (13.5 ± 8.4% versus 6.3 ± 7.0%, $P = 0.03$; Figure 1(d)), suggesting that the differences in collagen content may be explained by several factors.

The necrotic core is a hallmark component of the vulnerable plaque. To test whether CXCL10 inhibition reduces necrotic core formation, we analyzed both the number of necrotic cores in the lesions as well as their relative size. We found that CXCL10 inhibition resulted in fewer necrotic cores: 38.9 ± 22.1% versus 57.7 ± 20% of the sections covering the entire lesion that contained a necrotic core ($P = 0.02$). Moreover, also the relative size of the necrotic cores decreased following antibody treatment from 26.4 ± 11.4% to 15.6 ± 6.1% of the plaque surface area ($P = 0.01$; Figure 1(e)).

3.2. Patient Characteristics. For this study endarterectomy specimens of 106 patients were analyzed. An overview of the patient characteristics is provided in Table 1. Histological examples of the lesions are shown in a previous publication by Verhoeven et al. [18]. The CXCL10 concentration in the specimens ranged from undetectable to 384.8 pg/mL, with a median (interquartile range) of 38.34 pg/mL (14–39 pg/mL). To compare continuous CXCL10 levels to the categorical variables, patients were categorized into quartiles (Figure 2). The variables were then tested for changes across the quartiles. No differences were found comparing risk factors for atherosclerotic disease. The use of medication did not differ significantly between the quartiles.

3.3. High CXCL10 Concentrations Identify Patients with a More Vulnerable Plaque Phenotype. Several significant associations were found between plaque composition and CXCL10 levels. Atheromatous lesions were more prominent at higher CXCL10 concentrations than those classified as fibrous (Rank 53 versus 45, $P = 0.003$). Also, higher CXCL10 concentrations were associated with more macrophage-dominant plaques (Rank 56 versus 41, $P = 0.009$), fewer smooth muscle cells (Rank 39 versus 74, $P = 0.001$), and less collagen (Rank 37 versus 63, $P = 0.003$).

Subsequently, we analyzed the association between plaque composition and CXCL10 levels, by dividing patients into quartiles based upon CXCL10 concentration. More atheromatous lesions were represented in the higher quartiles, while fibrous lesions were more frequently present in the lower quartiles (Figure 3(a)). Macrophage-dominant lesions were mostly found in the top quartiles of CXCL10 content, while SMC dominance associated with lower CXCL10 concentrations (Figure 3(b)). However, no differences were seen in the macrophage staining between the quartiles (Figure 4(a)). This paradox was the result of an inverse relation between smooth muscle cell staining and increasing CXCL10 concentrations (Figure 4(b)). Additionally, more collagen staining was seen in the plaques in the lower quartiles (Figure 4(c)).

4. Discussion

This study may be summarized by its two main findings. First, we showed a functional role of CXCL10 in vulnerable plaque formation in an experimental mouse model for unstable atherosclerotic disease. We observed that inhibiting

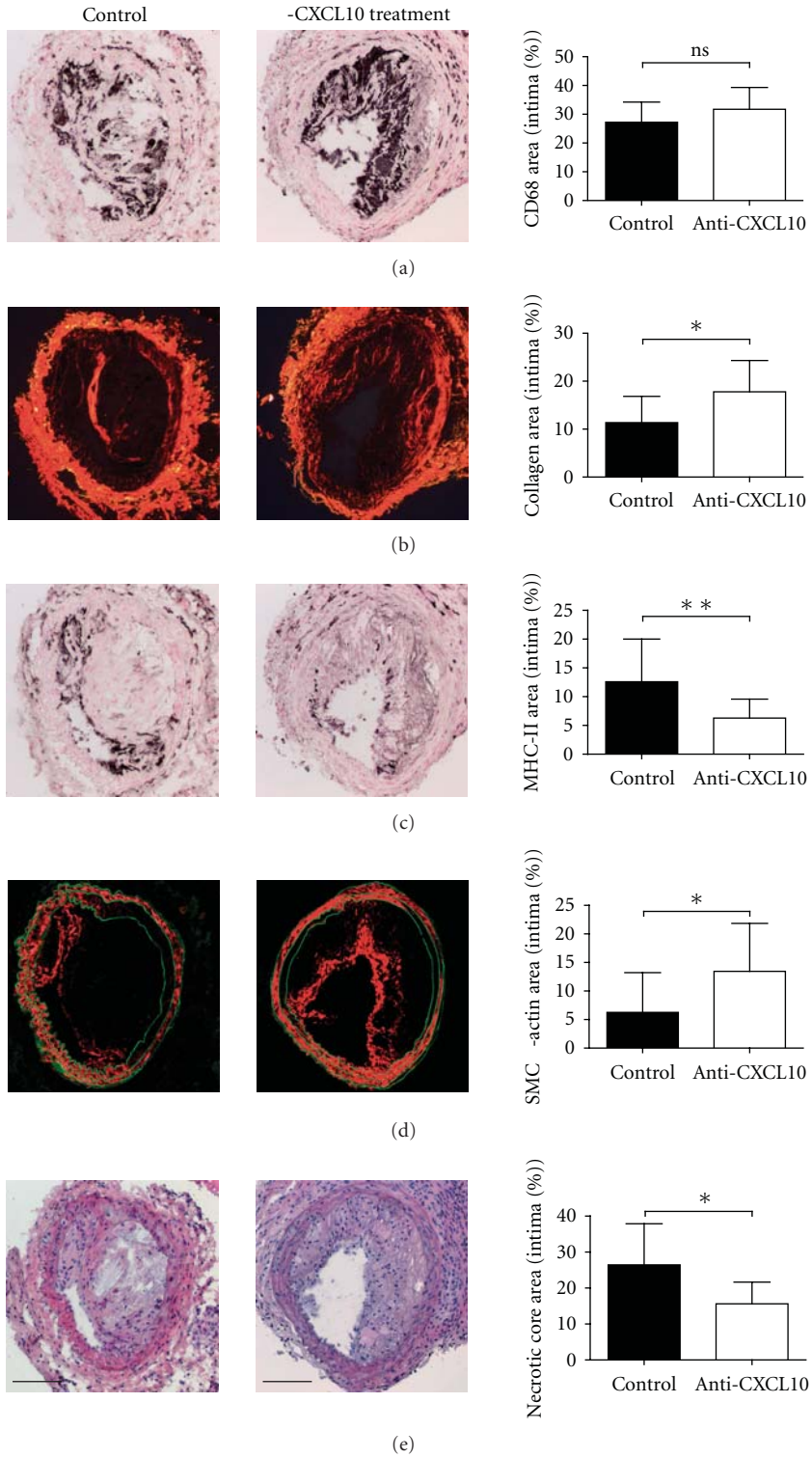

Figure 1: Anti-CXCL10 treatment in atherosclerosis susceptible mice results in a change into a more stable lesion phenotype. A flow-altering device around the common carotid artery induced atherosclerosis in ApoE$^{-/-}$ mice. From week 1 to 4 of lesion development, a bioactivity-neutralizing anti-CXCL10 antibody was injected. After 9 weeks, lesions were compared to untreated controls by histology. The pictures show representative histological sections of treated and control mice. All photographs have been made with the same magnification (100x). Scale bars are provided in (e) and represent 100 μm. Data in bar diagrams are the mean values ± SD of at least 8 sections from at least 10 different animals per group. CXCL10 inhibition resulted in a more stable morphology evidenced by unchanged amounts of lesion macrophages (a), increased amounts of collagen (b), decreased macrophage activation (c), increased numbers of SMC (d), and reduced necrotic core size (e). $^{*}P < 0.05$, $^{**}P < 0.01$. MHC-II: Major Histocompatibility Complex Class II, SMC: smooth muscle cell.

Table 1: Patient baseline measurements for carotid endarterectomy specimens.

Clinical characteristics	Total	Quartile of CXCL10 concentration				P value*
		1	2	3	4	
CXCL10 pg/mL	38.3 (14–40)†	0–11.3	11.8–18.4	19.3–37.2	37.6–382.6	0.000
Age (years)†	68.4 ± 8.9	66.0 ± 8.0	68.3 ± 10.2	67.7 ± 8.2	71.2 ± 8.5	0.157
Male (%)	73.6	73.1	70.4	66.7	84.6	0.316
Hypertension (%)	71.7	80.0	70.4	66.7	79.2	0.764
Smoker (%)	24.5	30.8	18.5	32.0	19.2	0.476
Symptomatic disease (%)	86.8	84.6	85.2	92.6	84.6	0.762
Statin (%)	56.6	66.7	55.6	55.6	56.0	0.530
β blocker (%)	39.6	37.5	33.3	56.0	41.7	0.321
ACE inhibitor (%)	39.6	42.3	33.3	40.7	42.3	0.881
Serum values†						
Total cholesterol (mmol/L)	4.9 ± 1.3	4.8 ± 0.9	5.2 ± 1.3	4.8 ± 1.7	4.6 ± 1.2	0.564
HDL (mmol/L)	1.2 ± 0.4	1.2 ± 0.4	1.4 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	0.156
LDL (mmol/L)	3.0 ± 1.1	2.7 ± 0.8	3.3 ± 1.0	3.2 ± 1.4	2.7 ± 1.0	0.392
Triglycerides (mmol/L)	1.9 ± 0.9	2.3 ± 1.0	1.8 ± 0.8	2.0 ± 1.1	1.7 ± 0.7	0.434
ApoB (mmol/L)	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	1.0 ± 0.3	0.9 ± 0.3	0.787
Glucose (mmol/L)	6.6 ± 1.9	5.8 ± 0.7	7.0 ± 2.0	7.0 ± 2.3	6.4 ± 1.9	0.129
CRP (mg/mL)	3.40 (1.6–7.3)	3.13 (1.4–5.8)	2.96 (1.8–5.8)	4.81 (1.7–9.1)	4.52 (1.6–7.3)	0.221

Baseline data of 106 patients were stratified into four quartiles according to plaque CXCL10 concentration. †Values are represented as mean ± SD or median (Inter Quartile Range) as appropriate. *P values based on ANOVA test between CXCL10 quartiles for continuous variables and nonparametric Mann-Whitney test for categorical variables.

CXCL10 with a bioactivity-neutralizing antibody resulted in plaques with less macrophage activation, with more SMC and a subsequent increase in collagen content compared to controls. A decreased number and size of necrotic cores in the lesions of the treated animals further substantiated these findings. This resulted in a lesion phenotype with increased intrinsic stability.

Second, we found that in human carotid plaques, CXCL10 levels are associated with lesion morphology. This is evidenced by an increased number of atheromatous plaques with increasing CXCL10 concentrations, and by the association with unstable plaque characteristics, such as macrophage dominance and reduced presence of smooth muscle cells and collagen.

Previous studies have shown that inhibition of CXCL10 by antibodies in an intestinal inflammation animal model is an effective way to inhibit its function [19, 20]. The findings in those studies have been ascribed to the reduced amount of Th1 cells that were recruited to the diseased sites and either reduced production of inflammatory cytokines [19] or resulted in inflammatory disease exacerbation [20], depending on the role of T cells in the pathogenesis of the disease.

The lesion-stabilizing effect we observed by inhibiting CXCL10 might be explained by a similar mechanism as described above, as it has been shown before that inhibition of the CXCL10-CXCR3 pathway decreases the amount of lesional T cells [16, 21]. Veillard et al. reported that genetic deletion of CXCR3 in ApoE knockout mice reduced early lesion formation, which correlated with an increase in lesional FoxP3-positive regulatory T cells [21]. Recently, it has been reported that genetic deletion of CXCL10 in atherosclerosis susceptible mice also resulted in smaller lesions, which contained fewer CD4+ T cells [16]. Surprisingly, despite the decrease in overall T-cell presence in the lesions, a higher expression of FoxP3 as well as of the anti-inflammatory cytokines IL-10 and TGF-β mRNA was found. This suggests a more prominent role for regulatory T cells in this model. A similar increase in FoxP3 and TGF-β expression was found in a study using a peptide antagonizing CXCR3 in LDL receptor knockout mice, resulting in smaller lesions in the aortic root and the aorta [22].

A general limitation of our experimental mouse model is that there are few T cells in the induced lesions after 9 weeks of cast placement, approximately 2–4 per lesion cross section [3]. As a consequence, we have been unable to find a difference in T-cell numbers following CXCL10 inhibition (data not shown). Nevertheless, the reduction in T cells might have taken place during the early phase of actual inhibition. We performed measurements at only one time point, while the presence of T cells in a lesion changes over time [23]. Also, the effect of small numbers of T cells might prove to be very strong [24].

The small numbers of T cells do not allow to produce sufficiently reliable data to enable making a comparison with the situation in mice that are deficient in CXCL 10 by gene targeting. This cannot be solved by mRNA analyses, as there was no RNA available from the endarterectomy samples that have been used. Besides facilitating recruitment of leukocytes, CXCL10 also induces and sustains a dominant

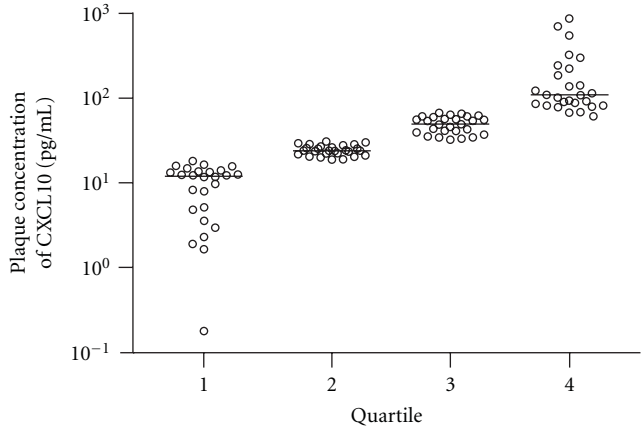

Figure 2: Distribution of CXCL10 measurements from each patient across quartiles. Atherosclerotic plaques were obtained from 106 patients during carotid endarterectomy. In the plaque segment directly adjacent to the culprit lesion, the content of the chemokine CXCL10 was measured by ELISA. Based on these measurements, patients were divided into one of four quartiles according to the CXCL10 concentration. Quartile 1 represents the lesions with the lowest CXCL10 values, whereas quartile 4 contains the highest. This figure shows the individual measurements for all patients and the distribution across each quartile of plaque CXCL10. Horizontal bars represent the median concentration of each quartile.

Th1-type response by increasing the production of IFN-γ, providing a positive feedback loop [25]. Neutralization of CXCL10 might therefore result in a decreased concentration of IFN-γ in the lesions, which is considered a key cytokine in disease progression [26] and macrophage activation [27]. This notion is in agreement with our findings regarding macrophage activation status as measured by MHC class II expression [27], SMC presence [27], and collagen accumulation [28].

An alternative or additional mechanism of plaque modification by CXCL10 inhibition might be operative as well. Not only Th1 and NK cells, as major IFN-γ producers, are responsive to CXCL10, but also plasmacytoid dendritic cells (PDC) express CXCR3 and are recruited via this receptor [29]. The numbers of circulating PDC are reduced in human atherosclerosis, while immunohistochemical analysis has indicated that they are recruited to advanced plaques [30, 31]. PDC are known to produce large quantities of IFN-α upon activation, and this cytokine functions as an inflammatory amplifier in the atherosclerotic plaque [32]. Additionally, a minor subset of monocytes has been shown to be recruited via CXCR3 to inflammatory environments [7] and might thus contribute to plaque instability as well.

Further research is needed to understand the mechanisms underlying the effects of CXCL10 inhibition. For example, apoptosis could have an important role in the observed differences in plaque composition and size. It could be of interest to study the role of SMC in more detail by staining serial sections for proliferation and apoptosis on the one hand and alpha actin on the other hand. In addition, in situ zymography studies could be performed to get a clearer idea of the possible role of collagenolytic

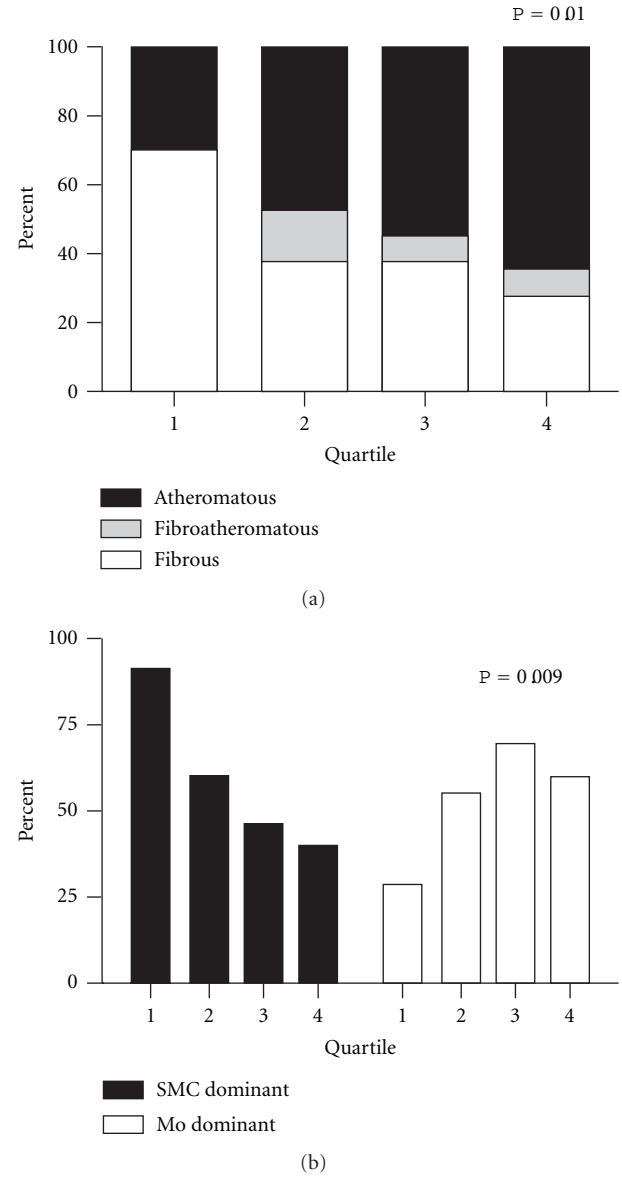

Figure 3: Plaque concentrations of CXCL10 are associated with lesion morphology. Individual values were divided over quartiles (see Figure 1). By using ANOVA, we tested to see if plaque CXCL10 concentrations associated with lesion morphology. Increasing plaque concentrations of CXCL10 were indeed associated with an increase in atheromatous-type lesions (a) and a decrease of SMC dominant lesions and an increase of macrophage dominant lesions (b).

activity in the lesions. It would be very interesting to know more about the role of the proinflammatory cytokine microenvironment in the plaque in the experiments with CXCL 10 inhibition. From the present data, we cannot exclude that by inhibiting CXCL10 we just postponed the process of the lesion formation and maturation rather than permanently altering it. For this argument and because of the ongoing discussion on the quality of animal models representing human disease states, we also performed a parallel study in humans.

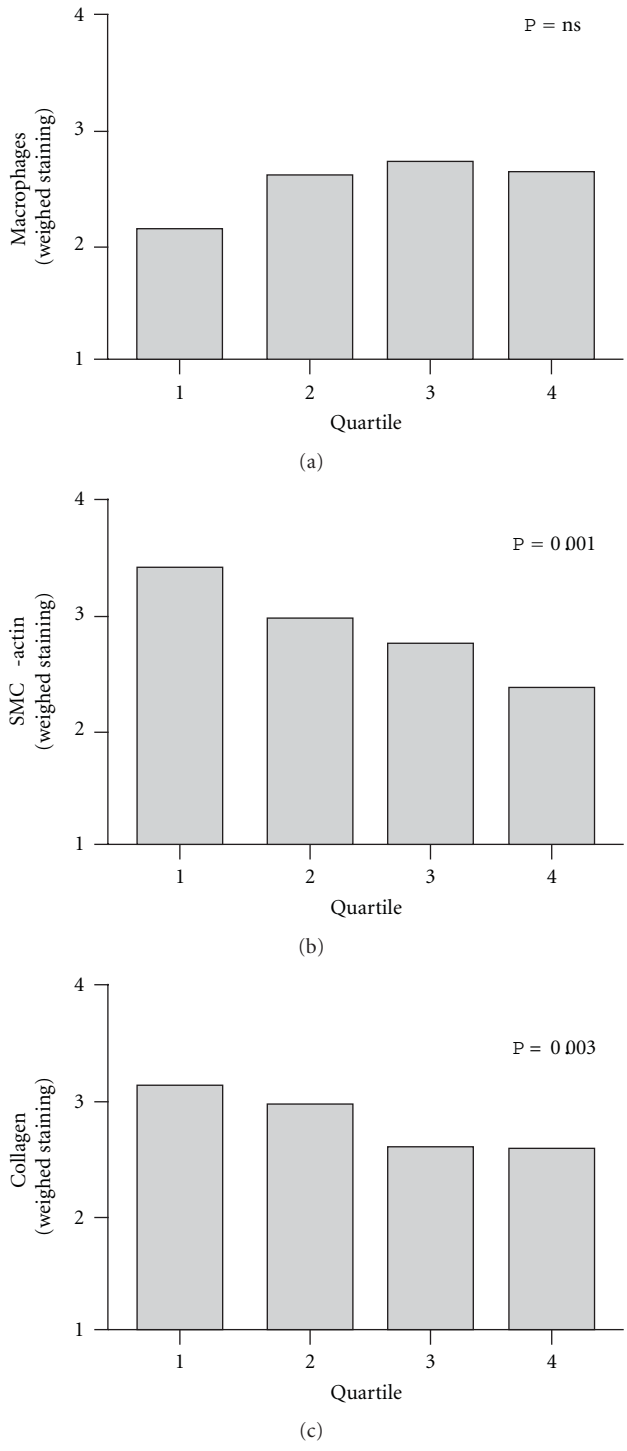

Figure 4: Increasing carotid plaque CXCL10 concentrations are associated with a less fibrous phenotype. Plaque concentrations of CXCL10 were associated with histological qualities of the lesion by ANOVA. Staining for macrophages, SMC, or collagen was quantified as none, minor, moderate, or high. Based on these qualifications a weighed value for average staining intensity was calculated (1, none; 2, minor; 3, moderate; 4, heavy staining). Macrophage staining was not associated with plaque CXCL10 (a), whereas high plaque concentrations of CXCL10 were associated with decreasing amounts of smooth muscle cells (SMC) (b), and decreasing collagen (c).

Mach et al. [15] were the first to associate CXCL10 with human atherosclerosis, reporting that CXCL10 was expressed in several stages of the disease. CXCL10 was shown to be present on the surface of endothelial cells, suggesting a role in the recruitment and adhesion of CXCR3 positive cells from the circulation. They also showed broad expression of CXCL10 by SMC and macrophages throughout the lesion. Little is known about the effects of CXCL10 on human atherosclerosis development and clinical outcome. Previous studies sought to correlate plasma CXCL10 levels with the occurrence of coronary heart disease (CHD). In a case-control study it was found that CHD risk was associated with an increase in serum CXCL10 [33]. A later prospective study showed that indeed increased levels of CXCL10 exist prior to CHD, but was not considered an independent risk factor [34]. Our study is the first one to correlate lesional CXCL10 protein content with plaque morphology in humans and to be a possible predictor of plaque vulnerability. Obviously, this cannot be measured directly in living patients, so it would be of interest to know the correlation between lesional and systemic levels of CXCL10. There were no plasma samples available to measure the systemic CXCL 10 levels and correlate them to the lesional levels, so this has to await future work.

In conclusion, we showed that the chemokine CXCL10 plays a functional role in the destabilization of atherosclerotic plaques in mice and is specifically upregulated in vulnerable plaques in humans. Since the experimental data were paralleled by similar findings in human unstable lesions, CXCL10 might be regarded as a new lead into understanding the process of plaque destabilization.

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Review Article

Lipid Chaperones and Metabolic Inflammation

Masato Furuhashi, Shutaro Ishimura, Hideki Ota, and Tetsuji Miura

Second Department of Internal Medicine, Sapporo Medical University School of Medicine, S-1, W-16, Chuo-ku, Sapporo 060-8543, Japan

Correspondence should be addressed to Masato Furuhashi, furuhasi@sapmed.ac.jp

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Over the past decade, a large body of evidence has emerged demonstrating an integration of metabolic and immune response pathways. It is now clear that obesity and associated disorders such as insulin resistance and type 2 diabetes are associated with a metabolically driven, low-grade, chronic inflammatory state, referred to as "metaflammation." Several inflammatory cytokines as well as lipids and metabolic stress pathways can activate metaflammation, which targets metabolically critical organs and tissues including adipocytes and macrophages to adversely affect systemic homeostasis. On the other hand, inside the cell, fatty acid-binding proteins (FABPs), a family of lipid chaperones, as well as endoplasmic reticulum (ER) stress, and reactive oxygen species derived from mitochondria play significant roles in promotion of metabolically triggered inflammation. Here, we discuss the molecular and cellular basis of the roles of FABPs, especially FABP4 and FABP5, in metaflammation and related diseases including obesity, diabetes, and atherosclerosis.

1. Introduction

Inflammation is classically characterized as heat (calor), pain (dolor), redness (rubor), and swelling (tumor) [1]. The short-term adaptive response of inflammation is crucial for integration of injury response and repair in cells and tissues. However, the long-term consequences of prolonged inflammation are often not beneficial. It has recently been shown that low-grade and chronic features of inflammation are observed in metabolic diseases including obesity, insulin resistance, type 2 diabetes, and cardiovascular disease [2, 3]. This atypical immune response emerging from metabolic tissues is referred to as metabolically triggered inflammation, "metaflammation," which is principally triggered by nutrients and metabolic surplus, resulting in the engagement of at least a subset of molecules and signaling pathways involved in classical and canonical inflammation [2].

A number of hormones, cytokines, and bioactive lipids function in both metabolic and immune responses. Metabolic and immune systems regulate each other by the same cellular machinery. In metabolically active cells such as adipocytes and macrophages, metaflammatory pathways can be initiated by not only extracellular mediators such as cytokines and lipids, particularly saturated fatty acids, but also by intracellular stresses such as endoplasmic reticulum stress and excess production of reactive oxygen species derived from mitochondria. Signals from all of these mediators converge on inflammatory signaling pathways, including signaling kinases: c-Jun N-terminal kinase (JNK), inhibitor of nuclear kappa B kinase (IKK), protein kinase R (PKR), and others. These pathways lead to the inhibition of insulin signaling [4–6] and a vicious spiral of additional production of inflammatory mediators through transcriptional regulation using activating protein-1 (AP-1) and nuclear factor-kappa B (NF-κB).

In this paper, we will focus on metabolically active cell-derived fatty acid-binding proteins (FABPs), which have been shown to regulate inflammatory and metabolic responses mainly in adipocytes and macrophages, and also discuss molecular and cellular links between FABPs and metaflammation, particularly in the context of metabolic diseases such as obesity, diabetes, and atherosclerosis.

2. Fatty Acid-Binding Protein (FABP) as a Lipid Chaperone

FABPs are a family of 14-15-kDa proteins that coordinate lipid trafficking and responses in cells [7]. FABPs can

reversibly bind to hydrophobic ligands, such as saturated and unsaturated long-chain fatty acids, eicosanoids, and other lipids, with high affinity and broad selectivity. To date, at least 9 different FABP isoforms have been identified. Different members of the FABP family are expressed most abundantly in tissues involved in active lipid metabolism. The family contains liver (L-FABP/FABP1), intestinal (I-FABP/FABP2), heart (H-FABP/FABP3), adipocyte (A-FABP/FABP4/aP2), epidermal (E-FABP/FABP5/mal1), ileal (Il-FABP/FABP6), brain (B-FABP/FABP7), myelin (M-FABP/FABP8), and testis (T-FABP/FABP9) isoforms. FABPs have been proposed to facilitate the transport of lipids to specific compartments in the cell, such as to the mitochondrion or peroxisome for oxidation, to the nucleus for lipid-mediated transcriptional regulation, to the endoplasmic reticulum for signaling, trafficking, and membrane synthesis, to cytoplasmic enzymes for activity regulation, and to the cytoplasm for storage as lipid droplets. However, regulatory mechanisms of tissue-specific expression and function of various FABPs are still poorly understood. Specific contribution of each type of FABP to cell biology, physiology, and lipid metabolism had not been demonstrated until FABP-deficient mice models were created.

3. Adipocyte/Macrophage FABPs

Among the FABPs, FABP4, known as adipocyte FABP (A-FABP) or adipocyte P2 (aP2), is one of best-characterized isoforms (Table 1). FABP4 is highly expressed in adipocytes, making up about 1% of all soluble proteins in adipose tissue [8]. FABP5, another FABP known as epidermal FABP (E-FABP) or mal1, is expressed most abundantly in epidermal cells of the skin but is also present in several other tissues and cells including adipocytes [7] (Table 1). FABP5 constitutes a minor fraction of FABPs in adipocytes, the amount being about 100-fold smaller than that of FABP4 in adipocytes [9]. These two proteins, FABP4 and FABP5, have 52% amino acid similarity and bind to a variety of fatty acids with similar selectivity and affinity [10]. Interestingly, both FABP4 and FABP5 are also expressed in macrophages and dendritic cells [11, 12]. The stochiometry of these two proteins appears to be approximately equal in macrophages under physiological conditions [11]. The content of FABP4 in adipocytes is about 10,000-fold higher than that in macrophages [13]. In a state of germline FABP4 deficiency, FABP5 expression exhibits a strong compensatory increase in adipose tissue but not in macrophages or dendritic cells [11, 12, 14]. It has been demonstrated that both FABP4 and FABP5 play important roles in the regulation of insulin sensitivity and the development of atherosclerosis and that their impacts differentially involve adipocytes or macrophages [11, 14–22].

3.1. FABP4 (A-FABP/aP2). Expression of FABP4 in adipocytes is highly regulated during differentiation of adipocytes and is transcriptionally controlled by fatty acids, peroxisome proliferator-activated receptor (PPAR) γ agonists, dexamethasone, and insulin [23–27]. Potential functional domains of FABP4 have been reported to include a nuclear localization signal, its regulation site, and a nuclear export signal [7, 62, 63]. The primary sequence of FABP4 does not demonstrate a readily identifiable nuclear localization signal or nuclear export signal. However, the signals could be found in the tertiary structure of FABP4. It has also been shown that there is a protein-protein interaction between FABP4 and hormone-sensitive lipase [28]. In this model, it has been postulated that FABP4 binds to and activates hormone-sensitive lipase in adipocytes, resulting in regulation of lipolysis. Adipocytes in FABP4-deficient mice exhibited reduced efficiency of lipolysis [29, 30]. Interestingly, during experimentally induced lipolysis, FABP4-deficient mice also revealed reduction in insulin secretion [29]. As another protein-protein interaction, ligand-bound FABP4 has been reported to bind to Janus kinase 2 (JAK2) and attenuate its signaling, indicating a new role for FABP4 as a fatty acid sensor affecting cellular metabolism [31]. It has also been reported that phosphatase and tensin homolog on chromosome 10 (PTEN), which negatively regulates the phosphoinositide 3-kinase pathway, interacts with FABP4, possibly regulating of lipid metabolism and adipocyte differentiation [32]. Interestingly, PTEN-null keratinocytes showed an elevated expression of FABP4, suggesting that PTEN plays a role in the transcriptional regulation of FABP4 expression [55].

In the whole body metabolic phenotype, FABP4-deficient mice exhibited an increase in body weight but reduced insulin resistance in the context of both dietary and genetic obesity [14, 15]. RNA interference-mediated *Fabp4* germline knockdown in mice on a high fat diet also increased body weight and fat mass but did not significantly affect glucose and lipid homeostasis [64], which is similar to phenotype of the diet-induced obesity in FABP4 heterozygous knockout mice [14] and indicates that residual FABP4 protein sustains some elements of its function in metabolic control.

In human and mouse monocyte cell lines, FABP4 expression is induced during differentiation from monocytes and by treatment with phorbol 12-myristate 13-acetate, lipopolysaccharide (LPS), PPARγ agonists, and oxidized low-density lipoprotein (ox-LDL) [11, 34–37]. FABP4 expression in macrophages was also elevated by advanced glycation end products (AGE) via engagement of the receptor for AGE (RAGE) [38]. Conversely, a cholesterol-lowering statin, atorvastatin, has been shown to suppress FABP4 expression in macrophages *in vitro* [39]. It has also been reported that metformin, an antidiabetic drug, inhibits forkhead box protein O1- (FOXO1-) mediated transcription of FABP4, leading to reduced lipid accumulation in macrophages [40].

In macrophages, FABP4 modulates cholesterol ester accumulation and foam cell formation via inhibition of the PPARγ-liver X receptor α (LXRα)-ATP-binding cassette A1 (ABCA1) pathway and induces inflammatory responses through activation of the IKK-NF-κB and JNK-AP-1 pathways [41, 42]. Deficiency of FABP4 protected against atherosclerosis in apolipoprotein E- (ApoE-) deficient mice with or without high-cholesterol-containing western diets [11, 16]. Bone marrow transplantation studies demonstrated that the protective effect of FABP4 deficiency on atherosclerosis is predominantly related to actions in macrophages rather than in adipocytes [11]. FABP4 in dendritic cells has been shown to regulate the IKK-NF-κB pathway

Table 1: Features of FABP4 and FABP5 in metaflammation and related diseases.

	Expression	Regulation and function	Connection to diseases	Reference
FABP4	Adipocyte	Induction by fatty acids, PPARγ agonists, dexamethazone, and insulin		[23–27]
		Lipolysis (interaction with HSL)		[28–30]
		Regulation of insulin secretion during lipolysis		[29]
		Fatty acid sensor (interaction with JAK2)		[31]
		Regulation of lipid metabolism and differentiation (interaction with PTEN)		[32]
		Protection from insulin resistance and diabetes in deficient mice	Insulin resistance, diabetes	[14, 15, 18, 19, 21]
		Protection from insulin resistance and diabetes by a FABP4 inhibitor	Insulin resistance, diabetes	[33]
	Macrophage	Induction by PMA, LPS, PPARγ agonists, ox-LDL, and AGE/RAGE		[11, 34–38]
		Reduction by atorvastatin and metformin		[39, 40]
		Activation of IKK-NF-κB pathway		[41]
		Activation of JNK-AP-1 pathway		[42]
		Inhibition of PPARγ-LXRα-ABCA1 pathway		[41]
		FOXO1-mediated transcription		[40]
		Association with ER stress		[22]
		Protection from insulin resistance and diabetes in double-deficient mice*	Insulin resistance, diabetes	[21]
		Protection from atherosclerosis in deficient mice	Atherosclerosis	[11, 16, 20]
		Protection from insulin resistance and atherosclerosis by a FABP4 inhibitor	Insulin resistance, atherosclerosis	[33]
	Dendritic cell	Activation of IKK-NF-κB pathway		[12]
		T-cell priming		[12]
	Endothelial cell	Expression in capillary and small vein but not in artery		[43]
		Regulation by VEGF-A/VEGFR2 and bFGF		[43]
		Induction in regenerated endothelial cells after balloon denudation of artery		[44]
		Induction by intermittent hypoxia		[45]
		FOXO1-mediated transcription inhibited by angiopoietin-1		[46]
		Expression in aortic endothelium of old ApoE-deficient mice		[47]
		Improvement of dysfunction in aortic endothelium by a FABP4 inhibitor	Endothelial dysfunction	[47]
		Association with oxidative stress and activation of NF-κB and P53 pathways	Cellular senescence	[48, 49]
	Bronchial epithelial cell	Induction by Th2 cytokines IL-4 and IL-13		[13]
		Suppression by Th1 cytokine interferon γ		[13]
		Noninduction by PPARγ agonists		[13]
		Protection from asthma in deficient mice	Asthma	[13]
	Lung	Detection in lung lavage cells obtained from patients	Bronchopulmonary dysplasia	[50]
		Detection in lung lavage cells obtained from patients	Sarcoidosis	[51]
	Ovary	Expression in granulosa cells inside atretic antral follicles		[52]
		Association with FABP4 gene polymorphisms	Polycystic ovary syndrome	[53]

Table 1: Continued.

	Expression	Regulation and function	Connection to diseases	Reference
	Spleen	Induction by dexamethazone		[54]
	T cell	Induction by dexamethazone		[54]
	Keratinocyte	Induction in PTEN-deficient keratinocytes		[55]
	Tumor	Detection in tumor	Lipoblastoma, liposarcoma	[56]
		Detection in tumor	Urothelial carcinoma	[57]
FABP5	Adipocyte	Lipolysis		[58]
		Protection from insulin resistance and diabetes in deficient mice	Insulin resistance, diabetes	[17–19, 21]
		Induction of insulin resistance in adipose-specific transgenic mice	Insulin resistance, diabetes	[17]
	Macrophage	Regulation by TLR agonists: LPS (TLR4) and zymosan (TLR2)		[59]
		Induction of inflammatory genes, COX2 and IL-6		[60]
		Protection from insulin resistance and diabetes in double-deficient mice*	Insulin resistance, diabetes	[21]
		Protection from atherosclerosis in deficient mice	Atherosclerosis	[20, 60]
	Liver	Induction by a high-cholesterol diet feeding in LDL-receptor-deficient mice		[61]
	Others	Expression in skin, dendritic cell, tongue, mammary gland, brain, intestine, kidney, lung, heart, skeletal muscle, testis, retina, lens, and spleen		[7]

ABCA1: ATP-binding cassette A1; AGE: advanced glycation end products; AP-1: activating protein-1; ApoE: apolipoprotein E; bFGF: basic fibroblast growth factor; COX2: cyclooxygenase-2; ER: endoplasmic reticulum; FOXO1: forkhead box protein O1; HSL: hormone-sensitive lipase; IKK: inhibitor of nuclear kappa B kinase; IL: interleukin; JAK2: Janus kinase 2; JNK: c-Jun N-terminal kinase; LDL: low-density lipoprotein; LPS: lipopolysaccharide; LXR: liver X receptor; NF-κB: nuclear factor-kappa B; ox-LDL: oxidized LDL; PMA: phorbol 12-myristate 13-acetate; PPAR: peroxisome proliferator-activated receptor; PTEN: phosphatase and tensin homolog on chromosome 10; RAGE: receptor for AGE; TLR: Toll-like receptor; VEGF-A: vascular endothelial growth factor-A; VEGFR2: VEGF-receptor-2.
*FABP4$^{-/-}$FABP5$^{-/-}$ mice.

and T-cell priming [12], which might contribute to the development of atherosclerosis since there is clear evidence for the involvement of both dendritic and T cells in the pathogenesis of atherosclerosis [65]. Involvement of FABP4 in atherosclerosis has also been indicated by clinical studies. In human endarterectomy samples of carotid stenosis, expression of FABP4 by macrophages was increased in unstable carotid plaques [66].

3.2. FABP5 (E-FABP/mal1). Transgenic mice with adipose tissue-specific overexpression of FABP5 exhibited enhanced basal and hormone-stimulated lipolysis and a decrease in insulin sensitivity in a high-fat diet model [17, 58]. Deletion of FABP5 resulted in a mild increase in systemic insulin sensitivity in genetic and dietary obesity mouse models [17]. Adipocytes in FABP5-deficient mice showed an increased capacity for insulin-dependent glucose transport. Except for increased FABP3 (H-FABP) in the liver [67], there was no compensatory increase in the expression of FABPs in tissues including adipose tissue in FABP5-deficient mice [17]. Interestingly, feeding a western-type high-cholesterol diet increased the expression of FABP5, but not that of FABP1 (L-FABP), in liver parenchymal cells of atherosclerotic LDL-receptor- (LDLR-) deficient mice together with an increase in plasma levels of atherogenic lipoproteins, VLDL and LDL [61]. These observations indicate a specific role of FABP5 in atherogenesis.

FABP5 expression in macrophages was increased by treatment with Toll-like receptor (TLR) agonists: LPS, a TLR4 agonist, and zymosan, a fungal product that activates TLR2 [59]. A recent study showed that macrophage FABP5 deficiency suppressed atherosclerosis in LDLR-deficient mice on a western-style diet through a reduction of the expression of inflammatory genes, cyclooxygenase-2 and interleukin 6, and macrophage recruitment in atherosclerotic lesions due to decreased CC chemokine receptor 2 expression [60].

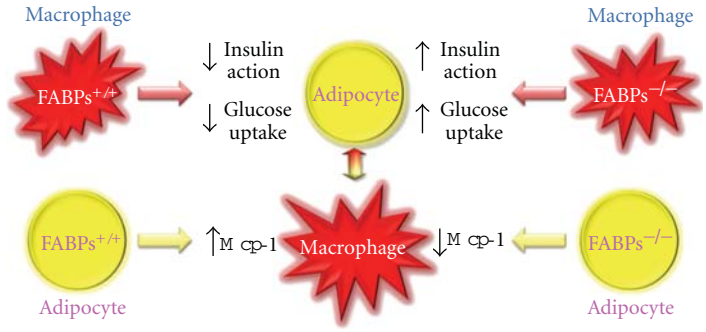


Figure 1: Interaction of adipocytes and macrophages. FABPs, FABP4, and FABP5, in adipocytes and macrophages, are critical for regulating inflammatory and metabolic responses in each type of cells and also interaction of the two types of cells.

3.3. Combined Deficiency of FABP4 and FABP5. Mice with combined deficiency of FABP4 and FABP5 (*Fabp4*$^{-/-}$ *Fabp5*$^{-/-}$) on a high-fat diet or in a genetic obesity model exhibit remarkably improved insulin resistance and protection against type 2 diabetes and fatty liver disease more than did FABP4- or FABP5-deficient mice [18, 19]. Furthermore, *Fabp4*$^{-/-}$*Fabp5*$^{-/-}$ mice intercrossed into an ApoE-deficient atherosclerosis model developed dramatically less atherosclerosis than that in FABP4-deficient or wild-type mice on the same background [20]. Interestingly, *Fabp4*$^{-/-}$*Fabp5*$^{-/-}$ *Apoe*$^{-/-}$ mice on a western-type hypercholesterolemic diet also had a significantly higher survival rate than that of *Apoe*$^{-/-}$ mice, presumably due to better plaque stability and good overall metabolic health [20].

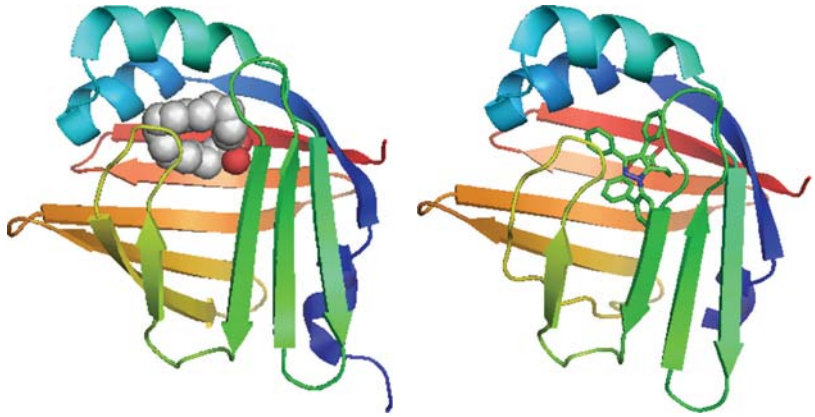
It has recently been suggested that macrophage infiltration and accumulation in adipose tissue is an important feature of metaflammation triggered by obesity [68, 69]. Although the impact of FABP4/FABP5 on atherosclerosis was shown to be mainly due to actions in macrophages [11, 60], cell-based coculture experiments with adipocytes and macrophages and bone marrow transplantation using wild-type and *Fabp4*$^{-/-}$*Fabp5*$^{-/-}$ mice showed that FABP actions in both adipocytes and macrophages have distinct roles in modulation of insulin sensitivity through inflammatory and metabolic responses as shown in Figure 1 [21]. In this setting, the predominant action was related to adipocyte FABPs with a more modest contribution from macrophages.

4. Therapeutic Target for Diabetes and Atherosclerosis

Since FABP4 and FABP5 act at the interface of metabolic and inflammatory pathways and play a significant role in the development of insulin resistance, type 2 diabetes, and atherosclerosis, it is expected that modification of the function of these FABPs may provide a new class of multi-indication therapeutic agents. In fact, several series of FABP4 inhibitors have recently been identified [70–75]. We previously demonstrated that chemical inhibition of FABP4 could be a therapeutic strategy against insulin resistance, diabetes mellitus, fatty liver disease, and atherosclerosis in experimental models using one of the specific FABP4 inhibitors, BMS309403 [33]. This compound is an orally active small molecule and interacts with the fatty acid-binding pocket within the interior of FABP4 to inhibit binding of endogenous fatty acids [7, 33, 72] (Figure 2). X-ray crystallographic studies identified the specific interactions of BMS309403 with key residues, such as Ser53, Arg106, Arg126, and Tyr128, within the fatty-acid-binding pocket as the basis of its high *in vitro* binding affinity and selectivity for FABP4 over other FABPs [72].

The FABP4 inhibitor, BMS309403, improved glucose metabolism and enhanced insulin sensitivity in both dietary (high fat-fed) and genetic (*ob/ob*) mouse models of obesity and diabetes [33]. Involvement of FABP4 inhibition in those beneficial effects was confirmed *in vivo* using wild-type and *Fabp4*$^{-/-}$*Fabp5*$^{-/-}$ mice. Although *Fabp4*$^{-/-}$ mice were not protected against fatty liver disease, inhibition of FABP4 suppressed fatty liver infiltration, similar to the liver phenotype of *Fabp4*$^{-/-}$*Fabp5*$^{-/-}$ mice. One possible explanation for the different effects between genetic deficiency of FABP4 and chemical inhibition of FABP4 is that there was no compensatory increase in FABPs in the adipose tissue of FABP4-inhibitor-treated mice. Furthermore, the FABP4 inhibitor markedly reduced the extent of atherosclerotic lesions in ApoE-deficient mice [33]. Cell-based studies showed that BMS309403 reduced macrophage foam cell formation with decreased cholesterol ester accumulation, increased cholesterol efflux, and decreased production of several inflammatory mediators in a target tissue-specific manner [33].

In high fat-diet-induced obesity models beginning at 4 weeks of age, treatment with the FABP4 inhibitor for 4 weeks improved insulin sensitivity in 24-week-old mice [33], which had severe macrophage infiltration in adipose tissue, but not in 20-week-old mice, which had much less macrophage accumulation in adipose tissue (Furuhashi M and Hotamisligil GS. unpublished data 2007). Recently, a similar pattern was also found in another study in which a different inhibitor was not effective in increasing insulin sensitivity [75]. It is difficult to completely inhibit whole FABP4 in adipocytes because the amounts of FABP4 in adipose tissue and adipocytes are very large [8], and these observations therefore raise the possibility that small molecules developed so far against FABP4 may be more effective in macrophages and hence their effects *in vivo* may be related to the extent of macrophage involvement with the disease process at



(a) (b)

Figure 2: FABP4 bound with a fatty acid or a small molecule inhibitor. (a) Human FABP4 binds to an endogenous fatty acid, palmitic acid, as a twisted U-shaped entity in the binding pocket (PDB code: 2hnx). (b) Human FABP4 crystallized in complex with BMS309403, a synthetic FABP4 inhibitor, is shown (PDB code: 2nnq). The molecule occupies the internal binding pocket of FABP4 and competitively inhibits binding of endogenous fatty acids. The figures were created using PyMOL.

the stage that these molecules are tested. Undoubtedly, future studies and alternative strategies to modulate FABP action, alone or in combination, in disease models should address these outstanding issues. Further studies are also needed to determine whether chemical or other modes of inhibition of FABP4 can be safely used in humans and to demonstrate their efficacy for metabolic diseases.

5. Ectopic Expression of FABP4

There is accumulating evidence to indicate that FABP4 is expressed in several cells other than adipocytes and macrophages under both special and physiological conditions (Table 1). For example, FABP4 expression was observed in endothelial cells of capillaries and small veins in several mouse and human tissues, including the heart and kidney [43]. FABP4 was significantly induced by treatment with vascular endothelial growth factor-A (VEGF-A) via VEGF-receptor-2 (VEGFR2) and by treatment with basic fibroblast growth factor (bFGF) in endothelial cells [43]. Conversely, knockdown of FABP4 in endothelial cells reduced proliferation both under baseline conditions and in response to VEGF-A and bFGF, suggesting that FABP4 is a target of the VEGF-A/VEGFR2 pathway and a positive regulator of cell proliferation in endothelial cells.

Interesting observations have been reported for roles of FABP4 in vascular injury. FABP4 was markedly upregulated in regenerated endothelial cells obtained after endothelial balloon denudation *in vivo* [44]. In human aortic endothelial cells, intermittent hypoxia increased FABP4 expression [45]. Anigiopoietin-1, which participates in blood vessel stabilization and remodeling together with angiopoietin-2, inhibited FOXO1-mediated expression of genes including FABP4 in endothelial cells [46]. FABP4 was expressed in the aortic endothelium of 12-week-old ApoE-deficient mice showing endothelial dysfunction, whereas FABP4 was not detected at the aortic endothelium in 8-week-old ApoE-deficient mice or in wild-type mice [47]. Chronic administration of BMS309403, a small molecule FABP4 inhibitor, significantly improved endothelial dysfunction in ApoE-deficient mice [47]. Notably, recent studies have shown possible involvement of FABP4/FABP5 in senescence of endothelial cells [48, 49]. These observations support the notion that pathological induction, but not physiological expression, of FABP4 in the endothelium significantly contributes to pathogenesis of atherosclerosis and other types of vascular injury.

Evidence is also accumulating as for involvement of FABP4 in respiratory diseases. Recently, FABP4 has been reported to be detected in lungs and bronchoalveolar samples from patients with bronchopulmonary dysplasia (BPD) [50]. Density of FABP4-positive endothelial cells was increased in peribronchial blood vessels, and FABP4 was also localized in a subset of macrophages in lung tissues. Several studies using lung lavage cells suggested that FABP4 gene expression is responsible for pathogenesis of sarcoidosis [51]. It is notable that expression of FABP4 in human bronchial epithelial cells is under regulation of cytokines. FABP4 expression in bronchial epithelial cells was enhanced by the Th2 cytokines IL-4 and IL-13, which are involved in development of asthma, and was suppressed by the Th1 cytokine interferon γ [13]. Interestingly, FABP4-deficient mice were protected from airway inflammation independently of bone marrow-derived elements, indicating possible protection against asthma through FABP action in stromal cells [13]. However, it should be noted that there are possible differences in response of FABP4 to stimuli depending on cell types. FABP4 expression in bronchial epithelial cells was significantly lower than that in adipocytes and macrophages, even after stimulation. In contrast to its effects in adipocytes and macrophages, PPARγ agonists could not induce FABP4 expression in bronchial epithelial cells. Such tissue-specific roles and response of FABP4 need to be taken into account for FABP4 modulating therapy.

In atretic antral follicles of the mouse ovary, FABP4 was detected in apoptotic granulosa cells [52], suggesting a possible relevance to polycystic ovary syndrome (PCOS), which often coexists with insulin resistance. Interestingly, association between FABP4 gene polymorphisms and the development of PCOS has been reported [53]. Additionally, dexamethasone treatment induced FABP4 in mouse spleen and in cultured T lymphocytes, and its distinct nuclear localization occurred with the dexamethasone-induced apoptosis process [54].

FABP4 expression was also detected in lipoblasts in lipoblastoma and liposarcoma but not in other benign adipose tissue or malignant connective tissue or in epithelial tumors [56]. Moreover, FABP4 expression has been linked to human urothelial carcinomas [57]. The significance of these associations remains to be elucidated but points to potential utility of FABP-based strategies to explore metabolic mechanisms related to tumorigenesis and related therapeutic possibilities.

6. Secretion and Circulating Concentrations of FABPs

In recent years, numerous studies have shown the presence of FABPs in circulation. Since these cytoplasmic proteins lack a secretory signal sequence, the presence of FABPs in serum is considered to be a biochemical marker of tissue injury in related cells that produce FABP proteins: FABP3 (H-FABP) for acute myocardial infarction and ongoing myocardial damage in heart failure, FABP7 (B-FABP) for brain injury, and FABP2 (I-FABP) for intestinal damage [76–78]. It has recently been reported that FABP4 is detected in serum and cultured adipocyte supernatants [79] and that the serum concentration of FABP4 is associated with obesity, type 2 diabetes, and cardiovascular diseases [79–82]. Similar findings have also been reported for FABP5 [83, 84]. Proteomics analysis using differentiated THP-1 macrophages revealed the presence of FABP4 and FABP5 in cell supernatants derived from macrophages [85]. However, the mechanisms and biological correlates of extracellular FABP4 and FABP5 remain unknown.

Serum levels of FABP4 were significantly increased in overweight and obese subjects compared to the level in lean controls and were positively correlated with waist circumference, blood pressure, and insulin resistance [79]. Similar to FABP4, circulating FABP5 levels were detected at the level of about one tenth or less of FABP4 concentrations and were associated with metabolic syndrome components [83, 84]. High serum levels of FABP4 at baseline independently predicted the development of metabolic syndrome during a 5-year follow-up period in a Chinese population [80]. A 10-year prospective study showed that high FABP4 concentration at baseline was a biomarker predicting development of type 2 diabetes, which was independent of obesity and insulin resistance [81]. Furthermore, it has also been reported that serum FABP4 levels are positively correlated with carotid intima-media thickness as an index of atherosclerosis [82]. These findings support the notion that FABP4 is a biomarker of ongoing atherosclerosis. Interestingly, serum levels of FABP4 could also represent noncardiovascular pathologic processes as well. A recent study has shown that FABP4 levels could be a novel and obesity-independent prognostic factor in patients with breast cancer [86].

Several drugs have been reported to modify FABP4 levels in blood. Atorvastatin, a HMG-CoA reductase inhibitor, and olmesartan, an angiotensin II receptor blocker, reduced circulating FABP4 levels [87, 88], whereas pioglitazone, an insulin-sensitizing thiazolidinedione (a PPARγ agonist), increased FABP4 concentrations [89], which could be explained through direct activation of PPARγ since the PPAR response element is present in the FABP4 gene promoter [90]. As general information for circulating FABPs, the concentrations of FABPs are influenced by renal clearance [91–93], and it might be necessary to evaluate the role of renal dysfunction in regulation of FABP level. Future studies should provide further insights into these phenomenon and how they contribute to disease progression in related FABP isoforms.

7. Lipokine

Meticulous lipidomic analyses using several samples including adipose tissue, liver, skeletal muscle, and blood from *Fabp4*$^{-/-}$*Fabp5*$^{-/-}$ and wild-type mice showed markedly increased *de novo* lipogenesis in adipose tissue resulting from activation/induction of fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1) [94]. Consequently, an unsaturated free fatty acid, palmitoleate (C16:1n7), was identified as an adipose tissue-derived lipid hormone, referred to as "lipokine," that strongly suppresses hepatosteatosis and stimulates glucose transport in skeletal muscle [94]. That study revealed a lipid-mediated endocrine network of tissues/organs, in which adipose tissue uses lipokines such as palmitoleate to communicate with distant organs, regulating systemic metabolic homeostasis. Absence of FABP4 in macrophages also resulted in an activation of *de novo* lipogenesis pathways particularly through LXRα-mediated activation of SCD-1 [22]. This enhanced lipogenesis induced production of bioactive lipids including palmitoleate and resistance to ER stress. These changes also translate into protection against atherosclerosis in mouse models [22]. Conversely, unsaturated fatty acids including palmitoleate repressed basal and LPS-induced FABP4 expression in macrophages via the modulation of histone deacetylation [95].

After results of animal studies on a lipokine were reported [94], palmitoleate in humans was examined in several studies in the context of metabolic disease, particularly in determining the risk for insulin resistance and type 2 diabetes. In a study that recruited 100 Caucasian subjects, circulating palmitoleate was positively correlated with insulin sensitivity assessed by euglycemic-hyperinsulinemic clamp studies, independent of age, gender, and adiposity [96]. Another study using 3630 subjects in the US showed that high concentrations of circulating *cis* isomer palmitoleate, which is primarily produced by the liver in humans, were associated with adiposity, carbohydrate consumption, and alcohol use [97]. However, theassociations between

circulating *cis* palmitoleate and metabolic risk factors were complex, perhaps related to divergent lifestyle determinants or tissue sources of endogenous palmitoleate synthesis from liver and adipose tissue: high fat- and carbohydrate-containing diet and fatty liver would confound or modify the ability to detect its metabolic effects [97]. Interestingly, it has recently been reported that circulating *trans* isomer of palmitoleate, an exogenous source of C16:1n7, is associated with markedly lower insulin resistance, higher HDL-cholesterol level, and lower incidence of diabetes, suggesting metabolic benefits of dairy product consumption [98]. Since this isoform is not related to endogenous production, the relation to reduced metabolic disease points to possibilities of the utilization of the *trans* isomer of palmitoleate as a potential strategy for intervention in human diseases.

8. Concluding Remarks

FABPs, especially FABP4 and FABP5, play significant roles in the regulation of glucose and lipid metabolism linked to inflammatory and metabolic processes through modulating critical lipid-sensitive pathways in target cells, adipocytes, and macrophages. There was no compromised phenotype of FABP4- or FABP5-deficient mice under normal physiologic conditions [14, 17]. However, the mice in the context of dietary or genetic obesity were protected from systemic pathologic stresses such as metaflammation, suggesting that the adipocyte/macrophage FABP genes may represent an example of the "thrifty" gene hypothesis [99]. FABPs have been evolutionarily preserved from invertebrates (lower eukaryotes) to vertebrates including humans [100], indicating that a close and conserved link between inflammatory and metabolic responses underlies the conservation of FABP function. The presence of these FABPs may have been beneficial for ensuring a strong macrophage immune response under pressure with pathogens or for maintaining adipose tissue energy stores as part of the "thrifty" phenotype to survive in famine. Under contemporary life-style accompanied by excessive caloric intake and decreased energy expenditure, presence or induction of adipocyte/macrophage FABPs may be rather disadvantageous for maintaining inflammatory or metabolic homeostasis. FABPs appear to be responsible for the development of obesity, diabetes, dyslipidemia, and atherosclerosis, and targeting the adipocyte/macrophage FABPs, particularly FABP4, offers highly attractive therapeutic opportunities for intervening metabolic derangements as an evolutionary bottleneck in humans. Much work is still needed to elucidate the precise biological functions of different forms of FABPs and to establish strategies to target these proteins for therapeutic purposes.

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Review Article

Toll-Like Receptors and Myocardial Inflammation

Yan Feng and Wei Chao

Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

Correspondence should be addressed to Wei Chao, wchao@partners.org

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Toll-like receptors (TLRs) are a member of the innate immune system. TLRs detect invading pathogens through the pathogen-associated molecular patterns (PAMPs) recognition and play an essential role in the host defense. TLRs can also sense a large number of endogenous molecules with the damage-associated molecular patterns (DAMPs) that are produced under various injurious conditions. Animal studies of the last decade have demonstrated that TLR signaling contributes to the pathogenesis of the critical cardiac conditions, where myocardial inflammation plays a prominent role, such as ischemic myocardial injury, myocarditis, and septic cardiomyopathy. This paper reviews the animal data on (1) TLRs, TLR ligands, and the signal transduction system and (2) the important role of TLR signaling in these critical cardiac conditions.

1. Introduction

Innate immune system represents the first line of defense against foreign pathogens. Toll-like receptors (TLRs) belong to the family of pattern recognition receptors (PRRs). PRRs recognize the conserved motifs in pathogens termed pathogen-associated molecular patterns (PAMPs) and trigger innate immune response [1, 2]. In addition to participating in the host defense against infectious pathogens, accumulating evidence suggests that TLRs also play an essential role in tissue inflammationand contribute to "noninfectious" tissue damage such as cardiac ischemia/reperfusion (I/R) injury, postischemic remodeling, and atherosclerosis [3–6]. Thus, understanding TLR signaling and their role in cardiovascular diseases may help to identify potential targets for intervention and have important clinical implications. This paper reviews TLR signaling and its critical roles in several inflammatory cardiac conditions: I/R injury, viral and autoimmune myocarditis, and septic cardiomyopathy.

2. Toll-Like Receptors

Toll means "amazing" and "fantastic" in German. In 1985, Anderson and colleagues coined it for a protein critical for early embryonic development of Drosophila [7, 8]. A decade later, Lemaitre et al. found that this protein was also essential to the host innate immunity against fungal infection in adult flies [9]. Subsequently, Medzhitov and colleagues identified a mammalian homologue of the Drosophila Toll protein in human and termed it "Toll-like receptor" [10]. Stimulation of TLR signaling leads to the activation of transcription factors such as NF-κB, one of the most important proinflammatory transcription factors. To date, 11 human and 13 mouse TLRs have been cloned. TLR1-TLR9 are conserved in both human and mouse, and all of them are functional to recognize diverse ligands [2]. However, mouse TLR10 has no function due to a retrovirus insertion, whereas human TLR10 may function as a TLR2 coactivator [2, 11]. Finally, TLR11, TLR12, and TLR13 are present in mouse but lost in human [2].

TLRs are type I single-spanning membrane glycoproteins with a leucine-rich repeat of extracellular domain, which mediates ligand recognition, and an intracellular TIR domain, which recruits adaptors and activates downstream signaling. According to the ligands and the subcellular location, TLRs can be divided into two subgroups (Figure 1). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are located

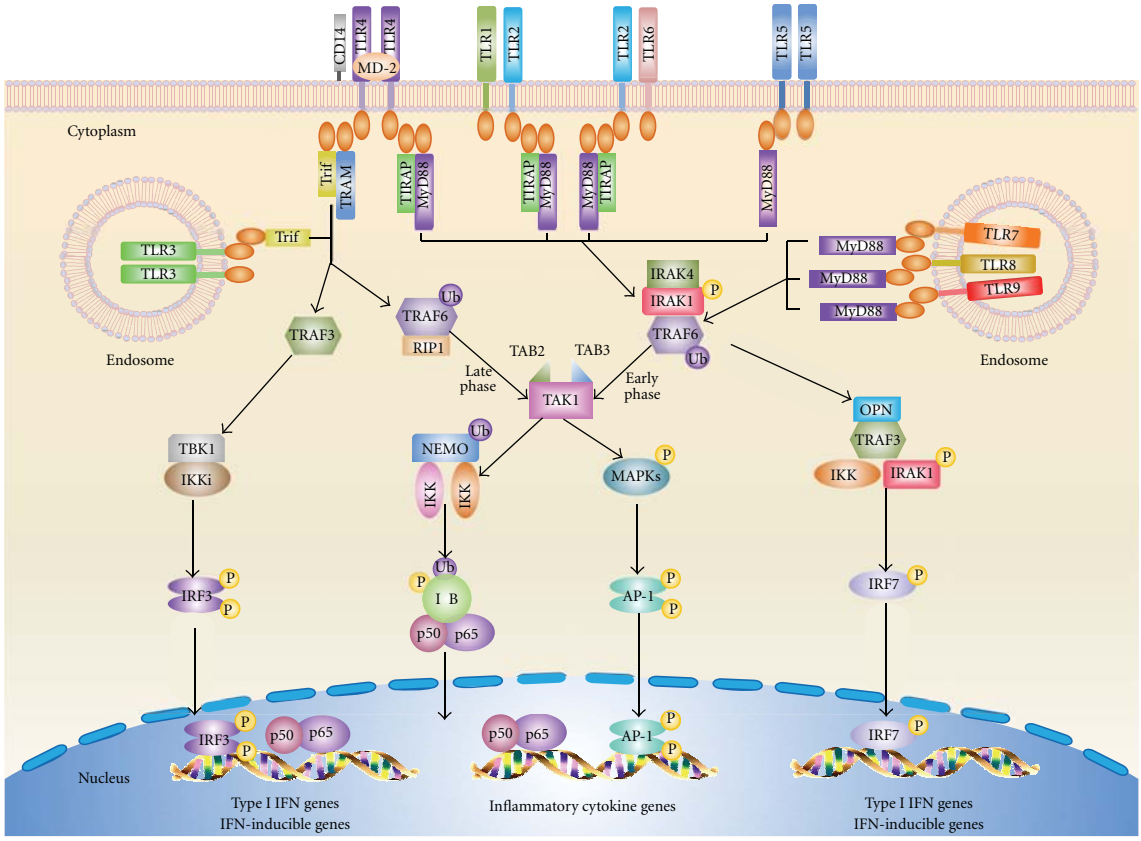

FIGURE 1: TLR signaling pathways. Upon respective ligands binding, TLRs form homo- or heterodimers and recruit one or more adaptor proteins, namely, MyD88, MAL/TIRAP, TRIF, or TRAM, to the cytoplasmic domains of the receptors through homophilic interactions between Toll/IL-1 receptor (TIR) domains present in each receptor and each adaptor. All TLRs with exception of TLR3 use the common MyD88-dependent pathway. TIRAP acts as a bridge to recruit MyD88 to TLR2 and TLR4 signaling, whereas TRIF is used in TLR3 signaling and, in association with TRAM, in TLR4 signaling. In MyD88-dependent pathway, MyD88 associates with IRAK4, IRAK1, and/or IRAK2. IRAK4 in turn phosphorylates IRAK1 and/or IRAK2 and promotes their association with TRAF6, which serves as a platform to recruit and activate the kinase TAK1. Activated TAK1 activates the IKK complex, composed of IKKα, IKKβ, and NEMO (IKKγ), which in turn catalyzes phosphorylation and subsequent degradation of IκB. IκB degradation lets NF-κB (*i.e.,* p50/p65) free to translocate from the cytoplasma to the nucleus, where it activates multiple gene expression. The transcription factor IRF7 is activated as the downstream signaling molecule of TLR 7, 8, and 9. It is directly phosphorylated by IRAK1 and then translocates into the nucleus to induce the expression of type I IFN and IFN-inducible genes. In the Trif-dependent pathway, Trif interacts with TRAF3 to activate TBK1 and IKKi, resulting in the dimerization and activation of IRF3, which then translocates into the nucleus activating the transcription of type I IFN and IFN-inducible genes.

primarily on the cell surface and recognize mainly microbial membrane components such as lipids, lipoproteins, and proteins. On the other hand, TLR3, TLR7, TLR8, and TLR9 reside on the membranes of intracellular compartments, such as endosomes, lysosomes, endolysosomes, and endoplasmic reticulum, and are responsible for the recognition of microbial nucleic acids [2, 12].

2.1. TLR Ligands: PAMPs versus DAMPs. As summarized in Table 1, TLRs consist of a family of receptors that specifically bind to a wide range of pathogens including bacteria, fungi, parasites, and viruses through "PAMPs" recognition [1, 2]. Accumulating evidence has indicated that TLR can also act as a stress sensor in response to noninfectious tissue injury and recognize a variety of endogenous stress molecules through "DAMPs" recognition [13].

PAMPs. TLR4 was first identified as the receptor for LPS, a component of outer membrane of Gram-negative bacteria [34, 35]. Its extracellular domain forms a complex with MD-2 and serves as the main LPS-binding site [55]. Additional proteins including LPS-binding protein and CD14 are also involved in modulating LPS binding [56, 57]. TLR2 is the most diverse TLR that recognizes a large number of PAMPs, such as lipopeptides from diverse bacteria [14], peptidoglycan [16, 17] and lipoteichoic acid [17] from Gram-positive bacteria, LPS from certain Gram-negative bacteria [21], lipoarabinomannan from mycobacteria [23, 24], zymosan

Table 1: TLR ligands: PAMPs versus DAMPs.

TLRs	PAMPs	Pathogens	Ref.	DAMPs	Ref.
TLR2	Lipopeptides	Bacteria	[14]	HSP60	[15]
	Peptidoglycan	Gram+ bacteria	[16, 17]	HSP70	[18, 19]
	Lipoteichoic acid	Gram+ bacteria	[17]	Gp96	[20]
	LPS	Leptospira interrogans	[21]	HMGB1	[22]
	Lipoarabinomannan	Mycobacteria	[23, 24]	bioglycan	[25]
	Zymosan	Fungi	[26]	versican	[27]
	Glycosylphosphatidylinositol anchors	Trypanosoma	[28]	Hyaluronan fragments	[29]
	Hemagglutinin protein	Measles virus	[30]		
TLR3	Double-stranded RNA	virus	[31]	mRNA	[32]
	Poly(I:C)		[31]		
	Small interfering RNAs		[33]		
TLR4	LPS	Gram− bacteria	[34, 35]	HSP60, HSP70, HSP72, HSP22, gp96	[15, 18–20, 36–39]
				HMGB1	[22]
				fibronectin, biglycan, tenascin-C, and versican	[25, 40, 41]
				Hyaluronan, lower molecular weight HA, and heparin sulfate	[42–44]
TLR5	flagellin	Bacteria flagella	[45]		
TLR7	Single-stranded RNA	virus	[46]	ssRNA	[48]
	Imidazoquinoline compounds		[47]		
	Guanine analogs		[49]		
TLR8	Single-stranded RNA	virus	[50]	ssRNA	[48]
TLR9	Unmethylated CpG DNA motif	Bacteria, virus	[51]	Chromatin-IgG complex	[52]
TLR11	Profilin-like molecule	Toxoplasma gondii	[53]		
		Uropathogenic bacteria	[54]		

from fungi [26], glycosylphosphatidylinositol anchors from Trypanosoma cruzi [28], and hemagglutinin protein from measles virus [30]. It usually forms heterodimers with TLR1 or TLR6. In general, TLR1/2 recognizes triacylated lipopeptides [58], whereas TLR2/6 heterodimer recognizes diacylated lipopeptides [59]. TLR5 recognizes flagellin from bacterial flagella [45], and TLR11 recognizes profilin-like molecule from the protozoan parasite Toxoplasma gondii [53] and

response to uropathogenic bacteria [54]. TLR3 senses dsRNA [31], synthetic analog of dsRNA, such as poly(I:C) [31], and certain small interfering RNAs [33]. It initiates antiviral immune responses through the expression of type I IFN and other inflammatory cytokines. TLR7 [46] and TLR8 [50] sense ssRNA from RNA viruses, imidazoquinoline compounds such as imiquimod and resiquimod (R-848) [47] and guanine analogs [49]. TLR9 senses unmethylated dinucleotides CpG DNA motifs, which are commonly present in bacteria and viruses but lacking in mammalian cells [51].

DAMPs. These endogenous ligands are ECM fragments or intracellular molecules produced either through release from preformed precursor or by *de novo* synthesis in response to tissue injury. DAMP-activated TLR signaling reportedly plays an important role in the pathogenesis of many inflammatory and autoimmune diseases. This topic is well reviewed by Piccinini and Midwood [13].

HSP60 was the first endogenous molecule linked to TLRs. Ohashi and colleagues found that similar to LPS, HSP60-induced TNFα expression and nitric oxide production were blocked in bone marrow-derived macrophages isolated from TLR4-deficient mice (C3H/HeJ strain) [36]. Since then, an increasing list of endogenous molecules has been identified to function as TLR ligands [2, 11, 13], including intracellular molecules released to extracellular environment after tissue injury, such as HSPs including HSP60 [15], HSP70 [18, 19, 37], HSP72 [38], HSP22 [39] and gp96 [20], and HMGB1 [22]. Others are ECM molecule such as fibronectin [40], biglycan [25], tenascin-C [41], versican [27], and fragments of ECM including oligosaccharides of hyaluronan [42], lower molecular weight hyaluronan [29, 43], and heparan sulfate [44]. In addition, chromatin-DNA and ribonucleoprotein complexes released from injured cells can activate intracellular TLRs. For example, mRNA exposure induces NF-κB activation and IL-8 production in stable TLR3-expressed HEK 293 cells. Meanwhile, TLR3 specific-antibody suppresses the activation of dendritic cells after stimulation with *in vitro* transcribed RNA or endogenous RNA released from necrotic cells [32]. In systemic lupus erythematosus, plasmacytoid dendritic cells could be activated to secrete type I IFN by RNA sequences through TLR7 and TLR8 [48]. Moreover, the ability to activate rheumatoid factor B cells in response to IgG2a-chromatin immune complexes was abolished in MyD88$^{-/-}$ mice, and the autoimmune complexes-induced activation was blocked by various inhibitors of TLR9 signaling [52].

2.2. TLR Signaling Pathways. As illustrated in Figure 1, upon activation, TLRs form dimers and initiate the downstream intracellular signaling. Heterodimerization occurs between TLR2 and TLR1 or TLR6 and between TLR4 and MD-2, whereas the other TLRs form homodimers. Ligand-induced homo-hetero dimerization of TLRs triggers the cytoplasmic signaling domains of the receptor to dimerize. The resulting TIR-TIR complexes trigger specific biological responses by initiating downstream signaling through a set of specific adaptors. So far, 5 adaptors have been identified [60]. They are MyD88, TIRAP, Trif, TRAM, and SARM [61]. TLRs interact with their respective adaptors *via* their TIR domain and the homologous domain present in these adaptors. Depending on the adaptors recruited, TLRs signaling can be divided into two distinct pathways: MyD88-dependent and Trif-dependent pathways. Mal acts as a bridge adaptor to help MyD88 recruiting to TLR2 and TLR4, whereas TRAM functions as a sorting protein that recruits Trif to TLR4 [2, 61].

MyD88-Dependent Pathway. MyD88-dependent pathway is activated by all TLRs with exception of TLR3. MyD88 signaling leads to inflammatory cytokine production by activating the transcription factor NF-κB and MAPKs. MyD88 recruits IL-1 receptor-associated kinases (IRAKs), such as IRAK1, IRAK2, IRAK4, and IRAK-M. IRAK4 is activated initially and followed by the activation of IRAK1 and IRAK2, leading to an interaction with TRAF6 [2]. The IRAK1-TRAF6 complex then activates TAK1 through a process involving cytosol translocation of TAK1 and two regulatory components TAK-binding protein 2 (TAB2) and TAB3 and the ubiquitination of TRAF6. Activated TAK1 then phosphorylates IKKβ, leading to phosphorylation and degradation of I-κB, which releases NF-κB and results in the nuclear translocation and DNA binding of NF-κB [2].

Trif-Dependent Pathway. Trif-dependent pathway is utilized by TLR3 and TLR4. It induces type I IFN and inflammatory cytokines through the activation of the transcription factor interferon regulatory factor 3 (IRF3) and NF-κB. Trif associates with TRAF3 and TRAF6. TRAF3 links a signaling complex involving the noncanonical IKKs, TRAF family member-associated NF-κB activator (TANK) binding kinase-1 (TBK1) and IKKi, which catalyze phosphorylation of IRF3 and induce its nuclear translocation and type I IFN expression. Moreover, Trif also recruits TRAF6 and receptor-interacting protein 1 (RIP1), with the help of TAK1, leading to the activation of NF-κB and MAPKs through ubiquitination-dependent mechanism similar to MyD88-dependent pathway [2, 12].

Of note, TLR4 reportedly activates both MyD88- and Trif-dependent pathways. After LPS binding, TLR4 initially triggers MyD88-dependent pathway on the plasma membrane and subsequently undergoes dynamin- and clathrin-dependent endocytosis and translocates to the endosome [2, 62]. This translocation is not only involved in degradation of TLR4, but also required for initiating Trif-dependent pathway [2, 62], which leads to IRF3 activation as well as late-phase activation of NF-κB [2, 62, 63].

3. TLR and Ischemic Myocardial Injury

TLRs are highly conserved and expressed ubiquitously throughout species including mammals, chicken, flies, and plants. In mammals, they are expressed differentially in immune cells such as monocytes/macrophage [64], neutrophil [65, 66], natural killer cells [67], dentritic cells [68], mast cells [69], specific T and B lymphocytes [70, 71], and non-immune cells, such as epithelial cells [72], skin keratinocytes [73], fibroblasts [74], and cardiomyocytes and endothelial cells in the heart [75–77]. Gene expression of TLR2, TLR3,

TLR4, TLR5, TLR7, and TLR9 has been reported in mouse heart tissue and in cardiomyocyte cell line [75–77]. Signaling *via* TLR2, TLR4, and TLR5, but not TLR3, TLR7, or TLR9, can initiate proinflammatory cytokines expression and inhibit cardiomyocyte contractility [75, 78]. Moreover, the mRNA expression of all 10 TLRs has been identified in the human heart tissue [79]. The one with highest expression is TLR4, whereas the lowest ones are TLR8, TLR9, and TLR10.

While tissue hypoxia is the initial cause of myocardial injury during transient ischemia, reperfusion-induced myocardial inflammation is an important contributor to ischemia-induced myocardial injury [80]. In fact, innate immune response is by far the most common cause of myocardial inflammation after I/R, characterized as proinflammatory cytokine release, endothelial cell activation, complement deposition, inflammatory cell infiltration, and increased vascular permeability [81–83]. Many of these inflammatory responses are regulated by NF-κB signaling pathway [84, 85]. Since TLRs are important upstream activators of NF-κB signaling, the role of TLRs in cardiac ischemic injury has been intensely studied in the past 10 years [3]. Among those TLRs expressed in the heart, TLR2 and TLR4 have been most investigated (Table 2).

3.1. TLR2. Several studies have indicated that TLR2 signaling is involved in myocardial I/R injury [86–89]. In an *ex vivo* model of I/R, TLR2$^{-/-}$ mice exhibited improved LV function compared to WT mice following I/R [86]. TLR2 is also involved in coronary artery endothelial dysfunction with impaired vessel relaxation induced by transient ischemia [87]. Similar to TLR4-deficient animals, TLR2$^{-/-}$ mice had reduced inflammatory responses and smaller MI sizes after I/R compared to WT control. Moreover, using chimeric TLR2 deletion models, Arslan and coworkers demonstrated that leukocyte TLR2 played a prominent role in mediating myocardial injury during I/R. They found that WT mice with circulatory cells derived from TLR2$^{-/-}$ mice were protected from I/R injury [88]. Administration of an anti-TLR2 antibody prior to reperfusion reduced MI sizes, preserved cardiac function, and decreased scar formation. Importantly, these cardiac benefits in TLR2$^{-/-}$ mice were associated with persistent attenuation of myocardial inflammation, such as reduced leukocytes infiltration and attenuated proinflammatory cytokines production. Interestingly, chemokines and adhesion molecules, which are essential for recruiting leukocytes to ischemic myocardium, were not changed.

3.2. TLR4. Several studies have demonstrated that TLR4 plays an important role in mediating immune cells infiltration, cytokine production, and complement activation during I/R. Oyama and colleagues [90] first demonstrated that after transient ischemia (1 h of coronary artery occlusion and 24 h of reperfusion), TLR4-deficient mice, C57/B10 ScCr and C3H/HeJ, had significantly smaller MI sizes with more than 50% reduction compared to their respective control mice, C57/BL10 ScSn and C3H/OuJ. C57/B10 ScCr mice have natural TLR4 gene deficiency, whereas C3H/HeJ mice have a spontaneous missense point mutation in the TIR domain. Furthermore, the decreased myocardial infarction in TLR4-deficient mice was associated with attenuated myocardial inflammation as evidenced by fewer neutrophil infiltration, less lipid peroxides production, and less complement 3 deposition in the heart [90].

In a similar, but shorter, *in vivo* protocol (1 h of ischemia followed by 2 h of reperfusion), Chong and colleagues [91] independently demonstrated a cardiac protection in C3H/HeJ mice with 40% reduction of MI compared to WT mice. I/R induced significant activation of ERK, p38 MAPK, and JNK, and translocation of NF-κB and AP-1 in WT mice. However, in C3H/HeJ mice, there was a significant reduction in JNK and NF-κB/AP-1 activity and mRNA levels of myocardial IL-1β, IL-6, and MCP-1 [91]. Moreover, blunting TLR4 signaling by eritoran, a specific TLR4 antagonist, also resulted in decreased MI sizes and attenuated myocardial inflammatory responses, such as reduced JNK phosphorylation, attenuated NF-κB nuclear translocation, and decreased gene transcripts of TNFα, IL-1β, IL-6, MCP-1, MIP-1α, and MIP-2 [92].

TLR4 may also mediate systemic cytokine production following myocardial I/R injury. Kim and colleagues measured the protein level of proinflammatory cytokines in the myocardium and serum after I/R [93]. They noticed a robust increase in the serum levels of TNFα, IL-1β, and IL-6 in response to 1 h of ischemia and 2 h of reperfusion *in vivo*. TLR4 deletion led to significant reduction in systemic inflammation, but only selective reduction in myocardial IL-6, and reduced MI sizes [93]. The authors thus speculated that systemic rather than local inflammatory response involving TLR4 signaling contributes to I/R injury.

In an isolated heart model of global I/R, which is devoid of circulating cells or other blood components, Cha and colleagues found that TLR4-deficient hearts had reduced levels of TNFα and IL-1β and improved cardiac contractile function compared to WT hearts [94]. Administration of TNFα and IL-1β to TLR4 defective heart, however, abrogated the beneficial effect of functional recovery in TLR4-deficient hearts after global ischemia [94], whereas functional recovery after ischemia was also improved in TNFα- and IL-1β-deficient hearts, as well as in wild-type hearts treated with TNF-binding protein or IL-1 receptor antagonist. These studies suggest that myocardial TLR4 signaling may contribute to cardiac dysfunction *via* TNFα- and IL-1β-dependent mechanisms after global I/R [94]. Interestingly, in a similar *ex vivo* model of I/R injury, Meng and colleagues found that 70-kDa heat shock cognate protein was released from ischemic myocardium and mediates, *via* a TLR4-dependent mechanism, myocardial NF-κB activation and cytokine/chemokine production in response to I/R [95, 96].

TLR4 signaling may also mediate inflammatory response and contribute to myocardial injury during heart transplantation. In a mouse model of heart transplantation, Kaczorowski and colleagues [97, 101] demonstrated that the serum myocardial injury marker, troponin I, was markedly increased in the recipient mice. This was associated with elevated serum inflammatory cytokines, such as TNFα, IL-1β, IL-6, and MCP-1. Similarly, myocardial inflammation was also dramatically induced in the graft. However, all

Table 2: Role of TLRs in myocardial inflammation and injury after acute ischemia/reperfusion.

TLRs	Mice strains	I/R models	I/R protocols	MI	Cardiac function	NF-κB activity	Inflammation				EC dysfunction/ROS ↑	Ref.
							Cytokine production			Myocardial neutrophil infiltration		
							Myocardium		Serum			
							mRNA	Protein				
TLR2	TLR2$^{-/-}$, TIRAP$^{-/-}$	*ex vivo*	30′ I/60′ R		↑			TNFα, IL-1β				[86]
	TLR2$^{-/-}$	*in vivo*	30′ I/1 h R	↓			IL-1β			↓	↓	[87]
	TLR2$^{-/-}$ chimeric, WT with Anti-TLR2	*in vivo*	30′ I/24 h R	↓	↑			TNFα, IL-1β, IL-10, M-CSF		↓		[88]
	TLR2$^{-/-}$, WT with Anti-TLR2	*in vivo*	20′ I/24 h R	↓						↓		[89]
TLR4	C57BL/10 ScCr C3H/HeJ	*in vivo*	60′ I/24 h R	↓						↓		[90]
	C3H/HeJ	*in vivo*	60′ I/2 h R	↓		↓	TNFα, IL-1β, MCP-1, IL-6					[91]
	WT with Eritoran	*in vivo*	30′ I/2 h R	↓			TNFα, IL-1β, MCP-1, IL-6, MIP-1, MIP-2					[92]
	C3H/HeJ	*in vivo*	60′ I/2 h, 24 h R	↓	NS		TNFα, IL-1β, IL-6		TNFα, IL-1β, IL-6			[93]
	C57BL/10 ScCr C3H/HeJ	*ex vivo*	20′ I/60′ R		↑	↓		TNFα, IL-1β				[94]
	WT with Anti-HSC70	*ex vivo*	20′ I/60′ R		↑		TNFα, IL-1β, IL-6	TNFα, IL-1β, IL-6				[95]
	C3H/HeJ	*ex vivo*	HSC70		↑	↓	TNFα, IL-1β, IL-6	TNFα, IL-1β, IL-6				[95]
	C3H/HeJ	*ex vivo*	20′ I/60′ R				KC, MCP-1	KC, MCP-1		perfused NE↓		[96]

Table 2: Continued.

TLRs	Mice strains	I/R models	I/R protocols	MI	Cardiac function	NF-κB activity	Inflammation Cytokine production Myocardium mRNA	Myocardium Protein	Serum	Myocardial neutrophil infiltration	EC dysfunction/ROS ↑	Ref.
	C3H/HeJ	*in vivo*	transplantation 2 h I/3 h, 24 h R			↓	TNFα, IL-1β, IL-6, ICAM-1		TNFα, IL-1β, IL-6, ICAM-1	↓		[97]
MyD88	WT with Ad-dnMyD88	*in vivo*	45′ I/4 h R	↓		↓						[98]
	$MyD88^{-/-}$	*in vivo*	30′ I/24 h R	↓	↑		MCP-1, KC, ICAM-1			↓		[99]
	$MyD88^{-/-}$	*ex vivo*	20′ I/40′ R	NS	NS							[99]
	$MyD88^{-/-}$ chimeric	*in vivo*	30′ I/24 h R	↓				NS		↓		[100]
Others	$CD14^{-/-}$, $MyD88^{-/-}$, $Trif^{-/-}$	*in vivo*	transplantation 2 h I/3 h, 24 h R			↓	TNFα, IL-1β, IL-6, ICAM-1		TNFα, IL-1β, IL-6, ICAM-1	↓		[101]

NS: no significant difference.

of these inflammatory responses were attenuated in TLR4-deficient mice subjected to the same transplantation protocol, suggesting that TLR4 signaling mediates myocardial injury and systemic and local inflammation during the transplantation.

3.3. MyD88. Given its critical role in TLR signaling, it is not surprising that MyD88 also plays a role in mediating myocardial innate immune response and contributes to injury after I/R. Employing genetically modified mouse models or local transgene expression of dominant negative MyD88 (dn-MyD88), investigators demonstrate that MyD88 signaling participates in I/R-induced myocardial inflammation and myocardial infarction [98, 99]. In a rat model of I/R injury, Hua and colleagues reported that adenoviral expression of dn-MyD88 three days prior to the onset of myocardial ischemia led to reduced infarct sizes and attenuated NF-κB activity, consistent with the notion that MyD88 signaling may contribute to ischemic myocardial injury by attenuating inflammatory response that is dependent on NF-κB signaling. One issue with adenovirus-mediated gene expression in the myocardium, however, is the well-documented innate immune response that may cause local inflammation rather than attenuate it [102]. The challenge would be to separate I/R-induced inflammation from adenovirus-mediated innate immune response. In a mouse model of I/R injury, Feng and colleagues found that compared to WT mice, mice deficient in MyD88 had markedly reduced myocardial infarction and significantly improved LV function between day 1 and day 7 after transient ischemia as measured by transthoracic echocardiography [99]. MyD88$^{-/-}$ mice also exhibited significantly reduced myocardial cytokines and chemokines [99, 100]. Flow cytometry analysis of cardiac cells isolated from the digested hearts demonstrated a robust increase in Gr-1+ neutrophils in the myocardium following I/R and a very small number of neutrophils in the myocardium of sham-operated mice. In contrast, there was a marked reduction in myocardial Gr-1+ neutrophils in MyD88$^{-/-}$ mice (Figure 2). Using an *in vivo* migration assay, the investigators found that MyD88$^{-/-}$ mice had markedly attenuated neutrophil migratory function, which was associated with decreased neutrophil CXCR2 expression and lower tissue KC, a neutrophil chemoattractant [100]. Interestingly, deletion of Trif, another innate immune adaptor, had no impact on myocardial neutrophil recruitment following I/R (Figure 2) or on neutrophil CXCR2 modulation [100]. In an effort to determine the specific contribution of myocardial MyD88 to cardiac injury following ischemia, Feng and colleagues tested whether or not MyD88 deficiency would have any effect on myocardial injury in isolated mouse hearts. Surprisingly, MyD88-deficiency had no significant impact on MI sizes and cardiac function in isolated hearts subjected to global I/R [99]. This finding is consistent with the notion that the cardiac benefits observed in MyD88$^{-/-}$ mice *in vivo* may require circulating blood components during I/R. Further studies in chimeric MyD88 deletion models demonstrated that compared to WT mice or WT mice transplanted with MyD88$^{+/+}$ bone marrow (WT⟶WT), WT mice transplanted with MyD88$^{-/-}$ donor bone marrow (KO⟶WT) had significantly decreased MI sizes (Figure 3). Collectively, these findings suggest that MyD88 signaling is essential for maintaining neutrophil migratory function and chemokine receptor expression. MyD88 signaling in bone marrow-derived neutrophils may play a specific and critical role in the development of myocardial I/R-induced injury (Figure 4) [103].

4. TLR and Myocarditis

Myocarditis is defined clinically as inflammation of the heart muscle and has been identified as a major cause of sudden, unexpected death in adults less than 40 years of age and young athletes, accounting for approximately 20% of such cases. It is estimated that the incidence of myocarditis in the general population ranges from 1.06% to 5.0% [104–106]. The causes of acute myocarditis include infection with various pathogens (viral, bacterial, and fungi), autoimmune disorders, systemic diseases, drugs, and toxins.

Viral Myocarditis. Viruses are the predominant cause of myocarditis in North America and Europe, whereas Trypanosoma cruzi and Chagas' disease are the major contributors to the high incidence of myocarditis in South America. While the exact role of various TLRs in the pathogenesis of viral myocarditis and cardiomyopathy is yet to be defined, both protective and detrimental effects have been reported (Table 3).

4.1. TLR3. TLR3 recognizes dsRNA and is involved in viral recognition. Hardarson and colleagues found that compared to WT mice, TLR3-deficient mice were susceptible to encephalomyocarditis virus (EMCV) infection with higher mortality, increased myocardial viral load, and more severe myocardial injury [107]. Importantly, myocardial inflammatory cell infiltration and cytokine mRNA expression, such as TNFα, IL-1β, and IFN-β, were significantly attenuated and delayed in TLR3$^{-/-}$ mice. These data suggest that EMCV infection induces a TLR3-dependent innate immune response in the heart, which represents a critical host protective mechanism against the virus-induced myocardial injury and mortality.

A similar protective role of TLR3 was reported in CV-induced myocarditis [108]. In that study, Negishi and colleagues demonstrated that compared to WT mice, TLR3$^{-/-}$ mice had higher mortality, higher systemic and myocardial viral replication, and depressed systemic as well as myocardial cytokine gene induction (IL-12p40 and IL-1β) after CV infection. Local myocardial production of IFN-γ, not IFN-β, was significantly reduced in TLR3$^{-/-}$ hearts (Figure 5). These studies demonstrate that type II IFN rather than type I IFN plays a critical role in the antiviral responses of TLR3 signaling [108].

4.2. TLR4. TLR4 mRNA was reportedly increased in endomyocardial biopsy samples from patients with clinically suspected myocarditis and from those with idiopathic dilated cardiomyopathy. Immunohistochemical analysis revealed that TLR4 was mainly expressed in infiltrated leukocytes

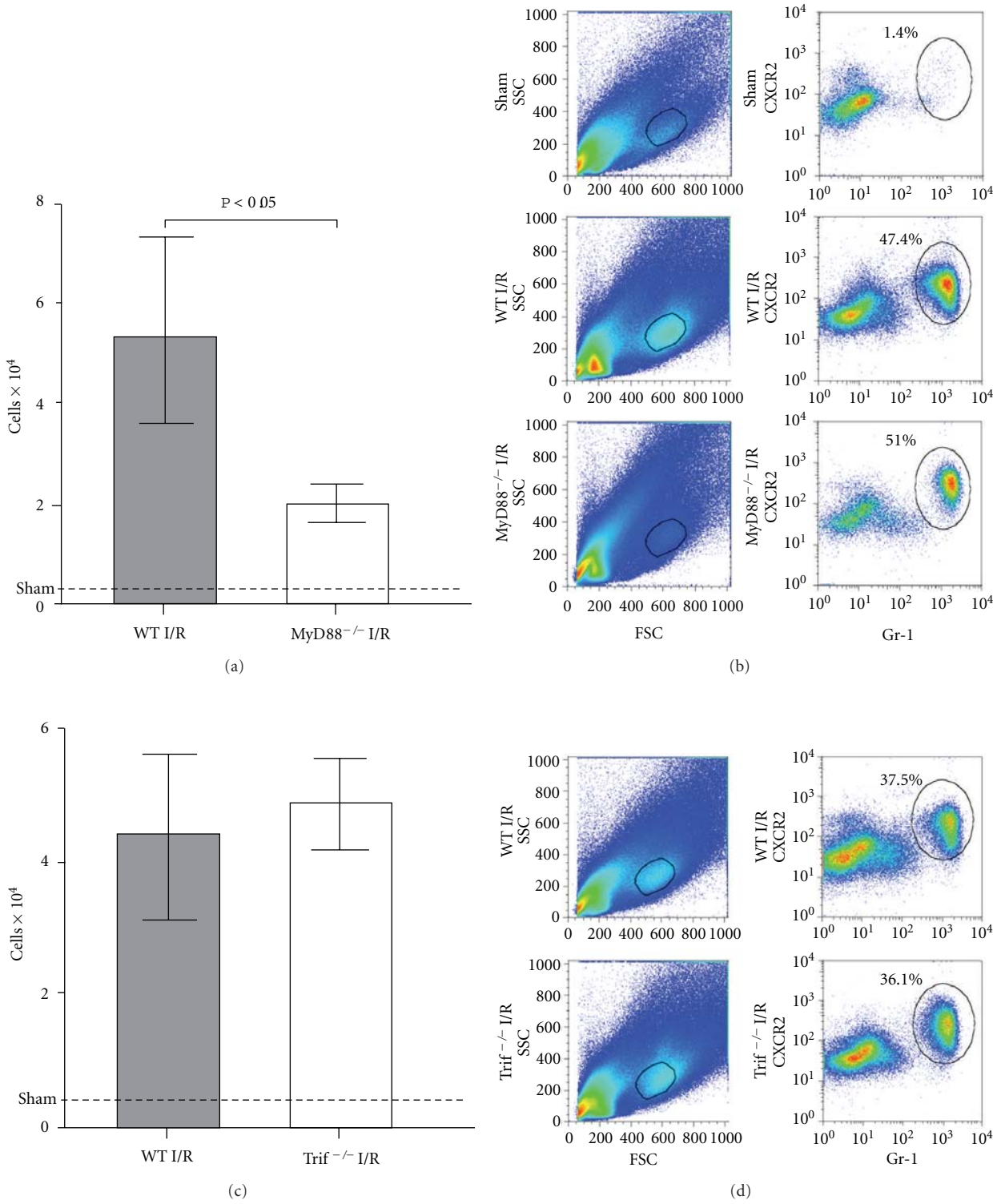

Figure 2: Myocardial neutrophil recruitment after I/R in MyD88$^{-/-}$ and Trif$^{-/-}$ mice. Twenty-four hours after 60 min of left anterior descending coronary artery (LAD) ligation, the hearts were isolated, perfused, and digested. After removal of the large cardiomyocytes through filtration, 50% of total cells were loaded onto flow cytometry and gated on Gr-1 and CXCR2. (a) Total Gr-1+ cells as measured by flow cytometry from the hearts subjected to I/R in MyD88$^{-/-}$ mice. Each error bar represents mean ± SD of 4 mice. A small number of neutrophils were recovered from the sham-operated hearts as indicated by the line. (b) A representative example of flow cytometry plots of myocardial infiltrating cells from sham, WT-I/R, and MyD88$^{-/-}$-I/R mice. (c) Total Gr-1+ cells as measured by flow cytometry from the hearts subjected to I/R in Trif$^{-/-}$ mice. Each error bar represents mean ± SD of 3 mice. A small number of neutrophils were recovered from the sham-operated hearts as indicated by the line. (d) A representative example of flow cytometry plots of myocardial infiltrating cells from WT-I/R and Trif$^{-/-}$-I/R mice. FSC, forward scatter; SSC, side scatter. (Feng et al., [100], used with permission).

Table 3: Role of TLRs in myocardial inflammation and injury during viral myocarditis.

TLRs	Mice	Virus	Viral replication	Survival	Myocardial injury marker cTnI	Cardiac function	Myocarditis (pathological change)	Inflammation Cytokine production Myocardial mRNA	Protein	Serum	IFN-γ	IFN-β	NF-κB activation	Ref.
TLR3	TLR3$^{-/-}$	EMCV	↑	↓	↑		↓	TNFα, IL-1β, IL-6, RANTES, IP-10↓, IFN-β ↑				↑		[107]
	TLR3$^{-/-}$	CVB3	↑	↓			↑	IL-12p40, IL-1β, IFN-γ ↓, IFN-β NS			↓	NS		[108]
	TLR3$^{-/-}$	CMV	NS		NS		NS							[109]
	TLR3$^{-/-}$	CVB4	↑	↓	↑		↑			TNFα and IFN-α ↓, IL-6 and IFN-γ NS	NS			[110]
TLR4	C3H/HeJ (BALB/c)	CVB3	d2↑d12↓				↓		IL-1β, IL-18↓, TNFα, IFN-γ, IL-12p40 NS		NS		↓	[111]
TLR7	TLR7$^{-/-}$	CMV	NS		NS		NS							[109]
TLR9	TLR9$^{-/-}$	CVB3	NS			↑	↓	TNFα, TGF-β, ICAM-1↓, IFN-β ↑				↑	↓	[112]
	TLR9$^{-/-}$	CMV	↑		NS		↑							[109]
MyD88	MyD88$^{-/-}$	CVB3	↓	↑			↓	IL-1β, TNFα, IFN-γ, IL-10 and IL-18↓, IFN-α and IFN-β ↑		IL-1β, TNFα, IFN-γ, IL-2, IL-6 and IL-10↓, IL-4 and IL-10 NS	↓	↑		[113]
	MyD88$^{-/-}$	CMV	↑		NS		↑							[109]
	MyD88$^{-/-}$	CVB4	NS	NS	NS		NS			TNFα NS, IFN-α, IL-6 and IFN-γ ↓	↓			[110]
Trif	Trif$^{-/-}$	CVB3	↑					IL-12p40, IFN-γ ↓, IFN-β NS			↓	NS		[108]
	Trif$^{-/-}$	CVB3	↑	↓		↓	↑	TNFα, IL-1β, IL-10, IL-18↑	TNFα, IL-1β, IL-18↑			d3↑, d7↓		[112]

NS: no significant difference.

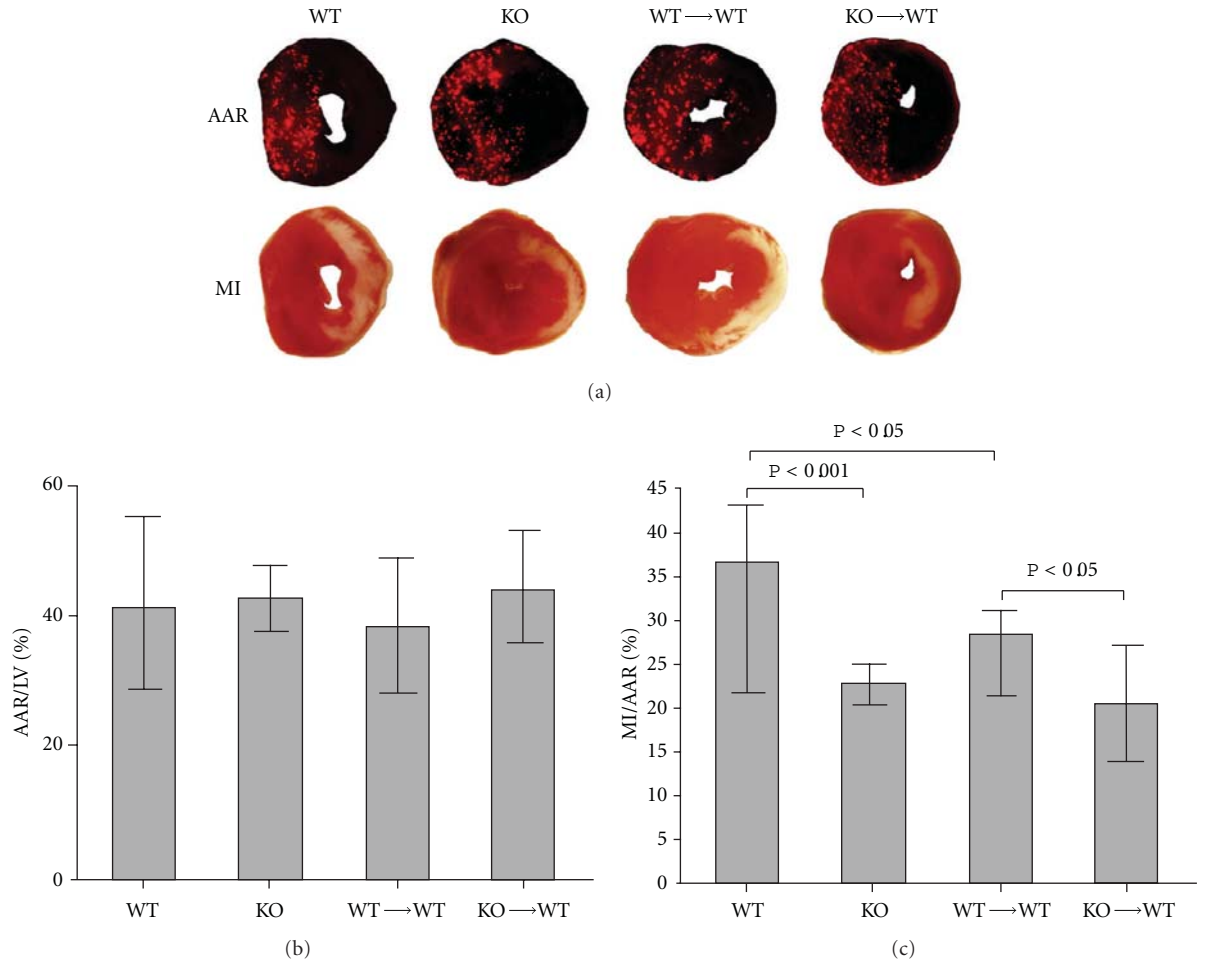

Figure 3: Decreased MI sizes in MyD88-knockout (KO) and KO → WT chimeric mice compared with wild-type (WT) and WT → WT chimeric mice. Mice were subjected to 30 min of ischemia and 24 h of reperfusion. At the end of reperfusion, animals were euthanized, and area-at-risk (AAR) and MI were analyzed. (a) Representative of triphenyltetra zolium chloride (TTC) staining (*bottom*) and fluorescent microsphere distribution (*top*) of myocardial sections from the 4 groups of mice. The nonischemic area is indicated by red fluorescent staining, area at risk (AAR) by area devoid of red fluorescent light, and infarct area by white. (b) Cumulative data of AAR/left ventricle (LV). (c) Cumulative data of MI/AAR. Each error bar represents mean ± SD of 6–9 mice. (Feng et al., [100], used with permission).

and cardiomyocytes. The increase in myocardial TLR4 mRNA expression was associated with enteroviral replication and cardiac dysfunction in human myocarditis [114]. In an animal model of myocarditis, investigators found that TLR4 and IL-12 receptor β1 exacerbated coxsackievirus replication and myocarditis, whereas IFN-γ protected against viral replication [111]. TLR4 signaling was also associated with increased proinflammatory cytokines (IL-1β and IL-18) expression in the infected hearts, suggesting these two cytokines play an important role in the pathogenesis of CV-induced myocarditis [111].

4.3. MyD88 and Trif. As noted above, MyD88 and Trif are two adaptors critical for TLR signaling, but their roles in the pathogenesis of viral myocarditis appear very much different. Fuse and coworkers found that within days after CVB3 inoculation, myocardial MyD88 and IRAK-4 expression was elevated. Moreover, compared to WT mice, mice deficient in MyD88 had less myocardial inflammation and injury, reduced CVB3 viral titers, and improved survival [113]. The myocardial cytokines (IL-1β, TNFα, IFN-γ, IL-10, and IL-18) was significantly decreased, but IFN-α and IFN-β were increased in MyD88$^{-/-}$ mice. This study established MyD88 signaling as a major contributor to CVB-induced myocardial inflammation and as a critical regulator in myocardial viral replication possibly *via* type I IFN-dependent mechanism [113]. On the other hand, Trif is the key adaptor essential for TLR3 signaling. Similar to TLR3$^{-/-}$ mice subjected to viral myocarditis, Trif$^{-/-}$ mice reportedly also had higher viral load, attenuated cytokine gene expression than WT mice [108, 112], and marked increase in mortality after CVB3 infection [112]. The antiviral protection of Trif signaling was probably mediated by type I IFN-β, since myocardial IFN-β expression was markedly suppressed in Trif$^{-/-}$mice and administration of IFN-β effectively reduced myocardial viral load and local inflammation and markedly improved the long-term survival rate in Trif-deficient animals [112].

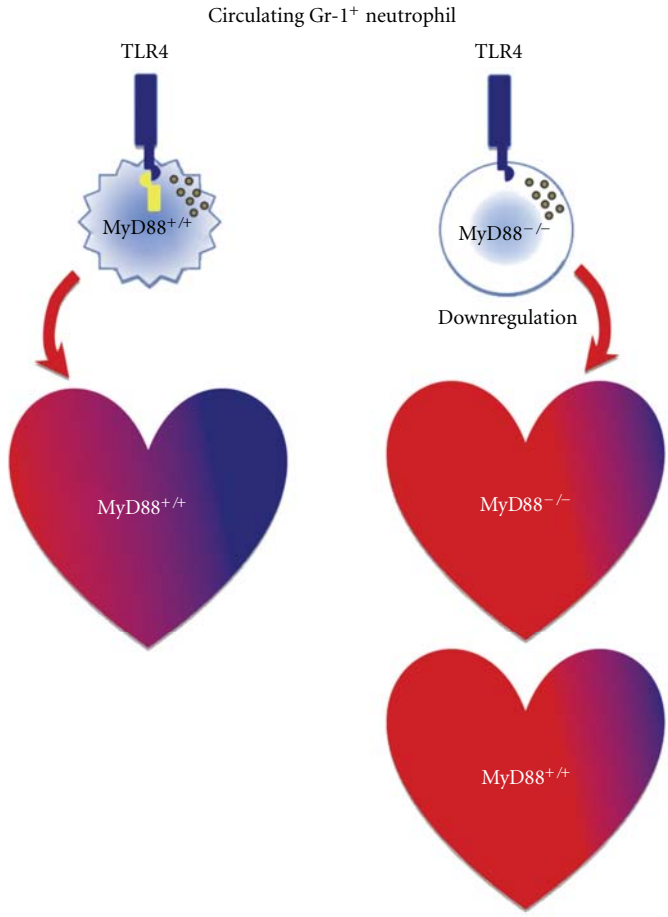

Figure 4: Schematic diagram of cardiac infarct size (blue region) after acute ischemia and reperfusion (I/R) and neutrophil CXCR2 downregulation by deletion of myeloid differentiation factor 88 (MyD88) globally (*right top*) and targeted to leukocytes only (*right bottom*). (Schmid-Schönbein, [103], used with permission).

Autoimmune Myocarditis. There is compelling evidence in a significant subset of patients with myocarditis and in several animal models of experimental autoimmune myocarditis (EAM) that host autoimmunity plays an important role in the pathogenesis of myocarditis and subsequent dilated cardiomyopathy [115]. TLR signaling activates the adaptive immune system by inducing proinflammatory cytokine production and upregulating costimulatory molecules of antigen presenting cells and is involved in autoimmune myocarditis.

In a mouse model of EAM, Nishikubo and colleagues [116] demonstrated that TLR4-induced Th1 immune response was required for the development of myocarditis induced by myosin and BCG. Similarly, in comparison to WT littermates, MyD88$^{-/-}$ mice were protected from myocarditis after immunization with α-myosin heavy chain-derived peptide (MyHC-α) and complete Freund's adjuvant [117]. This protection against EAM is due to impaired expansion of heart-specific CD4+ T cells after immunization. The serine/threonine kinase PKC-θ is required for certain T cell-driven autoimmune responses such as myocarditis. Mice deficient in PKC-θ did not develop EAM. However, TLR9 activation by CpG could overcome the PKC-θ deficiency and restored EAM in PKC-θ-deficient mice by activation of T cells [118]. To determine the role of the intracellular TLRs in EAM, Pagni and colleagues induced experimental EAM in mice deficient in TLR3, TLR7, and TLR9 by immunization with MyHC-α and complete Freund's adjuvant. They found that myocardial cellular infiltration and *in vitro* proliferation of MyHC-α-restimulated splenocytes were markedly reduced in TLR7$^{-/-}$ and MyD88$^{-/-}$ mice, while TLR3$^{-/-}$ and TLR9$^{-/-}$ mice showed similar myocardial inflammatory cell infiltration as WT mice. These data suggest that TLR7 and MyD88 signaling mediates myocardial inflammation and injury during the EAM [109]. Zhang and colleagues reported that human cardiac myosin could act as an endogenous ligand to directly activate human monocytes to release proinflammatory cytokines. This effect of human myosin is TLR2 and TLR8 dependent [119].

5. TLR and Septic Cardiomyopathy

Sepsis is defined as the systemic inflammatory response syndrome that occurs during infection. It has an estimated prevalence of 751,000 cases each year in the United States, and over 210,000 of them die [120]. Sepsis is the 10th leading

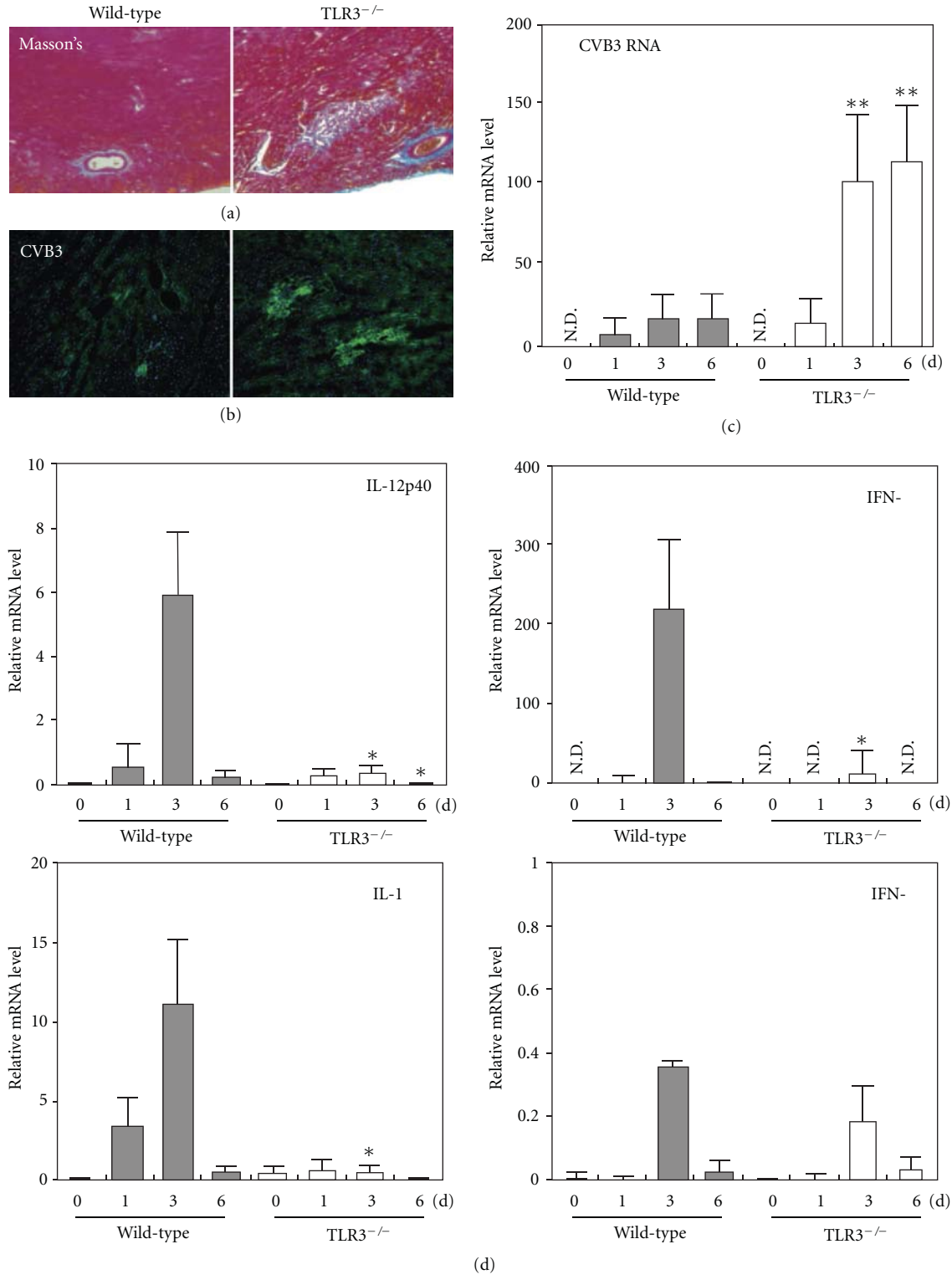

FIGURE 5: Myocarditis in TLR3$^{-/-}$ mice infected with CVB3. (a) Histopathologic results of hearts collected from TLR3$^{-/-}$ and wild-type mice 12 days after CVB3 infection was evaluated with Masson's trichrome staining. Data represent eight mice per group. (b) Immunofluorescence of hearts from TLR3$^{-/-}$ or wild-type mice 9 days after CVB3 infection followed by anti-CVB3 antibody staining. Data represent three mice per group. (c),(d) Real-time RT-PCR analysis of the expression of positive-strand CVB3 RNA (c) or the indicated cytokine genes (d) in hearts of TLR3$^{-/-}$ and wild-type mice on the indicated days after CVB3 infection. Data are presented as mean ± SD of triplicate determinations. All experiments were performed more than twice with similar results. $^{**}P < 0.01$; $^{*}P < 0.05$. (Negishi et al., [108], used with permission).

cause of death in the US [121]. Cardiovascular collapse induced by cardiac dysfunction and profound vasodilatation represents a main feature of septic shock and contributes to its high mortality. Since TLRs play an essential role in recognizing various microbial components such as LPS, lipoprotein, viral/bacterial DNA, these receptors play a pivotal role in the host innate immune defense and facilitate the adaptive immunity against foreign pathogens. On the other hand, inappropriate and imbalanced host immune response via TLR-dependent mechanisms may also contribute to the pathogenesis of sepsis.

5.1. TLR2. Knuefermann and colleagues [122] demonstrated that infusion of the Gram-positive bacteria *S. aureus* to isolated perfused heart activated myocardial IRAK-1 and NF-κB signaling, increased TNFα and IL-1β production, and induced marked contractile dysfunction under an *ex vivo* condition. The cardiac effects of *S. aureus* was dependent on myocardial TLR2, since TLR2-deficient hearts were protected from the above inflammatory responses and myocardial dysfunction. Zhu and colleagues [78] demonstrated that peptidoglycan-associated lipoprotein, a naturally occurring TLR2 agonist and a ubiquitous Gram-negative bacterial outer-membrane protein that is shed by Gram-negative bacteria (e.g., *E. coli*) into the circulation of septic animals [123], induced pro-inflammatory cytokine production and directly inhibited cardiomyocyte function (sarcomere shortening and Ca^{2+} transients) *in vitro.* Zou and colleagues [124, 125] demonstrate that TLR2 plays a critical role in myocardial inflammation, ROS production, and cardiac dysfunction during bacterial sepsis. In a mouse model of polymicrobial sepsis (cecum ligation and puncture, (CLP)), these investigators found that compared to WT mice, TLR2$^{-/-}$ mice had better survival, markedly improved cardiac function as measured by serial echocardiography, left ventricular pressure in isolated heart, and sarcomere shortening/Ca^{2+} transients in isolated cardiomyocytes (Figure 6), and depressed systemic and myocardial inflammatory cytokines production [124]. They further demonstrated that TLR2 activation by Pam3cys was sufficient to induce intracellular ROS production in neutrophils and cultured cardiomyocytes *in vitro* and that TLR2 deficiency markedly reduced intracellular ROS production in neutrophils isolated from polymicrobial peritoneal space [125]. While it remains unclear whether or not polymicrobial sepsis exerts cardiac dysfunction directly through TLR2 signaling *in vivo* [126], recent evidence appears to suggest that it is nonhematopoietic (parenchymal) TLR2 that plays a predominant role in mediating myocardial inflammation and cardiac dysfunction during polymicrobial sepsis [125] and as noted above, pathogenic ligand activation of TLR2 can induce direct functional depression of isolated cardiomyocyte *in vitro* [78].

5.2. TLR4. The role of TLR4 in sepsis-induced cardiac dysfunction has been studied mainly in endotoxemic models. LPS administration induces NF-κB activation [127] that leads to robust myocardial cytokines expression, such as TNFα, IL-1β, and myocardial dysfunction [128, 129]. LPS also reportedly upregulates TLR4 and CD14. Mice deficient in TLR4, CD14, and IRAK-1 were protected from endotoxic shock with reduced myocardial inflammation and improved cardiac function [129–131]. It is unclear, however, whether or not LPS elicits its cardiac depressive effect directly through myocardial TLR4. A few studies suggest that LPS-induced cardiac dysfunction may be an indirect effect secondary to immune cell TLR4 activation. For example, Tavener and colleagues [132] found that cardiomyocytes isolated from LPS-treated mice exhibited reduced sarcomere shortening and Ca^{2+} transients, whereas *in vitro* treatment with LPS failed to inhibit cardiomyocyte function. Further studies in chimeric mice suggest that TLR4 in bone marrow-derived hematopoietic cells is probably responsible for cardiac dysfunction during endotoxic shock [132–134]. However, using similar chimeric models, Fallach and colleagues recently found that, mice deficient in TLR4 in bone marrow-derived cells, but not in parenchymal tissues, remain to be sensitive to LPS challenge. They suggest that cardiomyocyte, not hematopoietic, TLR4 contributes to cardiac depression during endotoxemia [135].

It should be pointed out that while endotoxin models are highly reproducible and can provide great insight into inflammatory processes [136], these ligand-based models lack an infectious focus and do not closely mimic the pathophysiology observed in septic patients. On the other hand, bacterial infection models such as CLP closely resemble the clinical scenario of sepsis such as bowel perforation. Importantly, the contribution of TLR4 signaling in the two models of sepsis may differ significantly. For example, studies have demonstrated that TLR4 deletion confers a survival protection against endotoxin shock [35, 137] but no survival benefit in CLP model [138]. These data suggest that host mobilizes different innate immune defense mechanisms in endotoxemia and polymicrobial septic peritonitis [138]. Moreover, recent data indicate that endotoxemia and CLP utilize different signaling pathways to induce cardiac dysfunction and systemic inflammation. For example, MyD88, but not Trif, plays a predominant role in mediating cardiac dysfunction, systemic inflammation, and mortality during CLP, whereas MyD88 and Trif are both important for systemic inflammation, cardiac depression and mortality during endotoxin shock [139]. These data clearly illustrate the critical difference in the role of TLR4 signaling in these two models of sepsis.

5.3. TLR5. Rolli and coworkers first demonstrated that bacterial flagellin, a TLR5 ligand, induced marked myocardial inflammation and contractile dysfunction [140]. In cultured H9c2 cells and in primary rat ventricular cardiomyocytes, flagellin was found to activate NF-κB and MAPK and induce TNFα and MIP-2 expression. The flagellin-induced NF-κB activation was TLR5-dependent. *In vivo* administration of flagellin led to myocardial NF-κB activation, and expression of TNFα, IL-1β, IL-6, MIP-2, and MCP-1 increased myocardial neutrophil infiltration, and reversible cardiac dysfunction [140]. However, it is yet to be determined if TLR5 signaling plays a role in the pathogenesis of myocardial inflammation and cardiac dysfunction in more clinically relevant models of sepsis.

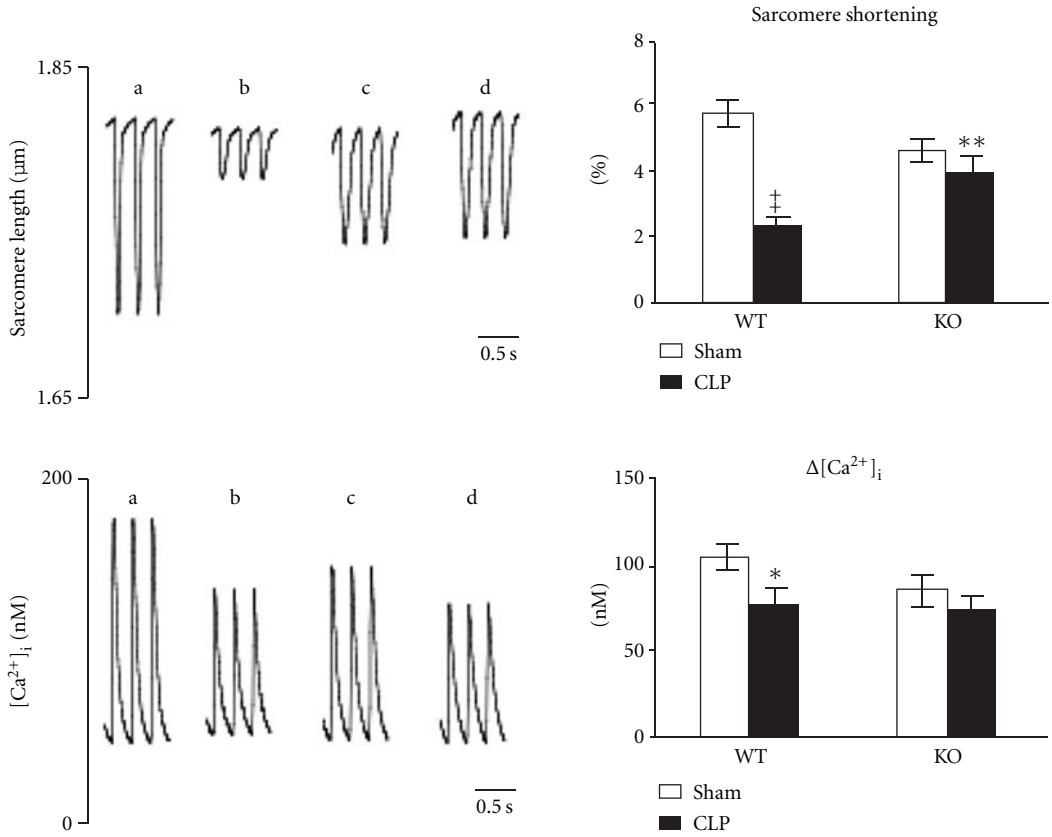

FIGURE 6: TLR2$^{-/-}$ mice have improved cardiomyocyte function after polymicrobial sepsis. Wild-type (WT) and TLR2$^{-/-}$ mice underwent sham or cecum ligation and puncture (CLP) procedures. Twenty-four hours later, the hearts were harvested and cardiomyocytes were isolated. A, Representative tracing of sarcomere shortening and Ca^{2+} transients in cardiomyocytes isolated from WT (a, b) and TLR2$^{-/-}$ (c, d) mice subjected to either sham (a, c) or CLP (b, d) surgeries. B, Accumulated data of sarcomere shortening and Ca^{2+} transients. Each error bar represents mean ± SE. The data in each group were recorded from 16 to 27 single adult cardiomyocytes isolated from more than four mice. $^{*}P < 0.05$ versus WT sham; $^{**}P < 0.01$ versus WT CLP; $^{‡}P < 0.001$ versus WT sham. KO, knockout (TLR2$^{-/-}$). (Zou et al., [124], used with permission).

5.4. TLR9. Paladugu and colleagues [141] demonstrated that bacterial DNA and RNA derived from clinically pathogenic *S. aureus* and *E. coli* isolates induced a concentration-dependent depression of maximum extent and peak velocity of contraction of rat ventricular cardiomyocytes. Significant, but more modest, depression was also induced by a nonpathogenic *Escherichia coli* isolate. Pretreatment with DNase or RNase abrogated this effect. Similarly, *in vivo* administration of synthetic DNA (CpG-ODN) caused myocardial NF-κB activation and inflammatory cytokine production (TNFα, IL-1β, and IL-6). *In vitro*, CpG-ODN inhibited sarcomere shortening of isolated mouse cardiomyocytes. Both the *in vivo* and *in vitro* effects of CpG were abolished in TLR9-deficient mice [142].

5.5. MyD88 and Trif. Using the CLP model [143] or a similar model [144], studies have established the critical role of MyD88 signaling in the pathogenesis of polymicrobial sepsis. In a colon ascendens stent peritonitis model, a highly inflammatory model, MyD88$^{-/-}$ mice were found to be protected with improved survival and attenuated systemic inflammation within the first 48 hours [144]. However, in a CLP model with a low grade of severity of peritoneal polymicrobial sepsis, MyD88$^{-/-}$ mice had worse survival compared with WT mice despite significantly attenuated systemic inflammation and reduced lymphocyte apoptosis in these mice [143]. In comparison, the role of Trif signaling in polymicrobial sepsis is not well understood. In a less severe sepsis model, Trif-deficient mice have reduced cytokine production including TNFα, IL-6, and IL-10 suggesting Trif signaling may contribute to systemic inflammation in a mild form of animal sepsis [143]. Feng and colleagues [139] compared the different role of MyD88- and Trif-signaling in endotoxemic and CLP models of sepsis. They demonstrate that MyD88 signaling is the dominant determinant in mediating inflammation, cardiac dysfunction, and mortality, whereas Trif signaling plays no major role, in the development of cardiac dysfunction and mortality in severe polymicrobial sepsis. But, as noted above, in endotoxemic model, MyD88 and Trif play an equally important role in mediating inflammation (IL-1β, IL-6, and TNFα), cardiac depression, and high mortality [139].

6. Summary and Perspective

During the past decade, studies characterizing the role of TLRs in the innate immunity and the immunopathology of human diseases have been extensive. A wide variety of microbial and nonmicrobial TLR ligands have been identified. These ligands act through their respective TLRs and elicit a variety of biochemical and proinflammatory responses *via* the distinct intracellular signal transduction systems. By medicating the critical and complex tissue inflammatory signaling, either protective or damaging in nature, TLRs play a pivotal role in the pathogenesis of cardiac critical conditions, such as acute ischemic myocardial injury, viral and autoimmune myocarditis, and septic cardiomyopathy. Several important future directions can be enumerated for characterization of the cellular and molecular mechanisms by which TLRs contribute to these cardiac conditions. While numerous studies have indicated the possible contributory role of TLRs in the development of ischemic myocardial injury, there are many unanswered questions that are critical for our ultimate understanding of the role of TLR signaling. For example, what are the specific contributions of *cardiac* versus *immune* cell TLRs to myocardial inflammation and infarction following I/R? How does the dual role of TLR4 signaling, *that is*, *proinflammatory* versus *antiapoptotic effect,* determine the final phenotypic outcome of myocardial injury [145]? How can we promote the protective *preconditioning* effect and at the same time prohibits the injurious *proinflammatory* effect of TLR signaling during myocardial ischemia [3]? Delineating these cellular and molecular details will help the future design of therapeutic strategy. Future studies will also be needed to delineate the role of *cardiac* versus *systemic* TLRs in the development of septic cardiac dysfunction and to define the intracellular mechanisms that control TLR-mediated deleterious cardiac dysfunction during sepsis. Without doubt, as we are developing new knowledge on the fine structure of the cellular and molecular mechanisms involved in these cardiac diseases, we will have better understanding on the essential role of TLRs in the human diseases. Dissecting the complex cellular and molecular pathways by which TLR signaling controls myocardial inflammation and cardiomyocyte injury will shed light on the mechanisms of these diseases and have significant clinical implications.

Abbreviations

AAR: Area-at-risk
AP-1: Activator protein 1
BCG: Bacille Calmette-Guérin
CLP: Cecum ligation and puncture
CpG: Cytidine-phosphate-guanosine
CV: Coxsackievirus
DAMPs: Damage-associated molecular patterns
dsRNA: Double-stranded RNA
EAM: Experimental autoimmune myocarditis
ECM: Extracellular matrix
EMCV: Encephalomyocarditis virus
ERK: Extracellular signal regulated kinase
HA: Hyaluronan
HMGB1: High-mobility group box-1
HSP: Heat shock protein
I/R: Ischemia/reperfusion
IFN: Interferon
IKK: I-κB kinase
IL: Interleukin
IRAK: IL-1 receptor-associated kinase
IRF: Interferon regulatory factor 3
JNK: C-Jun N-terminal kinase
KO: Knockout
LPS: Lipopolysacharide
LV: Left ventricle
Mal: MyD88-adaptor like protein
MAPKs: Mitogen-activated protein kinases
MCP-1: Monocyte chemotactic factor-1
MD-2: Myeloid differentiation factor-2
MI: Myocardial infarction
MIP: Macrophage inflammatory protein
MyD88: Myeloid differentiation factor 88
NF-κB: Nuclear factor kappa B
PAMPs: Pathogen-associated molecular patterns
PKC: Protein kinase C
poly(I:C): Polyinosine-polycytidylic acid
PRRs: Pattern recognition receptors
RIP1: Receptor-interacting protein 1
ROS: Reactive oxygen species
SARM: Sterile α- and armadillo-motif-containing protein
ssRNA: Single-stranded RNA
TAB: TAK binding protein
TAK1: Transforming growth factor-α activated kinase 1
TBK1: TRAF family member-associated NF-κB activator (TANK) binding kinase-1
TIR: Toll/interleukin-1 receptor
TIRAP: TIR domain-containing adaptor protein
TLRs: Toll-like receptors
TNFα: Tumor necrosis factor α
TRAF: TNF receptor-associated factor
TRAM: Trif-related adaptor molecule
Trif: TIR domain-containing adaptor inducing IFN-β—mediated transcription factor
WT: Wild-type.

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Review Article

Cellular Interplay between Cardiomyocytes and Nonmyocytes in Cardiac Remodeling

Norifumi Takeda[1,2] and Ichiro Manabe[2,3]

[1] *Department of Cell and Developmental Biology and Penn Cardiovascular Institute, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA*
[2] *Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan*
[3] *Global COE Program, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan*

Correspondence should be addressed to Ichiro Manabe, manabe-tky@umin.ac.jp

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Cardiac hypertrophy entails complex structural remodeling involving rearrangement of muscle fibers, interstitial fibrosis, accumulation of extracellular matrix, and angiogenesis. Many of the processes underlying cardiac remodeling have features in common with chronic inflammatory processes. During these processes, nonmyocytes, such as endothelial cells, fibroblasts, and immune cells, residing in or infiltrating into the myocardial interstitium play active roles. This paper mainly addresses the functional roles of nonmyocytes during cardiac remodeling. In particular, we focus on the communication between cardiomyocytes and nonmyocytes through direct cell-cell interactions and autocrine/paracrine-mediated pathways.

1. Introduction

Cardiac hypertrophy is an essential adaptive process, through which the heart responds to various mechanophysical, metabolic, and genetic stresses. On the other hand, the hypertrophy induced by sustained overload eventually leads to contractile dysfunction and heart failure. Cardiac hypertrophy involves cellular and molecular events within both cardiomyocytes and nonmyocytes. Cardiomyocytes show phenotypic modification that results in cellular hypertrophy accompanied by reexpression of several fetal genes, abnormal Ca^{2+} handling, oxidative stress and mitochondrial DNA damage, and cardiomyocyte death due to necrosis or apoptosis [1]. In addition to cardiomyocytes, the myocardium contains a variety of nonmyocytes, including vascular endothelial, and smooth muscle cells, fibroblasts and immune cells, which all appear to be crucially involved in the myocardial response to external and internal stress [2, 3]. During cardiac hypertrophy and the progression to heart failure, the myocardium exhibits complex structural remodeling involving rearrangement of muscle fibers, fibrosis, accumulation of extracellular matrix (ECM), cellular death, and angiogenesis [4]. Many of the processes underlying these phenomena are also seen in chronic inflammatory diseases and are mediated by cellular interactions among cardiomyocytes and nonmyocytes. In this paper, we will focus on the functional roles of nonmyocytes and the cellular communication ongoing during the development of cardiac hypertrophy and heart failure under noninfectious and noninfarction conditions, such as pressure overload.

2. Fibroblasts

Cardiac fibroblasts are critically involved in the development of cardiac fibrosis [4, 5]. They can produce a wide variety of ECM proteins, including interstitial collagens, proteoglycans, glycoproteins, and proteases [6]. Morphologically, fibroblasts are flat, spindle-shaped cells with multiple processes sprouted from the cell body, which lacks a basement membrane [7]. Fibroblasts play central roles in two types of fibrosis: reparative and reactive. Reparative (replacement) fibrosis or scarring accompanies cardiomyocyte death. Reactive fibrosis appears as "interstitial" or "perivascular" fibrosis

and does not directly associate with cardiomyocyte death [8, 9].

Increases in fibrosis result in mechanical stiffness and cardiac diastolic dysfunction [10]. In addition, by forming a barrier between cardiomyocytes, fibrosis can impair the electrical coupling of cardiomyocytes, leading to cardiac systolic dysfunction [11]. Moreover, perivascular fibrosis can increase oxygen and nutrient diffusion distances, leading to pathological remodeling [12]. Thus, fibrosis profoundly affects cardiomyocyte metabolism and performance, and ultimately ventricular function [13]. However, the functions of fibroblasts are not limited to producing ECM. Cardiac fibroblasts interact with other cell types, most notably cardiomyocytes. This interaction may be direct via physical contacts or indirect via paracrine factors. Thus fibroblasts are involved in much more than deposition of collagen [4, 7, 14].

In response to external stress, fibroblasts change their phenotype and become myofibroblasts [15, 16], which express several smooth muscle (SM) markers, including SM α-actin, SM22α, SMemb/nonmuscle myosin heavy chain-B, and tropomyosin [16–18]. However, more stringent SM markers (e.g., SM myosin heavy chains) are not expressed in myofibroblasts [19]. With the exception of heart valve leaflets, myofibroblasts are not found in normal cardiac tissue [4].

So far, no common definitive fibroblast-specific marker that could be used to identify fibroblasts in different tissues has been determined. In fact, fibroblasts in different tissues likely differ with respect to their cellular origins and functions. Several markers have been used to identify cardiac fibroblasts. Discoidin domain receptor 2 (DDR2) is specifically expressed by fibroblasts within the heart [20, 21], and, in a recent report, was successfully used as a marker in a flow cytometric analysis of cardiac cells [22]. This study found that fibroblasts represent a substantial portion of the cells in the mammalian heart. For example, the adult murine heart consists of approximately 55% cardiomyocytes and 45% nonmyocytes (~27% fibroblasts), and the adult rat heart consists of 30% cardiomyocytes and 70% nonmyocytes (~67% fibroblasts). Periostin [23] and thymus cell antigen-1 (Thy1/CD90) [14, 24] also have been used as markers of fibroblasts in developing and adult hearts. However, because fibroblasts can acquire heterogeneous phenotypes [25–27], those markers may not be capable of identifying all fibroblasts under all physiological and pathological conditions [5, 28]. Commonly used fibroblasts markers are reviewed elsewhere [28].

2.1. Origin and Phenotype of Cardiac Fibroblasts. The majority of resident cardiac fibroblasts are thought to arise from embryonic proepicardial organ (Figure 1) [23, 29]. Proepicardial and primitive epicardial cells undergo epithelial-mesenchymal transition (EMT) and then migrate into the myocardium, where they progressively differentiate into interstitial fibroblasts, perivascular fibroblasts, and coronary SMCs [30, 31]. These resident fibroblasts have traditionally been thought to be the sole source of cardiac fibroblasts, but more recently other cellular origins of cardiac fibroblasts have been proposed [23]. Endothelial cells and pericytes may also contribute to cardiac fibroblasts via endothelial-to-mesenchymal transition (EndMT) and EMT, respectively [32, 33]. In addition, bone marrow-derived cells may acquire fibroblast-like phenotypes [32, 34–37], and fibrocytes, circulating mesenchymal progenitor cells of bone-marrow origin, have been shown to be recruited to ischemic hearts and to express SM α-actin, collagen I, vimentin, and DDR2 [38]. Finally, it has been suggested that monocytes/macrophages represent another potential source of myofibroblasts in ischemic hearts [39]. How these various bone-marrow-derived cells are related to one another remains unclear, as does their precise lineage origins, in part, because of a lack of definitive markers for fibroblasts and the different myeloid cell subsets. Macrophages may also promote fibrosis by producing cytokines, such as TGF-β. It is therefore likely that bone-marrow-derived cells play multiple roles in cardiac fibrosis. Further studies will be needed to clarify precisely how these cells contribute to cardiac fibrosis.

2.2. Mediators of Intercellular Communications in Fibrogenic and Cardiomyocyte Hypertrophic Responses. Cardiomyocyte-specific deletion of genes has been shown to affect not only cardiomyocyte functionality but also the phenotypes and functions of fibroblasts [4]. Conversely, recent studies have shown that cardiac fibroblasts control cardiomyocyte proliferation in the developing ventricles during embryogenesis and that fibroblasts promote cardiomyocyte hypertrophy through paracrine factors and ECM [14, 24, 40]. These findings are indicative of the communication between cardiomyocytes and fibroblasts. Here we describe factors that mediate intercellular communication within the myocardium (Figure 2).

2.2.1. Angiotensin II. Ang II is a pleiotropic vasoactive peptide that plays key roles in the development of cardiac fibrosis and remodeling. Although most of the cardiovascular effects of Ang II are mediated via the Ang II type 1 receptor (AT1), the Ang II type 2 receptor (AT2) may be also important, as expression of both receptors is upregulated in various cardiac diseases [41–43]. Continuous infusion of Ang II into mice induces cardiac hypertrophy and fibrosis [44, 45]. Under these conditions, most of the proliferating fibroblasts were found to be surrounding cardiomyocytes carrying the AT1a receptor [46], suggesting that activation of cardiomyocytes via AT1a receptors also affects fibroblasts. Ang II also stimulates paracrine release of growth factors and cytokines, including TGF-β1 and endothelin-1 (ET-1) from cardiomyocytes [47–49]. On the other hand, AT1 receptor expression is greater in fibroblasts than cardiomyocytes [47], and Ang II directly stimulates fibroblast proliferation, collagen and ECM synthesis, and expression of fibroblast growth factor 2 (FGF2) [50]. It therefore seems likely that reciprocal interactions between cardiomyocytes and fibroblasts via paracrine factors are important for myocardial responses to Ang II. Consistent with these in vitro and in vivo findings in animal models are recent clinical studies demonstrating that blockade of the rennin-angiotensin system in patients, using a direct renin inhibitor, an angiotensin converting enzyme inhibitor, or an angiotensin receptor

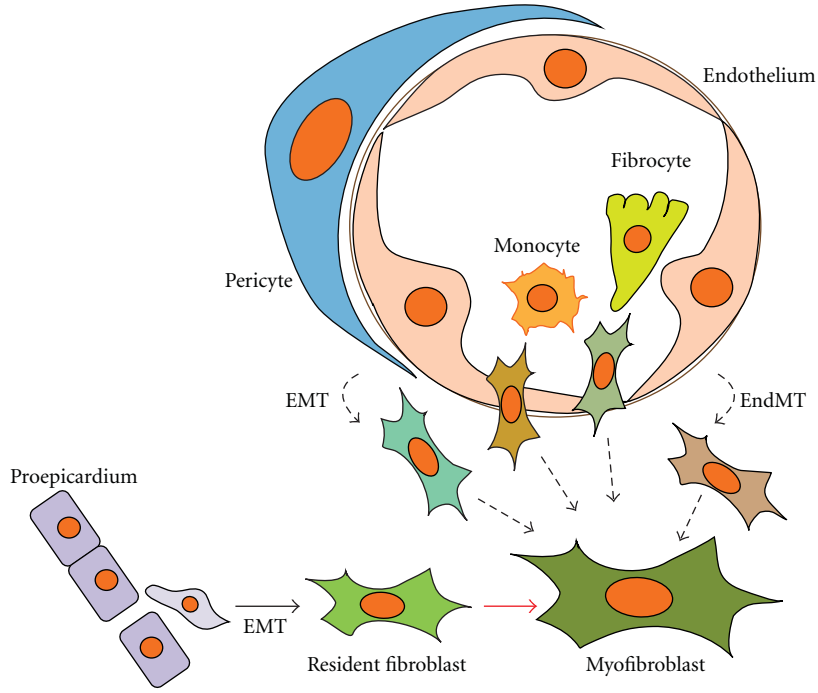

Figure 1: Diverse origins of cardiac fibroblasts. Resident cardiac fibroblasts are thought to arise from the proepicardium and embryonic epicardium during development. In response to fibrogenic stimuli, however, many other cell types, including bone marrow-derived cells, pericytes, and endothelial cells, may also acquire myofibroblast-like phenotypes. This scheme depicts the possible origins of cardiac fibroblasts. EMT: epithelial-to-mesenchymal transition; EndMT: endothelial-to-mesenchymal transition.

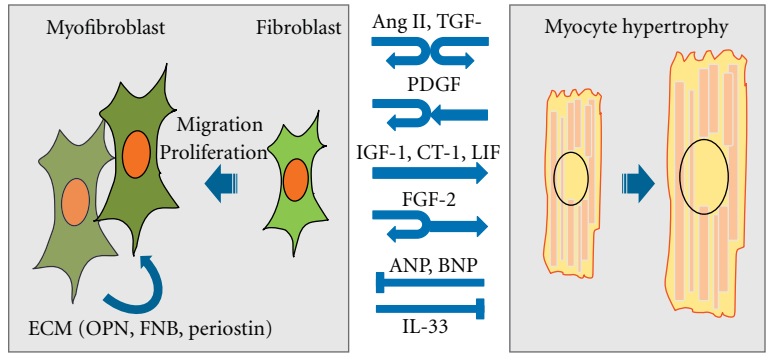

Figure 2: Reciprocal interactions between fibroblasts and cardiomyocytes. Many growth factors and cytokines have been shown to act in autocrine and/or paracrine fashion to induce hypertrophic responses in cardiomyocytes and activate fibroblasts. This scheme depicts only some of the factors identified. Ang II: angiotensin II; TGF-β: transforming growth factor-β; PDGF: platelet-derived growth factors; IGF-1: insulin-like growth factor-1; CT-1: cardiotrophin-1; LIF: leukemia inhibitory factor; FGF-2: fibroblast growth factor 2; ANP: atrial natriuretic peptide; BNP: brain natriuretic peptide; IL-33: interleukin-33; ECM: extracellular matrix; OPN: osteopontin; FBN: fibronectin.

blocker, effectively reduces cardiac fibrosis and remodeling in addition to reducing blood pressure [51].

AT2 receptor expression is also upregulated in failing human hearts, mainly in cardiac fibroblasts [43, 52]; however, the function of AT2 remains controversial. Initially, AT2 was reported to mediate effects opposing the growth-promoting signals mediated by AT1 [53], but since then there have been several reports that AT2 also stimulates prohypertrophic signaling [54, 55]. The function of AT2 may depend on the adaptor proteins recruited to the receptor and the pathophysiological conditions [56, 57].

2.2.2. Transforming Growth Factor-β. TGF-β exists in three isoforms (TGF-β1, TGF-β2, and TGFβ-3) that have distinct but overlapping functions in immunity, inflammation, and tissue repair, and TGF-β also has a central role in fibroblast activation and differentiation into myofibroblasts [58]. TGF-β is initially produced as a latent complex bound to latent TGF-β binding protein (LTBP) within the interstitium. It is activated physiochemically by altered pH, a large group of proteases and enzymes, high-energy ionizing radiation, or integrin-mediated mechanisms [59, 60]. Activated TGF-β binds to heterodimers comprised of TGF-β type 1 receptor

(TGF-βR1) and type 2 receptor (TGF-βR2) on both cardiomyocytes and fibroblasts [61]. TGF-βR1 (ALK5; activin-linked kinase 5) then phosphorylates receptor-regulated Smads (R-Smads: Smad2 and Smad3), which in turn associate with a common-mediator Smad (co-Smad: Smad4) and are translocated into the nucleus, where they act as transcription factors [62]. Smad3 is required for TGF-β to induce expression of collagen, fibronectin, and other ECM genes [63–66].

TGF-β promotes myofibroblast differentiation and ECM production by fibroblasts, and Ang II-induced cardiac hypertrophy is also mediated in part through TGF-β secreted from AT1-expressing fibroblasts [47]. TGF-β1-deficient mice subjected to chronic subpressor doses of Ang II showed no significant cardiac hypertrophy or fibrosis [67], which suggests that strategies to block TGF-β signaling may be useful for treating fibrogenic cardiac remodeling. Indeed, a TGF-β neutralizing antibody inhibited fibroblast activation and proliferation, and diastolic dysfunction in pressure-overloaded rats [68]. Similarly, an ALK5 inhibitor attenuated fibroblast activation and systolic dysfunction in an experimental rat model of myocardial infarction [69]. However, fibrosis was attenuated in Smad3-dificient mice subjected to experimental cardiac pressure overload, cardiac hypertrophy and heart failure were aggravated [70]. Moreover, TGF-β neutralizing antibody increased mortality and worsened cardiac remodeling, which correlated with reduction of ECM in a rat MI model [71]. These results indicate that the consequences of inhibiting TGF-β signaling can vary depending on the disease model and the timing of the inhibition, presumably because TGF-β signaling has an essential adaptive role in the myocardium under stress. Seemingly maladaptive functions, such as fibrosis, might also be essential for adaptation in other contexts. It will, therefore, be important to clarify the spatiotemporal functions of TGF-β signaling in different disease contexts if we are to develop effective therapeutic approaches involving TGF-β.

2.2.3. Fibroblast Growth Factor-2. FGF-2 is alternatively translated as a high molecular weight (Hi-FGF-2) and a low molecular weight (Lo-FGF-2) isoform from the single *Fgf2* gene [72]. The Hi-FGF-2 isoform contains nuclear localization sequences and is found predominantly within the nucleus, while the Lo-FGF-2 isoform is localized in the ECM and cytoplasm [72]. Cardiac fibroblasts predominantly express Hi-FGF-2, which acts in a paracrine fashion to promote cardiomyocyte hypertrophy [49]. Hi-FGF-2 also acts in an autocrine fashion on the fibroblasts themselves to stimulate release of other pro-hypertrophic factors, such as cardiotrophin-1 (CT-1) [49, 73, 74]. In addition, Lo-FGF-2 elicits cardioprotective effects against postischemic cardiac dysfunction [72].

2.2.4. Interleukins. The IL-6 family of cytokines, including leukemia inhibitory factor (LIF) and CT-1, are expressed by cardiac fibroblasts and cardiomyocytes. LIF and CT-1 secreted from fibroblasts mediate Ang II-induced cardiomyocyte hypertrophy [75, 76]. LIF was also shown to induce fibroblast hypertrophy but to inhibit myofibroblast transition and collagen deposition [77].

IL-33 is produced primarily by cardiac fibroblasts, and its expression is upregulated by cyclic strain [78]. IL-33 binds to a transmembrane form of the ST2 receptor (ST2L) on cardiomyocytes and inhibits the hypertrophic response of cultured cardiomyocytes to pro-hypertrophic stimuli. In vivo, IL-33 inhibits cardiomyocyte hypertrophy as well as fibrosis induced by pressure overload [40, 79].

2.2.5. Serotonin. Serotonin (5-hydroxytryptamine [5-HT]) acts via its 5-HT_{2B} receptor (5-HT_{2B}R) to contribute to cardiac hypertrophy. Indeed, plasma serotonin levels and cardiac 5-HT_{2B}R expression are both elevated in human heart failure. 5-HT_{2B}Rs mainly colocalize with AT1 receptors in fibroblasts [80, 81]. Isoproterenol (ISO) and Ang II-induced cardiac hypertrophy is suppressed in 5-HT_{2B}R-deficient mice, and this effect is accompanied by reduced production of cytokines (IL-6, IL-1β, TGF-β, and TNF-α) and reactive oxygen species in cardiac fibroblasts [82, 83]. Similarly, pharmacological blockade of 5-HT_{2B}Rs prevents ISO-induced murine cardiac hypertrophy [82]. Moreover, mice in which expression of 5-HT_{2B}R is limited to their cardiomyocytes are also resistant to ISO-induced cardiac hypertrophy and dysfunction, as well as to ISO-induced upregulation of the cytokines. This suggests that signaling through 5-HT_{2B}Rs on fibroblasts stimulates production of cytokines that promote cardiomyocyte hypertrophy [81, 84].

2.2.6. Platelet-Derived Growth Factors. PDGF-A and -B are secreted from cardiomyocytes and fibroblasts and play critical roles in cardiac fibrosis and angiogenesis through their interactions with the protein tyrosine kinase receptors PDGF receptor (PDGFR)-α and -β [85, 86]. PDGF signaling activates fibroblast proliferation and migration and ECM deposition. PDGF expression is significantly increased in cardiac hypertrophy and fibrosis [87], atrial fibrillation [88], and chronic rejection of cardiac allografts [89]. PDGF-C and -D may also contribute to cardiac fibrosis and remodeling. Transgenic mice exhibiting cardiomyocyte-specific expression of PDGF-C and -D develop hyperproliferation of myocardial interstitial cells, resulting in progressive fibrosis leading to dilated cardiomyopathy and heart failure [90–92].

PDGF signaling has been assessed as a therapeutic target for cardiac remodeling. The synthetic retinoid Am80 inhibited upregulation of PDGF-A by inhibiting the transcription factor Krüppel-like factor 5 (KLF5), thereby suppressing Ang II-induced cardiac fibrosis (Figure 3) [87, 93]. In addition, a neutralizing PDGFRα-specific antibody attenuated induction of pressure overload-induced atrial fibrosis and fibrillation [88]. However, several inhibitors of receptor tyrosine kinases, including PDGFRs, have been linked to the development of cardiomyopathy in some treated patients [94–97]. PDGFR-β on cardiomyocytes is indispensable for the cardiac response to pressure overload and may regulate angiogenesis [98]. For therapies targeting PDGF signaling to cardiac remodeling, it will be important to further clarify the precise roles played by PDGFs and PDGFRs under various pathological and physiological conditions.

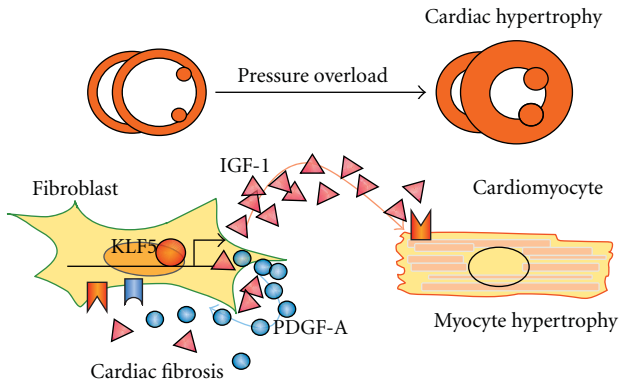

Figure 3: A model for the regulation of fibroblasts by KLF5 dur-ing cardiac hypertrophy. The transcription factor KLF5 controls *Igf1* and *Pdgfa* expression in cardiac fibroblasts. IGF-1 is a major cardiotrophic factor secreted from fibroblasts, and PDGF-A is primarily involved in mediating the migration and proliferation of fibroblasts.

2.2.7. Insulin-Like Growth Factor-1. IGF-1 exerts adaptive and cardioprotective effects in response to stress. The majority of serum IGF-1 is liver-derived and plays a critical role during normal body development. However, postnatal body growth is preserved, even in the complete absence of IGF-1 expression by hepatocytes; autocrine/paracrine IGF-1 appears to have important regulatory functions under these conditions [99]. In the heart, IGF-1 is mainly expressed in cardiac fibroblasts [14] and activates downstream signal transducers, such as phosphoinositide 3-kinase (PI3K), leading to cardiomyocyte hypertrophy [100]. We recently found that cardiac IGF-1 is transactivated by KLF5 (Figure 3) [14]. Although cardiac fibroblast-specific deletion of *Klf5* ameliorated the cardiac hypertrophy and fibrosis elicited by moderate-intensity pressure overload, it resulted in severe heart failure in high-intensity pressure overload. Similarly, administration of a peptide inhibitor of IGF-1 severely exacerbated heart failure induced by high-intensity pressure overload. These findings indicate that induction of IGF-1 is an essential cardioprotective response; that cardiac fibroblasts play a pivotal role in the myocardial adaptive response to pressure overload; that KLF5 controls IGF-1 expression in cardiac fibroblasts in response to stress [14].

2.2.8. Connective Tissue Growth Factor. CTGF (also known as CCN2) is expressed in fibroblasts and cardiomyocytes and regulates ECM deposition and wound healing [101, 102]. CTGF is induced by TGF-β, Ang II, and ET-1. By itself, CTGF only weakly promotes fibrosis and cardiomyocyte hypertrophy, but it appears that it may promote a more robust effect by acting as a cofactor for TGF-β [103, 104]. Transgenic mice exhibiting cardiomyocyte-specific expression of CTGF did not develop cardiac hypertrophy or fibrosis under baseline conditions but showed significantly increased fibrosis and contractile dysfunction in response to pressure overload [105]. Another group of transgenic mice developed age-dependent cardiac hypertrophy and dysfunction, though Ang II did not increase fibrosis in young transgenic mice [106]. Thus, the cardiac actions of CTGF will require further study.

2.2.9. Natriuretic Peptides. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are usually synthesized in the atria and ventricles, respectively [107]. Plasma levels of these peptide hormones are widely used as biomarkers when making a diagnosis or determining risk stratification in a variety of cardiac disease states. In addition, infusion of synthetic ANP or BNP is useful for treating cardiac heart failure and remodeling, mainly by optimizing intravascular volume and arterial pressure [108]. ANP and BNP also exert antihypertrophic and antifibrogenic effects on the heart, and knockout mice deficient in their common receptor, guanylyl cyclase-A (GC-A), showed cardiac hypertrophy and extensive interstitial fibrosis that was at least partially independent of blood pressure [109–111]. In fibroblasts, BNP inhibits TGF-β-regulated genes related to fibrosis (collagen I, fibronectin, and CTGF), proliferation (PDGF-A and IGF-1), and inflammation (COX2, IL-6, and TNF) [112], while ANP suppresses ET-1 expression and cell proliferation [113]. This suggests ANP and BNP secreted from cardiomyocytes suppress the fibrogenic activity of fibroblasts.

2.2.10. ECM Molecules. ECM serves as an important intermediary for intercellular communication by transducing intracellular signals via its receptor molecules (integrins and CD44) on myocardial cells [24, 114–116]. Production, degradation and modification of ECM components are dynamically regulated under both physiological and pathological conditions. Fibroblasts are a major source of nonbasement membrane collagen and other ECM proteins, and other cells in the myocardium, including cardiomyocytes, endothelial cells, and SMCs, also produce sets of ECM components [117]. Cardiac fibroblasts and macrophages are major producers of matrix metalloproteases (MMPs), which degrade ECM proteins.

The integrin family consists of 18 α and 8 β subunits, which form 24 known α-β-heterodimers. Integrins serve as cell-ECM and cell-cell adhesion molecules and also function as signal-transducing receptors for ECM proteins, including collagen, laminin, fibronectin (FBN), and osteopontin (OPN) [116, 118]. Cardiac-specific integrin signaling in genetically engineered animal models are reviewed elsewhere [116].

CD44 was originally described as a receptor for hyaluronan (HA), a ubiquitous constituent of the ECM, but is now known to interact with collagen, laminin, FBN, and OPN [119]. CD44-HA interactions play an important role in regulating leukocyte extravasation into sites of inflammation and in mediating efficient phagocytosis. CD44 also contributes to the resolution of inflammation through removal of matrix breakdown products, clearance of apoptotic neutrophils, and fibroblast migration [120]. In injured hearts, CD44 is upregulated in fibroblasts, leukocytes, and endothelial cells, particularly those cells surrounding and within the coronary arteries [120, 121]. *Cd44*-deficient mice subjected to myocardial infarction show increased myocardial infiltration by leukocytes and expression of proinflammatory cytokines,

followed by decreased fibroblast infiltration and fibrosis and enhanced dilative cardiac remodeling [120]. Finally, *Cd44*-deficient cardiac fibroblasts exhibit diminished proliferation and collagen synthesis in response to TGF-β. This suggests CD44 is important for resolution of postinfarction inflammatory processes and for regulation of fibroblast function.

FBN and OPN are upregulated in cardiac hypertrophy and by Ang II. They contain the arginine-glycine-aspartate (RGD) tripeptide integrin binding motif and activate integrin-mediated proliferation, survival, adhesion, differentiation, and migration of myocardial cells [122–124]. FBN is mainly expressed in fibroblasts and acts in a paracrine fashion to regulate cardiomyocyte proliferation through β1 integrin signaling during embryonic heart development [24].

OPN is strongly expressed in chronic inflammatory and autoimmune diseases and promotes the recruitment and retention of macrophages and T cells at inflamed sites [125]. Cardiomyocyte-specific overexpression of OPN results in dilated cardiomyopathy and severe fibrosis, with recruitment of activated T cells showing Th1 polarization [125]. Moreover, OPN (*Spp1*)-deficient fibroblasts are less proliferative and less adherent to ECM substrates, while *Spp1*-deficient mice exhibit less Ang II-induced cardiac fibrosis [123, 126]. Recently, OPN has emerged as a novel biomarker of various cardiac diseases [127–129].

Periostin is primarily expressed in myocardial fibroblasts, and its expression is upregulated by pressure overload and myocardial infarction. Periostin can serve as a ligand for $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 4\beta 6$ integrins [23] and can also directly interact with other ECM proteins, including FBN, tenascin-C, collagen I/V, and heparin [130]. Collagen fibrils from periostin (*Postn*)-null mice are reduced in size, somewhat disorganized, and less efficiently cross-linked, indicating that periostin facilitates proper organization of the ECM [23, 131, 132]. In addition, periostin is induced via TGF-β signaling and may then enable collagen realignment in response to TGF-β [130, 133, 134]. *Postn*-deficient hearts subjected to pressure overload or ischemic insult exhibited less fibrosis but more frequent rupture of the ventricular wall [132, 135].

3. Endothelial Cells

Vascular endothelial cells are also crucially involved in the development of cardiac hypertrophy, remodeling, and failure. Endothelial cells are capable of producing a wide variety of functional agonists and antagonists, including vasodilators and vasoconstrictors, procoagulants and anticoagulants, and inflammatory and anti-inflammatory factors. Endothelial cells maintain homeostasis by controlling the balance of these various mediators [136]; endothelial dysfunction disturbs that balance and leads to pathological inflammatory processes. For instance, activated endothelial cells express the adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which recruit and promote the infiltration of immune cells into the myocardium in response to various stimuli.

Endothelial cell-derived nitric oxide (NO), produced by endothelial NO synthase (eNOS), is a key regulator of vasodilation. NO also reduces vascular permeability and thrombogenesis, and it inhibits inflammation by suppressing signaling by adhesion molecules, proinflammatory cytokines, and NF-κB [136]. Under pathological conditions, the bioavailability of NO is diminished [137, 138]. For example, sustained pressure overload triggers eNOS uncoupling, which reduces NO signaling, increases levels of eNOS-derived reactive oxygen species, and promotes endothelial dysfunction. NO is also known to influence myocardial excitation-contraction coupling, substrate metabolism, and hypertrophy, as well as cell survival, which are at least in part dependent on eNOS and nNOS expression in cardiomyocytes [139]. For example, eNOS (*Nos3*) knockout mice develop concentric left ventricular hypertrophy and fibrosis [140], indicating the importance of the autocrine and paracrine effects of NO in cardiac remodeling.

Accumulating evidence indicates that impaired angiogenesis contributes to the transition of cardiac hypertrophy to heart failure. Hypertrophic stimuli induce expression of the angiogenic growth factors, vascular endothelial growth factor (VEGF), and angiopoietin 2 [141], which promote angiogenesis and blood flow in response to reductions in coronary perfusion pressure or ischemia. Blockade of VEGF action using an adenoviral vector encoding a decoy VEGF receptor or an anti-VEGF antibody promotes the transition from compensatory cardiac hypertrophy to failure in response to pressure overload in mice [142, 143]. Likewise, TNP-470, an inhibitor of angiogenesis, also induced cardiac dysfunction [144]. Conversely, VEGF treatment during prolonged pressure overload preserved contractile function [144, 145].

Within myocardium subjected to pressure overload, hypoxia-inducible factor-1-(HIF-1-) mediated transactivation of VEGF in cardiomyocytes plays an important role during induction of angiogenesis. Furthermore, it has been proposed that, in response to sustained pressure overload, p53 accumulates in cardiomyocytes and inhibits HIF-1 activity, thereby impairing cardiac angiogenesis and contractile function [144]. However, there are also conflicting data showing that ventricular deletion of HIF-1α prevents hypertrophy-induced activation of peroxisome proliferator-activated receptor-(PPAR-) γ and contractile dysfunction [146].

Endothelin-1 (ET-1) is a major growth factor secreted from endothelial cells that contributes to cardiac hypertrophy and fibrosis. ET-1 was originally identified as an endothelium-derived vasoconstrictor [147], but it is also expressed in various nonendothelial cells, including fibroblasts and cardiomyocytes, and it exerts both autocrine and paracrine effects that appear to be important for cardiomyocyte hypertrophy [47, 48, 148]. Although cardiomyocytes predominantly express ET_A ET-1 receptors, the hypertrophic response to Ang II and ISO is unaltered in cardiomyocyte-specific ET_A receptor-(*Ednra*-) deficient mice, suggesting the possible involvement of ET_A receptors on nonmyocytes and ET_B receptors in cardiomyocyte hypertrophy [48, 149]. Consistent with that idea, the combined ET_A/ET_B receptor antagonist bosentan inhibited Ang II-induced cardiac hypertrophy [150]. It was also shown that endothelium-derived ET-1 promotes cardiac fibrosis in diabetic heart [151].

4. Immune Cells

A variety of immune cells, including macrophages, T cells, and mast cells, reside in the myocardium under physiological conditions. They are also induced to infiltrate the myocardium under pathogenic conditions and to promote cardiac remodeling, in part by releasing cytokines, growth factors, and MMPs [152, 153].

Macrophages are essential effector cells involved in tissue remodeling and fibrosis. It is becoming increasingly clear that macrophages can have diverse phenotypes and functions [154]. In vitro studies have shown that Th1 cytokines, alone or in contact with microbial products, elicit classical M1 activation of macrophages, which then release proinflammatory cytokines and reactive oxygen species. Th2 cytokines (IL-4 and IL-13) elicit an alternative form of macrophage activation designated M2. M2 macrophages are thought to suppress immune responses and promote fibrosis and tissue remodeling, though M2 activation is a rather generic term often used to describe any form of macrophage activation other than classical M1. Previous studies have shown that macrophages are involved in cardiac hypertrophy and remodeling. For instance, Ang II-induced cardiac hypertrophy and fibrosis were diminished in macrophage-specific mineralocorticoid receptor-(MR-) deficient mice [155]. The MR-deficient macrophages exhibited M2-type activation and reduced expression of proinflammatory cytokines, suggesting it is M1-type macrophages that are involved in the cardiac hypertrophy and fibrosis induced by Ang II. Similarly, a monoclonal neutralizing anti-MCP-1 antibody attenuated not only macrophage accumulation, but also fibroblast proliferation and fibrosis, resulting in amelioration of cardiac diastolic dysfunction [156]. These results demonstrate the pathological involvement of macrophages in cardiac hypertrophy and fibrosis. By contrast, macrophage depletion using liposomal clodronate induces abundant infiltration of inflammatory cells, predominantly $CD4^+$ lymphocytes, and aggravates cardiac dysfunction in hypertensive rats harboring the mouse renin gene (*Ren2*) [157]. This suggests macrophages exert a protective effect against cardiac dysfunction induced by hypertension. Clearly further studies are needed to clarify the seemingly diverse functions of macrophages in cardiac hypertrophy and heart failure. It is very likely that macrophage function changes with time and in different pathological contexts [158]. It will therefore be important to characterize the different functions and phenotypes of macrophages at different times during the processes of cardiac hypertrophy and heart failure and to elucidate the underlying molecular mechanisms. These studies will be essential for the development of novel therapeutic interventions affecting macrophages.

Mast cells reside in the myocardium, and their numbers are increased in hypertrophied and failing hearts. Mast cells are an important source of an array of cytokines, growth factors, chemokines, and other mediators. Histamine is a major mediator released upon mast cell degranulation in the heart and may be involved in heart failure. Consistent with that idea, inhibition of histamine using the histamine H_2 receptor antagonist famotidine reportedly ameliorates heart failure [159, 160]. In addition, mast cells secrete the proteases renin and chymase, which, respectively, catalyze the conversion of angiotensinogen to angiotensin I and angiotensin I to Ang II and may thus activate the local rennin-angiotensin system in the heart [161]. Mast cell degranulation also releases preformed TGF-β, PDGF-A, and TNF-α [88, 161, 162], and inhibition of mast cells suppresses cardiac dysfunction and atrial fibrillation induced by pressure and volume overload [88, 163]. These results strongly suggest that mast cells are involved in inflammatory processes that contribute to remodeling in the heart.

T cells also reside in the myocardium. Although little is known about their function in cardiac pathology, Kvakan et al. recently reported that adoptive transfer of $CD4^+$ $CD25^+$ regulatory T cells suppresses cardiac hypertrophy and fibrosis induced by Ang II in mice, and this effect was accompanied by a marked reduction in infiltration of inflammatory cells [164].

5. Cardiac Progenitor Cells

In addition to fibroblasts and immune cells, the cardiac stroma contains a number of mesenchymal cell types. Previous studies have demonstrated that a fraction of these mesenchymal cells, referred to as cardiac progenitor cells (CPCs), have the potential to differentiate into cardiomyocytes under certain conditions [165]. There is also some evidence of cellular interactions between CPCs and their surrounding cells, including cardiomyocytes and fibroblasts. For instance, IGF-1 and Ang II produced by surrounding cells affect the survival of CPCs [166, 167]. In addition, human CPCs were shown to be connected to cardiomyocytes and fibroblasts via gap junctions and adherens junctions [168, 169], which may enable cellular communication via mediating molecules, including miRNA [170]. Communication between CPCs and surrounding cells through Notch-Notch ligand and Eph-ephrin signaling has also been shown [171, 172]. As such, cellular communication between mesenchymal cells, including CPCs, and their surrounding cells, including cardiomyocytes and fibroblasts, may contribute to the myocardial response to injury.

6. Conclusions

Both cardiomyocytes and nonmyocytes play essential roles in the processes involved in the development of cardiac hypertrophy, remodeling, and failure. The cellular and molecular processes that contribute to cardiac remodeling and failure share many features with chronic inflammatory processes in other tissues. Thus sterile stresses such as pressure overload and Ang II appear to activate pathways that are commonly used in inflammatory processes, including those involving immune cells in the myocardium. As we have seen here, dynamic cellular interactions among cardiomyocytes and nonmyocytes are a driving force for these inflammatory processes. In that regard, it will be important to further clarify the functional involvement of the different cell types residing in the myocardium and the underlying molecular control mechanisms. The function of a particular cell type

may change with time and in response to different insults. As discussed, the functions of some myocardial cells, such as fibroblasts and macrophages, appear maladaptive under certain conditions but are in fact essential for adaptive responses at different times and in different disease models. Elucidation of these complex processes could lead to identification of novel therapeutic targets for the treatment of cardiac hypertrophy and heart failure.

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Review Article

Adiponectin in Cardiovascular Inflammation and Obesity

Tamar R. Aprahamian[1] and Flora Sam[2,3]

[1] *Renal Section, Evans Department of Medicine, Boston University School of Medicine, Boston, MA 02118, USA*
[2] *Cardiovascular Section, Evans Department of Medicine, Boston University School of Medicine, Boston, MA 02118, USA*
[3] *Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA 02118, USA*

Correspondence should be addressed to Tamar R. Aprahamian, aprahami@bu.edu

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Inflammation is widely known to play a key role in the development and progression of cardiovascular diseases. It is becoming increasingly evident that obesity is linked to many proinflammatory and obesity-associated cardiovascular conditions (e.g., metabolic syndrome, acute coronary syndrome, and congestive heart failure). It has been observed that adipokines play an increasingly large role in systemic and local inflammation. Therefore, adipose tissue may have a more important role than previously thought in the pathogenesis of several disease types. This review explores the recently described role of adiponectin as an immunomodulatory factor and how it intersects with the inflammation associated with both cardiovascular and autoimmune pathologies.

1. Adiponectin

Adiponectin is a cytokine, "adipokine", produced almost exclusively in adipose tissue and is expressed at high levels by lean, healthy individuals. However, it has been reported that in pathological conditions such as coronary artery disease (CAD), diabetes mellitus, and hypertension that adiponectin levels decline [1–4]. The protein is present in human plasma at a range between 3 and 30 μg/mL; however, as body mass increases, serum adiponectin levels decrease [5, 6]. The larger adipocytes found in obese subjects produce lower levels of adiponectin but higher levels of proinflammatory cytokines, such as TNFα [7]. The adiponectin monomer (30 kDa) has a structure consisting of a globular head and a collagenous tail, and this monomer is able to multimerize to form several stable complexes of low-, medium-, and high-molecular weight. Adiponectin shares sequence homology with collagens VIII and X as well as complement factor C1q. It has previously been referred to as ACRP30 for adipose complement-related peptide of 30 kDa based upon its homology to C1q [8]. In addition to promoting adipocyte differentiation, PPAR-γ agonists are known to increase adiponectin expression both in vitro and in vivo [9]. Adiponectin inhibits the expression of TNF-α in adipocytes, and both TNF-α and IL-6 inhibit the production of adiponectin [9, 10]. Negative regulation of adiponectin expression also results from hypoxia and oxidative stress [11, 12]. It is interesting to hypothesize that any or all of these factors could contribute to the vascular breakdown that could create a vicious cycle of perpetual inflammation in an obese state.

Given that the levels of adiponectin vary in different inflammatory diseases as discussed above, these data suggest that the metabolic consequences observed in obesity may be related to an imbalance of pro- and anti-inflammatory cytokines.

Thus, adipokines contribute to the pathophysiology of obesity-linked disorders through their ability to modify proinflammatory and metabolic processes. Adipokines (such as leptin, TNF-α, PAI type 1, IL-1β, IL-6, and IL-8) are proinflammatory and increased in obesity [7]. In obese subjects, adiponectin levels are decreased, and the ability of adiponectin to inhibit the inflammatory processes is limited. Low adiponectin levels are inversely related to high levels of C-reactive protein (CRP) in patients with obesity, type 2 diabetes, and CAD [13–15].

Adiponectin also exerts anti-hypertrophic effects and protects against ischemia-reperfusion injury [16, 17].

Hypoadiponectinemia is a risk factor for patients with obesity-related complications such as CAD and hypertension [1–3, 18]. Hypoadiponectinemia also contributes to insulin resistance [19], impaired endothelium-dependent vasodilatation, impaired ischemia-induced neovascularization [20], salt-induced hypertension [21], and diastolic heart failure [22]. Thus, adiponectin mediates protective effects in obesity-related metabolic and vascular disease presumably by its anti-inflammatory actions and protects the heart against ischemia-reperfusion injury through its ability to suppress myocardial inflammation and apoptosis [23]. Similarly, lack of adiponectin exacerbates left ventricular hypertrophy and systolic heart failure and increases mortality after experimental aortic constriction [17, 24]. Hypoadiponectinemia appears to promote hypertension progression [2], but the mechanism by which this occurs is not entirely clear.

Adiponectin supplementation suppresses the progression of viral myocarditis in diabetic obese mice [25], inhibits atherosclerosis progression in vitro by NF-κB inhibition and phospho-Akt activation [26], and suppresses TNFα-induced IκBα phosphorylation [27]. Interestingly, chronic adiponectin overexpression increased subcutaneous fat mass and protected against diet-induced insulin resistance [28].

2. Shifting Paradigm of Adiponectin Expression in Disease

Obesity is considered a state of mild inflammation, and it is well documented that increasing adiposity results in a decrease in adiponectin production, thus, perpetuating an inflammatory state. This occurs in patients with metabolic syndrome, type II diabetes, and cardiovascular disease. In addition, low plasma adiponectin has been associated with myocardial infarction in young patients independent of other conventional risk factors [29]. Of interest, in diseases that are not necessarily related to obesity, adiponectin levels have recently been shown to be increased in chronic inflammatory and autoimmune diseases such as type I diabetes, SLE, rheumatoid arthritis, inflammatory bowel disease, and chronic systolic heart failure. The latter is contrary to the decrease in adiponectin levels in heart failure related to obesity [30]. Adiponectin levels are also increased in human patients with hypertrophic cardiomyopathy associated with diastolic dysfunction [31].

In this review, we will discuss the various conditions in which levels of adiponectin are known to be dysregulated and the impact that these scenarios of inflammation have on disease progression, in particular, the impact on the cardiovascular system (Figure 1).

3. Cardiac Remodeling, Hypertension, and Heart Failure

Hypertension is associated with left ventricular hypertrophy which can eventually lead to heart failure. In a mouse model of transverse aortic constriction, adiponectin deficiency results in concentric left ventricular hypertrophy and greater mortality at a short-time point; however, when the time course is extended, the results are limited to the prevention of left ventricular remodeling and preserved mitochondrial oxidative capacity [17, 32]. Consistent with this remodeling data, pioglitazone—a known modulator of inflamation and an activator or adiponectin—reduced cardiac hypertrophy and fibrosis in wild-type mice subjected to angiotensin-II-infusion. These beneficial effects were lost when the same injury and treatment were performed on adiponectin-deficient mice [33]. When adiponectin-deficient mice are maintained on a high-fat/high-sucrose/high-salt diet, the mice become obese, insulin resistant, and hypertensive [34]. In the KKAy mouse, a murine model to study metabolic syndrome [35, 36], adiponectin administration improved hypertension [21]. Antihypertensive drugs were used to determine adiponectin production in vitro, and it was found that there are varying degrees of adiponectin secretion which may impact the results of the drug efficacy [37]. With regards to human studies, significantly lower levels of adiponectin have been found in patients with essential hypertension compared to normotensive controls [38]. Taken together, these data suggest that adiponectin plays an important protective role in the development of hypertension.

While these animal models of heart failure have shown that adiponectin protects against the development of systolic dysfunction, adiponectin levels are increased in humans and may be predictive for mortality, in patients with chronic heart failure [39]. The exact cause of the upregulation is unknown, but several hypotheses are likely. First, it is possible that increased expression of adiponectin may be a compensatory response to the stress of heart failure, similar to the mechanism described for B-type natriuretic peptide (BNP) secretion [40]. Although the molecular mechanisms are unknown, BNP levels correlate with adiponectin levels in human heart failure [39, 40]. Furthermore, direct stimulation of human adipocytes with BNP has been shown to result in a cGMP-dependent release of adiponectin [41]. A second possibility for elevated adiponectin levels in cardiac disease could be the development of a condition termed "adiponectin resistance." Although the mechanism has not been elucidated, adiponectin resistance has been described in a small number of published studies in both human tissue and animal models [42–44]. Furthermore, as observed in systolic heart failure, increased levels of adiponectin may result as a compensatory response to aberrant expression of adiponectin receptors. Therefore, disrupted adiponectin signaling in target tissues (resulting from a change in adiponectin receptor expression) may act as a compensatory response and partially explain the observed increase in adiponectin levels in these disease states.

4. Coronary Artery Disease

The relationship between adiponectin levels and coronary artery disease and acute coronary syndrome is not so straightforward. There is an inverse relationship between serum adiponectin levels and nondiabetic patients with regard to the severity of coronary artery disease. In type I diabetic patients and nondiabetic controls, hypoadiponectinemia is associated with coronary artery calcification [45]. In

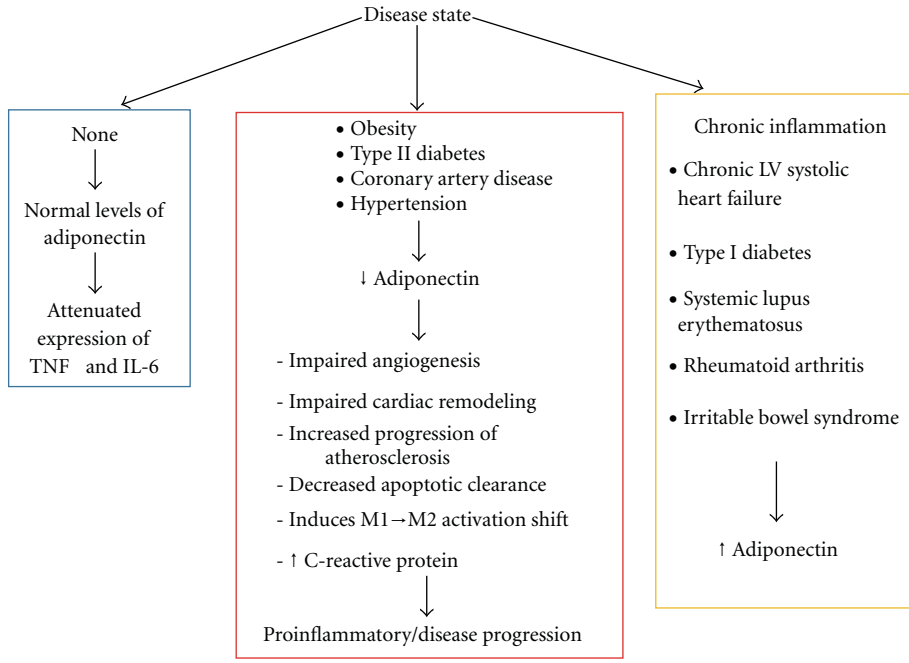

Figure 1: Adiponectin levels and inflammatory state. In healthy individuals, adiponectin maintains anti-inflammatory properties. Disease states where adiponectin levels decrease result in proinflammatory signaling and exacerbation of disease. Recent data has shown that adiponectin levels are increased in chronic inflammatory diseases, but the reason for this is incompletely understood.

addition, adiponectin levels are significantly lower in patients with acute coronary syndrome compared to patients with stable angina and healthy controls [46]. Furthermore, the development of atherosclerosis and coronary artery disease goes hand in hand. Adiponectin has anti-atherosclerotic, as well as anti-inflammatory properties that may play an important role in preventing the progression of coronary artery disease. Results from clinical surveys show that low adiponectin levels, while being a predictive marker for early-stage atherosclerosis, are also significantly associated with coronary artery disease [3].

5. Accelerated Atherosclerosis and Lupus

Accelerated atherosclerosis is believed to be a critical factor contributing to stroke and coronary heart disease (CHD), a leading cause of death among young women with SLE. Clinical studies have largely examined the relation between SLE and endpoint cardiac events including myocardial infarction and stroke [47, 48]. More recently, attention has shifted towards the causes of advanced cardiovascular diseases, the focus now being on the contribution of accelerated atherosclerosis in SLE patients. To complicate matters, it has been shown that there is an increased incidence of accelerated atherosclerosis, and as a result, increased endpoint cardiac events in patients with SLE. The exact cause is unknown, but chronic inflammation is likely a contributing factor.

Adiponectin plays a role in the inflammatory components of cardiovascular diseases. To begin with, lack of adiponectin in a mouse model of atherosclerosis leads to not only an increase in T-cell accumulation within the lesions, but also an increase in total atherosclerotic lesion area compared to adiponectin-sufficient controls [49]. Adiponectin is also known to be upregulated by PPARγ and treatment with PPARγ agonists results in decreased atherosclerosis [50]. A meta-analysis of studies to determine the renal protective effects of PPARγ agonists on diabetic patients showed that urine albumin excretion was decreased in patients receiving PPARγ agonists [51]. Based on this information, our lab hypothesized that the several immunomodulatory effects of PPARγ agonists could potentially be beneficial in lupus since glomerulonephritis is a major complication of lupus. Inducing adiponectin upregulation by maintaining two different mouse models on a PPARγ agonist-containing diet resulted in significantly less disease than mice receiving vehicle treatment. We further showed that these effects were mediated by adiponectin [52]. These data suggest that the increased level of adiponectin derived from upregulation by PPARγ plays a major role in the decrease of inflammation in these models.

Other studies have utilized mouse models to further elucidate the interaction between atherosclerosis and lupus. Several different lupus-like mouse models, including *gld*, *lpr*, and *Sle*1.2.3, have been used in the generation of various chimeras with either apoE−/− or LDLr−/− mice, which are prone to atherosclerosis. These models consistently report that lupus disease and vascular complications are worsened when simultaneously present, with inflammation likely being a contributing factor [53–56]. The involvement of adiponectin has not been examined in these models; however, given the importance of this adipokine in regulating SLE as well as inflammatory processes involved in atherosclerosis, it would be of great interest to study not only the levels of

adiponectin, but also to administer exogenous adiponectin to determine if the phenotypes could be rescued.

6. Pulmonary Disease and Pulmonary Hypertension

Cardiopulmonary complications are widely recognized causes of morbidity and mortality especially in obese adults [57]. Several instances have been described where adiponectin (or lack thereof) is involved in the progression of the disease. The presence of adiponectin in lung was first described by Summer et al. In this study, the authors show that alveolar macrophages from mice lacking adiponectin spontaneously produce increased levels of TNFα in vitro. In addition, these mice have increased expression of proinflammatory cytokines and matrix metalloproteinases, and the lungs display an emphysema-like phenotype including dilated air spaces, decreased tissue elastance, and increased lung displacement volume [58]. The same group has shown that adiponectin is found on the luminal side of the lung blood vessels and serves to inhibit TNFα-induced upregulation of E-selectin which is expressed when endothelial cells become inflamed. There was also evidence of pulmonary hypertension associated with the infiltration of perivascular inflammatory cells in an adiponectin-deficient mouse model [59]. In a similar vein, allergic airway inflammation, a model of chronic asthma, induced in adiponectin-deficient mice resulted in pulmonary vascular remodeling and pulmonary arterial hypertension [60]. Overexpression of adiponectin has direct effects on pulmonary artery smooth muscle cells in a murine model of inflammation-induced pulmonary hypertension. The result was reduced disease and improved pulmonary arterial remodeling [61]. Similarly, hypoxia-induced pulmonary arterial remodeling and right ventricular hypertrophy can be attenuated by overexpression of adiponectin [62]. More recently, it has been shown that patients with higher levels of plasma adiponectin after acute respiratory failure have a higher risk of mortality. Analysis of serum adiponectin at day 1 or day 6 after respiratory failure showed that low levels of adiponectin at the earlier timepoint were associated with increased survival [63]. Further study is required to explain the different results observed between human and animal studies. It is possible that, in humans with an acute illness, the increased levels of adiponectin could be a compensatory response or could be a result of resistance with relation to adiponectin receptors. Taken together, the mouse and human data suggest that adiponectin plays a role in pulmonary disorders and may play a role in modulating the acute response to critically ill patients, which is an area of research that should be further studied.

7. Angiogenesis

Prior data from our lab and others has shown that adiponectin modulates angiogenesis in vitro and in vivo. This has been demonstrated using mouse models of tissue ischemia and cardiac remodeling as well as rabbit corneal models of angiogenesis [20, 64, 65]. For example, pretreatment with adiponectin of endothelial progenitor cells for angiogenic cell therapy results in enhanced survival and proliferation of the cells in an ischemic hind-limb model, suggesting that high levels of adiponectin are essential for a robust angiogenic response [66]. Furthermore, adiponectin-deficient mice have a poor angiogenic response to ischemic hind-limb surgery; however, the systemic administration of adiponectin restores the angiogenic response to that seen in wild-type controls [20]. A mouse model of cardiac specific angiogenesis, transverse aortic constriction was performed in both adiponectin-deficient and wild-type mice, and a reduction in capillary density was observed as well as an increase in LVH, pulmonary congestion, and a reduction in LV systolic function in the mice lacking adiponectin. Once again, these data suggest that adiponectin is involved in angiogenesis related to cardiac remodeling [65]. One mechanistic explanation for the beneficial effects of adiponectin in angiogenesis has been demonstrated to occur through AMPK-dependent regulation. Another branch of the angiogenic regulatory pathway is implicated by in vitro and in vivo data linking adiponectin to a positive revascularization response to ischemia via COX-2 signaling which is known to be vasculo-protective [67]. Adiponectin supplementation to wild-type mice also resulted in an increased rate of recovery with regard to limb perfusion. Thus, adiponectin is able to act in several ways to promote a favorable angiogenic response.

8. Obesity, Vascularity, and Angiogenesis

Given its relationship to cardiovascular complications and the clinical importance of obesity in the United States, it is somewhat surprising that only recently has the importance of adipose tissue microenvironment been addressed in molecular studies related to metabolic dysfunction. Both animal models and obese human studies demonstrate that total fat mass does not necessarily provide an indicator for metabolic status. These types of studies have led to the thought that metabolic dysfunction is not related to the adipose tissue *quantity* alone, but rather, the *quality* of the individual adipocyte [28]. The factors contributing to the characteristics of the adipocyte in this context are likely related to the status of fat pad inflammation and its perfusion by the microvessels. It is possible that reducing the blood flow in the adipose tissue may result in spontaneous necrosis of the large adipocytes that are present in obese states, and this will contribute to inflammation. Numerous studies have shown that adiponectin deficiency leads to diminished tissue perfusion and that elevated adiponectin levels promote vascular growth in skeletal muscle and tumor vascularity. However, to date, there are no studies that have examined the role of adiponectin in adipose tissue perfusion. Capillary rarefaction in the fat pads has been observed in obese mice, and this has also been correlated with a decrease in the expression of the angiogenic growth factor VEGF, which can lead to adipose tissue hypoxia [68, 69]. This blood flow restriction in the adipose tissue could contribute to the

propagation of the inflammation. Recent studies have begun to determine the impact of fat pad expansion on adipose tissue hypoxia and capillary density in mouse models and humans. It has been suggested that spontaneous necrosis will occur in larger-sized adipocytes as a result of the limited oxygen from the circulation. Thus, there is a recruitment of macrophages to the adipose tissue in order to phagocytose the dying cells. It is possible that adiponectin, since it has a major role in both cardio-protective and anti-inflammatory processes, also plays a role in adipose tissue vascularity and inflammation.

9. Macrophage Phenotype and Obesity

Macrophages found in the adipose tissue contribute greatly to obesity-related metabolic dysfunction and chronic inflammation [69]. A correlation has been made in both humans and animal models showing that macrophage infiltration leads to the development of insulin resistance [12, 70, 71]. The accumulation of "crown-like" structures, which are markers of "inflamed fat", suggests that clearance of dead adipocytes by macrophages is impaired, and this may be due to dysregulated adipokine levels or another obesity-related factor [72].

Macrophages can be characterized as M1 or M2 depending on their activation phenotype, which is similar to Th1/Th2 polarization. "Classically activated" macrophages are referred to as having an M1 phenotype and can upregulate cytokines generally involved in pro-inflammatory processes such as TNFα, IL-6, and IL-12. In addition, M1-type macrophages can increase the production of reactive oxygen species and nitrogen intermediates. "Alternatively activated" macrophages are categorized as having an M2 phenotype. These macrophages secrete IL-10, which is an anti-inflammatory cytokine and partake mostly in the downregulation of pro-inflammatory cytokines. M2 macrophages can also upregulate arginase-1 which has the ability to diminish the inducible nitric oxide synthase reaction. Other functions of M2 macrophages include the ability to upregulate scavenger receptors, mannose receptors, and the IL-1 receptor antagonist [73–75]. Characterization of macrophages from adipose tissue shows differential activation phenotypes dependent on obesity. Diet-induced obesity in a mouse model results in adipose tissue macrophages that have an M1-type phenotype. However, in lean mice, macrophages express markers of the M2 phenotype suggesting a switch from an anti-inflammatory phenotype to a more pro-inflammatory phenotype [76]. Therefore, it is reasonable to suggest that diet-induced obesity is capable of shifting the activation characteristics of macrophages from the protective M2-phenotype, to the pro-inflammatory state leading to metabolic dysfunction characterized by the M1-phenotype.

Analysis of peritoneal macrophages and the stromal vascular fraction of cells from adipose tissue of adiponectin-deficient mice reveals a shift towards increased expression of cytokines related to macrophages with an M1-type pro-inflammatory phenotype. In contrast, exogenous overexpression of adiponectin can decrease the generation of reactive oxygen species, while also shifting the macrophage population to those of an M2-type phenotype [77].

Taken together, it is interesting to speculate that the power of adiponectin in shifting inflammatory properties of macrophages within adipose tissue combined with the data that adiponectin-deficient mice are unable to adequately respond to an ischemic event may be important in the role of the microenvironment of adipose tissue with relation to metabolic consequences and the progression of cardiovascular diseases.

10. Apoptotic Cell Clearance

Adiponectin is structurally similar to complement C1q, which is known for its role in the complement cascade and its ability to bind apoptotic bodies and facilitate their removal via a non-inflammatory process of phagocytosis by macrophage. C1q belongs to the family of collectin proteins and among their many anti-inflammatory functions, a major role is to facilitate clearance of apoptotic cells, thus, maintaining a quiescent, anti-inflammatory state. It was shown in vitro that adiponectin can similarly facilitate the clearance of apoptotic bodies via calreticulin receptor on macrophages, resulting in a "quiet" phagocytosis [78]. To further corroborate these findings in vivo [79], exacerbation of the lupus phenotype was observed in a lupus-prone mouse model lacking adiponectin, and this was shown to be partially due to impaired clearance of apoptotic material. Taken together, these data provide evidence that adiponectin can facilitate a non-inflammatory removal of apoptotic bodies. To extend these findings to another pathological condition, serum adiponectin levels are reported to decrease in patients with early loosening of hip replacement [80]. The authors hypothesize that decreased clearance of apoptotic cell remnants due to low adiponectin may contribute to the degeneration of the hip replacement; however, this is an area that is currently under investigation. These data provide further evidence that adiponectin could play a major role in both apoptotic cell clearance as well as bone destruction/creation.

With respect to obesity, the observation of "crown-like" structures (CLS) in adipose tissue is evidence of pro-inflammatory occurrences in obese subjects and animals [72, 81]. These CLS are rings of macrophages usually surrounding an adipocyte and could be the result of a lack of anti-inflammatory adipokines or the inability of macrophages to clear dead adipocytes, both of which are associated with obesity. Under normal conditions, clearance of early apoptotic cells is a non-inflammatory process. However, under obese conditions, the constant accumulation of dying adipocytes could be sensed by the macrophages to promote a proinflammatory response and, therefore, aggravate the inflammation. This impaired clearance of apoptotic cells in obesity is of particular interest since the lack of adiponectin could potentially play a role in driving inflammation resulting from the accumulation of CLS in obesity-related inflammation.

11. Concluding Statements

Adiponectin is a highly circulating adipokine that maintains its anti-inflammatory protective effects. Research continues to show its diverse functions in various disease states. It will be imperative to clarify the role of adiponectin in cardiovascular disease with relation to inflammation. This is complicated by the fact that low levels of adiponectin occur in obesity, type II diabetes, and metabolic disorders, whereas high levels of adiponectin are found in heart failure and hypertension, as well as chronic inflammatory autoimmune diseases such as SLE, type I diabetes, and rheumatoid arthritis.

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Research Article

Gene Profiling of Aortic Valve Interstitial Cells under Elevated Pressure Conditions: Modulation of Inflammatory Gene Networks

James N. Warnock,[1,2] Bindu Nanduri,[3] Carol A. Pregonero Gamez,[1] Juliet Tang,[2] Daniel Koback,[1] William M. Muir,[4] and Shane C. Burgess[2,3]

[1] *Department of Agricultural and Biological Engineering, Mississippi State University, Mississippi State, MS 39762, USA*
[2] *Life Science and Biotechnology Institute, Mississippi State University, Mississippi State, MS 39762, USA*
[3] *Department of Basic Sciences, Mississippi State University, Mississippi State, MS 39762, USA*
[4] *Department of Animal Sciences, Purdue University, West Lafayette, IN 47907, USA*

Correspondence should be addressed to James N. Warnock, jwarnock@abe.msstate.edu

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The study aimed to identify mechanosensitive pathways and gene networks that are stimulated by elevated cyclic pressure in aortic valve interstitial cells (VICs) and lead to detrimental tissue remodeling and/or pathogenesis. Porcine aortic valve leaflets were exposed to cyclic pressures of 80 or 120 mmHg, corresponding to diastolic transvalvular pressure in normal and hypertensive conditions, respectively. Linear, two-cycle amplification of total RNA, followed by microarray was performed for transcriptome analysis (with qRT-PCR validation). A combination of systems biology modeling and pathway analysis identified novel genes and molecular mechanisms underlying the biological response of VICs to elevated pressure. 56 gene transcripts related to inflammatory response mechanisms were differentially expressed. TNF-α, IL-1α, and IL-1β were key cytokines identified from the gene network model. Also of interest was the discovery that pentraxin 3 (PTX3) was significantly upregulated under elevated pressure conditions (41-fold change). In conclusion, a gene network model showing differentially expressed inflammatory genes and their interactions in VICs exposed to elevated pressure has been developed. This system overview has detected key molecules that could be targeted for pharmacotherapy of aortic stenosis in hypertensive patients.

1. Introduction

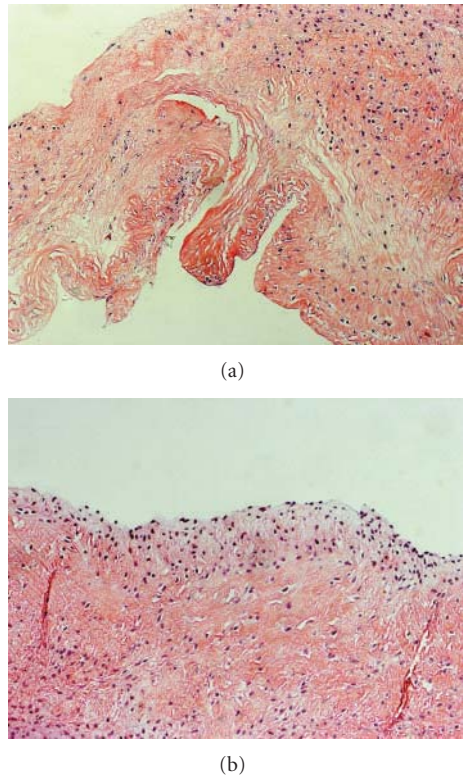
The pathogenesis of aortic stenosis (AS) is a largely understudied research area, compared to other cardiovascular diseases, which has major human health implications. Historically, AS has been considered an age-related, passive, degenerative disease. However, during the past 15 years, indisputable evidence has shown that AS is an active, cell-mediated process. Nonrheumatic AS is characterized by chronic inflammation, increased extracellular matrix (ECM) remodeling, proliferation, and differentiation of valvular interstitial cells (VICs) and the development of calcific lesions on the valve [1, 2]. Nonrheumatic AS is preceded by aortic sclerosis, a condition of valve thickening in which left ventricular outflow is not obstructed. Aortic sclerosis is associated with a 50% increase in death from all cardiovascular causes and increases the risk of myocardial infarction, heart failure, and stroke [3]. Progressive AS, in which obstruction to left ventricle outflow is present, produces left ventricular hypertrophy, left ventricular diastolic and systolic dysfunction, congestive heart failure, angina, arrhythmias, and syncope [4]. Severe symptomatic AS is associated with a life expectancy of less than 5 years [5]. In 2009, AS was directly responsible for over 13,752 American deaths and was an underlying factor in an additional 27,380 deaths and 49,000 hospital discharges. Though the disease is associated with significant clinical consequences, there is currently no effective therapy for valve disease other than surgical aortic valve replacement [6].

In vivo studies have shown that a causal link exists between hypertension and AS [7]. This is supported by numerous *in vitro* studies that have shown that elevated cyclic pressure plays an important role in valve ECM synthesis, proinflammatory and cathepsin gene expression [8–11]. In addition, it has been reported that transvalvular pressure has a direct effect on VIC stiffness and collagen synthesis [12]. The potential mechanisms connecting hypertension with initiation and progression of aortic valve disease include (1) hypertensive pressure raises the diastolic transvalvular pressure across the valve, increasing the mechanical strain experienced by the leaflets; (2) hypertension may disturb the hemodynamic environment (i.e., compression of the ECM, altered flow patterns), thus influencing valve cell behavior; and (3) hypertension may play a key role in the activation of several biological processes that induce aortic valve remodeling and disease [13].

We present here the first study of VIC gene expression profiling in an *ex vivo* model of elevated cyclic pressure. The data generated have enabled us to identify mechanosensitive gene networks, and we have also investigated VIC expression of a subset of genes associated with inflammation. It was hypothesized that expression of several proinflammatory genes, such as TNF-α and IL-6, would be significantly increased as clinical studies have shown these to colocalize with calcific regions in explanted aortic valves from prehypertensive patients [14]. TNF-α has also been associated with matrix remodeling through the expression of MMP-1 and -3 [15]. Additionally, TNF-α and other cytokines, such as IL-1β, express enzymes generating oxidants (O_2^-) capable of promoting low-density lipoprotein (LDL) oxidation [16]. Proteoglycans trap LDL in the tissue, and oxidative modification leads to endothelial expression of adhesion molecules (ICAM-1 and VCAM-1) and chemoattractants (MCP-1).

2. Materials and Methods

2.1. Tissue Harvest. Aortic valves were collected from six individual female Yorkshire/Hampshire pigs immediately after death. Animals were less than 6 months of age with a postslaughter weight of no more than 120 lbs (Sansing Meat Services, Maben, MS). Valves were rinsed twice in ice-cold sterile Phosphate Buffer Saline (PBS, Sigma, St Louis, MO) and transported to the laboratory on ice. Leaflets were cut one third of the distance from the annulus. Valve leaflets did not show any sign of degeneration, tearing or calcification. To ensure that only valve interstitial cells (VICs) were present in each sample, the endothelial cell layer was removed from each leaflet surface by immersion of valve tissue in collagenase II (2 μg/mL in serum free DMEM, Worthington Biochemical Corp.) for 5 minutes at 37°C and 5% CO_2. The leaflet surface was then gently swabbed. Confirmation that the EC had been removed was done through Hemotoxylin and Eosin staining (Figure 1). Valve leaflets were rinsed twice with PBS to remove excess collagenase II before being incubated overnight in DMEM supplemented with 10% Fetal Bovine Serum (FBS; Hyclone, Logan UT) and 1% antibiotic/antimycotic solution (Sigma). The tissue had approximate dimensions of 12 mm × 15 mm.

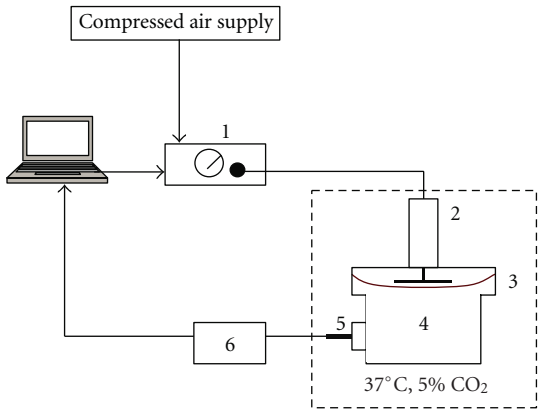


(a)

(b)

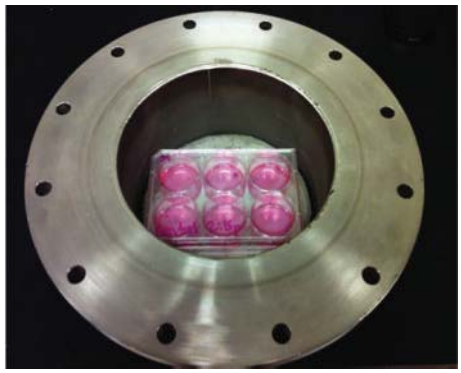
Figure 1: (a) H & E stain of a valve leaflet, confirming that the endothelium has been removed by 5-minute incubation with collagenase II followed by gentle swabbing. (b) Positive control sample showing an intact endothelium on a valve leaflet not treated with collagenase II and gentle swabbing. 10x magnification.

2.2. Pressure Studies. To investigate the effects of cyclic pressure, a custom-made, computer-operated dynamic pressure system was used. A schematic diagram and photos of the system are shown in Figure 2. Similar *ex vivo* systems have been used in the past to demonstrate changes in extracellular matrix protein synthesis and remodeling under elevated pressure conditions [8, 11]. Additionally, this system has been used to demonstrate a correlation between elevated pressure and proinflammatory gene expression in aortic valve interstitial cells [9]. Leaflet tissue was placed in a six-well tissue culture plate and immersed in 3 ml of culture medium. The tissue culture plates were placed in the pressure chamber and exposed to cyclic pressures of 80 mmHg or 120 mmHg, corresponding to diastolic transvalvular pressure in normotensive and hypertensive conditions [17], respectively, at a frequency of 1 Hz (sinusoidal wave; 0.6 sec influx, 0.4 sec outflux) for 24 hours. Figure 3 shows representative waveforms. At low-pressure conditions, pressure cycled between 35 mmHg and 80 mmHg, with amplitude of 45 mmHg. Under elevated pressure conditions, the maximum pressure was 120 mmHg and the minimum was 25 mmHg, providing amplitude of 95 mmHg. The pressure system exposed tissues to mechanical stimulation by increasing the air pressure above the supernatant media. To produce a change in the pressure within the chamber, the pneumatic piston moved downward in the chamber space,



(1) Electropneumatic regulator
(2) Pneumatic cylinder
(3) Silicone membrane
(4) Pressure chamber containing tissue culture plate
(5) Pressure transducer
(6) Load cell conditioner

(a)



(b)

(c)

Figure 2: (a) Schematic diagram of the pressure system. A computer controls an electropneumatic regulator (1), controlling the flow of compressed air to a pneumatic cylinder (2) the cylinder exerts a compressive force on a silicone membrane (3) reducing the volume in the pressure chamber (4) and subsequently increasing the atmospheric pressure surrounding the leaflets that are in tissue culture plates in the chamber. A pressure transducer (Omega Engineering, Inc, PX302-200GV) (5) records the internal pressure of the chamber, which is relayed back to the computer via a load cell conditioner (Encore Electronics, Inc., Model 4025-101) (6). The voltage and the pressure signal were acquired with a data acquisition card module (Measurement Devices, PMD1608) and monitored using an in-house developed LabVIEW graphical user program (LabVIEW, National Instruments). The system was placed inside an incubator to maintain a 37°C, 5% CO_2 humidified atmosphere. (b) Photograph of a six-well plate in the pressure chamber before being sealed. (c) Photograph of the sealed pressure chamber prior to being place in the 37°C incubator.

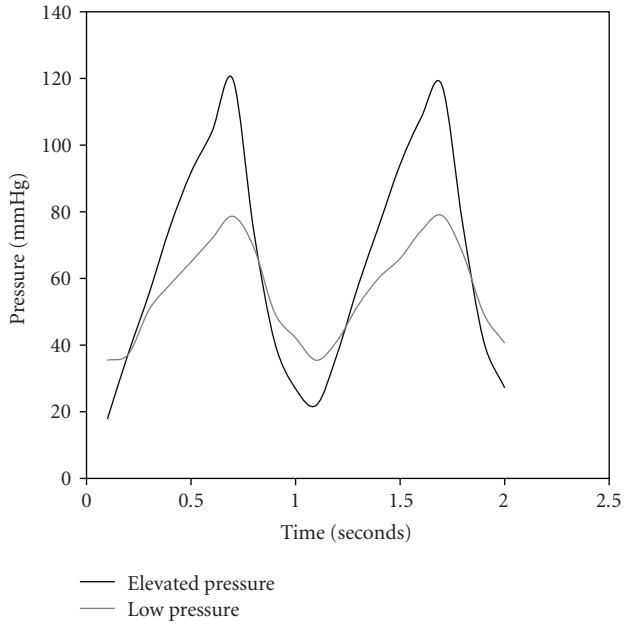


Figure 3: Pressure waveforms generated from the pressure system.

and the silicone gasket was stretched downward, reducing the volume of the chamber, and increasing the air pressure. During experiments, the pressure chamber was placed in an incubator at 37°C. The pH of the culture medium was measured prior to and after each test using a pH meter. pH readings of culture medium ranged between 7.3–7.4, indicating no significant changes. Each experimental group contained three biological replicates.

2.3. Array Experiments. Upon completion of pressure experiments, each leaflet was rinsed twice with sterile PBS, submerged in 1 mL of RNA later RNA Stabilization Reagent (Qiagen) to avoid changes in RNA expression and stored at −80°C until RNA extraction. Total RNA from porcine aortic valve interstitial cells (VICs) was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions and stored at −80°C. The quantity and quality of RNA was confirmed by spectrophotometry (A260/A280 ratio) and capillary electrophoresis (2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA) by using an RNA 6000 Picochip kit. Target preparation for microarray analysis was performed according to the manufacturer's established protocol (www.affymetrix.com). Briefly, a total of 100 ng of RNA from each sample was used for single-stranded cDNA synthesis with SuperScript II reverse transcriptase and T7-Oligo(dT) primer/Poly(A) controls (Affymetrix, Santa Clara, CA). The single-stranded cDNA was then converted to double-stranded cDNA by using *Escherichia coli* DNA polymerase I (Affymetrix) for the first cycle. From template cDNA, biotin-labeled cRNA was prepared in an *in vitro*

Table 1: Primer sequences used for qRT-PCR.

Gene	GenBank accession no.	Primer sequence	Size (bp)
MMP-3	NM_001166308.1	5′-TGTGGAGTTCCTGATGTTGG-3′(F) 5′-GGCTGAAGTCTCCGTGTTCT-3′(R)	240
MMP-1	NM_001166229	5′-TTTCCTGGGATTGGCAAC-3′(F) 5′-TCCTGCAGTTGAACCAGCTA-3′(R)	233
IL-6	NM_214399.1	5′-CACCAGGAACGAAAGAGAGC-3′(F) 5′-GTTTTGTCCGGAGAGGTGAA-3′(R)	204
PTX3	NM_002852.3	5′-GGGACAAGCTCTTCATCATGCT-3′(F) 5′-GTCGTCCGTGGCTTGCA-3′(R)	71
TNF-α	NM_214022.1	5′-GAAGACACACCCCCGAACAGGCA-3′(F) 5′-ACGTGGGCGACGGGCTTATCT-3′(R)	379

transcription (IVT) reaction by using the MEGAscript High-Yield Transcription Kit (Ambion Inc, Austin, TX). Following *in vitro* transcription, 600 ng of cRNA from each replicate was used for the second-cycle first-strand cDNA synthesis. The first-strand cDNA from the second cycle was then converted to second-strand cDNA. From second-strand cDNA template, biotin-labeled cRNA was prepared in an *in vitro* transcription (IVT) reaction by using GeneChip IVT Labeling Kit (Affymetrix) according to the manufacturer's instructions. Then 15 μg of biotin-labeled cRNA was fragmented in 1′ fragmentation buffer solution provided with the GeneChip Sample Cleanup Module (Affymetrix) at 94°C for 35 min. A total of 10 μg of fragmented biotin-labeled cRNA per replicate in hybridization mixture was then hybridized to Porcine Genome Array from Affymetrix GeneChips and incubated overnight at 45°C in a rotating hybridization oven, all according to the manufacturer's instructions (Affymetrix). After >16 h of hybridization, the mixture was removed, and, in several cycles, the chips were washed with nonstringent buffer and stained with streptavidin/phycoerythrin (SAPE) antibody solution according to the manufacturer's instructions by using an Affymetrix FS-450 fluidics station. The data were collected using Affymetrix GeneChip Scanner 3300 (Affymetrix). Three chips were used for each experimental condition and the RNA for each chip was obtained from three different leaflets.

2.4. Statistical Analysis of Array Data. Microarray data was analyzed using a mixture model approach as previously described [18]. Briefly, microarray analysis was performed for expression differences assuming that genes in alternative treatments are expressed or not in the following combinations: (i) not expressed in either condition, (ii) expressed only under the first condition, (iii) expressed only under the second condition, and (iv) expressed under both conditions, giving rise to 4 possible clusters with two treatments. The number of these combinations/clusters was determined by Akaike's Information Criterion (AIC) and the Bayesian Information Criterion (BIC) [19, 20].

2.5. Gene Expression Analysis by Quantitative Real-Time Polymerase Chain Reaction. To confirm the fold changes in gene expression from the array data, semi-quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was done to measure the relative change in mRNA expression. Real-Time qRT-PCR was carried out with 10 ng of total RNA using a Bio-Rad iCycler thermocycler and iScript one-step SYBR Green kit, following the manufacturer's instructions. Primer sequences (Table 1) were designed using Primer 3 software [14]. Sequences were selected that crossed intron/exon boundaries to ensure the elimination of genomic DNA. cDNA synthesis and PCR amplification were performed using the following steps: 50°C for 30 mins then the reaction mixture was heated to 95°C for 5 min; a 45 cycle two-step PCR was performed consisting of 95°C for 15 s followed by 1 min at 60°C. Following amplification, a melt curve was generated that confirmed primer specificity. Expression values for each gene were calculated relative to 18s mRNA levels. The mean fold change ($n = 5$) was calculated using the $2^{(-\Delta\Delta Ct)}$ method.

2.6. Network Modeling of Gene Expression Data. Affymetrix probe IDs that did not belong to the null distribution based on the mixture model analysis were mapped to Ensembl porcine gene accessions using Ensembl Biomart [21]. To identify the molecular functions, biological networks and signaling pathways in VICs responsive to cyclic pressure, pathway analysis using Ingenuity Pathways Analysis (IPA; Ingenuity Systems, California) was carried out as described previously [22]. Human orthologs for porcine genes (obtained from Biomart) were used in IPA. IPA generated networks that are no more than 35 genes/proteins in size. Based on the overlap between the genes in user dataset and a reference set (which is often the entire genome), IPA estimates the probability that genes in a network were found together due to chance. Networks scoring ≥ 2, with >99% confidence of not being generated by chance were considered to be significant. Annotations from scientific literature stored in the Ingenuity Pathways Knowledge Base (IPKB) were used to determine biological functions of the identified networks. Fisher exact test was used to calculate the P-value, the probability of each biological function/disease or pathway being assigned by chance. A $P \leq 0.05$ (adjusted for multiple testing for statistical rigorousness) was used to select highly significant biological functions and pathways represented in the gene expression datasets.

Table 2: Upregulated genes related to inflammation. All genes had a $P \leq 0.001$.

Ensembl Gene ID	Gene name	Description	Fold change
ENSSSCG00000014986	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	159.845
ENSSSCG00000015385	IL6	Interleukin 6 (interferon, beta 2)	117.817
ENSSSCG00000006286	SELE	Selectin E	74.336
ENSSSCG00000011727	PTX3	Pentraxin 3, long	41.052
ENSSSCG00000008087	IL1B	Interleukin 1, beta	20.185
ENSSSCG00000017482	CSF3	Granulocyte colony-stimulating factor Precursor (G-CSF)	15.267
ENSSSCG00000006588	S100A9	S100 calcium binding protein A9	9.664
ENSSSCG00000008959	CXCL2	CXCL2	9.498
ENSSSCG00000008090	IL1A	Interleukin 1, alpha	9.361
ENSSSCG00000017705	CCL5	Chemokine (C-C motif) ligand 5	9.339
ENSSSCG00000017721	CCL8	Chemokine (C-C motif) ligand 8	8.386
ENSSSCG00000006288	SELP	Selectin P (granule membrane protein 140 kDa, antigen CD62)	8.002
ENSSSCG00000014985	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	7.976
ENSSSCG00000008953	IL8	Interleukin 8	7.279
ENSSSCG00000008957	CXCL5	Alveolar macrophage chemotactic factor 2 Precursor (Alveolar macrophage chemotactic factor II) (AMCF-II)	7.189
ENSSSCG00000008975	CXCL9	Chemokine (C-X-C motif) ligand 9	6.687
ENSSSCG00000017723	CCL2	Chemokine (C-C motif) ligand 2	5.243
ENSSSCG00000012853	IRF7	Interferon regulatory factor 7	4.625
ENSSSCG00000013655	ICAM1	Intercellular adhesion molecule 1	4.041
ENSSSCG00000001050	EDN1	Endothelin 1	3.383
ENSSSCG00000004789	THBS1	Thrombospondin 1	3.113
ENSSSCG00000001404	TNF	Tumor necrosis factor Precursor (TNF-alpha)(Tumor necrosis factor Ligand superfamily member 2)(TNF-a)(Cachectin)	2.626
ENSSSCG00000010414	CXCL12	Chemokine (C-X-C motif) ligand 12	2.62
ENSSSCG00000000708	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	2.327
ENSSSCG00000009002	TLR2	Toll-like receptor 2	2.157
ENSSSCG00000003065	PLAUR	Plasminogen activator, urokinase receptor	1.937
ENSSSCG00000009477	EDNRB	Endothelin B receptor Precursor (ET-B)(Endothelin receptor nonselective type)	1.859
ENSSSCG00000011942	CD47	CD47 molecule	1.858
ENSSSCG00000001346	ABCF1	ATP-binding cassette subfamily F member 1	1.786
ENSSSCG00000010312	PLAU	Plasminogen activator, urokinase	1.747
ENSSSCG00000013181	SERPING1	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	1.71
ENSSSCG00000007440	CD40	CD40 molecule, TNF receptor superfamily member 5	1.674
ENSSSCG00000004156	IFNGR1	Interferon gamma receptor 1	1.6

Table 2: Continued.

Ensembl Gene ID	Gene name	Description	Fold change
ENSSSCG00000001849	ANPEP	Alanyl (membrane) aminopeptidase	1.547
ENSSSCG00000011322	CCR1	Chemokine (C-C motif) receptor 1	1.542
ENSSSCG00000000244	PPBP	Platelet basic protein Precursor (PBP)(C-X-C motif chemokine 7)(Small-inducible cytokine B7)	1.533
ENSSSCG00000013551	C3	Complement component 3	1.514

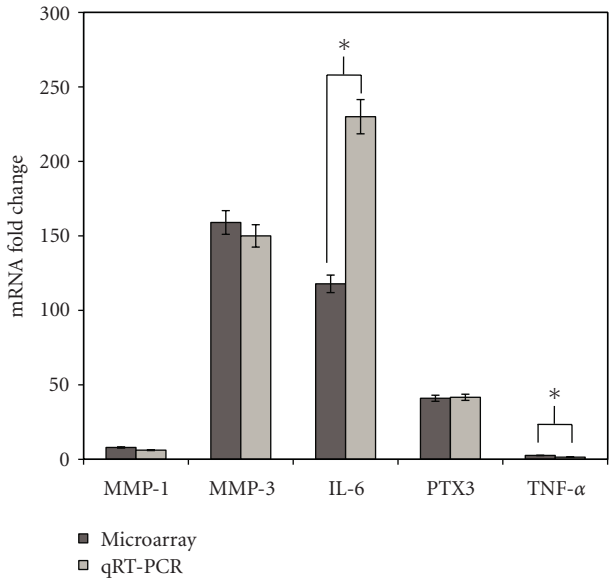


Figure 4: Confirmation of microarray data by real-time qRT-PCR. The fold change in gene expression was significantly higher in VICs exposed to elevated pressure compared to VICs exposed to normal cyclic pressure. *Represents a significant difference between microarray and qRT-PCR data.

3. Results

Transcriptome analysis of VICs was performed to determine the distinct genetic profile of VICs exposed to 80 mmHg (control) and 120 mmHg cyclic pressure. The mixture model method was used as a preliminary tool to cluster genes that could be important for the biology under investigation. In this analysis, 3 clusters with 49.8% of the differences in the null cluster were found, meaning that approximately 50% of the transcriptome was impacted by treatments to some degree. However, only ~6,000 of those genes could be differentiated from the null cluster with high probability ($P < 0.0001$). The microarray data was validated at the mRNA level by qRT-PCR (Figure 4). Based on the microarray data, we chose three genes known to be mechanosensitive and associated with aortic stenosis: MMP-1, MMP-3, and IL-6. Further, MMP3 and IL6 had the highest fold change detected by microarray. qRT-PCR was also performed on PTX3 and TNF-α, as these were key genes identified in the network analysis. Upregulation of all five genes in response to elevated pressure found in the microarray was confirmed by qRT-PCR. There was a significant difference in the fold change of IL6 and TNF-α; however, microarray and qRT-PCR both showed these genes to be upregulated in the presence of elevated pressure. A pathway analysis using IPA was completed to provide the basis for determining molecular functions, pathways, and networks that were important for the VIC response to altered pressure. IPA analysis showed that 56 genes related to inflammation were differentially expressed; 35 of these genes were up regulated and 21 genes were downregulated, as shown in Tables 2 and 3, respectively. At the chosen statistical thresholds, 16 networks were identified. The network centered on tumor necrosis factor (TNF-α) that included molecules involved in the inflammatory response is shown in Figure 5. This network included all genes differentially expressed by elevated pressure, as shown in Tables 2 and 3.

4. Discussion

The limited success of antiatherosclerotic therapies and the realization that distinct differences exist between the pathogenesis of atherosclerosis and AS suggests that innovative pharmacotherapies are needed. Analysis of gene expression changes in the context of response, networks, and pathways can expedite understanding of the molecular mechanisms that govern the VIC response to pressure. In the present study, novel molecular mechanisms that are activated in VICs during exposure to elevated pressure conditions were identified. Our results show that elevated pressure induces a gene expression pattern in cells that is considerably similar to that seen in aortic valve disease [1], in terms of altered expression of ECM proteins (MMP-1, MMP-3) [23, 24] and proinflammatory cytokines (IL-1β, IL-6) [15, 25, 26]. These results underline the key role of hypertension as an initiating factor in the onset of aortic valve pathogenesis. Modeling these genes to identify networks has facilitated the discovery of some very specific genes that could potentially be targeted for the treatment of aortic heart valve disease.

Previous histological studies of stenotic valves show that TNF-α and IL-6 both colocalize with ox-LDL. These tend to localize in the fibrosa at the vicinity of calcified areas [14]. Hence, the findings from the microarray data are supported by clinical observations. Creation of the gene network model provides a systems view of the molecular mechanisms and enables us to identify how various genes interact. TNF-α expression was increased under elevated pressure conditions and appears to be a key molecule in this network as it promotes expression of several adhesion molecules, including E-selectin, P-selectin, and ICAM-1.

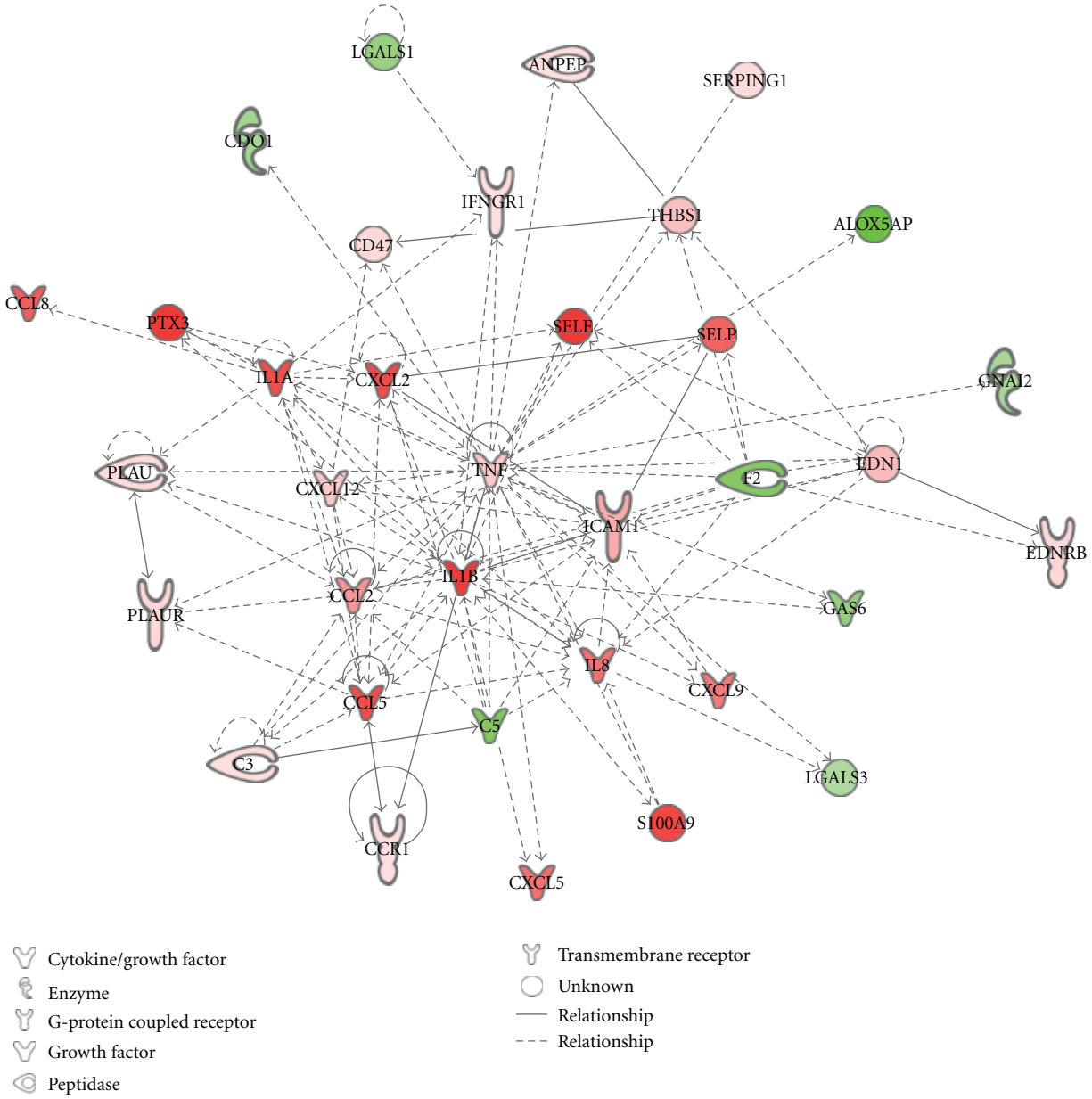


Figure 5: Inflammatory gene network associated with upregulation of TNFα. In the network, green represents genes with reduced expression while pink and red nodes denote a significant increase in gene expression.

Although adhesion molecules are more typically associated with endothelial cells, it is known that activated vascular smooth muscle cells can express adhesion molecules as part of the inflammatory process [27]. TNF-α also interacts with the cytokines IL-1α and IL-1β. Cytokine-targeting therapy that specifically targets TNF-α, IL-1, or IL-6 could be an effective antiinflammatory treatment for retarding disease progression. IL-1α and IL-1β promote the expression of long-chain pentraxin 3 (PTX3). Clinical studies have also shown that PTX3 levels are elevated in AS patients [28]. The short chain pentraxin, C-reactive protein, has been offered as an early diagnostic marker for cardiovascular diseases. However, because it can be produced in several organs, its reliability has been questioned. PTX3 could be a potential alternative, especially heart valve disease, and it has been proposed as a new candidate marker for acute and chronic heart diseases [29]. PTX3 is also involved in controlling inflammation and tissue remodeling and could therefore be a potential candidate for early AS therapy. Recent studies have shown that PTX3 has a nonredundant regulatory and cardioprotective role in acute myocardial infarction in mice [30].

The results of the present study are consistent with our global hypothesis that elevated pressure contributes to the development of aortic valve disease. However, certain limitations of the present study should be acknowledged. First, the small sample for microarray analysis may decrease the sensitivity of the study. However, the Mean-Difference-Mixture-Model (MD-MM) method used for statistical analysis is generally superior to other methods in most situations.

Table 3: Down regulated genes related to inflammation. All genes had a $P \leq 0.001$.

Ensembl gene ID	Gene name	Description	Fold change
ENSSSCG00000005055	LGALS3	Lectin, galactoside-binding, soluble, 3	−1.575
ENSSSCG00000011993	PROS1	Protein S (alpha)	−1.654
ENSSSCG00000011399	GNAI2	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	−1.69
ENSSSCG00000001408	AIF1	Allograft inflammatory factor 1 (AIF-1)(Ionized calcium-binding adapter molecule 1)(Protein G1)	−1.715
ENSSSCG00000014310	CXCL14	Chemokine (C-X-C motif) ligand 14	−1.73
ENSSSCG00000013030	PRDX5	Peroxiredoxin 5	−1.751
ENSSSCG00000014219	CDO1	Cysteine dioxygenase, type I	−1.791
ENSSSCG00000012840	CD151	CD151 molecule (Raph blood group)	−1.851
ENSSSCG00000015997	PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator pseudogene 1	−1.927
ENSSSCG00000006612	S100A10	S100 calcium binding protein A10	−1.937
ENSSSCG00000007982	MPG	N-methylpurine-DNA glycosylase	−1.949
ENSSSCG00000015570	IVNS1ABP	Influenza virus NS1A binding protein	−1.963
ENSSSCG00000014170	CAST	Calpastatin	−2.067
ENSSSCG00000006661	VPS45	Vacuolar protein sorting 45 homolog (S. cerevisiae)	−2.105
ENSSSCG00000000125	LGALS1	Lectin, galactoside-binding, soluble, 1	−2.133
ENSSSCG00000009565	GAS6	Growth arrest-specific 6	−2.193
ENSSSCG00000008428	MSH2	MutS homolog 2	−2.509
ENSSSCG00000013252	F2	Prothrombin Precursor (EC 3.4.21.5) (Coagulation factor II)	−2.746
ENSSSCG00000005512	C5	Complement component 5	−2.793
ENSSSCG00000017473	TOP2A	Topoisomerase (DNA) II alpha 170 kDa	−4.029
ENSSSCG00000009330	ALOX5AP	Arachidonate 5-lipoxygenase-activating protein	−4.058

The method is particularly advantageous in situations where there are few replicates, poor signal to noise ratios, or non-homogenous variances [18]. Second, the amount of total RNA isolated from tissue was insufficient for microarray analysis without nonlinear amplification. Therefore, a two-cycle amplification was necessary to increase the amount of cRNA for testing. Alternative methods of RNA isolation, such as the Qiagen lipid kit, may alleviate this limitation in future studies. As valve leaflets were only exposed to pressure, we did not address the potentially important role of other mechanical factors that are part of the valve mechanical environment *in vivo* such as tensile and compressive strain, shear stress, and flexure. When these forces are combined, they could potentially have an antagonistic or a synergistic effect. Another limitation in our study is our Pathway analysis using IPA. While IPA helped in identifying molecular networks responsive to elevated pressure in VICs, it failed to capture species-specific information that could be pertinent to effects of pressure on VICs. Furthermore, information within IPA knowledge base is obtained from scientific literature, and thus gene functions and interactions are subject to the last update of the software.

To obtain a pure population of VICs, the endothelium was removed from leaflets. Endothelial denudation may have multiple effects on the biology of the tissue. It has been proposed that alterations in the mechanical environment of the leaflets could be transduced into a pathobiological response via a two-way communication system between endothelial and interstitial cells. Denudation disrupts this communication and may expose the subendothelial interstitial cells to mechanical stimuli that they do not see when the endothelium is intact. This limitation would also be present in cell culture; however, by using an organ culture system, VICs were retained in their native three-dimensional ECM. The ECM is important for the transmission of mechanical signals to cells and thus, this system has a distinct advantage over mechanical studies performed with isolated cells. The alternative is *in vivo* studies. Although these have greater physiological relevance, they do not allow for strict mechanical characterization or isolation of the effects of pressure.

In conclusion, pressure-induced changes in the porcine aortic valve interstitial cell transcriptome are reported. This study provides rationale for further investigation of highly

connected and highly regulated genes as potential therapeutic targets.

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Review Article

Oxidative Stress and Inflammation in Heart Disease: Do Antioxidants Have a Role in Treatment and/or Prevention?

Fredric J. Pashkow[1, 2]

[1] *John A. Burns School of Medicine, University of Hawai'i, Honolulu, HI, USA*
[2] *Manoa Innovation Center, 2800 Woodlawn Drive, Honolulu, HI 96822, USA*

Correspondence should be addressed to Fredric J. Pashkow, fpashkow@cardaxpharma.com

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Inflammation triggered by oxidative stress is the cause of much, perhaps even most, chronic human disease including human aging. The oxidative stress originates mainly in mitochondria from reactive oxygen and reactive nitrogen species (ROS/RNS) and can be identified in most of the key steps in the pathophysiology of atherosclerosis and the consequential clinical manifestations of cardiovascular disease. In addition to the formation of atherosclerosis, it involves lipid metabolism, plaque rupture, thrombosis, myocardial injury, apoptosis, fibrosis and failure. The recognition of the critical importance of oxidative stress has led to the enthusiastic use of antioxidants in the treatment and prevention of heart disease, but the results of prospective, randomized clinical trials have been overall disappointing. Can this contradiction be explained and what are its implications for the discovery/development of future antioxidant therapeutics?

1. Introduction

While the importance of inflammation in illnesses where the phenomenon is overt, such as following trauma or infection has been recognized since ancient times, its presence and crucial role in the manifestation of many diseases never previously recognized as inflammatory is relatively recent. In such instances, the source of the inflammation is also often imperceptible [1]. This is especially relevant to the many pervasive chronic diseases that are still responsible for so much human suffering. We are currently achieving a major understanding of what is involved in the initiation of the inflammatory signaling cascade as well as the complex signaling pathways themselves that transcribe and counterregulate the molecular messengers (cytokines) that generate the biological combatants such as the inflammatory enzymes associated with the numerous relevant pathologies. In this paper we will overview some of the details of our emerging understanding of inflammation and its principal source, oxidative stress. Further, we will critically review the historical advocacy of antioxidants for the treatment and prevention of inflammatory-initiating oxidative stress, and proffer explanations for the failure to date of antioxidants to achieve therapeutic success. Finally, we will discuss the appropriateness of oxidative stress as a therapeutic target in cardiovascular disease [2] and the implications this has in us moving forward in the discovery and development of new safe and effective cardiovascular drugs.

2. Inflammation: A Major Cause of Human Disease

While inflammation occurring as a consequence of oxidative stress is not the only biological manifestation of excess ROS/RNS [3], inflammation resulting from oxidative stress is the cause of much human disease [4]. Typical examples are dyslipidemia [5], thrombosis [6, 7], metabolic syndrome [8], type 2 diabetes [9], nonalcoholic steatohepatitis (NASH) [10–12], macular degeneration [13], and neurodegenerative diseases such as Alzheimer's [14]. Inflammation is also a key factor in all aspects of coronary disease including the initiation and progression of atherosclerotic plaque, plaque rupture, and thrombosis (atherothrombosis), especially in recurrent thrombosis where oxidative stress is known to play a significant role [15] (Figure 4), including in those with normal cholesterol levels and in those being treated with

Table 1: Human proof-of-concept studies demonstrating effectiveness of various antioxidant regimens on cardiovascular endpoints.

Study	n	Intervention	Endpoint	Antiox Rx results	Placebo results	P value
SPACE [16]	196	Vit E 800 IU/day	Composite endpoint[1]	16%	33%	= 0.014
IEISS [17]	125	Vit A 50,000 IU/day, Vit C 1,000 mg/day, Vit E 400 mg/day, β-carotene 25 mg/day	Individual component scores[2]	20.6	30.6	"Sig. less"
VCE-MI [18]	61	Vit C&E 600 mg/day	SAECG[3]	No Δ	"Sig. Δ" [3]	<0.002
PART [19]	101	Probucol 1,000 mg/day	Restenosis p PCI	23%	58%	= 0.001
ASAP [20]	520	d-alpha-tocopherol 91 mg, Vit C 250 mg/day	Carotide IMT	0.011 mm/year-1	0.020 mm/year-1	= 0.008
MVP [21]	317	β-carotene 30,000 i.u., Vit C 500 mg/day, Vit E 700 IU/day, Probucol 500 mg/day	Restenosis p PCI	28.9%	38.9%	"Sig. less"

SPACE: Secondary Prevention with Antioxidants of Cardiovascular disease in Endstage renal disease; IEISS: Indian Experiment of Infarct Survival Study; VCE-MI: Vitamins C&E on Myocardial Infarction; PART: Probucol Angioplasty Restenosis Trial; ASAP: Antioxidant Supplementation in Atherosclerosis Prevention; MVP: Multivitamins and Probucol Study Group.
[1]Composite Endpoint: myocardial infarction (fatal and nonfatal), ischemic stroke, non-AV fistular peripheral vascular disease, and unstable angina.
[2]Individual Component Scores: mean infarct size (creatine kinase and creatine kinase-MB gram equivalents), serum glutamic-oxaloacetic transaminase, cardiac enzyme lactate dehydrogenase increased, and, QRS score in the electrocardiogram.
[3]SAECG: Signal-average electrocardiogram components consist of increase in mean QRS and low-amplitude (<40 microV) signal durations, a decrease in the root-mean-square voltage of the last 40 ms of the QRS complex.

"statins" and antiplatelet agents. This inflammation, caused by oxidative stress, could be a target for a great next wave of cardiovascular therapeutics.

3. Role of Oxidative Stress

Oxidative stress has been identified as critical in most of the key steps in the pathophysiology of atherosclerosis and acute thrombotic events, including dyslipidemia leading to atheroma formation, the oxidation of LDL, endothelial dysfunction, plaque rupture, myocardial ischemic injury, and recurrent thrombosis (i.e., the secondary, or subsequent clot that often occurs after initial thrombolysis). The role of oxidative stress in the connection between the various coronary disease risk factors such as elevated blood pressure, diabetes and cigarette smoking, and the clinical sequelae of disease associated with vasoconstriction, thrombosis, plaque rupture, and vascular remodeling has been recognized by Moreno and Fuster [22] but its recognition as a specific therapeutic target represents an elevation in its importance in the oxidative stress hypothesis [23]. Proinflammatory cytokines are also involved in cardiac muscle dysfunction and in the complex syndrome of heart failure [24–27]. Oxidative stress has been implicated as well in diabetic cardiomyopathy [28, 29], congestive cardiomyopathy [30], and hypertensive heart disease [31].

4. Potential Role of Antioxidants

Recently, progress has been made regarding the source of the oxidative stress and an understanding has been achieved regarding the role of the signaling cascade that moderates the resulting inflammatory process. However, as far back as the late 1940's (and perhaps before), antioxidants such as vitamin E have been suggested as potentially useful in the treatment of vascular disease [32]. Studies on the inhibition of experimental cholesterol arteriosclerosis in animals were published around 1949-1950 and specific discussions of the use of vitamin E in the treatment of cardiovascular disease appeared the same year [33, 34].

Over the years an oxidative stress hypothesis supported by epidemiologic and observational evidence that encouraged belief in and the use of antioxidants [35, 36]. For example, studies of fruit and vegetable consumption, those particularly rich in vitamin C and other antioxidants, correlated with a reduction in CVD mortality [37]. Further, the plasma level of vitamin E was inversely related to mortality from ischemic heart disease [38]. Numerous observational studies, such as the Nurses' Health Study, reported significantly reduced risk in those taking vitamin E [39].

5. Human Proof-of-Concept Studies Initially Encouraging

Up to the year 2000, several "smallish" trials using various combinations of antioxidant vitamins and drugs were reported as "positive." This produced optimism in the community of antioxidant advocates. The conclusions from a selection of these Phase 2-type studies are summarized in the remainder of this paragraph and Table 1. In hemodialysis patients with prevalent cardiovascular disease, supplementation with 800 IU/day vitamin E reduced composite cardiovascular disease endpoints and myocardial infarction according to Boaz and colleagues in Israel [16]. Singh and associates reported results from a study in India suggesting that combined treatment with antioxidant vitamins A, E, C,

and beta-carotene in patients with recent acute myocardial infarction might be protective against cardiac necrosis and oxidative stress and could be beneficial in preventing complications and in reduction of the cardiac event rate in ACS patients [17]. Chamiec and colleagues reported results supporting the hypothesis that in patients with AMI, oxygen-free radical-induced cellular damage contributes to alterations in electric function of the heart as seen on the signal-averaged ECG (SAECGs) and that vitamins C and E could reduce these alterations [18]. Yokoi et al. reported that probucol administered beginning 4 weeks before PTCA appears to reduce subsequent restenosis rates [19]. Salonen et al reported that combined supplementation with reasonable doses of both vitamin E and slow-release vitamin C could retard the progression of common carotid atherosclerosis in men [20]. And finally, Tardif and his colleagues reported from Montreal that the antioxidant probucol, with or without a combination of antioxidant vitamins, is effective in reducing the rate of restenosis after balloon coronary angioplasty [21].

It is important to note that these studies are all fairly modest in size with the exception of ASAP [20] which enrolled more than 500 subjects, and that with the exception of SPACE [16], use a surrogate measure for the primary endpoint. Note that all of the therapies tested, again with the exception of SPACE, involved subgroups where more than one antioxidant or combinations of therapy were used. Finally, while the statistical analyses suggest overall significance of the studies' findings, only those receiving the drug probucol with or without multivitamins demonstrated significant effect in the Mutivitamins and Probucol Study Group [21]. The implications of these observations will be discussed further below.

6. Larger Randomized Clinical Trials Unsupportive

But problems developed with the performance of larger randomized clinical trials [41]. An earlier meta-analysis of 6 large (>1,000 subjects) randomized trials of vitamin E with pooled data from over 77,000 subjects and 6 trials of β-carotene in over 131,000 subjects showed that the use of vitamin E was a "wash" ($P = 0.94$) (Figure 1(a)) and that β-carotene use was associated with a worse outcome ($P = 0.003$) (Figure 1(b)) [40].

The Cambridge Heart Antioxidant Study (CHAOS) buoyed hopes for believers in the oxidant stress hypothesis when it demonstrated a significant reduction in nonfatal MI ($P = 0.0001$) but offsetting that finding was an insignificant difference in cardiovascular deaths ($P = 0.78$) [42]. However, in a large, long-term trial of male physicians, neither vitamin E nor vitamin C supplementation reduced the risk of major cardiovascular events [43]. In women at high risk for CVD there were no overall effects of ascorbic acid, vitamin E or β-carotene on cardiovascular events [44]. Hence, there is a need for a better understanding and more scientific evidence of the relative contribution of major nutraceutical constituents to the inhibition of the progression of atherosclerosis and its clinical consequences [45].

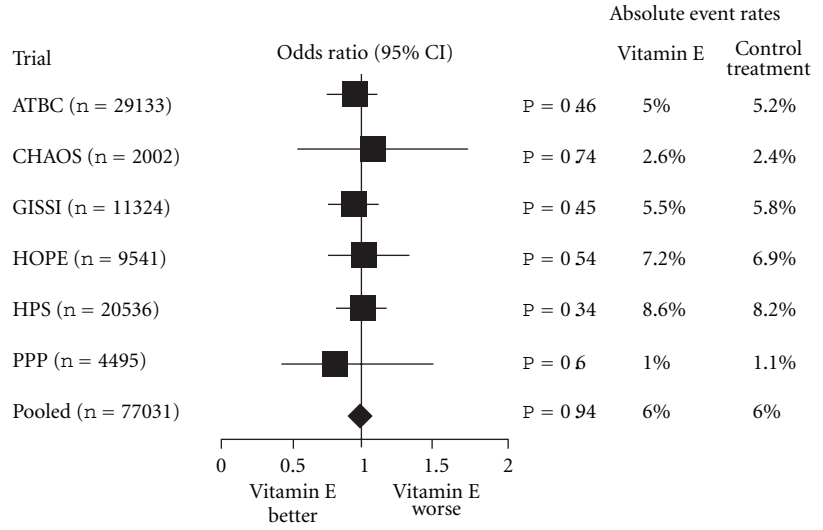
Studies looking at progression of atherosclerosis using vitamins E, C or β-carotene have also been inconclusive [47]. However, certain specific patient populations or clinical circumstances showed promise: for example, reduction in the development of transplant atherosclerosis [48]. In acute coronary syndrome, N-Acetylcysteine (NAC) appeared to produce a statistically significant improvement in cardiac index in STEMI patients treated with streptokinase and NTG [49], and in a more recent study, a significant reduction of in-hospital deaths in patients undergoing primary PCI [50].

7. Why the Failure of So Many Antioxidant Trials?

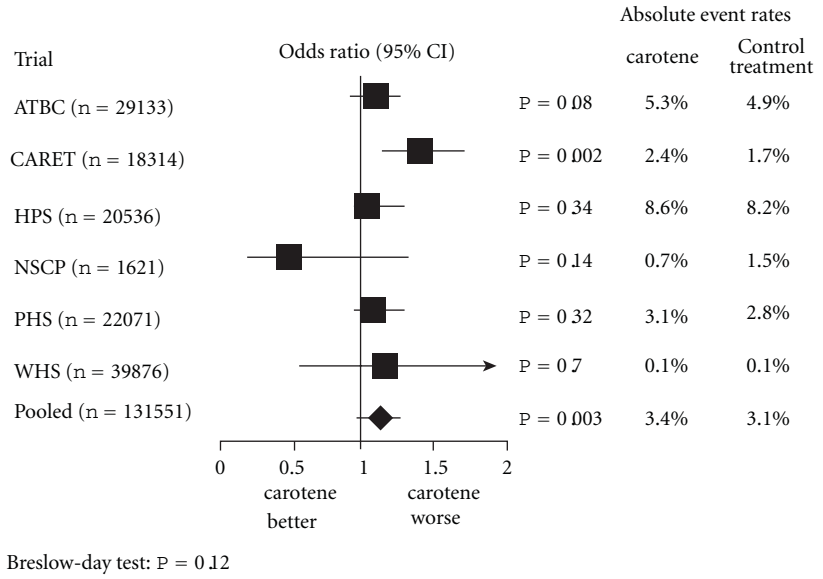
The question is, if oxidative stress is so critical in the development and manifestations of coronary heart disease, why is it that so many of the larger antioxidant trials have failed [52]? Before specifically attempting to answer this critical question, let us go back to basics for a moment. The utilization of oxygen as an integral part of the process for generating metabolic energy (i.e., mainly via the Electron Transport Chain or ETC on the inner membrane of the mitochondria) produces reactive oxygen species (ROS) [53, 54]. These reactive oxygen species can damage cells or components of cells by oxidizing DNA or proteins or starting chemical chain reactions such as lipid peroxidation which incidentally, occurs mainly *inside* the bilayer membrane of cells, nuclei, and mitochondria [55]. ROS can be quite destabilizing to membrane integrity, but they do have important useful functions, such as the maintenance of balanced intracellular redox signaling. The function of antioxidant systems is not to remove these oxidants entirely, but instead to keep them at a level below which they will trigger the inflammatory cascade, a series of intracellular and intranuclear signaling that results in the release of destructive inflammatory cytokines [56, 57].

8. Inflammation Is Complex

Pathological inflammation, a complex whole-cellular pathway, is a cascade that begins with the production of excess free radicals that frequently arise from mitochondria responding to internal or environmental stress and that trigger several signaling steps that endup producing the substances that actually cause the classical signs of redness, swelling, and pain in inflammation [1]. NF-kappa-B, one of the key signaling molecules, is a transcription factor that upregulates the production of downstream inflammatory mediators, including tumor necrosis factor-alpha (TNF-alpha) [58], inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and interleukein-1beta (IL-1beta). Recent studies have suggested that CD40-CD40L interactions themselves regulate oxidative stress and affect various signaling pathways in both the immunological and cardiovascular systems [59]. Normal cellular functions "suckup" these unwanted ROS allowing the signaling molecules, or cellular pathways such as NF-kappa-B, to operate normally along with the downstream products of these pathways, creating a balanced ROS environment and normal cellular health.



(a)



(b)

Figure 1: (a) Meta-analysis of large randomized trials of vit E versus placebo**.** Meta-analysis of 7 randomized trials involving 77,031 patients comparing the risk of cardiovascular death among those randomized to placebo or vitamin E (Breslow-Day test, $P = 0.73$). ATBC: α-Tocopherol, β-Carotene Cancer Prevention trial; CHAOS: Cambridge Heart Antioxidant Study; CI: Confidence Interval; GISSI – Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto; HOPE: Heart Ourcomes Prevention Evaluation; HPS: Heart Protection Study; PPP: Primary Prevention Project, modified from [40]. (b) Meta-analysis of Randomized Trials of β-carotene versus Placebo. Meta-analysis of 6 randomized trials involving 131,551 patients comparing the risk of cardiovascular death among those randomized to placebo or β-carotene (Breslow-Day test, $P = 0.12$). ATBC: α-Tocopherol, β-Carotene Cancer Prevention trial; CARET: β-Carotene and Retinol Efficacy Trial; HPS: Heart Protection Study; NSCP: Nambour Skin Cancer Prevention; PHS: Physicians' Health Study; WHS: Women's Health Study, modified from [40].

9. Erroneous Assumptions Influenced Trial Design

So why have so many antioxidant therapies failed when tested in randomized clinical trials? Studies were conducted based upon epidemiologic findings of benefit from surveys documenting increased intake of dietary fruits/vegetables [60]. The well-known biases of such studies aside, the assumption has been that "known" nutritional compounds that is, vitamin E, vitamin C, and β-carotene, are mainly responsible for the benefit. The favorable effects shown by some studies relating antioxidant dietary intake and cardiovascular disease may have been exerted by other chemicals present in foods. Flavonoids, for example, are ideal candidates, since they

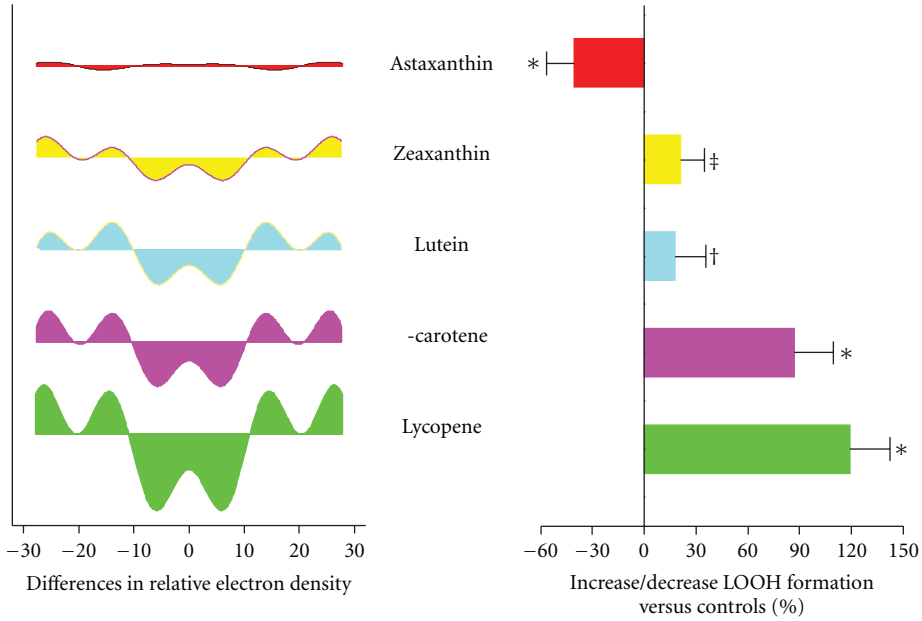


Figure 2: Antioxidant activity depends on molecular structure and localization ("Alignment") in cellular/mitochondrial Membrane. Extent of deviation in the X-ray crystallographic pattern correlates with an increase in lipid peroxide formation. Correlation between membrane structure changes and LOOH formation (LOOH, lipid peroxide). Differences in relative electron density as a function of treatment with various carotenoids in POPC membranes containing a C/P mole ratio of 0.2. For the peroxidation study, various carotenoids (10 μM) were incorporated into DLPC membranes and underwent lipid peroxidation at 37°C for 48 h. Expressed as percent increase or decrease in LOOH (lipid peroxide) formation compared to controls containing no carotenoids. $^{*}P < 0.001$ versus control; $^{\ddagger}P < 0.01$ versus control; $^{\dagger}P < 0.05$ versus control; $n = 5 \sim 6$. (POPC, 1-palmitoyl 2-oleoyl-3-sn-glycerophosphatidylcholine), adapted from McNulty et al. [46].

are plentiful in foods containing antioxidant vitamins (i.e., fruits and vegetables) and are potent antioxidants [61]. Other examples include cocoa [62, 63] and the active ingredients in wine [64, 65].

The naïve assumption is that all antioxidants are essentially the same. Nothing can be further from the truth. All antioxidants are not the same: they may work in substantially different ways (chain-breaking versus singlet oxygen quenching, e.g.), and in different locations (e.g., in the bilayer membrane versus the cytoplasm) and *very small* differences in molecular structure can have profound influence on biological activity. In the carotenoid family, for example, distinct effects occur in lipid peroxidation due to membrane structure changes. β-carotene, which misaligns when localized in the bilayer membrane is highly disruptive structurally and can be functionally pro-oxidant when compared to structurally similar members of the family that align completely (Figure 2) [46]. These contrasting effects of carotenoids on lipid peroxidation may explain the clinical outcomes observed in various randomized trials.

10. Understanding Antioxidant Mode of Action Is Critically Important

Further, lack of understanding of the mode of action [66] has led to erroneous clinical designs and patient selection. Little attempt was made to scale the antioxidant potential of the therapy to the underlying oxidative stress. Atherosclerosis is a multifactorial disease and LDL is oxidized by all major cells of the arterial wall during the development of atherosclerosis via more than one mechanism. The various LDL oxidation pathways produce several lipid peroxidation products such as isoprostanes from several fatty acids, oxysterols from unesterified and esterified cholesterol, hydroxy fatty acids, lipid peroxides, and aldehydes. Intervention trials should be accompanied by measurements of one or more of these relevant biomarkers at intervals during the study and the correlation of the biomarkers to the therapeutic intervention needs to be established. In addition to the markers in use for lipid peroxidation, there is a need to include markers for endothelial dysfunction, monocyte adhesion, macrophage uptake of lipoproteins, thrombotic, and inflammatory processes [67].

Our recognition of the connection between oxidative stress, inflammatory signaling, and such critical manifestations of atherosclerotic cardiovascular disease as atherothrombosis is growing. The cellular membranes of endothelial cells can possess oxidized phospholipids with protruding *sn*-2-oxidized fatty acid acyl chains into the extracellular space. This conformation renders them accessible to interact with scavenger receptors and other pattern recognition receptors on the surface of platelets or probing macrophages of the circulatory and the immune system (Figure 3) [51].

Oxidative stress is an important mediator of both abnormal platelet function and dysfunctional endothelium-dependent vasodilation in the setting of cardiovascular disease. Superoxide anion is an important source of oxidative stress, has direct effects, and limits the biological activity

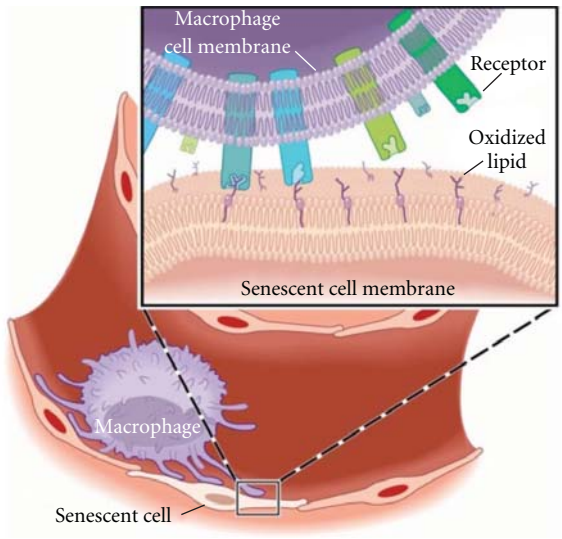


Figure 3: Oxidized phospholipids protrude from cell membranes in senescent endothelial cells forming oxidized lipid "whiskers." Schematic representation of the lipid whisker model. Cell membranes of senescent endothelial cells can possess oxidized phospholipids with protruding *sn*-2-oxidized fatty acid acyl chains into the extracellular space. This conformation renders them accessible to interact with scavenger receptors and other pattern recognition receptors on the surface of platelets or probing macrophages of the circulatory and the immune system, adapted from [51].

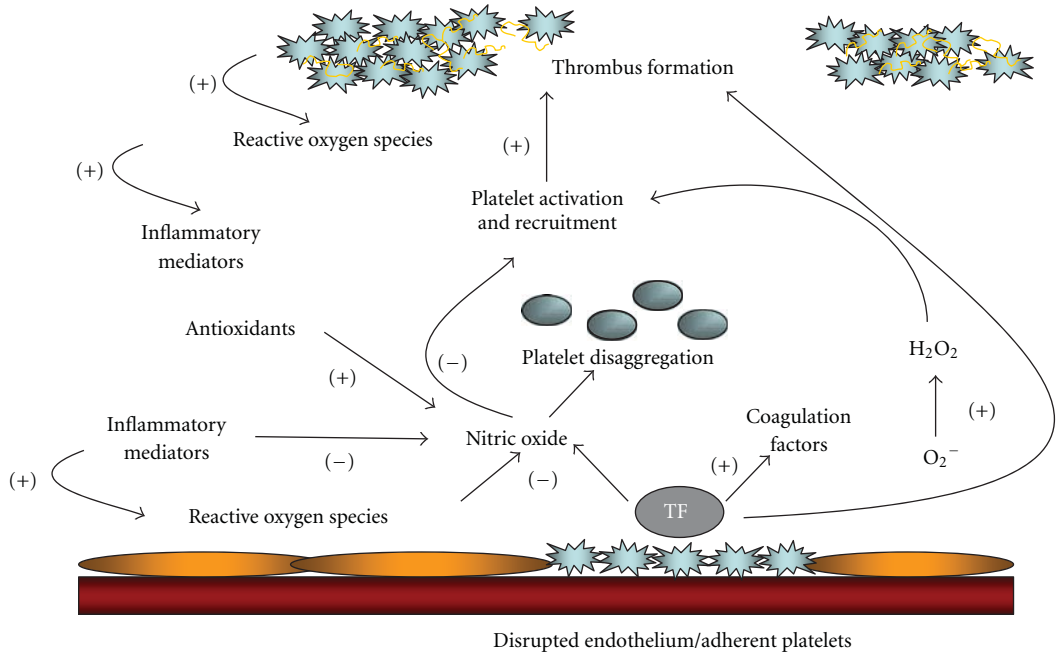


Figure 4: Oxidative stress mediates abnormal platelet function and dysfunctional endothelium-dependent vasodilation. Oxidative stress is an important mediator of both abnormal platelet function and dysfunctional endothelium-dependent vasodilation in the setting of cardiovascular disease. Superoxide anion is an important source of oxidative stress, has direct effects, and limits the biological activity of NO. Excessive vascular superoxide production drives further platelet activation and recruitment leading to greater thrombus formation. The occurrence of superficial intimal injury caused by endothelial denudation and deep intimal injury caused by plaque rupture expose collagen and Tissue Factor (TF) to platelets. Local platelet activation stimulates further thrombus formation and additional platelet recruitment by supporting cell-surface thrombin formation and releasing potent platelet agonists such as adenosine diphosphate (ADP), serotonin, and thromboxane A2. A thrombus forms as platelets aggregate via the binding of bivalent fibrinogen to GP IIb/IIIa. Platelet NO release influences platelet recruitment to the growing thrombus and impaired platelet-derived NO release is likely associated with acute coronary and stroke syndromes. Antioxidants may indirectly inhibit platelets through scavenging of reactive oxygen species, many of which alter platelet function. Despite the different subcellular locations of water- and lipid-soluble antioxidants, these antioxidant pathways in platelets are closely linked. Antioxidants may also indirectly inhibit platelets through the metabolism of reactive oxygen species, many of which alter platelet function. Inflammation is linked with the evolution of cardiovascular disease and acute coronary syndromes, adapted from [15].

of NO. Excessive vascular superoxide production drives further platelet activation and recruitment leading to greater thrombus formation. The occurrence of superficial intimal injury caused by endothelial denudation and deep intimal injury caused by plaque rupture expose collagen and tissue factor [62] to platelets. Local platelet activation stimulates further thrombus formation and additional platelet recruitment by supporting cell-surface thrombin formation and releasing potent platelet agonists such as adenosine diphosphate (ADP), serotonin, and thromboxane A2. A thrombus forms as platelets aggregate via the binding of bivalent fibrinogen to GP IIb/IIIa. Platelet NO release influences platelet recruitment to the growing thrombus and impaired platelet-derived NO release is likely associated with acute coronary and stroke syndromes (Figure 4) [15]. Thus, antioxidants may indirectly inhibit platelets through scavenging of reactive oxygen species, many of which alter platelet function. Despite the different subcellular locations of water- and lipid-soluble antioxidants, these antioxidant pathways in platelets are closely linked. Antioxidants may also indirectly inhibit platelets through the metabolism of reactive oxygen species, many of which directly alter platelet function.

Oxidative stress and inflammation are intimately linked with both the evolution of cardiovascular disease and acute coronary syndromes. It should be no surprise that ox-LDL levels show a significant positive correlation with the severity of acute coronary syndromes and that the more severe lesions also contain a significantly higher percentage of ox-LDL-positive macrophages. Such observations suggest that increased levels of ox-LDL relate to plaque instability in human coronary atherosclerotic lesions [68, 69].

11. Dose-Response Documentation Lacking

Implicit in the randomized trials is that the dose of antioxidant tested (usually vitamin E), effectively suppressed oxidative stress but this was never determined [70]. In fact, studies suggest that the dosages of the compounds tested and/or the duration of therapy was not adequate. In one time-course study, maximum suppression of plasma F2-isoprostane concentrations did not occur until 16 weeks of supplementation. In the dose-ranging study there was a linear trend between the dosage of vitamin E and percentage reduction in plasma F2-isoprostane concentrations which reached significance at doses of 1600 IU ($35 \pm 2\%$, $P < 0.035$) and 3200 IU ($49 \pm 10\%$, $P < 0.005$) [70]. Whether such dosages in human subjects would be safe and if the compound was administered early enough in the lifecycle of the disease process are other essential considerations.

12. Safety Now an Overarching Issue

Safety is the overarching issue in drug development today, but little was done historically to determine if the antioxidant being tested for an inflammatory-mediated cardiovascular manifestation is safely tolerated at the levels required to provide therapeutic relief [71, 72]. Since the high standards of chemistry, manufacturing, and controls (CMC) required for pharmaceutical drugs are unlikely to be applied to nutraceutical or dietary-supplement-type products, many of the questions regarding safety and efficacy of antioxidants will most likely be answered in the future related to the development of proprietary prodrugs seeking regulatory approval. These antioxidant drug candidates, incidentally, will most likely have greater druglikeness and bioavailability.

13. Conclusion

Despite the lack of significant randomized clinical trial data supporting their use, more than $20b is still being spent annually on the antioxidant vitamins A, C, and E with more than 6 million tons of the latter projected to be consumed annually on a global basis [73]. My belief is that antioxidants because of their provenance as "natural products" or "nutritional supplements" and their presumption of safety and efficacy generated from the results of epidemiologic and observational studies early-on, have not been subjected to the same stringent developmental requirements that are applied to new pharmaceutical drug candidates. Biologically active compounds, formulated properly, administered in appropriate amounts for an appropriate duration to the right patients will be required to achieve all the requirements that truly define therapeutic success.

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Review Article

Inflammatory Concepts of Obesity

Viviane Zorzanelli Rocha[1] and Eduardo J. Folco[2]

[1] *Lipid Clinic, Heart Institute, University of Sao Paulo, 05403-900 Sao Paulo, SP, Brazil*
[2] *Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*

Correspondence should be addressed to Eduardo J. Folco, ejfolco@rics.bwh.harvard.edu

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Obesity, long considered a condition characterized by the deposition of inert fat, is now recognized as a chronic and systemic inflammatory disease, where adipose tissue plays a crucial endocrine role through the production of numerous bioactive molecules, collectively known as adipokines. These molecules regulate carbohydrate and lipid metabolism, immune function and blood coagulability, and may serve as blood markers of cardiometabolic risk. Local inflammatory loops operate in adipose tissue as a consequence of nutrient overload, and crosstalk among its cellular constituents-adipocytes, endothelial and immune cells-results in the elaboration of inflammatory mediators. These mediators promote important systemic effects that can result in insulin resistance, dysmetabolism and cardiovascular disease. The understanding that inflammation plays a critical role in the pathogenesis of obesity-derived disorders has led to therapeutic approaches that target different points of the inflammatory network induced by obesity.

1. Introduction

Atherothrombosis is the basis of most coronary, peripheral, and cerebral arterial disease and is a critical health burden and major cause of death worldwide [1]. Despite the undeniable importance of cardiovascular disease in morbidity and mortality in most regions of the world, control of risk factors and advances in the treatment of atherothrombosis have significantly reduced age-adjusted cardiovascular events in USA and Western Europe. However, all this progress in the war against cardiovascular disease has been threatened by the dramatic increase in the prevalence of obesity, an important risk factor for both atherogenesis and increased coagulability [1].

The significant advance of the obesity epidemic worldwide and the association between atherothrombosis and obesity have attracted great interest from the scientific community, contributing importantly to increase the understanding of the pathophysiology of excess adiposity. Indeed, several concepts related to obesity pathophysiology have changed in the last 2 decades [2]. The hypothesis of obesity as a low-grade chronic and systemic inflammatory disease gradually replaced the idea of a mere lipid deposit disease characterized by inert adipose tissue and passive accumulation of fat in the context of weight gain [2, 3]. Several research groups demonstrated that adipose tissue of obese animals and humans produces increased amounts of inflammatory mediators and presents higher number of inflammatory cells compared to adipose tissue of lean controls [2, 3]. This recently recognized endocrine role of adipose tissue likely provides a crucial mechanistic link between obesity and atherothrombosis.

2. Inflammatory Mechanisms of Obesity

2.1. The Local Inflammatory Network in Adipose Tissue. The first clues supporting the involvement of inflammation in obesity came to light almost half a century ago, including a report that described increased plasma levels of fibrinogen in obese patients [4]. However, the inflammatory view of obesity started attracting interest in the 1990s, particularly after the demonstration of enhanced expression of tumor necrosis factor-alpha (TNF-α) in adipose tissue of obese rodents and the amelioration of insulin resistance after neutralization of

this potent cytokine [5, 6]. Since the publication of these reports, several other groups demonstrated the production and secretion of multiple cytokines, chemokines, hormones, and other inflammatory mediators by adipose tissue, collectively referred to as adipokines, culminating with the recognition of adipose tissue as one of the greatest endocrine organs in the body [7]. Besides the aforementioned cytokine TNF-α, macrophage chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6), leptin, and adiponectin are remarkable examples of adipokines differentially expressed by obese adipose tissue, with potentially important roles in the pathophysiology of obesity, either locally or systemically [7–11].

Adipocytes constitute the major cell type of adipose tissue, being a major source of several bioactive products secreted by this tissue. Indeed, they are responsible for the production of several adipokines, among which adiponectin and leptin are likely exclusively elaborated in adipocytes. Pioneer work published in 2003 demonstrated that adipocytes are not alone and that macrophages also accumulate in adipose tissue of obese animals (Table 1 and Figure 1), coinciding with increased expression of inflammatory markers and preceding a significant increase in circulating insulin levels [12, 13]. Since then, extensive work has supported the involvement of macrophages in the inflammatory network of adipose tissue. Studies on the contribution of MCP-1 and its receptor, chemokine (C-C motif) receptor 2 (CCR2), both important mediators of macrophage recruitment to sites of inflammation (Figure 1), demonstrated reduced macrophage accumulation in adipose tissue of diet-induced obese mice deficient in either of those genes [22, 23]. These knockout animals exhibit decreased insulin resistance and hepatic steatosis, suggesting that adipose tissue macrophage influx contributes to the local and systemic metabolic effects of obesity [22, 23]. However, the role of the MCP-1/CCR2 duo in obesity-induced inflammation remains incompletely understood, as not all studies found the influence of MCP-1 deficiency relevant to macrophage accumulation in adipose tissue or insulin sensitivity [24, 25].

Macrophages are not all equal in the inflamed adipose tissue, according to differential phenotypic patterns and chemokine receptor usage [26]. Whereas the so-called resident macrophages, the dominant subtype in lean fat tissue, predominantly express markers of alternative activation or M2 (such as mannose receptor C type I), the infiltrative macrophages, widely present within the obese adipose tissue, are characterized by their enhanced expression of classic activation or M1 markers, such as TNF-α and inducible NO synthase (iNOS) [14]. Interestingly, adipose tissue-derived macrophages from obese CCR2-deficient mice present significantly less expression of M1 markers than their wild-type counterparts, with M2 markers at levels comparable to those from lean mice [14]. Thus, although CCR2-deficient animals have a less prominent subset of infiltrative macrophages within their adipose tissue, their population of adipose tissue-resident macrophages remains intact, suggesting the usage of distinct chemokine receptors by different subsets of macrophages and local operation of various chemotactic systems [14].

In the last five years, other inflammatory cell types have also been gathering attention in the pathophysiology of obesity. T cells, although less numerous than macrophages, also accumulate in adipose tissue of obese mice (Table 1 and Figure 1). We and others showed the presence of both T cell subpopulations, $CD4^+$ and $CD8^+$, in fat tissue [15–18]. Nishimura and colleagues reported that mice fed a high-fat diet have an increased number of CD8 cells in adipose tissue and that depletion of these cells reduced macrophage infiltration and adipose tissue inflammation and improved systemic insulin resistance [18].

The classic Th1 cytokine interferon-gamma (IFN-γ) also figures importantly in the inflammatory circuit that operates in obese adipose tissue (Figure 1). In one study, high-fat diet promoted a progressive IFN-γ bias among adipose tissue-derived T cells in mice [27]. Furthermore, IFN-γ or IFN-γ-receptor deficiencies lower adipose tissue expression of inflammatory genes and ameliorate metabolic parameters in obese animals [16]. In humans, a positive association of $CD3^+$cells and IFN-γ mRNA expression in adipose tissue with waist circumference in a cohort of patients with type 2 diabetes mellitus suggests the involvement of the Th1 arm of adaptive immunity in obesity-related metabolic disorders [17].

Whereas proinflammatory T cells appear enriched in obese adipose tissue, the pool of anti-inflammatory T cells, $CD4^+$ $Foxp3^+$ T regulatory (Treg) cells, decreases in fat tissue of obese animals compared to their lean counterparts [19] (Table 1). Using loss-of-function and gain-of-function approaches, Feuerer and colleagues revealed that Treg cells influence the inflammatory state of adipose tissue and insulin resistance. The higher number of Treg cells in lean fat tissue may be one important factor to restrain inflammation and keep local homeostasis [19, 27]. In humans, Th1 cells expressing the transcription factor Tbet outnumber $Foxp3^+$ T cells with a ratio of approximately 12 : 1 in visceral adipose tissue of obese individuals, compared to 6 : 1 in lean ones [27].

Recent studies highlighted potential roles for other immune cells, such as mast cells and natural killer T (NKT) cells, in adipose tissue inflammation [20, 21] (Table 1). Genetic deficiency of mast cells or their pharmacological stabilization in diet-induced obese mice reduce weight gain, adipose tissue, and systemic inflammation and improve glucose metabolism and energy expenditure [20]. Mice lacking NKT also present less adipose tissue inflammation and glucose intolerance than wild-type control animals when fed a high-fat diet [21]. A recent study has reported an important role of eosinophils in the maintenance of metabolic homeostasis [28]. These cells are major producers of IL-4 that contribute to sustain alternatively activated macrophages in adipose tissue [28].

2.2. Systemic Inflammatory Loops. The global endocrine profile of adipose tissue appears to reflect the interactions among its paracrine loops. In other words, local crosstalks involving adipocytes, endothelial cells, and immune cells result in the production of a wide repertoire of bioactive substances that can act in a paracrine fashion, further

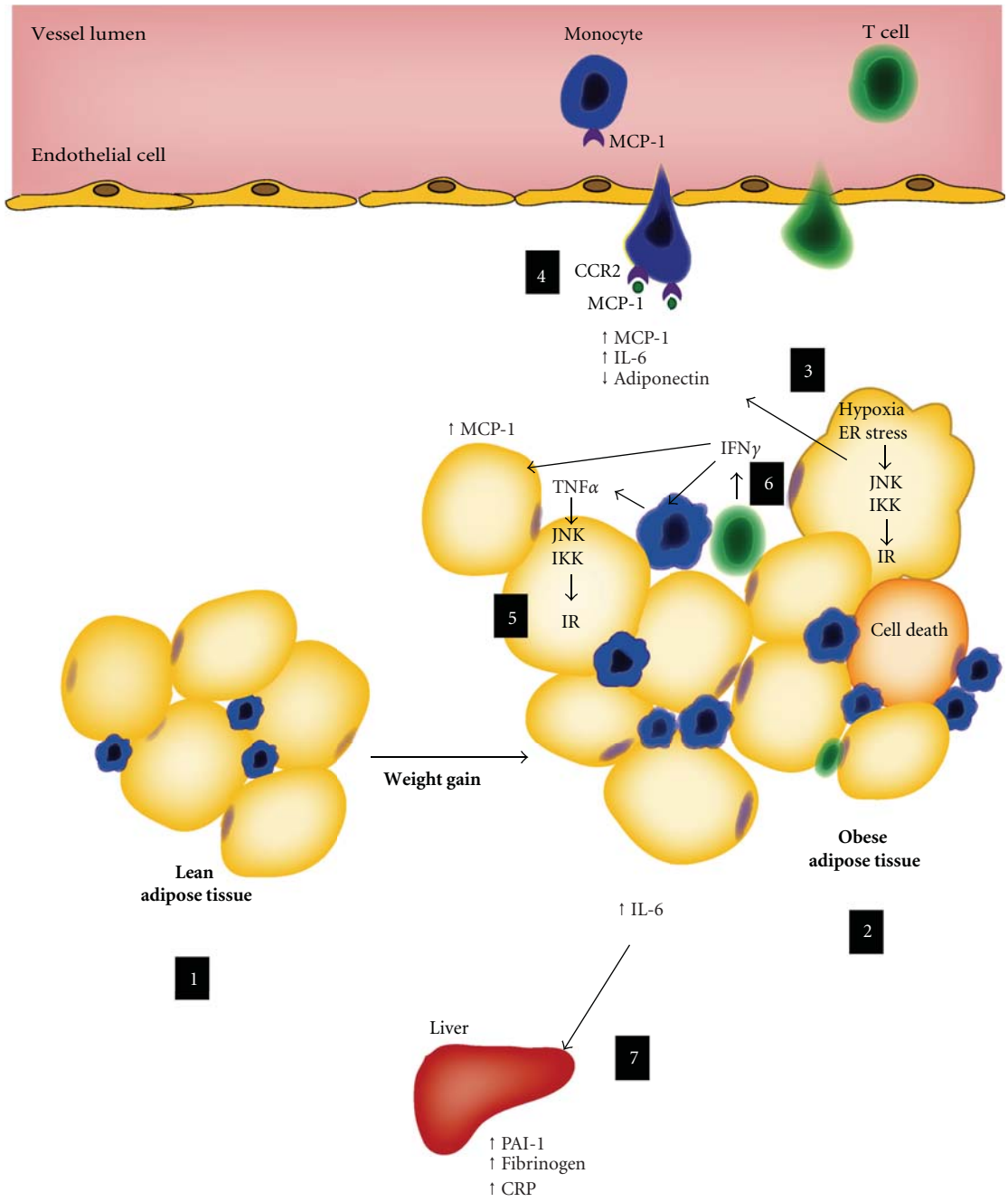

Figure 1: Adipose tissue inflammation in obesity. Whereas lean adipose tissue contains a population of resident inflammatory cells (1) and secretes various active substances, the obese adipose tissue (2) accumulates higher numbers of macrophages and T cells, producing copious amounts of inflammatory mediators, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6), and less adiponectin (3). In the context of nutrient surplus and hypoxia, expanding adipocytes present endoplasmic reticulum (ER) stress (3), important trigger of inflammatory kinases, such as JNK and IKK, which can ultimately inhibit insulin signaling (further detail in the text) and activate inflammatory cascades and the production of inflammatory mediators. Existing evidence suggests that higher production of chemokines, such as MCP-1, within the obese adipose tissue could enhance local macrophage accumulation (4). Once in the tissue, monocyte-derived macrophages can be a fundamental source of tumor necrosis factor-alpha (TNFα), among other mediators. Cytokines like TNFα and other stimuli can cause further activation of inflammatory kinases (5). Several studies have demonstrated that T cells also accumulate in adipose tissue in the obese state (6). Interferon-gamma (IFNγ), a typical T-helper 1 cytokine, likely regulates local expression of TNFα, MCP-1, and other inflammatory mediators, suggesting a role for adaptive immunity in obesity pathophysiology. The spillover of adipokines, such as IL-6, into the circulation can also promote important systemic effects (7), such as increased production of liver-derived acute-phase inflammatory mediators and coagulation-related factors, most of them likely correlated with atherothrombosis.

Table 1: Inflammatory cell types in adipose tissue.

Inflammatory cells in adipose tissue network of obesity	Comments
Macrophages	Accumulate in obese versus lean AT. Presence correlated with ↑ expression of inflammatory mediators in AT and metabolic disturbances [12, 13].
	Infiltrative macrophages and M1 markers predominate over resident macrophages and M2 markers in obese AT [14].
T cells	Fewer than macrophages, they also accumulate in obese versus lean AT [15–17].
	Depletion of CD8 lymphocytes from DIO-mice ↓ macrophage accumulation in AT and ↓ systemic IR [18].
	$CD4^+$ $Foxp3^+$ T regulatory cells (Treg) decrease in AT of obese versus lean mice. Treg cells may keep homeostasis and limit inflammation in lean AT [19].
Mast cells	Genetic deficiency of mast cells or their pharmacological stabilization in DIO-mice ↓ weight gain, AT, and systemic inflammation, and improve glucose metabolism and energy expenditure [20].
NKT cells	DIO-mice lacking NKT cells present less AT inflammation and glucose intolerance than wild-type control animals [21].

AT: adipose tissue; DIO-mice: diet-induced obese mice.

amplifying inflammation within the adipose tissue [29]. At the same time, the spillover of adipokines into the circulation can also promote important systemic effects, and specific mediators such as adiponectin, leptin, plasminogen activator inhibitor-1 (PAI-1), and IL-6 may even serve as blood markers of cardiometabolic risk.

2.2.1. Adiponectin. The most abundant and one of the most extensively studied adipokines is adiponectin. Unlike most other adipokines, adiponectin plasma levels are lower in obese than in nonobese individuals [30]. It circulates in the plasma at levels of 3–30 mg/mL and forms three major oligomeric complexes with distinct biological functions: trimer, hexamer, and high-molecular-mass form, the latter likely being the most bioactive form in vascular cells (reviewed in [31]). There is also a bioactive proteolytic product of adiponectin that includes its C1q-like globular domain, which circulates at low concentration in plasma [32]. The adiponectin receptors, AdipoR1 and AdipoR2, activate signaling molecules such as AMP-activated protein kinase (AMPK), peroxisome-proliferator-activated receptor (PPAR)-α, and p38 mitogen-activated protein kinase (MAPK) [33]. Targeted disruption of AdipoR1 and AdipoR2 simultaneously abrogates adiponectin binding, causing insulin resistance and glucose intolerance [34].

Numerous experimental studies support the idea that adiponectin has antidiabetic properties [31]. Adiponectin-deficient ($APN^{-/-}$) mice exhibited late clearance of free fatty acids from plasma and diet-induced insulin resistance [35], whereas adiponectin delivery via adenovirus in those knockout animals improved insulin sensitivity [35]. In another study, ob/ob mice overexpressing adiponectin showed improved glucose tolerance and reduced triglyceride levels compared to their nontransgenic ob/ob littermates in spite of being morbidly obese [36]. Nevertheless, the interpretation of these results is often difficult from a mechanistic standpoint, because different studies used distinct forms of the recombinant protein [32, 37].

Vast literature also suggests anti-inflammatory and antiatherogenic properties of adiponectin. Studies have shown anti-inflammatory effects of adiponectin on most cells involved in atherogenesis, including endothelial cells and macrophages [38, 39]. Physiological levels of adiponectin attenuate the attachment of monocytes to the endothelium in culture by reducing TNF-α-induced expression of adhesion molecules [38]. Pretreatment of human macrophages with adiponectin attenuates lipopolysaccharide- (LPS-) induced expression of TNF-α, and of a trio of T-lymphocyte chemoattractants associated with atherogenesis: interferon- (IFN-) inducible protein 10 (IP-10/CXCL10), IFN-inducible T-cell alpha chemoattractant (I-TAC/CXCL11), and monokine induced by IFN-gamma (MIG/CXCL9) [39]. Whereas the anti-inflammatory mechanisms elicited by adiponectin are not completely understood, recent work from our laboratory shed some light on this arena. We demonstrated that pretreatment of human macrophages with adiponectin inhibits phosphorylation of nuclear factor κB inhibitor (IκB), c-Jun N-terminal kinase (JNK), and p38 MAPK induced by either LPS or TNF-α as well as signal transducer and activation of transcription 3 (STAT3) phosphorylation induced by IL-6 [40]. Interestingly, treatment of human macrophages with adiponectin alone induced sustained phosphorylation of IκB, JNK, p38, and STAT3 but prevented further activation of these signaling molecules upon addition of pro-inflammatory agonists [40]. These findings and others from additional studies suggest that adiponectin may induce some degree of inflammatory activation that likely mediates tolerance to further treatment with pro-inflammatory stimuli [40–42].

Several animal studies have confirmed the anti-inflammatory and antiatherogenic properties of adiponectin. Adenovirus-mediated delivery of adiponectin to apolipoprotein-E-deficient ($ApoE^{-/-}$) mice, an atherosclerotic murine model, reduced plaque formation in the aortic sinus [43]. Transgenic mice expressing globular adiponectin crossed

with ApoE$^{-/-}$ mice also had less atherosclerotic burden than ApoE$^{-/-}$ control animals, despite similar plasma glucose and lipid levels[44]. Additionally, APN$^{-/-}$ mice exhibited a 5-fold increase in leukocyte adhesion in the microcirculation, in association with decreased NO levels and augmented expression of adhesion molecules in the endothelium [44]. Yet, a recent study that used APN$^{-/-}$ mice and transgenic mice with chronically elevated adiponectin levels crossed to ApoE$^{-/-}$ or LDLR$^{-/-}$ mice found no correlation between adiponectin levels and atheroma development [45].

Multiple clinical studies have correlated hypoadiponectinemia with insulin resistance and type II diabetes mellitus in various populations [46, 47]. Recently, a meta-analysis of prospective studies that involved 14,598 subjects demonstrated that higher adiponectin concentrations associate with lower risk of type II diabetes [48]. A recent study that compared metabolic parameters in insulin-resistant versus insulin-sensitive obese individuals demonstrated that the strongest predictors of insulin sensitivity were macrophage infiltration in adipose tissue and circulating levels of adiponectin [49]. Genetic studies have also provided a link between adiponectin and metabolic disorders. Genetic mutations that likely reduce adiponectin plasma concentrations [50, 51] and adiponectin multimerization [52] associate closely with type II diabetes. These findings, although correlational in nature and thus not proving direct causality, support a primary role for adiponectin in preventing metabolic disease in humans.

Abundant data from several epidemiological studies have also reported an inverse correlation between adiponectin plasma levels and incidence of hypertension [53], dyslipidemia [54, 55], and cardiovascular disease [38, 56, 57]. Patients with coronary artery disease (CAD) have lower adiponectinemia than controls [38]. Similarly, men with both type II diabetes and CAD have lower circulating levels of adiponectin than patients with diabetes without CAD [56]. Another study showed a 2-fold increase in the prevalence of CAD among male patients with low plasma concentrations of adiponectin, independent of classic risk factors [57]. Prospective data have also demonstrated that high plasma adiponectin concentrations associate with lower risk of myocardial infarction in healthy men [58], and decreased CAD risk in diabetic men [59]. However, there are prospective studies that have not observed a correlation between adiponectinemia and risk of future CAD [60, 61]. Furthermore, a large prospective study and meta-analysis found a weak association between CAD and plasma adiponectin levels [62]. Intriguingly, two secondary prevention studies associated adiponectin with an increased risk of recurrent cardiovascular events [63, 64]. These conflicting studies indicate that further prospective analyses of various populations are still necessary to examine whether adiponectinemia can independently predict cardiovascular disease.

2.2.2. Leptin. Leptin, a 16 kDa hormone product of the *ob* gene [65], is predominantly released by adipocytes [66] to control body weight centrally through its cognate receptor in the hypothalamus [67]. Leptin circulates in the plasma at levels proportional to total body adiposity [68] and immediate nutritional state. In fed states, leptin levels increase and, via a central action in the brain, inhibit appetite and stimulate thyroid-mediated thermogenesis and fatty acid oxidation. In a fasting situation, leptin levels fall, and thus appetite increases, and thermogenesis becomes limited.

Leptin deficiency associates with increased appetite and marked obesity in mice and humans, a scenario completely abrogated by treatment with recombinant leptin [69]. Interestingly, human obesity only rarely associates with leptin deficiency or leptin receptor mutations. In the common form of obesity, leptin concentrations are actually increased in proportion to body adiposity [70], and the response of body weight to recombinant leptin is modest [71], defining a state of leptin resistance [72].

In addition to its role in energy homeostasis, leptin participates in other energy-demanding physiological processes such as reproduction [73], hematopoiesis [74], and angiogenesis [75]. Several studies have also shown that leptin has an important immunomodulatory role [76]. In monocytes or macrophages, it increases phagocytic function and proinflammatory cytokine production [77]. In polymorphonuclear cells of healthy subjects, it stimulates the production of reactive oxygen species [78] and chemotaxis [79]. Leptin is also involved in processes of cell development, proliferation, activation, and cytotoxicity of NK cells [80]. In adaptive immunity, leptin polarizes Th cytokine production toward a proinflammatory phenotype (Th1) [76].

Leptin signaling occurs typically via Janus tyrosine kinases (JAKs) and STATs. After binding to its functional receptor (ObRb), which is expressed not only in hypothalamus, but also in all cell types of innate and adaptive immunity [76, 81, 82], leptin recruits JAKs and activates the receptor, which serves as a docking site for adaptors such as STATs [83]. STATs translocate to the nucleus and induce gene expression. Several studies in human peripheral blood mononuclear cells have demonstrated that although the JAK-2-STAT-3 pathway constitutes an important pathway mediating leptin's function on immune cells, there are other pathways involved in this activity, such as the MAPK, the insulin receptor substrate 1, and the phosphatidyl-inositol 3′-kinase pathways [84].

2.2.3. Plasminogen Activator Inhibitor (PAI-1). Obesity and metabolic syndrome promote a hyperthrombotic state through distinct pathways that involve hypofibrinolysis, hypercoagulability, and platelet activation [85]. One of the most remarkable abnormalities of the haemostatic system in the context of obesity and metabolic syndrome is the increased circulating concentrations of PAI-1 [86, 87], which seems particularly associated with higher production of this factor by the fatty liver, ectopic adipose tissues, and dysfunctional endothelium. PAI-1 correlates with all components of the insulin resistance syndrome, and weight loss associates with a significant decrease in PAI-1 levels [88].

PAI-1 is a serpin that regulates both tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), likely representing the principal physiological inhibitor of plasminogen activation [89]. Thus, the increase in PAI-1

levels observed in obesity and metabolic syndrome impairs fibrinolysis and may influence the risk of atherothrombosis.

In addition to hypofibrinolysis, obesity and metabolic syndrome appear associated with enhanced platelet activity [87]. Moreover, higher concentrations of vitamin K-dependent coagulation factors and fibrinogen may also accompany excess adiposity, at least partially due to its inflammatory component [86]. Endothelial dysfunction, likely an important element in obesity, may also contribute to the thrombotic diathesis observed in this condition [87], through increased expression of proinflammatory and haemostatic products such as microparticles and von Willebrand factor.

2.2.4. Interleukin-6 (IL-6). IL-6 is an inflammatory cytokine with distinct pathophysiologic roles in humans. It stimulates the hypothalamic-pituitary-adrenal axis, being under negative control by glucocorticoids and positive regulation by catecholamines [90]. IL-6 is a potent inducer of the acute-phase reaction [90] that circulates at high levels during stress, inflammatory and infectious diseases. In mice, the metabolic role of IL-6 is not fully understood. Mice genetically deficient in IL-6 develop mature-onset obesity, partly reversed with IL-6 administration, and abnormalities in carbohydrate and lipid metabolism [91]. On the other hand, mice chronically exposed to IL-6 develop hepatic insulin resistance [92]. In either case though, IL-6 is likely a critical metabolic modulator.

Several immune cell types, including but not limited to monocytes, constitute typical sources of IL-6 [93]. In the last decades, the study of adipose tissue as an endocrine organ unraveled its abundant production of IL-6. Indeed, subcutaneous adipose tissue appears to release approximately 25% of circulating IL-6 in humans [94]. The obese state associates with increased secretion of IL-6 and, therefore, higher hepatic release of acute-phase reactants, C-reactive protein (CRP) likely being the major one [95]. A large bulk of evidence, including experimental and cross-sectional data, suggests that both IL-6 and CRP correlate with hyperglycemia, insulin resistance, and type 2 diabetes mellitus [96–100]. A prospective study found strong correlation between baseline IL-6 and CRP levels and risk of developing type 2 diabetes mellitus [101]. After multivariate analysis and adjustment for adiposity measurements, there was significant attenuation of the association between IL-6 and risk of diabetes, but CRP persisted as an independent predictor of incident diabetes [101].

Excess adiposity, particularly central adiposity, also associates with elevated levels of CRP. Indeed, besides the higher CRP levels in obese than in nonobese individuals, there is a positive correlation between CRP and waist-to-hip ratio (a measurement of visceral obesity) even after adjustment for BMI [102]. CRP, a liver-derived pentraxin, also correlates well with other risk factors and increased cardiovascular risk in the absence of acute inflammation. It has emerged as one of the most promising biomarkers for future cardiovascular events since 1997, when the Physicians Health Study demonstrated a strong and independent correlation between systemic levels of CRP and future occurrence of myocardial infarction and stroke in apparently healthy men [103]. After this work, several other prospective studies corroborated the CRP capacity of predicting cardiovascular events, even beyond traditional risk factors [104–110]. Besides the evidence in primary prevention, several studies also suggested that CRP levels predict efficiently new cardiovascular events in individuals who already had a myocardial infarction [111, 112]. CRP levels also seem to correlate with cerebrovascular events [113] and development of peripheral vascular disease [114].

Despite the large bulk of evidence on the CRP prediction capacity, the utility of this biomarker in risk stratification beyond traditional risk factors is not consensual, with opponents arguing that it only has a modest ability of stratifying the risk beyond conventional scores according to the C statistic [115, 116].

A recent meta-analysis including individual records of 160,309 people without a history of vascular disease from 54 long-term prospective studies showed that $\log(e)$ CRP concentration was linearly associated with several conventional risk factors and inflammatory markers, and nearly log-linearly with the risk of ischemic vascular disease and nonvascular mortality [117]. However, the risk ratios for coronary heart disease, ischemic stroke, vascular mortality, and nonvascular mortality per 1-SD higher $\log(e)$ CRP concentration decreased when adjusted for age and sex and even further for conventional risk factors [117]. These results suggest that a great proportion of the vascular risk associated with CRP depends on its correlation with traditional risk factors. On the other hand, CRP still kept its ability of predicting vascular events (and even non-vascular mortality) despite the adjustment for several conventional risk factors [117]. Interestingly, after further adjustment for other markers of inflammation, such as fibrinogen, the relative risk declined significantly, suggesting that CRP might be indeed an efficient marker of systemic inflammation but probably not a causal factor of atherothrombosis *per se*. A genetic study on the polymorphisms in the CRP gene also suggests a noncausal link between CRP levels and the risk of ischemic vascular disease [118]. In this work, genetic variants associated with lifelong elevations in circulating CRP, and therefore, with a theoretically predicted increase in the risk of ischemic vascular disease, failed to show the expected risk [118].

Research has also tested the utility of CRP beyond risk stratification. JUPITER (justification for the use of statins in primary prevention: an intervention trial evaluating rosuvastatin) for example, tested the value of CRP as a therapeutic guide for the initiation of statins [119]. This study found a significant reduction in cardiovascular events among individuals with low LDL-cholesterol levels (LDL <130 mg/dL), but high CRP levels (CRP >2 mg/L) receiving Rosuvastatin 20 mg per day compared to placebo [119]. The noninclusion of a group of people with low LDL-cholesterol and low CRP levels and the early interruption of the study figure among its limitations and most frequent critics.

Multiple studies have also showed that several other adipokines, such as resistin, retinoid binding protein 4 (RBP4), visfatin, and omentin, present varied potential

effects on glucose homeostasis. Extensive reviews on these bioactive mediators exist elsewhere [120, 121].

3. Potential Links between Inflammation and Insulin Resistance

Ample literature supports that insulin resistance precedes the development of overt hyperglycemia and type 2 diabetes [122]. Genetic burden and acquired conditions, including obesity as one of the most relevant, may play a role in the origin of insulin resistance [122]. While pancreatic β-cells properly respond to insulin resistance with substantial secretion of insulin, glucose metabolism stays equilibrated. Once the pancreas fails in overcoming insulin resistance with more insulin release, hyperglycemia, and eventually diabetes mellitus ensue.

In adipose tissue and skeletal muscle, insulin promotes cellular glucose uptake through a system of intracellular substrates [123]. Upon insulin binding to its receptor at the cell surface, members of the insulin receptor substrate (IRS) family become tyrosine phosphorylated. This process leads to downstream signaling which triggers translocation of GLUT-4 from intracellular stores to the cellular membrane and, therefore, glucose transport into the cell [124]. The phosphorylation of particular serine residues of IRS-1 may instead abrogate the association between IRS-1 and the insulin receptor, impairing insulin signaling [125]. Inflammatory mediators such as TNF-α or elevated levels of free fatty acids may activate serine kinases, such as c-Jun NH_2-terminal kinase (JNK) and IκB kinase (IKK) (Figure 1), which phosphorylate serine residues of IRS-1 and disrupt insulin signaling [126–129]. Both JNK and IKK, which are members of two major pro-inflammatory cascades, are likely activated in insulin-resistant states, and thus provide potential connections between inflammation and insulin resistance [127, 130, 131].

Experimental research with JNK-1-deficient mice showed substantial reduction of IRS-1 serine phosphorylation and amelioration of insulin sensitivity [127]. Besides serine phosphorylation of IRS-1, IKK-β can also influence insulin function through phosphorylation of the NFκB inhibitor (IκB), leading to activation of NFκB. The stimulation of this potent inflammatory pathway culminates with further production of several inflammatory substances including TNF-α, which can maintain and potentiate inflammatory activation [132]. Experiments involving rodents with targeted disruption of IKK-β demonstrated significant attenuation of insulin resistance [130]. There are also other kinases, such as mammalian target of rapamycin (mTOR), protein kinase R (PKR), and protein kinase θ (PKθ) that can induce inhibitory phosphorylation of IRS-1, and thus contribute to nutrient and inflammation-related disruption of insulin signaling [133].

Although obesity-associated inflammation probably initiates in adipocytes, where nutrient overload is first sensed, adipose tissue is not the only organ characterized by local inflammation, nor is it solely responsible for systemic inflammation and glucose metabolism abnormalities in obesity [3]. The liver, as another major metabolic site in the body, also participates importantly in the systemic inflammatory networks of obesity by experiencing activation of inflammatory pathways within its local cells [3]. The liver contains a resident population of macrophage-like cells, the Kupffer cells, which can secrete inflammatory mediators upon activation. However, unlike adipose tissue, the liver does not accumulate macrophages or other immune cells in the context of obesity. Animal studies involving liver-specific gain-of-function or loss-of-function of IKK-β increased our understanding on the role of obese liver in inflammation-related insulin resistance [132, 134]. Hepatocyte-specific deletion of IKK-β protects the liver but not muscle or adipose tissue of obese mice from insulin resistance [134]. On the other hand, transgenic mice with constitutively active IKK-β in hepatocytes display increased hepatic production of inflammatory cytokines, severe hepatic insulin resistance, and moderate systemic insulin resistance [132]. Intriguingly, specific deletion of JNK-1 in hepatocytes deteriorated glucose homeostasis in mice [135], whereas administration of a cell-permeable JNK-inhibitory peptide had the opposite effect, improving insulin sensitivity and glucose tolerance in diabetic mice [136]. In summary, although adipose tissue may be the organ where energy surplus exerts its first effects, presenting a pivotal role in the inflammatory and metabolic derangements of obesity, other metabolic organs, such as the liver, also seem to participate in this loop although in a somehow more localized manner.

4. The Origin of the Inflammatory Response in Obesity

Despite the large bulk of data available on the local and systemic inflammatory networks operating in obesity, the precise triggers of inflammation in this condition remain unidentified. However, several recent studies have brought potential answers to fundamental questions in this fascinating area of research. A reasonable hypothesis posits that nutrient overload in metabolic cells such as adipocytes induces intracellular stress which results in activation of inflammatory cascades [3, 137] (Figure 1). The endoplasmic reticulum (ER), an organelle specialized in protein folding, maturation, storage, and transport, senses nutrient levels in the cell. Under conditions of cellular stress induced by nutrient surplus, misfolded or unfolded proteins accumulate in the ER and activate the so-called unfolded protein response (UPR) pathway [138]. The UPR functioning depends essentially on three main ER sensors: PKR-like eukaryotic initiation factor 2α kinase (PERK), inositol requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF-6) [139]. Once activated, the UPR leads to increased activity of the kinases JNK and IKK-β, serine-phosphorylation of IRS-1, and activation of the NFκB pathway, leading to enhanced proinflammatory cytokine expression and impaired insulin signaling [3, 140–142].

The ER also responds to other conditions of cellular stress, such as hypoxia, another element of obese adipose tissue [124]. As obesity evolves and enlargement of adipose tissue ensues, the expanding adipocytes get relatively hypoxic. Regions of microhypoxia within the adipose tissue

also exhibit increased activation of inflammatory pathways [143, 144].

It is also possible that, in excess, nutrients can themselves directly activate immune pathogen-sensors in the cell such as toll-like receptors (TLRs), which likely respond to excess fatty acids and may contribute to insulin resistance [145]. TLR4 deficiency improves insulin sensitivity in high-fat diet-induced obese mice [145, 146]. PKR, another pathogen-sensor in the cell, is likely activated in mice during lipid infusion and in obesity [147] and, as discussed above, is also able to activate JNK and IKK and disrupt insulin signaling.

5. Obesity, Inflammation, and Cardiovascular Disease

There are numerous potential mechanisms by which increased adiposity may induce atherothrombosis and cardiovascular disease [85]. Obesity, predominantly visceral, often associates with other morbid conditions, such as insulin resistance, glucose and lipid abnormalities, and hypertension, each one an independent cardiovascular risk factor *per se*. Moreover, the inflammatory state that characterizes obesity is likely an important connection between this condition and the other cardiovascular risk factors, and at the same time, a possible direct link to atherothrombosis [85]. Indeed, as aforementioned, excess adiposity often correlates with abnormal production of several mediators which often associate with cardiovascular events. The adipokine imbalance characterizing obesity, including low levels of adiponectin, high levels of leptin, inflammatory mediators (IL-6 and TNF-α) and antifibrinolytic factors (PAI-1) may induce oxidative stress and endothelial dysfunction, initial steps of atherogenesis. Moreover, the insulin-resistant state of obesity frequently involves high circulating levels of non-esterified fatty acids, which cause lipotoxicity, and therefore further oxidative stress and endothelial dysfunction. As described above, numerous studies also support an independent association between circulating levels of CRP, an inflammatory marker potently induced by IL-6 and TNF-α (in excess in the obesity state), and cardiovascular events.

6. Insights into Anti-Inflammatory Therapies

All the evidence linking obesity-related metabolic disorders to inflammation raises the question whether modulation of inflammatory pathways would have a beneficial impact on metabolism. Several studies have targeted inflammation at different points of the inflammatory network of obesity, ranging from inhibition of circulating cytokines to suppression of intracellular inflammatory cascades and ER stress.

The approval of anti-TNF-α compounds for clinical use in patients with specific inflammatory conditions motivated studies with these reagents in obese and insulin-resistant subjects. These trials have yielded conflicting results on the improvement of insulin sensitivity by TNF-α blockers, such as etanercept [148, 149], and final conclusions warrant further studies.

Another promising anti-inflammatory approach in obesity and insulin-resistant states targets kinases and intracellular inflammatory pathways. Salsalates, nonacetylated members of the group of salicylates, appear to modulate inflammation through suppression of IKK action [29]. Clinical studies have shown decreased inflammation and improved metabolism, including glucose and lipid parameters, in diabetic subjects under therapy with salsalates [150]. Studies involving JNK antagonists, available for mice but still lacking for human treatment, also yielded positive results in glucose metabolism [136].

Since ER stress may represent one major source of inflammatory signals within the cell, it provides a potential therapeutic target for modulation of inflammation and metabolic derangements. Indeed, attenuation of ER stress through use of chemical chaperones restores glucose homeostasis in a mouse model of type 2 diabetes [151]. In humans, studies are still limited, but already reveal positive metabolic results [152].

Thiazolidinediones (TZDs) are peroxisome proliferator-activated receptor-γ (PPARγ) agonists, which possess significant anti-inflammatory effects and insulin-sensitizing properties [153]. PPARγ is a nuclear transcription factor, member of the nuclear-receptor superfamily, with important regulatory effects on inflammatory processes. As a synthetic ligand of PPARγ, TZDs may act via repression of inflammatory gene promoters [154]. They can also exert PPARγ-independent stimulation of glucocorticoid receptors [155]. By activating PPARγ, these compounds can also regulate genes related to adipocyte differentiation, lipid metabolism, and glucose uptake, each of which can contribute to their beneficial metabolic effects. However, changes in fluid balance and in myocardial function and predisposition to fractures render the therapeutic use of TZDs, more specifically rosiglitazone, challenging in specific groups of patients, particularly in patients with heart failure or increased cardiovascular risk [156–158].

The use of nutrients with anti-inflammatory properties may constitute another promising weapon against obesity. Mice receiving high fat diet supplemented with omega-3 polyunsaturated fatty acids (n-3 PUFAs) had reduced adipose tissue inflammation and improved insulin sensitivity [159]. In type 2 diabetic individuals, supplementation with omega-3 fatty acids has beneficial effects on serum triglycerides, HDL-cholesterol, lipid peroxidation, and antioxidant enzymes, which may lead to reduced rate of occurrence of vascular complications in those patients [160]. In diabetic women, high consumption of fish and long-chain omega-3 fatty acids supplementation associated with decreased cardiovascular risk [161].

Finally, cell-based immunomodulation of obesity inflammation has recently attracted attention although it is still incipient. Several animal studies, including some discussed above, demonstrated significant metabolic benefits as a consequence of depletion or stimulation of specific cell pools. Whereas deficiency or inhibition of CD11c-positive cells [162], CD8 T cells [18], mast cells [20] and NKT cells [21] improves metabolic parameters, an increase in the pool of $CD4^+$ $Foxp3^+$ T cells following CD3-specific antibody

treatment likely produce similar beneficial metabolic effects [27].

7. Conclusions

Far beyond a mere inert lipid storage, adipose tissue represents a site of plural activities involving secretion of a wide gamma of active substances, a great proportion of them portending inflammatory actions and/or highly regulated by inflammation. By imposing a state of nutrient overload, obesity significantly boosts inflammation in adipose tissue and other metabolic organs, with consequent impairment of insulin signaling and abnormalities in glucose metabolism. Progressive understanding of all inflammatory mechanisms operating in obesity is mandatory for further therapeutic advances.

Abbreviations

CCR2: C-C receptor 2
CRP: C-reactive protein
ER: Endoplasmic reticulum
IKK: IκB kinase
IR: Insulin resistance
IFN-γ: Interferon-gamma
IL-6: Interleukin-6
JNK: c-Jun NH_2-terminal kinase
MCP-1: Monocyte chemoattractant protein-1
PAI-1: Plasminogen activator inhibitor-1.

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Review Article

Animal Models of Calcific Aortic Valve Disease

Krista L. Sider,[1] Mark C. Blaser,[1] and Craig A. Simmons[1,2,3]

[1] *Institute of Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, Toronto, ON, Canada M5S 3G9*
[2] *Department of Mechanical and Industrial Engineering, University of Toronto, 5 King's College Road, Toronto, ON, Canada M5S 3G8*
[3] *Faculty of Dentistry, University of Toronto, 124 Edward Street, Toronto, ON, Canada M5G 1G6*

Correspondence should be addressed to Craig A. Simmons, c.simmons@utoronto.ca

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Calcific aortic valve disease (CAVD), once thought to be a degenerative disease, is now recognized to be an active pathobiological process, with chronic inflammation emerging as a predominant, and possibly driving, factor. However, many details of the pathobiological mechanisms of CAVD remain to be described, and new approaches to treat CAVD need to be identified. Animal models are emerging as vital tools to this end, facilitated by the advent of new models and improved understanding of the utility of existing models. In this paper, we summarize and critically appraise current small and large animal models of CAVD, discuss the utility of animal models for priority CAVD research areas, and provide recommendations for future animal model studies of CAVD.

1. Introduction

Valvular heart diseases account for over 23,000 deaths annually in the United States, with the aortic valve being the most frequently affected [1]. The aortic valve is composed of three semilunar cusps or leaflets that passively open and close over 25 million times per year to maintain unidirectional blood flow from the left ventricle to the systemic and coronary circulations. To meet its functional requirements under these demanding conditions, the thin, pliable leaflet tissue is organized into three distinct layers: (1) the fibrosa on the aortic side of the leaflet, composed primarily of collagen; (2) the spongiosa in the middle, composed mainly of proteoglycans; and (3) the ventricularis on the ventricular side, composed of collagen and elastin [2]. The cellular components of the aortic valve include a monolayer of valvular endothelial cells (VECs) on the outer surface of the leaflets and valvular interstitial cells (VICs), which populate each of the three layers of the leaflet. VICs are a heterogeneous population of mostly fibroblasts [3], a subpopulation of which are mesenchymal progenitor cells [4]. VICs play the critical role of remodelling and organizing the valve extracellular matrix (ECM) to maintain valve integrity [3, 5].

In disease, ECM structure and organization are disturbed, resulting in valve dysfunction. The most common valvular disease is calcific aortic valve disease (CAVD) [1]. CAVD encompasses early sclerosis, characterized by leaflet thickening without left ventricular outflow obstruction, to late stenosis in which leaflets stiffen, flow is obstructed, and cardiac function is compromised. The consequences of CAVD are significant: sclerosis is associated with a 50% increased risk of cardiovascular death and myocardial infarction [6], and the prognosis for patients with stenosis is very poor [7]. Because CAVD is a slowly progressing disease taking several decades to develop, it was once thought to be a passive "degenerative" or "senile" process. However, CAVD is now recognized to be an active pathobiological process that shares many risk factors with atherosclerosis, including hypercholesterolemia, smoking, hypertension, diabetes, chronic renal disease, and male gender [8–10].

Also similar to atherosclerosis, chronic inflammation is a predominant feature of CAVD. This is reflected in human disease by the presence of macrophages and T cells [11–16]; subendothelial oxidized low-density lipoprotein (LDL) deposits [11–13, 16, 17]; elevated superoxide and hydrogen peroxide [18, 19]; active mast cells [17, 20]; complement activation [16]; elevated expression of tumor necrosis factor-α (TNF-α) [14], matrix metalloproteinases (MMP-1, -2, -3, -9) [14, 21], interleukin-2 (IL-2) [22], angiotensin converting enzyme (ACE), angiotensin II (AngII), angiotensin II type-1

receptor (AT1R), and chymase [20, 23]; and VEC expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) [24, 25], and E-selectin [25]. Inflammatory processes are associated with, and may drive, the alterations in the valve ECM that are hallmarks of CAVD, including leaflet thickening [13, 14, 21]; collagen turnover and fibrosis [13, 24, 26]; accumulation of proteoglycans and hyaluronan [27, 28]; fragmentation of elastin [29, 30]; and calcification [11–13, 15, 17, 22, 24]. Ultimately, it is maladaptation of the valve ECM (which is often localized to the fibrosa layer [11, 13]) that results in valve stiffening and dysfunction.

While many features of human CAVD are well described (particularly for late-stage disease), specific pathobiological mechanisms are not fully understood. Some insight may come from similarities with atherosclerosis, but less than 40% of patients with CAVD have clinically significant coronary atherosclerosis [31], suggesting distinct processes. Thus there is an unmet scientific need to determine pathobiological mechanisms of CAVD and identify new approaches to treat CAVD. Animal models are emerging as vital tools to this end, facilitated by the advent of new models and improved understanding of the utility of existing models. In this paper, we summarize and critically appraise current small and large animal models of CAVD, discuss the utility of animal models for priority CAVD research areas, and provide recommendations for future animal model studies of CAVD.

2. Animal Models of CAVD

Animal models are an important platform for studying the initiation and progression of CAVD in vivo, as well as for judging the effectiveness of therapeutic interventions. To be most effective, models should mimic human disease or at least important facets thereof and the conditions in which human CAVD develops. The most common species used to model CAVD are mouse, rabbit, and swine. Of these, only swine develops CAVD naturally with age, but this process is slow and is usually accelerated by diet-induced hypercholesterolemia. Rabbits are not naturally susceptible to CAVD but are responsive to diet-induced hypercholesterolemia, and mice require a genetic predisposition to promote advanced disease.

2.1. Mouse Models. In the study of CAVD, a large proportion of contemporary animal work is performed in mouse models, as mice are uniquely suited to mechanistic studies of the root causes of aortic valve pathology. Mouse models of CAVD offer a number of advantages, including their small size, easy husbandry, and cost effectiveness. In addition, short generation time, resultant ease of genetic manipulations, and availability of clonal samples allow specific investigation of key molecular mediators of CAVD [32, 33]. A summary of the critical elements of mouse models of CAVD is presented in Table 1.

Despite these advantages, mouse models do suffer from several important limitations. Firstly, the anatomical structure of mouse aortic valves differs drastically from that of humans. Mice do not have the trilayer aortic valve tissue morphology characteristic of human, pig, or rabbit valves; rather their leaflets are usually only ~5–10 cells thick and do not exhibit segregated layers [62]. Secondly, and most importantly, wild-type (WT) mice on standard diets do not exhibit spontaneous calcification [32], and consequently the study of CAVD in mouse models requires dietary [34] and/or genetic [34, 38, 42, 43] or other interventions [46, 47] to induce a valvular calcium burden.

Furthermore, the development of atherosclerosis in inbred mice is dependent on the background strain in use. When comparable hyperlipidemia is induced in C57BL/6J mice, this strain exhibits considerably greater atherosclerotic lesion formation than the FVBN or C3H strains [63–65]. While strain-specific susceptibility to CAVD has not been explored in detail, similarities between atherosclerosis and valve disease imply that comparable strain-specific susceptibility to CAVD is present as well. In a related issue, while the C57BL/6J mouse is the most atheroprone strain and most commonly used background in mouse models of CAVD, their valve leaflets contain black pigmentation that may be melanocytes [66] or lipofuscin-containing granules [35]. Regardless of their identity, these dark particulates can appear in the valve interstitium and are easily mistaken as positive von-Kossa calcification staining, thus requiring the use of alizarin red to accurately determine the extent of calcification in C57BL/6J models of CAVD [62].

2.1.1. Diet-Induced and Genetic Mouse Models. As WT mouse strains do not spontaneously calcify, dietary and/or genetic insults are often employed. Most commonly, mice deficient in the low-density lipoprotein (LDL) receptor (*Ldlr*−/−) are used. *Ldlr*−/− mice with only apolipoprotein B (ApoB)-100 (*Ldlr*−/−;$Apob^{100}$-only) spontaneously develop mild hypercholesterolemia (~270 mg/dL), drastic reductions in valve orifice diameter (>50%), elevated transvalvular systolic pressure gradients, and left ventricular hypertrophy when aged for 17–22 months without dietary insult [38]. Furthermore, ageing is associated with superoxide development indicative of the onset of oxidative stress and abundant von Kossa staining in the aortic valve [38].

Typically, the Harlan Teklad TD.88137, or "Western" diet, is used in models with a dietary induction component. This adjusted diet derives 21.2% weight and 42% of total calories from fat, with 0.2% [36, 42–44, 46] or 0.25% [39, 40] cholesterol (mouse diet fat content is typically expressed as percent of total calories from fat). Towler and colleagues first reported extreme hyperlipidemia (~1040 mg/dL), hyperinsulinemia/hyperglycemia, and mineral deposition in *Ldlr*−/− mice fed TD.88137 for 16 weeks [36]. *Ldlr*−/− mice fed a similar high-cholesterol diet (0.15%) develop increases in valve thickness, macrophage accumulation, superoxide production, activated myofibroblasts and osteoblasts, and mineralization [37]. When mice are regularly exercised while the high-cholesterol diet is fed, these pathological symptoms are significantly reversed [37]. The *Ldlr*−/−;$Apob^{100}$-only mouse has also been modified to incorporate a conditional knockout of the microsomal triglyceride transfer protein (*Mttp*) under the control of an

Table 1: Characteristics of mouse models of CAVD.

Model	Insult*, duration, (treatment)	Total chol†	Results, (treatment)	Study
		Mouse		
WT C57BL/6J	58.7% fat, <0.1% cholesterol, 4 mo	166 ± 4	↑T, TV, M, Tc, C; ↓OA	Drolet et al. 2006 [34]
WT C57BL/6J	9% fat (w/w), 1.25% chol, 0.5% cholic acid, 15–22 wk	—	↑M on high-shear ventricular face, no Δ in L or LPR	Mehrabian et al. 1991 [35]
Ldlr−/−	42% fat, 0.2% chol., 4 mo	1040 ± 37	↑mineral deposition reported	Towler et al. 1998 [36]
Ldlr−/−	42% fat, 0.15% chol., 4 mo	1958 ± 235	↑M, SO, My, O, C	Matsumoto et al. 2010 [37]
Ldlr−/−;Apob$^{100/100}$	normal diet, 20 mo	271 ± 12	↑LV hypertrophy, C, SO; ↓OA	Weiss et al. 2006 [38]
Ldlr−/−;Apob$^{100/100}$/*Mttp*$^{fl/fl}$/Mx1-*Cre*$^{+/+}$, "Reversa"	42% fat, 0.25% chol., 6–12 mo, (Reversal)	997 ± 87	↑L, M, My, PFS, Col, PCS, SO, C; ↓OA, (↓ all but Col; ↓TC)	Miller et al. 2009 [39]
"Reversa", as above	42% fat, 0.25% chol., 14 mo, (Reversal)	571 ± 54	↑PFS, PCS, SO, C; ↓OA, (↓ all but PFS, SO; does not ↑OA; ↓TC)	Miller et al. 2010 [40]
Apoe−/−	Normal diet, 30 mo	—	↑TV, M, Tc, My, O, C	Tanaka et al. 2005 [41]
Apoe−/−	42% fat, 0.15% chol., 4 & 16 mo	—	↑macrophage-targeted NIRF signal	Aikawa et al. 2007 [42]
Apoe−/−	42% fat, 0.2% chol., 5 mo	588 ± 47	↑T, M, My, O, MMP-2/9, microC, ALP, cathepsinB/K, activated VECs	Aikawa et al. 2007 [43]
Apoe−/−	42% fat, 0.2% chol., 125 μg/day leptin, 2 mo	4709 ± 247	↑O, C, ALP	Zeadin et al. 2009 [44]
Apoe−/−	Normal diet, 2.5 mg/kg/day acrolein, 8 wk	564 ± 17	↑M, L, activated VECs, TC, VLDL, PF4; no ↑My or SO	Srivastava et al. 2011 [45]
Apoe−/−;Ctss−/−	42% fat, 0.2% chol., 5/6 nephrectomy, 5 mo	660 ± 25	↓M, C, O, elastolytic activity, elastin fragment versus *Ctss*+/+	Aikawa et al. 2009 [46]
Apoe−/−	Normal diet, 5/6 nephrectomy, 6 mo	397 ± 25	↑M, microC, osteoporosis	Hjortnaes et al. 2010 [47]
Mgp−/−	Normal diet, 2-3 wk	n/a	↑valve calcification reported	Luo et al. 1997 [48]
Fibulin4−/−	Normal diet, 4-5 mo	n/a	↑T, functional impairment, PFS, PCS, immune response gene	Hanada et al. 2007 [49]
Eln+/−	Normal diet, 1 wk–17 mo	n/a	↑proliferation of VICs, regurgitation	Hinton et al. 2010 [50]
Notch1+/−	42% fat, 0.2% chol., 10 mo	—	↑C by 5x, ALP; no bicuspid aortic valves	Nigam and Srivastava 2009 [51]
RBPJk+/−	1.25% chol., 0.5% sodium cholate, 5 IU VitD3, 4 mo	~200	↑T, M, Col, TV, PFS, PCS, C; ↓FS and EF; no bicuspid aortic valves	Nus et al. 2011 [52]
Postn−/−	Normal diet, 10 mo	n/a	↑bicuspid-like aortic valves, PCS, C	Tkatchenko et al. 2009 [53]
Postn−/−	57% fat, 4 mo	~145	↓T, M, My, annular fibrosis, MMP-2/13 versus WT on HF diet	Hakuno et al. 2010 [54]
ChmI−/−	Normal diet, 21–24 mo	n/a	↑T, L, C, AVA, VEGF-A, angiogenesis	Yoshioka et al. 2006 [55]
Nos3−/− (eNOS)	Normal diet, 25–30 g	n/a	~50% bicuspid aortic valve incidence	Lee et al. 2000 [56]
Madh6−/−	Normal diet, 6 wk	n/a	↑valve hyperplasia, aortic ossification	Galvin et al. 2000 [57]
persistent Twist1	Normal diet, 1 wk & 6.5 mo	n/a	↑T, *Col2a1*, periostin, MMP-2/13; ↓OA	Chakraborty et al. 2010 [58]
hypomorphic *Egfr*	Normal diet, 1 wk–12 mo	n/a	↑M, C, O, congenitally enlarged valves	Barrick et al. 2009 [59]
Il1rn−/−	Normal diet, 4–40 wk	~90	↑T, M, My, C, TV, TNF-α	Isoda et al. 2010 [60]

* % fat: % kcal from fat unless otherwise noted.

† mg/dL. Normal total cholesterol ranges: human: 197 ± 23 mg/dL [61], mouse: 90–110 mg/dL, rabbit: 30–60 mg/dL, swine: 60–75 mg/dL.

↑: increased compared to control, ↓: decreased compared to control, ALP: alkaline phosphatase activity, AVA: aortic valve area, C: calcification, Col: collagen, EF: ejection fraction, FS: fractional shortening, HF: high fat, L: lipid deposition, LPR: lipoprotein infiltration, LV: left ventricle, M: macrophage, My: myofibroblast, n/a: not applicable, OA: opening area, O: osteoblast, PCS: procalcific signalling, PFS: profibrotic signalling, PF4: platelet factor 4, SO: superoxides, T: thickness, Tc: T-cell, TC: total cholesterol, TV: transvalvular velocity, VEC: valve endothelial cells, VIC: valve interstitial cells, VLDL: very low-density lipoprotein, and WT: wild type.

inducible Mx1-*Cre* transgene. Known as the Reversa model, these *Ldlr*−/−/$Apob^{100/100}$/$Mttp^{fl/fl}$/Mx1-$Cre^{+/+}$ mice offer *Cre*-mediated loss of *Mttp* activity (required for ApoB lipoprotein secretion) and allow controllable normalization of serum cholesterol levels [39]. In conjunction with the TD.88137 diet, the Reversa mouse develops robust calcific aortic stenosis. Serum cholesterol rises to 800–1000 mg/dL in 6–12 months, and lipid deposition and macrophage infiltration are significantly increased. Profibrotic signalling and myofibroblast activation as measured by pSmad2 and α-smooth muscle actin (αSMA) are elevated, as is procalcific signalling (pSmad1/5/8, Msx2, β-catenin, Runx2, osterix) and superoxide levels, leading to oxidative stress, valvular mineralization, and positive von Kossa/alizarin red staining [39, 40]. Importantly, *Cre*-mediated loss of *Mttp* activity at six months ("reversal") normalizes serum cholesterol levels, decreases valvular lipid deposition and macrophage infiltration, prevents further calcification, lowers pSmad2/αSMA, lowers pSmad1/5/8/Msx2/Runx2, attenuates oxidative stress, and results in functional improvements in cusp separation [39]. Interestingly, reversal after 12 months does not lower pSmad2 or superoxide levels and does not improve leaflet cusp separation distance [40]. The on-demand "switching" of cholesterol levels in this model allows, for the first time, regression studies in dietary-induced CAVD independent of pharmacological intervention.

Diets with substantially elevated cholesterol levels are, however, not always employed as initiators of mouse CAVD. One such study employed the use of a high-fat, high-carbohydrate diet, where 35.5% of weight and 59% of calories were derived from fat, but no cholesterol was added (<0.1% present) [34]. In WT C57BL/6J mice on this diet, total cholesterol levels are mildly elevated to 166 mg/dL, while *Ldlr*−/− mice exhibit drastic hypercholesterolemia at 722 mg/dL and develop overt diabetes mellitus. Most interestingly, the high-fat, low-cholesterol diet is able to induce early markers of CAVD even in WT mice: thickened leaflets, black particulates which may be von-Kossa-positive calcification, decreased aortic valve opening area, and increased transvalvular blood velocities are reported, along with CD68-positive macrophage and T-lymphocyte infiltration into the valvular interstitium. These macrophages infiltrate primarily on the high-shear ventricular side of the mouse valve [35]. The development of these disease hallmarks in WT mice with only mild hypercholesterolemia may prove to be more relevant to human CAVD.

In addition to the *Ldlr*−/− model, a second common genetically manipulated model is the endogenously hyperlipidemic [63] ApoE-deficient (*Apoe*−/−) mouse [41–44, 46, 47]. ApoE allows receptor-mediated removal of very-low density lipoprotein (VLDL) from the circulation. However, ApoE also regulates T-cell proliferation and macrophage function and modulates lipid antigen presentation as well as general levels of inflammation and oxidation [67]. In this way, deletion of *Apoe* may significantly impact the inflammatory response to CAVD in a manner distinctly unrelated to hypercholesterolemia and/or pathogenesis of the human disease. This potential for differential disease progression remains to be studied in detail. Without dietary intervention, *Apoe*−/− mice develop hypercholesterolemia (~490 mg/dL) [68], and their increasing age up to 2.5 years is correlated with increases in transvalvular velocity, mild aortic regurgitation, αSMA and osteocalcin (OCN) expression, macrophage and T-cell infiltration, and nodular calcification [41]. Administration of the TD.88137 diet to *Apoe*−/− mice for 4-5 months induces accelerated early disease with a substantial increase in serum cholesterol to ~588 mg/dL, thickened leaflets, activated endothelial cells, and subendothelial lesions rich in macrophages (colocalized with MMP-2/9, cathepsin B, αSMA, ALP, Runx2, and OCN expression) [42, 43]. Importantly, there is no evidence of von Kossa or alizarin red staining at this early time point, though a bisphosphonate-conjugated imaging particle shows signs of early microcalcification and colocalizes with cathepsin K [42, 43]. Another version of the *Apoe*−/− model includes coadministration of the adipocytokine leptin, a known cardiovascular risk factor [44]. Leptin treatment does not induce hypercholesterolemia nor does atherosclerotic lesion size change, but von Kossa and ALP-positive staining is significantly increased in leptin-treated *Apoe*−/− mice, and osteopontin (OPN) and OCN expression is also increased. While on a normal diet, oral exposure of *Apoe*−/− mice to acrolein, a dietary aldehyde generated during inflammation and oxidative stress, induces hypercholesterolemia, macrophage and lipid infiltration, and platelet and endothelial activation [45]. Interestingly, such treatment does not induce fibrosis, nor does it provoke a systemic oxidative stress response.

Along with these two common hyperlipidemic models, there exist other interesting genetic models that recapitulate some aspects of CAVD. Knockout of the mineral binding ECM protein matrix GLA protein (*Mgp*−/−) produces spontaneous ectopic apatite formation in the arterial collagen fibrils and von-Kossa-positive calcification in the aortic valve [48]. An insufficiency of elastin (*Eln*+/−) produces proliferation of VICs and aortic regurgitation [50]. Hypomorphic expression of fibulin-4, an ECM stabilizing protein, results in thickened leaflets, significant functional impairments, and positive pSmad2, pSmad1/5/8, and von Kossa staining [49]. Mutant tissue displays increased transforming growth factor-β (TGF-β) and bone morphogenic protein (BMP) signalling, while an Affymetrix microarray showed differential expression of a number of immune response genes between the WT and fibulin-4 deficient animals. Of specific interest to the role of inflammation in spurring the onset of CAVD are mice deficient in the antiinflammatory cytokine interleukin-1 receptor antagonist (*Il1rn*−/−). These animals develop thickened aortic valves infiltrated by macrophages and containing differentiated myofibroblasts, while aged *Il1rn*−/− mice develop calcified lesions with functional impacts on transvalvular blood velocity [60]. Circulating levels of TNF-α rise dramatically in this model, and the importance of this chemokine to CAVD pathogenesis is underscored by double knockout *TNF-α*−/−;*Il1rn*−/− mice which do not develop CAVD [60].

2.1.2. Congenital and Developmental Mouse Models. The presence of a congenital bicuspid aortic valve is associated with drastically increased risk of CAVD [69]. Mutations in the transcriptional regulator *NOTCH1* have been shown to cause bicuspid valves and CAVD development in humans. The normally developing mouse valve displays higher *Notch1* levels than during postnatal growth [70]. Mice heterozygous for *Notch1* (*Notch1*+/−) fed a Western diet with 0.2% cholesterol for 10 months exhibit fivefold greater aortic valve calcification than WT controls, but do not exhibit bicuspid valves [51]. Recently, mice haploinsufficient for the primary nuclear Notch effector protein recombining binding protein suppressor of hairless (RBPJK) were challenged with a high-cholesterol/cholate diet for a shorter 4-month period [52]. These mice present normal trileaflet aortic valves, and develop thickened and calcified leaflets, macrophage infiltration, collagen deposition, and profibrotic/osteogenic signalling. Interestingly, *Notch1*+/− mice display relatively little functional impairment when compared to *RBPJk*+/− mice, implying that other Notch effectors contribute to valvular homeostasis [52]. Periostin is highly expressed in the endocardial cushion during embryogenesis, and its deletion (*Postn*−/−) induces overexpression of delta-like 1 homolog (*Dlk1*), a negative regulator of *Notch1* [53]. By 10 months of age, the valves of *Postn*−/− mice exhibit a severely deformed bicuspid-like morphology displaying expression of Runx2, OPN, and OCN, along with significant valvular calcification (von Kossa). Paradoxically, when *Postn*−/− mice are fed a high-fat diet for four months, they display decreased valve thickness, macrophage infiltration, myofibroblast differentiation, annular fibrosis, and MMP-2/13 expression when compared with WT mice fed the same diet, possibly reflecting a reduced ability of myofibroblasts and macrophages to adhere to and infiltrate the ECM [54]. Periostin expression is mutually exclusive to that of chondromodulin-I (ChmI), an antiangiogenic factor. Aged *ChmI*−/− mice display increases in valve thickness, lipid deposition, calcification, VEGF-A, and angiogenesis [55]. In humans, expression of endothelial nitric oxide synthase (eNOS) in the valvular endothelium is drastically reduced in bicuspid valves [71], and mice lacking eNOS (*Nos3*−/−) display a high incidence (∼50%) of bicuspid aortic valve [56]. The susceptibility of this mouse model to CAVD and inflammatory processes is as of yet unexplored. Paradoxically however, *Nos3*−/− mice do not develop atherosclerosis when fed a high-cholesterol atherogenic diet [72], a phenomenon which may be the result of reductions in eNOS-driven LDL oxidation in the vasculature [73].

Signalling pathways normally associated with embryonic development of the valve have recently been implicated in CAVD pathogenesis, and have become the focus of several mouse models of the disease [5]. While epidermal growth factor receptor (EGFR) has been implicated in the development of cancer, and targeted for inhibition by cancer therapeutics, mice carrying a hypomorphic allele of *Egfr* (*Egfr*$^{wa2/wa2}$) exhibit congenitally enlarged valves, and mature mice display valvular OPN expression, macrophage infiltration, and extensive von Kossa-positive calcification [59]. Interestingly, these results imply that cancer patients with congenital valve defects or other cardiovascular risk factors should avoid EGFR inhibition. The Smad6 inhibitory protein regulates TGF-β signalling and mediates endocardial cushion transformation, and mutation of the Smad6 gene (*Madh6*−/−) in mice produces valvular hyperplasia, outflow tract septation defects, elevated blood pressure, and aortic ossification [57]. Endocardial cushion development is also promoted by the Twist1 transcription factor and is upregulated in human CAVD. Mice engineered with persistent Twist1 (*CAG-CAT-Twist1;Tie2Cre* mice) develop two- to threefold increases in area, length, and thickness of the aortic valve [58]. Expression of collagen-II, periostin, and the matrix remodelling enzymes MMP-2/13 are also elevated [58].

2.2. Rabbit Models. In CAVD research, rabbits are the most common large animal model used. Rabbits are desirable for a number of reasons as they (1) have a trilayer valvular morphology [74]; (2) respond to dietary cholesterol [74–86]; (3) are relative easy to manage due to their medium size; (4) have some similarities to human lipoprotein metabolism [87–89]; (5) are susceptible to accelerated calcification with vitamin D2 (VitD2) [80, 82]; (6) are available as transgenic [90–92] and natural mutant strains [93, 94].

On the other hand, there are a number of dissimilarities with human disease. Rabbits do not form spontaneous atherosclerotic lesions and therefore require very high cholesterol levels to induce more advanced disease [78–80, 84, 85], unless very long-term studies [74, 83, 86] or VitD2 supplementation [80, 82] are used. Rabbits also have significant differences in their lipid metabolism from humans [87–89], which can result in their development of cholesterol storage syndrome while on high-cholesterol diets (0.5–3%), with cholesterol deposited in regions such as the liver, adrenal cortex, and reticuloendothelial and genitourinary systems. Rabbits have also been reported to form atherosclerotic lesions that do not resemble those in humans [74, 88, 102]. A summary of the critical elements of rabbit models of CAVD is presented in Table 2.

2.2.1. Diet-Induced Rabbit Models. The standard rabbit model is the New Zealand white rabbit (NZWR) with a starting weight of 1.6–3 kg [74, 76–86] and hypercholesterolemia induced by one of four main diet categories (1) moderate-to-high cholesterol with [75–77, 81] or without fat [78, 79, 84, 85, 95, 103]; (2) moderate cholesterol and VitD2 [19, 80, 82]; (3) low cholesterol [74, 83, 86]; or (4) VitD2 only [82, 98]. (Below, rabbit diet additions are expressed as additional weight percentage added.)

Moderate-to-high cholesterol diets (0.5–2%) induce very high total blood cholesterol levels, ranging from approximately 1000–3500 mg/dL [79, 80, 84, 85], and sometimes induce extreme hypertriglyceridemia [79]. After one week on this diet, lipids infiltrate into the subendothelial region of the fibrosa prior to macrophage presence [75] and associate with collagen fibres and the proteoglycans that connect them [76, 81]. This is paralleled by hyperplasia of the basal lamina, including fragmentation of the elastin and collagen bundles, and accumulation of proteoglycan in the fibrosa [75], with

Table 2: Characteristics of rabbit and swine Models of CAVD.

Model	Insult*, duration, (treatment)	Total chol†	Results, (treatment)	Study
		Rabbit		
NZWR	2% chol., 10% soybean, 2 & 3 wk	—	↑L associated with Col and PG fibres	Nievelstein-Post et al. 1994 [76]
NZWR	2% chol., 10% soybean, 2 wk	—	↑L associated with Col, Fc	Haberland et al. 2001 [77]
NZWR	2% chol., 10% soybean, 4 wk	—	↑L associated with Col in clusters, fusing	Zeng et al. 2007 [81]
Chinchilla	0.5% or 2% chol., 5% butter, 2–10 wk	—	↑L, ECM fragment, followed by ↑M and Fc, to ↑Col and C	Filip et al. 1987 [75]
NZWR	1% chol., 12 wk	2235 ± 162	↑HDL, TG, Fc, apoptosis of M and Fb	Rajamannan et al. 2001 [79]
NZWR	1% chol., 8 wk, (atorvastatin)	3235 ± 328	↑ALP, Runx2, OPN, M, PCNA, hsCRP, T, (↓all but hsCRP; ↓TC)	Rajamannan et al. 2002 [78]
JWR	1% chol., 8 wk, (ARB)	1510 ± 265	↓VEC integrity, eNOS; ↑L, M, OPN, My, Runx2, ACE, (all trends reversed)	Arishiro et al. 2007 [95]
NZWR	1% chol., 8 wk, (eplerenone)	1206 ± 160	↑C, L, Fc, M, ACE, (↓all; ↑aldosterone levels)	Gkizas et al. 2010 [96]
NZWR	0.5% chol., 3 mo, (atorvastatin)	1725 ± 637	↑hsCRP, T, C; ↓nitrate, (↓hsCRP, T, C, TC; ↑eNOS, nitrate)	Rajamannan et al. 2005 [85]
NZWR	1% chol., VitD2 50,000 IU [daily], 12 wk	1670 ± 150	↑T, D, Col, L, C, Cl, M, My, TF, TVG; ↓ADV	Marechaux et al. 2009 [97]
NZWR	0.5% chol., VitD2 50,000 IU [daily], 12 wk	~1737	↑TVG, serum calcium, C; ↓AVA	Drolet et al. 2003 [80]
NZWR	0.5% chol., VitD2 50,000 IU [3 times/wk], 10 wk	—	↑TVG, E, C, Tc, M, My, serum calcium & phosphate; ↓AVA	Drolet et al. 2008 [82]
NZWR	0.5% chol., VitD2 100,000 IU [daily], 12 wk, (tempol) (Lipoic acid)	1363 ± 90	↑M, T, Cl, Col, C, E, OPN, Runx2, OC, SO, HP, N/OS, OS, SCP, SC; ↓AVA, (↑C, OC, HP, OS; ↓SO, Cl, N/OS) (↓C, E, SO, HP, N/OS)	Liberman et al. 2008 [19]
NZWR	0.25% chol., 20 & 40 wk	822 ± 61	↑T, L, Col, reorganization of Col & elastin, M, OPN	Cimini et al. 2005 [74]
NZWR	0.12–0.25% chol., 15 & 30 mo, (atorvastatin)	500 titrated	↑L, M, Tc, T, Col, C, OPN, R, (↓L, M, Tc, T, OPN)	Hamilton et al. 2011 [83]
NZWR	VitD2 25,000 IU[4 times/wk], 8 wk	—	↑TV, TVG, E, L, C, M, Lc, TXNIP, SCP, TC, SC, LVH; ↓AVA, VEC fn	Ngo et al. 2008 [98]
NZWR	VitD2 25,000 IU[4 times/wk], 8 wk, (ramipril)	—	Same as above, (↓TV, E, LVH, TXNIP, SCP; ↑VEC fn)	Ngo et al. 2011 [99]
WHHL	0.25% chol., 6 mo, (atorvastatin)	1329 ± 141	↑T, Lpr5, PCNA, OPN, p42/44, C, My, (↓all & TC)	Rajamannan et al. 2005 [84]
		Swine		
Yorkshire	normal (N) or chol, fat, diabetes(HCD), 6 mo	—	HCD has L and C; N Aortic VEC are antioxidative, non-I, and permissive to C	Simmons et al. 2005 [100]
Yorkshire	1.5% chol., 15% lard, 2 wk & 6 mo	549 ± 114	↑L, C; Aortic VEC are anti-I, and anticalcific	Guerraty et al. 2010 [101]

*Rabbit and porcine diets are expressed as % weight added over base diet.

†mg/dL. Normal total cholesterol ranges: human: 197 ± 23 mg/dL [61], mouse: 90–110 mg/dL, rabbit: 30–60 mg/dL, WHHL: 284 ± 29 mg/dL, swine: 60–75 mg/dL.

↑: increased compared to control, ↓: decreased compared to control, ACE: angiotensin-converting enzyme, ADV: aortic Doppler velocity, ALP: alkaline phosphatase activity, ARB: angiotensin receptor blocker, AVA: aortic valve area, C: calcification, Cl: cellularity, Col: collagen, D: ECM disorganization, E: echogenicity, ECM: extracellular matrix, Fb: fibroblasts, Fc: foam cells, fn: function, HDL: high density lipoproteins, HP: hydrogen peroxide, hsCRP: highly sensitive C-reactive protein, I: inflammatory, JWR: Japanese white rabbit, L: lipid deposition, Lc: leukocyte (CD45), LVH: left ventricle hypertrophy, M: macrophage, My: myofibroblast, n/a: not applicable, N/OS: nitro/oxidative stress, NZWR: New Zealand white rabbit, OC: osteoclasts, OPN: osteopontin, OS: NAD(P)H oxidase subunits, PCNA: proliferating cell nuclear antigen, PG: proteoglycans, R: regurgitant flow, SC: serum creatinine, SCP: serum calcium-phosphate, SO: superoxide, T: thickness, Tc: T-cell, TC: total cholesterol, TF: tissue factor, TG: triglycerides, TVG: transvalvular pressure gradient, TV: transvalvular velocity, and VEC: valve endothelial cells.

both VECs and VICs increasing their protein production. By eight weeks there is macrophage infiltration, myofibroblast presence, and increased proliferation, ACE, OPN, and osteoblast gene expression (alkaline phosphatase, *OPN*, and *Runx2*) [78, 95]. Highly sensitive C reactive protein (hsCRP) in the blood is also increased, indicating an inflammatory state [78], and there is impaired endothelial integrity on the aortic side with decreased eNOS expression [95]. Foam cells and lipid uptake by VICs have also been seen prior to 12 weeks [75]. By 12 weeks, some calcification is present [85], and there is a slight increase in apoptosis of macrophages and proliferating fibroblasts (0.1% of cells) [79]. With the addition of VitD2 to 0.5–1% cholesterol diets, by 10–12 weeks there is enough calcification and thickening to cause decreased aortic valve opening area [19, 80, 82] and aortic Doppler velocity index [97], and increased transvalvular velocities [80, 82, 97] and echogenicity [19]. There are also macrophages present in the subendothelial spaces [19, 82, 97], and T cells [82] and smooth muscle actin positive cells found near the calcification [82, 97]. These valves are thickened due to increased collagen, cellular infiltrates, calcification [19, 97], and lipid deposition [97] in the fibrosa, with increased expression of OPN and Runx2, and the presence of osteoclasts. Reactive oxygen species (ROS) are also present around calcified areas (superoxides, hydrogen peroxide, indicators of nitro/oxidative stress and NAD(P)H oxidative subunits) [19], as well as tissue factor [97].

Low-cholesterol diets (0.125–0.25% cholesterol) maintain more moderate cholesterol levels of around 500–800 mg/dL [74, 83, 86]. By five months, lipid deposition and collagen disarray are seen in the fibrosa, with macrophages and OPN in the lesion. By 10–15 months there is an increase in macrophages, OPN, collagen area, thickness, and lipid deposition. However, no calcification is seen [74, 83] until 30 months, which is accompanied by an increase in macrophages, T cells, and collagen deposition, and the presence of regurgitant flow [83].

VitD2-only treatment has been used in some models to induce advanced CAVD [82, 98]. By 8–10 weeks, functional changes indicative of mild stenosis including increased peak transvalvular velocity and pressure gradients, increased echogenicity of the valve [82, 98], and decreased aortic valve area [98] occur. Histologically, these valves display increased burden of lipids, leukocytes, macrophages [98], myofibroblasts, T cells [82], and calcification [82, 98]. Serum levels of cholesterol, calcium, phosphate, creatinine [98], and calcium-phosphate product [82] were also seen to increase. There is also the indication that VitD2 might increase the oxidative stress (increased thioredoxin-interacting protein) and impair VEC function (increased plasma asymmetric dimethylarginine, an NO inhibitor) [98]. This indicates that VitD2 alone is capable of inducing CAVD resulting in functional impairment of the valve [98]. However, use of a hypercholesterolemic diet in combination with VitD2 at 12 weeks produces greater valvular functional impairment than VitD2 alone [82].

2.2.2. Genetic Rabbit Models. Rabbit models utilizing spontaneous mutations as well as transgenic manipulations are available. These models primarily have alterations in the LDLR and/or apolipoproteins that result in hypercholesterolemia when on a cholesterol-free, limited fat diet. Such models include (1) Watanabe heritable hyperlipidemic (WHHL) rabbits, which have a spontaneous *LDLR* mutation [84, 93, 104, 105]; (2) St. Thomas Hospital rabbits, which acquire hypertriglyceridemia as well as hypercholesterolemia [94]; (3) rabbits with altered lipid profiles, such as induced human ApoB100 [90, 94] or Apo(a) [91]. Of these, only the WHHL rabbit has been used in a study of CAVD to show that hypercholesterolemia-induced calcification may be mediated in part by the LPR5/β-catenin pathway [84]. Comprehensive reviews of genetically altered models of rabbit atherosclerosis have been done by Brousseau and Hoeg [87] and Fan and Watanabe [92].

2.3. Porcine Models. Porcine models are regarded as excellent animals for atherosclerosis research [88, 89, 106–108] and more recently for the study of CAVD [32]. Swine have many similarities to humans, including similar systemic hemodynamic variables and heart anatomy [89], including trilayered aortic valve leaflets. They also have similar lipid profiles [61] and lipoprotein metabolism [106, 108] to humans, though their high-density lipoprotein (HDL) level does rise with hypercholesterolemic diets. The porcine genome is of a comparable size to that of humans and is homologous in both sequence and chromosomal structure [32]. Swine naturally develop atherosclerotic lesions, which are accelerated by high-cholesterol/high-fat diets and result in human-type lesions [108–111]. The size of swine also makes them ideal for studies that characterize leaflet mechanical properties and for studies requiring blood analysis.

Size is also the primary limitation of swine, as there is increased complexity and expense in maintaining them. In many cases, this has led to the use of mini- [106, 112, 113] and micro- [114] swine breeds for atherosclerosis studies, instead of full sized Yorkshire swine. Standard weights at six months are around 33 kg for Yucatan mini, 24 kg for Sinclair mini, and 20 kg for Yucatan microswine. These are significantly smaller than Yorkshire swine (approximately 115 kg at six months). Smaller breeds also develop human-type atherosclerosis lesions ranging from early (3-4 months) [106, 113] to advanced lesion (8 months) with a necrotic core, fibrous cap, haemorrhage, calcification, and medial thinning [112]. They may also be very good models for CAVD investigations, but have not been used for this purpose to date. A summary of the critical elements of porcine models of CAVD is presented in Table 2.

2.3.1. Diet-Induced Porcine Models. Though porcine models have been principally used in atherosclerosis research, they have recently been employed to study CAVD. Typical hypercholesterolemic swine diets consist of a standard corn/soybean diet with an additional 1.5–2% cholesterol and 10–20% fat, sometimes with 0.7–1.5% sodium cholate (porcine diet additions are expressed as additional weight percentage added). There is some indication that diets started prior to sexual maturity may be more effective at producing advanced disease [108]. Standard lipid profiles for studies ranging

from 2 weeks to 12 months in length show total cholesterol of ~300–500 mg/dL (2- to 8-fold increase), LDL ~200–500 mg/dL (4- to 11-fold increase), HDL increases of 1.5- to 4-fold, and heterogeneous triglyceride (TG) levels ranging from twofold increase to twofold decrease.

Initial CAVD studies show evidence that swine on hypercholesterolemic diets develop human-type disease. Macroscopic focal areas of increased opacity are seen by six months on a hypercholesterolemic diet with [100] or without diabetes [101]. Small early calcific nodules are also seen histologically at 6-7 months of age either with diet for two weeks or six-months [101] and with six month diet and diabetes [100]. Subendothelial lipid infiltration is seen only within the fibrosa layer, increasing with diet duration [100, 101]; however there is no frank inflammation seen after two weeks or six months of diet [101]. In normal valves it has been found that the VECs on the aortic side of the valve have an antioxidative, noninflammatory, and calcification permissive phenotype [100]. After treatment with a hypercholesterolemic diet, the aortic VECs display a protective phenotype described as antiinflammatory, antiapoptotic, and anticalcific [101]. Notably, VECs on the aortic side are more responsive to the diet. More investigation is needed to explain the mechanistic foundations of side-specific VEC phenotypes.

Atherosclerotic swine exhibit a standard human-type inflammatory response in the vasculature [108], so it is notable that the same is not observed in the valve. However, early human CAVD often does not have many inflammatory cells present [11]. In leaflets with mild disease changes, macrophages were only found in 20% of the lesions and T cells in 55% [13], with greater amounts appearing in stenotic valves [12, 13, 15], ranging from 59% [15], to 75% [13] of valves analyzed.

2.3.2. Genetic Porcine Models. Naturally occurring mutations have also been exploited in swine to develop models of nondiet-induced hypercholesterolemia for atherosclerosis research; they may also be suitable as models of CAVD. These models have mutations in the *LDLR* and/or apolipoprotein genes. Some common models include (1) inherited hyper-low-density lipoprotein and hypercholesterolemia (~250 mg/dL) from mutant alleles Lpb^5, Lpr^1, and Lpu^1 for ApoB and ApoU with normal LDLR [115, 116]; (2) familial hypercholesterolemia (130–490 mg/dL) due to an *LDLR* mutation with altered lipid profiles [117, 118]; or (3) familial hypercholesterolemia due to mutations relating to ApoB and LDLR (<300 mg/dL) [119]. These models are capable of achieving complex atherosclerotic lesions by two to three years of age without diet induction [115].

2.4. Other Animal Models. Many other animals have been used historically or in certain niche areas as models of atherosclerosis. They include rats, hamsters, pigeons, guinea pigs, cats, dogs, or nonhuman primates and have been reviewed by Moghadasian et al. [88, 107]. Occasionally these animals have been used for CAVD studies [75, 120, 121] and some may be suitable for studying certain comorbidities that directly relate to CAVD [122, 123]. However, they are not standard models for CAVD currently.

3. Emerging CAVD Research Themes and the Role of Animal Models

Increased appreciation and understanding of CAVD as an active, cell-mediated process has renewed interest in valve (patho)biology and the possibility for therapeutic intervention. To this end, priority research areas were recently identified by the National Heart and Lung and Blood Institute Aortic Stenosis Working Group [124] and include (1) improving understanding of the basic biology of CAVD, including signalling pathways and the roles of inflammation and biomechanics in disease initiation and progression; (2) determining the unique contributions of comorbidities to disease development; (3) developing highly sensitive imaging modalities to identify early and subclinical CAVD; (4) determining the feasibility of earlier pharmacological intervention. Research in each of these areas currently benefits from animal models and will continue to require the use of appropriate models in the future, as discussed below.

3.1. Basic Biology of CAVD. The biology of CAVD is complex, involving multiple cellular and molecular regulators, genetic and environmental cues, and interacting signalling cascades [125]. Clearly animal models, notably transgenic mice, are important for dissecting specific signalling pathways and their functional consequences; this topic was recently reviewed [126]. Here we highlight inflammation and biomechanics as two factors that are associated with CAVD and likely influence signalling pathways, but have not been well studied. Animal models are well suited to study inflammation and biomechanics and thus will be critical tools to this end.

3.1.1. Inflammation. Despite the strong association of CAVD with inflammation, the effects of inflammatory cytokines and related factors on valve pathobiology have not been thoroughly studied. Many animal models of CAVD demonstrate inflammation and may therefore be well suited to dissecting the role of inflammation in CAVD pathogenesis. Evidence of inflammation in mice includes significant macrophage [34, 37, 39, 41–43, 46, 47, 59] and T-cell [34, 41] infiltration in some models. Others have seen increased expression of immune response genes [49], MMPs [43, 58], and cathepsins [42, 43, 46]. Lastly, oxidative stress as measured by superoxide production has been exhibited in a number of models [37–40]. In rabbits, macrophages [74, 75, 77–79, 82, 83, 95], foam cells [75, 79], T cells [82, 83], and increased MMP-3 expression [103] are reported in valves along with increased hsCRP levels in the blood [78, 85]. In porcine leaflets, VECs on the disease-prone aortic surface progress from a noninflammatory phenotype in normal valves [100] to an antiinflammatory and antiapoptotic phenotype with early disease [101].

While inflammation has been clearly demonstrated in both animal and human histopathological data, studies are

typically limited to characterization of the inflammatory response and not the mechanistic causes. Mouse models of CAVD are well suited for studying pathological mechanisms, as conditional knockouts and other genetic manipulations in this species are relatively straightforward. The *Il1rn*−/− mouse exemplifies this approach, as studies with this model have demonstrated a protective role for the interleukin-1 receptor antagonist in preventing CAVD onset [60]. However, the lack of a trilayer leaflet morphology in mice differs from that of the human, rabbit, and swine and may impact the relevance of findings in mice to human disease. In addition, care must be taken when employing the common *Apoe*−/− mouse model to specifically study inflammatory CAVD mechanisms, as recent work has implicated ApoE as a regulator of inflammation [67]. Rabbit and porcine valves have three layers and are large enough to allow study of biomechanical forces and their impact on valvular inflammation. For example, altered fluid flow-induced shear stress induces endothelial expression of VCAM-1, ICAM-1, BMP-4, and TGF-β1 [127] and elevated mechanical stretch upregulates MMP and cathepsin activity in porcine leaflets ex vivo [128].

Future animal studies will need to better characterize inflammatory processes at various stages of disease development and begin to dissect the regulatory mechanisms that link inflammation to ECM remodelling and valve dysfunction. The validity and utility of various animal models to study inflammation in CAVD will be further clarified by improved temporal resolution of the pathogenesis of human valvular disease.

3.1.2. Biomechanics. The aortic valve exists in a highly dynamic mechanical environment where it is exposed to significant blood flow-induced shear stresses, pressure loads, flexural deformations, and mechanical resistance from the ECM. Each of these mechanical stimuli regulates valve cell biology and therefore likely contributes to both homeostasis and disease [129, 130]. However, much of what is known about mechanoregulation of valve biology is based on in vitro or ex vivo studies and the use of normal, nondiseased tissue sources. Animal models are an important, but largely unexploited source of diseased tissue for ex vivo and in vivo studies of valve biomechanics and mechanobiology in CAVD.

To date, the mechanical properties of *normal* porcine valves have been extensively characterized at multiple length scales: whole leaflets [131–137], individual layers [138, 139], or at higher spatial resolution, focal regions within individual layers [140]. Normal porcine aortic valves have also been used to study the effects of aberrant mechanical forces on valve pathobiology ex vivo [127, 128, 131, 136, 141–143]. The similarity of the trilayer structure, size, and anatomy of the porcine valve to human valve makes them excellent models for structural and biomechanical studies.

To date, the mechanical properties of *diseased* aortic valve tissue from humans, swine, or other species have not been reported, likely due in part to the limited availability of tissue samples and lack of well-characterized large animal models of CAVD. Such information is critical to defining the role of ECM mechanics in disease regulation [144] and should be a focus of future research. The suitability of porcine valves for biomechanical testing lends promise to the use of porcine CAVD models for the study of biomechanical changes with disease progression. Rabbit models may also be suitable, but their smaller size in comparison to porcine valves makes them less desirable. Mouse valves lack the trilayer human-like leaflet morphology and are difficult to test mechanically due to their small size (~500 μm long and 50 μm thick) [62]. However, the speed at which advanced disease can be obtained in mice and the feasibility of studying the effect of genetic knockouts on valve mechanics do provide significant benefit. To do this end, micromechanical test methods, like micropipette aspiration (MA), have been adapted recently to characterize mouse leaflet properties [145]. MA also shows promise for its ability to measure focal, microscale tissue properties. Recently, this technique was used to demonstrate that there is significant spatial heterogeneity in the local elastic modulus within individual layers of normal intact porcine aortic valves, but that on average, the fibrosa layer is stiffer than the ventricularis layer, with distinctly stiff and soft regions in the fibrosa and ventricularis, respectively [140]. The use of MA with animal models of CAVD promises to enable high spatial resolution biomechanical testing that is able to address the focal nature of CAVD.

Ultimately, the gap between in vitro/ex vivo biomechanics studies and human disease will need to be bridged by in vivo biomechanical models of CAVD. The valvular mechanical environment and forces experienced by valve cells are defined in part by the external hemodynamic forces that shear and deform the valve leaflets throughout the cardiac cycle. Manipulation of these forces to test direct causal effects of mechanics on valve biology is challenging because of confounding factors that result from invasive manipulation of the valve or perivalvular tissues (e.g., hypertension). As a result, the causal effects of hemodynamic forces on CAVD have yet to be investigated in vivo. Some advances in this area have been made in the study of the effects of hemodynamics on valve *development*, which were elegantly studied in the zebrafish by using microbeads to impair blood flow [146]. It is believed that aspects of adult valve disease may recapitulate developmental processes [147], and therefore the zebrafish and other developmental models may in fact have some utility for studying the link between hemodynamics and adult valve biology in vivo.

3.2. Contributions of Comorbidities to CAVD. CAVD often coexists with other conditions, including hypercholesterolemia, diabetes mellitus, chronic renal disease, hypertension, metabolic syndrome, and disorders of calcium or phosphate metabolism [148–150]. It is likely that these comorbidities uniquely contribute to the initiation and/or progression of CAVD [124]. A variety of animal models have been used to investigate comorbidities and CAVD, most coupled with a diet that induces hypercholesterolemia to accelerate disease.

A frequently studied comorbidity is familial hypercholesterolemia, an inherited form of hypercholesterolemia that can be caused in humans by more than 800 different mutations [151]. Familial hypercholesterolemic models do

not require diet induction to induce and accelerate disease, and provide a medium for deeper investigation into specific elements of the disease, especially lipoprotein metabolism. In mice, the *Apoe*−/− and *Ldlr*−/−;*Apob*^{100}-only models contain mutations which mimic familial hypercholesterolemia [38, 41]. As discussed in earlier sections, the WHHL rabbit [84, 93, 104, 105], the St. Thomas Hospital rabbit [94], and a number of porcine models [115–119] also mimic familial hypercholesterolemia. Of these, only the *Apoe*−/− and *Ldlr*−/−;*Apob*^{100}-only mice models [38, 41] and WHHL rabbit [84] have been used to study CAVD to date.

The impact of a variety of other comorbidities on *vascular* disease has been studied in various species in which hypercholesterolemia was induced by diet. The unique imbalances caused by diseases such as diabetes, chronic renal disease, hypertension, metabolic syndrome, and high serum minerals likely also influence CAVD progression and could lead to necessary unique insights into disease pathways and inciting factors. To date, however, these models have only been applied to study CAVD in mice. For example, the *Apoe*−/− mouse model has been combined with surgically induced chronic renal disease (CRD) to recapitulate the accelerated CAVD which accompanies human CRD [152]. This 5/6 surgical nephrectomy model with normal diet induces serum hypercholesterolemia after six months (∼400 mg/dL), as well as prototypically high serum phosphate, creatinine, and cystatin C levels. Valve leaflets stain strongly for microcalcification and macrophages, as detected by near-infrared imaging of targeted nanoparticles [47]. The *Apoe*−/−;5/6 nephrectomy model has also been combined with the TD.88137 diet and a knockout of the elastolytic proteinase cathepsin S (*Ctss*−/−). Knockout of cathepsin S reduced valvular calcification, macrophage accumulation, osteogenic and elastolytic activity, and elastin fragmentation [46]. Metabolic syndrome has been recapitulated in WT C57BL/6J mice with the use of a high-fat, high-carbohydrate diet which includes no added cholesterol and produces mild hypercholesterolemia [34], while exercise has been shown to mitigate the effects of the TD.88137 diet in *Ldlr*−/− mice [37]. The development of diabetes is common in many mouse models of aortic valve disease [34, 39], but it is typically a side effect of the studied insult and rarely investigated as a primary initiator of disease [36].

CAVD comorbidities have not been studied in other species, although appropriate putative models do exist. For example, rabbit comorbidity models include diabetes [153], hypertension [154], metabolic syndrome [155, 156], and high serum calcium [80, 82], along with hypercholesterolemia. Porcine models have also combined diabetes [106, 108, 110–112] or hypertension [157] with diet-induced hypercholesterolemia. All of these models have been applied to study atherosclerosis, and presumably would be appropriate and effective for studying CAVD, perhaps offering some advantages over murine models.

3.3. Development of Sensitive Imaging Modalities for Early CAVD Detection. The ability to arrest or slow CAVD progression will likely require early disease detection, before significant calcium burden and hemodynamic dysfunction have occurred. For this reason, there is significant interest in developing sensitive imaging modalities for early stage detection. Targeted fluorescent nanoparticles are one promising strategy. Commercially available nanoparticles specifically targeting cathepsin S, cathepsin K, cathepsin B, macrophages, VCAM-1, MMP-2/9, and hydroxyapatite have been imaged with intravital dual channel fluorescence imaging in a number of animal studies of CAVD [42, 43, 46, 47]. These or other molecular imaging techniques are promising approaches to detect early human CAVD, and clearly preclinical animal models will be critical to their validation.

Beyond their application in clinical medicine, sensitive noninvasive imaging methods are also important for animal studies, as they enable tracking of disease progression and measurement of cardiac function in vivo over the duration of an experiment. Due to their relatively large size, imaging of rabbits and pigs is typically performed with clinical machines [80, 82, 114]. A possible future trend is to combine nanoparticles with standard imaging modalities for targeted detection. For example, iron oxide (MION-47) and MRI imaging were used to detect early CAVD lesions in rabbits fed a low-cholesterol diet by targeting invading macrophages. However, in these experiments, the MION-47 was also taken up by myofibroblasts in control and cholesterol-fed animals, and therefore more specific targeting may be needed [86].

Many mouse studies continue to employ clinical echocardiography using frequency ranges of ∼10–15 MHz to noninvasively examine the functional state of the mouse aortic valve [34, 37–41, 49]. Measurements such as transvalvular blood flow velocity, valve opening time, cusp separation distance, and valve opening area are routinely performed. However, due to the high heart rate of an adult mouse, these frequency ranges only capture 10–20 frames per cardiac cycle [158]. More recently, a high-frequency ultrasound system better suited to imaging the mouse heart has been developed [159]. This system operates at 20–40 MHz, offers a ∼fourfold higher spatial resolution and ∼twofold higher temporal resolution than clinical echo machines [160] and has been validated by magnetic resonance imaging (MRI) as an accurate means to image functional and anatomical parameters of the mouse heart [161]. Increasingly, investigators are taking advantage of this improved technology to delve into the impacts on cardiac function in mouse models of CAVD [50, 53, 59]. Of note, however, is that the higher frequency range of this technique impairs penetration depth and can cause difficulties in successfully imaging aged and obese mice. An alternative is to use high-strength (5–15 T) MRI to image mouse cardiac valves directly [39, 43].

3.4. Evaluation of Pharmacological Interventions. Currently, there is no medical treatment for CAVD, and clinical trials have yielded mixed, but generally discouraging, results that motivate the identification and development of new pharmacological interventions to slow or stop the calcific process in CAVD [162]. Clearly, animal models of CAVD are and will continue to be essential to evaluating therapeutics. As is clear from the summary below, the efficacy of various pharmacological interventions depends largely on the stage

of the disease at the time of drug administration. Thus, translation of results from animal models to humans will be challenging (as CAVD is intentionally accelerated in most models), but must remain a priority.

3.4.1. HMG-CoA Reductase Inhibitors. HMG-CoA reductase inhibitors (statins) were one of the first class of drugs to be directly tested for the treatment of CAVD, with mixed results in both clinical studies [163–166] and NZW rabbit models [78, 83, 85]. In the rabbit studies, statins were administered either concomitant with diet initiation [78, 85] or 15 months after diet initiation, by which point there was established sclerosis but no calcification or functional abnormalities [83]. In the former case, eight weeks of simultaneous atherogenic diet and statin treatment resulted in decreased total cholesterol, leaflet thickness, OPN, macrophage infiltration, cell proliferation, and Runx2 expression compared with diet-only samples [78]. However, the high-cholesterol diet used in this study induced lesions throughout the leaflet, which does not mimic the fibrosa specificity of human disease. In a subsequent study using a lower cholesterol diet, three months of statin treatment (initiated at the same time as dietary insult) decreased the amount of calcification and increased eNOS expression [85]. In WHHL rabbits, atorvastatin attenuated hypercholesterolemia-induced calcification when administered concomitantly with diet, in part through the LDLR LRP5/β-catenin pathway [84]. In contrast, when statins were administered for 15 months after disease had already been established in NZW rabbits, treatment decreased inflammatory cell infiltration and OPN expression but did not prevent calcification or collagen deposition, reduce lipid burden, or prevent the functional impairment that occurred without statin treatment [83].

Statins have not been extensively tested in mouse models of CAVD, though preliminary studies with pitavastatin in *Il1rn*−/− mice are reported to have no therapeutic benefit [60]. However, aggressive lipid lowering has been more directly achieved with the Reversa mouse model, which significantly normalizes plasma cholesterol levels with the use of a genetic switch (~800 → ~200 mg/dL) [39, 40]. Early intervention after six months on the TD.88137 diet was able to reverse nearly all pathological signs of CAVD, but reversal at 12 months after significant calcium burden had already developed did not produce measureable improvements in aortic valve function.

In total, preclinical and clinical trials to date do not support statin therapy as a primary treatment for patients with valvular heart disease to slow its progression [124]. The inconsistent results among the clinical trials likely reflect many differences, including enrolment criteria and timing of therapy [124], which are reflected in the animal studies by the extent of disease at the time of treatment. Thus, it is important that animal models mimic the progression and severity of human disease and experimental designs match the clinical situation as closely as possible, so that preclinical data can be translated to inform the design and interpretation of future clinical trials of statins or other pharmacological interventions.

3.4.2. Renin-Angiotensin-Aldosterone System Inhibitors. The renin-angiotensin-aldosterone system (RAAS) controls blood pressure and fluid and electrolyte balances, and members of this family may have roles in normal physiological repair of the valve. Dysregulation of RAAS components can have proatherothrombotic effects resulting in pathologic fibrosis and calcification of the aortic valve [167]. The major members of this family implicated in CAVD are ACE, AngII, and AT1R [20, 23]. Aldosterone receptors have also been shown to be present in rabbit aortic valves [96]. AngII, produced by ACE and mediated by the AT1R, may contribute to CAVD by promoting macrophage lipid accumulation and inflammation, increasing oxidative stress, impairing fibrinolysis, and stimulating production of the proteoglycan biglycan, which can retain lipoproteins [125].

ACE inhibitors, angiotensin receptor blockers (ARBs), and aldosterone receptor antagonists (ARAs) have begun to be investigated for treatment of CAVD. ACE inhibitors have shown mixed results in retrospective clinical studies with one showing a strong association between the use of ACE inhibitors and decreased valve calcification [168] and the other finding no effects [169]. In NZWR, treatment with the ACE inhibitor ramipril concomitantly with induction of CAVD by VitD2 at 25000 IU (four days per week) for eight weeks retarded the development of CAVD [99]. The ARB olmesartan decreased macrophage and myofibroblast accumulation, decreased *OPN*, *ACE*, and *Runx2* mRNA expression, and maintained endothelial integrity when administered to NZWR during the last four weeks of an eight-week 1% cholesterol diet treatment [95]. The ARA eplerenone showed no effect in clinical trials of moderate- to severe stenosis [170]. However, administration of eplerenone to NZWR for the last four weeks of an eight-week 1% cholesterol diet elevated aldosterone levels without altering blood pressure and decreased macrophage accumulation, ACE expression, and calcification within the leaflets [96].

3.4.3. Antioxidants. Reactive oxygen species are present in both human [20, 23] and rabbit [19] valves during CAVD. The antioxidative compounds tempol and lipoic acid (LA) were tested individually by concomitant administration with a CAVD-inducing diet of 0.5% cholesterol and VitD2 in NZWR. Tempol decreased superoxide presence but led to an increase in hydrogen peroxide, NAD(P)H oxidase subunits, and calcification. LA decreased both superoxide and hydrogen peroxide and led to a decrease in calcification and echogenicity of the valve. These preliminary results showed that ROS may potentiate calcification, particularly in relation to hydrogen peroxide and possibly NAD(P)H oxidase activity, and pharmacological prevention of ROS may prevent or decrease calcific burden [19].

4. Conclusions and Recommendations

When properly chosen and implemented, animal models are a vital tool for the investigation of pathobiological mechanisms of CAVD and potential therapeutic interventions. Many animal models have been shown to recapitulate

important aspects of human CAVD pathobiology, thus enabling detailed investigations that are otherwise unfeasible or impossible to conduct in humans. The development of new models and our improved understanding of the utility of existing models have quickly advanced the impact that animal model-based studies are making within this field. Mouse, rabbit, or swine models each offer species-specific advantages for the study of CAVD and represent a fundamental step forward in the translation of basic scientific research into clinically relevant and impactful knowledge.

Moving forward, there is a need for improved model characterization to enable direct comparisons between multiple studies and aid in interpretation of findings as they related to human CAVD. At a minimum, the following model parameters necessary for interstudy comparisons should be provided: (1) animal starting age and weight; (2) full diet composition, including % kcal contributions; (3) feeding regime (ad libitum, weight adjusted, or fixed amount, and growth rate); (4) full plasma lipid profiles (TC, HDL, TG, LDL); (5) background strain(s) used in generating knockout mice; (6) age and weight at sacrifice. Models should have full histological characterization of lesion progression, including lesion composition and anatomical location, and valvular function should be impaired in advanced disease models. Some mice and rabbit models acquire hemodynamically significant CAVD, but the same has yet to be shown in swine. However, some swine models develop advanced atherosclerotic lesions and vascular stiffening after eight months of high-cholesterol diet [112], and therefore valvular stiffening and dysfunction may simply take longer than has been studied to date [101].

In order to truly understand the relevance of animal models to human valvular disease, a better understanding of human CAVD pathogenesis is required. As new insights into human disease are revealed, animal models and the data they generate need to be critically reinterpreted. A priority is to determine whether the extreme hypercholesterolemia induced in many standard CAVD models faithfully represents the full progression of human disease. In the future, ageing models of disease may prove to be the best at inducing human-type disease, although time and expense remain prohibitive. To date, there are two aged mouse models [38, 41] and one aged rabbit model [83] that maintain moderate cholesterol levels and induce functional disease. Swine models of familial or mild diet-induced hypercholesterolemia could provide the best match with cholesterol levels in humans and when aged, may produce advanced disease.

Perhaps the most promising application of well-characterized animal models is to investigate CAVD-initiating events and the mechanisms of its progression to late-stage functional impairment. Animal models clearly offer a significant advantage in this context, as it is difficult to clearly identify disease stage or control for confounding factors in human autopsy or transplant samples. CAVD initiation and early progression have largely been ignored to date, but would provide the richest insight into therapeutic targets to arrest calcification before the putative "point of no return" when calcific burden cannot be reversed. Inflammation, ECM adaptation, and early procalcific signalling likely play important roles, but have not yet been studied in detail. There has been little focus on, for example, the mechanisms that regulate macrophage/T-cell infiltration, inflammatory signalling cascades, or layer-specific ECM changes and their role in modulating VIC phenotype. It is prospective investigations of molecular regulators of valve homeostasis and disease progression like these that will benefit from well-characterized and validated animal models and are most likely to lead to the discovery of novel treatments for CAVD.

Author's Contribution

Krista L. Sider and Mark C. Blaster contributed equally to this paper.

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Review Article

Inflammatory Regulation of Valvular Remodeling: The Good(?), the Bad, and the Ugly

Gretchen J. Mahler and Jonathan T. Butcher

Department of Biomedical Engineering, Cornell University, 304 Weill Hall, Ithaca, NY 14853, USA

Correspondence should be addressed to Jonathan T. Butcher, jtb47@cornell.edu

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Heart valve disease is unique in that it affects both the very young and very old, and does not discriminate by financial affluence, social stratus, or global location. Research over the past decade has transformed our understanding of heart valve cell biology, yet still more remains unclear regarding how these cells respond and adapt to their local microenvironment. Recent studies have identified inflammatory signaling at nearly every point in the life cycle of heart valves, yet its role at each stage is unclear. While the vast majority of evidence points to inflammation as mediating pathological valve remodeling and eventual destruction, some studies suggest inflammation may provide key signals guiding transient adaptive remodeling. Though the mechanisms are far from clear, inflammatory signaling may be a previously unrecognized ally in the quest for controlled rapid tissue remodeling, a key requirement for regenerative medicine approaches for heart valve disease. This paper summarizes the current state of knowledge regarding inflammatory mediation of heart valve remodeling and suggests key questions moving forward.

1. Introduction

The heart valves are the sole mediators of unidirectional flow through the cardiovascular system. These valves flex open and close 30 million times per year, subjecting the thin and flexible cusps or leaflets to demanding tissue strains and hemodynamic stresses. The fact that these tissues thrive can only be attributed to the remarkable stamina and remodeling capacity of the indigenous valve endothelial and interstitial cells that populate these valves. Over the past decade, many exciting discoveries have been made regarding the unique phenotypes of these cells, yet it has only framed the beginning of our understanding of valve function and dysfunction. Heart valve disease remains a serious and increasing clinical problem for which no solution exists save prosthetic replacement. These come in the form of mechanical or processed biological tissue valves. While providing over 20 years of function in elderly patients, these technologies perform dismally in children and young adults, with undesirable lifestyle restrictions and significant medical requirements. Tissue engineering has the potential to alleviate these limitations by providing a living valve conduit that can grow and remodel with the patient. Current results in animal trials are very promising, but human trials to date suggest that there is still much more to learn. Foremost among these needs is to understand how valve tissue remodels in the midst of the complex mechanical and biological signaling environment in which it resides. This includes natural tissue remodeling that occurs over embryonic development into adulthood, homeostatic and pathological adaptation over the course of life and disease, and with different living tissue replacement strategies. Being a biological structure that evolves and adapts over the entire lifespan, it seems likely that similar signaling mechanisms would be utilized across this continuum. While literally hundreds of regulatory genes have been identified in valve phenotypes and discussed in several reviews, this review will focus on the role of inflammation. While it is well appreciated that inflammation is a major driver of valve pathology, recent evidence suggests that inflammatory cytokines are present in embryonic development and in remodeling valves, which suggests its presence may not be singularly negative.

2. Inflammation and Wound Healing

The general healing response to tissue injury involves three phases: inflammation, tissue formation, and tissue remodeling [1]. The healing response begins when the tissue is injured, blood comes into contact with collage or other components of the extracellular matrix, and a blood clot forms. The blood clot platelets release chemotactic factors that recruit leukocytes to the injury site and initiate the inflammation phase [2]. These leukocytes then secrete chemokines and inflammatory cytokines to enhance the inflammatory response [2]. Next, neutrophils enter the wound site to remove foreign materials, bacteria, and damaged tissue; macrophages follow to continue the process of phagocytosis [3]. Fibroblasts deposit new extracellular matrix in the tissue formation phase [3]. In the remodeling phase, the newly deposited extracellular matrix is cross-linked and organized [3]. There are many cell signaling events required for this tightly controlled repair process to take place. The cytokine transforming growth factor-β (TGF-β) has been shown to play a part in all three phases of healing [4].

The inflammatory phase is initiated when TGFβ and other growth factors are released from platelets [5]. TGFβ has been shown to be chemotactic and mitogenic for neutrophils, lymphocytes, monocytes, macrophages, and fibroblasts [6]. During the tissue formation phase inflammatory cells migrate to the wound and secrete additional TGFβ, which at higher concentrations may induce the expression of other growth factors and increase cell proliferation at the wound site, stimulate angiogenesis, and promote collagen deposition [6–8]. During tissue remodeling TGFβ continues to promote extracellular matrix production and inhibit its breakdown, which has been implicated the cytokine in scar formation [6, 9]. Scars are fibrous tumors characterized by overabundant collagen deposition [10]. Treatment with TGFβ has been shown to increase endogenous TGFβ production, collagen deposition, and scar formation; while exposure to anti-TGFβ antibody decreases endogenous TGFβ production, collagen deposition, and scarring [9–11].

3. Inflammation and Valve Homeostasis

Each valve is comprised of thin, fibrous leaflets or cusps that are attached to a relatively rigid annulus or root [12]. The atrioventricular valves are further supported by tendinous chords that connect the leaflet free edge to the papillary muscles [12]. The leaflets/cusps are a multilayer composite of collagen, elastin, and glycosaminoglycans that assist in its efficient biomechanical function [12]. The surfaces of these tissues are lined with endothelial cells (VEC) while the underlying matrix is populated with interstitial cells (VIC)—a constellation of subphenotypes with incompletely understood individual roles [13]. In general, the endothelial cells are responsible for sensing and integrating biological and mechanical signals from the blood, and transmitting signals to the interstitial cells [14]. The VIC in turn proliferate and remodel the surrounding matrix [15]. Both cell types are also affected by other microenvironmental cues, such as mechanical strain, tissue stiffness, and the presence of other cell types such as inflammatory macrophages and circulating cells [16]. Mitral valve cell biology is much less understood because the tissue is much more heterogeneous in structure and composition, potentially reflective of its more diverse mechanical environment [17]. The mitral valve is unsurprisingly susceptible to a much wider range of dysfunctional conditions (explained later). Therefore, this section will discuss results from aortic valves.

The inflow (atrialis/ventricularis) surface is exposed to a rapid, pulsatile, unidirectional shear stress with cycle averaged magnitude of 20 dynes/cm^2 [18]. The outflow (fibrosa) surface experiences a much lower magnitude, nearly oscillatory shear stress [19]. VEC align perpendicular to the direction of flow *in vitro* and *in vivo* whereas vascular EC align parallel [20]. Microarray comparisons between aortic valvular and vascular endothelium in static culture and under fluid flow identify hundreds of significantly different genes, suggesting that VEC are a distinct endothelial phenotype [21]. Steady shear stress was protective against pro-oxidation and proinflammation in both cell types, but VEC were inherently less inflammatory than arterial endothelial cells [21]. Simmons and colleagues probed side-specific differences in aortic VEC gene expression [22]. Aortic side endothelial cells showed significantly less expression of multiple inhibitors of cardiovascular calcification, enhanced antioxidative gene expression, and a lack of differential expression of proinflammatory molecules; suggesting that the aortic side endothelium may be primed to protect against inflammation and lesion initiation in the normal valve. In a follow-on study, Guerraty et al. investigated the side-specific aortic valve endothelial gene expression of hypercholesterolemic pigs [23]. They identified differential expression on the aortic side of caspase 3, peroxisome proliferator-activated receptor-γ, tumor necrosis factor-α (TNF-α), and nuclear factor-κB- (NF-κB-) related pathways that were consistent with a protective endothelial phenotype that persisted at 6 months. In contrast to these results, Sucosky et al. showed that high magnitude pulsatile shear stress applied to fibrosa-side VEC (an "altered" shear stress state) induced upregulation of inflammatory receptors and expression of BMP-4 [24]. Similar to shear stress, cyclic mechanical strain or pressure can induce and modulate an inflammatory phenotype in aortic VEC [25–27]. Cyclic tissue strain at 5% or 20% magnitude increased inflammatory cytokine expression in aortic valve endothelium, but decreased at 10–15% [25, 26].

VIC are a heterogeneous cell population with up to five different cell phenotypes (fibroblasts, smooth muscle cells, myofibroblasts) in adult aortic valves [13, 15, 28–31], but generally exhibiting fibroblastic phenotypic characteristics. Normal aortic valve interstitial cells secrete and turnover proteins and glycosaminoglycans at a dramatically increased rate in comparison to other cell types *in vivo*, with a significantly higher index of proliferation [32]. This suggests that VIC continually repair mechanically induced tissue microdamage to enable long-term durability. VIC freshly isolated from higher pressure left-sided valves (aortic, mitral) were significantly stiffer and had more collagen biosynthesis than cells isolated from right-sided valve (tricuspid,

pulmonary) [33]. Collagen synthesis by valve interstitial cells was shown to be dependent upon the degree and duration of stretch by Ku et al., as there was a significant increase in [^{3}H]-proline incorporation into stretched valve cells at 10%, 14%, and 20% stretch [34]. These results indicate that VIC-mediated matrix remodeling is regulated in part by the magnitude of local mechanical signaling. VIC exposed to 15% "pathological" cyclic strain increased expression of α-smooth muscle actin (α-SMA), bone morphogenic protein (BMP)-2/4, matrix metalloproteases (MMP) and cathepsin activity, apoptosis, and osteoblastic protein expression, but not at 10% [27]. Hypertensive (170 mmHg) cyclic pressure also increased expression of VCAM-1 and downregulated osteopontin [35]. VIC-VEC cocultures within 3D type I collagen scaffolds suggest that VEC help maintain a quiescent VIC fibroblastic phenotype [36, 37]. The presence of endothelial cells stabilized VIC proliferation, promoted a quiescent VIC phenotype, increased VIC protein synthesis, and decreased glycosaminoglycan loss [37]. The addition of steady shear stress to the cocultures further enhanced the effects of the endothelial cells, including a further decrease in myofibroblastic markers and increase in protein synthesis [37].

4. Inflammation and Calcific Aortic Valve Disease

The cause of calcific aortic valve disease (CAVD) is not completely defined, but inflammation plays a lead role in the initiation and progression of CAVD. Adhesion molecules, such as ICAM-1, VCAM-1, PECAM-1, CD34, and E-selectin, promote the participation of endothelial cells (EC) in both physiological and pathological inflammatory responses through the recruitment of leukocytes [38]. EC become activated, which is a phenotypic change characterized by the production of a variety of biologically active products (cytokines, growth factors, proteolytic enzymes, adhesion molecules), an increase in adhesion molecule expression, endothelial-leukocyte interaction, and permeability, in response to stimuli including circulating inflammatory cytokines, lipopolysaccharides, activation of the renin-angiotensin system, hypercholesteremia, CD40/CD40 ligand interactions, ischemia-reperfusion, physical trauma, diabetes, and hemodynamic forces [38–41]. In diseased aortic valves, VEC upregulate ICAM-1, VCAM-1, and E-selectin, and this occurs preferentially on the fibrosa surface of the valve [24, 38, 40]. The fibrosa or aortic side of the valve is also exposed to disturbed, oscillatory flow, high bending stresses, and is where calcific degeneration initiates [19, 42–45]. The hemodynamics on the valve fibrosa may make the cells more susceptible to inflammatory cell infiltration. *In vitro*, disturbed flow has been shown to cause pro-inflammatory cytokine release (BMP-2/4) and a pro-oxidant phenotype (NADPH, ROS) in VEC [21, 24, 46]. Oxidative, inflammatory, and chondrogenic/osteogenic gene expression profiles are upregulated *in vitro* in VEC grown under static conditions, which mimic hemodynamic conditions on the fibrosa side, when compared with steady shear stress conditions, which recreate ventricularis side hemodynamics [21, 47]. Evidence of leaflet stress promoting CAVD is the discrepancy in age at the time of presentation with tricuspid and bicuspid valves [48]. Patients with bicuspid valves, which are subjected to higher mechanical stresses, on average present with CAVD two decades younger than those with tricuspid valves [49, 50].

Atherosclerotic risk factors, such as increased low-density lipoprotein cholesterol, increased lipoprotein(a), male gender, cigarette smoking, hypertension, elevated body mass index, and diabetes, increase the incidence of aortic stenosis and likely contribute to valve endothelial dysfunction [44, 51, 52]. The dysfunctional, activated VEC in early valve disease have increased permeability and upregulated adhesion molecule expression. Monocytes attach to adhesion molecules, migrate into the subendothelial space of the valve, and differentiate into macrophages [38, 40, 53]. Macrophages and T-lymphocytes have been shown to be present in aortic valve lesions [54–56]. These invading inflammatory cells, and likely the resident activated endothelial cells, secrete a number of cytokines and/or chronic inflammation effector molecules (e.g., HLA-DR IL-1β/2/6, TNF-α, TGFβ-1, BMP-2/4/7) [43, 56–59]. In addition, circulating low density lipoproteins (LDL) are able to migrate through the permeable endothelial layer, and oxidized forms are capable of deep tissue penetration [60, 61]. Sub-endothelial LDL accumulation can recruit additional inflammatory cells by mechanisms including the induction of macrophage chemoattractants, adhesion molecules, and cytokines [62, 63]. Circulating LDL particles may also deliver angiotensin converting enzyme (ACE) to valve lesions [64]. Angiotensin II, which is generated from angiotensin I by ACE, can compound the inflammatory responses already present by stimulating inflammation and macrophage cholesterol accumulation, increasing oxidant stress, and impairing fibrinolysis [65].

The cytokines and inflammatory effector molecules secreted into the subendothelium and fibrosa by immune cells and activated endothelial cells contribute to a local biochemical environment that promotes VIC differentiation, matrix remodeling, neovascularization, fibrosis, and calcification. TGFβ-1, BMP-2/4/7, IL-1β, TNF-α, lipopolysaccharide, and peptidoglycan have been shown to induce myofibroblastic differentiation, the expression of proinflammatory mediators, or the upregulation of osteogenesis-associated factors in VIC when applied individually *in vitro* [43, 57, 59, 66–69]. Impaired anti-inflammation mechanisms may also contribute to VIC-mediated pathogenesis of aortic stenosis as interleukin-1 receptor antagonist, which is the antagonist of interleukin-1β, was shown to be abundant in nonstenotic aortic valve leaflets and nearly absent in leaflets from stenotic valves [70]. Myofibroblastic activation is characterized by an increase in myofibroblast markers such as vimentin, α-SMA, and embryonic nonmuscle myosin heavy chain (SMemb); and increased cell migration, proliferation, and contractility [13, 29, 31, 71–73]. Activated VIC and inflammatory cells secrete MMP, and cathepsins that progressively destroy the primarily collagen and elastin valve matrix ultrastructure [71, 74–79]. Enzymatic cleavage of ECM can release bound growth factors such as TGFβ-1, which further promotes VIC

myofibroblast differentiation and MMP expression [80–83]. MMP and cathepsin inhibitors, such as TIMPs and cystatin C, are also expressed by activated VIC, but their role in CAVD is not yet known [77, 84]. Following healthy ECM destruction, activated VIC deposit a remodeled, fibrotic matrix characterized by disorganized collagen bundle accumulation, proteoglycan degradation, and fragmentation and stratification of elastin fibers [84–86]. This ECM remodeling results in a stiff aortic valve that is prone to restricted movement, stenosis, and eventual calcification [85].

5. Myxomatous Degeneration of the Mitral Valve

Myxomatous degeneration of the mitral valve or mitral valve prolapse (MVP) is a condition diagnosed with echocardiographically and characterized by abnormally thickened, redundant, floppy leaflets that are displaced into the left atrium during systole [87]. MVP is estimated to affect 1–3% of the US population, and some serious complications of the condition include progressive heart failure, thromboembolism, infective endocarditis, and sudden death [71, 87–90]. The mechanisms for the changes within the valve leaflets are unknown, but myxomatous valvular degeneration is characterized by collagen degradation, proteoglycan accumulation, and elastin fragmentation [85, 87]. This valve matrix remodeling allows stretching of the leaflets, resulting in a floppy valve that is prone to prolapse and regurgitation. It has been hypothesized that the leaflet remodeling may be a response to repeated mechanical stress [91].

There are several connective tissue inherited disorders that are associated with mitral valve dysfunction including Marfan syndrome (fibrillin-1 mutations), Williams syndrome (elastin mutations), osteogenesis imperfecta (collagen 1A1 and 1A2 mutations), Ehlers-Danlos syndrome (mutations in collagen 1A1, 3A1, 1A2, 5A1, and tenascin-x), and Stickler syndrome (collagen 2A1 and collagen 11A1 mutations) [92–100]. MVP is generally sporadic, however, and it is unlikely that more than 1-2% of MVP cases are associated with a connective tissue disorder [87]. A Marfan syndrome mouse model has indicated that increased TGF-β signaling contributes to collagen dysregulation and loss of valve matrix integrity in Marfan syndrome-related and possibly other forms of MVP [101]. Fibrillin-1 interaction with latent TGF-β binding proteins (LTBP) regulates TGF-β activation [102, 103]. LTBP forms a bridge between matrix microfibrils and latency-associated peptide (LAP), which remains noncovalently linked to TGF-β and aids in matrix sequestration. Work by Ng et al. suggests that TGF-β sequestered in the TGF-β, LAP, and LTBP complex is stabilized and/or less prone to activation due to interaction with fibrillin-1 and potentially other components of the extracellular matrix [101]. Pharmacological inhibition of TGF-β signaling with losartan has been shown to reduce Marfan syndrome pathology in mice and humans [104, 105]. Increased TGF-β signaling also supports the interstitial cell activated myofibroblast phenotype and excessive proteolytic activity found in myxomatous valves, as TGF-β has been shown to activate valve interstitial cells and upregulate the expression of ECM-degrading enzymes [71, 82].

6. Rheumatic Heart Valve Disease and Infective Endocarditis

Rheumatic fever (RF) is an inflammatory complication that may develop after a untreated throat infection by the group A β-hemolytic streptococcal bacteria *Streptococcus pyogenes* in susceptible children and teenagers [106]. Carditis, which is one of the most serious RF complications, occurs about 20 days after the infection in 40–50% of patients and can lead to valvular heart disease, heart failure, or death [107–109]. The streptococcal cell structures include the cell wall, capsule, fimbriae, peptidoglycans, cytoplasmic membrane, group-specific carbohydrates, and the M, T, and R antigenic proteins [110]. The streptococcal M protein and hyaluronic acid capsule have been established as the most important virulence factors in human infections, as both confer antiphagocytic properties upon the streptococcal cell and patients with acute RF have a high level of antibodies to streptococcal M protein [111–115]. The M protein is attached to the bacterial cell wall and membrane and extends from the cell surface as an alpha-helical coiled-coil dimer [116, 117].

RF-related cardiac complications are the result of an autoimmune reaction induced by molecular mimicry of human tissues by streptococcal M proteins [108]. The alpha-helical coiled-coil dimer streptococcal M protein has been shown to be structurally and immunologically similar to cardiac myosin, a known mediator of inflammatory heart disease, and other alpha-helical coiled-coil molecules [118]. The antistreptococcal/antimyosin monoclonal antibody mAb 3B6 from rheumatic carditis has shown that the group A streptococcal M protein *N*-acetylglucosamine, which is the dominant group A carbohydrate epitope, and cardiac myosin in the myocardium are the cross-reactive antigens involved in antibody deposition on the valve [119]. Cardiac myosin is not part of the valve, but mAB 3B6 was also shown to recognize laminin, a valvular extracellular matrix protein with alpha-helical coiled-coil domains that are highly homologous with streptococcal M proteins and cardiac myosins [118, 119].

In acute rheumatic carditis host M-protein antibodies bind the valve surface endothelium and/or the valve basement membrane structure protein laminin, which upregulates the endothelial expression of inflammatory adhesion molecules such as VCAM-1 [120]. The inflamed valvular endothelium leads to T-cell recruitment and infiltration through the endothelial layer [118]. T-lymphocytes enter the valve interstitium and cause further inflammation, degeneration, and remodeling. The resulting valve pathologies include neovascularization, chronic inflammation, commissural fusion, thickening, calcification, and thickened and shortened chordae in the atrioventricular valves [108].

Infective endocarditis (IE) is inflammation of the endocardial surfaces of the heart, most commonly the heart valve, caused by the presence of bacteria in the bloodstream and bacterial vegetations forming on valve leaflets [121].

The bacterial strains that cause IE include staphylococci, streptococci, and enterococcus, and the complications from this type of infection include severe valvular dysfunction, congestive heart failure, and death [122]. If there is altered blood flow around the valves or the valves have been damaged, from mitral valve regurgitation and thickening due to rheumatic fever, for example, the risk of bacterial attachment increases [121].

The bacterial vegetations that form on the valve surface are composed of platelets, fibrin, microorganisms, and inflammatory cells and are the result of mechanical or inflammatory valve lesions [121, 123]. Mechanically denuded endothelial layer lesions promote microbial adherence to the endothelium when bacteria is present in the bloodstream [123]. Endothelial denudation results in direct contact between blood and proteins within the valve interstitium, which include extracellular matrix proteins, thromboplastin, and tissue factor, and causes blood coagulation [123]. The damaged endothelium is colonized when microorganisms bind to the fibrin and platelet-containing blood clots and initiate monocyte activation and production of cytokines and tissue factor activity (TFA) [124]. The vegetation grows when cytokines and TFA activate coagulation cascades, attract and activate blood platelets, and induce cytokine, integrin, and TFA production from nearby endothelial cells [123]. Inflammatory lesions are the result of endothelial cells responding to local inflammation by expressing β1 integrins, including very late antigen (VLA) [125]. β1 integrins bind fibronectin to the valve endothelial surface [123]. IE-associated pathogens have fibronectin-binding proteins on their surface, therefore β1 integrins provide an adhesive surface for the circulating microorganisms [123]. Following pathogen adhesion, endothelial cells internalize the bacteria; which causes endothelial TFA and cytokines production, blood clotting, the extension of inflammation, and vegetation growth [123, 126]. Internalised bacteria eventually lyse endothelial cells by secreting membrane active proteins such as hemolysins [123]. The vegetation growth and tissue damage caused by mechanical or inflammatory lesions can result in abscess formation and septic emboli may circulate to other organs [127].

7. Serotonin Metabolism-Related Valve Disorders

Clinical studies have identified an increased incidence of fibrotic aortic valve disease in patients using several classes of drugs that are structurally similar to serotonin (5-hydroxytryptamine, 5-HT) and in patients with carcinoid syndrome, which can result in a high serotonin concentration on the right side of the heart [128–131]. External serotonin administration *in vitro* and *in vivo* has been shown to increase VIC proliferation and α-SMA, collagen, and TGFβ expression, which are all indicative of myofibroblastic activation and fibrotic matrix remodeling [132–135]. No studies have directly linked aortic valve calcification and elevated serotonin levels; however, serotonin may mediate intermediate stage valve fibrosis. Recent evidence supports serotonin synthesis by valve cells [136]. This may contribute to valve calcification through increased TGFβ signaling by serotonin receptor antagonists or transporter agonists in mitral valves [136]. The effects of serotonin on VEC are less well understood, but endothelial function is required for serotonin-mediated valve relaxation [137, 138]. These results suggest that controlling serotonin metabolism may be a novel means of selectively modulating valve cell phenotype *in vivo*.

8. Bone Marrow Stem Cell Contribution to Valve Repair and Disease

Bone marrow-derived mesenchymal stem cells (BMSCs) are found in adult circulation at a low concentration and are thought to regulate the immune response in settings such as tissue injury, transplantation, and autoimmunity [139]. Mouse and human valve studies have shown that at least 10% of VIC are BMSCs, but the specific role of BMSCs in valve disease is not well understood [140, 141]. The progenitors appear to be recruited with other inflammatory cells into the valve interstitium, but whether they initiate repair or promote disease is unclear. Tanaka et al. identified bone marrow-derived cells expressing myofibroblast and osteoblast markers near calcific nodules in aged mice, which was supported in humans by Helske et al. [79]. Understanding the recruitment and function of circulating stem cells in valve homeostasis and pathogenesis could have clinical benefits, as autologous BMSCs may be treated to express antifibrotic and anticalcific proteins before their mobilization to valves [142].

9. Inflammation and Valve Tissue Engineering

Tissue engineered heart valves could provide a great clinical benefit to patients with valve disease, especially children who require valve growth and younger patients who cannot tolerate the side effects of nonliving prosthetics [143, 144]. Understanding the role of host inflammatory response is essential to the successful implantation of tissue engineered valves, however. Repopulation and regeneration are the two main heart valve tissue engineering approaches that have been attempted [145]. A repopulated heart valve is created when a patient's cells repopulate an implanted decellularized porcine aortic valve [145]. A heart valve is regenerated by implantation of a resorbable matrix that remodels *in vivo*, resulting in a functional valve composed of the patient's cells and connective tissue proteins [145]. Neither of these approaches have achieved clinical success, however.

Long-term sheep implantation studies with decellularized heart valves and acellular valves seeded with cells before implantation showed significant tissue overgrowth, infiltration with inflammatory cells, and dilatation [146, 147]. The SynerGraft valve by CryoLife is a decellularized pulmonary valve that has been implanted in humans. Adult clinical trials have been encouraging, but in children, rapid inflammatory reactions caused death in 3 out of 4 patients [148, 149]. All pediatric valves displayed severe inflammation, including fibrosis, encapsulation, perforation, and deterioration of the leaflet tissues [149]. Matrix P plus valves, which are decellularized pulmonary porcine

valves, displayed early obstruction following implantation in pediatric patients [150]. A foreign body-type reaction accompanied by severe fibrosis and massive neointima formation around the decellularized porcine valve wall was found. Examination of the explanted valves showed inflammatory infiltrates, composed of T cells, B cells, plasma cells, dendritic cells, macrophages, and mast cells, in the tissue surrounding the porcine matrix. *In vitro* studies have shown that the inflammatory mechanism of decellularized valves may involve porcine collagen type I, but not porcine elastin [151].

In one of the few long-term *in vivo* studies with polymeric tissue engineered heart valves, composite biodegradable polymer valves were seeded with mesenchymal stem cells, cultured for 4 weeks, and implanted into a sheep model for up to 20 weeks [152]. *In vivo* monitoring showed leaflet coaptation, and explanted valves showed near-native trilaminar matrix striation with both endothelial-like and interstitial-like cell phenotypes. Elevated transvalvular pressure gradients, however, suggest that the cusps are still too stiff. Interestingly, these valves exhibited a marked inflammatory activation during their early remodeling period, characterized by the presence of endothelial inflammatory receptors (VCAM-1, ICAM-1), matrix metalloproteases, and interstitial cell myofibroblastic activation. Early matrix structure was virtually absent and disorganized. After 2-week *in vitro* conditioning, and further enhanced with *in vivo* implantation, the progression to organized tissue striation correlated inversely with the degree of activated valve cell phenotypes. Collectively, these results demonstrate that the degree of inflammation has a significant role on the fate of engineered tissues, and suggests that progressively reduced inflammation can help develop and maintain quiescent tissue.

10. Inflammatory Regulation of Valve Formation

The morphogenesis of the atrioventricular and semilunar valves is a complex process that occurs along with the changing cardiac morphology and hemodynamics of the growing embryo. The early embryonic heart is a tube of endocardium surrounded by myocardium [153]. Soon after the linear heart tube begins to loop the myocardium secretes cardiac jelly, a hyaluronan and chondroitin sulfate rich gelatinous matrix, into the atrioventricular (AV) junction and outflow tract (OFT) lumen [154–157]. At chick stage HH14 (mouse E9.0, human day 20), valvulogenesis begins when a subset of endocardial cells receive growth factor signals from the myocardium that initiate a cascade of signal pathways resulting in an endocardial to mesenchymal transformation (EMT) [153, 158]. EMT is characterized by endocardial cell activation; three antigens expressed only by activated endocardial cells include ES130, JB3/fibrillin, and TGFβ3 [159–161]. Activated endocardial cells lose cell-cell contacts proteins (PECAM1, NCAM1, DS-CAM), gain cell-matrix adhesions (integrins), gain mesenchymal markers (α-smooth muscle actin, α-SMA), and the acquire cell migratory and invasive capacity (transformation) [153, 157, 158, 162, 163]. The transformed endocardial cells invade the cardiac jelly and form the endocardial cushions that will eventually develop into mature atrioventricular and semilunar valves [157, 162].

Few studies have been performed to determine the role of inflammation on valve development. A recent study showed that leptin, a member of the IL-6 superfamily, mediates embryonic EMT [164]. TNF-α serum levels were found to be high in children with congenital heart disease and TNF-α and IL-6 levels were elevated in the myocardium of infants with tetralogy of Fallot or ventricular septal defects [165, 166]. Neonates with hypoplastic left-heart syndrome (HLHS) have evidence of an activated inflammatory response, as IL-6 levels are significantly elevated when compared with controls [167]. Fetal aortic valve endothelial cells express ICAM-1 and VCAM-1, but whether this is due to inflammation is unknown [41]. VCAM-1 deficient mice survive to E11.5–12.5 and display severe heart abnormalities including a reduction of the compact layer of the ventricular myocardium and intraventricular septum, blood in the pericardial space, and an absent epicardium [168].

Abnormal inflammatory signaling may play a role in the development of congenital heart defects. Children born to mothers' with the connective tissue disease lupus erythematosus (LE) have an increased risk for congenital heart block (CHB), a type of arrhythmia, and valve regurgitation [169, 170]. CHB can result in early death from delayed pacemaker placement or hemodynamic compromise from associated congenital heart disease [171]. The fetal heart defects resulting from maternal LE are thought to be the result of transplacental passage of certain maternal autoantibodies, including immunoglobulin G (IgG) antibodies to SSA/Ro and SSB/La ribonucleoproteins [169, 172]. These autoantibodies damage the fetal AV conduction tissue by inflammation in the early stage and later by fibrosis [173]. Maternal inflammatory insult has also been shown to result in fetal cardiac dysfunction [174]. Administration of intra-amniotic lipopolysaccharide caused an increase in maternal TNF-α and IL-6 serum levels, and the placenta showed severe maternal vascular dilatation and congestion [175]. No inflammatory activation was found in the fetal tissues, and the amniotic fluid revealed no significant increase in cytokines; but ultrasonographic examination of the fetal hearts showed that 65% of the fetuses exhibited atrioventricular valve regurgitation [175]. The maternal inflammatory insult was shown to activate placental labyrinthine macrophages, which then leads to an acute increase in placental vascular resistance and fetal cardiac dysfunction [175].

Abnormal nuclear factor-Kappa B (NF-κB) activation may play a role in congenital heart defects. NF-κB is a transcription factor that coordinates inflammation and cellular proliferation. Upregulated NF-κB activation was found in the myocardium of children with congenital heart disease and in the myocardium of infants with tetralogy of Fallot or ventricular septal defects [165, 166]. The inhibition of NF-κB during chicken heart development led to impaired OFT development and resulted in interventricular communication, double outlet right ventricle, and valvular

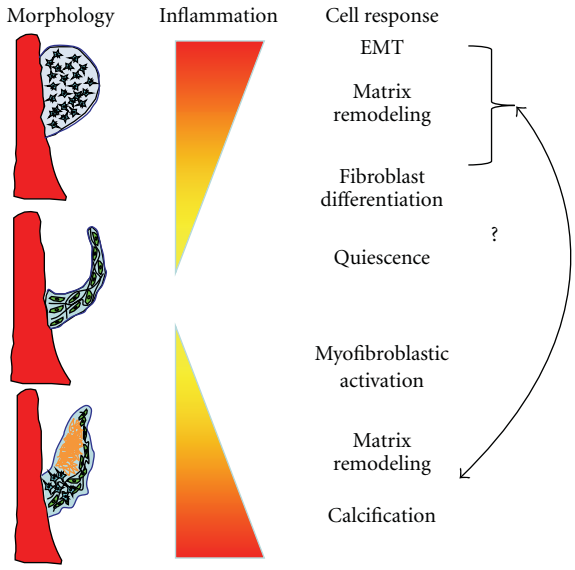

Figure 1: Inflammation and its signaling partners are involved in every stage of the heart valve life cycle, from embryonic morphogenesis (top), quiescent adult valve homeostasis (middle), and in valve pathology (bottom). Many structural and biological features of diseased valves resemble those from embryonic/fetal valves, suggesting similar signaling pathways are reactivated. Identifying and controlling these pathways represents an important and challenging task with potential for large payoff for heart valve disease diagnosis and treatment.

and great arteries stenosis [176]. Nuclear factor of activated T cells (NF-ATc) is a transcription factors that may be needed for cytokine gene expression in activated lymphocytes. Mice lacking NF-ATc died *in utero* from congestive heart failure at days 13.5–17.5 of gestation due to the absence pulmonary and aortic valve leaflets [177]. Adults with congenital heart disease were found to have elevated levels of endotoxin and of the inflammatory cytokines TNF-α and IL-6 when compared to controls, which indicates that adults with congenital heart disease could potentially benefit from novel anti-inflammatory therapies [178].

11. Conclusions

These studies highlight our evolving understanding of the complex role of inflammation in heart valve remodeling, and underscore how much more is left to be learned. While it is true that inflammation mediates the ugliest of pathological valve remodeling, the fact that it is also present during normal embryonic development suggests a potential benefit. Indeed, inflammation-mediated tissue formation, for example, during foreign body encapsulation, is among the most rapid processes available in our natural arsenal. Embryonic morphogenesis progresses on a significantly more rapid scale than wound healing, yet instead of fibrosis results in scar-free, mature tissue architectures. It is therefore time to reconsider the myopic view of inflammation as the harbinger of destruction, but rather as a universal agent of tissue remodeling, for good or ill. In this light, many more questions are revealed rather than answered (Figure 1).

How does inflammation promote positive remodeling, and what are the triggers for pathological change? Are there signaling paradigms in embryonic valve formation that are reactivated in pathological valve remodeling? If so, how are these controlled by inflammation? The answers will likely be heavily dependent on the microenvironmental context (patient age, gender, underlying inflammatory state, gene mutations, mechanical stress, etc.). However, this transformative notion suggests a new avenue to promote and control tissue remodeling in adaptive and regenerative heart valve applications. It is not yet known whether or how this may be achieved in tissue engineering applications, but strongly suggests that complete cessation of inflammatory signaling may not be the best approach. We hope with future research in this area these answers will lead to new clinically translatable therapies for patients with valve disease.

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Review Article

Inflammation in Cardiovascular Tissue Engineering: The Challenge to a Promise: A Minireview

Agneta Simionescu, Jason B. Schulte, George Fercana, and Dan T. Simionescu

Biocompatibility and Tissue Regeneration Laboratory, Department of Bioengineering, Clemson University, 304 Rhodes Center, Clemson, SC 29634, USA

Correspondence should be addressed to Dan T. Simionescu, dsimion@clemson.edu

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Tissue engineering employs scaffolds, cells, and stimuli brought together in such a way as to mimic the functional architecture of the target tissue or organ. Exhilarating advances in tissue engineering and regenerative medicine allow us to envision *in vitro* creation or *in vivo* regeneration of cardiovascular tissues. Such accomplishments have the potential to revolutionize medicine and greatly improve our standard of life. However, enthusiasm has been hampered in recent years because of abnormal reactions at the implant-host interface, including cell proliferation, fibrosis, calcification and degeneration, as compared to the highly desired healing and remodeling. Animal and clinical studies have highlighted uncontrolled chronic inflammation as the main cause of these processes. In this minireview, we present three case studies highlighting the importance of inflammation in tissue engineering heart valves, vascular grafts, and myocardium and propose to focus on the endothelial barrier, the "final frontier" endowed with the natural potential and ability to regulate inflammatory signals.

1. Introduction

Biomedical engineers in the cardiovascular tissue engineering (CVTE) arena dare to "boldly go where no man has gone before"; they mix scaffolds with cells, add mechanical stimuli, growth factors, and other ingredients, culture the constructs *in vitro* for maturation and ... voilá: a newly created surrogate structure ready to replace an inflamed, thrombotic, atherosclerotic, calcified, or infected cardiovascular tissue [1]. Tissue engineering and regenerative medicine are at the footsteps of clinical translation and hold great therapeutic potential. However, progress in the field is critically hampered by uncontrolled, chronic implant-host interactions and more specifically by chronic inflammation.

When challenged by an implanted biomaterial, the body selects one or more of its three defense mechanisms existent in the "armamentarium": hemostasis/coagulation, immune reactions, and inflammation [2]. While we have the ability to control the first two mechanisms relatively well using drugs, chronic cardiovascular inflammation is more difficult to manage. Moreover, the clinical consequences of chronic inflammation including uncontrolled cell proliferation, fibrosis, calcification, and sclerosis are almost impossible to treat pharmaceutically [3, 4].

As we will describe in this minireview, technical challenges in CVTE are plentiful, but the "final frontier" is the healthy, quiescent endothelium [5]. This monolayer of cells that naturally covers all blood-contacting tissues acts as a dynamic and selective barrier by maintaining a nonthrombogenic surface, controls the transfer of molecules across the vascular wall, modulates blood flow and vascular resistance, regulates immune and inflammatory reactions and also interacts with underlying cells to regulate their growth and proliferation. The activation of the endothelium by cytokines, bacterial products, hemodynamic forces, lipids, and other agents induces expression of a new and radically different cell phenotype. Activated endothelium expresses new adhesion molecules on its surface and secretes chemokines, growth factors, vasoactive mediators, and coagulation proteins. Dysfunctional endothelium becomes

adhesive to inflammatory cells, exposes thrombogenic surfaces, and thus promotes inflammation, atherosclerosis, and thromboembolism [6–8] Activation of other cardiovascular cells such as vascular smooth muscle cells and cardiac fibroblasts also contributes significantly to cardiovascular pathology by initiating intimal hyperplasia [9] and cardiac fibrosis [10], respectively.

Overall, the presence, integrity, and state of activation of an endothelial surface at the implant-host interface can "make or break" a tissue-engineered cardiovascular device. It is, thus, clear that the secret to successful CVTE is gaining control over inflammation by modulating the endothelium, the "ultimate interface".

2. Inflammation in Cardiovascular Tissue Engineering

After implantation, cardiovascular devices typically undergo a process similar to wound healing [3]. Following an initial blood-material interaction where fibrin is deposited on the luminal surface, inflammatory processes occur around the implanted construct. In initial stages, neutrophils and monocytes migrate to the interface between the implant surface and the injured tissue. During the granulation phase, phagocytes remove debris due to trauma and then provide signals for fibroblasts and smooth muscle cells to start remodeling. This phase lasts 2-3 weeks in humans and ideally will culminate with complete healing. However, the inflammatory response may continue for months or years and, thus, may lead to chronic inflammation. The consequences of this deleterious process include intimal thickening, tissue overgrowth (*pannus* formation), foreign body reactions, granulation, fibrosis, and ectopic calcification. The mechanisms of these pathological phenomena are not fully understood, but it is known that monocytes/macrophages are observed within implanted constructs until they fully degrade or, if the implant is not degradable, for the life of the implant [4]. Mineralization of synthetic or biologic scaffolds is end-stage pathology, generally irreversible and untreatable. It is also commonly accepted that calcification may not occur if scaffolds degrade slowly and the matrix is capable of remodeling [11]. In most studies on chronic inflammation reported to date, a "diffuse mononuclear infiltrate" was described together with "frustrated macrophages" a.k.a., foreign body giant cells [12]. The latter secrete large amounts of proteases and reactive oxygen species which in turn promote implant degradation, loosening, and eventual failure. Recent evidence suggests that infiltrated monocytes/macrophages produce important cytokines, growth factors, and matrix-remodeling proteases (specifically metalloproteinases). Monocytes/macrophages express markers typical of proinflammatory macrophages (type M1) or remodeling macrophages (type M2) depending on whether the implanted scaffolds are degradable or not [13–16]. Additionally, recruited monocytes release vascular endothelial growth factors and thus may be involved in undesired neovascularization of CVTE implants [17].

Clearly, a better understanding of the underlying pathways appears crucial for controlling the fate of implants and modulating inflammatory reactions in such a way as to induce implant healing and remodeling as compared to fibrosis and/or degeneration. One way of approaching, this issue is developing imaging modalities and discovering new biomarkers of inflammation which would help further understanding of inflammatory diseases and discerning events related to inflammation in CVTE implants [18]. Examples include the detection of vascular cell adhesion molecules, markers for proteases, integrin labeling and uptake of oxidized low-density lipoprotein in atherosclerotic lesions [19–22]. Circulating C-reactive protein has become particularly favored as an inflammatory marker due to its long half-life and chemical stability [23–25] in addition to its ability to predict cardiovascular events [26] and evaluate the effectiveness of clinical anti-inflammatory treatment options [7, 27].

3. Scaffolds for Cardiovascular Tissue Engineering

Tissue engineering (TE), aided by emerging stem cell technology, holds immense potential for the treatment of cardiovascular (CV) diseases, as progress has been made in engineering the various components of the CV system, including blood vessels, heart valves, and cardiac muscle [28–31]. The goal of CVTE is to create or regenerate a functional structure populated with cells capable of continuously remodeling the extracellular matrix (ECM). Optimal replacements for failed CV components would be biocompatible tissues that have the potential to rapidly restore the lost function and slowly regenerate by remodeling. This is a bioengineer's dream come true and increasingly more reports show that we are slowly getting closer to viable solutions. The specific concepts of TE include the creation of a suitably shaped scaffold, repopulation with the appropriate cells (endothelium, smooth muscle cells, fibroblasts), ensuring that cells are placed in their appropriate tissue "niche" (e.g., lumen, media, adventitia), promotion of neovascularization (where needed), and dynamic mechanical conditioning [32] to slowly adapt cells to loads and prepare constructs for implantation. Scaffolds for CVTE must be noncytotoxic, biocompatible, biodegradable with safe by-products, and highly porous yet mechanically stable for the appropriate functions [33]. Bioscaffolds derived from xenogeneic ECM as well as synthetic polymers have been used in numerous TE applications. ECM is the natural scaffold for tissue and organ morphogenesis, maintenance, and reconstruction following injury, and is associated with constructive tissue remodeling. The 3D organization of its components and the complexity of the composition clearly distinguish the ECM from synthetic scaffolds [34]. The processing of ECM biomaterials for medical use involves decellularization of mammalian tissues in order to remove all epitopes associated with cells, especially the terminal galactose alpha 1,3 galactose (alpha-Gal) [35], expressed on the cell surface of all mammals except those of humans and

old world primates [36]. It is known from earlier studies that pure collagen scaffolds degrade slowly and do not calcify in subdermal implantation models [37–39]. Aldehyde cross-linked ECM calcifies after implantation [40], thus, limiting the use of glutaraldehyde-treated valve bioprostheses. Notably, increasing the extent of matrix cross-linking by adding amine bridges [41] significantly reduced calcification when tested in a variety of animal models [42–45].

At early stages, scaffolds are infiltrated by macrophages which degrade ECM slowly by means of secreted matrix metalloproteinases (MMPs); at later stages scaffolds are infiltrated by fibroblasts which initiate repair and remodeling processes. Although degrading collagen scaffolds do not accumulate calcium deposits in vivo, degrading elastin scaffolds have a natural tendency to calcify unless stabilized [46]. While the mechanisms of this process are still under investigation, it is evident that matrix cytokines ("matrikines") such as elastin peptides can induce osteogenic responses in smooth muscle cells and fibroblasts and thus mediate calcification [47]. Thus it is apparent that there is a dire need for a method to stabilize elastin. Polyphenols such as tannic acid and its more stable core compound, pentagalloyl glucose (PGG) was shown to bind tightly to elastin and in doing so stabilizes elastin fibers sufficiently to reduce degeneration and calcification in animal models of aneurysms and in bioprosthetic heart valves [48–51]. In recent years we have extended the use of PGG to ECM scaffold stabilization for tissue engineering heart valves [37], and vascular grafts [38]. Notably, when tested *in vivo*, PGG-stabilized acellular porcine pericardium and carotid arteries degraded at a slower rate than unstabilized scaffolds, did not calcify and underwent remodeling, suggesting that PGG could serve as an ideal stabilization agent for biological scaffolds [37, 38].

4. Challenges in Cardiovascular Tissue Engineering

Effective clinical application of the TE scenario described above raises a series of challenges linked to (1) the preexisting pathology, (2) the surgical procedure, and (3) the nature of the implant.

Little is known about the effect of the pathological status of a tissue, organ, or patient on the fate of CVTE devices and constructs. However it is reasonable to believe that the preexisting pathology or existing risk factors would influence long-term outcomes of device implantation. For example, implantation of a vascular graft in an atherosclerosis-prone patient results in decreased patency [52].

The surgical procedure may also affect host reactivity. In the first several weeks, surgery-related tissue trauma (much unrelated to the implant itself) induces an expected inflammatory response coupled to a wound healing reaction. The response follows the known pathways of inflammation followed by repair and healing. This portion of the implant-host interaction time line is necessary to heal the excised tissues and establish a "working interface" between the implant and host. Since the extent of the host response depends on the degree of surgical injury, more and more CVTE approaches envision devices that could be delivered and implanted using less traumatic, minimally invasive (percutaneous, laparoscopic, endovascular) approaches [53].

The nature of the implant could evidently have a great impact on its clinical outcome. Ideally, the CVTE implant should not be immunogenic, nor should it induce thromboembolic complications, or excessive and prolonged inflammation. Unfortunately, few biomaterials exist which can be considered completely inert. For example, ePTFE and Dacron vascular grafts function well in large diameter graft applications without endothelial cell coverage but when used in peripheral applications, one half of them occlude within the first five years of implantation [54].

A viable endothelium can drastically alter the outcomes of synthetic prostheses; this was demonstrated by *in vitro* endothelialization of ePTFE grafts, procedure pioneered by Prof. Peter Zilla in South Africa more than 20 years ago [55]. In this process, the patients' own endothelial cells were harvested and grown on synthetic ePTFE grafts prior to implantation. This resulted in an improvement in graft patency to values similar to those obtainable with autologous vessels [56].

Thus, to meet these challenges, novel CVTE approaches should take into account preexisting pathology by "personalizing" implants to each patient. This includes use of autologous cells and specific implants developed for specific disease states. We also need to develop implants that attempt to reduce surgical trauma to a minimum and prepare noninflammatory materials which are protected by a healthy endothelial layer. The following sections provide more details on inflammation and other challenges associated with use of TE heart valves, vascular grafts and myocardium.

5. Heart Valve Tissue Engineering

The hallmarks of valve pathology are fibrosis in rheumatic heart disease and calcification in aortic stenosis [11]. Post-inflammatory scarring is one of the most cited mechanisms of valvular pathology; this denotes correlations with inflammation, but very little is known about the specific mechanisms involved in the onset and progression of heart valve pathology [6]. Jian et al. showed that as a response to injury, calcific degeneration in human valves is characterized by localized endothelial damage, macrophage infiltration, MMP secretion and activation, interstitial cell transformation into an osteogenic phenotype, apoptosis and calcification of devitalized cells and some processes possibly mediated by TGF-beta1 [47, 57]. Information obtained from human patient samples are just a snapshot in time and in most circumstances reflect the end stages of valvular disease. Currently, there is no effective medication available to limit, progression of heart valve diseases, and the most common treatment is their surgical replacement with mechanical devices or valves made from biological tissues. It is estimated at present that 275,000 valve replacements are performed annually worldwide using mechanical valves and tissue valves. However, the most physiological prostheses

are the pulmonary autograft valves (transposition of the living patient's own pulmonary valve into the aortic position) and the allograft valves (sterilized, cryopreserved human cadaveric valves). Allografts exhibit excellent durability after implantation but are not readily available and represent only a small percentage of total valve replacements.

The main issue that emerged during clinical investigations of replacement heart valves was their limited durability which limits their use in young patients. Reoperation following valve replacement surgery, for the purpose of retrieving and replacing the defective device, is a relatively common event and occurs within 10–15 years after initial valve surgery [58]. Newer tissue valve models claim better performances [59], but this remains to be determined. Histological, ultra structural, and biochemical aspects of degenerated explanted tissue valves are similar to those of native human-diseased heart valves. The major processes that contribute to this "new pathology" of replacement heart valves are tissue calcification and mechanical damage. Calcification may occur independent of mechanical damage [60] but may also be accompanied by tissue abrasion, tearing and perforations. There is a startling paucity of basic knowledge on calcification mechanisms, as well as action of treatments that presumably mitigate calcification [40].

The goal of heart valve tissue engineering (HVTE) is to reestablish proper valve functions while allowing for slow regeneration of a new valve tissue. These two processes have to occur simultaneously without influencing each other or promoting thrombosis, coagulation or inflammation. Immediately upon implantation, the device needs to function properly, to withstand the harsh biomechanical regime of the aortic valve environment and induce left ventricular remodeling. While performing these functions, the device is expected to be perfectly "tuned" so that endothelial and interstitial cells slowly remodel the ECM without altering mechanical properties in the process. This is a challenging task, as the balance between MMPs and their inhibitors is very delicate [58].

Strategies for HVTE fall into several categories [61]. A first approach is implantation of decellularized valve matrices and relying on host cells to repopulate and remodel the scaffolds. This approach has been limited by the fact that scaffolds are not porous enough, cells do not readily infiltrate the scaffolds and human acellular valves are in short supply. Porcine acellular valves have been implanted in recent years in several clinics, but their use is currently limited due to early failure of several valves in clinical studies [62]. Some valves exhibited severe inflammation after only 2 days of implantation, leading to structural failure at 7 days and degeneration associated with severe foreign body reaction dominated by early neutrophils and macrophage infiltration and late lymphocyte reactions. These reactions were the result of incomplete cell removal and other manufacturing problems [62]. A second alternative would be to repopulate decellularized valve matrices with appropriate cell types *in vitro* before implantation. Recently we have shown that *in vitro* endothelialization of acellular porcine aortic valves is possible and that surface-seeded endothelial cells withstood bioreactor testing in pulmonary conditions for 3 weeks showing excellent retention and viability [63]. A third strategy involves assembly of synthetic biodegradable matrices populated by cells and bioreactor conditioning to express adequate properties before implantation [29, 64]. While this approach seems appealing, the polymeric matrices lack sufficient mechanical strength [65], develop fibrosis [64] and have not yet withstood the test of time under arterial pressures.

5.1. Prognosis. Tissue engineering of heart valves is one of the most daunting tasks in the field of regenerative medicine. Technologically, there are numerous hurdles to overcome before implantable living heart valves become a reality. Valvular scaffolds have to function immediately after implantation, endure significant mechanical stress, and also maintain stable matrix homeostasis while undergoing adaptation to altered hemodynamics and somatic growth. The presence of living interstitial cells and a continuous layer of endothelial cells covering the scaffolds would prove highly beneficial ensuring proper matrix homeostasis and protection from inflammation. Further studies are needed to make the dream of HVTE a reality.

6. Vascular Tissue Engineering

Current treatment options for vascular diseases, regardless of location in the vasculature, include balloon angioplasty, stent placement, graft bypass surgery, and use of pharmacological agents [66–70]. The total need for vascular grafts has been estimated to be more than 1.4 million in the USA alone [71]. These can be divided into three categories, in order of decreasing diameter. The large and medium caliber synthetic grafts are used in the thoracic and abdominal cavities with good long-term outcomes. Almost 1,200,000 small-caliber grafts (<6 mm) are used every year for vascular access, to relieve lower limb ischemia and for coronary bypass surgery. Autologous veins or arteries are the "gold standard" for replacement of small caliber arteries, but in 30–40% of patients these are not available due to prior harvesting or preexisting conditions. In these latter cases synthetic grafts are used, but they provide poor outcomes, as 50% of these will occlude within 5 years [72], potentially leading to amputation.

Tissue engineering vascular grafts (TEVGs) entails the growing of living vessels, by using cells and scaffolds, alone or in combinations. The functional requirements must include the following: nonthrombogenicity, adequate burst pressure and compliance, appropriate remodeling responses, and vasoactivity. Equally important is the propensity to elicit inflammation and to resist proinflammatory *milieus* when implanted in compromised patients. Traditionally, TEVGs have been constructed from both biological and synthetic scaffolds [73] with or without repopulation with autologous cells [72]. For these purposes, mesenchymal stem cells derived from a patient are purified, expanded, and exposed to specific biological cues to incite differentiation into desired cell types, and the TEVGs are then seeded with cells [74, 75]. Cell-seeded grafts are then cultured in bioreactors to adapt

to increasing loads and increase overall performance [76]. Excellent results have been reported in animal testing and also limited number of clinical trials [77]; however, the long-term efficacy of these grafts is still uncertain. Attaining a proper balance between scaffold degradation and remodeling is still under investigation. Rapid degradation is clearly detrimental and could have dramatic consequences. Slow degradation of scaffolds is a prerequisite for successful TEVGs; however, scaffold degradation products (collagen, elastin, fibronectin and laminin peptides) in synergism with TGF-beta and other cytokines may induce the differentiation of smooth muscle cells and fibroblasts into osteoblast-like cells and myofibroblasts, respectively [47, 78–80].

6.1. Existing Inflammation: The Bane of Tissue-Engineered Vascular Grafts. One of the most complicated aspects of designing a replacement for diseased tissue is incorporating measures which prevent the device from succumbing to the same fate as the diseased tissue it is replacing. The clinical experience with the transplantation of vascularized organs has uncovered several aspects useful for TEVGs. In heart transplants, it has been reported that as the duration of time after-implant increases, patient mortality also increases [81]. The culprit for this problem has been denoted as graft arterial disease (GAD) [82], characterized by intimal hyperplasia, the denudation of the medial layers, fibrosis of adventitial layers, and vasoconstriction [83]. GAD differs from atherosclerosis by not being related to fatty streak deposition and by developing circumferentially and not focally [84]. Shimizu and Mitchell [82] also reported that symptoms of ischemia are often masked in transplanted tissues due to the lack of innervation, only furthering the convolution of the disease. Therefore, even following successful organ transplantation and the necessary immunosuppression medication that ensues, GAD can arise and threaten the health of both the transplanted organ and the patient. Placing TEVGs into a compromised site would likely decrease the life of the vascular construct, especially due to the expression of chemokines that recruit inflammatory cells which are key to allograft rejection [85, 86]. GAD has been reported to incite host ingrowth of endothelial cells and intimal smooth muscle-like cells, which are reported to be different from medial-smooth muscle cells [87], into the allograft vasculature [88–90]. On the upside, such cellular ingrowth properties could potentially be harnessed to further TE strategies for vascular constructs.

Atherosclerosis has similarities to GAD but ultimately differs in the involvement of lipoproteins and foam cells entrapped within the intima, which form the characteristic localized fatty streaks [23]. After several decades of research, we now know that atherosclerosis is a consequence of chronic inflammation [91] with elements of immune system activation [92]. The main questions that bioengineers have to address now are how to develop a TEVG that would be protected from atherosclerosis after implantation in an atherosclerotic prone patient? Will an endothelium that is healthy at implantation become dysfunctional under systemic insults, and, therefore, not be capable of preventing local inflammatory signals? Is control of systemic inflammation in fact the critical step to success? Regardless of the mechanisms perpetuating disease progression, studies into pathogenesis of atherosclerosis and other inflammatory diseases point to the activation of endothelial cells as the critical starting point for the disease [23, 93]. Thus, knowledge of mechanisms of endothelium activation and its role in promoting inflammation would need to be incorporated into future TEVGs [23].

6.2. Prognosis. The field of vascular tissue engineering necessitates advances in cell seeding, cell ingrowth, elastin biosynthesis, and nutrient perfusion before any large clinical breakthroughs are to be expected. Scaffolds need to be altered so that they are less inflammatory and also that they resist and perform well in proinflammatory environments. For these objectives, testing TEVGs in healthy and pathological animal models would be useful. The ability of vascular grafts to fully remodel into *de novo* formed arteries still needs to be evaluated extensively.

7. Myocardial Tissue Engineering

Aside from replacements for ailing valves and devices used to occlude septal defects, applications for tissue engineering which deal solely with the myocardium are very limited. A major area of interest, however, is the restoration of cardiac muscle functionality in patients who have suffered an acute myocardial infarction (AMI) and are at risk for developing congestive heart failure (CHF) [94–97]. The acute inflammatory response which occurs subsequently to the death of ischemic cells is culpable for scar tissue development, which, in turn, is responsible for global remodeling of the heart and, ultimately, development of CHF [24, 94, 96]. This scenario is different from inflammation in heart valves or blood vessels because the post-AMI inflammatory response is a consequence of or response to, ischemia. Injury leads to the activation of inflammation, ECM degradation and MMPs and TGF secretion which in turn activate cardiac fibroblasts to initiate fibrosis [98–100]. Clearly, vascular endothelium is the main barrier that controls tissue reactivity to cytokines and determines the outcome of tissue inflammation [101]. Tissue engineering approaches to the regeneration of the myocardium following AMI strive to attenuate the geometrical alterations (dilated or ischemic cardiomyopathy) which take place in the ventricles, concurrently slowing or halting progression towards CHF [95, 102].

The remainder of this section will discuss only efforts to regenerate the myocardium which involve the use of engineered heart tissue (EHT) [103] constructs, defined as consisting of a scaffold composed of synthetic material, natural material, or any combination thereof and at least one type of cell attached to the scaffold. Ideally, the EHT should be prevascularized, posses similar mechanical properties to ventricular myocardium, be contractile and integrate well within existing tissues. More importantly it should be

noninflammatory and also resist the proinflammatory environment surrounding the infarcted area. We will specifically address several points which are relevant to the feasibility of using EHT to treat ischemic cardiomyopathy, including the interaction of EHT with existing scar tissue, the host response to the construct and the challenges of implanting EHT in patients with poor cardiovascular health or other preexisting conditions.

7.1. Complications from Existing Scar Tissue. The inflammatory response following AMI occurs rapidly. It is estimated that necrosis of cardiomyocytes begins 20–30 minutes after the initial blockage of the coronary vasculature [104]. The current gold standard in clinical care after-AMI is percutaneous coronary intervention (PCI), a transcatheter procedure wherein a balloon is used to dilate the vessel lumen immediately adjacent to the site of thrombotic occlusion, thus restoring flow to the downstream tissues [105]. Currently, chest pain centers and cardiac care units have set a benchmark "door-to-balloon" time of 90 minutes in order to limit the extent of damage produced by AMI [106, 107]. While minimizing door-to-balloon time is critical, it is difficult to imagine achieving the reperfusion of the ischemic tissue before cardiomyocyte necrosis sets in.

When necrosis occurs, so will inflammation. And with a lack of intrinsic regenerative capability in the myocardium, the necrotic core will be completely remodeled into fibrous tissue in 2–6 weeks after-AMI [97, 108, 109]. The implications of this abnormal remodeling are twofold: (a) tissue-engineering strategies currently hold the most promise for regeneration of the myocardium because they introduce new, viable cells into an environment that is not conducive to regeneration on its own [94]; (b) tissue engineered constructs will have to be placed over or adjacent to scar tissue unless the construct is implanted immediately following AMI (before scar tissue can be formed) or the scar tissue is surgically resected prior to the implantation of the construct [102]. In order to implant a construct immediately after AMI, it would need to be ready before the event, in an "off the shelf" fashion. This possibility is precluded by complications that arise due to autologous cell sourcing and the need for *in vitro* cell proliferation, seeding, and preconditioning in a bioreactor. Such a process could take several weeks, if not months, to complete and could not benefit the patient immediately unless the AMI was predicted [95, 102, 108].

Whether or not grafting EHT [103] directly to the fibrotic scar inhibits proper adherence, and the perfusion of the graft with the host tissue through an inflammatory response is unclear. A number of studies have reported positive outcomes when implanting EHT on top of infarct scars created in animal models [110–112]. When EHT constructs are not placed directly on the epicardium over the scar, but rather at the endocardial surface (by means of a Dor procedure) [113] or in a depression made by the resection of the scar tissue, solid engraftment has been repeatedly achieved [114–116]. These opposing implantation methods will need to be compared for efficacy by performing both with a single type of EHT, then perhaps the difference, if one exists, can be elucidated.

Equally conflicting results were reported by two groups who implanted "prevascularized" EHT in a nude rat model (without infarct). One group reported that their construct integrated well, forming microvasculature which perfused with the host's and resembled viable myocardium [117], while another group observed fibrotic encapsulation of their EHT graft. Ironically, the latter occurred in rats which were on an immunosuppression regimen [97]. What is clear, however, is that cell-free constructs composed of synthetic material can be at risk for poor performance when implanted in or adjacent to the myocardium, possibly due to inflammation. Stuckey et al. reported that patches of titanium oxide-reinforced poly(ethylene terephthalate)/dimer fatty acid implanted onto infarcted rat hearts produced extensive necrosis in the adjacent healthy host tissue, most likely due to micromotions between the graft and host caused by compliance mismatch [94]. Similarly, another group reported extensive foreign body response, thrombus formation, necrosis, and calcification associated with an ePTFE graft that was implanted on the endocardial surface of the infarcted heart in a pig model [115]. It is evident that control of inflammation in EHT is of essence for the success of implanted scaffolds and that this might be enhanced by the preexisting "inflamed" status of the ischemic myocardium.

7.2. Characterizing the Host Response. Positive outcomes in designing EHT are measured by criteria such as the attenuation of ventricular wall remodeling or dilation, perfusion of the graft by the host vasculature, electrical coupling of the cardiomyocytes in the graft with those of the host (via the formation of connexins or gap junctions), and the avoidance of inflammation, encapsulation, or calcification [95, 102, 108]. Tissue engineering for this specific application is still in its early stages. Most implantation studies have not been run for longer than 3 months and have focused on establishing proof of principle rather than studying the long-term performance of EHT constructs [110–112, 114–116]. As such, there is very little data available in regards to chronic inflammatory responses or calcification with which to make a prognosis for the long term viability of these constructs.

The first ever clinical trial (MAGNUM-trial) involving an EHT construct was begun in 2003 and used autologous bone marrow cells (BMC) seeded onto a 3D collagen type I scaffold [118]. The construct was implanted over the epicardial surface of the postinfarct scar in a group of 10 patients while they were undergoing coronary artery bypass grafting surgery. Followup at 10 months indicated prevention of cardiac remodeling and thickening of the scar (via cell ingrowth) with functional, healthy tissue. These were assessed using echocardiography and single-photon emission computed tomography [119]. There were no indications of immune or inflammatory reactions, no arrhythmias, and the EHT appeared to integrate well with the host tissue. It is important to note that this particular EHT construct was not intended to be functional, meaning it was neither contractile

nor vascularized [102]. Its mechanism of action was one of providing "passive" support, and the collagen matrix most likely acting as a stable microenvironment from which the BMC could secrete paracrine signals to the healthy cells in the peri-infarct tissue. A construct of this type, implanted in this fashion, would not be at risk for developing either GAD or atherosclerosis, because the cells were autologous and the neovasculature formed within it would not consist of vessels large enough to be afflicted with such conditions [120, 121]. Calcification, however, might develop in this construct because type I collagen is known to predispose to forming nucleation sites for calcium deposits [122]. Furthermore, the mechanical stresses and deformations imposed on the construct via the continuous, repetitive contraction of the heart might accelerate mineralization [122]. It is noted that the authors of the MAGNUM trial make no mention of calcification being observed in the followup, especially since echocardiography can be used to image calcium [123]. In the case of the MAGNUM trial, it is probable that more time must elapse before evidence of the activity of the typical culprits in the failure of tissue-engineered constructs emerges.

7.3. Engineered Heart Tissue in the Compromised Patient. The vast majority of patients who could benefit from a clinically feasible and cost-effective EHT have some form of cardiovascular disease [95]. When developing EHT, researchers should rid themselves of the illusion that potential candidates for their therapy will be a *tabula rasa* of health. Rather, they should prepare EHT constructs to withstand a hostile, diseased environment. In the MAGNUM trial discussed earlier, for example, 11 of the 20 participants were hypertensive, 13 were hypercholesterolemic, 7 were diabetic, and 10 were smokers [118].

In terms of inflammatory processes, a few of the most threatening conditions to the long-term viability of EHT are atherosclerosis, GAD, and arteriolosclerosis. Atherosclerosis is the most well known of these diseases, as it is implicated in having a causal role in both CHD and peripheral artery disease. Characteristic atheromatous plaques or lesions also indicate the possibility of tissue calcification [120, 124, 125]. GAD has been observed to occur predominantly in implanted allografts, which contain immunogenic cell constituents [121]. EHT constructs, however, should ideally consist of nonimmunogenic ECM proteins seeded with autologous cells, rendering them free from concerns about GAD or transplant vasculopathy [121]. Finally, arteriolosclerosis, or the thickening of the intimal layer in arterioles due to hyperplasia or hyaline matrix deposition, could occur in EHT [120]. EHT grafts implanted in hypertensive or diabetic patients would be at an increased risk of contracting arteriolosclerosis because these risk factors are closely interrelated [120]. Both atherosclerosis and arteriolosclerosis could have a major impact upon the viability of EHT constructs, because such constructs will most likely contain a preformed or preserved vascular network that will stenose or occlude if these diseases are present, leading to ischemia and necrosis of the cells in the construct.

7.4. Prognosis. It is difficult to make conjectures about the ultimate success or failure of EHT constructs. Given the requirements placed upon them and the mechanisms or processes that might be at work *in vivo*, producing an EHT construct that remains viable for some time will be a challenging task. In the future, more clinical trials of EHT constructs need to be conducted so that a larger data set can be formed and the efficacy of their outcomes assessed. The pathways which mediate the inflammatory processes of atherosclerosis and arteriolosclerosis, the fibrous remodeling process, and the unfriendly microenvironment of patients with these conditions need to be elucidated so that drug discovery efforts can generate new prophylactic drugs. While such drugs could reduce the patient population in need of EHT treatments, the current demand is so great that the need for them would still be obviated [95]. Until such drugs are available, however, EHT needs to be developed so as to withstand the hostile environs in patients compromised by diseases. Short of this, the best case scenario is that patients receiving EHT grafts will cease or seek treatment for risky behaviors such as smoking, hypertension, hypercholesterolemia, or diabetes and initiate beneficial behaviors like engaging in physical activity or adjusting dietary intake and nutrition. Sadly, inflammatory processes are self perpetuating in patients afflicted with cardiovascular disease. The progression to CHF can begin with one chronic inflammatory condition which begets another acute inflammatory response, constituting a chain of causality. If researchers working to develop EHT can overcome the inherent challenges associated with it, they can alleviate the devastation CVD causes so many patients and their families throughout the world.

8. Final Remarks

CVTE holds great potential to solve some of the biggest current health issues. The prospects of using scaffolds, cells, and chemical or mechanical stimuli to create functional tissues such as valves, arteries and myocardium are hugely exciting as we attain "magical powers" previously unimaginable. After an initial period of hype and hope, we are now closer to clinical application of CVTE; however, we need to increase our control over inflammation and its clinical consequences. One approach could involve making scaffolds from carefully screened, intact molecules (synthetic of biologic), and repopulating the scaffolds with interstitial cells and endothelial cells *in vitro*. This should be followed by conditioning the constructs in bioreactors until cells "gain control" over the remodeling process and the constructs show clear signs of regeneration. Only then would tissue engineering products be ready for implantation in the targeted dynamic cardiovascular sites. While we continue to trek towards the unknown at warp speed, we need to focus more on the endothelium, the "final frontier". This will eventually determine whether novel CVTE devices will successfully help thousands of patients in need of tissue regeneration and repair.

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Review Article

Adipose Tissue Remodeling as Homeostatic Inflammation

Michiko Itoh,[1] Takayoshi Suganami,[1] Rumi Hachiya,[1] and Yoshihiro Ogawa[1,2]

[1] *Department of Molecular Medicine and Metabolism, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan*
[2] *Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo 113-8510, Japan*

Correspondence should be addressed to Yoshihiro Ogawa, ogawa.mmm@mri.tmd.ac.jp

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Evidence has accumulated indicating that obesity is associated with a state of chronic, low-grade inflammation. Obese adipose tissue is characterized by dynamic changes in cellular composition and function, which may be referred to as "adipose tissue remodeling". Among stromal cells in the adipose tissue, infiltrated macrophages play an important role in adipose tissue inflammation and systemic insulin resistance. We have demonstrated that a paracrine loop involving saturated fatty acids and tumor necrosis factor-α derived from adipocytes and macrophages, respectively, aggravates obesity-induced adipose tissue inflammation. Notably, saturated fatty acids, which are released from hypertrophied adipocytes via the macrophage-induced lipolysis, serve as a naturally occurring ligand for Toll-like receptor 4 complex, thereby activating macrophages. Such a sustained interaction between endogenous ligands derived from parenchymal cells and pathogen sensors expressed in stromal immune cells should lead to chronic inflammatory responses ranging from the basal homeostatic state to diseased tissue remodeling, which may be referred to as "homeostatic inflammation". We, therefore, postulate that adipose tissue remodeling may represent a prototypic example of homeostatic inflammation. Understanding the molecular mechanism underlying homeostatic inflammation may lead to the identification of novel therapeutic strategies to prevent or treat obesity-related complications.

1. Introduction

The metabolic syndrome is a constellation of visceral fat obesity, insulin resistance, atherogenic dyslipidemia, and hypertension, which all independently increase the risk of atherosclerotic diseases [1–5]. The adipose tissue secretes a number of bioactive substances or adipocytokines, and unbalanced production of pro- and anti-inflammatory adipocytokines in obese adipose tissue may critically contribute to many aspects of the metabolic syndrome [1–5]. Obesity is now viewed as a state of systemic, chronic low-grade inflammation [1–4]. In contrast to acute inflammation which resolves by an active termination program, chronic inflammation may involve persistent stress and/or impaired resolution process, thereby resulting in functional maladaptation and tissue remodeling [6]. On the other hand, during the course of obesity, adipose tissue is characterized by adipocyte hypertrophy, followed by increased angiogenesis, immune cell infiltration, and extracellular matrix overproduction [1, 2, 7, 8], which may be referred to as adipose tissue remodeling.

Pathogen sensors or pattern-recognition receptors (PRRs), which are important for the recognition of pathogen-associated molecular patterns (PAMPs) in innate immunity, are also capable of recognizing endogenous ligands, damage-associated molecular patterns (DAMPs) or danger signals (Figure 1) [6, 9, 10]. Interaction between endogenous ligands and pathogen sensors may play a role in the basal homeostatic state as well as diseased tissue remodeling, which has been referred to as homeostatic inflammation [6, 11]. This paper summarizes the molecular mechanism and pathophysiologic implication of adipose tissue remodeling as a prototypic example of homeostatic inflammation.

2. Adipose Tissue Inflammation and Adipose Tissue Remodeling

In addition to lipid-laden mature adipocytes, the adipose tissue is composed of various stromal cells, including

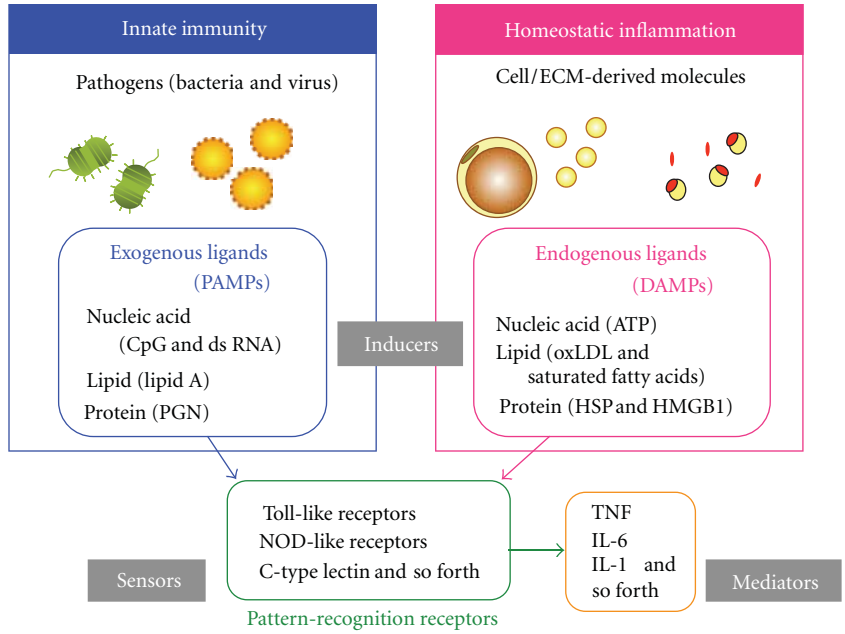

Figure 1: Adipose tissue inflammation as homeostatic inflammation. In innate immunity, exogenous ligands (pathogen-associated molecular patterns; PAMPs) are sensed by pattern-recognition receptors (PRRs), thereby inducing inflammatory changes. On the other hand, damage-associated molecular patterns (DAMPs) released from damaged or stressed cells and tissues can activate PRRs, thereby inducing homeostatic inflammation ranging from the basal homeostatic state to diseased tissue remodeling. For instance, free fatty acids (FFAs) released from hypertrophied adipocytes can report, as a danger signal, their diseased state to macrophages via Toll-like receptor 4 (TLR4) complex during the course of obesity. dsRNA, double-strand RNA; PGN, peptidoglycan; ATP, adenosine tri-phosphate; oxLDL, oxidized low-density lipoprotein; HSP, heat shock protein; HMGB1, high-mobility group box-1.

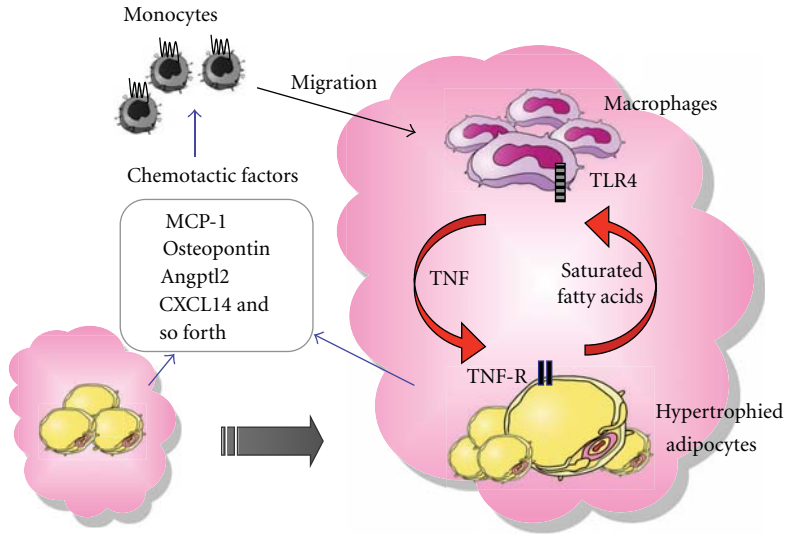

Figure 2: Molecular mechanism underlying adipose tissue inflammation. During the course of obesity, adipose tissue secretes several chemotactic factors to induce macrophage infiltration into adipose tissue. Circulating monocytes migrate and infiltrate into adipose tissue through adhesion process to endothelial cells. Macrophages enhance the inflammatory changes through the crosstalk with parenchymal adipocytes. For example, the macrophage-derived tumor necrosis factor-α (TNFα) induces the release of saturated fatty acids from adipocytes via lipolysis, which, in turn, induces inflammatory changes in macrophages via TLR4. Such a paracrine loop between adipocytes and macrophages constitutes a vicious cycle, thereby further accelerating adipose tissue inflammation. TNF-R, TNFα receptor.

preadipocytes, endothelial cells, fibroblasts, and immune cells [12]. Obese adipose tissue exhibits functional and morphological changes, thereby leading to unbalanced production of pro- and anti-inflammatory adipocytokines [1, 2, 7, 8]. The morphological changes found in obese adipose tissue are reminiscent of the chronic inflammatory responses in atherosclerotic vascular walls termed vascular remodeling, which arise from the complex interactions among vascular endothelial cells, vascular smooth muscle cells, lymphocytes, and monocyte-derived macrophages [4]. Vascular remodeling is considered to be an adaptive process in response to long-term changes in hemodynamic conditions and lipid metabolism, thereby contributing to the pathophysiology of vascular diseases [13]. Thus, the dynamic changes seen in obese adipose tissue can be referred to as adipose tissue remodeling. Notably, macrophage infiltration and inflammation-related gene expression in the adipose tissue precedes the development of insulin resistance in animal models [14, 15], suggesting that macrophages should play a central role in adipose tissue remodeling. It is, therefore, important to know the pathophysiologic role of macrophages infiltrated into the adipose tissue during the course of adipose tissue remodeling.

3. Macrophage Infiltration into Obese Adipose Tissue

Evidence has accumulated that adipocytes *per se* secrete pro-inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), during the course of adipocyte hypertrophy [1–3]. Increased production of chemokines in obese adipose tissue has been implicated in the regulation of monocyte recruitment to adipose tissue [14]. The involvement of MCP-1/chemokine receptor 2 (CCR2) pathway has been extensively studied as the mechanism underlying macrophage infiltration into obese adipose tissue (Figure 2) [16–19]. Moreover, several reports have suggested the role of other chemotactic factors in obesity-induced macrophage infiltration: osteopontin, angiopoietin-like protein 2 (Angptl2), and CXC motif chemokine ligand-14 (CXCL14) (Figure 2) [20–22]. Inhibition of macrophage infiltration into obese adipose tissue through genetic and/or pharmacologic strategies has improved the dysregulation of adipocytokine production, thereby leading to the amelioration of obesity-induced adipose tissue inflammation and insulin resistance. Indeed, macrophage infiltration and inflammation-related gene expression in the adipose tissue precedes the development of insulin resistance in animal models [14, 15]. Understanding the molecular mechanisms underlying increased macrophage infiltration into obese adipose tissue may lead to the identification of novel therapeutic strategies to prevent or treat obesity-induced adipose tissue inflammation.

4. Interaction between Adipocytes and Macrophages

The adipose tissue macrophages also represent a major source of pro-inflammatory cytokines, which play important roles in chronic inflammatory responses in obese adipose tissue. Using an *in vitro* coculture system composed of adipocytes and macrophages, we have demonstrated that a paracrine loop involving saturated fatty acids and TNFα derived from adipocytes and macrophages, respectively, establishes a vicious cycle that augments the inflammatory changes (Figure 2) [23]. Among numerous cytokines derived from infiltrated macrophages in obese adipose tissue, TNFα acts on TNF receptor in hypertrophied adipocytes, thereby inducing pro-inflammatory cytokine production and adipocyte lipolysis via nuclear factor-κB- (NF-κB-) dependent and independent (possibly mitogen-activated protein kinase- (MAPK-) dependent) mechanisms, respectively [24]. On the other hand, saturated fatty acids released from adipocytes serve as a naturally occurring ligand for Toll-like receptor 4 (TLR4) complex, which is essential for the recognition of lipopolysaccharide (LPS), to induce NF-κB activation in macrophages [24].

The interaction between adipocytes and macrophages results in marked upregulation of pro-inflammatory adipocytokines and significant downregulation of anti-inflammatory adipocytokines, which lead to development of obesity-related complications in multiple organs, such as atherosclerosis and hepatic steatosis [1–4]. For instance, adiponectin is a well-established anti-inflammatory adipocytokine, which is markedly downregulated in obese adipose tissue, and supplementation of adiponectin in obese mice effectively reverses insulin resistance in the skeletal muscle and liver [25, 26]. On the other hand, overproduction of MCP-1 induces macrophage infiltration into the adipose tissue and directly induces insulin resistance in the skeletal muscle and liver [17, 18, 27]. Thus, dysregulation of adipocytokine production as a result of inflammatory changes in the adipose tissue may be involved in the pathogenesis of metabolic derangements in obesity.

5. Heterogeneity of Adipose Tissue Macrophages

Recent studies have pointed to the phenotypic change of macrophages in lean and obese adipose tissue; M1 or classically activated (pro-inflammatory) macrophages and M2 or alternatively activated (anti-inflammatory) macrophages (Figure 3) [28]. Adipocytes in lean adipose tissue produce humoral factors that induce M2 activation of macrophages, such as interleukin-4 (IL-4) and interleukin-13 (IL-13), and M2 activated macrophages release anti-inflammatory mediators, such as interleukin (IL-10) [29]. On the other hand, hypertrophied adipocytes secrete pro-inflammatory saturated fatty acids, cytokines, and chemokines to induce M1 polarization of macrophages [29]. Activated M1 macrophages in turn produce pro-inflammatory cytokines and

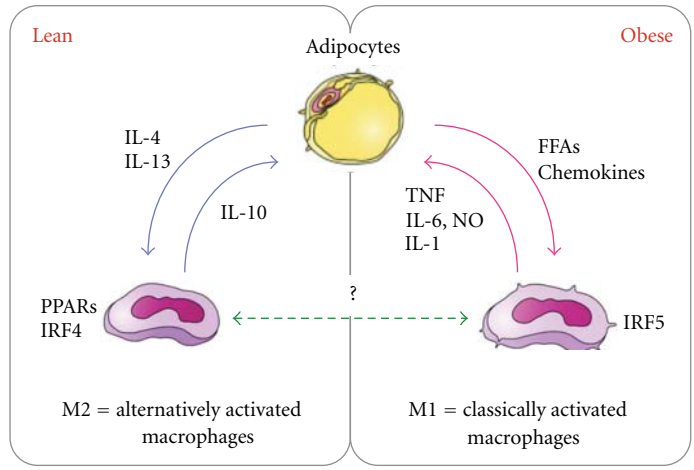

Figure 3: Regulation of macrophage polarity in adipose tissue. Recent evidence has also pointed to the heterogeneity of adipose tissue macrophages, that is, M1 or classically activated (pro-inflammatory) macrophages and M2 or alternatively activated (anti-inflammatory) macrophages. Under lean condition, adipocytes secrete factors that promote M2 activation of macrophages, such as interleukin-4 (IL) and interleukin-13 (IL-13). M2 macrophages secrete anti-inflammatory mediators. On the other hand, adipocytes secrete pro-inflammatory FFAs, chemokines, and cytokines under obese condition. Activated M1 macrophages produce large amounts of pro-inflammatory cytokines, thereby accelerating inflammatory responses in adipose tissue through paracrine interaction between adipocytes and macrophages.

chemokines, thereby accelerating adipose tissue inflammation.

We have recently identified activating transcription factor 3 (ATF3), a member of ATF/cAMP response element-binding protein family of basic leucine zipper-type transcription factors, as a target gene of saturated fatty acids/TLR4 signaling in adipose tissue macrophages and found that ATF3 attenuates obesity-induced macrophage activation in obese adipose tissue [30]. On the other hand, peroxisome proliferator-activated receptor γ (PPARγ) and peroxisome proliferator-activated receptor β/δ (PPARβ/δ) can stimulate M2 polarization of adipose tissue macrophages and thus systemic insulin sensitivity [31–34]. Indeed, the activation of PPARγ by pioglitazone, a thiazolidinedione class of insulin sensitizer, improves the unbalanced M1/M2 phenotype of adipose tissue macrophages in diet-induced obese mice [35]. Interestingly, circulating blood monocytes, precursors of infiltrated macrophages to the site of chronic inflammation, also express both M1 and M2 markers [36, 37]. Moreover, monocytes in obese mice and/or obese type 2 diabetic patients show significantly higher expression of M1 markers and lower expression of M2 markers relative to normal-weight controls [36]. Thus, pioglitazone treatment improves the unbalanced M1/M2 phenotype of monocytes, which may contribute to its antidiabetic and antiatherogenic effect [36, 37].

Recent studies have found other molecules that regulate macrophage polarization; that is, Jumonji domain containing-3 (Jmjd3) is essential for M2 activation through demethylation of interferon-regulatory factor 4 (IRF4) under infectious condition [38], and interferon-regulatory factor 5 (IRF5) is crucial for conversion from M2 to M1 activation in response to LPS [39]. It is interesting to know their importance in the regulation of macrophage polarization and plasticity during the course of obesity. Modulating macrophage activation state in obese adipose tissue would be a novel therapeutic target to treat or prevent the progression of obesity-induced complications such as diabetes and atherosclerosis.

6. Adipose Tissue Remodeling and Ectopic Lipid Accumulation

The adipose tissue is primarily an energy reservoir that stores fatty acids in the form of triglyceride, which is facilitated by insulin. However, obesity induces insulin-resistant state and inflammation in the adipose tissue, both of which lead to increased fatty acid release from the adipose tissue [1, 23, 29]. Moreover, recent studies have suggested that increased expression of genes related to ECM components and fibrotic changes in the adipose tissue from obese subjects and animals [40–43]. It is reported that adipose tissue fibrosis is negatively correlated with adipocyte diameters in human adipose tissue [44], suggesting that increased ECM components may limit adipose tissue expandability. Indeed, Khan et al. reported that mice lacking collagen VI, which is expressed predominantly in the adipose tissue, exhibit the uninhibited adipose tissue expansion and substantial improvements in whole-body energy homeostasis during a high-fat diet feeding [43]. It is conceivable that the rigid extracellular environment limits adipocyte expansion, and triggers adipocyte cell death and inflammatory responses through MAPK activation by increased shear stress and membrane stretching [43, 44]. Recent evidence suggests that impaired lipid storage in the adipose tissue may contribute to ectopic lipid accumulation in the skeletal muscle, liver, and pancreatic β-cells, where lipotoxicity impairs their metabolic functions [45–47]. This discussion supports the emerging view that metabolic problems associated with obesity become overt when adipose tissue cannot fully meet demands for

additional lipid storage in addition to the dysregulation of adipocytokine production.

7. Adipose Tissue Remodeling as Homeostatic Inflammation

TLR4 is a pattern-recognition receptor essential for the recognition of LPS, which is reported to play an important role in obesity-induced adipose tissue inflammation and systemic glucose and lipid metabolism *in vivo* [24, 48–50]. In obese adipose tissue, TLR4 expressed in macrophages is capable of sensing saturated fatty acids (FAs) released from adipocytes to induce chronic inflammatory responses [24, 51, 52], suggesting that saturated fatty acids could be a danger signal. On the other hand, free fatty acids (FFAs) are an important energy source mobilized from triglycerides stored in the adipose tissue, particularly under starvation conditions. Kosteli et al. have recently suggested that FFAs released from adipocytes during fasting recruit macrophages into the adipose tissue, which may be involved in the regulation of local lipid concentrations [53]. In this regard, FFAs, when released physiologically during fasting or starvation via adipocyte lipolysis, may be involved in the regulation of metabolic homeostasis within the adipose tissue rather than a danger signal. Under overnutrition conditions, increased concentrations of FFAs also activate inflammatory pathways to maintain adipose tissue homeostasis such as tissue repair and regulation of metabolism. When cellular and/or tissue stresses are excessive and/or sustained and adaptive responses are no longer possible, inflammatory responses are prolonged (i.e., chronic inflammation), thereby leading to diseased tissue remodeling [6].

Recently, we have reported that macrophage-inducible C-type lectin (Mincle; also called Clec4e and Clecsf9), a pathogen sensor for pathogenic fungi and Mycobacterium tuberculosis, is induced in adipose tissue macrophages in obesity at least partly through the saturated fatty acid/TLR4/NF-κB pathway, thereby suggesting its pathophysiologic role in obesity-induced adipose tissue inflammation [54]. Yamasaki et al. reported that Mincle serves as a receptor for SAP130, a component of small nuclear ribonucleoprotein released from damaged cells, to sense cell death and induce pro-inflammatory cytokine production [55]. Since dead adipocytes are surrounded by macrophages in the adipose tissue of obese humans and mice (crown-like structure) [7, 8, 56], it is conceivable that Mincle plays a role in sensing adipocyte-derived endogenous ligand(s) during adipocyte death.

The above discussion supports the concept that interaction between endogenous ligands and pathogen sensors in the adipose tissue involves multiple stages of adipose tissue remodeling, ranging from normal metabolic homeostasis to diseased tissue remodeling, which may be referred to as homeostatic inflammation. It is interesting to identify other endogenous danger signals and pathogen sensors that contribute to the pathophysiology of adipose tissue inflammation.

8. Homeostatic Inflammation and Other Metabolic Disorders

Recent evidence has provided new insight into the interaction between endogenous ligands and pathogen sensors in a variety of chronic inflammatory diseases such as atherosclerosis, diabetes mellitus, malignant cancers, autoimmune diseases, and even neurodegenerative diseases. Similar to the interaction between saturated fatty acids and TLR4, oxidized low-density lipoprotein (LDL), known as a ligand for the scavenger receptor CD36, is reported to trigger inflammatory signaling through a newly identified heterodimer of TLR4 and TLR6 in macrophages [57] and also to trigger CD36-TLR2-dependent apoptosis in macrophages under endoplasmic reticulum stress [58]. On the other hand, Schulthess et al. reported that CXC motif chemokine ligand-10 (CXCL10), when upregulated in diabetic pancreatic islet, is capable of binding to TLR4 in β cells in pancreatic islets to induce apoptosis [59].

In Nod-like receptor family, the NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome is well characterized. The NLRP3 inflammasome is a cytosolic protein complex consisting of the regulatory subunit NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and the effector subunit caspase-1. It is activated by pathogen-derived DNA and endogenous DAMPs such as components of necrotic cells and damaged tissues [60, 61]. Several lines of evidence have suggested that the NLRP3 inflammasome plays an important role in the pathogenesis of obesity-related diseases. NLRP3 deficient mice show improved glucose tolerance and insulin sensitivity [62]. It is also reported that ceramide and islet amyloid polypeptide (IAPP) activate as danger signals for NLRP3 inflammasome in adipose tissue macrophages and pancreatic islets, respectively, which results in insulin resistance [63–65]. In atherogenesis, it is reported that crystalline cholesterol acts as an endogenous danger signal and its deposition in arteries or elsewhere is an early cause rather than a late consequence of inflammation [66]. A better understanding of the molecular basis underlying homeostatic inflammation would allow more efficient multidisciplinary approach to and a better assessment of the metabolic syndrome.

9. Concluding Remarks

Obesity may be viewed as a chronic low-grade inflammation as well as a metabolic disease. Although considerable progress has been made in understanding the cellular and molecular events that are involved in acute inflammation caused by infection, there is no clear understanding of their physiological counterpart of the systemic chronic inflammatory state, which could be referred as homeostatic inflammation. The interaction between parenchymal and stromal cells through a number of endogenous ligands and pathogen sensors may contribute to inflammatory responses in obese adipose tissue as well as other metabolic organs. Understanding

the molecular mechanism underlying adipose tissue remodeling as homeostatic inflammation may lead to novel therapeutic strategies to prevent or treat obesity-related complications.

Abbreviations

Angptl2: Angiopoietin-like protein 2
ASC: Apoptosis-associated speck-like protein containing a caspase-recruitment domain
ATF3: Activating transcription factor 3
CCR2: Chemokine receptor 2
CXCL10: CXC motif chemokine ligand-10
CXCL14: CXC motif chemokine ligand-14
DAMP: Damage-associated molecular pattern
ECM: Extracellular matrix
FFA: Free fatty acid
IAPP: Islet amyloid polypeptide
IL-4: Interleukin-4
IL-6: Interleukin-6
IL-10: Interleukin-10
IL-13: Interleukin-13
IRF4: Interferon-regulatory factor 4
IRF5: Interferon-regulatory factor 5
Jmjd3: Jumonji domain containing-3
LDL: Low-density lipoprotein
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MCP-1: Monocyte chemoattractant protein-1
NF-κB: Nuclear factor-κB
NLRP3: NACHT, LRR and PYD domain-containing protein 3
PAMP: Pathogen-associated molecular pattern
PPARγ: Peroxisome proliferator-activated receptor γ
PPARβ/δ: Peroxisome proliferator-activated receptor β/δ
PRR: Pattern-recognition receptor
SVF: Stromal vascular fraction
TLR4: Toll-like receptor 4
TNFα: Tumor necrosis factor-α.

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Review Article

Hemodynamics and Mechanobiology of Aortic Valve Inflammation and Calcification

Kartik Balachandran,[1] Philippe Sucosky,[2] and Ajit P. Yoganathan[3]

[1] *Wyss Institute for Biologically Inspired Engineering, School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA*
[2] *Department of Aerospace and Mechanical Engineering, University of Notre Dame, 143 Multidisciplinary Research Building, Notre Dame, IN 46556-5637, USA*
[3] *The Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology, 313 Ferst Drive, Suite 1121, Atlanta, GA 30332-0535, USA*

Correspondence should be addressed to Ajit P. Yoganathan, ajit.yoganathan@bme.gatech.edu

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Cardiac valves function in a mechanically complex environment, opening and closing close to a billion times during the average human lifetime, experiencing transvalvular pressures and pulsatile and oscillatory shear stresses, as well as bending and axial stress. Although valves were originally thought to be passive pieces of tissue, recent evidence points to an intimate interplay between the hemodynamic environment and biological response of the valve. Several decades of study have been devoted to understanding these varied mechanical stimuli and how they might induce valve pathology. Here, we review efforts taken in understanding the valvular response to its mechanical milieu and key insights gained from *in vitro* and *ex vivo* whole-tissue studies in the mechanobiology of aortic valve remodeling, inflammation, and calcification.

1. Introduction

Cardiac valves are dynamic, sophisticated structures which interact closely with the surrounding hemodynamic environment. The aortic valve located between the left ventricle and the aorta and the pulmonary valve positioned between the right ventricle and the pulmonary artery are designated as semilunar valves and prevent the backflow of blood from the respective outflow tracts into the ventricles. The atrioventricular valves that ensure the blood to flow from the atria to the ventricles consist of the mitral valve located between the left atrium and left ventricle and the tricuspid valve lying between the right atrium and the right ventricle. Although these four valves present different anatomies and different opening/closing characteristics, they allow the unidirectional flow of blood while maximizing flow rate and minimizing resistance to flow. Although valves were originally thought to be passively moving due to blood flow, it is now acknowledged that the mechanisms ensuring the proper structure and function of the heart valves are essentially controlled by the interaction between the valve, its cells, and the surrounding hemodynamic or mechanical environment. Understanding the effect of the mechanical environment on heart valve biology, that is, its mechanobiology, is therefore critical to better understand normal valve function and disease progression. This paper presents a detailed review of the hemodynamics and mechanobiology of the cardiac valve as it relates to valve pathology, with an emphasis on the aortic valve. The paper is divided into two main sections. The first section summarizes the hemodynamic forces experienced by normal and diseased semilunar valves and closes with a detailed description of the hemodynamic forces experienced by the aortic valve. The second section reviews results from recent *in vitro* and *ex vivo* studies on the effects of these hemodynamic forces on aortic valve biology and disease. This paper therefore provides a comprehensive description of the hemodynamics and mechanobiology of the valve in both normal and pathologic conditions, focusing primarily on the aortic valve.

2. Organ-Level Semilunar Heart Valve Hemodynamics

A study of valve mechanobiology will be incomplete without first analyzing the mechanical and hemodynamic milieu of the valve. The purpose of this section therefore is twofold: (I) to qualitatively and quantitatively outline, as best possible, the complex mechanical environment of the valve (Figure 1) and (II) to provide an overall sense of the changes in mechanics that occur due to valve disease. Table 1 consolidates the relevant mechanical benchmarks in terms of practical parameters that can be simulated experimentally.

2.1. Normal Valve Hemodynamics. The aortic valve opens during systole when the ventricle is contracting and then closes during diastole as the ventricle relaxes (Figure 1). In healthy individuals, blood flows through the aortic valve accelerating to a peak value of 1.35 ± 0.35 m/s [1]. The valve closes near the end of the deceleration phase of systole with very little reverse flow through the valve. The adverse axial pressure difference causes the low inertia flow in the developing boundary layer along the aortic wall to decelerate then to reverse direction resulting in vortices in the sinuses behind the aortic valve leaflets [2]. This action forces the belly of the leaflets away from the aortic wall and toward the closed position. When this force is coupled with the vortices that push the leaflet tips toward the closed position, a very efficient and fast closure is obtained. *In vitro* studies have shown that the axial pressure difference alone is sufficient to close the valve [2]. Thus, without the vortices in the sinuses, the valve still closes, but its closure is not as efficient.

The velocity profile at the level of the aortic valve annulus is relatively flat. However there is a slight skew towards the septal wall (less than 10% off the center line) caused by the orientation of the aortic valve relative to the long axis of the left ventricle [9]. The flow patterns just downstream of the aortic valve (in the outflow tract) are of particular interest because of their complexity and relationship to arterial disease. Highly skewed velocity profiles and corresponding helical flow patterns have been observed in the human aortic arch using magnetic resonance phase velocity mapping [9]. *In vitro* flow quantification experiments (via laser Doppler anemometry) have shown that these flow patterns are dependent on the valve geometry and thus can be used to evaluate function and fitness of the heart valve [10].

2.2. Diseased Valve Hemodynamics. Aortic valve pathology, long thought to be due to passive degenerative valve disease caused by increasing longevity coupled with rheumatic and infective endocarditis [11], is now acknowledged to result from active disease processes such as inflammation and modulation of cell phenotype [12–15]. Current surgical interventions include valve repair or replacement depending on the diagnosis. Tremendous progress has been achieved during the last century on the development and improvement of prosthetic valves, but, to date, there is no ideal replacement valve available. Because knowledge of the hemodynamics could be invaluable in the treatment of such pathologies, studies have been done on the characterization of the fluid environment in the vicinity of diseased semilunar valves.

Aortic valve stenosis is a condition characterized by the incomplete opening of the valve. The partial opening of the valve produces an obstruction that limits the forward blood flow from the left ventricle to the aorta. Yoganathan [6] carried out some flow measurements on bioprosthetic valves mimicking different degrees of aortic stenosis *in vitro*. Under physiologic conditions (heart rate of 70 beats per min, systolic duration of 300 ms, and mean aortic pressure of 90–100 mmHg), flow visualization demonstrated that the fluid exits from the stenotic valve as an asymmetric, angulated jet. As the degree of stenosis increased, the jet diameter at the base of the aorta decreases and the flow field becomes more disturbed and chaotic (Figure 2). In addition, flow measurements showed that as compared with the evenly distributed flow field obtained at peak systole in the normal aortic valve (maximum axial velocity of 1.20 m/s), the stenotic valve is characterized by a jet-type flow field (maximum axial velocity of 7 m/s) with regions of separation located around the jet and highly turbulent shear layers (maximum rms axial velocity of 2.0 m/s) (Figure 2). The elevated levels of turbulence measured downstream of the stenotic valves are high enough to cause damage to the blood elements (red blood cells and platelets) and the endothelial cells lining the wall of the ascending aorta.

3. Mechanical Forces Experienced by the Aortic Valve

3.1. Pressure. *In vivo*, the pressure on the leaflet varies from systole to diastole, changing the stress and, consequently, the length of the leaflets. Under normal physiological conditions, the closed valve supports a transvalvular pressure of 80–120 mmHg acting perpendicular to the leaflet area (normal stress). This force is supported by the lamina fibrosa layer of the leaflet and is transmitted from the collagen fibers to the cells within the tissues that are aligned with the collagen fibers. The pressure acting on the leaflets is usually estimated in terms of stresses assuming the tissue to be homogeneous [3]. In reality, the leaflet is inhomogeneous, anisotropic, nonlinear, and viscoelastic with a complex geometry.

In vivo studies using a marker-fluoroscopy technique with radiopaque markers placed on canine aortic valve leaflets were conducted as early as in 1980. The stresses were estimated from the change in position of these markers using equations for membrane stress assuming a cylindrical geometry. The membrane stresses in the circumferential direction of the leaflet were 0.167 kPa during systole and 2.4 kPa during diastole [3]. In another study, finite element formulation was used to analyze the stresses. Based on a pressure of 114.7 mmHg and a human aortic valve leaflet thickness of 0.6 mm, the maximum principle stress was found to be 2.19 kPa, which is comparable to the *in vivo* study [16].

3.2. Fluid Shear Stress. Aortic valve fluid shear stress is an important factor in the synthetic activity of the valvular cells and also influences cell adhesion of macrophages and other

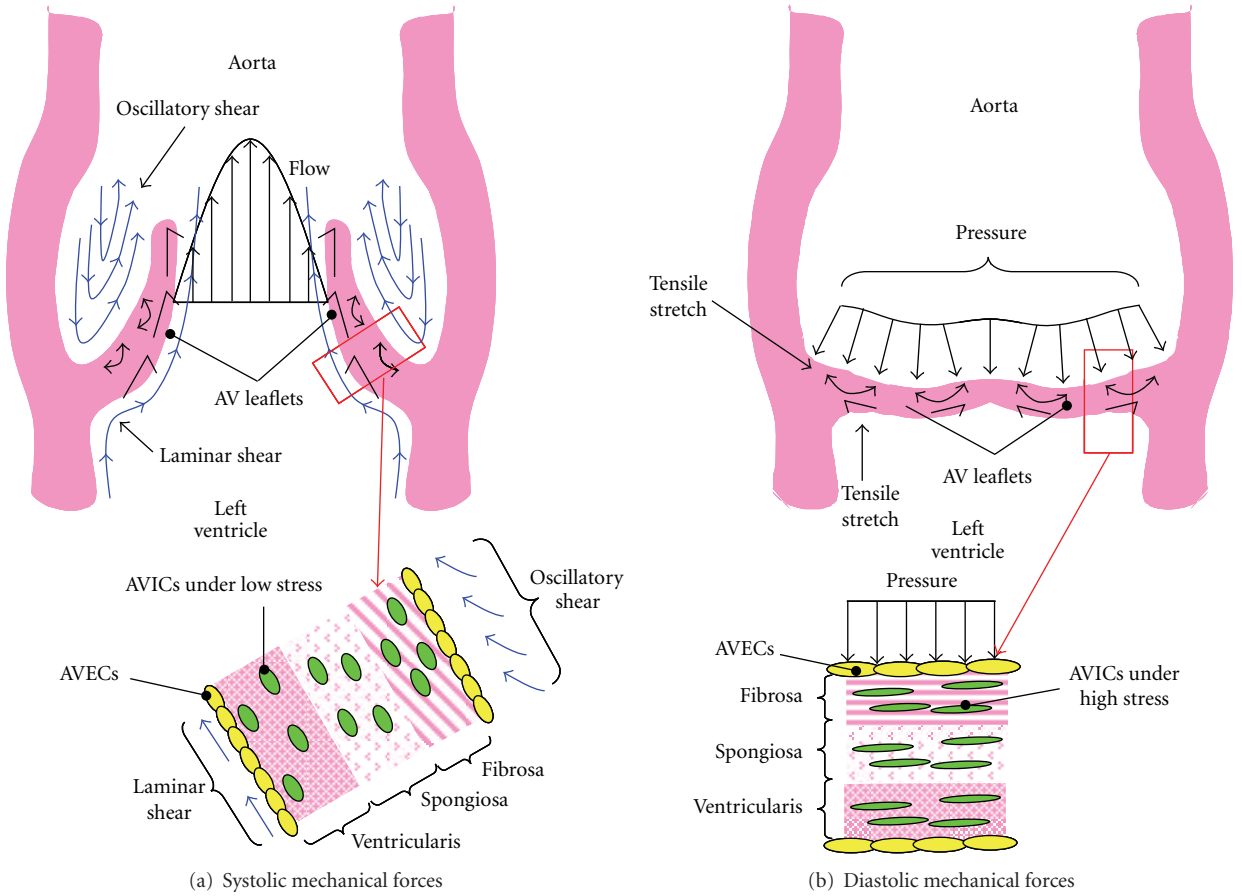

Figure 1: Schematic of mechanical forces experienced by the aortic valve during peak systole (a), and peak diastole (b). Insets depict qualitatively the effect of these forces on valve cells.

Table 1: Table outlining the current state of knowledge of valve hemodynamic/mechanical parameters. Knowledge of these parameters will aid the design of *in vitro* or *ex vivo* experimental studies to study mechanobiology.

Mechanical parameter	Normal	Diseased	Notes
Pressure	120/80 mmHg	Hypertensive: >120/>80 mmHg	
Membrane stress	Systole: 0.167 kPa Diastole: 2.4 kPa		[3]
Shear stress	Aortic side: peak 20 dyn/cm^2 Ventricular side: peak 64–71 dyn/cm^2		[4, 5]
Peak flow velocity	1.20 m/s	7.0 m/s (Jet-like flow)	[6]
Bending strain and stress	Systole: 14.5% and 1.22 MPa Diastole: 8.3% and 0.71 MPa		[7]
Tensile strain and stress	Circumferential: 9–11% Radial: 13–25%	Circumferential: >15% Radial: 15–31%	Porcine AV [8]

factors in the bloodstream. Shear stress is experienced by the ventricular surface of the leaflets when blood flows past the leaflets during systole and on the aortic surface when blood pools into the sinuses during diastole. An estimate of these stresses aids in understanding the effect of stresses on leaflet cellular function and in elucidating cellular responses [17]. Due to its apparent significance to atherosclerosis, the effects of shear stress on vascular endothelial cells have been extensively studied. One of the earliest recognized effects of shear stress is the elongation and realignment of endothelial cells. Cultured endothelial cells exposed to steady laminar shear stress elongate in the direction of flow, while valvular endothelial cells align perpendicular to flow [18, 19]. Actin stress fibers in the cytoskeleton are also

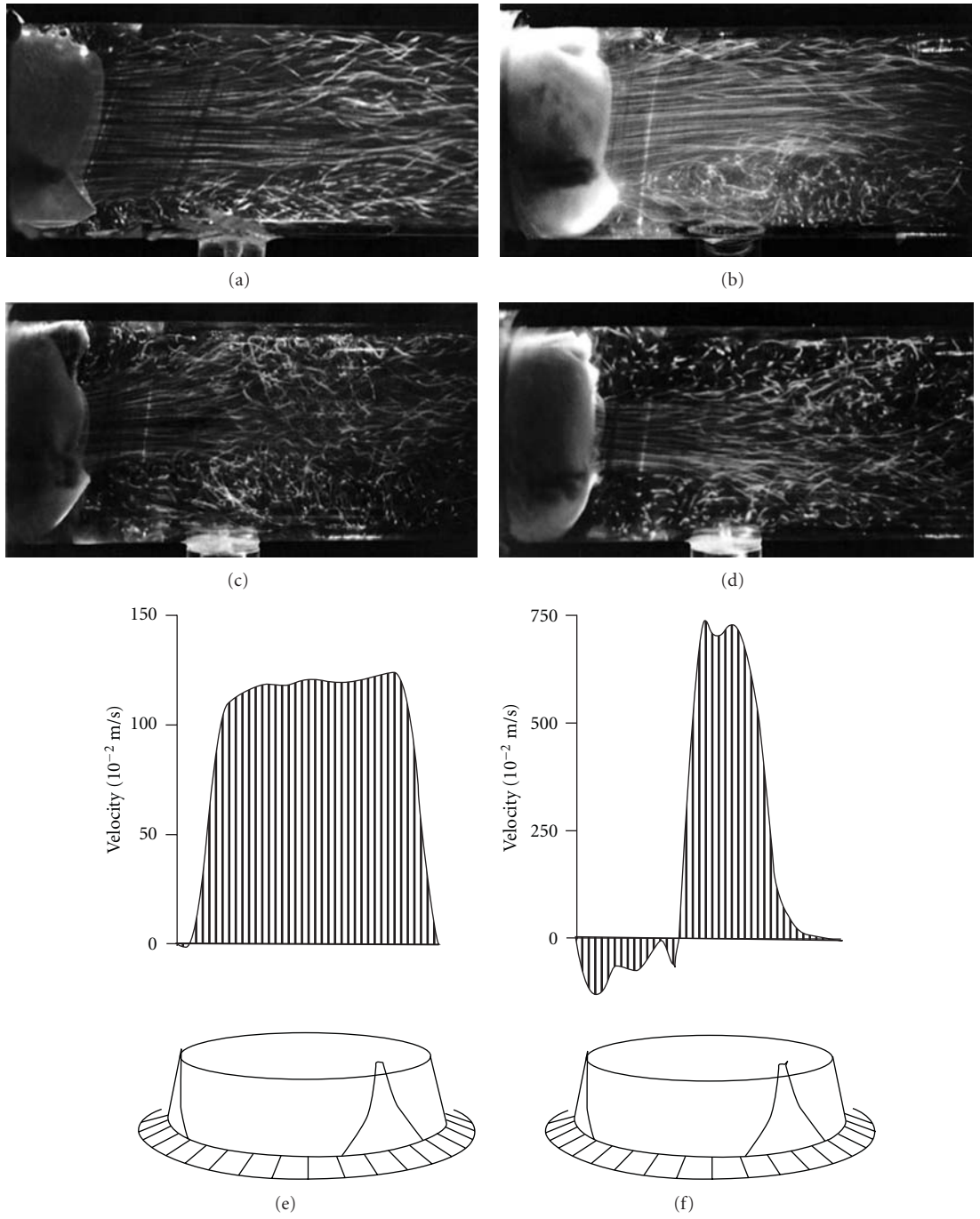

Figure 2: Flow patterns created at peak systole by different stenotic bioprosthetic aortic valves: (a) normal; (b) mildly stenotic; (c) moderately stenotic; (d) severely stenotic valves [6]. Schematic velocity profile at peak systole distal to (e) the normal aortic valve and (f) severely stenotic aortic valve [6].

subject to realignment with flow and increase in number with increasing shear stress [20]. Tissue degradation and failure due to calcification of the leaflets has been associated with regions of high shear and bending stresses in the leaflets during valve opening and closing [21]. Recently, Yap et al. quantified fluid shear stresses on the aortic and ventricular sides of the aortic valve leaflet and have reported differences in peak magnitudes and qualitative profiles of the shear stresses of both sides [4, 5].

3.3. Bending Stress. The change in leaflet curvature during the cardiac cycle gives rise to bending stresses, shearing, or buckling [3]. The collagen chords in the leaflet structure are free to bend in the circumferential direction without significant resistance from the elastic fibers aligned in the radial direction. Bending stress is both tensile and compressive with the leaflet on the convex side experiencing tensile stress while the concave side experiences compressive stress. During bending, the belly of the leaflet undergoes reversal of

curvature due to loading and unloading of the valve while the zone of attachment acts as a hinge facilitating leaflet movement. The bending stress increases with an increase in leaflet stiffness causing early failure of some bioprosthetic valves. Thubrikar used the radiopaque marker technique to calculate the bending strains *in vivo* in canine aortic valves. The bending strains, calculated from modulus of elasticity, thickness and radius of the leaflet, were found to be 2% during systole and 2.2% during diastole in the circumferential direction [3]. *In vitro* experiments using dip-cast polyurethane trileaflet valves were performed to determine values for bending strain and stress at the free edge of the leaflet under physiological pulsatile conditions. The bending was greatest during the opening phase corresponding to a maximum strain and stress of 14.5% and 1.22 MPa, respectively. During the closing phase, the maximum strain and stress were 8.3% and 0.71 MPa, respectively [7]. It is likely that this large difference between *in vitro* and *in vivo* results is due to differing thicknesses of the canine valve leaflets and polymeric trileaflet valves, and further study is needed to accurately characterize the bending strains experienced by the valve *in vivo*.

3.4. Axial Stretch. Stretching is important for a cusp as it allows the cusp to extend and form a coaptive seal with the other two cusps during diastole [22–24]. It is required for the maintenance of an adequate coaptation area [22]. Leaflet stretch may be lost at a relatively rapid rate for reasons that are not yet understood [3]. The first and most rapid change starts in late adolescence. The stretch during this period is halved from 80% to 40% over a time span of 15 to 25 years. This corresponds to a linearized reduction of approximately 4% per year in stretch rate. Between the ages of 25 and 40, the stretch remains approximately constant at a value of about 40%. After the age of 40, the stretch continues to decline at a slower linearized rate of about 1% per year until age 58 [25]. Thus, a valve from 15-year-old donor has about four times more stretch than one from a 58-year-old donor. The tissues become less extensible with increasing age because collagen fibrillogenesis increases the diameter of some of the constituent fibrils in discrete steps. Thus, larger numbers of thick collagen fibrils will require greater force to produce the same extension, causing a reduction in stretch.

In vivo studies done by Thubrikar measured the change in leaflet length in the circumferential and radial direction during the cardiac cycle in canine aortic valves. He observed that the leaflet in both the circumferential and radial directions is longer during diastole than during systole. The leaflets elongate by 11% in the circumferential direction and 31% in the radial direction from systole to diastole [3]. This is because the collagen in the circumferential direction provides greater tensile strength than that in the radial direction, which is mainly composed of elastic structures. These *in vivo* results agree well with the *in vitro* results published recently by Yap et al. [8] demonstrating circumferential strain of ~ 11% in the circumferential direction and ~28% in the radial direction. Additionally, Christie and Barratt-Boyes [25] have measured the biaxial properties of fresh and glutaraldehyde-fixed pulmonary and aortic valve leaflets during extension. For the pulmonary leaflets, radial stretch was greater than that in the aortic leaflets, and circumferential stretch was similar. Thus, the ratio of radial to circumferential stretch was 6.0 ± 1.1 for the aortic leaflets and 9.0 ± 1.8 for the pulmonary leaflets. After fixation in 0.2% glutaraldehyde, the ratios in the aortic leaflets were the same except with significantly reduced stretches in both directions.

4. Mechanobiology of Aortic Valve Remodeling, Inflammation and Calcification

As evident now, native heart valves—the aortic and mitral valves in particular—function in a high-magnitude and complex surrounding hemodynamic environment to which the valvular structure constantly responds. The mechanical environment varies spatially and temporally over the cardiac cycle. Close correlations between mechanical stresses and heart valve biology have long been documented by clinical observations and animal studies [26–30]. The cellular and molecular events involved in these processes, however, still remain unclear. Moreover, the biological response and the mechanotransductive signaling pathways appear to be different from the extensively studied vascular cell counterparts [19, 31].

According to various early studies, the structural components of the aortic valve undergo constant renewal in response to mechanical loading [26], and the sites of protein and glycosaminoglycan synthesis in the leaflets correlate with the areas of functional stress [27]. Changes in mechanical loading in turn alter the biosynthetic behavior of valve cells. For example, collagen synthesis in mitral valve leaflets was enhanced as a result of altered stress distribution due to left ventricular infarctions [32]. Other studies indicate that abnormal hemodynamics experienced by the valve leaflets cause tissue inflammation, which can lead to calcification, stenosis, and ultimate valve failure [33–37]. The common feature in valvulopathy appears to be the expression of an activated myofibroblast phenotype in the valve interstitium [38], which is absent in the quiescent leaflet, but abundant during disease initiation and progression [39, 40]. The expression of particular phenotypes of valvular endothelial and interstitial cells appears to depend not only on a combination of intrinsic genetically programmed biology, but also on local hemodynamic environmental factors, one family of which is the stresses induced by blood flow and structural strain due to leaflet deformation. It is therefore hoped that a detailed understanding of valve mechanobiology and disease regulation will allow development of better treatment options for valve disease.

Traditional benchtop studies can be categorized as either *ex vivo* or *in vitro* studies. *In vitro* studies approach the biological problem at the cell level and have tremendous utility in elucidating signaling mechanisms. However, in the valve where several cell types exist, paracrine signaling between endothelial and interstitial cells is key, and *in vitro* single-cell studies are limited in their ability to explain. It is only recently that advances are being made in developing 2D and 3D *in vitro* coculture models for the valve [41].

Also, critical cell-extracellular matrix interactions cannot be holistically modeled *in vitro*. *Ex vivo* whole-tissue studies can fill this critical gap.

Bioreactors are the standard means of imposing mechanical forces on cells or tissues. Commercially available ones include the Flexercell series of tension, compression, or flow devices that are primarily used for cells in culture. Several research groups have also designed customized bioreactors for the culture of sections of valve tissue or the whole valve apparatus. These include devices to subject tissues to stretch, shear stresses [42], bending stresses [43], and pressure [44, 45]. More recently, bioreactors subjecting valve tissue to combined mechanical forces [43, 46] and "benchtop heart simulators" [47–49] that subject the entire valve explant to hemodynamic forces have become more prevalent. The following sections discuss key findings from benchtop *in vitro* and *ex vivo* work on the pathological changes experienced by the aortic valve exposed to various hemodynamic stimuli organized by the different mechanical forces.

4.1. Effect of Static and Pulsatile Pressure. The effects of pressure on cultured cells have been studied extensively with vascular endothelial cells, smooth muscle cells, cartilage chondrocytes, and other cell types [50–54]. The affected properties include cell proliferation, apoptosis, synthetic activity, and gene expression, suggesting that the effects of pressure on cellular function are complex. Also, there was evidence of two different mechanoregulatory mechanisms for static and pulsatile pressures as indicated by the work of Sukhova et al. [54]. The effects of changes in static and pulsatile cyclic pressure have been investigated on whole valve tissue using pressurized chamber bioreactors. For the most part, either compressed air or a piston was used to alter the pressure within the pressurized chamber in a controlled manner. Xing et al. investigated the effects of static and cyclic pressure on aortic valve cusps with a focus on pressure magnitude and pulse frequency [44]. A wide range of pressures (80–120, 120–160, and 150–190 mmHg) and pulse frequencies (0.5, 1.167, and 2 Hz) were studied. Elevated static pressure caused an increase in collagen synthesis that was more significant at elevated pressures, while no significant difference in DNA or sGAG synthesis was observed. A notable decline in α-SMA, a standard marker for valve interstitial cell activation, was observed over the course of these experiments although no significant difference was observed between the pressure and control groups. It was concluded that elevated pressure caused a proportional increase in collagen synthesis of porcine aortic valve leaflets and had a downward effect on valve cell activation. Culture under pulsatile pressure revealed that increases in pressure magnitude (with the frequency fixed at 1.167 Hz) resulted in significant increases in both collagen and sulfated glycosaminoglycan (sGAG) synthesis, while DNA synthesis remained unchanged. Responses to pulse frequency (with the mean magnitude fixed at 100 mmHg) were more complex. Collagen synthesis and sGAG synthesis were increased at 0.5 Hz, but were not affected at 1.167 and 2 Hz. In contrast, DNA synthesis increased at 2 Hz, but not at 0.5 and 1.167 Hz. Under extreme hypertensive pressure conditions (170 mmHg, 2 Hz), collagen synthesis and sGAG synthesis were increased but to a lesser degree than at 170 mmHg and 1.167 Hz. As with the static pressure studies, a notable decline in α-SMA was observed over the course of the experiments. In a subsequent study, Warnock et al. [55] reported significant increases in VCAM-1 expression under elevated pressures suggesting an early inflammatory response.

These results suggest that cyclic pressure affects biosynthetic activity of aortic valve leaflets in a magnitude- and frequency-dependent manner. Collagen synthesis and sGAG synthesis were positively correlated and more responsive to pressure magnitude than pulse frequency. DNA synthesis was more responsive to pulse frequency than pressure magnitude. However, when combined, pressure magnitude and pulse frequency appeared to have an attenuating effect on each other. Intriguingly, the activated contractile phenotype of the valve interstitial cell, measured by α-SMA expression, was not expressed when cultured under isolated pressure. Taken together, an observed increase in proinflammatory expression suggests an important role for transvalvular pressure in mediating valve disease and valve cell activation.

4.2. Effect of Fluid Shear Stress. Early studies primarily focused on the effects of steady shear stress on aortic valve biology [56]. These studies were mostly conducted in parallel plate chamber bioreactors and reported that steady shear stress altered the biosynthetic activity of aortic valve cusps and was unable to preserve α-SMA expression in cells [56]. Although the cellular mechanisms triggering this specific response to shear stress were not well understood, it was hypothesized that the valvular endothelium plays an important role. Butcher et al. [19] reported that valve endothelial cells aligned parallel to steady, laminar flow in contrast with vascular endothelial cells, underscoring the difference between these two cell types. It was also postulated that aortic valve endothelial cells cocultured with smooth muscle cells can affect the properties of smooth muscle cells through the release of paracrine factors [41]. *Ex vivo* experiments from the Yoganathan lab demonstrated that valvular responses to shear in the absence of an endothelium were remarkably different from responses of intact leaflets (Figure 3). Collagen synthesis in the intact leaflets was enhanced under shear stress, but not changed in the endothelium-denuded leaflets at the same shear stress; sGAG content was not affected by shear stress in the intact leaflets, but was upregulated by shear stress in the denuded leaflets. These results indicate that aortic valve interstitial cells respond to shear stress in the absence of aortic valves' endothelial cells, but the presence of aortic valves' endothelial cells mediates these responses.

Matrix metalloproteinases and cathepsins are known to play an important role in the remodeling of various types of tissues and are often expressed early in the disease progression of aortic valves [57, 58]. This was demonstrated elegantly using molecular imaging by Aikawa et al. [59]. Cathepsin L activity has been shown to decrease in response to steady laminar shear stress [60]. Since the function of cathepsin L is to degrade collagen, the observed higher

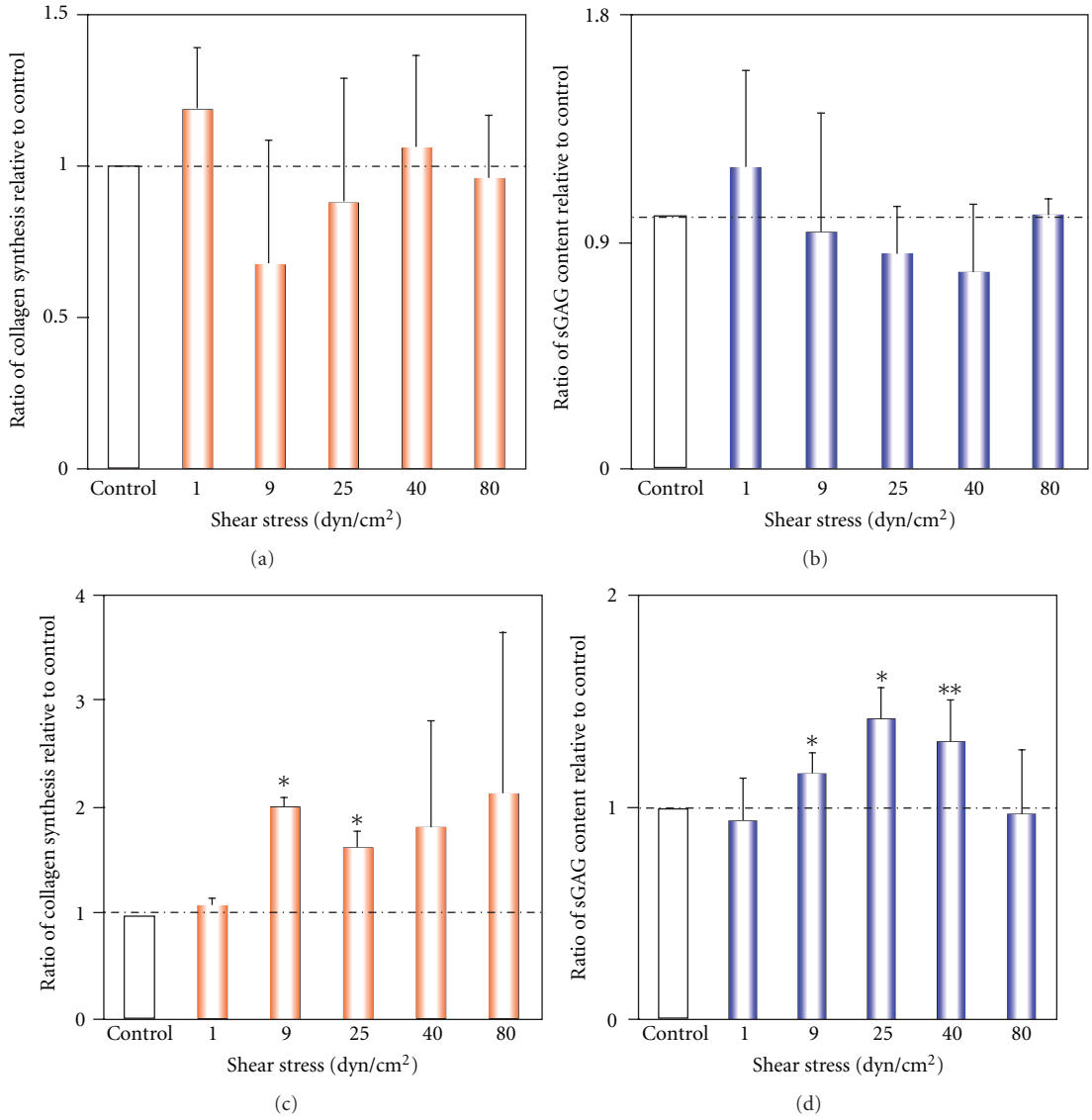

Figure 3: Effects of steady shear stress on aortic valve biology. Collagen synthesis and sulfated glycosaminoglycan content in intact (a and b, resp.) and denuded (c and d, resp.) aortic valve leaflets under steady shear stress.

collagen content under shear stress conditions is likely due, at least partly, to an increase in the overall collagen content due to a downregulation of the degradation process. The resulting collagen pool in the valve tissue is therefore a dynamic balance between new synthesis and degradation by collagenases such as cathepsins, which are regulated by mechanical factors such as shear stress. In addition, the inhibitory effects of shear stress on cathepsin L activity do not seem to depend on the presence of an intact endothelium as suggested by an *ex vivo* study [60]. The reduction of cathepsin L activity in endothelium-denuded leaflets is almost similar to that observed in intact leaflets exposed to fluid shear stress. This implies that the shear stress regulation of cathepsin activity might be achieved through a pathway that does not require the participation of the endothelium.

Shear stress is also an important hemodynamic force that directly regulates inflammation, calcification, and ossification, which are common features of aortic valve diseases [52, 53, 61]. Although the events leading to these disease states share some similarities with bone miner-alization [34, 54], their molecular mechanisms remain vastly understudied [62]. Aortic valve diseases preferentially occur on the aortic side of the valvular leaflets where they are exposed to complex and unstable hemodynamic conditions [58, 63]. The reasons for this side-specific response potentially associated with the local shear stress environment are not completely understood. Although many studies have been carried out to characterize the response of vascular endothelial cells to shear stress [64, 65], studies on valvular endothelial cells are few. The exposure of valvular endothelium to steady unidirectional shear stress has been shown to result in the alignment of the endothelial cells perpendicularly to the flow whereas vascular endothelial cells align parallel to the flow [19]. In addition, the transcriptional profiles of both

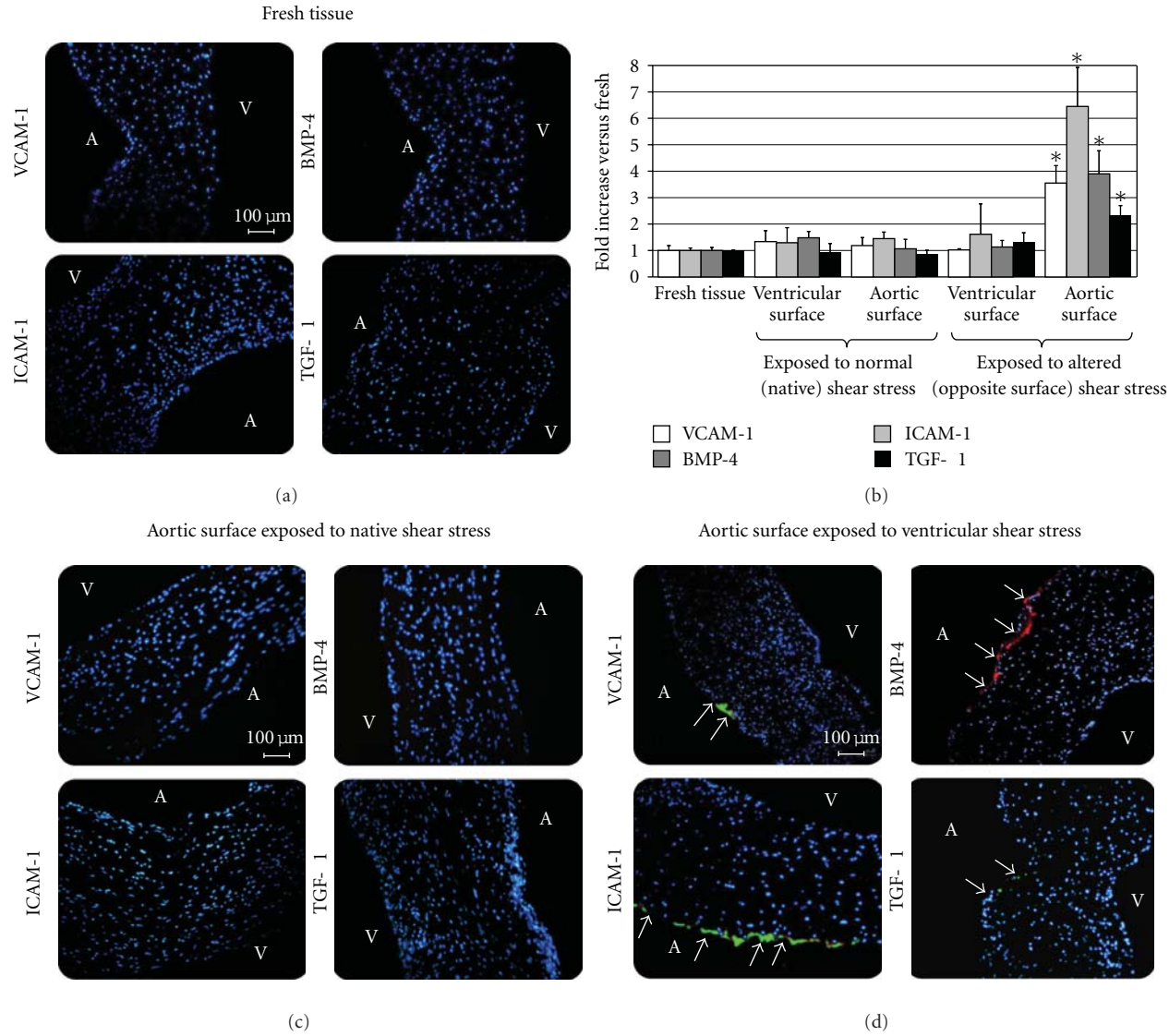

Figure 4: Inflammatory response of AV leaflets after exposure of the fibrosa to normal (native) and altered (ventricular) shear stress: immunostaining (blue: cell nuclei; green: ICAM-1/VCAM-1/TGF-β1; red: BMP-4); quantitative results ($^*P < .05$ versus fresh).

cell types have been compared under static and shear stress conditions, and up to 10% of the genes considered in that study were found to be significantly different [66], suggesting clear phenotypic differences between these two cell types in response to shear stress. Despite those differences, it has been shown that the pathological inflammatory responses of the two cell types involve similar mediators such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) [67]. In the context of the valvular response, these mediators are expressed preferentially on the aortic side of the leaflet. In addition to this side specificity, aortic valve calcification and inflammation are associated with the expression of transforming growth factor-beta1 (TGF-β1) [68]. TGF-β1 is a polypeptide member of the TGF-β superfamily which consists of TGF-βs, inhibins, bone morphogenic proteins (BMPs), growth differentiation factors, anti-Mullerian hormone, activins, and myostatin [69]. TGF-β1 has been shown to trigger calcification in sheep aortic valve interstitial cells by increasing alkaline phosphatase activity [70]. Although cell and clinical studies have suggested a potential role for TGF-β1 in the initiation and progression of calcification in aortic valve interstitial cells, studies at the tissue level are lacking. In addition, although it has been shown that exposure of vascular endothelial cells to oscillatory shear stress induces inflammatory responses by the BMP-dependent mechanisms [71, 72], it is not clear whether BMP plays a role in inflammatory responses in aortic valve leaflet in response to altered mechanical environment.

Sucosky et al. studied the effects of pulsatile and oscillatory shear stresses on aortic valve leaflet samples in a modified cone-and-plate bioreactor [42, 73]. The main objective of this study was to understand the effect of altered hemodynamics on aortic valve cellular response. Exposure of the aortic surface to pulsatile shear stress (i.e., the non-physiological or altered hemodynamic force) increased the

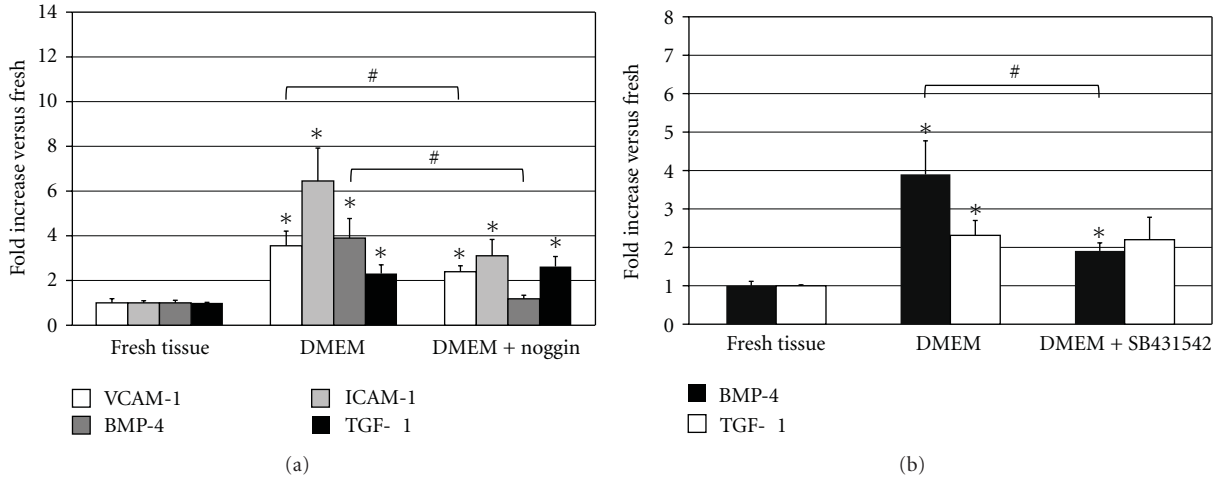

Figure 5: Cytokine and cell-adhesion molecule expressions after exposure of the fibrosa to altered shear stress in culture medium supplemented with (a) noggin and (b) SB-431542 ($^{*}P < .05$ versus fresh; $^{\#}P < .05$).

expression of the inflammatory markers (Figure 4). In contrast, neither pulsatile nor oscillatory shear stress affected the expression of the inflammatory markers on the ventricularis surface. The shear-stress-dependent expression of VCAM-1, ICAM-1, and BMP-4, but not TGF-β1, was significantly reduced by the BMP inhibitor noggin, whereas the TGF-β1 inhibitor SB431542 blocked BMP-4 expression (Figure 5) on the aortic surface exposed to pulsatile shear stress. These results therefore demonstrate that altered hemodynamics stimulates the expression of AV leaflet endothelial adhesion molecules in a TGF-β1- and BMP-4-dependent manner, providing some potential directions for future drug-based therapies for AV diseases.

As an extension of this work, another *ex vivo* study investigated the isolated effects of alterations in shear stress magnitude on valvular endothelial activation [74]. The fibrosa of porcine leaflets was subjected to subphysiologic, physiologic, and supraphysiologic magnitudes of native oscillatory shear stress in the same cone-and-plate apparatus. Under mild and severe supra-physiologic shear stress conditions, VCAM-1 and ICAM-1 expressions were observed on the endothelial lining of the fibrosa while positive BMP-4 and TGF-β1 staining was detected in both the endothelial and subendothelial layers (Figure 6(a)). In contrast, exposure of the fibrosa to physiologic and subphysiologic shear stress did not elicit any positive staining. Those results demonstrate the shear stress magnitude dependence on the leaflet pathological state in response to hemodynamic alterations. In addition, the synergistic effects of BMP-4 and TGF-β1 in shear stress-induced valvular endothelial activation were investigated by silencing those cytokines through the use of a culture medium supplemented with both the BMP antagonist noggin and the TGF-β1 inhibitor SB-431542. Under supra physiologic shear stress, the combined noggin+SB-431542 treatment significantly reduced cytokine and cell-adhesion molecule expressions as compared to the levels measured in tissue exposed to similar conditions in standard medium and brought them back to the levels measured in fresh controls (Figure 6(b)). Those results demonstrate that (1) exposure of the fibrosa to supra-physiologic shear stress stimulates cytokine and cell-adhesion molecule expression within 48 hours, (2) BMP-4 and TGF-β1 interact to synergistically regulate endothelial activation in response to elevated shear stress, and (3) TGF-β1 plays a dominant role in the shear-stress-induced pathological response of AV leaflets.

4.3. Effects of Cyclic Stretch. As mentioned before, cyclic stretch is one of the forces experienced by the aortic valve during the cardiac cycle that allows the valve cusps to extend and form a coaptive seal during diastole [22, 23]. The valve, under normal physiological conditions, experiences approximately 10% stretch during diastole [3, 75]. Utilizing an *in vitro* flow loop and a native porcine aortic valve, Yap et al. have demonstrated that for every 40 mmHg increase in pressure, there is a 5% increase in cyclic stretch [8]. The effects of cyclic stretch on the aortic valve have been investigated on valve cells as well as on whole valve tissue. Most *in vitro* studies have been conducted using the Flexercell device and primarily as single-cell models studying either the endothelial or interstitial cell [76–78]. *In vitro* coculture models for cyclic stretch have been lacking, while custom made bioreactors have been used to study the whole valve. One such device is the tensile stretch bioreactor, which has been used to culture whole valve samples under a defined stretch waveform [79–81].

Batten et al. reported that cyclic stretch upregulated collagen synthesis in valve interstitial cells as well mesenchymal stem cells [78], which was also demonstrated recently by Balachandran et al. on whole valve cusps utilizing the aforementioned tensile stretch bioreactor [79]. This study reported that collagen content increased with increasing stretch, while sGAG content was reduced. Cyclic stretch also was shown to upregulate the contractile phenotype of interstitial cells, which is in contrast to the result from the pressure studies. In addition, stretch studies suggest that α-smooth muscle actin is preferentially expressed on the

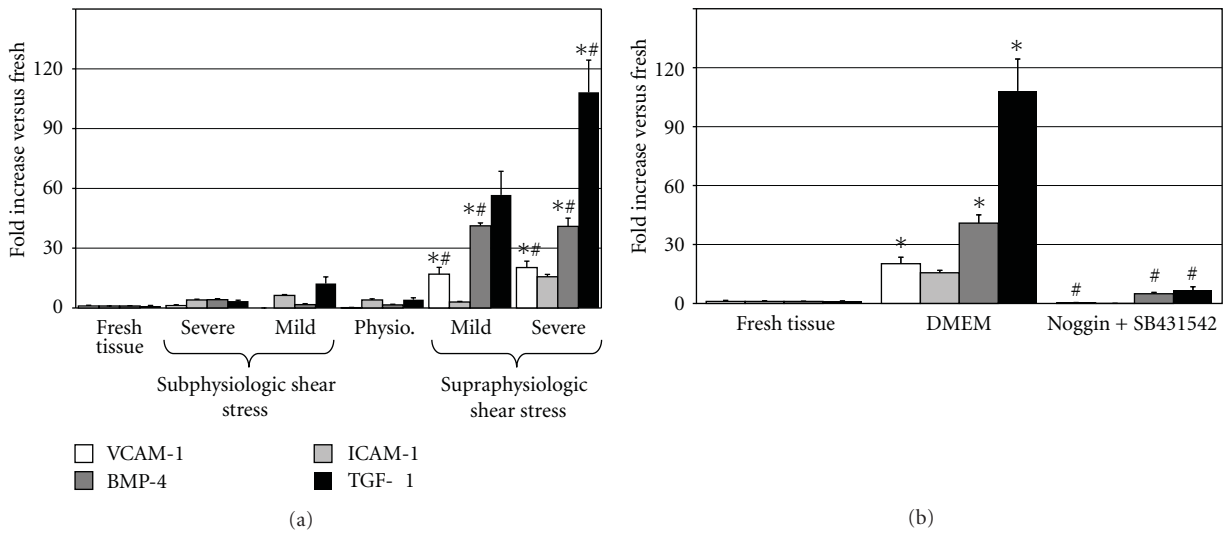

Figure 6: Cytokine and cell-adhesion molecule expressions after exposure of the fibrosa to (a) various shear stress magnitudes ($^{*}P < .05$ versus fresh; $^{\#}P < .05$ versus physiologic/subphysiologic) and (b) severe supraphysiologic shear stress in medium supplemented with SB-431542 and noggin ($^{*}P < .05$ versus fresh; $^{\#}P < .05$ versus standard medium (DMEM)).

ventricular side of the stretched aortic valve leaflet [79]. Merryman et al. reported that valve interstitial cells respond to local tissue stresses by altering cellular stiffness via collagen biosynthesis [82]. Indeed, it has been suggested that valve stiffness may be an important regulator of calcification [83]. Hopkins et al. also reported that the presence of cytokines such as TGF-β1 in a cyclic stretch environment could potentially result in altered matrix architecture and compromised valve function, underlining the importance of cyclic stretch in regulating valve structure, function, and disease progression [81].

Aortic stenosis and regurgitation, which are clinical manifestations of aortic valve disease, have been correlated in several patients with overexpression of proteolytic enzymes such as matrix metalloproteinases (MMPs), their tissue inhibitors (TIMPs), and cathepsins [84]. During normal homeostasis in the aortic valve, there is balance between extracellular matrix biosynthesis and degradation maintained by these enzymes [85]. A perturbation of this delicate equilibrium can lead to pathological remodeling of the tissue matrix and compromised valve function [84]. A number of these cathepsins, MMPs and TIMPs, are also involved in key cellular processes such as apoptosis, proliferation, and cell differentiation and have demonstrated roles in valve disease pathways [86]. Cathepsins K, L, and S, which are potent elastolytic proteases, have been associated with atherosclerotic plaque progression [53] and myxomatous heart valves [87].

Sucosky et al. [80] studied the effects of three levels of cyclic stretch (10%-physiologic, 15%—pathologic, 20%—hyperpathologic) on aortic valve extracellular matrix remodeling in porcine aortic valves with a focus on the aforementioned MMPs, TIMPs, and cathepsins. Immunohistochemical staining revealed that Cathepsin S and K expressions were upregulated by 15% cyclic stretch, while Cathepsin L expression was downregulated when compared with controls. Gelatin zymography and reverse zymography revealed modulation of MMP and TIMP activity in a time- and magnitude-dependent manner. TIMP activity was reduced significantly by all levels of cyclic stretch when compared with fresh controls. Collagenase activity was increased significantly compared to fresh controls after 15% and 20% stretch. These results suggest that activity of certain proteolytic enzymes (Cathepsin S, K, MMP-2,9) may be mechanosensitive and have an important role in the progression of valvulopathy under altered mechanical loading.

Probing deeper into the mechanisms behind stretch-induced valve disease, we sought to understand the effects of stretch on calcification in valve tissue [88]. Utilizing an osteogenic medium to stimulate rapid valve inflammation and calcification on the benchtop, Balachandran et al. observed that tissue mineralization occurred in a stretch-magnitude-dependent manner, which was inhibited by the bone morphogenic protein antagonist noggin, in a noggin-concentration-dependent manner. These results therefore highlight that as in fluid shear stress, valve inflammation and calcification are modulated by cytokines such as TGF-β1 and BMP-4 and that the signaling pathways induced by these molecules can be highly mechanosensitive. Smith et al. [77] reported the anti-inflammatory effects of cyclic strain on aortic valve interstitial cells. The apparent difference with the results reported by Balachandran et al. is thought to be due to the lack of endothelial cells in the former study, highlighting the importance of endothelial-interstitial cell crosstalk. Additionally, Ferdous et al. [89] demonstrated that stretch-induced mechanisms for valve interstitial cells differ from those of vascular smooth muscle cells. Further study in this area is therefore required to identify unique target candidate molecules for gene and molecular therapy in order to prevent or slow down valve inflammatory disease.

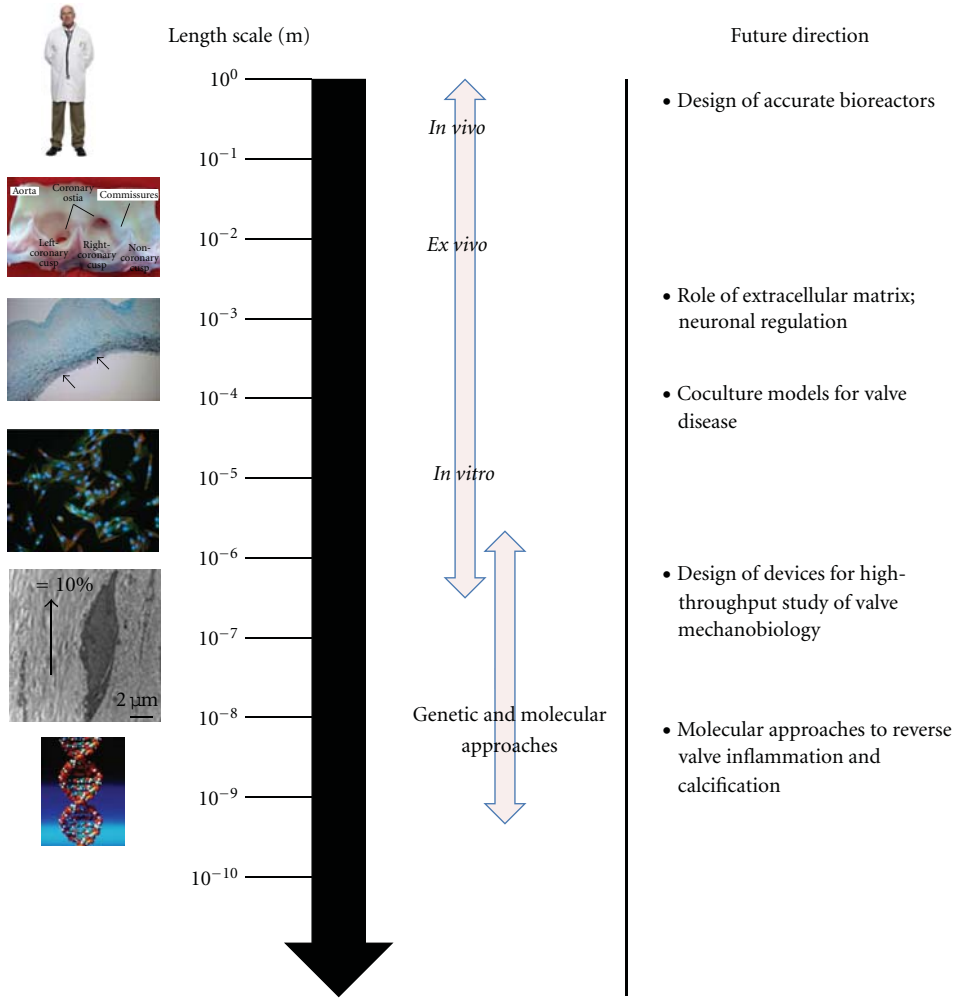

Figure 7: Multiscale, hierarchical approach that is recommended for approaching the study of valve mechanobiology and mechanopathology.

4.4. Complex Loading Regimes. Due to the complex interplay among the various mechanical forces experienced by the valve, there has been a much needed trend towards the design of novel bioreactors that can simulate combined mechanical forces on valve tissue. Engelmayr et al. developed the flex-stretch-flow bioreactor to study the combined effects of bending, stretch, and shear stress on tissue samples [43]. Thayer et al. developed a stretch-pressure bioreactor and reported the nonsynergistic regulation of aortic valve cell phenotype by the two mechanical forces (i.e., opposing effects of stretch versus pressure), suggesting the importance of the combined gamut of mechanical stimulation for the maintenance of the valve phenotype [46]. Indeed, Xing et al. demonstrated the need for all the *in vivo* mechanical forces in the normal homeostatic maintenance of the valve [90]. Recently, Barzilla et al. developed a novel splashing bioreactor [49] for the mitral valve that also demonstrated the importance of mechanical stimulation for normal cardiac valve function. The same group also demonstrated the utility of these whole organ culture models in studying serotonergic valve disease [48]. Taken as a whole, it appears that the response of the valve to combined mechanical stimulation is not just a simple sum of the effects of each of the individual forces, but a more complex response.

5. Summary and Future Direction

It is evident that the field has made tremendous progress toward understanding the mechanoresponse of valve cells and how it relates to disease. Research into the mechanobiology of cardiac valve disease is relatively recent, and we are only now beginning to get a deeper understanding of the complex interplay between valve cells, the extracellular matrix, and the surrounding mechanical environment. Early work was focused on understanding the mechanical environment and developing bioreactors to perform benchtop studies. Future work must devote greater attention to detail in the design of accurate bioreactors that simulate the combined mechanical forces of the native tissue environment. Coculture (interstitial and endothelial cells) *in vitro* models also need to be further explored [91], as *ex vivo* studies alone cannot provide the level of detail in the

mechanoregulation of signaling pathways. Additionally, the role of the extracellular matrix [83] and potential neuronal regulation [12, 92] of valve function has emerged as important in the interplay between valve structure, mechanics, and function and should not be overlooked. With the goal of developing pharmacological and gene therapies for valve inflammation and calcification, there is also a dire need for devices that can perform high-throughput testing of valve cells or tissues and their mechanobiological responses. Moraes et al. demonstrate such a device while probing for the mechanoregulation of wnt/β-catenin signaling in valve cells [93]. In addition, new evidence for regional/focal variation in valve mechanics [94] has potential implications for understanding the regional variation of cellular response to these varied mechanical stimuli. Finally, we currently have no way of reversing the effects of valve calcification once diagnosed. Heart valve replacement is often the only option for these patients, and it would be extremely valuable to be able to apply findings from the mechanobiology towards the development of therapies that can stimulate the cells to resorb or degrade calcific lesions.

It has become clear as we delve deeper in our understanding of valve mechanobiology that an interdisciplinary, hierarchical approach (Figure 7) toward its study is required. Approaches based on the intersection of biology, mechanics, bioinformatics, and micro/nanoengineering among others are key. Intimate crosstalk between the different length scales will allow for a complete understanding of the mechanoresponse of the valve. With this understanding, one can work towards the ultimate goal of developing treatments and functional replacements for diseased valves.

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Review Article

Oxidative Stress in Cardiovascular Inflammation: Its Involvement in Autoimmune Responses

Elisabetta Profumo, Brigitta Buttari, and Rachele Riganò

Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy

Correspondence should be addressed to Elisabetta Profumo, elisabetta.profumo@iss.it

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Recently, it has become clear that atherosclerosis is a chronic inflammatory disease in which inflammation and immune responses play a key role. Accelerated atherosclerosis has been reported in patients with autoimmune diseases, suggesting an involvement of autoimmune mechanisms in atherogenesis. Different self-antigens or modified self-molecules have been identified as target of humoral and cellular immune responses in patients with atherosclerotic disease. Oxidative stress, increasingly reported in these patients, is the major event causing structural modification of proteins with consequent appearance of neoepitopes. Self-molecules modified by oxidative events can become targets of autoimmune reactions, thus sustaining the inflammatory mechanisms involved in endothelial dysfunction and plaque development. In this paper, we will summarize the best characterized autoantigens in atherosclerosis and their possible role in cardiovascular inflammation.

1. Introduction

Atherosclerosis was initially believed as a process caused by the passive accumulation of lipids in the vessel wall. Recently, however, it has become clear that atherosclerosis is a complex condition and that multiple pathogenic factors contribute to trigger and sustain vessel wall damage [1]. Experimental studies have clearly demonstrated that atherosclerosis can be considered a chronic inflammatory disease of the arterial wall and that inflammation plays a key role in all stages of the pathogenic process, including formation, progression, and rupture of atherosclerotic plaque [2, 3]. In early lesions, chemokines and adhesion molecules expressed by endothelial cells recruit inflammatory cells, in particular monocytes and T cells, to the arterial intima [4]. If the acute inflammatory response does not resolve and macrophages and T cells continue to accumulate in the intima, a fibrotic repair process activates, leading to the formation of an atherosclerotic lesion [5]. This process is driven by the inflammatory cells that secrete cytokines and growth factors and stimulate smooth muscle cells to migrate to the intima, where they proliferate and produce extracellular matrix proteins [4]. In the advanced lesion, matrix metalloproteinases secreted from activated macrophages degrade the connective tissue in the fibrous cap causing plaque rupture.

Accelerated atherosclerosis has been reported in patients with various autoimmune diseases, suggesting an involvement of autoimmune mechanisms in atherogenesis [6–9]. This phenomenon is attributed to the presence of traditional risk factors for atherosclerosis, but could be also the result of autoimmune and inflammatory mechanisms that are aggravated in these diseases [6].

The first evidence for the involvement of autoimmune responses in atherosclerosis was provided by Jonasson et al. [10] who demonstrated the expression of MHC class II molecules and the presence of activated T cells in human atherosclerotic plaques. Analysis of plaque-infiltrating T cells revealed that these cells primarily showed the proinflammatory Th1 phenotype [11]. Subsequent studies on different animal models demonstrated that activation of Th1 responses contributes to a more aggressive progression of atherosclerosis [4]. Different studies have strongly suggested that atherosclerosis to some extent can be viewed as an autoimmune disease in which the adaptive immune system

is targeted against self-antigens modified by biochemical factors such as oxidative stress and hypercholesterolemia [12]. In this respect, atherosclerosis shares some similarities with other diseases characterized by an autoimmune etiology, such as type I diabetes and rheumatoid arthritis. In this contest, identifying the antigens responsible for the activation of immune responses that promote and sustain inflammation in atherosclerosis may be relevant.

2. Mechanisms of Autoimmunity Generation

Although infectious agents have been associated with the activation of immune mechanisms, evidence exist that the main antigenic targets in atherosclerosis are modified endogenous structures [12]. Atherosclerotic plaques express autoantigens that are targeted by both IgM and IgG. It is likely that these autoimmune responses initially have a beneficial effect facilitating the removal of potentially harmful antigens [13]. However, studies performed on hypercholesterolaemic mice deficient in different components of innate and adaptive immunity uniformly indicate that the net effect of immune activation is proatherogenic [13, 14] and that atherosclerosis, at least to some extent, should be regarded as an autoimmune disease.

In general, three mechanisms have been proposed to explain the autoimmune phenomena. (i) The molecular mimicry theory proposes that the autoimmune response is triggered by the initial activation of lymphocytes recognizing epitopes derived from microorganisms that share sequence homology with self-molecules. Therefore, autoimmunity resulting from epitope mimicry may be a consequence of the antipathogen immune response that can lead to disease in predisposed individuals [15]. (ii) Impairments in apoptosis execution and clearance can potentially render apoptotic cells as a source of autoantigens normally sequestered in the intracellular environment. Structural changes occurring during cell death may influence the immunogenicity of clustered self-antigens at the surface of apoptotic bodies [16]. (iii) Autoimmune responses may be directed against self-structures altered by high-affinity ligand binding or by chemical damage due to environmental events, such as oxidative stress [17].

3. Oxidative Stress in Atherosclerosis

Excessive oxidative stress and chronic inflammation are both major characteristics of atherosclerosis [18, 19]. However, the mechanisms by which oxidative stress mediates cardiovascular diseases are not clear [20]. Oxidative stress is the major event causing protein structural modification and appearance of neo-/cryptic epitopes [21, 22]. Several systems that generate reactive oxygen species (ROS) catalyze a variety of oxidative damage to nucleic acids, lipids, and proteins [23]. In physiological conditions oxidative stress is well compensated. When there is an overproduction of ROS or a deficiency of antioxidant enzyme activity, a biological damage with vascular lesion formation and functional defects may occur [24].

Oxidative stress and inflammation may determine the modification of self-structures also favouring the formation of advanced glycation end products (AGEs). Chronic oxidative stress causes an accumulation of AGEs [25]. The generation of AGEs and augmentation of proinflammatory mechanisms in the vessel provide a potent feedback loop for sustained oxidant stress, ongoing generation of AGEs, and vascular perturbation.

Epidemiological studies have demonstrated an inverse association between risk of stroke and intake of several antioxidant-rich food items [26, 27]. Intake of high total antioxidant capacity (the cumulative capacity of food components to scavenge free radicals) has been related to reduced inflammation and increased circulating antioxidants in both cross-sectional and randomized intervention studies [28, 29]. Nevertheless, clinical trials with anti-oxidants in humans have shown conflicting results in protecting against detrimental cardiovascular outcomes [20]. In particular, most antioxidant vitamin trials have failed to reduce cardiovascular morbidity and mortality [30]. In contrast, other investigators sustain that the beneficial effects of lipid-lowering and antihypertensive treatments are in part due to their antioxidant properties [31, 32]. Furthermore, recent findings suggest that antioxidants may play a role in reducing the risk of cerebral infarction [33], thus confirming the pathogenetic role of oxidative stress in inflammation and cardiovascular pathology. Collectively, these studies suggest that more rigorous clinical trial designs are needed to evaluate the usefulness of an antioxidant-based therapeutic approach to cardiovascular diseases [34].

4. Autoantigens Implicated in Cardiovascular Diseases

4.1. Oxidized Low-Density Lipoproteins. Oxidized low-density lipoproteins (LDLs) is the best characterized autoantigen in atherosclerosis. LDL modifications caused by oxidation and enzymatic attacks result in the release of proinflammatory phospholipids and lipid peroxides, which rapidly activate an inflammatory response in surrounding cells [35, 36]. The oxidation process is also associated with major structural modifications of LDL determining the formation of new antigenic epitopes which can be presented by dendritic cells and give rise to clonal expansion of oxidized LDL-specific T cells [12]. About 10% of all T cells present in human atherosclerotic plaques specifically recognize oxidized LDL [17], and these cells are also present in the circulation [37]. Furthermore, autoantibodies specific for oxidized LDL have been detected in humans and associated with cardiovascular disease, but their role is controversial [38, 39]. Pilot studies in animal models have provided promising results for the development of vaccines based on oxidized LDL antigens [12].

4.2. Heat Shock Proteins. A second category of autoantigens that have been implicated in atherosclerosis are the stress-induced heat shock proteins (HSPs) [40]. HSPs act as molecular chaperons facilitating refolding of denatured proteins

in stressed cells. Interestingly, some of them have also been implicated in loading of immunogenic peptides to major histocompatibility class (MHC) I and II molecules [41]. Besides being expressed in cells under physiological conditions, HSPs increase in response to many environmental stresses, including oxidative stress [42]. Under stress conditions, HSPs are expressed not only within cells, but also on the cell surface and can be released into the intercellular space. Intracellular HSPs have cytoprotective functions whereas extracellular located or membrane-bound HSPs mediate immunological functions [43]. In atherosclerotic lesions, human HSPs appear to stimulate an immune response leading to the development and progression of atherosclerosis [44, 45]. Antibody levels against HSP-60/65 are increased in subjects with established cardiovascular disease and predict further development of the disease [46]. These antibodies specifically react with cells in atherosclerotic plaques and mediate lysis of stressed endothelial cells and macrophages *in vitro* [47]. Recently, we demonstrated that human HSP90 is overexpressed in plaque and serum from patients with carotid atherosclerosis and induces humoral and cellular immune responses in these patients, implicating this self-protein as a possible target autoantigen in the pathogenesis of carotid atherosclerosis [48, 49]. In particular, we detected HSP90-specific T lymphocytes within the atherosclerotic plaque; these cells showed a predominant Th1, pro-inflammatory profile, suggesting a role for HSP90 in sustaining the inflammatory mechanisms responsible for the thrombogenicity of the atherosclerotic lesion [49]. Major questions for future research are the possible role of circulating anti-HSP90 antibodies and perhaps HSP90-specific peripheral blood T cells as diagnostic and prognostic markers of disease.

4.3. Beta2-Glycoprotein I. Beta2-glycoprotein I (β2-GPI) is a plasma protein involved in the haemostatic system that has been detected in carotid atherosclerotic lesions. Previous studies in animal models demonstrated that the transfer of lymphocytes obtained from β2-GPI-immunized mice was associated with the presence of larger fatty streaks within the recipients compared with animals that received lymphocytes from control mice, suggesting that T cells specific for β2-GPI are capable to increase atherosclerosis and that β2-GPI is a target autoantigen in this disease [6].

β2-GPI is also the most common target for antiphospholipid antibodies (aPLs). These autoantibodies are associated with thrombotic events and with the incidence of accelerated atherosclerosis in patients with antiphospholipid antibody syndrome and systemic lupus erythematosus [50–52]. In a previous study, we observed that β2-GPI, as several plasma proteins involved in the haemostatic system, undergoes major structural and functional changes upon exposure to oxidative stress and that such modification renders this self-molecule able to activate immature monocyte-derived dendritic cells (DCs) [21]. We demonstrated that DCs stimulated with the oxidized form of human β2-GPI presented a mature phenotype, produced cytokines that support T-cell activation, and were able to activate a Th1-type response by allogenic naïve T cells, characterized by interferon (IFN)-γ production. These findings are in accordance with our recent study demonstrating that β2-GPI is a target antigen of Th1 cellular immune response in patients with carotid atherosclerosis [53]. In this study, we used the native form of the plasma protein, but our previous evidence that β2-GPI spontaneously undergoes oxidative modification in *in vitro* culture conditions [21] led us to hypothesize that T-cell reactivity detected in these patients is directed to the oxidized form of the protein.

More recently, we have demonstrated that a glycated form of β2-GPI is able to activate monocyte-derived immature DCs through RAGE engagement and to trigger a proinflammatory signaling pathway mediated by the activation of mitogen-activated protein kinases and nuclear factor-κB [54].

Our findings underline the role of glycation/glycoxidation in rendering this self-protein able to activate the immune system and suggest that chronic activation of autoimmune reactions against β2-GPI modified by oxidative events may contribute to local and systemic inflammation, thus sustaining endothelial dysfunction and promoting thrombotic events in patients with cardiovascular diseases.

4.4. Oxidized Haemoglobin. Microvascular haemorrhage in atherosclerotic plaques is a common event in advanced lesions [55]. Intraplaque haemorrhage is an event characterizing high-risk "vulnerable" plaques, which are prone to rupture and associated with acute thrombotic events. Intraplaque haemorrhage causes the deposition of blood products into the extravascular space, particularly red blood cells (RBCs) and haemoglobin (Hb). In physiological conditions, Hb released within the vascular compartment from destroyed RBCs dimerizes and is rapidly bound by the serum protein haptoglobin. The haptoglobin-Hb complex is recognized by the scavenger receptor CD163 on the surface of mononuclear phagocytes, that promotes endocytosis and degradation of the complex [56]. Complicated plaques are characterized by repetitive haemorrhage events and hemolysis, accompanied with the release of large amounts of Hb into the atherosclerotic lesions. In these conditions, the capacity of Hb scavenging mechanisms is saturated. The proinflammatory and pro-oxidant microenvironment within the plaque may predispose free Hb to oxidative modifications. Free Hb or its oxidized forms may represent dangerous autoantigens, unwanted target of immune responses initially directed to the removal of escaped or modified proteins. Following this hypothesis, we have examined the presence of T cells specific for oxidized Hb in the peripheral blood of patients with advanced carotid atherosclerosis [57]. We used an oxidized Hb preparation predominantly containing hemichromes. Hemichromes represent Hb oxidation products characterized by a cross-linking between globin and heme that is responsible for molecule distortion and structural changes. We observed that the mean frequency of IFN-γ-secreting T lymphocytes specific for oxidized Hb was significantly higher in patients with carotid atherosclerosis than in healthy subjects, suggesting that the oxidized forms of Hb display immunodominant T-cell epitopes that are able

to stimulate adaptive immunity. Furthermore, our oxidized Hb preparation enhanced phenotypic maturation of LPS-stimulated DCs, as demonstrated by the appearance of the DC maturation marker CD83 and by the upregulation of the costimulatory molecules CD80, CD40, HLA-DR, and CD86, and significantly increased the production of the pro-inflammatory cytokines interleukin (IL)-12p70 and tumor necrosis factor (TNF)-α by DCs [57]. In a more recent study, we extended our results and demonstrated the presence of T lymphocytes specific for oxidized Hb within human atherosclerotic plaques. These cells produced high levels of the pro-inflammatory Th1 cytokines IFN-γ and TNF-α [58].

Previous studies demonstrated that Hb exerts a pro-inflammatory and oxidative action on endothelial cells [59, 60]. In particular, oxidized Hb upregulates the transcription rate of several pro-inflammatory genes, that is, E-selectin, ICAM-1, and VCAM-1, on vascular endothelial cells through the activation of the NF-κB family of transcription factors [61]. Our data implicate oxidized Hb as a possible antigenic target of cell-mediated immune responses contributing to tissue damage in the pathogenesis of carotid atherosclerosis. We can hypothesize that intraplaque haemorrhage in complicated lesions determines the release of large amounts of free Hb. In these conditions, haptoglobin and CD163, getting overwhelmed, are unable to clear efficiently the protein which accumulates in the extracellular compartment [55, 62]. The pro-oxidant microenvironment within the atherosclerotic plaque may promote oxidative modifications of Hb determining the generation of novel antigenic epitopes on this self-molecule. These epitopes can activate specific T lymphocytes with a predominant Th1 profile that contribute to inflammation. In physiological conditions, the oxidized forms of Hb do not reach consistent levels *in vivo* because oxidative stress is well compensated. Of note, we observed the presence of T-cells specific for oxidized Hb also in the peripheral blood from healthy subjects, suggesting that T cell clones specific for this rare molecule are not deleted within the thymus as, in basal conditions, they do not encounter the target antigen necessary for their activation.

Overall, these results indicate that Hb, in particular conditions, can sustain endothelial dysfunction interacting with endothelial cells and/or with immune cells, thus promoting the pathogenetic mechanisms involved in the progression of cardiovascular diseases.

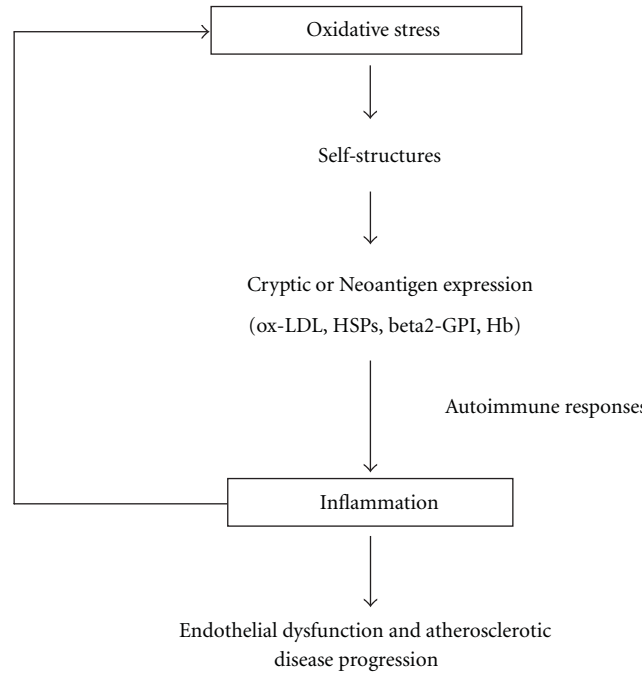

Figure 1: Schematic representation of the relationship between oxidative stress and inflammation in cardiovascular diseases. Patients with atherosclerosis are characterized by strong oxidative stress conditions. Prooxidant compounds that form may interact with molecular and cellular components thus determining expression of cryptic antigens, such as HSPs, and/or structural modifications of self-molecules and generation of neoepitopes, such as oxidized LDL. These cryptic or neoantigens may trigger autoimmune reactions, thus sustaining the inflammatory mechanisms involved in endothelial dysfunction and plaque progression. Of note, molecular and cellular mediators of inflammatory mechanisms sustain oxidative stress, thus creating a pathogenic system that amplifies itself. Ox-LDL: oxidized low-density lipoproteins; HSPs: heat shock proteins; beta2-GPI: beta2-glycoprotein I; Hb: haemoglobin.

5. Conclusions

Excessive oxidative stress and low-grade chronic inflammation are major pathophysiological factors contributing to the development of cardiovascular diseases. The strong oxidative and inflammatory conditions occurring in patients with atherosclerosis trigger the generation of a series of oxidation byproducts that may contribute to the pathogenesis of the disease. In addition to pro-inflammatory properties, self molecules modified by oxidative events can become targets of autoimmune reactions, thus sustaining the inflammatory mechanisms involved in endothelial dysfunction and plaque development (Figure 1). More studies aimed to clarify these aspect might be useful for designing novel preventive strategies in cardiovascular diseases. Modulation of the immune system could represent a useful approach to prevent and/or treat these diseases. A vaccination approach might be a useful, effective tool in the modern arsenal of cardiovascular therapy and could possibly be used on a large scale at a low cost. Several modalities of vaccines have been tested against oxidized LDL, β2-GPI, HSPs, cholesterol, and other molecules, with promising results. Nevertheless, a deeper understanding of the role of immunization in atherosclerosis will be essential to the use of vaccines in clinical medicine [63, 64].

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Review Article

Endoplasmic Reticulum Stress-Related Inflammation and Cardiovascular Diseases

Tomomi Gotoh,[1,2] Motoyoshi Endo,[1] and Yuichi Oike[1]

[1] *Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan*
[2] *Department of School Health, Faculty of Education, Kumamoto University, Kumamoto 860-8555, Japan*

Correspondence should be addressed to Tomomi Gotoh, tomomi@gpo.kumamoto-u.ac.jp

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The endoplasmic reticulum (ER) is the site of synthesis and maturation of proteins designed for secretion or for localization on the cell membrane. Various types of stress from both inside and outside cells disturb ER function, thus causing unfolded or misfolded proteins to accumulate in the ER. To improve and maintain the ER functions against such stresses, the ER stress response pathway is activated. However, when the stress is prolonged or severe, apoptosis pathways are activated to remove damaged cells. It was recently reported that the ER stress pathway is also involved in the inflammatory response, whereby inflammation induces ER stress, and ER stress induces an inflammatory response. Therefore, the ER stress response pathway is involved in various diseases, including cardiovascular diseases such as atherosclerosis and ischemic diseases, in various ways. The ER stress pathway may represent a novel target for the treatment of these diseases.

1. Introduction

The endoplasmic reticulum (ER) is the site of synthesis and maturation of proteins destined for secretion, the cell membrane, Golgi apparatus, and lysosomes [1–4]. The ER has several additional important functions, including assisting the maturation processes of newly synthesized proteins, such as cleavage, glycosylation, formation of disulfide bonds, folding, and assembly. These processes are assisted by ER chaperone proteins, such as BiP (an ER-resident Hsp70 family molecule), Grp94 (an ER-resident Hsp90 family molecule), calreticulin, calnexin, and protein disulfide isomerase (PDI). The protein maturation processes are dependent on intraluminal circumstances such as the Ca^{2+} concentration, oxygen supply, and redox homeostasis. High concentrations of Ca^{2+} in the ER are necessary for the functions of several ER chaperones, such as calreticulin, calnexin, and PDI. The formation of disulfide bonds is influenced by the redox circumstances in the ER. Unfolded or misfolded proteins cannot be delivered to the Golgi apparatus and are retained in the ER. The accumulation of these abnormal proteins in the ER disturbs the ER function, and cell survival can thus be threatened. When cells are subjected to ER stress due to accumulation of unfolded proteins in the ER, the ER stress response (also known as unfolded protein response) pathways are induced to protect cells [1] (Figure 1). However, when the ER functions are severely impaired, apoptosis occurs to remove damaged cells and protect the surrounding cells. When ER stress-induced apoptosis causes the loss of a large number of cells, the functions of tissues or organs are impaired, resulting in various pathological conditions, such as diabetes mellitus, hepatic steatosis, inflammatory diseases, ischemic diseases, and neurodegenerative diseases [2–21]. Interestingly, it was recently shown that the ER stress pathway is directly involved in the induction of not only apoptosis but also inflammation and autophagy [4–9].

Inflammation is a reaction of the host following exposure to various stimuli, and it is an attempt to eliminate the causal factors. There are two types of inflammation, acute inflammation and chronic inflammation [4, 22–24]. Chronic inflammation is caused by the failure to eliminate the

causal factors and/or by the failure to restore the damaged tissues to normal conditions. Macrophages and lymphocytes play major roles in chronic inflammation. Recently, chronic inflammation has been considered as the underlying pathology of various diseases including cardiovascular diseases, cancer, and metabolic diseases. It has been demonstrated that the ER stress response pathway is involved in the pathogenesis of various chronic inflammatory diseases [2, 4, 8, 9, 20].

ER stress-induced transcription factor CHOP/GADD153 is involved in ER stress-mediated cell damage, and it is also involved in the metabolic and inflammatory processes [6, 10, 11, 20, 25] (Figure 2). Therefore, CHOP plays crucial roles in the development and progression of cardiovascular and metabolic diseases. In this paper, the inflammation-related roles of the ER stress pathway including CHOP in cardiovascular diseases are discussed.

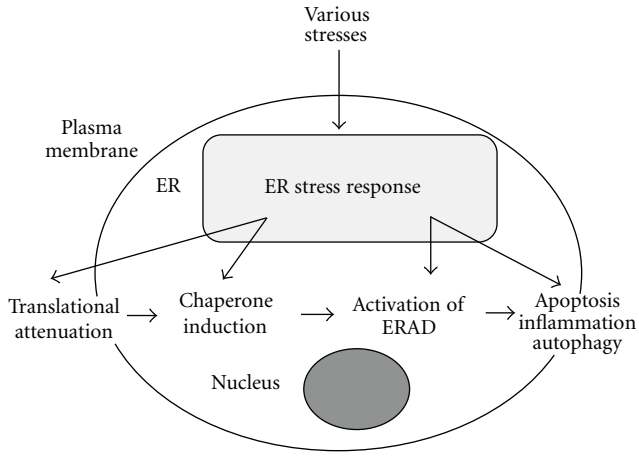

Figure 1: An overview of the ER stress response pathway. See the text for details.

2. Molecular Mechanisms of the ER Stress Response

There are three main ER stress sensors (Ire1, ATF6, and PERK) on the ER membrane [1–4, 9, 26] (Figure 2). Various stresses, including hypoxia and oxidative stresses, disturb ER function, and unfolded or misfolded proteins are accumulated in the ER. When unfolded or misfolded proteins are accumulated in the ER, Ire1 and PERK are activated by dimer formation and then are autophosphorylated. The inactive form of ATF6 (p90ATF6) is transported to the Golgi apparatus and is activated by a two-step cleavage by Site-1 protease (S1P) and Site-2 protease (S2P), to produce the active form of ATF6 (p50ATF6). The active form is then transported to the nucleus and functions as a transcriptional activator for ER stress-related genes [3, 27]. Four distinct ER stress response phases have been identified (Figure 1). (1) The first phase involves translational attenuation to reduce the load of newly synthesized proteins through phosphorylation of eIF-2α (α-subunit of eukaryotic initiation factor 2) by activated PERK. (2) The second involves the induction of ER chaperones such as BiP to enhance folding activity in the ER. (3) The third is degradation of unfolded or misfolded proteins in the ER through the ubiquitin-proteasome system in the cytosol. This system is called the ER-associated degradation (ERAD). (4) When ER stress-inducing stresses are too severe or prolonged to allow for recovery of ER function, the apoptosis pathway is activated to remove damaged cells [2, 10, 28–30]. At least three pathways are involved in the ER stress-mediated apoptosis [2, 10, 28–31]. The first is transcriptional activation of the gene for CHOP. The second is activation of the Ire1-TRAF2-ASK1-MAP kinase pathway. The third is activation of ER-associated caspase-12.

CHOP is expressed at low levels under physiological conditions, but it is strongly induced at the transcriptional level in response to ER stress [1, 2, 10, 25, 32]. The transcription of the *chop* gene is activated by all three ER stress sensors (Ire1, ATF6 and PERK) signaling pathways (Figure 2). The PERK-ATF4 signaling pathway plays a dominant role in the induction of CHOP over that of the ATF6 and Ire1 signaling

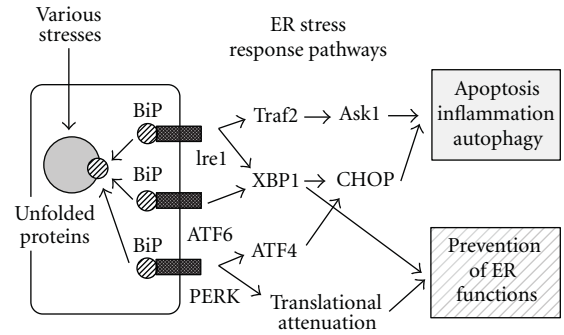

Figure 2: The major signal transduction pathway in the ER stress response. See the text for details.

pathways, although the presence of all three signaling pathways is required to achieve the maximal induction of CHOP [10, 25]. ER stress-induced apoptosis is suppressed in both CHOP null cells and *chop* knockout mice. Marciniak et al. reported that CHOP inhibits ER stress-induced attenuation of protein synthesis by dephosphorylation of eIF-2α through the induction of GADD34 [33]. They showed that the *de novo* protein synthesis, induced by CHOP under ER stress conditions, led to the accumulation of a high molecular weight protein complex in the ER and impaired ER function. It is also reported that the apoptosis signal induced by CHOP is transmitted to the mitochondria through activation of the proapoptotic molecule Bax and induction of the proapoptotic BH3-only type Bcl-2 family member Bim [34, 35].

The c-Jun N-terminal kinase (JNKs) and p38 MAP kinases are classified as members of the stress-responsive MAP kinase family [1, 36]. Apoptosis signal-regulating kinase 1 (ASK1) belongs to the MAPKKK family and activates both JNK and p38 pathways by directly phosphorylating and activating MAPKK family molecules, SEK1/MKK7 and MKK3/MKK6 (Figure 2). ASK1 is activated by the treatment of cells with TNFα or Fas ligand. In the case of activation by TNFα treatment, this activation is regulated by

TNF receptor-associated factor 2 (TRAF2). TRAF2 directly interacts with ASK1, activating ASK1 in a redox-dependent manner. Under unstressed conditions, thioredoxin (Trx) directly binds to ASK1 and inhibits its kinase activity. Treatment with ROS, including H_2O_2, oxidizes Trx, thus leading to its dissociation from ASK1. Under ER stress conditions, activated Ire1, one of the ER stress sensors, recruits TRAF2; then ASK1 directly binds to TRAF2 and is activated. It is thought that the mitochondria pathway is involved in ASK1-mediated apoptosis.

Caspase-12 belongs to the ICE (caspase-1) subfamily of caspase cysteine protease family [1, 28, 29]. Pro-caspase-12 is localized on the cytosolic side of the ER membrane and is activated by ER stress. However, the mechanism underlying the caspase-12 activation under ER stress conditions has not been confirmed. *Caspase-12* knockout cells are partially resistant to ER stress-induced apoptosis [28]. However, the human *caspase-12* gene is nonfunctional (pseudogene). Therefore, caspase-12 does not appear to be involved in the ER stress-induced apoptosis pathway in human cells.

As mentioned above, there are several signaling pathways involved in ER stress-induced apoptosis. It has been speculated that these pathways function in parallel under ER stress conditions, and the pathway that plays the major role is cell type and stress dependent [1].

Autophagy is a degradation pathway responsible for the bulk turnover of intracellular proteins and organelles via lysosomal degradation [21, 37]. Autophagy is induced by proteasome inhibition under ER stress condition. It has been reported that autophagy is directly induced through the Ire1-JNK/p38 pathway and the ATF4 pathway under ER stress [7].

3. Inflammation-Related Roles of the ER Stress Pathway

Inducible type NO synthase (iNOS) is induced in activated inflammation-related cells such as macrophages, and NO is involved in various inflammation-related diseases [2, 38]. Treatment with NO activates the ER stress pathways including CHOP, probably through disturbance of Ca^{2+} homeostasis in the ER, or enhancing ROS production by inhibiting mitochondrial respiration system through nitrosylation of cytochrome *c* oxidase, or inhibiting PDI through NO-induced S-nitrosylation [10, 38, 39]. The production of other ROS is also elevated under inflammatory conditions and plays a role as an ER stress inducer in inflammatory lesions [40]. The electrons generated by disulfide-bond formation in the ER do not recycle to reduce compounds in the cytoplasm but are transferred from reduced ERO1 to molecular oxygen [41]. ERO1 is an ER-resident oxidase and is involved in the formation of disulfide bonds. Therefore, biosynthesis of secreted proteins causes a net loss of reducing equivalents in the ER. In addition, many proteins synthesized in the ER are retrotransported to the cytoplasm to be degraded in a process of ERAD, because of the protein quality system in the ER. In this process, disulfide bonds are reduced to go through membrane channel. ERAD system is therefore a net consumer of reducing equivalents. Totally, the ER is a net consumer of reducing power. As already mentioned, ERAD is enhanced in the ER stress response to preserve ER function. It is also reported that treatment with ROS disturbs ER functions including intraluminal Ca^{2+} homeostasis and protein maturation processes [42, 43]. Therefore, oxidative stress induces ER stress, and ER stress produces oxidative stress.

Recently, it is reported that Toll-like receptor (TLR) 2 and 4-mediated signals specifically activate Ire1-XBP-1 pathway and are involved in host defense against bacterial pathogen [44]. XBP-1 is an ER stress-induced transcription factor and is involved in ER function-protective factors. We also reported that treatment with LPS activates Ire1-XBP1 pathway, but not PERK pathway [6, 45]. Therefore, the ER stress pathway is involved in various inflammatory diseases. Not only does inflammation induce the ER stress pathway, but the ER stress pathway is also involved in the induction of inflammatory responses. When mice are given LPS intratracheally, the ER stress-CHOP pathway is induced in activated macrophages in the lungs [6]. Under this condition, caspase-11, which is needed for the activation of procaspase-1 and pro-IL-1β, was induced by LPS in a CHOP-dependent manner, both in the lungs and in primary cultured macrophages [6]. In fact, LPS-induced IL-1β secretion and inflammatory responses in lung tissues and primary cultured macrophages were evidently suppressed in *chop* knockout mice. Moreover, Park et al. reported that the CHOP is involved in regulating the expression of proinflammatory cytokine IL-8 through activation of NF-κB [46]. As already mentioned, activated ER stress sensor Ire1 activates JNK/p38 MAP kinase through the Ire1-TRAF2-ASK1 pathway [30]. The activated JNK/p38 MAP kinase phosphorylate c-Jun, a component of AP-1 transcription factor, then AP-1 activity is enhanced. Activated AP-1 is involved in the transcriptional induction of inflammation-related genes, such as TNF-α, IL-6, IL-8, and MCP-1 [47]. The IRE1α·TRAF2·IKK complex induces degradation of IκBα, activation of NF-κB, and the transcription of inflammatory genes such as TNF-α, TGF-β, IL-2, IL-6, and IL-8 [5, 48, 49]. Therefore, the ER stress pathway plays crucial roles in inflammatory response.

As mentioned above, severe or prolonged ER stress-inducing stresses activate the ER stress-CHOP pathway, and CHOP induces apoptosis. Under inflammation-related ER stress conditions, CHOP induces caspase-11 and activates the IL-1β activation pathway, but not the apoptosis pathway [6]. Therefore, CHOP plays different roles in response to different types of stimulation. We recently reported the differences between the precise induction of the ER stress-CHOP pathway by inflammatory stimuli and the typical ER stress-induction stimulus [45]. The transcription of the *chop* gene is regulated through all three ER stress sensor (ATF6, Ire1, and PERK) signaling pathways under ER stress-mediated apoptosis [10, 41]. When cells are exposed to inflammatory stimuli, ATF6 and Ire1 are activated, but PERK is not activated. Therefore, the induction of CHOP by LPS is delayed in comparison to that induced by the ER stress-inducer, thapsigargin, even though ER function-protective molecules such as BiP are induced during the

early stage of response [45]. XBP-1 is an ER stress-induced transcription factor. The active form of XBP1 is induced by the combination of the ATF6 and Ire1 pathways. The active forms of ATF6 and XBP1 play crucial roles in the induction of the protective factors for ER functions, including BiP and ERAD-related molecules. Therefore, the ER stress-enhancing and apoptosis-inducing effect of CHOP is suppressed in LPS-treated macrophages, because the ER protective system is already enhanced before CHOP expression is induced. More detailed molecular analyses of the ER stress sensors are needed to elucidate the precise mechanisms of the starting point of the ER stress response pathway and how the three pathways are activated and regulated [50].

4. Roles of the ER Stress Pathway in Atherosclerogenesis

Atherosclerosis is mainly caused by metabolic disorders and is deeply involved in life-threatening ischemic diseases, such as myocardial infarction and cerebral infarction [20, 51–53]. Therefore, clarification of atherosclerogenesis is one of the most emergent issues in the field of medical science. For infarction, we need to distinguish the mechanisms of the progression of atherosclerosis and the disruption of blood flow. Acute coronary syndrome (ACS), including myocardial infarction and unstable angina, is most frequently caused by occlusive coronary thrombosis at the site of a preexisting atherosclerotic plaque. The formation of coronary thrombosis is generally the result of the rupture of an atherosclerotic plaque, followed by the aggregation of platelets and the formation of fibrin. It is thought that metalloproteinases secreted by macrophages and apoptosis of macrophage-derived foam cells affect the stability of plaques. Therefore, monocytes/macrophages play a key role in the instability of atherosclerotic plaques. Activations of the ER stress pathways including CHOP are detected in atherosclerotic lesion at all stage of atherosclerogenesis, especially in macrophage-derived cells [54] (Figure 3(a)). Myoishi et al. reported the induction of apoptosis and the activation of the ER stress pathway, including the induction of CHOP, to be detected in macrophages and smooth muscle cells within ruptured plaques, but not in stable fibrous plaques in humans [52]. They also reported the levels of ER stress in atherectomy specimens from patients with unstable angina pectoris to be higher than those from patients with stable angina pectoris. These results suggest that ER stress-induced apoptosis of macrophages affects the vulnerability of plaques to rupture. Thorp et al. reported the progression of atherosclerosis to be suppressed in two distinct models of advanced atherosclerosis in the mice lacking CHOP [55]. These results show that the ER stress-CHOP pathway plays crucial roles in atherosclerogenesis, which is a specific type of chronic inflammation of arterial wall induced by high levels of intracellular cholesterol, fatty acids, oxidative stress, and NO. Accumulation of free cholesterol in ER membrane is thought to be one of the main causes of the activation of the ER stress response pathway in atherosclerotic lesion [20, 56]. During the process of atherosclerogenesis, the iNOS expressed by macrophages invading the arterial walls plays a crucial role [2, 38]. Therefore, iNOS-derived NO is one of the major candidate ER stress-inducers in atherosclerotic lesions. TLR-mediated signals are also involved in atherosclerogenesis [57]. As already mentioned, TLR-mediated stimulation and ROS also can be the cause of the activation of the ER stress response pathway. The ER stress-CHOP pathway is activated in macrophage-derived cells at all stage of atherosclerosis, but apoptosis becomes prominent at the late stage (partly because of the rapid disposal of apoptotic cells at the early stage). Therefore, it can be speculated that the role of CHOP changes at the late stage. Li et al. showed the induction of the ER stress pathway by the accumulation of free cholesterol to be involved in the induction of TNF-α and IL-6 [5]. They also showed that the CHOP branch of the ER stress response is required for IL-6 induction and demonstrated that other ER stress-related pathways are responsible for the activation of p38, JNK1/2, NF-κB, and TNF-α. Therefore, the ER stress pathway contributes to the progress of atherosclerogenesis, through activation of inflammatory cells in arterial wall. Although it appears that CHOP plays a crucial role in atherosclerogenesis, its role in the final rupture of the plaque and subsequent thrombosis is still undefined. We recently showed that the ER stress-induced apoptotic pathway in macrophages contributes to the formation of the rupture of atherosclerotic plaques [56]. Large numbers of CHOP-expressing macrophages showed apoptosis in advanced ruptured atherosclerosis lesions in wild-type mice, while few apoptotic cells were observed in *chop* knockout mice. The rupture of atherosclerotic plaques was significantly reduced in high cholesterol-fed *chop*/*apoe* double knockout mice compared to *chop* wt, *apoe* knockout mice. Using primary cultured macrophages, we further showed that the unesterified free cholesterol derived from incorporated denatured LDL was accumulated in the ER and induced ER stress-mediated apoptosis in a CHOP-Bax pathway-dependent manner. We concluded that the ER stress-CHOP-Bax-mediated apoptosis in macrophages contributes to the instability of atherosclerotic plaques. In addition, the formation of a fibrous cap of plaques in the brachiocephalic artery was enhanced in *chop*/*apoe* double knockout mice compared to *chop* wt, *apoe* knockout mice. As already mentioned, CHOP is involved in the induction of the inflammatory response, and the CHOP-mediated extracellular matrix degeneration pathway may be induced in the final stage of atherosclerotic lesion rupture.

It has been suggested that the suppression of acyl–coenzyme A and cholesterol acyltransferase- (ACAT-) mediated cholesterol esterification and/or activation of neutral cholesteryl esterase, are induced during the progression of atherosclerosis [58–60], and unesterified free cholesterol is accumulated in foam cells. Under physiological conditions, the cholesterol content of the ER membrane is low, and it is thought that increases in the cholesterol content of the ER membrane may alter the stiffness of the membrane, thus leading to a disturbance in the function of ER membrane proteins, including Ca^{2+} pumps such as sarcoplasmic reticulum ATPase (SERCA) [59]. ACAT is an ER membrane protein, therefore, accumulation of free cholesterol in the ER membrane may further disturbs the

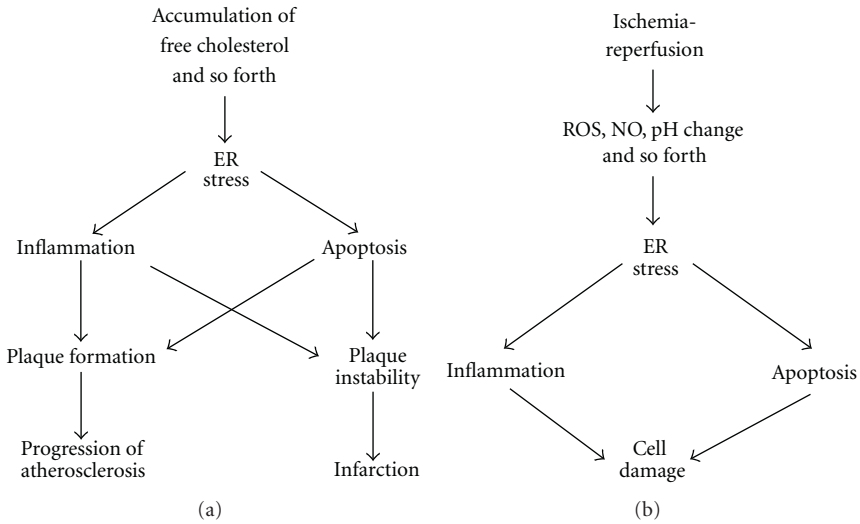

Figure 3: Involvement of ER stress response in atherosclerosis (a) and ischemia-reperfusion injury (b). See the text for details.

ACAT activity [59]. As a result, the ER stress pathway may be activated by ER dysfunction. We directly showed that free cholesterol is transported to the ER and activates the ER stress pathway in primary cultured macrophages [56]. Seimon et al. reported that oxidized phospholipids, saturated fatty acids, and lipoprotein (a) induce apoptosis in ER-stressed macrophages via the CD36 and TLR2-mediated pathway [61]. As already mentioned, ER stress-mediated inflammatory pathway is induced by the TLR2-mediated pathway. Therefore, it can be speculated that TLR-ER stress-mediated inflammation is also involved in the process of atherosclerogenesis.

The ER stress pathway therefore may be a key therapeutic target related not only to atherosclerotic lesion progression, but also to the instability of existing atherosclerotic plaques.

5. Roles of the ER Stress Pathway in Ischemia-Reperfusion Injury

Ischemic stress, including hypoxia and hypoglycemia, is an inducing factor for the ER stress pathway, probably because of the disturbance of protein modification processes such as disulfide bond formation and N-glycosylation of newly synthesized proteins in the ER [3, 7, 40] (Figure 3(b)). Therefore, the ER stress pathway is also involved in the pathogenesis of ischemic diseases, such as cerebral infarction and myocardial infarction, most likely through induction of apoptosis or inflammation. Azfer et al. reported that ER stress-associated genes, including CHOP, are induced in the heart of an ischemic heart disease mouse model [62]. Szegezdi et al. reported that the ER stress pathway is induced by deprivation of serum glucose and oxygen in primary cultured rat neonatal cardiomyocytes [63]. Under those conditions, CHOP was induced by prolonged treatment alone, which induced apoptosis. Therefore, they concluded that CHOP was involved in ischemia-induced cardiomyocyte loss. The ER stress-CHOP or JNK/p38 MAP kinase pathway can be induced and play negative roles in ischemic tissues during the preapoptotic period, and it is also likely involved in the induction of inflammation. Mutant KDEL receptor (a retrieval receptor for ER chaperones in the early secretory pathway) transgenic mice show dilated cardiomyopathy, enhanced expression of CHOP, and apoptosis in cardiomyocytes [64]. Therefore, the normal function of the ER is essential for the physiological function of the heart.

In the case of cerebral infarction and acute myocardial infarction, early and successful reperfusion is the most effective therapy to reduce infarct size and improve the clinical outcome. However, the recanalization of the occluded artery can paradoxically reduce the beneficial effects by inducing further cellular damage, termed ischemia-reperfusion (I/R) injuries, and the clarification of the precise mechanism underlying this damage is one of the major urgent issues in the field of cardiovascular medicine [19, 21, 65, 66]. Several potential mediators have been proposed as the cause of I/R injury, such as ROS generation, intracellular Ca^{2+} disturbance, rapid pH changes, and inflammation. It is likely that all of these factors are involved in mediating neuronal cell and cardiomyocyte death, and inflammation, and they are also thought to be the potential causes of ER stress. Then, the ER stress response leads to further apoptosis and inflammation. In the case of cerebral infarction, the ER stress responses were shown to be activated by I/R in mouse brains, and I/R injury was suppressed in the *chop* knockout mouse brain [13]. Several studies have demonstrated that the ER stress response is activated in rodent models of myocardial I/R [66–69]. Moreover, overexpression of ATF6, one of the ER stress sensors on the ER membrane, induced the expression of ER function-protective ER stress-related genes such as BiP and Grp94 without a significant induction of CHOP, showing protective effects against reperfusion injury [67]. In addition, the general inhibition of the ER stress response by a δ-protein kinase C (δPKC) inhibitor also resulted in reduced injury size or cardiomyocyte apoptosis [69]. Therefore, ER stress appears to regulate myocardial

I/R injury variably, either attenuating or exacerbating the condition, possibly depending on the balance between prosurvival and proapoptotic ER stress responses. It is reported that ischemic preconditioning or postconditioning reduces cardiac damage, probably through induction of ER function-protective molecules including PDI [70]. Recently, Miyazaki et al. showed that myocardial I/R activates the ER stress-induced, CHOP-mediated pathway partially through ROS overproduction occurring early after reperfusion, and that the pathway subsequently exacerbates myocardial I/R injury not only by inducing cardiomyocyte apoptosis but also by enhancing tissue inflammation [66]. Furthermore, an *in vitro* analysis showed the potential and complexity of the CHOP-related proinflammatory signaling under inflammatory conditions. Using a myocardial I/R model (not a chronic ischemia model), it was demonstrated that reperfusion-dependent ROS production is upstream of ER stress and that CHOP exhibits proinflammatory activities, such as induction of IL-1β and IL-6, in the reperfused tissue [66]. Additionally, the time-dependent activation of ER stress-related molecules, such as BiP, CHOP, and XBP-1 has been directly demonstrated *in vivo*. It is therefore suggested that the CHOP-mediated pathway enhances myocardial inflammation, possibly by the transcriptional induction of specific cytokine genes, such as IL-6. It was demonstrated that CHOP deficiency almost completely suppressed cardiomyocyte apoptosis in the reperfused myocardium and neuronal cell death in the reperfused mice brain, thus suggesting that the CHOP-mediated pathway also plays a potentially important role in inducing cardiomyocyte and neuronal cell apoptosis after I/R [13, 66]. Another study showed that either a deficiency in stress-activated MAP kinase JNK1 or JNK2 reduces cardiomyocyte apoptosis by approximately 40% [71]. It was also shown that intravenous administration of edaravone (a free radical scavenger) immediately before reperfusion significantly suppresses the superoxide overproduction and subsequent activation of the ER stress pathway, leading to reduced injury size in a mouse I/R model heart [66].

These experimental results suggest that the ER stress pathway may represent a new target for the prevention of I/R injuries, which include the apoptosis pathway and inflammation pathway, and that scavengers of ROS can be the promising agents that can reduce ER stress and suppress I/R injuries.

6. Conclusions

The ER stress-related Inflammation and apoptosis are involved in the pathogenesis of various diseases, including cardiovascular diseases. Therefore, the ER stress pathway can be a new target for the treatment of those diseases. However, the molecular mechanisms underlying the activation, regulation, and execution of the ER stress response have not been fully clarified. Recently, Iwawaki et al. reported that ER stress sensor IRE1α in the placenta is essential for placental development through induction of VEGF-A and other factors [72]. IRE1α-deficient mouse show embryonic lethality, because of reduced proliferation of trophoblasts. They also showed that IRE1α-deficient embryos supplied with functionally normal placentas can be born alive. The molecular mechanisms how IRE1α is involved in the secretion of VEGF-A and the placental angiogenesis remain to be elucidated. It is therefore necessary to investigate the molecular mechanisms by which the ER stress pathway exerts different effects in different situations, to elucidate the pathological roles of this pathway and the potential of targeting this pathway for the prevention or treatment of human diseases.

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