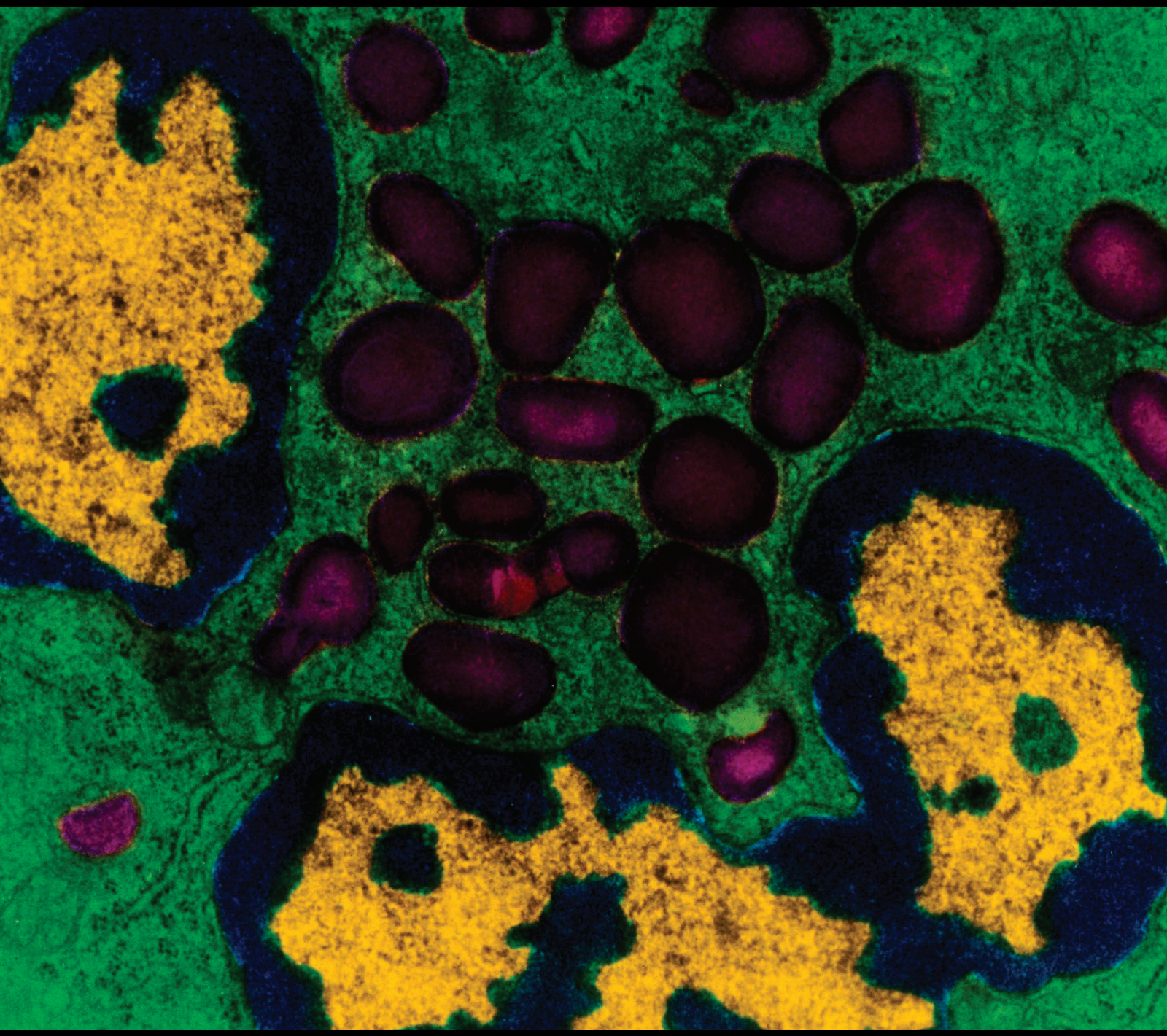
Mediators of Inflammation

Danger Signals in Cardiovascular Disease

Guest Editors: Stefan Frantz, Claudia Monaco, and Fatih Arslan



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Editorial Board

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 395278, 2 pages
http://dx.doi.org/10.1155/2014/395278

Editorial

Danger Signals in Cardiovascular Disease

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Received 16 February 2014; Accepted 16 February 2014; Published 6 April 2014

More than ten years ago, the "danger theory" challenged conservative immunology. At that time, the consensus was that the immune system is activated by antigens recognized as nonself. However, the self-nonself theory gave no explanation why, for example, a fetus with obvious foreign antigens does not lead to maternal immune activation whereas transplanted organs do. In an attempt to resolve these apparent paradoxes, the danger theory postulated that the immune system is triggered by "danger signals" released upon tissue injury and stress alerting the immune system that there is risk to the host [1]. The "danger theory" is supported by the growing number of endogenous ligands that can activate innate immune receptors such as toll-like receptors, RIG-I-like receptors, NOD-like receptors, and the inflammasome. However, it poses the challenge of identifying such signals and the mechanisms of their generation rigorously. Danger signals or danger associated molecular patterns (DAMPs) identified so far include factors like high-mobility group protein B1, mitochondrial DNA, heat shock protein (HSP), interleukin-1α, adenosine triphosphate, reactive oxygen intermediates, and uric acid [2].

A common feature of cardiovascular diseases, like myocardial infarction, heart failure, atherosclerosis, and so forth, is a robust inflammatory response. The reason for an immunologic reaction in mostly nonimmune diseases is not very well defined. However, the danger theory offers a good explanation: tissue damage, for example, in myocardial infarction, could lead to the release of danger signals and thereby cause an immune response. Indeed, in the current issue, several aspects of this process are highlighted: after a general introduction into DAMPs in the cardiovascular system [3], M. Ashri et al. discuss the theory of cardiotrophin-1 as a secondary DAMP in obesity "*Update on the pathophysiological activities of the cardiac molecule cardiotrophin-1 in obesity*," whereas A. Schiopu et al. review S100A8 and S100A9, members of the calgranulin family, as potential DAMP in cardiovascular disease "*S100A8 and S100A9: DAMPs at the crossroads between innate immunity, traditional risk factors, and cardiovascular disease*." F. van den Akker et al. highlight that danger signals might influence the phenotype of mesenchymal stem cells and secondarily outcome after myocardial infarction "*Mesenchymal stem cell therapy for cardiac inflammation: immunomodulatory properties and the influence of toll-like receptors*." A few original articles deal with the role of oxidative stress as DAMP "*Berberine protects against palmitate-induced endothelial dysfunction: involvements of upregulation of AMPK and eNOS and downregulation of NOX4*" and "*Natural antioxidant-isoliquiritigenin ameliorates contractile dysfunction of hypoxic cardiomyocytes via AMPK signaling pathway*" and with actin or chitinase 3-like 1 as a trigger of immune activation in patients with advanced atherosclerotic plaques "*Actin is a target of T-cell reactivity in patients with advanced carotid atherosclerotic plaques*" and "*Increased expression of chitinase 3-like 1 in aorta of*

patients with atherosclerosis and suppression of atherosclerosis in apolipoprotein E-knockout mice by chitinase 3-like 1 gene silencing" or complement factor C3 as marker of danger signal activation in patients with heart failure "*Complement c3c as a biomarker in heart failure*." All manuscripts underline the importance of danger signals in cardiovascular disease in basic as well as clinical science.

Clinical Implications. DAMPs may have great diagnostic, prognostic, and therapeutic potential. In theory, DAMPs may indicate active tissue injury. Since DAMP levels are related to the extent of injury, they may have prognostic implications. When DAMPs are the most important trigger for immune activation, pharmaceutical interference should allow tailoring an immune response. However, it has to be beard in mind that the activation of the immune system in the context of tissue injury makes evolutionary sense and is not necessarily negative. For example, after myocardial infarction depletion of macrophages causes the scar not to be cleared of cell debris and left ventricular thrombi to develop leading to adverse outcome in animals and potentially also in humans (*Monocytes/macrophages prevent healing defects and left ventricular thrombus formation after myocardial infarction*). Thus, an initial immune activation is necessary for a coordinated pathophysiologic and beneficial response to injury. However, a chronic immune activation might be detrimental, as has been shown by several groups. Therefore, timing will be crucial when interfering with DAMPs.

In conclusion, a better understanding of DAMPs in cardiovascular disease might give us dual benefit: it will help us to identify and treat patients at the very core of the pathophysiological process. However, markers and potential drug targets warrant further research.

Stefan Frantz
Claudia Monaco
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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2014, Article ID 905463, 12 pages
http://dx.doi.org/10.1155/2014/905463

Research Article

Increased Expression of Chitinase 3-Like 1 in Aorta of Patients with Atherosclerosis and Suppression of Atherosclerosis in Apolipoprotein E-Knockout Mice by Chitinase 3-Like 1 Gene Silencing

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Received 2 July 2013; Revised 27 November 2013; Accepted 23 January 2014; Published 4 March 2014

Academic Editor: Claudia Monaco

Introduction. The purpose of this study was to investigate the changes of chitinase 3-like 1 (CHI3L1) in the aorta of patients with coronary atherosclerosis and to determine whether inhibition of CHI3L1 by lentivirus-mediated RNA interference could stabilize atherosclerotic plaques in apolipoprotein E-knockout ($ApoE^{-/-}$) mice. *Methods*. We collected discarded aortic specimens from patients undergoing coronary artery bypass graft surgery and renal arterial tissues from kidney donors. A lentivirus carrying small interfering RNA targeting the expression of CHI3L1 was constructed. Fifty $ApoE^{-/-}$ mice were divided into control group and CHI3L1 gene silenced group. A constrictive collar was placed around carotid artery to induce plaques formation. Then lentivirus was transfected into carotid plaques. *Results*. We found that CHI3L1 was overexpressed in aorta of patients with atherosclerosis and its expression was correlated with the atherosclerotic risk factors. After lentivirus transduction, mRNA and protein expression of CHI3L1 were attenuated in carotid plaques, leading to reduced plaque content of lipids and macrophages, and increased plaque content of collagen and smooth muscle cells. Moreover, CHI3L1 gene silencing downregulated the expression of local proinflammatory mediators. *Conclusions*. CHI3L1 is overexpressed in aorta from patients with atherosclerosis and the lentivirus-mediated CHI3L1 gene silencing could represent a new strategy to inhibit plaques progression.

1. Introduction

Coronary artery disease (CAD) has become the principal cause of death in the world. As a systematic disease which usually affects large- and medium-sized elastic and muscular arteries all over the body, atherosclerosis is the underlying pathology of most CADs. Substantial evidence supports the concept that atherosclerosis is a chronic inflammatory disease characterized by the deposition of fibrous matrix and lipids in the arterial wall. According to the "response-to-injury" hypothesis, the endothelial denudation and endothelial dysfunction caused by some risk factors are the first step in the development of atherosclerosis [1]. The activated endothelial cells facilitate monocytes infiltration into the vessel wall. Then, these monocytes differentiate into macrophages, which accumulate lipids from the circulation and remain in the vessel wall, thereby becoming foam cells. These cells mentioned above can synthesize and release proinflammatory molecules such as tumor necrosis factor α (TNF-α), monocyte chemoattractant protein 1 (MCP-1), and interleukin 1 (IL-1), which can induce further accumulation of monocytes and migration and proliferation of vascular smooth muscle cells (SMCs) [2].

Chitinase 3-like 1 (CHI3L1), also called cartilage glycoprotein 39 or YKL-40 in human and breast regression protein 39 in mice, is a 40 kDa chitin-binding glycoprotein without chitinase activity, and it has been shown to act as an important regulator of acute and chronic inflammation [3, 4]. It is secreted by a variety of cells, including SMCs and macrophages and is found in tissues with inflammation and extracellular tissue remodeling. Up to now, several

studies have shown an important link between CHI3L1 and inflammation or metabolic diseases, including asthma [5], hypertension [6], diabetes mellitus [7, 8], insulin resistance [9], and atherosclerosis [10, 11], and naturally believe that CHI3L1 may be a potential biomarker and therapeutic target for the related diseases.

Although the relationship between CHI3L1 and CAD is important, there is a controversy in the association between blood CHI3L1 levels and the severity of atherosclerosis. One study investigating the role of CHI3L1 in patients with peripheral arterial disease showed that severity of atherosclerosis is associated with higher blood CHI3L1 levels [12], and another paper concluded that circulating CHI3L1 was not specifically related to the size of atherosclerotic stenosis [13]. These conflicting results may be due to the differences in the study participants and diagnostic modality for evaluation of coronary artery and severity of atherosclerosis. In order to elucidate the relationship between CHI3L1 and CAD and furthermore verify the therapeutic value of CHI3L1, we designed this study. First, we investigated the correlation between CHI3L1 expression and pathogenesis of atherosclerosis by measuring the changes of CHI3L1 in the aortic tissues of patients undergoing coronary artery bypass graft (CABG) surgery. Second, we constructed lentiviral vectors, which can efficiently deliver small interfering RNAs (siRNAs) due to their stable transduction of both dividing and nondividing cells, and aimed at knocking down CHI3L1 to explore the mechanisms of CHI3L1 in atherosclerosis in apolipoprotein E-knockout ($ApoE^{-/-}$) mice as a potential target for treatment.

We found that the expression of CHI3L1 was enhanced in aorta of patients with coronary atherosclerosis and its expression was significantly correlated with the atherosclerotic risk factors and the severity of CAD. In addition, the interference with CHI3L1 expression resulted in an improvement of atherosclerotic burden and plaque stability in $ApoE^{-/-}$ mice.

2. Methods

2.1. Study Population. From 2011 to 2012, 39 patients with CAD scheduled for CABG surgery were investigated and were defined as a research group. After admission, a detailed disease history, physical examination, and routine laboratory tests were carried out to establish a clinical diagnosis. Special attention was paid to the atherosclerotic risk factors including status of smoking, hypertension, and diabetes mellitus. All CAD patients were confirmed by coronary angiography (CAG). CAG was obtained using 5F catheters with Judkins method. The Gensini score system was used to assess the CAD severity, according to the distribution, extent, and severity of coronary artery stenosis. The exclusion criteria were acute or chronic infection, stroke, acute, or chronic liver or kidney disease, autoimmune disease, neoplasm, and trauma. Eleven normal subjects who donated kidneys were investigated as the control group. All kidney donators were blood relatives of recipients. They did not suffer from any diseases including CAD, hypertension, diabetes mellitus, trauma, malignancies, and acute or chronic inflammatory status. In addition, they were nonsmokers and reported no long-term drug use.

Aortic specimens were obtained from the aorta that was routinely removed during CABG surgery as a button hole. In addition, the discarded renal arterial tissues without atherosclerotic lesions were collected from the 11 subjects who donated kidneys. In each group, the arterial tissues were randomly distributed for histological analysis and western blot analysis.

This study protocol conforms to the principles of the Declaration of Helsinki and was approved by the ethics committee of the Shandong Provincial Qianfoshan Hospital. All patients and normal subjects who participated in this study signed the informed consent forms.

2.2. Biochemical Analysis. Venous blood samples of patients in the research group and normal subjects in the control group were obtained after a 12 h fasting for measurement of serum triglycerides (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), lipoprotein (a) [Lp (a)], and apolipoprotein A (ApoA), as well as apolipoprotein B (ApoB). All the items mentioned above were measured with standard laboratory techniques by the Department of Clinical Chemistry, Shandong Provincial Qianfoshan Hospital.

2.3. Cell Culture. The 293T human embryonic kidney cell line, which expresses simian virus 40 large T antigen and facilitates the optimal production of viruses, and the RAW264.7 mouse macrophage cell line were purchased from the Chinese Academy of Typical Culture Collection cell bank (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100U/mL penicillin, and 100 ug/ml streptomycin. All cells were incubated in a 37°C humidified incubator with 95% air and 5% CO_2.

2.4. Lentivirus Construction and Target Screening for RNAi. Four different sequences (sites A, B, C, and D) of CHI3L1 gene in mice were designed as the target for RNA interference (RNAi) (Genepharma, Shanghai, China). The sequence of site A was 5′-GCGACAACATGCTTAGCACATTTCAAGAGAATGTGCTAAGCATGTTGTCGCTT-3′; the sequence of site B was 5′-GGCCATTGACACTGGCTATGATTCAAGAGATCATAGCCAGTGTCAATGGCCTT-3′; the sequence of site C was 5′-GCACTGGATTTGGATGATTTCTTCAAGAGAGAAATCATCCAAATCCAGTGCTT-3′; the sequence of site D was 5′-GCCAGAAGGACACTAGGTTTGTTCAAGAGACAAACCTAGTGTCCTTCTGGCTT-3′. As a control, the scrambled sequence (mock siRNA) was 5′-GTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAACTT-3′. Then the pShuttle vectors containing the mouse CHI3L1 RNAi sequences were constructed. A lentivirus was produced by cotransduction of the siRNA expression pShuttle vectors pGag/Pol, pRev, and pVSV-G into the 293T cells. Then lentiviruses were used to transfect RAW264.7 cells. To screen the target for the most effective gene

interference, RAW264.7 cells were collected for the following polymerase chain reaction (PCR) and western blot experiment at 72 h and 96 h after transduction, respectively. Nonlentivirus and lentivirus containing mock siRNA transduction served as controls.

2.5. Animal Experiment. We obtained 50 male ApoE$^{-/-}$ mice, 8 weeks old, from the Beijing University Animal Research Center (Beijing, China). All mice were housed five per cage and were fed a high-fat diet (15% cocoa butter and 0.25% cholesterol) with free access to water. The mice were divided into 2 groups ($n = 25$ each): control group and CHI3L1 lentivirus silenced group. The atherosclerotic model was as previously described [14]. In brief, after anesthesia by intraperitoneal injection of pentobarbital sodium (40 mg/kg), a constrictive silica collar (inner diameter, 0.3 mm; outer diameter, 0.5 mm; and length, 2.5 mm) was placed on the right common carotid artery of mice. Eight weeks after surgery, the carotid collars were removed and the proximal right common carotid artery and the distal right internal and external carotid arteries were temporarily ligated. Then 20 μL of lentiviral suspension at 1×10^9 TU/mL was instilled into the right common carotid artery, left *in situ* for 15 min before closure of the skin incision [15].

All animal procedures were performed in accordance with the institutional guidelines of the Shandong Provincial Qianfoshan Hospital.

2.6. Tissue Preparation and Histological Analysis. Immunohistochemical analyses were performed according to routine laboratory methods [16]. For arterial tissues obtained from CABG surgery and kidney donators, the specimens were fixed in 4% buffered formalin overnight at 4°C, then dehydrated in an ascending ethanol series, routinely embedded in paraffin, and sectioned at 3 μm. After conventional deparaffinage, hydration, and antigen retrieve, endogenous peroxidase was inactivated by 3% hydrogen peroxide. The sections were incubated with the rabbit antihuman CHI3L1 polyclonal antibody (diluted to 1 : 200, Bioss, Beijing, China) at 4°C for 12 h. After washings with phosphate-buffered saline (PBS), the sections were incubated with the goat anti-rabbit IgG polymer at room temperature for 30 min. Then the sections were visualized with 3,3-diaminobenzidine to produce a brown product and then counterstained with hematoxylin, dehydration, transparention, and fixation by neutral resins. As negative controls, rabbit nonimmune serum was applied parallel with rabbit anti-human CHI3L1 polyclonal antibody.

Mice were sacrificed by intraperitoneal injection of pentobarbital sodium (200 mg/kg) 4 weeks after transduction and were perfused with PBS through the left ventricle. The right common carotid artery was carefully excised and immersed in 4% formaldehyde. Six cross-sections in each mouse were used for a particular type of staining. One section was stained with hematoxylin and eosin (H&E). Another section was immunostained with rabbit anti-mouse CHI3L1 polyclonal antibody (diluted to 1 : 300, Santa Cruz, sc:98954). Collagen and lipids deposition in plaques were identified by Sirius red staining and oil red O staining, respectively. SMCs and macrophages were immunostained with anti-α-actin antibody (diluted 1 : 300, Boshide, Wuhan, China) and macrophage-specific antibody (diluted 1 : 200, Boshide, Wuhan, China), respectively. An automated image analysis system (Image-Pro Plus 5.0, Silver Spring, MD) was used for quantitative measurements. The positive-staining area of collagen, lipids, SMCs, and macrophages was quantified by computer-assisted color-gated measurement, and the ratio of positive-staining area to intimal area was calculated. The vulnerability index was calculated by the following formula: positive-staining area of (macrophages + lipids)/positive-staining area of (SMCs + collagen).

In addition, small part of fresh mice arterial tissues was used to undergo electron microscope examination. In brief, the arterial tissues were placed in 2.5% glutaraldehyde and 2% paraformaldehyde for 1 h; then the vessels were cut into approximately 1 mm × 1 mm × 1 mm and returned to the fixative for another 1 h. The tissues were postfixed in 1% osmium tetroxide for 1 h followed by staining with 2% uranyl acetate for 1 h. Then the tissues were dehydrated through ethanol and were embedded in Spon812. Finally, 50 nm sections were stained with uranyl acetate followed by lead citrate and examined in a JEM-1010 electron telescope (JEOL, Japan).

2.7. Quantitative Real-Time RT-PCR Analysis. The mRNA expression levels of β-actin and CHI3L1 in RAW264.7 cells, β-actin, CHI3L1, TNF-α, MCP-1, IL-8, and matrix metalloproteinase 9 (MMP-9) in plaque tissues were quantitatively analysed using real-time RT-PCR according to the routine methods [17]. In brief, RNA was extracted with the use of TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. The housekeeping gene β-actin was quantified as an internal RNA control. The forward and reverse primers were as follows: 5′-AGGCTTTGCGGT-CCTGAT-3′ and 5′-CCAGCTGGTGAAGTAGCAGA-3′ for CHI3L1; 5′-CACCACGCT CTTCTGTCTACTGAAC-3′ and 5′-CCG GACTGCGTGATGTCTAAGTACT-3′ for TNF-α; 5′-CAGCCAGATGCAGTTAACGC-3′ and 5′-GCCTAC-TCATTGGGATCAT CTTG-3′ for MCP-1; 5′-ACTGAG-AGTGATTGAGAGTGGAC-3′ and 5′-AACCCTCTGCAC-CCAGTTTTC-3′ for IL-8; 5′-CCTGGAACTCACACG-ACATCTTC-3′ and 5′-TGGAAACTCACACGCCAGAA-3′ for MMP-9; 5′-CACTGTGCCCATCTACGA-3′ and 5′-GTAGTCTGTCAGGTCCCG-3′ for β-actin. All values obtained were normalized to mouse β-actin and relative expression analysis involved the $2^{-\Delta\Delta CT}$ method.

2.8. Western Blot Analysis. The protein expression levels of CHI3L1, MAPK, AKT, GAPDH, and β-actin in human arterial tissues, RAW264.7 cells, and plaque tissues were assayed by western blot analysis [18]. In brief, equal amounts of protein were separated on sodium dodecyl sulfate-14% polyacrylamide gels and transferred to nitrocellulose membrane. After blocking with 5% nonfat milk, the blots were washed with PBS containing 0.1% Tween 20 and incubated with an appropriate primary antibody at 4°C for 12 h. The blots were probed with antibodies against human CHI3L1 (diluted

Table 1: Baseline characteristics and serum lipid levels of subjects and patients in the two groups.

	Control group ($n = 11$)	Research group ($n = 39$)	P
Age, yrs	57 ± 5	60 ± 6	0.144
Male sex, n (%)	7 (64%)	27 (69%)	0.745
Smoker, n (%)	0	8 (20%)	
Hypertension, n (%)	0	17 (44%)	
Diabetes, n (%)	0	15 (38%)	
TG (mmol/L)	1.31 ± 0.46	1.95 ± 1.16	0.078
TC (mmol/L)	3.55 ± 0.26	4.99 ± 1.47	<0.01
LDL-C (mmol/L)	2.05 ± 0.21	3.15 ± 1.24	<0.01
HDL-C (mmol/L)	1.78 ± 0.25	1.26 ± 0.36	<0.01
Lp (a) (mg/dL)	19.87 ± 7.31	30.45 ± 23.53	0.025
ApoA (g/L)	1.42 ± 0.18	1.08 ± 0.25	<0.01
ApoB (g/L)	0.85 ± 0.13	0.91 ± 0.28	0.245

1 : 500, Bioss, Beijing, China), mice CHI3L1 (diluted 1 : 1000, Santa Cruz), mice p44/42 MAPK (ERK1/2) (diluted 1 : 1000, CST, 4695), phospho-ERK1/2 (diluted 1 : 1000, CST, 4370), AKT (diluted 1 : 1000, CST, 4691), phospho-AKT (diluted 1 : 1000, CST, 4060), GAPDH, or β-actin (diluted 1 : 500, Zhongshan, Beijing, China). Then the blots were washed with Tris-buffered saline with Tween 20 and incubated with appropriate secondary antibody conjugated to horseradish peroxidase. Blots were processed for enhanced chemifluorescence using a Pierce ECL Western blotting substrate. The housekeeping gene GAPDH or β-actin was quantified as an internal control.

2.9. Statistical Analysis. Statistical analysis was performed with Statistical Package for the Social Science (SPSS 12.0) and quantitative variables are expressed as mean ± standard deviation. After testing for normal distribution of variables, Student's t-test was used to analyze continuous normally distributed variables. Correlations between two variables were performed by linear correlation analysis. In general, a 2-tailed $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline Characteristics. The baseline characteristics of the two groups are summarized in Table 1. The preoperative serums TC, LDL-C, and Lp (a) of 39 patients in research group were significantly elevated, whereas serums HDL-C and ApoA were significantly decreased, compared with the control group ($P < 0.05$). In addition, the 11 kidney donors in the control group had no history of hypertension or diabetes mellitus. Moreover, all were nonsmokers.

3.2. Coronary Atherosclerotic Lesions in the Research Group. CAG of 39 patients undergoing CABG showed that 19 patients were with left main coronary artery lesions, 6 patients were with two coronary arteries lesions, and 14 were patients with all three coronary arteries lesions. Significant calcification and stenosis were observed in coronary arteries of all the patients in the research group. The average Gensini score of the 39 patients was 62.25 ± 21.77.

3.3. Presence of CHI3L1 in the Human Arterial Tissues. As shown in Figure 1(a), in the arterial tissues of healthy donors little CHI3L1 expression could be demonstrated according to the immunohistochemical staining. However, the expression of CHI3L1 was elevated in the arterial specimens of CAD patients. Western blot analysis was used to evaluate the expression of CHI3L1 protein. As shown in Figure 1(b), there were significant differences of CHI3L1 expression levels between control group and research group.

3.4. Correlation between the Arterial CHI3L1 Expression and the Clinical Criteria of Atherosclerosis. We investigated the correlation between the relative expression levels of CHI3L1 and clinical criteria of atherosclerosis, including gender, smoking, hypertension, and diabetes mellitus. The expression levels of CHI3L1 did not differ significantly between males and females, but they differ significantly among smokers and nonsmokers, hypertensives and nonhypertensives, and diabetics and nondiabetics. As shown in Figure 1(c), the expression levels of CHI3L1 were increased in smokers and patients with hypertension or diabetes mellitus ($P < 0.05$), whereas gender had no significant effect. As shown in Figure 1(d), the linear correlation analysis revealed that arterial CHI3L1 expression levels were significantly correlated with coronary severity Gensini scores, which ranged from 24 to 120 ($r = 0.611$, $P < 0.05$).

3.5. Effects of Lentiviral Transduction In Vitro. The RAW264.7 cell line was transfected with lentivirus expressing different CHI3L1 siRNAs, and gene silencing analysis showed that site C lentivirus was the most effective vector in blocking CHI3L1 expression. As shown in Figure 2(a), CHI3L1 knockdown clones A, B, C, and D exhibited 38, 18, 64, and 14% reduction, respectively, in protein expression and 32, 17, 65, and 30% reduction, respectively, in mRNA expression. Then site C lentivirus and mock lentivirus were selected and produced at

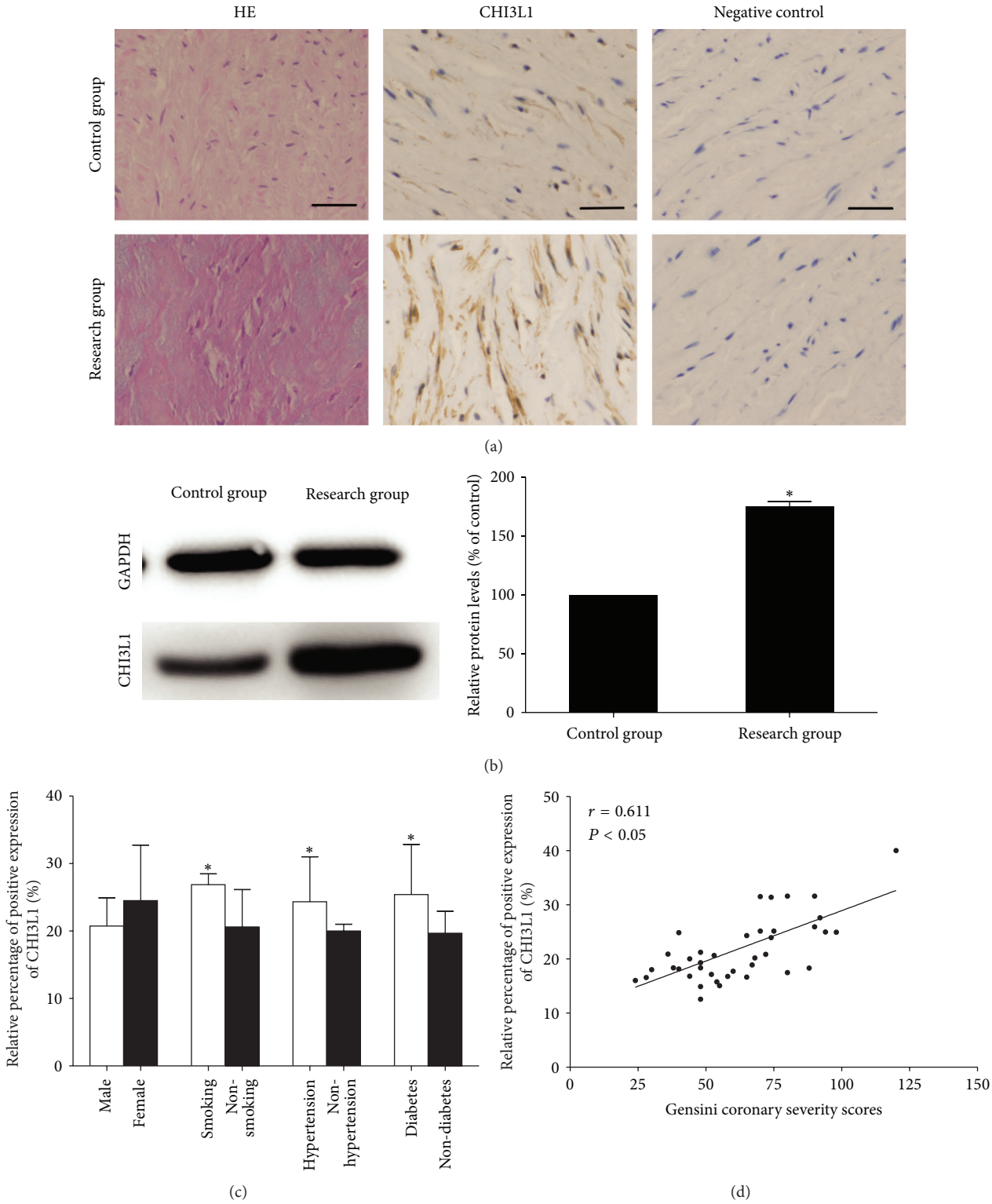
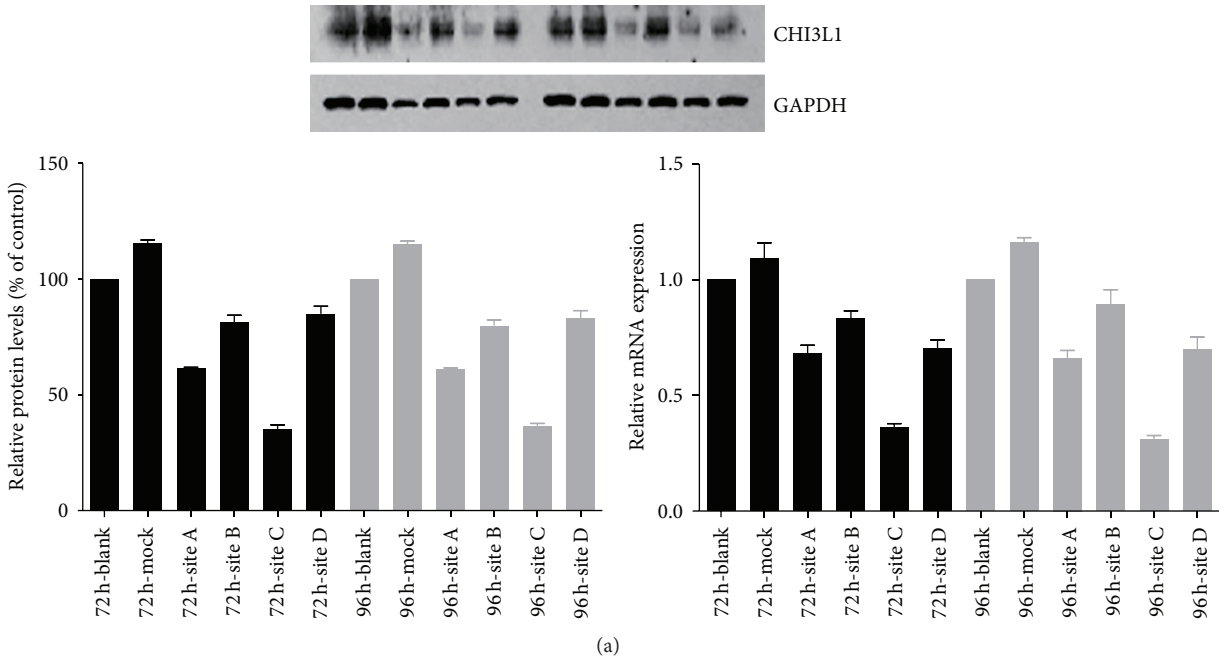
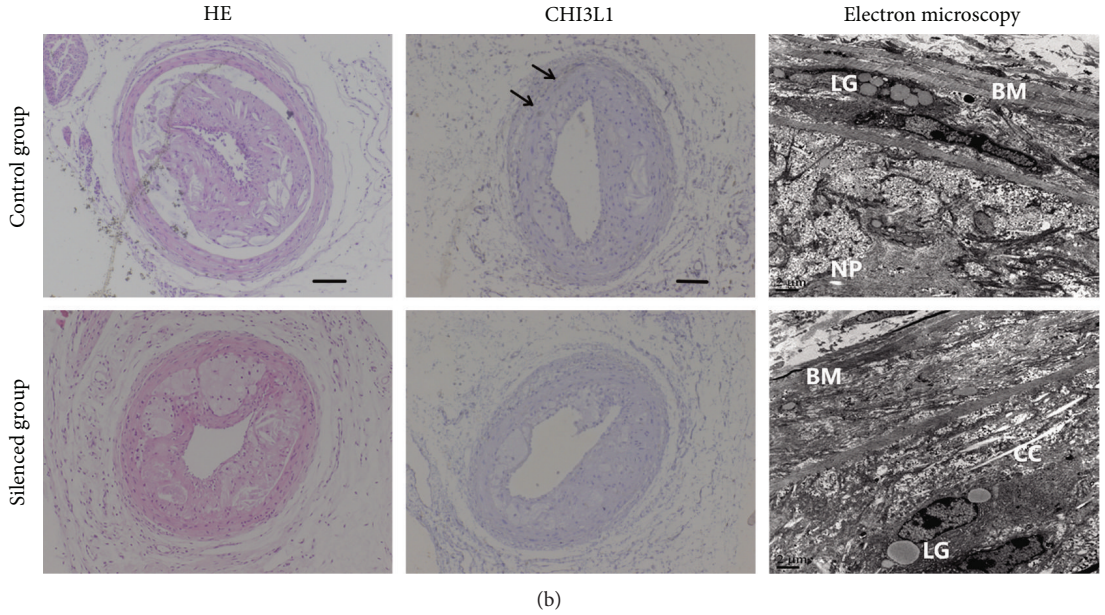


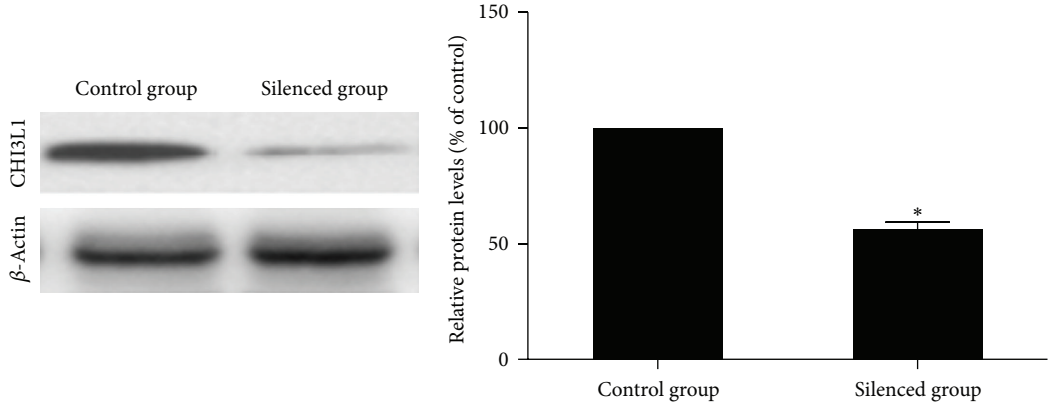
Figure 1: (a) Immunohistochemical staining of CHI3L1 on sections of arterial vessels in control group and research group. The expression of CHI3L1 was increased in the arterial specimens of CAD patients in research group. (scale bars = 100 μm) (b) Western blot analysis and quantification of CHI3L1 protein expression in control group and research group. The levels of CHI3L1 protein expression were higher in research group than in control group. $^{*}P < 0.05$ versus control group. (c) Quantitative analysis of arterial CHI3L1 expression in research group patients according to gender, smoking, hypertension, and diabetes mellitus. The expression levels of CHI3L1 were elevated in smokers and patients with hypertension or diabetes mellitus, whereas gender had no significant effect. $^{*}P < 0.05$. (d) Relationship between arterial CHI3L1 expression and coronary severity scores. The arterial CHI3L1 expression levels were significantly correlated with coronary severity Gensini scores. Each point represents one patient.



(a)

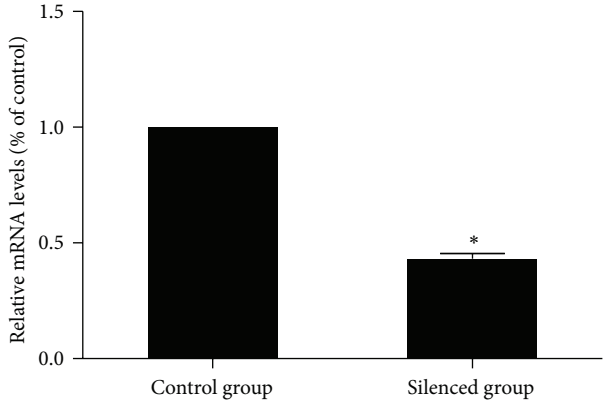


(b)



(c)

Figure 2: Continued.



(d)

Figure 2: (a) Target site screening for CHI3L1 by western blot analysis and real-time RT-PCR in RAW264.7 cells. The RAW264.7 cell line was transfected with lentivirus expressing different CHI3L1 siRNAs, and gene silencing analysis showed that site C lentivirus was the most effective vector in blocking CHI3L1 expression. (b) The immunohistochemical staining of CHI3L1 and electron microscopy in control group and silenced group. In the control group CHI3L1 expression (arrow) could be demonstrated according to the immunohistochemical staining. However, little CHI3L1 was expressed in silenced group. For electron microscopy, in control group most of the endothelial cells denudated and there were a large number of lipid granules (LG) under the basement membrane (BM) in the vessel wall. The atherosclerotic plaques were occupied with necrotic particles (NP), calcification crystals (CC), and cellular debrises. However, in silenced group the number of lipid granules was relatively decreased. (scale bars = 100 μm) (c) Western blot analysis and quantification of CHI3L1 protein expression in control group and silenced group. The levels of CHI3L1 protein expression were higher in control group than in silenced group. (d) Real-time RT-PCR quantification of CHI3L1 mRNA expression in control group and silenced group. $^{*}P < 0.05$ versus control group.

a viral titer of 1×10^{9} TU/mL (Genepharma, Shanghai, China) for further *in vivo* studies.

3.6. Effects of Lentiviral Transduction on CHI3L1 Expression in Plaques. To evaluate the efficacy of lentivirus-mediated gene silencing *in vivo*, the changes of CHI3L1 histology, protein, and mRNA expression in atherosclerotic plaques were measured. As shown in Figure 2(b), in the control group CHI3L1 expression could be demonstrated according to the immunohistochemical staining. However, little CHI3L1 was expressed in the silenced group. For electron microscopy, in the control group most of the endothelial cells denudated and there were a large number of lipid granules under the basement membrane in the vessel wall. The atherosclerotic plaques were occupied with necrotic particles, calcification crystals, and cellular debrises. However, in the silenced group the number of lipid granules was relatively decreased. A regenerating endothelial cell was seen partially covering the denuded surface. Collagen bundles and elastic fibers were seen on the vessel side of the endothelium. SMCs migrated into plaque tissues. Western blot analysis was used to evaluate the expression of CHI3L1 protein in plaque tissues. As shown in Figure 2(c), compared with control group, the silenced group showed a reduction in CHI3L1 protein expression of 50%. In addition, there were significant differences of CHI3L1 mRNA expression levels between control group and silenced group (Figure 2(d)).

3.7. Effects of Lentiviral Transduction on Plaque Composition. The relative content of lipids, collagen, SMCs, and macrophages in plaque tissues was derived by histological and immunohistochemical staining (Figure 3). The relative content of lipids in plaque tissues of the control group and the silenced group was 48.8% and 35.2%, and it was significantly lower in the silenced group than in the control group ($P < 0.05$). The relative reduction of lipids content in plaque tissues of silenced group was 27.5% as compared with control group. The relative content of collagen in plaque tissues of the control group and silenced group was 19.5% and 29.8%, and it was significantly increased in silenced group more than in control group ($P < 0.05$). The relative increase of collagen content in plaque tissues of silenced group was 53.2% as compared with control group. The relative SMCs content in plaque tissues of the control group and silenced group was 14.8% and 22.5%, and it was higher in silenced group than in control group ($P < 0.05$). The relative increase of SMCs content in plaque tissues of silenced group was 51.2% as compared with control group. The relative macrophages content in plaque tissues of the control group and silenced group was 11.9% and 7.5%, and it was decreased in silenced group more than in control group ($P < 0.05$). The relative reduction of macrophages content in plaque tissues of silenced group was 36% as compared with control group. The vulnerability index for the control group and silenced group was 1.76 ± 0.25 and 0.81 ± 0.13, and it was decreased in silenced group more than in control group ($P < 0.05$). The relative reduction in vulnerability index in silenced group was 53.8% as compared with control group.

3.8. Effects of CHI3L1 Gene Silencing on Inflammatory Mediators within Lesions. To elucidate the molecular mechanisms by which CHI3L1 gene silencing inhibits plaques progression and stabilizes the plaques, the mRNA expression changes

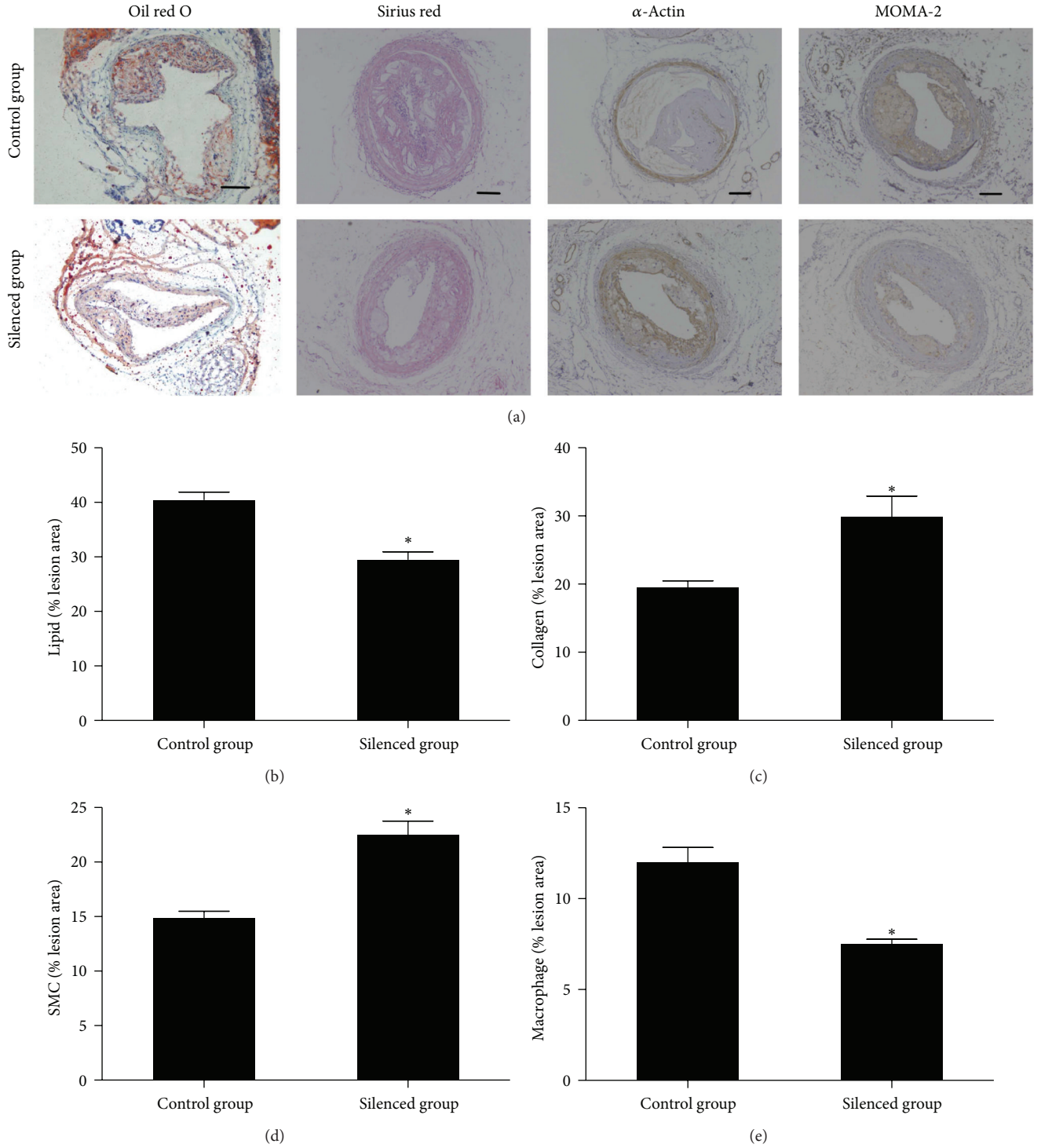


Figure 3: CHI3L1 gene silencing influenced plaque composition and stability. (a) Cross-sections of mice carotid arteries in the control group and silenced group were stained for lipids (oil red O), collagen (Sirius red), SMCs (α-actin), and macrophages (MOMA-2). The relative content of lipids (b), collagen (c), SMCs (d), and macrophages (e) in the plaque tissues. The relative contents of lipids and macrophages in plaque tissues were significantly lower in silenced group than in control group. However, the relative collagen and SMCs contents in plaque tissues were increased in silenced group than in control group. $^{*}P < 0.05$ versus control group.

of the proinflammatory molecules including TNF-α, MCP-1, IL-8, and MMP-9 were investigated in the mice carotid arteries. As shown in Figure 4, the silenced group showed lower mRNA expression levels of TNF-α, MCP-1, IL-8, and MMP-9, compared with the control group ($P < 0.05$), and inhibition of CHI3L1 reduced the inductions of proinflammatory cytokines. In addition, we studied the protein expression levels of ERK1/2, phospho-ERK1/2, AKT, and phospho-AKT in the mice carotid arterial tissues and found that the protein expression levels of phospho-ERK1/2 and phospho-AKT were decreased in the silenced group compared with that in the control group.

4. Discussion

In the present study we found that the expression of CHI3L1 was augmented in aorta of patients with coronary atherosclerosis and its expression was significantly correlated with the atherosclerotic risk factors and the severity of CAD as quantified by coronary angiograph. More importantly, the interference of CHI3L1 resulted in an improvement of atherosclerotic burden and plaque stability in ApoE$^{-/-}$ mice.

Inflammation and endothelial dysfunction are thought to be key processes in the progression of atherosclerosis. Several proinflammatory cytokines, acute phase-reactants, and cell adhesion molecules seem to play an important role in the development of low grade inflammation, and there is substantial evidence supporting the role of TNF-α, IL-6, and MCP-1 as cardiovascular risk factors and participants in the pathogenesis of atherosclerosis [1, 14, 16].

As a member of the chitinase-like proteins, CHI3L1 is highly conserved and is produced by a variety of cells such as macrophages, neutrophils, SMCs, cancer cells, and arthritic chondrocytes. Although mammals are not able to synthesize or metabolize chitin and the exact function of CHI3L1 remains unknown, recent studies have implicated CHI3L1 in different biological processes such as inflammation, tissue remodeling, fibrosis, and angiogenesis [19].

Using human arterial tissues, Boot et al. studied the CHI3L1 mRNA expression in the atherosclerotic plaques [20]. Similar to their research, we studied the CHI3L1 protein expression in the aorta of patients with coronary atherosclerosis. More importantly, to identify the potential role of CHI3L1 in atherogenesis, we investigated the relationship between CHI3L1 and cardiovascular risk factors. Gender, smoking, hypertension, diabetes mellitus, and dyslipidemia are all risk factors that contribute to the development of atherosclerosis and some evidence indicates that circulating levels of CHI3L1 have relationships with smoking [5], hypertension [6], and diabetes mellitus [7, 8]. Our results showed that aortic CHI3L1 expression had a positive correlation with smoking, hypertension, and diabetes mellitus. These results indicate that the main cardiovascular risk factors may promote the expression of CHI3L1 and CHI3L1 can influence atherosclerosis by regulating these risk factors. The Gensini scoring system is a useful tool to estimate the severity of CAD based on CAG findings. We found that there was a significant correlation between aortic CHI3L1 expression and coronary artery severity suggesting the important role of CHI3L1 in angiogenesis and in the process of atherosclerotic plaque formation.

RNA interference is an effective method for silencing mRNA, and it has been used in the treatment of several diseases [21, 22]. The use of siRNAs is more efficient than other gene-specific targeting approaches. As one kind of viral vectors, adenovirus is commonly applied in the RNA interference. However, it can lead to a marked immunogenic response, limiting associated gene expression. The lentivirus has several advantages than adenovirus, such as the high efficiency of gene transduction, long-term infection due to gene integration into the chromosome of host cells, and the absence of toxicity or immune response. Thus, lentiviral vectors expressing siRNAs was applied in our study.

Some phenotypic characteristics of atherosclerotic plaques, such as fibrous cap thickness, collagen content, and macrophage number have been widely used as indicators of plaque stability. Plaques with a thin fibrous cap and a large lipid core are considered vulnerable. In addition, in the process of acute coronary syndrome, the plaque component is more important than the plaque size. We investigated the effect of CHI3L1 gene silencing on advanced atherosclerotic lesions and found that CHI3L1 was important in the development of atherosclerotic plaques, as the CHI3L1 gene silenced group consistently showed a decreased content of macrophages and lipids and an increased content of collagen and SMCs. The morphological changes mentioned above led to a decreased plaque vulnerability index in the CHI3L1 gene silenced group compared to the control group, indicating that the plaque rupture is not apt to occur.

Studies have demonstrated that increased expression of CHI3L1 in the human atherosclerotic lesions is associated with production and activation of inflammatory factors [11, 20]. Although the membrane receptor specific for CHI3L1 binding has not been identified, the heparin-binding affinity of CHI3L1 appears to be essential for its activity, resembling the heparin-binding property of vascular endothelial growth factor. CHI3L1 can initiate MAPK and phosphoinoside-3 kinase (PI3K) by phosphorylation of the ERK1/2 and AKT, respectively, and thereby mediate signalling cascades [17, 18, 23, 24]. Both pathways have well-established roles in the propagation of mitogenic signals and play an important role in the process of atherosclerotic plaque formation. In our study, we found that the protein expression levels of phospho-ERK1/2 and phospho-AKT were decreased in silenced group compared with that in control group. As the major proinflammatory factors, TNF-α, MCP-1, IL-8, and MMP-9 have been detected in the human atherosclerotic lesions [25–27]. TNF-α, MCP-1, and IL-8 play important roles in the recruitment and activation of monocytes, macrophages, and SMCs. In turn, activated macrophages are involved in the secretion of inflammatory factors such as MCP-1. MMP-9 in the atherosclerotic plaques can degrade extracellular matrix, therefore contributing to thinning of the fibrous cap of plaques. In the present study, the mRNA expression levels of TNF-α, MCP-1, IL-8, and MMP-9 were decreased in silenced group compared with that in control group. These results suggested that a reduced level of these cytokines induced

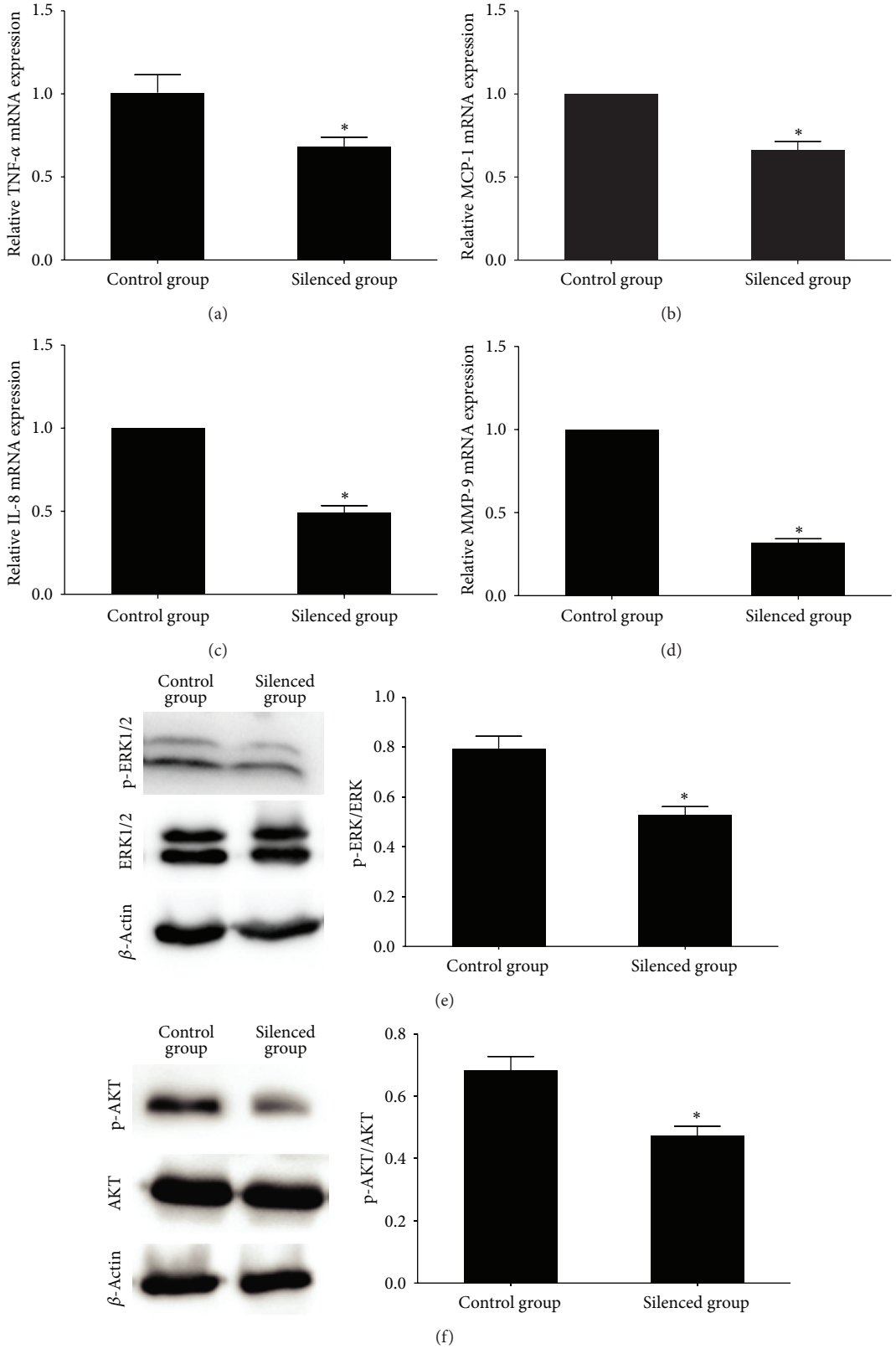


Figure 4: (a)–(d) Real-time RT-PCR quantification of inflammatory cytokines mRNA expression in mice carotid plaques in control group and silenced group. The silenced group showed lower mRNA expression levels of TNF-α, MCP-1, IL-8, and MMP-9, compared with the control group, and inhibition of CHI3L1 reduced the inductions of proinflammatory cytokines. (e)-(F) Western blot analysis and quantification of ERK1/2, phospho-ERK1/2, AKT, and phospho-AKT protein expression in control group and silenced group. The protein expression levels of phospho-ERK1/2 and phospho-AKT were decreased in silenced group compared with that in control group. $^{*}P < 0.05$ versus control group.

by interference of CHI3L1 contributed to the stabilization of atherosclerotic plaques.

5. Limitations

It should be mentioned that this study included a relatively small number of patients, normal subjects, and ApoE$^{-/-}$ mice, limiting the statistical power. Although we used the Gensini scoring system to estimate the severity of coronary atherosclerosis, this score is determined using CAG and does not reflect the actual volume of atherosclerotic plaque. Thus, other diagnostic methods, such as intravascular ultrasound and angioscopy, may be needed to evaluate plaque volume. Another major limitation in the human arterial tissues study is the normal subjects, considering that healthy human aortic tissues can rarely be ethically obtained. Consequently, we selected discarded arteries from kidney donors as the control group. All kidney donors were blood relatives of the recipients. To our knowledge, up to now there are no data to show any difference in CHI3L1 expression between human ascending aorta and renal arteries. It should also be mentioned that collar-induced carotid atherosclerosis is different from natural aortic atherosclerosis in the ApoE$^{-/-}$ mice. The carotid lesions occur in a region proximal to the constrictive collar with a prominent plaque burden, whereas the aortic lesions develop in the ascending aorta with scattered plaques of small size. Nonetheless, the carotid lesions in ApoE$^{-/-}$ mice resemble advanced human atherosclerosis and represent the reproducible and reliable model for studies of vulnerable plaques. Finally, the local lentivirus carrying small interfering RNA can be absorbed into the circulation and cause the general effect.

6. Conclusions

In conclusion, the expression of CHI3L1 was enhanced in the aorta of patients with coronary atherosclerosis and its expression was significantly correlated with the atherosclerotic risk factors and the severity of CAD. More importantly, silencing of CHI3L1 diminished atherosclerotic burden and increased plaque stability in ApoE$^{-/-}$ mice, and it might provide a new therapeutic approach to the treatment of atherosclerosis.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This study was supported by the Shandong Provincial Natural Science Foundation (no. Y2007C066-2009). The authors thank Dong Wang, Degang Sun, Wei Lu, Yao Wang, Bo Li, and Zhi Yang for their technical assistance and they are indebted to all the patients for their kind collaboration.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 716902, 7 pages
http://dx.doi.org/10.1155/2013/716902

Clinical Study

Complement C3c as a Biomarker in Heart Failure

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Received 6 October 2013; Revised 8 December 2013; Accepted 9 December 2013

Academic Editor: Fatih Arslan

Introduction. Experimental data indicates an important role of the innate immune system in cardiac remodeling and heart failure (HF). Complement is a central effector pathway of the innate immune system. Animals lacking parts of the complement system are protected from adverse remodeling. Based on these data, we hypothesized that peripheral complement levels could be a good marker for adverse remodeling and prognosis in patients with HF. *Methods and Results.* Since complement activation converges on the complement factor C3, we measured serum C3c, a stable C3-conversion product, in 197 patients with stable systolic HF. Subgroups with normal and elevated C3c levels were compared. C3c levels were elevated in 17% of the cohort. Patients with elevated C3c levels exhibited a trend to better survival, slightly higher LVEF, and lower NTpro-BNP values in comparison to patients with normal C3c values. No differences were found regarding NYHA functional class. Significantly more patients with elevated C3c had preexisting diabetes. The prevalence of CAD, arterial hypertension, and atrial fibrillation was not increased in patients with elevated C3c. *Conclusion.* Elevated C3c levels are associated with less adverse remodeling and improved survival in patients with stable systolic heart failure.

1. Introduction

Patients with heart failure frequently exhibit a chronic low-grade activation of the immune system as indicated by increased levels of cytokines, chemokines, and inflammatory proteins [1–3]. For many years it remained unclear how in heart failure, a primarily noninfectious disease with the rare exception of infectious myocarditis, the immune system could be triggered. However in recent years, it became clear that these reactions might be due to activation of the innate immune system via endogenous "danger signals" [4–6]. These danger signals are released, for example, by dying cells and include factors like heat shock proteins.

Effects of immune activation on heart failure development are time dependent. After an event of acute cardiac injury, like myocardial infarction, activation of the innate immune system is a prerequisite for adequate healing [7, 8]. However, long-term chronic innate immune activation is detrimental leading to adverse left ventricular remodeling and aggravation of heart failure [3, 4, 9].

Complement might be an important mediator in this context. The complement system is one of the key components of the innate immune system [10, 11]. It has a dual role: on the one hand, it is a receptor, for example, for host infection. On the other hand it is also an effector protein that can efficiently attract inflammatory cells and also directly destroy cells by the membrane attack complex. From noncardiac diseases we know that inappropriate complement activation is pathologic and leads to various autoimmune diseases [12, 13].

The complement system features more than 20 different serum proteins that are produced by a variety of cells. The interaction of these proteins constitutes a meticulously regulated cascade of activation steps. Finally, all activation steps converge on the complement factor C3. In the heart, activated C3 (C3a) caused tachycardia, impairment of atrioventricular conduction, left ventricular contractile failure, coronary vasoconstriction, and histamine release after injection into isolated guinea pig hearts [14]. Thus, C3 seems to be not only a good indicator for overall complement activation but might also be of pathophysiological relevance in

the cardiovascular system. We therefore hypothesized that C3 would be a good marker of innate immune activation and might also be associated with adverse cardiac remodeling and mortality in patients with stable heart failure.

A problem with using complement components as biomarker is that due to their aggressive nature complement factors have a very short half-life. C3c is the stable conversion product of C3, which develops out of C3 within one hour at body temperature. For the detection of complement function, we used therefore C3c [15, 16], in a study cohort of patients with stable heart failure.

2. Methods

2.1. Patients. Patients participating in the Interdisciplinary Network for Heart Failure Study (INH Study, http://www.controlled-trials.com/, ISRCTN23325295) were eligible. The INH Study investigated the effects of a telephone-based nurse intervention on clinical outcome and enrolled consecutive adults hospitalized for congestive cardiac failure at nine hospitals in Bavaria and Baden-Württemberg. Inclusion criteria were informed written consent, left ventricular ejection fraction (LVEF) ≤40%, and typical signs and symptoms of heart failure at the time of inclusion. Exclusion criteria were logistic or health reasons precluding participation in a telephone-based intervention. Approval of the INH study protocol was obtained from the responsible Ethics Committees [17, 18].

2.2. Data Collection and Follow-Up. Prior to discharge, patients underwent a standardized evaluation including detailed medical history, physical examination including assessment of New York Heart Association (NYHA) functional class, blood chemistry, 12-lead electrocardiogram, and echocardiography. All assessments were repeated at 6-month intervals after hospital discharge. Survival status after 3 years was ascertained by contacting patients themselves or their general physician [17].

2.3. Laboratory Tests, C3c Sampling, and Measurement. Consecutive serum C3c levels of 197 patients were collected between January 2009 and June 2011 during an ambulatory outpatient visit, about 3 (1–7) years after the inclusion in the original study. Due to heart failure associated mortality and impossibility to attend the ambulatory follow-up, we could assess C3c values in 197 patients of the originally included 1022 patients. After rapid centrifugation of the venous blood sample collected between 8 and 11 AM, C3c was immediately measured in the Central Laboratory of the University Hospital Würzburg that accords with rigid external control. In clinical routine, complement C3c is measured instead of complement C3 [19–21] using turbidimetry (Cobas c 502, Roche, Mannheim, Germany). In our laboratory, the reference interval in adults spans from 75 to 140 mg/dL.

2.4. Definition of C3 Status. According to assay dependent reference intervals the patients were subdivided into three groups: low C3c (<75 mg/dL), normal C3c (75–140 mg/dL), and elevated C3c (>140 mg/dL). We evaluated the clinical characteristics, the survival, the echocardiographic status, and the laboratory parameters of the patients according to the predefined subgroups.

2.5. Data Analysis. Patients were grouped according to normal (75–140 mg/dL) versus elevated (>140 mg/dL) C3c levels; 33 (16.8%) of all evaluated heart failure patients had elevated C3c levels. Only one patient had a C3c level below the reference range and was excluded from the following analyses. Thus, C3c levels of 196 patients were included in the present analysis. Data are expressed as mean (standard deviation), median (quartiles), or n (%), as appropriate. Group-wise comparisons were performed using Fisher's exact test, chi-square test, Mann-Whitney U-test, or Kruskal-Wallis test, as appropriate. Kaplan-Meier curves were constructed to investigate the prognostic value of C3c and were tested by log rank test. Adjustment for potential confounders for the association between C3c and survival was performed using Cox proportional hazards regression, and hazard ratios (HR) with 95% confidence intervals (CI) were reported. Patients with normal C3c values served as reference. Reported P values are two-sided, and P values < 0.05 were considered statistically significant. All tests were performed using commercial software (SPSS Inc, Chicago, Illinois version 20.0).

3. Results

3.1. Study Subjects. A total of 196 patients with a median age of 69.0 years (range 32–87 years) were included in the analysis. No differences regarding age were found in patients with normal versus elevated C3c values ($P = 0.77$). Baseline characteristics of the entire sample are shown in Table 1. Forty-two (27%) of the patients were female, with a trend for a higher proportion of women in the group with elevated C3c (30% versus 20%, $P = 0.174$; Table 1). No differences were found regarding the prevalence of arterial hypertension ($P = 0.636$), coronary artery disease ($P = 0.565$), chronic obstructive pulmonary disease ($P = 0.525$), malignant disease ($P = 0.523$), and peripheral artery occlusive disease ($P = 0.582$) in patients with elevated C3c compared to those with normal C3c. Twenty-seven (82%) patients with elevated C3c had sinus rhythm and 5 (15%) atrial fibrillation on the actually performed ECG. The one remaining patient (3%) had a pacemaker dependent rhythm. A trend towards more frequent sinus rhythm (82% versus 66%; $P = 0.079$) was observed in patients with elevated C3c. No differences were observed regarding the frequency of atrial fibrillation (15% versus 24%; $P = 0.272$). Interestingly, the prevalence of diabetes mellitus was higher in patients with elevated C3c compared to those with normal C3c (61% versus 42%, $P = 0.047$). Patients with elevated C3c values had a slightly higher frequency of ICD and CRT device implantations at the C3c evaluation (27.3% versus 19.6% and 27.3% versus 11.0%). No differences in cardiac medication were found in patients with normal versus elevated C3c (Table 1).

3.2. Echocardiography. We found a trend towards smaller end-diastolic (59 ± 11 mm versus 63 ± 10 mm; $P = 0.059$)

Table 1: Baseline characteristics of study participants.

	All patients$^{\infty}$ (n = 197)	Normal C3c (n = 163)	Elevated C3c (n = 33)
Age (years)	67 (12)	68 (11)	63 (13)
Female sex	42 (21)	32 (20)	10 (30)
Duration of follow-up for survivors [months]	20 (6)	20 (6)	22 (4)
All-cause mortality	23 (12)	22 (14)	1 (3)*
Diagnosis of heart failure known			
≤5 years	77 (39.1)	63 (38.7)	14 (42.4)
5 years	103 (52.3)	87 (53.4)	15 (30.7)
Predominant cause of heart failure			
Coronary artery disease	92 (46.7)	78 (48)	13 (39.4)
Dilated cardiomyopathy	64 (32.5)	50 (31)	14 (42.4)
Hypertension	19 (9.6)	17 (10)	2 (6.1)
Other	22 (11.2)	18 (11)	4 (12.1)
NYHA functional class			
I/II	144 (73.1)	118 (72.4)	25 (75.8)
III/IV	53 (26.9)	45 (27.6)	8 (24.2)
Left ventricular ejection fraction (%)	44.2 (13.2)	43.5 (13.2)	48.0 (12.8)
Medical history			
Current smoker	16 (8.1)	14 (8.6)	2 (6.1)
Myocardial infarction	81 (41.1)	69 (42.3)	11 (33.3)
Comorbidities†			
Atrial fibrillation	44 (22.3)	39 (23.9)	5 (15.2)
Peripheral vascular disease	35 (17.8)	28 (17.2)	7 (21.2)
Hypertension	168 (85.3)	138 (84.7)	29 (87.9)
Diabetes mellitus	89 (45.2)	68 (41.7)	20 (60.6)*
COPD	31 (15.7)	27 (16.6)	4 (12.1)
Anemia	36 (18)	29 (18)	6 (18)
Renal dysfunction	96 (48.7)	78 (47.9)	17 (51.5)
Uncured malignancy	2 (1)	2 (1.2)	0
Devices			
ICD	41 (20.8)	32 (19.6)	9 (27.3)
CRT	27 (13.7)	18 (11.0)	9 (27.3)*
Medication			
ACEi and/or ARB	189 (96)	156 (96)	32 (97)
β-blocker	182 (92)	148 (91)	33 (100)
Aldosterone antagonist	108 (55)	87 (54)	21 (64)
Diuretic	170 (86)	139 (85)	30 (91)
Amiodarone	19 (9.6)	16 (9.8)	3 (9.1)
Digitalis	72 (37)	59 (36)	13 (39)

Values are mean (SD) or n (%). $^{\infty}$All patients imply results for all patients with the C3c measurement, including the only one with the diminished C3c value. *P value < 0.05 in Mann-Whitney U test, the comparison between the group with normal C3c and elevated C3c. $^{\circ}$Heart rate according to electrocardiogram. †Comorbidities: atrial fibrillation: diagnosed from the electrocardiogram. Hypertension: sitting blood pressure > 140/90 mmHg or history of hypertension prior to the onset of heart failure or hypertensive heart disease accepted as predominant cause of heart failure. COPD: chronic obstructive pulmonary disease: history of this condition requiring bronchiolytic treatment or newly diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease criteria [43]. Anemia according to WHO criteria: haemoglobin <12 g/dL in women and <13 g/dL in men [44]. Renal dysfunction: estimated glomerular filtration rate < 60 mL/min/1.73 m^2 [45]. ICD: implantable cardioverter-defibrillator. CRT: cardiac resynchronization therapy with a biventricular defibrillator; ACEi: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker.

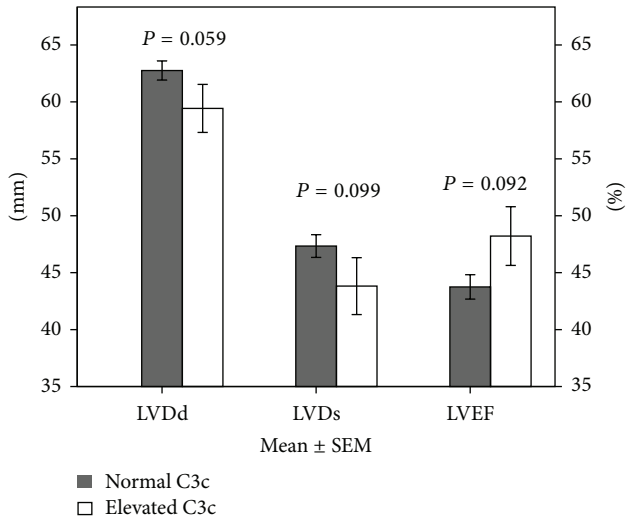

Figure 1: Comparison of echocardiographic parameters in patients with normal versus elevated C3c values. There is a trend towards smaller diastolic (59 ± 11 mm versus 63 ± 10 mm; $P = 0.059$) and systolic (44 ± 13 mm versus 47 ± 13 mm; $P = 0.099$) diameter of the left ventricle and higher left ventricular ejection fraction (48.0 ± 12.83% versus 43.5 ± 13.2%; $P = 0.092$) in patients with elevated complement C3c. LVDd, diastolic left ventricular diameter; LVDs, systolic left ventricular diameter; LVEF, left ventricular ejection fraction.

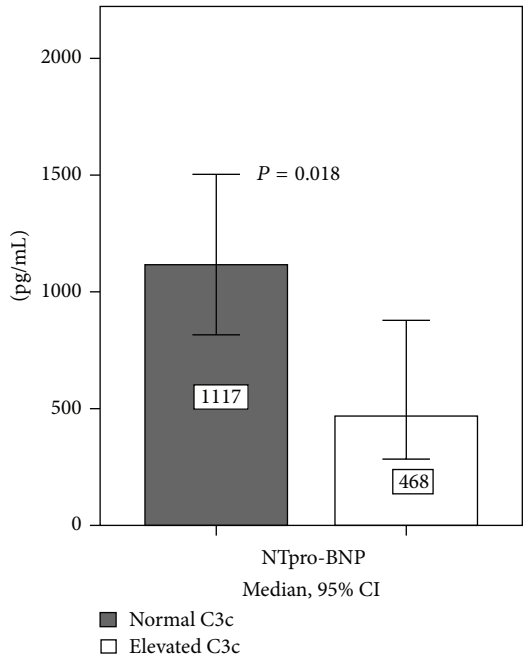

Figure 2: Comparison of NT-proBNP values in patients with normal versus elevated C3c values. Lower values of NTpro-BNP were found in patients with elevated C3c (468 [246; 1182] pg/mL versus 1117 [385; 2662] pg/mL; $P = 0.018$). NT-proBNP: N-terminal prohormone of brain natriuretic peptide.

and end-systolic (44 ± 13 mm versus 47 ± 13 mm; $P = 0.099$) diameters of the left ventricle and towards a higher left ventricular ejection fraction (48.0 ± 12.83% versus 43.5 ± 13.2%; $P = 0.092$) in patients with elevated complement C3c (Figure 1). No relevant differences could be found regarding diastolic function ($P > 0.05$) and systolic tricuspid valve gradient ($P > 0.05$).

3.3. Laboratory Parameters. Lower values of NTpro-BNP were found in patients with elevated C3c (468 [246; 1182] pg/mL versus 1117 [385; 2662] pg/mL; $P = 0.018$; Figure 2). C3c was negatively correlated with NTproBNP ($R^2 = -0.266$; $P = 0.001$). Atrial fibrillation is known to be associated with BNP levels and was underrepresented in patients with elevated C3c values. Nonetheless, two-way ANOVA did not show relevant interactions between C3c group allocation, occurrence of atrial fibrillation, and NTpro-BNP distribution.

C-reactive protein (CRP) values above 0.5 mg/dL (i.e., the upper threshold of normal range according to our Hospital Laboratory) were considered elevated. CRP levels were significantly higher in patients with elevated C3c (0.91 ± 0.18 mg/dL versus 0.55 ± 0.08 mg/dL; $P < 0.001$). We did not find any association between LV function or morphology in patients with normal versus elevated CRP (LV enddiastolic diameter: 62±10 versus 62±10 mm, $P = 0.973$; LV endsystolic diameter: 46 ± 12 versus 47 ± 12 mm, $P = 0.770$; LVEF: 45 ± 13 versus 44 ± 14%, $P = 0.459$). Kaplan Meier survival analysis showed a tendency for a better survival in patients with normal versus elevated CRP, independent of C3c status (Log rank 0.081).

We found relevant alterations in the blood profile of patients with elevated C3c: higher values for platelets (252 ± 108 versus 202±53 ∗ 1000/μL; $P = 0.001$), erythrocytes (4.73± 0.46 versus 4.50 ± 0.58 ∗ 10$E6$/μL; $P = 0.040$), and leukocytes (8.45 ± 2.76 versus 7.45 ± 3.92 ∗ 1000/μL; $P = 0.016$) but no differences regarding haemoglobin (13.94 ± 0.14 versus 14.14 ± 0.25 g/dL; $P = 0.70$). Ferritin levels were significantly higher (227 [152; 441] versus 161 [84; 325] μg/L; $P = 0.044$) in patients with elevated C3c and were accompanied by corresponding alterations of transferrin saturation (22 ± 8 versus 26 ± 10%; $P = 0.118$). No differences were observed regarding the renal and liver function between the two groups.

3.4. Survival Analysis. During the follow-up period (median 21 months; range 3–43 months) 23 (11.7%) patients died. The follow-up duration for survivors comprised at least 9 months (median 22; range 9–43). Twenty-two patients (13.5%) in the group with normal C3c compared to only 1 (3.0%) patient in the group with elevated C3c died during the follow-up. Mortality risk tended to be increased in the group with normal C3c values compared to elevated C3c values (log rank 0.078; Figure 3). This corresponded to an unadjusted hazard ratio of 5.0 (95% CI 0.68–37.3, $P = 0.114$). This trend for an increased mortality risk in patients with normal C3c values was only slightly attenuated after adjustment for age (HR 4.13, 95% CI 0.55–30.8, $P = 0.166$) and sex (HR 3.98, 95% CI

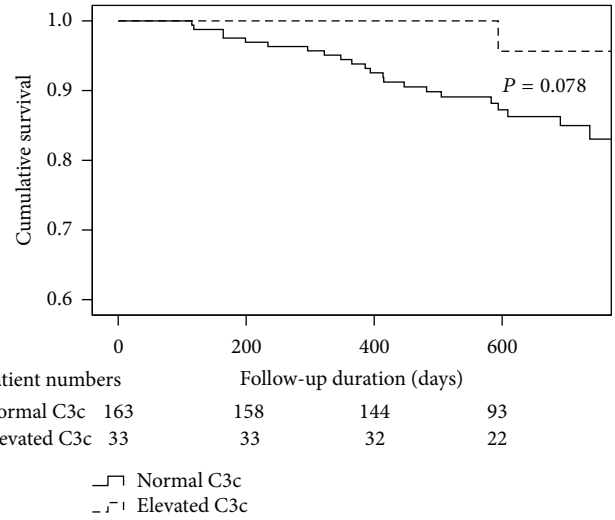

Figure 3: Kaplan-Meier estimates all-cause mortality risk by C3c values. Mortality risk tended to be increased in the group with normal ($n = 163$) C3c values compared to subjects with elevated ($n = 33$) C3c values (log rank test, $P = 0.078$).

0.52–30.8, $P = 0.185$) but notably attenuated after adjustment for NYHA functional class (HR 3.4, 95% CI 0.44–25.1, $P = 0.242$).

4. Discussion

Since there is good experimental evidence from studies in small animals that complement activation is mechanistically involved in adverse cardiac healing and remodeling after myocardial infarction [22], we hypothesized that complement plasma levels may be indicative for heart failure progression in humans. However, here we present data that elevated complement C3c levels in patients with heart failure are associated with a trend towards improved survival and better cardiac reverse remodeling (i.e., reduced left ventricular volume, increased ejection fraction, and reduced NT-proBNP values).

4.1. Complement—A Prognostic Marker in Heart Failure? Several reports confirm that complement is activated after myocardial infarction [23–25]. Hill and Ward demonstrated C3 cleavage in the infarcted myocardium and documented a role for the complement system in leukocyte infiltration [26]. Moreover, complement inhibition consistently attenuated leukocyte recruitment following myocardial infarction highlighting the critical role of the complement cascade in triggering inflammation in the ischemic myocardium [25, 27]. Its role as a prognostic marker in heart failure was only addressed by a very limited number of investigations: Gombos et al. recently presented data from 182 patients indicating an association between activated complement C3a and a combined endpoint consisting of all-cause mortality or rehospitalization due to progression of heart failure [28]. Aukrust et al. found systemic complement activation in 39 patients with chronic heart failure; treatment with intravenous immunoglobulin reduced complement activation and increased left ventricular function during the 5-month follow-up period [29, 30]. However, both reports have major weaknesses: the patient cohort of Gombos et al. appears rather heterogeneous since the authors included not only outpatients but also inpatients after best possible cardiac recompensation. Moreover, variables allowing the assessment of left ventricular remodeling were not presented in this study. In contrast, our patients were all stable outpatients undergoing long-term follow-up in a clinical study with in-depth characterization of cardiac remodeling by echocardiography. The main limitation of the study by Aukrust et al. was the low number of patients studied. A further methodological problem is in both studies related to the complement measurements. Upon activation of complement factor C3, C3 is cleaved in two fragments: C3a and C3b. In the cited studies either C3a or C3b was measured. However, both components are unstable and are degraded upon freezing making a more or less immediate measurement necessary [16, 19–21]. In both studies samples were frozen prior to analysis, thus rendering the obtained complement plasma levels questionable. We circumvented this problem in two ways. First, we measured complement levels routinely without freezing our samples. Second, we used C3c, a stable conversion product which develops out of C3 within one hour at body temperature [15]. The disadvantage of C3c measurement is that we determined total C3 and cannot distinguish between activated and non-activated complement.

Our sample patients were clinically stable, under long-term heart failure medication and still under observation 3 to 4 years after the inclusion in the original study. So, one potential limitation of our study could be that our patients were not sick enough to develop immune activation. However, activation of an immune response in our cohort could be demonstrated by an association of C3c and increased leucocytes and C-reactive protein levels. Moreover, our study may be biased since our assessment was performed only once, while cardiac remodeling is an ongoing process. Ischemic cardiac disease is the predominant cause for heart failure in our patient cohort. For this reason, our data are underpowered to assess the role of C3c in patients with alternative causes of heart failure. Finally, natriuretic peptides might directly cause proinflammatory protein release [31–33]. Thus, one has to consider that inflammatory markers might not necessarily be independent markers of heart failure progression.

4.2. Are Peripheral Complement Levels a Marker of Intracardiac Complement Activation? Heart failure is associated with activation of immune system [34]. Peripheral levels of important innate immune cytokines like tumor necrosis factor (TNF)-α are associated with adverse outcome [2, 3, 25, 35]. On the other side, infusion of TNF-α or intracardiac overexpression of TNF-α in animals leads to heart failure [36, 37]. This suggests that peripheral levels of TNF-α reflect a local, intracardiac activation of the immune system. However, this assumption cannot be generalized. For example, we know from our own studies that intracardiac levels of extracellular

matrix proteins are not mirrored in the plasma [38]. In rheumatoid arthritis activation of complement by immune complexes in the joint space of patients results in local depression of C4 [39]. However, serum levels of C4, and more specifically C3, are elevated in serum of patients with rheumatoid arthritis [40, 41]. Thus, peripheral blood complement levels can only partly reflect complement involvement in the disease development and healing processes in the target tissue.

Moreover, in the clinical routine low complement levels are usually an indicator of high tissue complement turnover, for example, in glomerulonephritis [42]. Thus, higher peripheral levels of complement could also reflect a decreased activation of complement in the tissue. This could also explain why patients with higher peripheral—and potentially lower local—complement activation had a better clinical outcome in our study.

5. Conclusion

We present data that elevated C3c levels seem to be associated with less adverse remodeling and improved survival in patients with stable systolic heart failure. It is unlikely that plasma complement mirrors intracardiac complement activation.

Conflict of Interests

The authors declare no conflict of interests, including specific financial interests and relationships relevant to the subject of this paper.

Acknowledgment

The main sponsor of this study was the Federal Ministry of Education and Research (BMBF, Berlin, Germany, Project 01GL0304). The authors received additional financial support from the German Competence Network Heart Failure (CNHF, Berlin, funded by the BMBF, Project 01GI0205) and the Comprehensive Heart Failure Center Würzburg and the study was also supported by Grants from the Federal Ministry of Education and Research (BMBF, Berlin, Germany, Project 01EO1004) as well as the SFB 688. None of the funding sources interfered with the collection, management, and interpretation of the data nor requested review of the paper.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 828354, 10 pages
http://dx.doi.org/10.1155/2013/828354

Review Article

S100A8 and S100A9: DAMPs at the Crossroads between Innate Immunity, Traditional Risk Factors, and Cardiovascular Disease

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Received 3 October 2013; Revised 21 November 2013; Accepted 21 November 2013

Academic Editor: Stefan Frantz

Amplification of innate immune responses by endogenous danger-associated molecular patterns (DAMPs) promotes inflammation. The involvement of S100A8 and S100A9, DAMPs belonging to the S100 calgranulin family, in the pathogenesis of cardiovascular disease is attracting an increasing amount of interest. S100A8 and S100A9 (also termed MRP8 and MRP14) preferentially form the S100A8/A9 heterodimer (MRP8/14 or calprotectin) and are constitutively expressed in myeloid cells. The levels of circulating S100A8/A9 in humans strongly correlate to blood neutrophil counts and are increased by traditional cardiovascular risk factors such as smoking, obesity, hyperglycemia, and dyslipidemia. S100A8/A9 is an endogenous ligand of toll-like receptor 4 (TLR4) and of the receptor for advanced glycation end products (RAGE) and has been shown to promote atherogenesis in mice. In humans, S100A8/A9 correlates with the extent of coronary and carotid atherosclerosis and with a vulnerable plaque phenotype. S100A8/A9 is locally released following myocardial infarction and amplifies the inflammatory responses associated with myocardial ischemia/reperfusion injury. Elevated plasma levels of S100A8/A9 are associated with increased risk of future coronary events in healthy individuals and in myocardial infarction survivors. Thus, S100A8/A9 might represent a useful biomarker and therapeutic target in cardiovascular disease. Importantly, S100A8/A9 blockers have been developed and are approved for clinical testing.

1. Introduction

Inflammation plays a central role in the development of atherosclerosis and in plaque vulnerability [1]. The chronic, low-grade inflammatory process characteristic of atherosclerosis development in the arterial wall is sustained by a constant interplay between innate and adaptive immunity [2]. The primary function of the innate immune system is to combat pathogen invasion, but it can also be activated by endogenous ligands under conditions of immunological stress [3]. Neutrophils and monocytes, central components of innate immunity, express pattern recognition receptors (PRRs) on their surface that bind evolutionarily conserved structures such as bacterial pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs), leading to cell activation [3]. DAMPs, also known as alarmins, are intracellular molecules that involved in cellular function under normal homeostasis, which are released after cell death, signaling tissue damage [3, 4].

The S100 proteins form a calcium-binding cytosolic protein family defined by their common ability to dissolve in 100% ammonium sulphate [5]. Several S100 proteins have so far been identified as DAMPs, including S100A7 [6], S100A8, S100A9, S100A12 [5, 7], and S100A15 [6]. S100A8, S100A9, and S100A12 are produced by cells of myeloid origin [8] and have been linked with cardiovascular disease (CVD) [9, 10]. Clinical data show clear correlations between S100A12 and the severity of coronary and carotid atherosclerosis [10–12], but mechanistic studies on the role of S100A12 in CVD are hampered by the absence of this protein in mice. The present review will attempt to summarize the increasing body

of evidence demonstrating the involvement of S100A8 and S100A9 in atherogenesis, plaque vulnerability, myocardial infarction (MI), and heart failure.

S100A8 and S100A9 are also known as calgranulins A and B or myeloid-related proteins (MRP) 8 and 14. S100A8 and S100A9 are constitutively expressed in neutrophils, monocytes [8], and dendritic cells [13] but can also be induced upon activation in other cell types such as mature macrophages [14–16], vascular endothelial cells [17–19], fibroblasts [20], and keratinocytes [21]. In neutrophils, S100A8 and S100A9 constitute ~45% of all cytosolic proteins, compared to only about 1% in monocytes [8]. S100A8 expression seems to differ between subsets of human monocytes, as higher levels of S100A8 mRNA were detected in classical $CD14^{++}CD16^{-}$ monocytes compared to their nonclassical $CD14^{+}CD16^{++}$ counterparts [22]. S100A8 and S100A9 exist as homodimers but preferentially form the S100A8/A9 heterodimer (also called calprotectin) in the presence of Zn^{2+} and Ca^{2+}. Intracellularly, S100A8/A9 promotes phagocyte migration by promoting tubulin polymerization and stabilization of tubulin microfilaments in a calcium dependent manner [23].

Extracellular S100A8/A9 is primarily released from activated or necrotic neutrophils and monocytes/macrophages and is involved as an innate immune mediator in the pathogenesis of various diseases with an inflammatory component [24, 25]. We have recently studied the correlations between S100A8/A9 and the circulating numbers of neutrophils, lymphocytes, platelets, total monocytes, and different monocyte subpopulation in human blood. Our data suggests that neutrophils seem to be the main source of systemic S100A8/A9, as neutrophils were the only cell population that strongly and independently correlated with plasma S100A8/A9 levels [26]. Interestingly, both pro- and anti-inflammatory functions of S100A8, S100A9, and S100A8/A9 have been reported, suggesting that the functions of S100A8/A9 might be concentration-dependent and influenced by the cellular and biochemical composition of the local milieu [27]. S100A8, S100A9, and S100A8/A9 promote neutrophil and monocyte recruitment by activating the microvascular endothelium [28] and by stimulating phagocyte Mac-1 expression, affinity and binding to ICAM-1, fibronectin, and fibrinogen [29–32]. However, other authors failed to reproduce the chemotactic activity of S100A8 and S100A9 and demonstrate instead a fugitactic (repellent) effect on neutrophils at picomolar concentrations, which may contribute to resolution of inflammation and tissue repair [33, 34]. Oxidant scavenging [35], matrix metalloproteinase (MMP) inhibition by Zn^{2+} chelation [36] and inhibition of reactive oxygen species production in phagocytes [37–39] are additional anti-inflammatory and tissue protective mechanisms that were proposed for S100A8, S100A9 and S100A8/A9.

The toll-like receptor 4 (TLR4) and the receptor for advanced glycation endproducts (RAGE) have so far been suggested as innate immune receptors of S100A8/A9 [40–42]. S100A8/A9 binding triggers MyD88-mediated TLR4 signaling, leading to NF-kB activation and secretion of pro-inflammatory cytokines such as TNFα and IL-17 [40, 43, 44]. The S100A8/A9-TLR4 interaction has been shown to be involved in the pathogenesis of systemic infections, autoimmune diseases, malignancy, and acute coronary syndrome [40, 43, 45–48]. Similarly, S100A8/A9 binding to RAGE leads to MAP kinase phosphorylation and NF-kB activation, promoting leukocyte production in the bone marrow [49], carcinogenesis [50–52], cardiomyocyte dysfunction [53] and postischemic heart failure [54]. RAGE activation by S100A8/A9 or other ligands leads to further enhancement of S100A8/A9 production, creating a putative positive feedback loop in chronic inflammation [55, 56]. Interestingly, it has recently been shown that, in contrast to neutrophils, S100A9-defficient dendritic cells secrete increased amounts of inflammatory cytokines upon TLR4 stimulation, suggesting that S100A9 might function as an innate immune suppressor in this particular cell population [13].

S100A8/A9 binds heparan sulphate proteoglycans and carboxylated glycans on endothelial cells [57, 58] and triggers endothelial activation, characterized by enhanced production of inflammatory cytokines and chemokines [28, 56], increased expression of adhesion molecules [28, 56], and increased platelet aggregation at the surface of the endothelium [28]. Additionally, endothelial cells treated with S100A8/A9 were shown to downregulate antiapoptotic genes and genes responsible for the integrity of the endovascular monolayer [28, 59]. Extended S100A8/A9 exposure leads to endothelial cell dysfunction and increased endothelial permeability [59]. These effects are partly mediated by RAGE [41] and exacerbated by hyperglycemia [56, 60].

Oxidative modifications of S100A8 and S100A9 induced by reactive oxygen species mainly target cysteine and methionine residues and have been shown to regulate function. The different reversible and irreversible oxidative modifications of S100 proteins described to date and their potential functional consequences have been expertly reviewed elsewhere [27, 61]. Oxidation of methionines 63 and 83 on S100A9 and of cysteine 42 on S100A8 inhibits both the chemotactic and the repellent effects of the proteins on neutrophils, whereas the oxidation-resistant mutants were shown to retain function [33, 34]. Conversely, oxidation of these residues was found to be required for the antifungal activities of S100A8/A9 [62]. HOCl induced oxidation of S100A8 and S100A9 generates stable cross-linked dimers, trimmers, and S100A8-S100A9 complexes of different sizes that were found in human carotid plaques [18]. Oxidized S100A8 was also found to predominate in sputum from asthmatic patients compared to native S100A8 [35], suggesting that these mechanisms are involved *in vivo* in the pathogenesis of inflammatory disease in humans. S100A8 and S100A9 are much more sensitive to oxidation compared to low-density lipoproteins and albumin and the authors propose that the high amounts of S100 proteins present in atherosclerotic plaques might contribute to oxidant scavenging and protect other proteins and tissue components from oxidative damage during inflammation [18]. Interestingly, S100A9 is less susceptible to oxidation compared to S100A8 [18] and has a much higher affinity for TLR4 and RAGE compared to S100A8 and S100A8/A9 [42]. It is tempting to speculate that under mild oxidative conditions, S100A8/A9 oxidation releases S100A9 from the

heterocomplex, leading to TLR4 and RAGE binding and activation. This hypothesis would explain the lack of widespread receptor activation under steady-state physiological conditions despite the presence of large amounts of circulating S100A8/A9. However, other authors propose S100A8 to be the main active component of the S100A8/A9 complex [40], so this issue remains controversial. The influence of S100A8/A9 oxidation on TLR4 and RAGE binding and activation has not been investigated and it needs further clarification.

2. S100A8/A9 and Cardiovascular (CV) Risk Factors

Diabetes mellitus, obesity, smoking, and hyperlipidemia are traditional CV risk factors that have been associated with increased levels of S100A8/A9 in plasma. An overview of the interplay between S100A8/A9, traditional CV risk factors, circulating phagocytes, and vascular inflammation is presented in Figure 1. Hyperglycemia induces the production of reactive oxygen species (ROS) in human endothelial cells *in vitro* and in aortic endothelial cells of diabetic mice *in vivo*, leading to overexpression of S100A8 and RAGE [17]. Similarly, hyperglycemia-induced expression of ROS in neutrophils leads to increased S100A8/A9 secretion [49]. S100A8/A9 binds RAGE on common myeloid progenitors and macrophages in the bone marrow and stimulates production of growth factors, leading to accelerated myelopoiesis and increased release of neutrophils and inflammatory $Ly6C^{hi}$ monocytes into the circulation [49]. As a result, hyperglycemic diabetic mice have higher concentrations of S100A8/A9 in plasma and increased numbers of circulating leukocytes. This phenotype can be reversed by pharmacological reduction of systemic glucose levels or by knocking out the RAGE receptor [49]. In diabetic $LDLR^{-/-}$ mice, accelerated recruitment of $Ly\text{-}6C^{hi}$ monocytes into the atherosclerotic plaques leads to impaired lesion regression, which might explain the increased severity of atherosclerosis found in diabetic patients [49]. Neutrophil recruitment into the arterial wall was not assessed in this study. These experimental results are supported by clinical data demonstrating elevated S100A8/A9 levels in patients with type 2 diabetes or impaired glucose tolerance compared with nondiabetic controls [63]. Additionally, plasma S100A8/A9 was found to positively correlate with measures of impaired glucose metabolism such as insulin resistance, fasting blood glucose [63], and glycosylated hemoglobin A1c (HbA1c) [26].

Body-mass index (BMI) is an independent determinant of S100A8/A9 concentrations [26, 63]. Among nondiabetics, plasma S100A8/A9 was found to be higher in obese versus non-obese individuals [63–65]. This effect could not be observed in diabetic subjects [63, 64], suggesting the presence of partially overlapping mechanisms responsible for increased production of S100A8/A9 in obesity and diabetes. Weight loss in obese nondiabetic subjects leads to significantly decreased S100A8/A9 alongside insulin resistance and plasma lipids [63]. Interestingly, the reduction in circulating S100A8/A9 levels was not associated with lower blood leukocyte counts, suggesting that obesity is associated with increased leukocyte activation and S100A8/A9 production rather than increased leukocytosis [63].

As previously discussed, neutrophils seem to be the main source of circulating S100A8/A9 [26] and blood neutrophil counts correlate strongly with plasma S100A8/A9 concentrations [26, 63]. Smoking and hyperlipidemia stimulate granulopoiesis and S100A8/A9 production. Active smoking is a strong stimulus for neutrophilia in apparently healthy individuals [66] and smokers have elevated S100A8/A9 levels [26]. Similarly, hyperlipidemia stimulates neutrophilia through increased granulopoiesis and enhanced neutrophil release from the bone marrow [67]. Our own observations in a cohort of apparently healthy individuals show that LDL positively and HDL negatively influence plasma S100A8/A9 concentration independently of BMI, smoking, and glycemic control [26]. Thus, several traditional cardiovascular risk factors increase systemic S100A8/A9 levels either directly by phagocyte activation and S100A8/A9 release or indirectly by stimulation of neutrophil and monocyte production in the bone marrow.

3. S100A8/A9 and Atherosclerosis

S100A8/A9 is an active mediator in the pathogenesis of various autoimmune and inflammatory conditions [24, 25]. In recent years, the involvement of neutrophils and S100A8/A9 in CV disease (CVD) has attracted an increasing amount of interest [9]. S100A8/A9 is thought to accelerate atherogenesis through increased recruitment and activation of neutrophils and monocytes in the arterial wall (Figure 1). Despite early controversy, the proatherogenic role of neutrophils, the main source of circulating S100A8/A9, is now firmly established [67–70]. S100A8 and S100A9 are present in atherosclerotic plaques in both mice and humans (Table 1) [18, 71–73] and S100A8/A9 has been proposed as a potential target for plaque-directed accumulation of gadolinium nanoprobes in imaging and therapeutic applications [71]. Signaling through TLR4 and RAGE, the endogenous receptors of S100A8/A9 have been shown to be proatherogenic. Plaque size is reduced in atherosclerotic mice deficient in TLR4 or its adaptor protein MyD88 [74, 75] and RAGE deficiency is associated with delayed plaque progression and attenuated vascular inflammation in hyperlipidemic $ApoE^{-/-}$ mice [41]. RAGE is overexpressed in atherosclerotic plaques collected from diabetic patients and from mice rendered diabetic by streptozotocin treatment [76, 77]. The diabetic $ApoE^{-/-}$ mice have elevated plasma S100A8/A9 levels and develop larger atherosclerotic lesions characterized by increased content of S100A8/A9, advanced glycation endproducts (AGEs), activated NF-kB, VCAM-1, and MCP-1 [77]. These effects were abrogated in the absence of RAGE [77], suggesting that RAGE and its ligands play important roles in the accelerated atherogenesis associated with diabetes.

The $S100A9^{-/-}$ mouse strain has facilitated important advances in the understanding of the role of S100A8/A9 in myeloid cell function and in vascular disease [78]. S100A8 is unstable in the absence of S100A9, so these mice lack

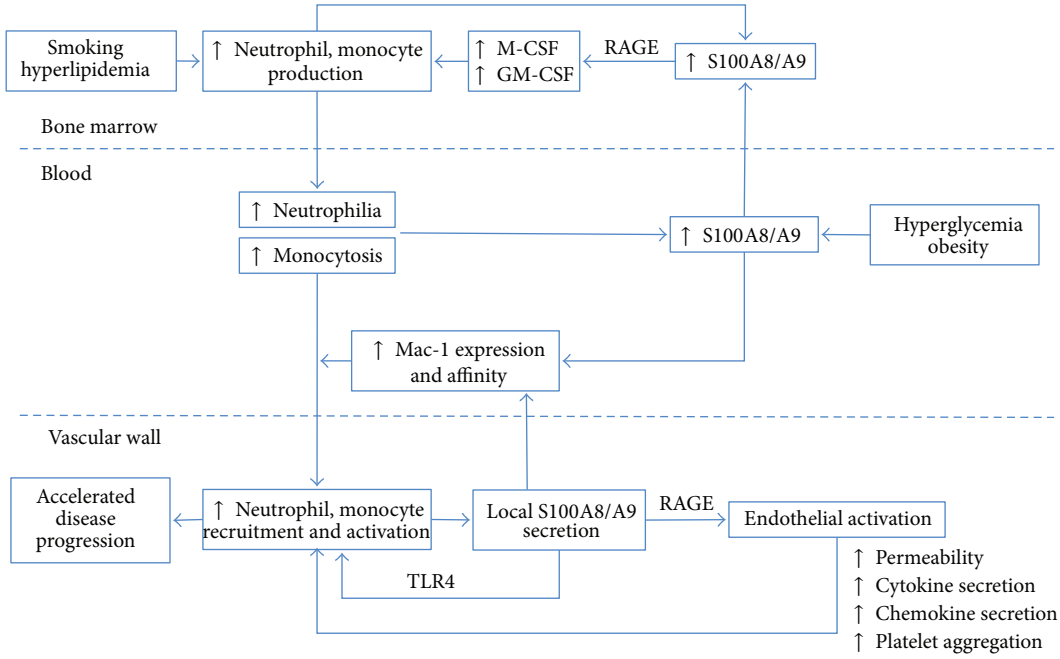

Figure 1: Overview of the interplay between S100A8/A9, traditional CV risk factors, circulating phagocytes, and atherogenesis. Smoking, hyperlipidemia, hyperglycemia, and obesity induce elevated S100A8/A9 production either directly or indirectly by stimulating neutrophilia and monocytosis. S100A8/A9 enhances phagocyte production in the bone marrow and facilitates their recruitment into the vascular wall through endothelial activation and increased Mac-1 expression and affinity. These effects are primarily mediated by RAGE and accelerated by hyperglycemia. In the vascular wall, S100A8/A9 binding to TLR4 triggers phagocyte activation and secretion of inflammatory cytokines, further contributing to phagocyte recruitment and accelerated atherogenesis. M-CSF: macrophage colony stimulating factor; GM-CSF: granulocyte-macrophage colony stimulating factor; RAGE: receptor for advanced glycation end products; TLR4: toll-like receptor 4.

both S100A8 and S100A9 proteins [78]. S100A8 mice are not viable [79]. It has been shown that S100A9 deficiency impairs the migratory capacity and cytokine production of neutrophils and monocytes/macrophages [13, 23, 80–82]. Leukocyte recruitment and lesion size were significantly reduced in S100A9$^{-/-}$ mice undergoing femoral artery wire injury [82]. The hyperlipidemic ApoE$^{-/-}$S100A9$^{-/-}$ double knockout mice develop smaller atherosclerotic lesions with lower macrophage infiltration compared to their ApoE$^{-/-}$ counterparts [82]. Unexpectedly, atherosclerosis development was not delayed in hyperlipidemic LDLR$^{-/-}$ mice reconstituted with S100A9$^{-/-}$ bone marrow, suggesting that local S100A9 expression in nonmyeloid cells might play an important role [13]. In an attempt to explain these contrasting findings, the authors have found opposite effects of S100A9 deficiency in neutrophils and dendritic cells. While S100A9$^{-/-}$ neutrophils secreted markedly less TNFα and MCP-1 in response to LPS stimulation, inflammatory cytokine production in dendritic cells was exacerbated by S100A9 deficiency and exogenous S100A8/A9 was shown to inhibit the ability of activated DCs to induce T cell proliferation *in vitro* [13].

The link between S100A8/A9 and atherosclerosis is further supported by clinical studies demonstrating a positive relationship between plasma S100A8/A9 and the severity of coronary artery disease (CAD) in type 1 and type 2 diabetic patients (Table 1) [49, 83]. S100A8/A9 was also shown to correlate with carotid intima-media thickness (IMT) in a small subgroup of diabetic patients without CAD [84] and in middle-aged individuals with no previous history of CVD [26]. Circulating neutrophil numbers presented similar associations with carotid IMT, independently of traditional CV risk factors [26]. Detailed immunohistochemical and biochemical analysis of human carotid plaques have demonstrated an increased amount of S100A8/A9 in vulnerable lesions characterized by large lipid cores, intense macrophage infiltration, low collagen content, and elevated concentrations of inflammatory cytokines and matrix metalloproteinases [72]. The authors found an increased number of S100A8 and S100A9 positive macrophages in rupture-prone atheromas [72], consistent with experimental data showing that S100A9 positive monocytes are preferentially recruited into atherosclerotic plaques [73]. Ultrasound analysis of carotid plaques in type 2 diabetic patients demonstrated that the presence of echolucent plaques, generally considered to belong to the vulnerable phenotype, is associated with increased plasma levels of S100A8/A9 [85]. In patients undergoing carotid endarterectomy, high concentrations of S100A8/A9 in plasma and in the carotid plaques were associated with the incidence of acute CV events (fatal or nonfatal) during follow-up, independently of the classic CV risk factors and CRP [86]. Associations between plasma S100A8/A9 and CV risk have also been found to be valid in healthy individuals and in systemic lupus erythematosus (SLE) patients. Healy et al. reported that apparently healthy postmenopausal women with S100A8/A9 levels within the highest quartile

Table 1: S100A8/A9 in cardiovascular disease.

S100A8/A9 and atherosclerosis	
Mouse studies	Present in mouse atherosclerotic plaques [71, 73]
	Reduced atherosclerotic lesions in hyperlipidemic ApoE$^{-/-}$ S100A9$^{-/-}$ mice [82]
	No effect on atherosclerosis in hyperlipidemic LDLR$^{-/-}$ mice reconstituted with S100A9$^{-/-}$ bone marrow [13]
	Reduced neointima formation in S100A9$^{-/-}$ mice following femoral artery wire injury [82]
	Elevated plasma and plaque S100A8/A9 in diabetic ApoE$^{-/-}$ mice [77]
Clinical studies	Present in human atherosclerotic plaques [18, 72]
	Associated with histological and ultrasound measures of plaque vulnerability [72, 85]
	Correlates with the severity of CAD in type 1 and 2 diabetic patients [49, 84]
	Correlates with carotid IMT in healthy diabetics and nondiabetics [26, 84]
S100A8/A9 in acute coronary syndrome	
Mouse studies	Accumulates into the myocardium following coronary ischemia [54]
	Triggers RAGE-mediated phagocyte activation, recruitment, and inflammatory cytokine production [54]
	Aggravates the development of post-MI heart failure [54]
Clinical studies	Increases rapidly in plasma following an ischemic coronary event [90]
	Released into the circulation from the site of the coronary occlusion [90]
	Upregulated in infiltrating neutrophils and monocytes in the infarcted myocardium and in the occluding thrombus [90, 92]
	Higher in MI patients compared to stable and unstable angina [87, 92]
	Remains elevated for several weeks after the event and correlates with peak white cell and neutrophil counts [92]
S100A8/A9 and CV risk	
Clinical studies	Correlates with short- and long-term risk for CV events in apparently healthy women independently of traditional CV risk factors [26, 87]
	Associated with the incidence of subsequent CV events in patients undergoing carotid endarterectomy [86]
	Elevated S100A8/A9 at 30 days after a coronary event is associated with increased risk for recurrent events during the following 30 day period [97]
	Associated with all-cause 1-year mortality in elderly patients with severe heart failure [98]
	Elevated in SLE patients with CV disease—retrospective study [89]

run a 3.8 times higher risk to develop acute CV events during a median follow-up period of 3 years, independently of other CV risk factors [87]. Recently published data from our group demonstrate that the independent associations between elevated S100A8/A9 in apparently healthy women and the incidence of coronary events and CV death are paralleled by similar associations for circulating neutrophil numbers [26]. SLE is a chronic inflammatory disease associated with increased CV risk [88]. Serum S100A8/A9 was found to be elevated in patients with clinically inactive SLE and prevalent CVD [89], but it remains to be determined whether S100A8/A9 can predict incident CV events in this population in prospective studies.

4. S100A8/A9 in Acute Coronary Syndrome

The demonstrated associations between S100A8/A9 and the incidence of acute CV events have prompted further research into the role of S100A8/A9 as potential disease mediator and prognostic biomarker in coronary artery disease. Plasma S100A8/A9 was found to be highly increased during the ischemic event in acute coronary syndrome patients compared with stable angina or with individuals with angiographically assessed normal coronary artery morphology (Table 1) [90]. As cardiac myocytes subjected to ischemia do not upregulate S100A8 and S100A9 mRNA and protein levels [91], S100A8/A9 is probably released from activated monocytes and neutrophils recruited to the site of the injury. This hypothesis is supported by an elegant study by Altwegg et al. demonstrating that in ST-elevation MI patients, S100A8/A9 is markedly increased at the site of the coronary occlusion compared to the systemic circulation [90]. Additionally, the presence of S100A8/A9-positive neutrophils and macrophages was confirmed both in the occluding thrombus and in the infarcted myocardium [90, 92]. In myocardial infarction (MI) patients, plasma S100A8/A9 levels increase before the classical markers of myocardial injury such as troponin T or creatine kinase [90] and are higher compared with patients suffering from unstable angina [87, 92]. However, S100A8/A9 is a poor diagnostic biomarker for MI in

patients presenting at the emergency department with acute chest pain and does not offer additional information to the already established model based on cardiac troponin [93]. Interestingly, microarray and RT-PCR analysis of platelet mRNA revealed strikingly elevated S100A9 mRNA levels in ST-elevation MI patients compared to patients with stable angina [87]. As the platelet transcriptome is directly derived from megakaryocyte mRNA, this is likely to reflect platelet composition prior to the acute event and might be responsible for differences in platelet function between MI patients and controls. However, the presence of S100A9 mRNA in platelets has been debated by other studies [94] and it is unclear whether the activated platelets contribute to local S100A8/A9 release, as platelets in the occluding thrombus did not express the S100A8/A9 protein [90].

Compared with cardiac troponin, which is acutely released from necrotic cardiomyocytes and peaks within hours after the ischemic injury, S100A8/A9 peaks after 3–5 days and continues to be elevated for several weeks after the event [92]. S100A8/A9 levels correlate with peak white blood cell and neutrophil counts [92], possibly related to the ability of S100A8/A9 to stimulate neutrophil production in the bone marrow [49]. Human monocytes isolated from MI patients are particularly responsive to S100A8/A9-induced TLR4 upregulation and secrete increased amounts of TNFα and IL-6 [48, 95]. Monocyte TLR4 expression and the levels of inflammatory cytokines in plasma remain elevated for more than 14 days after the acute event and correlate with the development of heart failure [95]. These results are supported by experimental data demonstrating that TLR4 deficiency is protective against the development of cardiac dysfunction after coronary ischemia [96]. In a mouse model of coronary artery occlusion, S100A8/A9 binding to RAGE on phagocytes was shown to trigger NF-kB activation, inflammatory cytokine production, and enhanced immune cell recruitment into the myocardium [54]. Thus, S100A8/A9 amplifies the local myocardial inflammation associated with ischemia/reperfusion injury, facilitating myocardial remodeling and the development of heart failure [54].

To date, the only study assessing the value of S100A8/A9 as a potential prognostic biomarker in the immediate post-ACS period has been performed by Morrow et al. in 237 case-control pairs selected from the PROVE IT-TIMI 22 statin therapy trial cohort [97]. S100A8/A9 was measured 30 days after the acute event and found to be elevated in patients who suffered a recurrent event (MI or CV death) during the subsequent 30 day period [97]. Patients with S100A8/A9 values within the top quartile had a 2 times higher risk to develop a recurrent event compared to the lowest quartile, independently of diabetes, hypertension, previous CV disease, heart failure, and hsCRP. S100A8/A9 and hsCRP provided additive prognostic information in this population and the intensive statin therapy (atorvastatin 80 mg) lowered plasma S100A8/A9 levels compared to the moderate therapy group (pravastatin 40 mg) after 30 days of treatment [97]. S100A8/A9 was found to be increased in patients suffering from severe heart failure (NYHA class III-IV) compared to patients with hypertension or healthy subjects, in a group of elderly individuals (>70 years of age). In the heart failure group, S100A8/A9 was positively correlated with IL-6 and IL-8 and predicted all-cause mortality in 1 year [98]. However, it is unclear whether progressive heart disease was the main cause of death in this group and it remains to be determined whether S100A8/A9 is actively involved in the pathogenesis of heart failure in humans.

5. S100A8/A9 as Therapeutic Target

Due to its potential involvement in atherogenesis, plaque vulnerability, ischemia-associated myocardial inflammation, and heart failure, S100A8/A9 represents an attractive target in CVD. Quinoline-3-carboxamides are orally active chemical compounds with potent anti-inflammatory properties in various models of autoimmune disease such as SLE, experimental autoimmune encephalomyelitis, and collagen arthritis [99–102]. The molecular targets and therapeutic mechanisms of these compounds have initially been unknown. Recently, Björk at al. have identified S100A9 as the elusive target of quinoline-3-carboxamides [42]. The quinoline-3-carboxamide ABR-215757 binds both mouse and human S100A9 and S100A8/A9 in a Ca^{2+} and Zn^{2+} dependent manner and blocks their interaction with RAGE and TLR4 [42]. This effect is biologically relevant *in vivo*, as ABR-215757 inhibits TNFα production in response to LPS challenge in a mouse model, to a similar extent as a Fab antibody fragment specific for the interaction site of S100A9 with its receptors [42]. Additionally, oral ABR-215757 treatment was shown to delay disease progression in lupus-prone mice [99]. Testing quinoline-3-carboxamides as potential therapeutic principles in CVD is particularly appealing, as several of these compounds have already been approved for human use and have generated promising preliminary results in multiple sclerosis [103], juvenile type 1 diabetes [104], SLE [99], and castration-resistent prostate cancer [105]. ABR-215757 blocks S100A12 as well as S100A8/A9 and a proof-of-principle study in S100A12 transgenic hyperlipidemic $ApoE^{-/-}$ mice demonstrated that ABR-215757 treatment reduced atherosclerotic plaque size, inflammation, and vulnerability features [106].

6. Conclusions and Future Directions

The experimental and clinical studies presented in the present review have demonstrated a promising potential for S100A8/A9 as a clinical biomarker and treatment target in CVD. As biomarker, S100A8/A9 correlates with the extent of subclinical carotid and coronary artery disease, increases rapidly in plasma during myocardial ischemia and necrosis, and is associated with unfavorable prognosis in MI and heart failure patients and in patients undergoing carotid arterectomy (Table 1). However, several issues remain to be elucidated before the use of S100A8/A9 can enter clinical practice. As discussed above, S100A8/A9 amplifies inflammatory processes commonly involved in the pathogenesis of atherosclerosis and autoimmune diseases. The incidence of CVD is distinctly elevated in patients with autoimmune rheumatic diseases [107] and S100A8/A9 is

increased in SLE patients with CVD [89]. Prospective studies are required to determine whether S100A8/A9 measurement can offer independent information for CV risk stratification in this particular patient group. The ability of S100A8/A9 to independently predict recurrent events following an ischemic coronary event [97] needs to be compared to other biomarkers of myocardial necrosis, overload, phagocyte activation, and vascular inflammation. As the sustained inflammatory response associated with myocardial necrosis following an MI is absent in unstable angina, these patient groups should be assessed separately with regard to the prognostic value of S100A8/A9 in secondary prevention. Mouse experiments have demonstrated that S100A8/A9 is actively involved in the development of heart failure secondary to ischemia/reperfusion injury [54] and elevated S100A8/A9 concentrations are associated with increased mortality in heart failure patients [98]. Robust prospective clinical studies are required to explore whether S100A8/A9 is involved in the pathogenesis of heart failure in humans and whether plasma S100A8/A9 levels in the pre- and post-infarct period are associated with loss of cardiac function.

The main obstacle related to the use of S100A8/A9 as a therapeutic target in CVD is the relative abundance of this protein in human circulation, with median values of approximately 5 mg/L in healthy individuals, rising up to 15 mg/L in ACS patients [90, 97]. However, treatments with nontoxic doses of S100A8/A9 blockers have demonstrated encouraging results in experimental and clinical interventional studies on autoimmune disease and cancer, suggesting that complete systemic S100A8/A9 inhibition is probably not required for therapeutic effect. Topic S100A8/A9 blockade in the vulnerable atherosclerotic plaques and in the injured myocardium might provide increased efficacy and decreased systemic toxicity and represent exciting alternative approaches that need to be explored.

To conclude, S100A8/A9 seems to play a central role in the complex interactions between innate immunity, traditional CV risk factors, and CVD. Activated neutrophils and monocytes are the main sources of extracellular S100A8/A9 and diabetes, dyslipidemia, obesity, and smoking are associated with elevated circulating protein levels. S100A8/A9 seems to be involved in atherogenesis, plaque vulnerability, and post-ischemic myocardial damage. Pending further investigation, S100A8/A9 might serve as a clinical biomarker and therapeutic target in CVD, with S100A8/A9 blockers readily available and approved for clinical testing.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 181020, 13 pages
http://dx.doi.org/10.1155/2013/181020

Review Article

Mesenchymal Stem Cell Therapy for Cardiac Inflammation: Immunomodulatory Properties and the Influence of Toll-Like Receptors

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Received 4 October 2013; Accepted 14 November 2013

Academic Editor: Stefan Frantz

Background. After myocardial infarction (MI), the inflammatory response is indispensable for initiating reparatory processes. However, the intensity and duration of the inflammation cause additional damage to the already injured myocardium. Treatment with mesenchymal stem cells (MSC) upon MI positively affects cardiac function. This happens likely via a paracrine mechanism. As MSC are potent modulators of the immune system, this could influence this postinfarct immune response. Since MSC express toll-like receptors (TLR), danger signal (DAMP) produced after MI could influence their immunomodulatory properties. *Scope of Review*. Not much is known about the direct immunomodulatory efficiency of MSC when injected in a strong inflammatory environment. This review focuses first on the interactions between MSC and the immune system. Subsequently, an overview is provided of the effects of DAMP-associated TLR activation on MSC and their immunomodulative properties after myocardial infarction. *Major Conclusions*. MSC can strongly influence most cell types of the immune system. TLR signaling can increase and decrease this immunomodulatory potential, depending on the available ligands. Although reports are inconsistent, TLR3 activation may boost immunomodulation by MSC, while TLR4 activation suppresses it. *General Significance*. Elucidating the effects of TLR activation on MSC could identify new preconditioning strategies which might improve their immunomodulative properties.

1. Introduction

Ischemic heart disease occurs in approximately 40% of the population above 40 years and is the leading cause of death worldwide [1]. During ischemia a shortage of oxygen and nutrients is present in the heart, leading to apoptosis and necrosis of cardiomyocytes and endothelial cells [2–4]. Subsequent restoration of blood flow is currently the most effective therapy, yet results in additional damage of the myocardium, which is also known as ischemia/reperfusion injury (I/R injury) [5–7]. Both ischemia and reperfusion-induced cell death trigger a strong inflammatory reaction [8–10]. After initiation, this response is propagated by the damaged cells and matrix via the release of chemokines, cytokines, and a variety of endogenous proteins, referred to as danger-associated molecular patterns (DAMPs). DAMPs can subsequently bind toll-like receptors (TLR) on immune and other cells to activate them, resulting in a strong inflammatory environment. This results in an influx of neutrophils, subsequently followed by infiltrating monocytes and lymphocytes [10]. The immune response is essential to clear up the debris caused by the infarct, but also to initiate the wound healing process and the formation of proper scar tissue [10, 11]. The balance between inflammatory and reparative phases is delicate and needs proper fine-tuning in order to prevent excessive inflammation or inadequate stimulation of repair. Eventually this leads to adverse remodeling and subsequently decreased heart functions [5, 12, 13].

The resolution of postinfarct inflammation is considered to be an active process, influenced by factors released by the matrix and local cells, including surviving cardiac cells and infiltrated immune cells [13]. As the regulation of the

inflammatory reaction seems inefficient after massive damage, a special interest has developed for the induction of anti-inflammatory or regulatory subtypes of immune cells. This includes alternatively activated (type 2) macrophages and regulatory T-cells, both of which have been claimed to control the progression of the immune response. In time, sturdy scar tissue forms in the damaged areas. The matrix deposition in the scar impedes optimal contraction of the heart, resulting in further loss of cardiac function, which can progressively develop into heart failure [9, 14–17]. Activation of autoreactive T-lymphocytes has been reported at later stages. This negatively influenced cardiac remodeling and cardiac function long after the initial infarction response had occurred [18–21]. This indicates that the influence of immune cells persists long after the initial phase of damage. No curative therapy for heart failure exists besides cardiac transplantation or assistant devices, which is not without risk and many patients die waiting for a heart due to a lack of donor organs [1].

Although the effect of inflammation after MI has been known for many decades, no treatment options currently exist in a clinical setting to properly control this response [10]. Different avenues of treatment have been thoroughly investigated. General suppression of the whole immune system, using cortisone or cytostatic drugs, has shown detrimental effects on overall survival in both animal models and clinical studies. These treatments inhibited scar formation, which greatly increased the chance of cardiac rupture [22–26]. Similar results were found in studies investigating the use of nonsteroidal anti-inflammatory drugs (NSAIDs), where decreased wound healing led to fragile scar formation and decreased survival [27]. Depletion studies for circulating immune cells demonstrated that macrophages were essential for initiating healing after myocardial infarction [12, 28]. Meanwhile, depletion of neutrophils showed a reduction in final scar size without adverse effects on cell survival or cardiac function, demonstrating that their role was unnecessary for healing and only led to additional damage [12, 29–31]. Additionally, a recent rat study investigated T-cell depletion after MI, using antithymocyte globulin to induce T-cell apoptosis. They showed smaller infarcts with reduced remodeling, maintenance of cardiac function, and increased neoangiogenesis [32]. Depletion of B-cells using an anti-CD20 antibody also has beneficial effects on infarct size and heart function, by limiting myocardial inflammation [33]. These studies are only the first steps towards understanding, as for other cells of the immune system or their interactions the role in clearing of cardiac cell debris and stimulation of reparative responses remains largely unknown. What we have learned from these experiments, however, is that a general suppression of the immune response is not a therapeutic answer for modulating post-MI processes.

Recently, mesenchymal stem cells (MSC) emerged as potent modulators of the immune system. Interactions with several cells of the immune system have been described and many reports showed that MSC suppress white blood cells or trigger them into specific anti-inflammatory subsets. Treatment of post-MI inflammation using MSC could therefore provide a new approach of modulating the immune response, shifting the balance towards the reparative phase and reducing inflammation. Although these hypotheses seem to hold for the ideal *in vitro* situation, during post-MI inflammation many danger signals are released which will trigger toll-like receptors (TLR) not just on immune cells, but also on the injected MSC. The effect of TLR activation on MSC function is still largely unknown. It is possible this influences the MSC paracrine signaling capacities, thereby altering their ability to suppress the immune system. This review will focus first on the effects of MSC on the different types of immune cells after MI. Subsequently an overview will be provided of the current knowledge on the effects of DAMP-mediated TLR activation on MSC functioning.

2. Stem Cell Therapy against Cardiac Inflammation

Cardiac cell transplantation therapy is a new treatment option using stem cells for myocardial repair [34, 35]. The goal was to stabilize or reverse progressive heart failure by replacing myocardial scar tissue with contractile cells. Stem cells transplanted in the heart are suggested to reduce initial damage after infarction, promote activation of the endogenous regenerative potential of the heart, and integrate in the regenerated tissue [35, 36]. However, despite favorable results on cardiac function obtained in both animal and human studies, only few stem cells were reported to survive in the heart upon injection [34, 37–41]. This indicates that stem cell differentiation and direct contractile contribution are at most a minor explanation for the observed myocardial effects. The release of supportive or paracrine factors by the injected cells is more likely to be responsible—a theory called the paracrine hypothesis [42, 43].

Mesenchymal stem cells (also known as mesenchymal stromal cells or mesenchymal progenitor cells) are a heterogeneous group of stromal cells, which can be isolated from nearly all tissues of mesodermal origin. They are most prevalent in the bone marrow and adipose tissue, but can also be isolated from umbilical cord blood, placenta, dental pulp, and synovium [36, 44, 45]. Despite ongoing efforts, no single marker has yet been found that characterizes a pure MSC population with a homogenous functional profile. MSC are therefore characterized and defined by the minimal criteria described by the International Society for Cellular Therapy [46]. These criteria include (1) adherence to plastic, (2) expression of surface markers CD105, CD73, and CD90, while lacking the expression of CD45, CD34, CD14, or CD11b, CD79alpha or CD19, and HLA-DR surface molecules, and (3) differentiation *in vitro* into osteoblasts, adipocytes, and chondroblasts. In addition to these criteria, differentiation into hepatocytes and cardiomyocytes has been described. However, the *in vivo* occurrence of cardiomyocyte differentiation is rare and is *in vitro* only effective in young cell sources [36, 42, 47, 48].

MSC are especially known for their secretion of paracrine factors, which have beneficial effects on angiogenesis, cell survival, and inflammation. MSC have been shown to regulate the activation and differentiation of many cells of the immune system, including T-cells, B-cells, NK cells, monocytes, dendritic cells, and neutrophils [10]. MSC transplantation is

considered safe and has been widely tested as treatment for neurological, immunological, and cardiovascular diseases with promising results [45]. Animal and clinical studies using MSC therapy after MI reported beneficial effects, such as increased ejection fraction and reduced remodeling. However, cell retention in the heart is declining rapidly, with only 10% present after four hours and approximately 1% 24 hours after injection [36, 49, 50]. No long term engraftment and subsequent vascular differentiation have been reported [36]. Interestingly, currently there are about 40 registered trials investigating the effect of MSC therapy for cardiac disease only (clinicaltrials.gov) and many more exist for other diseases, based on their paracrine effectiveness.

3. Modulation of the Immune System by MSC

The discovery that MSC could modulate the immune system was initiated over a decade ago when it was observed that MSC abrogated T-cell proliferation *in vitro* [51]. These observations were quickly transferred to the clinic, where treatment of patients with therapy-resistant acute severe graft-versus-host-disease (GVHD) improved after multiple MSC infusions [52, 53]. In the next phase, MSC were administered simultaneously with hematopoietic stem cells (HSC) to reduce the chances of developing GVHD [54]. The successes obtained in these studies sparked investigations into MSC therapy against graft rejection and autoimmune disease, as both conditions also depend heavily on T-cell activation [55–57]. In the vast majority of these studies, MSC therapy had a favorable effect on inflammation status, disease progression, and functional outcome of the different organs [58–63]. Most research on the immunomodulatory properties of MSC have focused on their interaction with T-cells. To better understand the interactions between MSC and different immune cells, a short overview of the current knowledge will be given for each cell type and is also summarized in Figure 1.

3.1. T-Cell Proliferation. T-cells are a heterogeneous group of cells, consisting of many subtypes of which the T-helper cells (T_H-cells; CD4+), cytotoxic T-cells (T_C-cells; CD8+), and the regulatory T-cells (T_{reg}; CD4+ or CD8+, CD25+ FoxP3+) are best known. Both T_H and T_C-cells recognize a specific antigen, but while T_C-cells directly induce apoptosis of the cell displaying that particular antigen, T_H cells mobilize macrophages and B-cells to attack the antigen-displaying cell. T_{reg} are regulators of the immune response and capable of terminating T-cell mediated immunity. Upon MI, antigen-specific T-cells form against endogenous cardiac myosin and actinin, which leads to a continuous assault of T_H-cells and T_C cells on the remaining myocardium [18, 19, 64].

Several authors showed that MSC are quite potent suppressors of T-cell proliferation, although there is a lot of donor variability [51, 65–67]. As shown in Figure 1, MSC affect both T_H- and T_C-cells, by inducing cell cycle arrest of the T-cells in the G0/G1 phase [68]. Many different pathways were found to play a role in this interaction between MSC and T-cells, of which most studied are indoleamine-pyrrole 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) [10]. IDO is an enzyme involved in the tryptophan metabolism, which is upregulated in MSC in coculture with T-cells. This leads to tryptophan depletion and local accumulation of metabolites KYNA and kynurenine, all of which are thought to be able to reduce T-cell proliferation [69]. Alternatively, induction of COX-2 expression also occurs in these cocultures resulting in increased secretion of PGE2, thereby inhibiting T-cell proliferation [67, 70]. Another pathway possibly involved is the interaction of inhibitory molecule programmed death 1 (PD-1) and the ligands PD-L1 and PD-L2 [71]. The PD-1/PD-L1/PD-L2 pathway is a regulatory mechanism which controls T-cell-receptor-mediated lymphocyte proliferation and cytokine secretion [71]. MSC expressing PD-L1 or PD-L2 can activate the PD-1 receptor on the target T-cell. This results in a decrease in production of proinflammatory cytokines IFN-γ, TNF-α, and IL-2 and subsequent T-cell cycle arrest [65, 71]. Another way in which T-cells could be kept inactive is related to the (inducible) expression of MHC (or HLA) molecules on MSC. With these molecules, MSC can play the role of antigen-presenting cell, which would normally activate T-cells [72]. However, due to the absence of an indispensable costimulatory signal from CD80, CD86, or CD40, T-cells might go into anergy instead of being fully activated [45, 73, 74]. In this state, T-cells are still alive, yet unable to be activated and therefore unable to mount a functional immune response.

3.2. T-Cell Differentiation. MSC are also able to influence differentiation of T-cells into different subtypes. In addition to the aforementioned pathways, several paracrine factors including HGF, TGF-β1, IL-6, and IL-10 have been implicated in this process, although the exact mechanisms still remain unknown [10]. MSC suppress the formation of T_H1 and T_H17 lymphocytes, which are essential for the activation of cytotoxic T-cells and the boost of phagocytic capacity of neutrophils and macrophages [75, 76]. Meanwhile, MSC enhance the formation of T_H2 lymphocytes, which have a more immunotolerant phenotype and produce large amounts of IL-4 and IL-10 [10, 76, 77]. Although these T_H2 cells normally induce B-cells, there are reports that the costimulatory molecules are downregulated on the T_H cells, resulting in a reduction in B-cell activation [78]. Besides reducing T-cell proliferation, MSC also induce formation of regulatory T-cells [75, 76]. This provides a negative feedback loop for T-cell activation and proliferation and helps to regain a tolerance for autoantigens, such as myosin [79]. These regulatory T-cells are suggested to be formed via IDO-expression, secretion of PGE2, and TGF-β by interacting MSC. Interestingly, an increase in regulatory T-cells has been shown to attenuate ventricular remodeling after MI [80].

3.3. NK Cells. Natural Killer (NK) cells are the innate immune system's subtype of cytotoxic lymphocytes. They usually react in response to viral antigens presented on MHC-I molecules, but can also recognize and lyse stressed cells, which many cardiac cells are shortly after MI [81]. MSC can suppress the proliferation of NK cells, as well as reduce the cytotoxic activity and pro-inflammatory cytokine profile (Figure 1) [82]. Proliferation of NK cells is sharply reduced

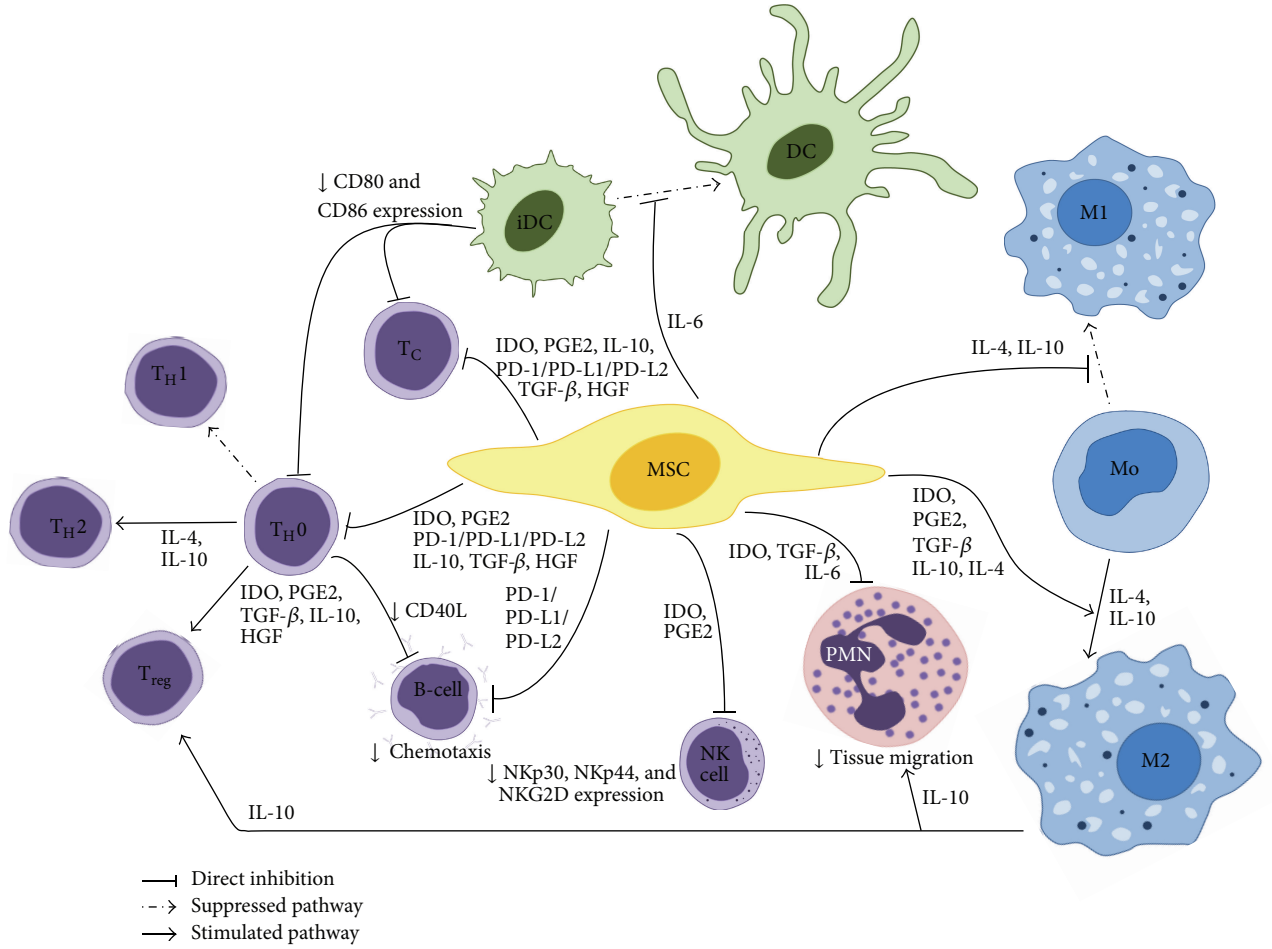

FIGURE 1: Schematic overview of the interactions between MSC and the immune system. Mesenchymal stem cells influence the functioning of many immune cells. Via multiple possible pathways MSC suppress proliferation of both helper (T_H) and cytotoxic T-cells (T_C). In addition, differentiation to T_H2 and regulatory T-cells (T_{reg}) is triggered, resulting in an anti-inflammatory environment. Maturation of immature dendritic cells (DC) is inhibited via IL-6, blocking upregulation of CD40, CD80, and CD86, which in turn can reduce T-cell activation. Monocytes are triggered by MSC to differentiate towards the M2 phenotype. Different mechanisms appear to be involved in this process, amongst which IDO, TGF-β, IL-10, and PGE2 are the most important ones. IL-10 produced by these M2 macrophages can boost the formation of T_{reg}, while reducing tissue migration of neutrophils. Neutrophils (polymorphonuclear granulocytes; PMN) are allowed a longer life span by MSC-derived IL-6, while ROS production is decreased. Natural killer cell (NK cells) proliferation is suppressed, as well as cytotoxic activity and cytokine secretion. B-cell proliferation is inhibited and the production of antibodies is reduced.

in the presence of both IDO and PGE2, thereby pointing to the possible synergistic effect of these two pathways [82–84]. MSC can also downregulate the NK activating receptors NKp30, NKp44, and NKG2D [83]. As NK receptors are correlated with the function of the NK cell, the downregulation of activating receptors leads to an altered cytotoxic activity and reduces secretion of pro-inflammatory cytokines [83]. The reduction in IL-2 and IFN-γ secretion leads to further suppression of NK cell proliferation [68].

3.4. B-Cells. B-cells are part of the adaptive immune response and responsible for the production of antibodies during inflammation. The antibodies cover the cell displaying the specific antigen and allow easy engulfment by phagocytic cells, such as macrophages and neutrophils [85]. After MI, mature B-cells release Ccl7 to attract the pro-inflammatory M1 macrophages to the heart, which decreases cardiac function by enhancing tissue injury [33]. MSC were found to arrest B-cells in the G0/G1 phase, while simultaneously reducing the chemotactic capacity of these cells, as depicted in Figure 1 [68, 74, 86, 87]. How this is exactly regulated remains unclear. MSC can interact with B-cells via the PD-1 pathway as seen for T-cells, hereby reducing activation and proliferation of B-cells [71]. The costimulatory molecule CD40L is mainly present on activated T-cells and plays a role in B-cell activation [77]. If this costimulatory signal is not obtained, B-cells activation will be reduced and antibody secretion will diminish. A reduction in T_H-cell activation by MSC, and especially the existence of T-cell anergy, could lead to decreased B-cell activity *in vivo* [45, 88]. Finally, some research showed that MSC were able to suppress the production of antibodies by B-cells [86]. It is important to note

here that some reports have also been published that MSC stimulate B-cell proliferation and differentiation [89, 90].

3.5. Dendritic Cells. Dendritic cells (DC) are the most potent antigen presenting cells of our immune system and after MI they present cardiac antigens, which activate the adaptive immune system [91]. Coculture of MSC with DC progenitors, whether CD34+- or monocyte-derived, prevented differentiation into mature DC, despite the fact that cells were grown in lineage-specifying growth conditions [84, 92–94]. MSC also blocked maturation of DC, leading to a reduced expression or absence of antigens and co-stimulatory molecules CD40, CD80, and CD86, subsequently necessary to activate T-cells (Figure 1) [84, 93, 95]. This process is at least in part regulated via secretion of IL-6 by MSC [60]. MSC induce the production of IL-10 while suppressing IL-2, IL-12, IFN-γ, and TNF-α by DC, resulting in impaired maturation, migration, antigen capture, and processing [65, 68, 92]. These cytokines are also crucial for the activation of lymphocytes, which was therefore impaired as well. This suggests that MSC may induce a suppressive phenotype of DC which reduced the effector T-cells, but enhanced regulatory T-cell responses [45, 68, 87, 92, 96].

3.6. Monocytes/Macrophages. Monocytes, which can differentiate into tissue macrophages, have a dual role in inflammation, and tissue repair. After MI, two major subsets of macrophages can be found in the heart at different time points. Shortly after MI, the classically activated M1 macrophage (inducible nitric oxide synthase (Nos2, iNOS), MHC Class II, CD80, CD86) is present in the heart, which is strongly associated with the clearing of debris, inflammation, and the production of pro-inflammatory cytokines, such as IL-1β, TNF-α, and IFN-γ [97]. After about five days the more prevalent type has switched to the alternatively activated M2 macrophage (Arginase 1 (Arg1); macrophage mannose receptor (Mrc1, CD206); Macrophage scavenger receptor (Msr1, SR-A, CD204)) [97]. This macrophage subtype has an anti-inflammatory phenotype, reducing the release of pro-inflammatory cytokines, while stimulating cardiac reparative pathways, scar formation, and angiogenesis [10, 97–99]. In the presence of MSC, differentiation of macrophages into the M2 subtype was boosted (Figure 1). Many pathways have been indicated in this process, such as IDO, PGE2, and MSC-derived IL-4 and IL-10 [67, 100–102]. MSC also secrete TGF-β1, which together with PGE2 were found to reduce the production of pro-inflammatory cytokines by the macrophages, such as IL-1β, IL-6, TNF-α, and IFN-γ [101, 102]. Meanwhile, anti-inflammatory cytokine IL-10 was strongly increased, which in turn is said to boost formation of regulatory T-cells [100, 101]. No negative effects on macrophage phagocytosis were observed in the presence of MSC, meaning their debris-clearing functions were still intact [13, 102].

3.7. Neutrophils. Neutrophils kill microorganisms and infected cells by production of reactive oxygen species (ROS) and clearance of the subsequent debris. They are also activated in response to local chemokines and DAMPs after sterile tissue damage, such as MI [68]. Within an hour an influx of neutrophils in the heart is visible and they remain the most prominent cell type for 1-2 days [10]. MSC produce high levels of IL-6, which activates STAT3 transcription factors, resulting in a longer life span of the neutrophils, as indicated in Figure 1 [10]. Although this appears counterintuitive at first, IL-6 also attenuates the neutrophil respiratory burst, so the neutrophils are less harmful to their environment [103]. MSC are able to suppress the degranulation of the enzyme—containing granules of neutrophils. Among others, IDO is found to inhibit the secretion of defensin-α (also known as human neutrophil peptide 1–3), which is stored in secreted granules of the neutrophils and has various pro-inflammatory characteristics which can become cytolytic [104] at high concentrations. The effect of MSC on neutrophil tissue migration remains unclear, with few contradicting reports [103, 105, 106]. PGE2 produced by MSC stimulates monocytes and macrophages to produce IL-10, which can prevent neutrophils entering damaged tissue [77, 100]. Likewise, TGF-β and IL-10 trigger endothelial cells to reduce their E-selectin expression, which is essential for immune cell extravasation [13, 107].

Even though in most literature researchers try to identify one major pathway which regulates immunomodulation, it is more likely, considering the heterogeneity of the MSC and the numerous parallel systems in immunology, that a combination of pathways provide the optimal effect [10].

The effects described above make MSC appear to be an ideal anti-inflammatory effector, but most of these studies have been performed *in vitro* under artificial inflammatory conditions. When considering using these cells against cardiac inflammation, it is necessary to investigate the environment these cells will encounter after injection. Especially when injected or infused in the heart shortly after MI, MSC will be surrounded by an unfavorable pro-inflammatory environment [5, 10]. What the effect is of this inflammatory environment on the functions of MSC and their effects is worth looking into before commencing large scale clinical trials.

4. Effects of TLR-Signaling on MSC

The innate immune system is constantly surveying the body for the presence of so-called "pathogen-associated molecular patterns" (PAMPs), which are detected by highly conserved receptors known as Pattern Recognition Receptors (PRR). Binding of a PAMP to one of these receptors triggers the activation of signaling pathways, ultimately leading to the activation of transcription factors, mainly NF-κB. Subsequently, this leads to inflammatory cell maturation and activation and the production of inflammatory cytokines and chemokines [44, 108, 109]. Toll-like receptors (TLR) are among the best-described receptors of these PRR. TLR are type I transmembrane glycoproteins expressed by many cell types [44, 110, 111]. In addition, intracellular TLR exist which recognize nucleic acids, such as RNA or DNA of pathogens. Receptor activation can control cell surface expression levels, allowing for both positive and negative regulation [112]. In humans, ten different analogues of TLR exist (TLR1-10), while mice express TLR 1–13 [105]. Each receptor is activated by its own specific set of ligands, resulting in the recognition of a wide variety of ligands [108, 113]. In the cell, the TLR-domain interacts

with several adaptor molecules (MyD88, TIRAP, TRIF, and TRAM). Activation of TLR leads to nuclear localization of NF-κB, resulting in the transcription of various chemokines, cytokines, and several genes involved in cell maturation [108, 109]. The resulting immune response is intended to clear the pathogen and activate the repair mechanisms of the injured tissue. Interestingly, TLR do not only get activated in response to pathogen challenges, but also in response to signals released during sterile tissue damage, for example, due to ischemia upon MI [36, 114, 115]. These signals are called damage-associated molecular patterns (DAMPs) and include heat shock protein (HSP) 60 and 70, fibronectin extra domain A (-EDA), uric acid, oxidized LDL, intracellular components of fragmented cells, hyaluronan fragments, members of the S100 protein family, eosinophil-derived neurotoxin, myeloid-related proteins-8 and 14, and human defensin-3 [15, 44, 116].

TLR are not only present on immune cells but on a variety of other cell types, including all cardiac cell types, epithelium, and mesenchymal stem cells [108, 116, 117]. MSC express several TLR and it is essential to determine whether MSC capacities might be altered after stimulation of TLR in response to DAMPs released upon MI [118]. Although there are minor disagreements, it is generally accepted that TLR 1–6 are present on human MSC from different origins, such as the bone marrow, adipose tissue, umbilical cord blood, dental pulp or follicle, and Wharton jerry's MSC [44]. Meanwhile, reports on TLR7-10 are less consistent [44, 108, 109, 119–123]. The presence of TLR7, TLR9, and TLR10 on human bone marrow derived MSC has been reported by some groups, while expression of TLR8 has never been detected [108, 122, 123]. Murine MSC were found to express all TLR mRNA, except for TLR9 [123]. There appear to be only small differences in TLR expression between humans, and mice MSCs. Although this would hint that results might be extrapolated from murine to human studies, one should keep in mind that complex immunological processes might still mechanistically run differently.

Variations in expression between MSC from the same origin can of course be due to donor variations, cell isolation method, culture conditions, and whether RNA or protein expression was measured. For example, Delarosa and Lombardo found that hypoxia caused an increase in expression of TLR 1,2,5,9, and 10 in MSC [108], while Tomchuck et al. did not find any effect of hypoxia on TLR levels [120]. In an inflammatory environment TLR2, 3, and 4 appear to be upregulated on MSC, while TLR6 expression decreases slightly [119]. Of all TLR expressed by MSC, TLR3 and TLR4 have the highest expression, making them an interesting subject for study [109, 121, 123, 124]. Upon TLR-stimulation on MSC, different processes have been studied that could be affected for their functional effects, including migration, proliferation, and differentiation, but also their immunosuppressive potential.

4.1. Proliferation, Differentiation, and Migration. Most studies investigating the effect of TLR stimulation on MSC found little to no effect on proliferation. In two studies, a slight reduction in MSC proliferation was found after TLR9 activation by using CpG-ODN and TLR3 activation by poly(I:C) [113, 125]. Another *in vitro* study demonstrated increased proliferation upon TLR2 stimulation with Pam3Cys, while blocking all differentiation [122]. Unfortunately, these three studies used MSC from different origins, namely, umbilical cord [113], adipose tissue [125], and bone marrow [122], which made direct comparisons harder. *In vivo* murine TLR4 knockout (KO) MSC were found to have a higher proliferation rate than their wild-type (WT) counterparts [118], while MSC propagation from TLR2 KO mice was reduced [126].

In addition to proliferation, differentiation is one of the most important hallmarks of MSC. In these studies contradicting results were obtained with regard to differentiation, ranging from no effect to general suppression of differentiation. [109, 122, 124]. Osteogenic differentiation could be increased by activation through LPS, PGN, Pam3CSK4, and poly(I:C) [125, 127–129]. Chondrogenic differentiation in response to TLR signaling could be decreased via poly(I:C), or increased via Pam3CSK4, or remain unchanged via poly(I:C) and LPS [109, 124, 128]. Lastly, adipogenesis remained undisturbed in many studies, although a few studies showed suppression of differentiation after stimulation of TLR2, TLR3, and TLR4 [109, 113, 122, 124, 125, 127, 128]. Interestingly, TLR3 activation has also once been described to induce adipogenic differentiation [113].

Relatively few studies investigated the effect of TLR stimulation on MSC migration. MSC are known to home damaged tissues, which permits parenteral administration, so changes in migratory capacity could be very important. Studies show that TLR activation with poly(I:C), LPS and CpG-ODN improved MSC migration [120, 124, 130], although this effect was temporal for TLR3 and TLR4 and this was no longer noticeable at 24 h [124].

4.2. Immunomodulation In Vitro. Similar to the other MSC cell processes, contradicting effects of TLR stimulation on immune-modulatory capacities of MSC were reported. Since TLR3 and TLR4 are highly expressed on MSC, most studies focused on them but currently the role of these two molecules in immunomodulation by MSC is still largely unknown. In the few completed studies only T-cell proliferation was investigated as a measure of immunomodulation by MSC, despite the known interactions with nearly all cells of the immune system. TLR4 activation of MSC by LPS reduced immunomodulatory abilities of MSC in a small majority of the studies [109, 119, 124, 131], although other studies found no effect of TLR4 activation [113, 127, 132] and other studies found an improvement in immunosuppression [121, 131]. Of special interest is the study by Tomic et al. [131]. They found that immunosuppression by MSC derived from dental follicle was boosted following LPS exposure, while it was inhibited in dental-pulp-derived MSC. Although both cells fulfilled all the MSC characteristic requirements, the origin of the cell strongly influenced the effect of LPS on the paracrine potential.

Activation of TLR3 by poly(I:C) resulted in the majority of studies in an increase of immunosuppressive capacities of MSC [113, 120, 121, 124, 131], although some studies also reported no effect [127] or a decrease in suppressive capacity [109, 119]. While some claimed that immunosuppressive

pathways, including IDO and PGE2, were induced in BM-MSC [121], other reported these to be reduced [119]. Studies with other ligands indicated that activation of TLR9 can augment immunosuppressive capacities [113], while TLR2 activation appeared to have no effect [122].

In conclusion, there are many inconsistent results regarding the role of TLR on MSC on their immunomodulatory potential. Based on the reports published so far, activation of TLR3 by poly(I:C) might have beneficial effects, while TLR4 activation could slightly decrease immunomodulative functions. It is important to note, however, that the T-cell suppressive capacity of MSC varied strongly between studies (between ±20 and ±80% in untreated conditions) and the effects of TLR activation were often minor, with only few exceptions [124].

4.3. Immunomodulation In Vivo. Naturally the normal physiological environment and the cross-talk between different cell types are absent in *in vitro* studies. To get a better idea of the effect of TLR signaling in MSC after MI, a small number of animal models were examined. Acute ischemia reperfusion injury was induced in a TLR-2 KO rat in an *ex vivo* isolated heart perfusion system [126]. Treatment with wild-type (WT-) MSC improved left ventricular recovery, while TLR-2 KO MSC did not. This is possibly caused by the lower MSC proliferation rate for the TLR-2 KO, as well as a reduction in vascular endothelial growth factor (VEGF) secretion [126]. A second study performed by the same group had a similar set-up, but used a TLR-4 KO rat heart [118]. Cardioprotection was enhanced in the TLR-4 KO heart, mediated by increased activation of STAT-3. These two studies suggested TLR2 presence and activation could be essential for cardiac recovery, while TLR4 activation would have harmful effects. Unfortunately, as this model contained no immune system, the effect of TLR2 and TLR4 activation on immunomodulation by MSC remains unclear. Others used poly(I:C) preconditioning on MSC before injection into a hamster model of heart failure [133]. The TLR3-preconditioned MSC secreted more IL-6, VEGF, hepatocyte growth factor (HGF), and stromal derived factor 1 (SDF-1) and more proliferating CD34/GATA4 positive progenitor cells were found. Meanwhile, infiltrated immune cells were reduced in number and cardiac function was significantly improved [133]. These outcomes correspond with the *in vitro* studies which suggested TLR3 could boost the MSC immunosuppressive potential, while TLR4 has negative influence of cardiac recovery.

It has been observed that cell injections 7 days after MI give slightly (but not significantly) better outcomes [40]. It is as of yet unclear what might be the cause of this. One of the possibilities is of course the reduced release of DAMPs and therefore a different polarization of the MSC. However, several DAMPs, such as HSP70, remain elevated for at least 14 days [134]. Meanwhile, many other factors could also play a role in the improved results. The healing phase will have started after a week. MSC could influence processes at play at this time after MI, such as scar formation and angiogenesis. It is plausible that DAMPs also influence these processes, although this has not yet been investigated.

5. Conclusions

The inflammatory response after MI is essential to initiate reparative pathways and clear debris, yet these activated immune cells cause a lot of short and long term damage to the myocardium. Broad immunosuppressive drugs were only detrimental by reducing both the damaging and healing pathways. Stem cell therapy after MI could improve cardiac function, most likely by the production of paracrine factors. One of the systems influenced by these paracrine factors is the immune system. Basically every immune cell was reported to be affected by MSC in different degrees and subtypes are induced which in turn can influence other immune cell functioning. The mechanisms by which MSC achieve these effects remain unclear, with many groups supporting various effector molecules and pathways. In all studies, however, MSC can influence the immune system via different pathways, thereby having a range of possible effects on their target cells. One of the obvious reasons why different outcomes are still observed is due to the heterogeneity of the MSC and the differences in donor, origin, isolation, culture, and coculture conditions with immune cells. A broad definition for MSC has been defined, but this does not mean all these cells are identical. MSC from different origins can have different capacities and can react differently to similar stimuli [131]. Even when cells are isolated from identical origins according to a strict protocol, strong variations still exist between donors [109, unpublished own observations]. Likewise the timing, concentration, and duration of the stimulation with TLR-ligands can influence observed effects, and the outcome after one hour of stimulation might be entirely different from results after a day of stimulation [124].

Additionally, immunosuppression assays show a lot of variation. Some groups worked with peripheral blood mononuclear cells (PBMC), while others worked with isolated fractions of CD3+ or CD4+ T-cells. It is difficult to compare these results directly with each other, for in a PBMC mixture many other immune cells are present which influence their environment, as shown in Figure 1. Add to this that the immune-suppressing effects on PBMC or T-cell proliferation in the untreated groups varied strongly between groups, it becomes clear that universal protocols are needed to perform this type of assays. In addition, the experiments need to be performed with various different MSC and immune cell donors to make the outcomes more robust.

Although a great effort has been undertaken to identify the effects of TLR activation on MSC, many inconsistencies still remain. Despite the many contradicting reports, some similarities can be found and some clues provided insights into possible mechanisms. Many groups have established the expression of TLR by MSC, although at protein level they are sometimes hard to detect. The effect of TLR activation on proliferation is probably minimal, while differentiation can be interfered with. Although few studies have looked at migration, improved migration might help honing in immunomodulative stem cell therapy and should be investigated further. Initial reports indicate an increase in migration, at least in the acute phase [124]. Regarding the immunomodulatory capacities of MSC, much ambiguity remains. *In vitro*

and *in vivo* work seem to indicate that TLR3 activation with poly(I:C) can boost the immunosuppressive potential of MSC, while TLR4 activation with LPS could reduce it. Experimental studies showed TLR2 and TLR4 become activated after MI and are correlated to ischemia-reperfusion injury and LV dysfunction [135–137]. The TLR4 activation can create an unfavorable environment for MSC, reducing their effectiveness as immunomodulatory therapeutics after MI. This in turn would make preconditioning of MSC by using TLR3 ligands to boost immunomodulation an interesting target. These divergent effects of TLR3 and TLR4 signaling have prompted Waterman et al. to suggest MSC can be polarized into inflammatory and anti-inflammatory subtypes by differential TLR activation [124]. However, due to the many contradictory findings, more research will be necessary to validate this hypothesis.

The vast majority of the studies discussed in this review did not focus on cardiac inflammation, but on auto-immune diseases or organ transplantation. This justifies the work with PAMPs, as the main concern will be an infectious threat to a patient with a suppressed immune system. In the setting of inflammation after myocardial infarction, the inflammatory signals consist of DAMPs. It is unlikely that DAMPs and PAMPs activate the same receptors in exactly the same way. There are likely more receptors (PRRs) on the MSC that can recognize these ligands and it could very well be a combined activation of receptors that leads to the activation of a specific pathway in the cell, which could differ between PAMPs and DAMPs. To study the effectiveness of MSC therapy for post-MI inflammation, it would be advisable to investigate the effect of TLR activation on MSC using DAMPs that are released after MI. Only by investigating it this way can the role of TLR activation on MSC in the cardiac setting be truly elucidated.

Abbreviations

MSC:	Mesenchymal stem cell
T_H:	T-helper cell
T_C:	Cytotoxic T-cell
T_{reg}:	Regulatory T-cells
iDC:	Immature dendritic cell
DC:	Dendritic cell
Mo:	Monocyte
M1:	Classically activated type 1 macrophage
M2:	Alternatively activated type 2 macrophage
PMN:	Polymorphonuclear cells
NK-cell:	Natural killer cell
IDO:	Indoleamine-pyrrole 2,3-dioxygenase
PGE2:	Prostaglandin E2
PD-1:	Programmed death 1
PD-L1/2:	Programmed death ligand 1 or 2
TGF-β:	Transforming growth factor β.

Conflict of Interests

The authors declare they have no conflict of interest.

Acknowledgments

This work was supported by a grant from the Alexandre Suerman Program for MD/PhD students of the University Medical Center Utrecht, the Netherlands, and the Netherlands Cardiovascular Research Initiative: the Dutch Heart Foundation, Dutch Federation of University Medical Centers, the Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 260464, 8 pages
http://dx.doi.org/10.1155/2013/260464

Research Article

Berberine Protects against Palmitate-Induced Endothelial Dysfunction: Involvements of Upregulation of AMPK and eNOS and Downregulation of NOX4

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Received 23 July 2013; Revised 12 September 2013; Accepted 10 November 2013

Academic Editor: Claudia Monaco

Endothelial dysfunction is a critical factor during the initiation of cardiovascular complications in diabetes. Berberine can ameliorate endothelial dysfunction induced by diabetes. However, the underlying mechanisms remain unclear. The aim of this study was to investigate the protective effect and mechanism of berberine on palmitate-induced endothelial dysfunction in human umbilical vein endothelial cells (HUVECs). The cell viability of HUVECs was determined by MTT assays. Nitric oxide (NO) level and production of reactive oxygen species (ROS) were determined in supernatants or in the cultured HUVECs. The mRNA level of endothelial nitric oxide synthase (eNOS) was measured by RT-PCR, and the protein levels of eNOS, p-eNOS, Akt, p-Akt, AMPK, p-AMPK, and NADPH oxidase (NOX4) were analyzed. The results demonstrated that berberine significantly elevated NO levels and reduced the production of ROS. The expressions of eNOS were significantly increased, while NOX4 protein expression was decreased in berberine-treated HUVECs. Moreover, berberine upregulated the protein expression of AMPK and p-AMPK in palmitate-treated HUVECs, but had no effect on the levels of Akt. Therefore, berberine ameliorates palmitate-induced endothelial dysfunction by upregulating eNOS expression and downregulating expression of NOX4. This regulatory effect of berberine may be related to the activation of AMPK.

1. Introduction

Cardiovascular complications are main causes of high mortality and morbidity induced by obesity, diabetes, and metabolic syndrome. Endothelial dysfunction has been known as a critical factor and main pathological change during the development of vascular complication [1]. Lipid metabolic disorder plays a vital role in the pathogenesis of endothelial dysfunction in obesity, insulin resistance, and diabetes. An abnormality in patients with all of these disorders is an increase in the plasma concentration of free fatty acids (FFA) [2]. Elevated FFA may cause a series of pathophysiological changes in the endothelium, including endothelial nitric oxide synthase (eNOS) uncoupling, intracellular accumulation of reactive oxygen species (ROS), and cell apoptosis, which in turn contribute to accelerating the endothelium dysfunction associated with excessive acceleration of atherosclerosis. Studies showed that high concentration of FFA impair the eNOS activity and reduce the production and bioactivity of NO in endothelial cells. FFA overload attenuates Ca^{2+} signaling and eNOS activity, reduces NO production, and indirectly leads to endothelial dysfunction in endothelial cells [1]. Ye-rong found that elevated FFA could inhibit eNOS phosphorylation and its gene expression, decrease endothelium-derived NO production, and thus lead to an impairment of vasodilation in metabolic syndrome [3].

Moreover, FFA-induced endothelium dysfunction is related to the activity of NADPH oxidase, the most important enzyme for the production of O_2^- within the vascular wall. As O_2^- inactivates NO to form peroxynitrite ($ONOO^-$), it triggers a series of harmful events such as decreasing NO bioavailability, reducing the production of NO, and causing impaired vasodilatation [4]. Inoguchi et al. reported high glucose level and FFA (palmitate) stimulate ROS production through PKC-dependent activation of NAD(P)H oxidase in cultured aortic smooth muscle cells and endothelial cells, which in part accounted for the excessive acceleration of atherosclerosis in patients with insulin resistance and diabetes [5]. Elevated FFAs not only inhibit the eNOS/NO signal pathway and decrease NO production, but also activate NADPH oxidase, increase production of O_2^-, and reduce NO bioactivity during the development of atherosclerosis and thrombosis in vascular complications associated with obesity and diabetes. As matter of relevance, it also has been established that impaired eNOS activity upon palmitate stimulation may be linked to toll like receptor 4 (TLR4) signaling, which is a critical mediator of palmitate-induced IKKβ and NF-κB activation, and subsequent decreases in insulin signaling and NO production in endothelial cells [6, 7].

Decreasing lipotoxicity may be a key component to prevent and treat cardiovascular complications of metabolic syndrome. *Rhizoma Coptidis* (root of *Coptis chinensis* from Ranunculaceae) has been used in traditional Chinese medicine for more than 1000 years. Berberine, an isoquinoline alkaloid, a major active component of *Rhizoma Coptidis* [8], has been well reported with pleiotropic pharmacological activities, including antibacterial, antibiotic, anti-inflammatory, and antioxidant properties, as well as ameliorating effects on hyperlipidemia and hyperglycemia. Recently, both animal and clinical studies have demonstrated that berberine improves insulin resistance, decreases blood glucose levels, regulates lipid metabolism, and inhibits the progression of obesity and diabetes [8–11]. Whether berberine can improve endothelium dysfunction and prevent the cardiovascular complications associated with these disorders causes a great interest to researchers. Tang et al. reported that berberine had antioxidant effects and could increase the protective effect on diabetic complications [12]. Hao et al. demonstrated that berberine ameliorates diabetic microendothelial injury induced by the combination of high glucose and advance glycation end products *in vitro* [13]. Our previous study indicated that berberine not only modulates glucose and lipid metabolism but also ameliorates endothelial dysfunction in diabetic rats induced by high fat diet combined with streptozotocin injection. However, the underlying mechanism through which berberine improves the endothelial dysfunction to prevent the vascular complications in obesity and diabetes mellitus is still unclear. There has been no report about the effect of berberine on the lipotoxicity in the endothelium dysfunction yet. Therefore, the present study was to elucidate the protective effects and underlying mechanism of berberine on endothelial dysfunction induced by high doses of palmitate, which could provide evidence for berberine's clinical applications in the future.

2. Materials and Methods

2.1. Materials. Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC, USA). RPIM-1640 medium and other culture reagents were obtained from Gibco Life Technologies (Gibco, Grand Island, NY, USA). Berberine was kindly provided by Northeast General Pharmaceutical Factory (Changchun, China). Palmitate, N^G-nitro-L-arginine (L-NA), 2,7-dichlorodihydrofluorescein diacetate (DCHF-DA), and thiazolyl blue (MTT) were purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA). Bovine serum albumin (BSA, fatty acid free) was purchased from Wako pure chemical industries (Japan). Kit for measuring NO was provided from Nanjing Jiancheng Chemical Factory (Nanjing, China). eNOS primers were synthesized by Lianxing Biotechnology (Dalian, China). Polyclonal antibodies of eNOS, Akt, AMPK, and NOX4 were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA). Chemical agents for western blot and RT-PCR were obtained from Sigma Aldrich. All other chemical reagents were purchased from commercial source.

2.2. Cell Culture. HUVECs were cultured in RPIM-1640 medium supplemented with 10% fetal bovine serum (FBS) and passaged according to the recommended procedures of ATCC. Cells in passages 4–8 were used for experiments; cells were exposed to exogenous free fatty acid (0.5 mmol/L palmitate) for 12 h or 24 h treated with or without different concentrations of berberine (1.25, 2.5 and 5 μmol/L).

2.3. Preparation of Free Fatty Acid-Albumin Complexes. Lipid-containing media were prepared by conjugation of FFA to BSA using a modified method described [14]. Briefly, palmitate was dissolved in 0.1 M NaOH solution in 70°C water bath for fully dissolving. Then the 0.1 M sodium palmitate was mixed with 5% fatty acid-free BSA at 1 : 9 ratio and left for one hour in 37°C incubator. 10 mM palmitate stock solution was stored at −4°C. Before experiment, the stock solution was diluted in the complete culture medium to the required concentration, adjusted to a pH value of 7.5, and filter sterilized. The control solution containing fatty acid-free BSA was prepared in the same way.

2.4. Cell Viability Assays. The viability of the HUVECs was determined by MTT assays. Briefly, cells were plated for 24 h in a 96-well plate at a density of 1×10^4 cells per well in 200 μL medium. When cells grew to 60% to 70% confluence, the medium was changed to one containing 2% FBS and 0.5 mmol/L palmitate (sovled in 4.5% free fatty acid free BSA) or treated with different concentrations of berberine (1.25, 2.5, and 5 μmol/L). Each treatment was repeated in 6 wells. The cells were incubated for 20 h at 37°C in a humidified chamber. MTT reagent (20 μL, 5 mg/mL in PBS) was added to each well and incubated for 4 h. The microplate containing the cells was centrifuged at 1,800 rpm for 5 min at 4°C. The MTT solution was removed from the wells by aspiration. The formazan crystals were dissolved in 150 μL

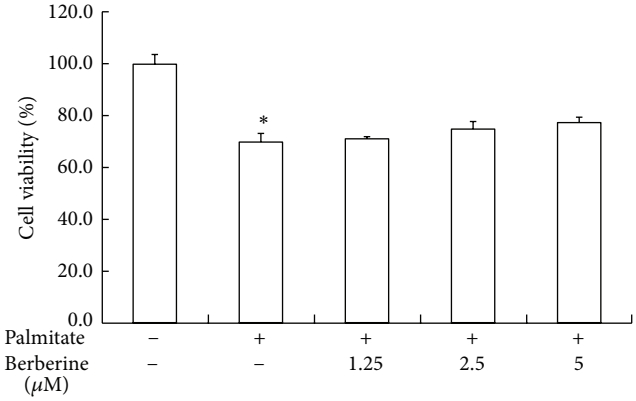

Figure 1: The effects of berberine on viability of HUVECs exposed to palmitate. HUVECs were cultured in RPMI-1640 containing 0.5 mmol/L palmitate and treated with 1.25, 2.5, and 5 μmol/L berberine for 24 h. Cell viability was measured by MTT assay and normalized to cells incubated in control medium. Data were expressed as mean ± S.E.M. $^*P < 0.05$ versus control.

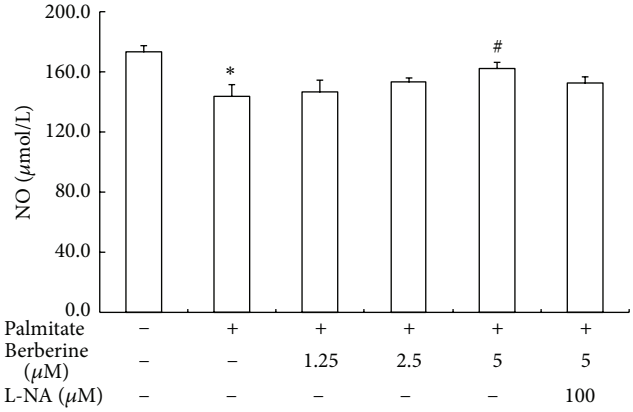

Figure 2: The effects of berberine on NO production in HUVECs exposed to palmitate. HUVECs were cultured in RPMI-1640 containing 0.5 mmol/L palmitate and treated with 1.25, 2.5, and 5 μmol/L berberine for 24 h. The control group was not stimulated with 0.5 mmol/L palmitate. L-NA: N^G-nitro-L-arginine. Data are expressed as mean ± S.E.M. $^*P < 0.05$ versus control group, $^\#P < 0.05$ versus palmitate treated group.

DMSO. Absorbance was recorded at 570 nm wavelength using a Microplate Reader. Cell viability was calculated as follows: cell viability (100%) = absorbance of experiment group/absorbance of control group × 100%.

2.5. Measurement of NO Level. HUVECs were grown in 96-well dishes. When cells grew to 60% confluence, the medium was changed to one containing 2% FBS. The cells were treated with 0.5 mmol/L palmitate and various concentrations of berberine dissolved in ethanol for 24 h. The same volume of ethanol was included in each control group. NO release in cultured supernatants was determined by the Griess method [15].

2.6. Measurement of ROS Level. HUVECs were plated in 24-well dishes at a density of 6×10^4 cells per well in 500 μL of complete medium. When cells grew to 60% confluence, the medium was changed to one containing 2% FBS. The cells were treated with 0.5 mmol/L palmitate and various concentrations of berberine for 12 h. 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA, 10 μM, Sigma) staining was employed for ROS analysis as described previously [16].

2.7. RNA Extraction and Semiquantitative RT-PCR. Total RNA was extracted from HUVECs using Trizol reagent (Invitrogen). RNA samples were quantified by spectrophotometry, and the integrity was assured by 1.5% agarose gel electrophoresis and ethidium bromide staining. The first-strand cDNAs were synthesized from 5 g total RNA, using SuperScript reverse transcriptase and oligo deoxythymidine primers. The reverse transcription products were amplified by PCR, using Taq DNA polymerase and specific primers for Human eNOS (forward: 5′-GTGATGGCGAAGCGAGTGAAG-3′; reverse: 5′-CCGAGCCCGAACACACAGAAC-3′, 422 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, forward: 5′-CCATGGAGAAGGCTGGG-3′; reverse: 5′-CAAAGTTGTCATGGATGACC-3′, 194 bp). The cycling conditions were 94°C melting, 60°C annealing, and 72°C extensions for 30 sec (30 cycles for eNOS and 28 cycles for GAPDH). The amplification conditions were optimized in preliminary studies to result in amplification within the linear range. PCR products were visualized on 1.5% agarose gels by ethidium bromide staining and gels were photographed under UV light. Relative gene expression was quantified by being densitometrically analyzed using image software. GAPDH transcript abundance was considered as an internal control to which eNOS transcript abundance was normalized.

2.8. Western Blot Analysis. Protein samples were prepared from cultured HUVECs with ice-cold cell protein lysates. Protein concentrations were measured using Bradford assay (Bio-rad protein assay kit). The protein samples (60 μg) were denatured by boiling for 5 min, separated by 10% SDS-polyacrylamide gel, and then electroblotted at 4°C and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were blocked in 5% (w/v) nonfat milk for 2 h at room temperature and then incubated with rabbit polyclonal antibodies (eNOS, 1 : 800; Akt, 1 : 1000; AMPK, 1 : 1000; NOX4, 1 : 1000, Santa Cruz Biotechnology) with gentle agitation overnight at 4°C. The membranes were washed 3 times for 10 min each with 15 mL of TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.1% (v/v) Tween-20) and then incubated with the second antibody (1 : 1000 goat Anti-rabbit IgG Horseradish Peroxidase Conjugate, Santa Cruz Biotechnology) at room temperature for 2 h. The protein was then visualized with enhanced chemiluminescence solution and X-ray film. To correct for differences in protein loading, the membranes were washed and reprobed with 1 : 2000 dilution goat polyclonal antibody to actin (Santa Cruz Biotechnology). An imaging densitometer was used to scan the protein bands and quantify them using the image analysis software.

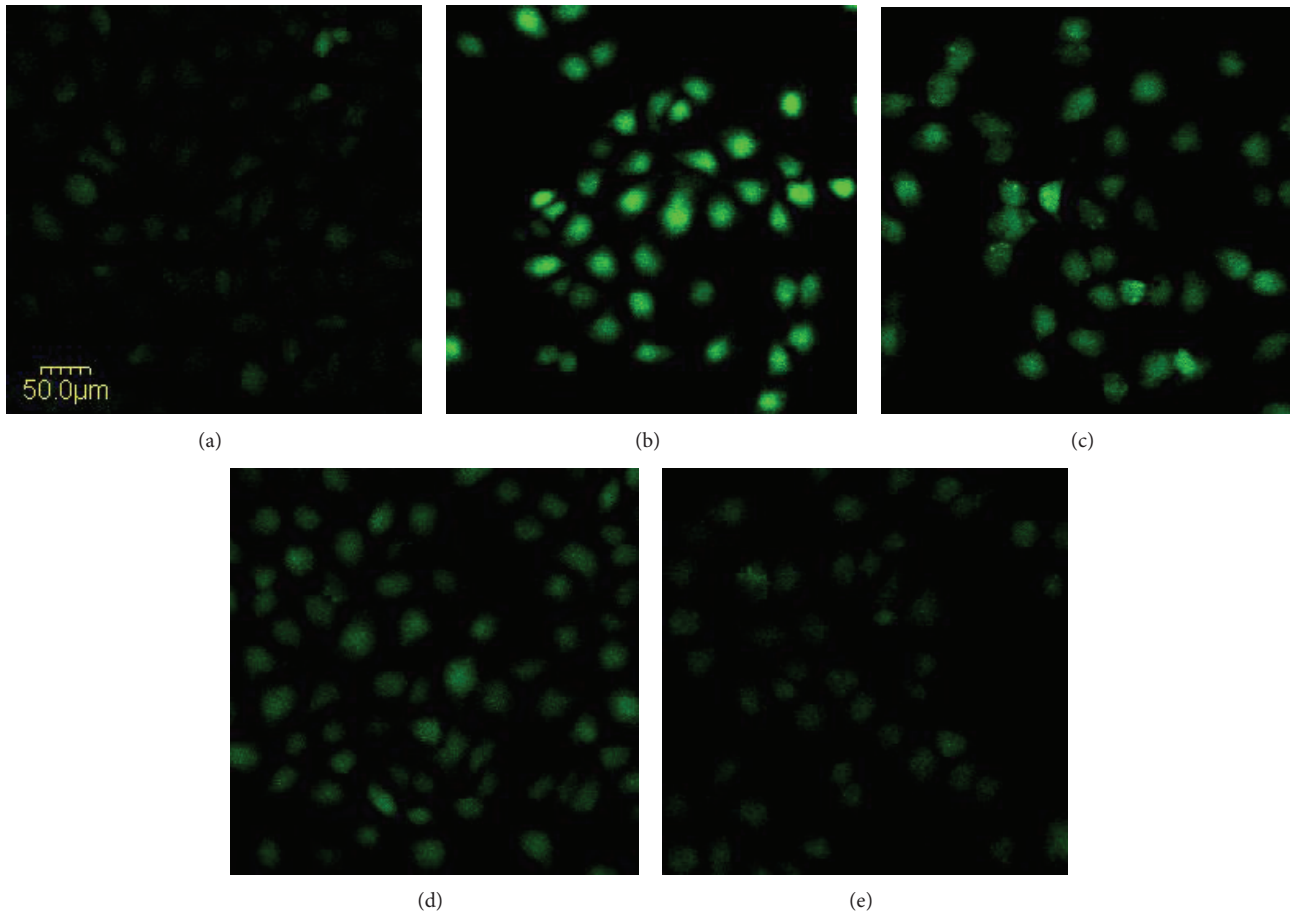

Figure 3: The effects of berberine on ROS in HUVECs exposed to palmitate. HUVECs were cultured in RPMI-1640 containing 0.5 mmol/L palmitate and treated with 1.25, 2.5, and 5 μmol/L berberine for 12 h. HUVECs were labeled with DCFH-DA for 20 min and ROS generation was analyzed by fluorescence detection with Confocal microscopy at 200x. (a) HUVECs were cultured in RPMI 1640 without palmitate; (b) HUVECs were stimulated by 0.5 mmol/L palmitate; ((c), (d) and (e)) HUVECs were stimulated by 0.5 mmol/L palmitate and treated with 1.25, 2.5, and 5 μmol/L berberine, respectively.

2.9. Statistical Analysis. All data were expressed as mean ± S.E.M. The "n" denoted the sample size in each group. The statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test. SPSS software (version 13.0 for Windows) was used for the statistical analysis. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of Berberine on HUVECs Viability. HUVECs viability in the palmitate treated group fell to 70.03 ± 3.06% compared with that in the group without palmitate treatment. After berberine (1.25~5.0 μmol/L) treatment in HUVECs, the cell viability was increased from 71.27 ± 3.05% to 77.27 ± 2.70% (Figure 1). Although this difference did not reach statistical significance, but the increased trend could be observed. In preliminary experiments, we found that higher concentrations of berberine (>5.0 μmol/L) induced toxicities to HUVECs (data not shown). Accordingly, different concentrations of berberine (1.25~5.0 μmol/L) were selected in the following studies.

3.2. Effect of Berberine on NO Levels in Cultured Medium of HUVECs. HUVECs cultured with 0.5 mmol/L palmitate displayed a remarkable decrease in NO release compared with that of HUVECs without palmitate treatment (Figure 2). Berberine treatment significantly increased NO release as compared with untreated palmitate HUVECs. Berberine 5.0 μmol/L had the strongest effect on NO release. eNOS inhibitor L-NA partially inhibited the effect of berberine on NO release. These results suggest that palmitate could reduce NO synthesis and release in cultured HUVECs, while berberine could significantly rescue the NO production which might be related to eNOS, the key enzyme of NO synthesis in endothelial cells.

3.3. Effect of Berberine on ROS in HUVECs. As shown in Figure 3, the green fluorescent intensity in HUVECs cultured with palmitate was significantly enhanced compared with that in the control group, which suggested that intracellular ROS levels in palmitate stimulated HUVECs were markedly increased. Berberine treatment decreased intracellular fluorescence intensity in a dose-dependent manner compared

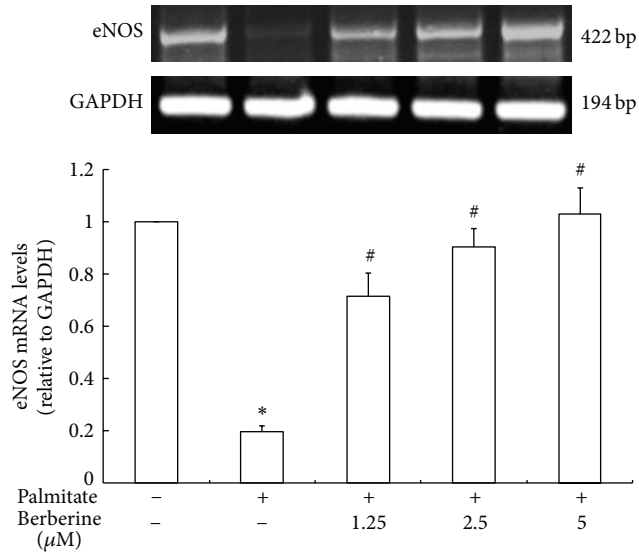

Figure 4: The effects of berberine on eNOS mRNA expression in HUVECs exposed to palmitate. HUVECs were cultured in RPMI-1640 containing 0.5 mmol/L palmitate and treated with 1.25, 2.5, and 5 μmol/L berberine for 24 h. Total RNA was extracted and RT-PCR was performed. All values were normalized to 100% for the value of the control and were expressed as the percentage of the control. Data are mean ± S.E.M. The control group was not stimulated with 0.5 mmol/L palmitate. All presented results are representative of at least 3 independent experiments. $^{*}P < 0.05$ versus control group. $^{\#}P < 0.05$ versus palmitate treated group.

with palmitate group. These results indicate that palmitate could stimulate significantly the increase in ROS production and release from HUVECs, which might be related to cell injury caused by oxidative stress. It was believed that berberine treatment could reduce the production of ROS induced by high palmitate cultured HUVECs and play a protective effect on endothelial cells.

3.4. Effect of Berberine on eNOS mRNA Expression. Palmitate treatment significantly reduced eNOS mRNA expression in HUVECs versus control group (Figure 4), while intriguingly, berberine treatment can rescue the eNOS mRNA in palmitate-HUVECs in a dose-dependent manner ($P < 0.05$).

3.5. Effect of Berberine on Signaling of eNOS, Akt, and AMPK. To investigate whether berberine treatment could activate eNOS and its upstream kinase, Akt and AMPK in cultured HUVECs, eNOS, Akt, and AMPK protein expression were analyzed by western immunoblotting (Figure 5). The protein expression of eNOS and phosphorylation of eNOS were significantly reduced in HUVECs stimulated by palmitate compared to that of controls cells (Figure 5(a)). Berberine significantly increased eNOS and phosphorylation of eNOS protein expression. Compared with the group without palmitate, protein expression of total Akt had no significant change in HUVECs cultured with palmitate, while phosphorylation of Akt expression markedly decreased. Berberine treatment did not change the expression of Akt and p-Akt in HUVECs stimulated by palmitate (Figure 5(c)). However, the protein expression of AMPK, another upstream kinase of eNOS in endothelial cells, was significantly lowered in HUVECs cultured with palmitate compared with control group (without palmitate). Berberine increased not only the protein expression of total AMPK but also the phosphorylation of AMPK (Figure 5(b)). These results indicate that palmitate could downregulate eNOS expression in cultured HUVECs. Berberine treatment could reverse this change which might contribute to the activation of AMPK, promoting eNOS phosphorylation. While Akt/eNOS signaling pathway might not be involved.

3.6. Effect of Berberine on Protein Expression of NOX4. In contrast to eNOS expression, NOX4 protein expression, a main subunit of NADPH oxidase in vascular endothelium, was markedly enhanced in HUVECs stimulated by palmitate (Figure 6). Berberine treatment decreased the protein expression of NOX4 in HUVECs cultured with palmitate compared with control group (without palmitate). It suggests that berberine could reduce ROS levels by downregulating NOX4 expression.

4. Discussion

Increased oxidative stress and reduced NO bioavailability are important contributing factors and are closely related with inflammatory signaling pathways such as toll like receptor 4 signaling in the pathogenesis of endothelial dysfunction, hypertension, and cardiovascular and renal diseases [17]. Our previous studies showed that berberine restored endothelial vasodilation function under diabetic condition by enhancing NO bioavailability. In the present study, the direct effect of berberine on NO and ROS production was further observed in palmitate-induced endothelial injury of HUVECs. The results showed that the survival rate of HUVECs cultured with 0.5 mM palmitate for 24 h was significantly decreased. Moreover, berberine treatment significantly increased NO content in the supernatant of HUVECs cultured with palmitate. These results indicated that the protective effect of berberine on endothelial dysfunction induced by FFA might be associated with the elevation of NO levels.

eNOS is a key enzyme that produces NO in vascular endothelial cells. Studies on endothelial cells have demonstrated that FFA elevation in the culture medium can significantly decrease the activity of eNOS [18]. Palmitate and oleic acid could inhibit the phosphorylation of eNOS ser1177 sites, in turn reduce the NO production, which may contribute to endothelial dysfunction and the occurrence of atherosclerosis [18]. Healthy SD rats were administered fat emulsion and heparin in intravenous infusion; the elevated FFA level inhibited eNOS activity and expression and reduced endothelium-derived NO production in turn [19]. It has been reported that endothelium-derived NO production was mediated by Akt/eNOS signaling pathway [20]. In accordance with these findings, our results showed that palmitic acid significantly decreased the expression of eNOS in cultured HUVECs. Berberine treatment upregulated the expression of eNOS mRNA and total protein and promoted the phosphorylation

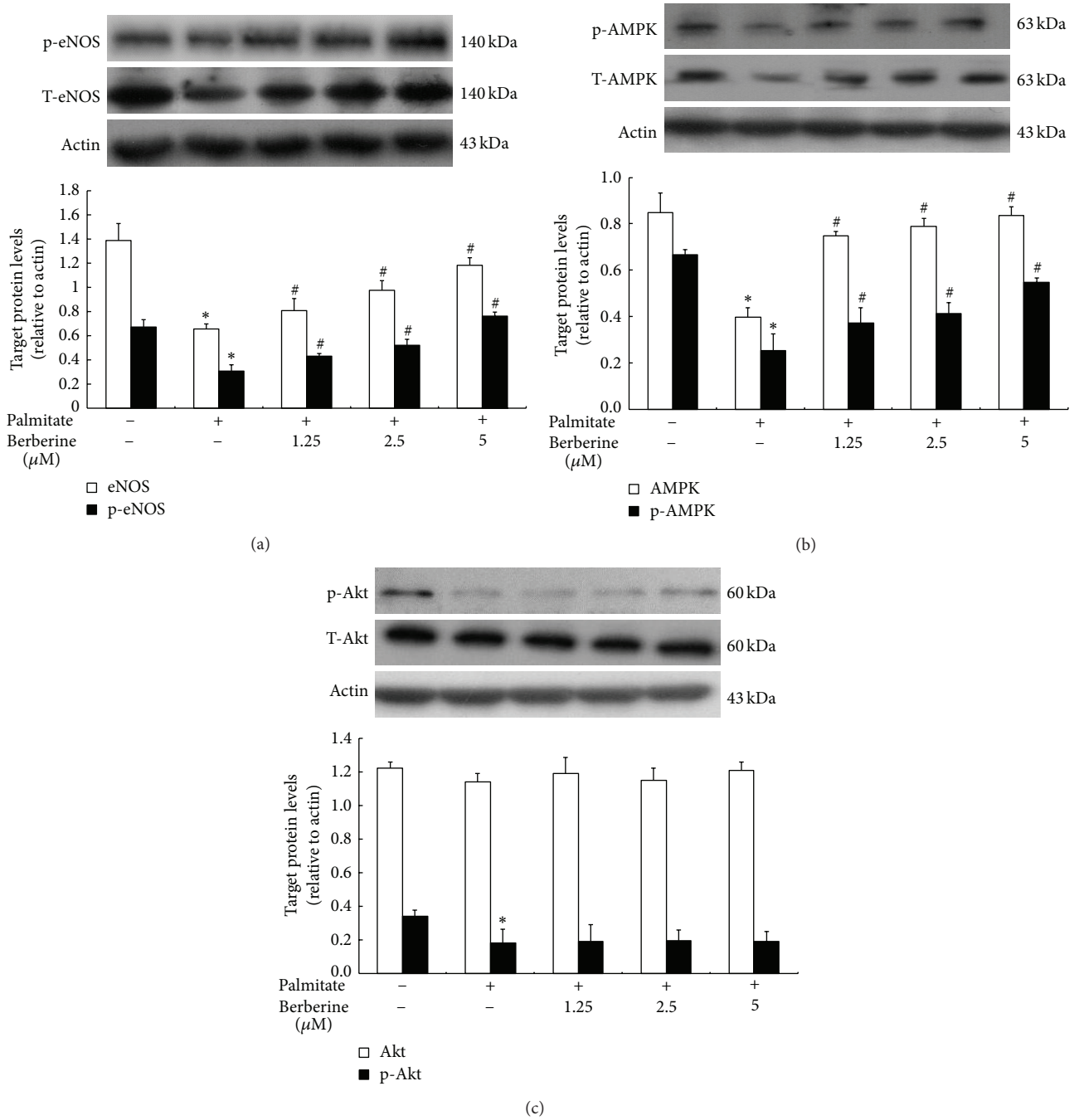

Figure 5: The effects of berberine on protein expression of eNOS, p-eNOS, Akt, p-Akt, AMPK, and p-AMPK in HUVECs exposed to palmitate. HUVECs were cultured in RPMI-1640 containing 0.5 mmol/L palmitate and treated with 1.25, 2.5, and 5 μmol/L berberine for 24 h. Total protein was prepared and separated by SDS-PAGE. Expression and relative quantification of eNOS, Akt, and AMPK and phosphorylation of eNOS, Akt, and AMPK protein levels were expressed relative to the amount of actin. Control group was not stimulated with 0.5 mmol/L palmitate. Data are mean ± S.E.M. All presented results are representative of at least 3 independent experiments. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus palmitate treated group.

of eNOS at ser1177 sites, thereby increasing the NO synthesis. Moreover, L-NA, one of eNOS inhibitors, partially attenuated NO production stimulated by berberine. Furthermore, it can be seen in the present study that palmitic acid remarkably reduced the phosphorylation of Akt in HUVECs. However, berberine did not affect the expression of Akt and its phosphorylation in HUVECs cultured with palmitic acid. It might indicate that berberine might exert its regulatory effect on eNOS activity by other ways.

Adenosine monophosphate-activated protein kinase (AMPK), as an intracellular energy receptor, has attracted more attention and become a new target for the treatment

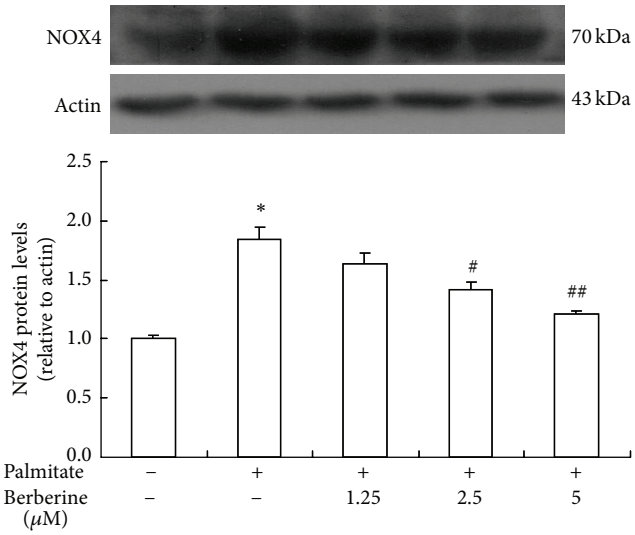

FIGURE 6: The effects of berberine on protein expression of NOX4 in HUVECs exposed to palmitate. HUVECs were cultured in RPMI-1640 containing 0.5 mmol/L palmitate and treated with 1.25, 2.5, and 5 μmol/L berberine for 24 h. Total protein was prepared and separated by SDS-PAGE. Expression and relative quantification of NOX4 protein levels were expressed relative to the amount of actin. Control group was not stimulated with 0.5 mmol/L palmitate. Data are expressed as mean ± S.E.M. All presented results are representative of at least 3 independent experiments. $^{*}P < 0.01$ versus control group, $^{\#}P < 0.05$ versus palmitate treated group, and $^{\#\#}P < 0.01$ versus palmitate treated group.

of diabetes and its cardiovascular complications due to its regulatory effect on endothelial cell function and energy homeostasis in recent years. AMPK plays an important role in regulating function of NO synthesis signaling pathways in endothelial cells. AMPK, an upstream kinase of eNOS, promotes the phosphorylation of eNOS Ser1177 site. AMPK can promote the formation of eNOS and HSP90 complex as well, thereby activating eNOS [17, 21]. Our results showed that berberine could significantly upregulate the expression levels of AMPK and p-AMPK protein of HUVECs cultured with palmitic acid, but had no effect on the expression of Akt and p-Akt protein. Accordingly, we speculated that the regulatory effect of berberine on eNOS activity and NO production might be related to the activation of AMPK partially.

Furthermore, endothelial dysfunction is also correlated with the production of ROS in vascular endothelial cells besides the decrease of NO production. It has been broadly accepted that elevated ROS levels are primarily derived from the action of NADPH oxidase (NOX). Activation of NOX could improve the ROS formation and contribute to endothelial dysfunction [22]. NOX4 is a subtype of NADPH oxidase expressed mainly in vascular endothelial cells, and it is the main source of O_2^- production in the endothelial cells. In the present study, ROS production and NOX4 protein expression were measured in cultured HUVECs. Our results showed that palmitic acid significantly increased ROS production and the expression of NOX4 protein in cultured HUVECs. While berberine reduced ROS production and decreased protein expression of NOX4. These results are consistent with our previous findings from the diabetes animal models.

Several lines of studies indicated that AMPK is an important inhibitor of NADPH oxidase in cardiovascular cells. The activation of AMPK reduced ROS production by inhibiting the activity of NADPH oxidase and finally prevented the endothelial cell apoptosis induced by palmitic acid [23]. AMPK activators such as metformin may exert their cardiovascular protective function through NOX inhibition [24]. AMPK activation suppresses NOX activity may either block NOX phosphorylation and translocation to cell membrane or inactivate transcription factors including NF-κB and STAT [25]. Therefore, berberine might prevent endothelial dysfunction from FFA-induced ROS generation by activation of AMPK. Taken together, berberine inhibits eNOS activation and NOX4-derived ROS accumulation in the HUVECs treated with FFA through AMPK activation, which may contribute to the protective effects of berberine on endothelial function. However, in our experiments, the specific inhibitor of AMPK was not used. It was still unknown whether the effect of berberine on the regulation of eNOS and NOX4 can be blocked by AMPK inhibitor, which should be explored to clarify the molecular mechanisms in the future studies. Furthermore, it has been reported that berberine could inhibit the TLR4-NFκB pathway in LPS-induced intestinal injury in mice, a pathway involved in the impairment of eNOS expression and NO production. This might be another mechanism involved in the protective effect of berberine on endothelial dysfunction and still needs further investigation [26].

In summary, the present study investigated the protective effect of berberine on the vascular endothelial function in cell injury model induced by palmitic acid incubation, and revealed the underlying mechanism through which berberine can significantly ameliorate the endothelial dysfunction. Berberine could upregulate eNOS expression, enhance eNOS activity, and promote NO production. Meanwhile, berberine could downregulate NADPH oxidase expression and inhibit its activity to reduce ROS production and then inhibit NO inactivation as well. Thereby, berberine treatment could enhance the biological activity of NO to protect the vascular endothelial cell function. In our study, we also found that the regulatory effect of berberine on eNOS and NADPH oxidase activity may be related to the activation of AMPK. These results provide an important theoretical evidence for the application of berberine in the prevention and treatment of obesity, diabetes, and their cardiovascular complications.

Authors' Contribution

Ming Zhang and Chun-Mei Wang equally contributed to the paper.

Acknowledgments

The research in this study was supported by a Grant from National Natural Science Foundation of China (81170745, 81200598) and Opening Project of State Key Laboratory of

Supramolecular Structure and Materials of Jilin University under Grant no. SKLSSM201317.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 206039, 13 pages
http://dx.doi.org/10.1155/2013/206039

Review Article

Danger Signals in the Initiation of the Inflammatory Response after Myocardial Infarction

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Received 4 October 2013; Accepted 4 November 2013

Academic Editor: Stefan Frantz

During myocardial infarction, sterile inflammation occurs. The danger model is a solid theoretic framework that explains this inflammation as danger associated molecular patterns activate the immune system. The innate immune system can sense danger signals through different pathogen recognition receptors (PRR) such as toll-like receptors, nod-like receptors and receptors for advanced glycation endproducts. Activation of a PRR results in the production of cytokines and the recruitment of leukocytes to the site of injury. Due to tissue damage and necrosis of cardiac cells, danger signals such as extracellular matrix (ECM) breakdown products, mitochondrial DNA, heat shock proteins and high mobility box 1 are released. Matricellular proteins are non-structural proteins expressed in the ECM and are upregulated upon injury. Some members of the matricellular protein family (like tenascin-C, osteopontin, CCN1 and the galectins) have been implicated in the inflammatory and reparative responses following myocardial infarction and may function as danger signals. In a clinical setting, danger signals can function as prognostic and/or diagnostic biomarkers and for drug targeting. In this review we will provide an overview of the established knowledge on the role of danger signals in myocardial infarction and we will discuss areas of interest for future research.

1. Introduction

In 1994, Matzinger postulated a theory that the immune system may not be evolved to distinguish between self and non-self, but rather sense "danger" [1]. Danger signals, besides pathogen associated molecular patterns (PAMPs), can be intracellular molecules that are normally not exposed to the immune system, for example, cardiac myosin and mitochondrial DNA, but also proteins that are only upregulated during injury, such as heat shock proteins (HSP). Danger signals can therefore be divided into constitutive and inducible. Furthermore, danger signals can be classified as truly primal initiators, which do not require previous activation of antigen presenting cells (APC) or positive feedback signals, which can amplify or convert an ongoing inflammatory response [2]. This danger model explains the inflammatory response following myocardial infarction (MI), a situation where danger associated molecular patterns (DAMPs), and not pathogens, activate the immune system. For instance, extracellular matrix breakdown products released by the damaged myocardium and constituents of dying cardiomyocytes serve as danger signals in the infarcted myocardium, activating an inflammatory reaction. A certain amount of inflammation is required for proper healing and scar formation of the damaged myocardium. However, a prolonged presence of active leukocytes can be deleterious for the injured heart and can ultimately result in heart failure.

In the last decades we gained a lot of knowledge about danger signals, their receptors, and signaling pathways in different disease models. Also the inflammatory reaction in the heart is intensively studied and many DAMPs and their signaling pathways have been elucidated. Nevertheless, the precise actions of certain DAMPs in the heart remain unknown. In this review we will shortly address the concept of the danger model with its modulators and receptors. Subsequently, we summarize the current knowledge on danger

signals after MI and discuss potential therapeutic possibilities and clinical applications.

2. Sensing Danger

The primary mechanism by which the innate immune system can detect the presence of DAMPs is via pattern recognition receptors (PRRs). Ligands for these PRRs include molecules released by dying cells such as high mobility box 1 (HMGB1) and HSPs but also self-DNA and RNA and different extracellular matrix components. There are different classes of PRRs which sometimes share the same ligand and signaling pathways. In this review we will mainly focus on the membrane-bound Toll-Like Receptors (TLRs), the intracellular nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), and the Receptor for Advanced Glycation End-Products (RAGE). Both TLR and NLR can be activated through either PAMPs or DAMPs. Interaction with coreceptors like CD24-Siglec-G/-10 [3] or CD14/MD2 [4] allows the PRRs to discriminate between DAMPs and PAMPs and subsequently influence the level of inflammation [5]. In general, activation of PRRs results in the production of proinflammatory cytokines and recruitment and activation of immune cells (Figure 1).

2.1. Toll-Like Receptors. TLR is one of the best-described PRRs families. They are transmembrane receptors that can be divided into two groups, based on ligands and subcellular location. TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, and TLR11 are located on the cell surface and scan the extracellular environment. TLR3, TLR7, TLR8, and TLR9 are present on the membrane of endosomal compartments of the cell and responsible for the recognition of, for example, microbial nucleic acids or self-DNA/RNA from dying cells.

TLR activation results in dimerization of the cytoplasmic signaling domains of TLRs. This subsequently initiates an intracellular signaling pathway involving specific adaptor molecules like MyD88 or TRIF. The MyD88 pathway can be used by all TLRs except TLR3 [6] and results in a cumulative activation of NF-κB that mediates the transcription of proinflammatory cytokines. The TRIF pathway, independent of MyD88, can be activated via TLR3 and TLR4 [7] and results in the synthesis of interferon (IFN) (Figure 1).

The first article demonstrating an interaction between TLR and DAMPs was in 2000 by Ohashi who demonstrated that HSP60 could bind to and activate TLR4 [8]. Since then, the list of DAMPs that can activate TLRs is expanding rapidly. Depending on their biological background, TLRs can be activated by different types of DAMPs (Table 1).

In cardiac ischemic injury the role of TLRs has been intensively investigated [9, 10] and been linked to noninfectious tissue injury. TLR2 and TLR4 are the most extensively studied receptors in myocardial ischemic injury. TLR2 knockout (KO) mice have a reduced infarct size, improved cardiac function, and attenuated myocardial inflammation which is mediated via leucocytic TLR2 expression [11, 12]. Inhibition of TLR2 via an anti-TLR2 antibody also reduces infarct size and preserves cardiac function [13, 14]. In addition, TLR4 has a proinflammatory function during myocardial injury. TLR4 KO mice show reduced infarct sizes, attenuated adverse remodeling, and decreased inflammation [15, 16].

2.2. NOD-Like Receptors. NLRs are a class of intracellular receptors that recognize a variety of PAMPs and DAMPs and are highly conserved between species. So far, 22 different members have been identified in human, though the function of many remains unknown. All NLRs share the central nucleotide-binding and oligomerization (NACHT) domain, which is flanked by C-terminal leucine-rich repeats (LRRs) and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains. Based on phylogenetic studies and similarities on domain structures, the NLR family can be divided into 3 subfamilies: the NODs, the NLRPs, and the IPAF.

The best known members of the NOD family are NOD1 and NOD2. Both initiate proinflammatory signaling via mitogen-activated protein kinase (MAPK) and NF-κB pathways [17, 18]. There are many studies demonstrating a role for NOD1 and NOD2 in the recognition of peptidoglycan. However, there is still no evidence for direct binding to their ligands. In contrast to other NLRs, no endogenous ligands have been described for NOD1 and NOD2 so far.

Many of the IPAF and NLR subfamily members are well known for their capability to form large multiprotein complexes called inflammasomes that control caspase-1 activity. These include IPAF (or NLRC4) and NAIP (or NLRB1) from the IPAF subfamily and NLRP1, NLRP3, IPAF, and AIM2 from the NLRP family. Activation of the inflammasome involves a signaling complex consisting of a NLR protein, the adaptor ASC (apoptotic speck-containing protein with a CARD), and procaspase-1 and finally results in the formation of the pro-inflammatory cytokines IL-1β and IL-18 (Figure 1). For a more detailed description of inflammasome function, we refer to excellent review articles from Latz and Schroder [66, 67]. Notable, the important role of inflammasomes in myocardial ischemic injury has been described in several studies [68–70]. There are many different endogenous ligands that can activate inflammasomes (Table 1). For example, the AIM2 inflammasome can sense cytoplasmic DNA [56, 71] and the NLRP3 inflammasome can be activated via C3a [72], extracellular acidosis [73], and extracellular Ca^{2+} [74].

Although they have not been studied as extensively as TLR, there are a number of studies that demonstrate a role for NLR in myocardial ischemic injury. Already in 2001, it was demonstrated that caspase-1 inhibition reduces myocardial ischemia injury [75], whereas activation of NOD1 induces cardiac dysfunction and modulates cardiac fibrosis and cardiomyocyte apoptosis [76]. More recently, studies with KO mice demonstrate the direct role of NLR in myocardial ischemic injury. NLRP3 KO mice show improved cardiac function and decreased infarct size [69]. Similar results are found using either ASC or caspase-1 KO mice [68].

2.3. Receptor for Advanced Glycation End-Products. RAGE is the only AGE receptor that has been implied to play a role in DAMP recognition. It is a membrane bound multiligand

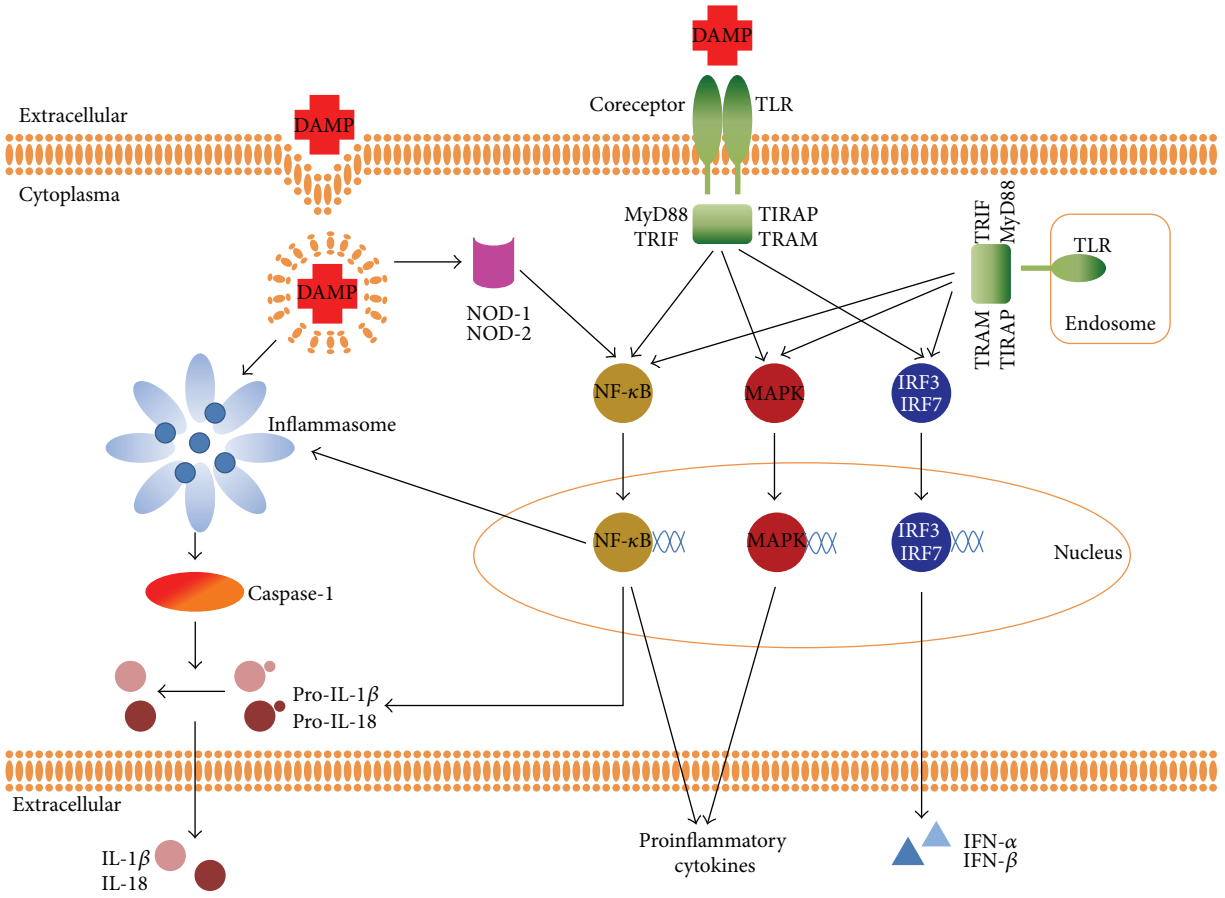

Figure 1: DAMP signaling through different PRRs. TLR activation by DAMPS triggers adaptor proteins MyD88, TRIF, TIRAP, or TRAM to activate various transcriptions factors. The subsequent translocation of NF-κB and MAPK leads to the production of several proinflammatory cytokines. TRIF-dependent activation of transcription factors IRF3 and IRF7 results in the induction of type I interferon. Additionally, the TLR- NF-κB pathway can induce the transcription of pro-IL-1β, pro-IL-18, and other components of the inflammasome pathway. Inflammasome activation is considered to depend on two distinct signals. The first signal via TLR and this might be the rate limiting step for inflammasome assembly and activity; the second signal via NLR which is responsible for inflammasome assembly, caspase-1 activation, and secretion of IL-1β and IL-18. Activation of NOD receptors results in activation of the NF-κB pathway.

receptor that can recognize, besides AGE, multiple ligands including HMGB1, amphoterin, and several S100 proteins [77, 78]. Recently, several secreted isoforms of RAGE have been described that lack the transmembrane domain and the cytosolic tail which might act as a "decoy" receptor [79–81].

RAGE signaling appears to be detrimental after MI, since recombinant HMGB1 or recombinant S100A8/A9 worsened ischemia-/reperfusion injury. Furthermore, RAGE KO mice show reduced tissue damage and less inflammation after MI [40, 82].

2.4. Synergy and Cross-Talk. There is a high level of interplay between the different PRRs family members and they also share several common ligands like HMGB1, S100A8/A9 complex, and β-sheet fibrils [40, 41, 53, 54, 83]. It is generally accepted that IL-1β release by the inflammasome requires two distinct signals where the first signal primes the cell via TLR. As most cells do not constitutively express high amounts of pro-IL-1β, TLR activation and subsequent NF-κB translocation to the nucleus results in increased expression of pro-IL-1β, pro-IL-18, and other inflammatory components like NLRP3 [69, 84–86]; the secondary (endogenous) stimulus then promotes inflammasome assembly, activation, and subsequent secretion of IL-1β and IL-18. The necessity of costimulation via two receptor types might function as a fail-safe mechanism to make sure that only in the presence of a real stimulus such as tissue injury, the activation of the proinflammatory pathways occurs.

Another example of the interaction between different PRRs family members is demonstrated after costimulation of both TLR2 and NOD1 [87] which result in enhanced proliferation, expansion, and effector function of T cells. In contrast, costimulation with TLR2 and NOD2 is responsible for an augmented inflammatory response [88]. Interestingly, there can also be a negative regulation when TLR2 and NOD2 are simultaneously activated, NOD2 has also been described to play a suppressive function in TLR2 signaling [89].

There is also evidence that endogenous ligands can interact with each other to enhance or dampen the inflammatory

Table 1: DAMPs and their receptors.

	Endogenous ligand	TLR	NLR	Others	References
Proteins, peptides	Amyloid-β	TRL2, TLR4/6	NLRP3		[19–21]
	Complement membrane attack complex		NLRP3		[22]
	α and β defensins	TLR4	NLRP3		[23, 24]
	Eosinophil-derived neurotoxin	TLR2			[25]
	Fetuin A	TLR4			[26]
	Fibrinogen	TLR4			[27]
	Fibronectin-EDA	TLR2, TLR4			[11, 28–30]
	HMGB1	TLR2, TLR4, and TLR9		RAGE	[31–34]
	HSP60	TLR2, TLR4			[35–37]
	HSP70	TRL2, TLR4/6			[38]
	Osteopontin	TLR9 (MyD88)			[39]
	S100A8/A9	TLR4	NLRP3	RAGE	[40–42]
	Tenascin-C	TLR4			[43]
	TNF-α	NLRP3			[44]
Proteoglycans, Glycosaminoglycans	Biglycan	TLR2, TLR4	NLRP3		[45, 46]
	Hyaluronic acid fragments	TLR2, TLR4			[47, 48]
	Versican	TLR2/6			[49]
Fatty acids, lipoproteins	Cholesterol crystals		NLRP3		[50]
	Oxidized LDL	TLR2,TLR4, and TLR4/6			[20, 51]
	Saturated fatty acids	TLR4			[52]
	Serum amyloid A	TLR2, TLR4	NLRP3		[53–55]
Nucleic acids	Mitochondrial DNA	TLR9	AIM2, NLRP3		[54, 56, 57]
	mRNA	TLR3			[58]
	ss RNA	TLR7, TLR8			[59]
Protein-nucleotide complexes	IgG-chromatin complexes	TLR9			[60]
	HMGB1-nucleosome complex	TLR2			[61]
Purine metabolites	ATP		NLRP1b, NLRP3		[62, 63]
	Uric acid	TLR2, TLR4	NLRP3		[64, 65]

response that they elicit. A classic example is HMGB1 that was first identified as a DAMP. However, several studies demonstrated recently that the formation of complexes with other proinflammatory ligands results in enhanced inflammation instead of HMGB1 alone [90]. For example, HMGB1 can facilitate the transfer of LPS to CD14 [91] and enhances nucleosome binding to TLR2 [61] and dsDNA binding to TLR9 [92].

3. High Mobility Box 1

High mobility box 1 (HMGB1, also known as HMG1, amphoterin, or p30) was discovered as a nonhistone DNA binding protein, involved in stabilization of DNA and promotion of gene transcription. Recent discoveries established the inflammatory role of HMGB1. Scaffidi et al. demonstrated that necrotic cells release HMGB1 and hereby elicit inflammation. On the other hand during apoptosis HMGB1 is firmly attached to the chromatin, thus preventing its release and subsequent immune responses [34]. HMGB1 exhibit specific danger signal functions, because it is only released by damaged cells and activates immune responses.

HMGB1 signals through RAGE, TLR2, and TLR4, thereby stimulating macrophages, monocytes, and neutrophils to secrete the proinflammatory cytokines TNF-α, IL-1, IL-6, IL-8, and macrophage inflammatory protein (MIP) [31–34]. Furthermore, HMGB1 induces the expression of adhesion molecules, for example, intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) on endothelial cells. HMGB1 has been studied as an inflammatory mediator in a range of diseases, such as ischemia in the liver [93] and brain [94]. Also after MI there is an immediate increase of plasma HMGB1 levels in rat and

human [95, 96]. In the infarcted myocardium of rodents the expression of HMGB1 is upregulated after 2 [82] or 3 days [95] depending on the model used. Furthermore, the elevated levels of HMGB1 in patients with acute coronary syndrome [95, 96] are associated with a decreased heart rate recovery, a marker of autonomic function defined as the fall in heart rate during the first minutes of exercise [97], and adverse LV remodeling [98] and predict secondary events, such as pump failure and cardiac rupture [95]. This might reflect that increased amounts of HMGB1 are detrimental. Surprisingly, injection of HMGB1 in rat hearts after permanent coronary ligation improved cardiac function by modulating inflammation via reducing the accumulation of dendritic cells [99] and HMGB1 delivered to the heart by a hydrogel induced vascularization and improved cardiac function [100]. Furthermore, treatment of anti-HMGB1 showed enhanced adverse LV remodeling, although it prevented the upregulation of the cytokines TNFα and IL-1β and the influx of macrophages [95]. In addition, in a mouse model of permanent coronary ligation, local injection of exogenous HMGB1 improved myocardial function [101] and in transgenic mice overexpressing HMGB1 survival and cardiac function was improved after MI [102]. However, there are also studies showing opposite effects. For example, Andrassy et al. demonstrated that systemic injection of an antagonist of HMGB1 improves cardiac function after ischemia-reperfusion in WT mice and recombinant HMGB1 worsened cardiac function. Both the antagonist or the recombinant protein had no effect on RACE KO mice, which suggest that HMGB1 signaling through RAGE inhibits the reparative response after MI. Also, administration of ethyl pyruvate, which inhibits the release of HMGB1, preserves cardiac function after extended myocardial ischemia followed by reperfusion [103]. Interestingly, preconditioning with HMGB1 shows protection against ischemia-reperfusion injury [104].

These contradictory results can partly be explained by the different models that are used. In permanent coronary ligation models angiogenesis is a prominent mediator of cardiac remodeling and improves cardiac repair. HMGB1 appears to have beneficial effects in this model, which can be assigned to the role of HMGB1 in angiogenesis [102]. The detrimental effects of HMGB1 are observed in ischemia-reperfusion models, in which inflammation plays a great role and might be aggravated by HMGB1. However, the amount of leukocytes is comparable in early time points after permanent ligation or reperfusion injury [105]. Thus in some cases the improved cardiac function in the different models may be explained by the route and time point of administration. Local injection after MI with HMGB1 improved cardiac function and in contrast systemic HMGB1 just before MI worsened cardiac function. Furthermore, low dose HMGB1 seems to be beneficial and high dose of HMGB1 to be harmful [106]. Apart from the great knowledge we already have about the role of HMGB1 as a danger signal, further research is required to solve the disagreement whether HMGB1 is deleterious or beneficial in ischemic heart diseases and how this can be implemented in the clinic. It should be taken into account that ischemia-reperfusion models are more clinically relevant, since all patients undergo reperfusion therapy (either pharmacologically or mechanically) in the setting of acute MI.

4. Heat Shock Protein 60 and 70

HSPs gain their name because their expression is upregulated as a result of high temperatures. Later it became clear that various kinds of stress responses can enhance the expression and release of HSPs. During homeostasis HSPs are expressed by numerous cell types and function as chaperones in protein folding and translocation; however, upon injury HSPs can function as danger signals. For instance, in rats, HSP27, HSP72, and HSP60 were significantly induced following coronary artery ligation, whereby the expression of HSP60 was correlated with the development of heart failure [107, 108]. In human, HSP27 and HSP60 expressions are increased in the myocardium of patients with ischemic cardiomyopathy [109] and circulating HSP70 levels are increased after acute MI [38, 110, 111]. HSP60 and HSP70 are widely studied as danger signals after MI. Endogenous and exogenous HSP60 signals via TLR4-MyD88-p38/NF-κB in cardiomyocytes and augments pro-inflammatory cytokine production, such as IL-1β, TNF-α, and IL-6 [36, 37]. Furthermore, in patients with acute coronary syndrome pro-inflammatory HSP60-reactive $CD4^{+}CD28^{null}$ T cells are found [112], which indicates that these T cells are activated by HSP60 stimulated APC. Likewise, this suggests that HSP60 functions as a primal danger signal after acute coronary syndrome. Similarly, HSP70 is elevated after MI and related to inflammation and TLR4 signaling [38, 111]. Moreover, HSP70 can activate monocytes through CD14, which results in a release of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 [113]. Surprisingly, rats administered with bimoclomol, which increases HSP70 levels, exhibit decreased infarct size after coronary ligation. However, bimoclomol was given before MI induction, which is not a good clinically relevant model [114]. More studies are necessary to establish the exact role of HSP70 as a danger signal and how this can be used in the clinic.

In summary, HSP60 and HSP70 are both upregulated after MI. HSP60 has a well-established role as a danger signal, while HSP70 has only been associated with inflammation. More studies are warranted to define the role of HSPs in the clinical setting.

5. Mitochondrial DNA

It is known that bacterial DNA has robust immune properties. The CpG sequence abundantly present on prokaryotic DNA serves as a PAMP and activates B cells, macrophages, and DCs through the intracellular TLR9 [115]. Mitochondrial mtDNA, originating from bacteria, contains the same CpG sequence and can thereby function as a DAMP. Zhang et al. show that traumas, for example, myocyte injury, trigger the release of mtDNA and that circulating mtDNA provokes inflammation in polymorphonuclear neutrophils [116]. Interestingly, Oka et al. show that mtDNA can also autonomously activate TLR9 by escaping from autophagy-mediated degradation and in this

way aggravate pressure-overloaded heart failure [117]. As a response to this paper, Konstantinidis and Kitsis postulate that this pathway may be of greater importance after MI, because inflammation is more pronounced in MI compared to chronic heart failure [118]. In addition, patients suffering from MI show increased levels of circulating mtDNA [119]. Despite the lack of studies on the effects of mtDNA in ischemic heart diseases, it can be speculated that mtDNA, released by necrotic cells or escaped from autophagy, serves as a danger signal after MI.

6. Fibronectin-EDA

Fibronectin (FN) is a dimeric glycoprotein found in the ECM. Different isoforms exist due to alternative splicing. The FN-EDA splice variant is highly expressed during embryogenesis and upregulated upon injury. FN-EDA can bind the integrins $\alpha 9\beta 1$ and $\alpha 4\beta 1$, thereby mediating cell adhesion [120]. Furthermore, FN-EDA can activate leukocytes through TLR2 and TLR4 in vitro [28, 29]. FN-EDA is upregulated after MI in mice [121] and human (unpublished data). Moreover, EDA KO mice show less inflammation, reduced monocyte recruitment, and improved cardiac function after MI [121]. In addition, in ischemic stroke, constitutive expression of FN-EDA significantly increased neutrophil and macrophage infiltration, inflammatory cytokines, and brain injury. Interestingly, treatment with a specific TLR4 inhibitor abolished these effects, which suggests that FN-EDA by signaling through TLR4 promotes inflammation and subsequent injury [30]. Although some evidence is still lacking, it can be speculated that EDA functions as an inducible danger signal after MI by attracting and activating leukocytes through TLR and/or integrin signaling.

7. Matricellular Proteins as Danger Signals

Matricellular proteins are nonstructural proteins expressed in the ECM and are upregulated upon injury. Many matricellular proteins are shown to be upregulated after MI and play an important role in the reparative response. An excellent review has been published about the role of matricellular proteins in the infarcted myocardium [122]. Some matricellular proteins also show characteristics of an inducible danger signal and those will be discussed here.

7.1. Tenascin-C. Tenascin-C (TN-C) is a glycoprotein mostly expressed in the ECM during development [122] and is normally not abundantly expressed in adult tissue. TN-C is upregulated under pathological conditions, such as pulmonary fibrosis [123] and MI [124–127] and is closely associated with inflammation [123]. Despite the fact that TN-C is upregulated in inflammatory diseases, not much is known yet about its role in vivo and whether TN-C functions as a primal danger signal. In vitro, TN-C supports lymphocyte tethering and rolling under flow conditions [128, 129], and soluble TN-C has also been shown to inhibit T cell activation and proliferation [129, 130] through the $\alpha 5\beta 1$ integrin [131]. In a model of rheumatoid arthritis, TN-C shows to signal through TLR4, thereby increasing inflammation [43]. Moreover, human macrophages secrete more of the proinflammatory cytokines IL-6, IL-8, and TNF upon TN-C stimulation through TLR4 [43].

TN-C KO mice show no cardiac dysfunction in the absence of injury, suggesting that TN-C does not play a significant role in homeostasis of the heart. However, TN-C KO mice show less fibrosis and remodeling after MI. Unfortunately, in this study the inflammatory actions of TN-C were not studied. Future research should focus on the immune modulatory actions of TN-C in the infarcted myocardium to investigate whether TN-C might be an interesting candidate in controlling the inflammatory response.

7.2. Osteopontin. Osteopontin (OPN or Eta-1) was originally identified as a bone matrix protein. Later it became clear that OPN is also a cytokine, secreted by many immune cells. OPN is constitutively expressed by macrophages [132] and is upregulated in numerous cells types upon injury [133]. In macrophages OPN has been shown to function in the migration [134], activation [135], phagocytosis [133], and inflammatory cytokine production [133, 136]. Furthermore, OPN acts as a chemoattractant for neutrophils and DCs [136, 137]. Interestingly, OPN can activate DCs to produce IL-12 and TNF-α, which suggest that OPN functions as a primal danger signal. Additionally, OPN activated DCs stimulate a Th1 response when cocultured with naïve T cells [138–140].

OPN is upregulated in experimental models of infarction in mice [141, 142], rats [143], dogs [142], pigs [144], and in human patients suffering an acute MI [132, 145]. OPN KO mice show excessive dilation and reduced collagen deposition of the LV upon MI [141]. Unfortunately, the mechanisms behind the decreased collagen deposition and the role of inflammatory cells are not studied, so whether OPN functions as a danger signal in MI cannot yet be defined. In patients, OPN levels are increased after MI [145] and are predictive for long-term outcome. Furthermore, their role as an immune modulator has been established in many other diseases [133, 137, 146], so it can be speculated that OPN functions as a danger signal. However, more research is required to unravel the role and function of OPN in cardiac ischemic injury and subsequent repair, as this might lead to new therapeutic options.

7.3. CCN1. The CCN family obtained its name from the first members described, cysteine-rich protein 61(CYR61), connective tissue growth factor and nephroblastoma overexpressed protein. CCN are considered as matricellular proteins and have been shown to be involved in many cellular processes such as adhesion, migration, and proliferation, mainly via modulating signaling of other molecules [122]. CCN1, also known as CYR61, is highly upregulated in the infarcted myocardium in mice [147, 148] and human [148]. Furthermore, CCN2 and CCN4 are upregulated after MI, but little is known about their inflammatory actions. Interestingly, CCN1 can activate proinflammatory genes in macrophages by binding to $\alpha_M\beta_2$ and syndecan-4 [149]. However, CCN1 inhibits the migration of macrophages and

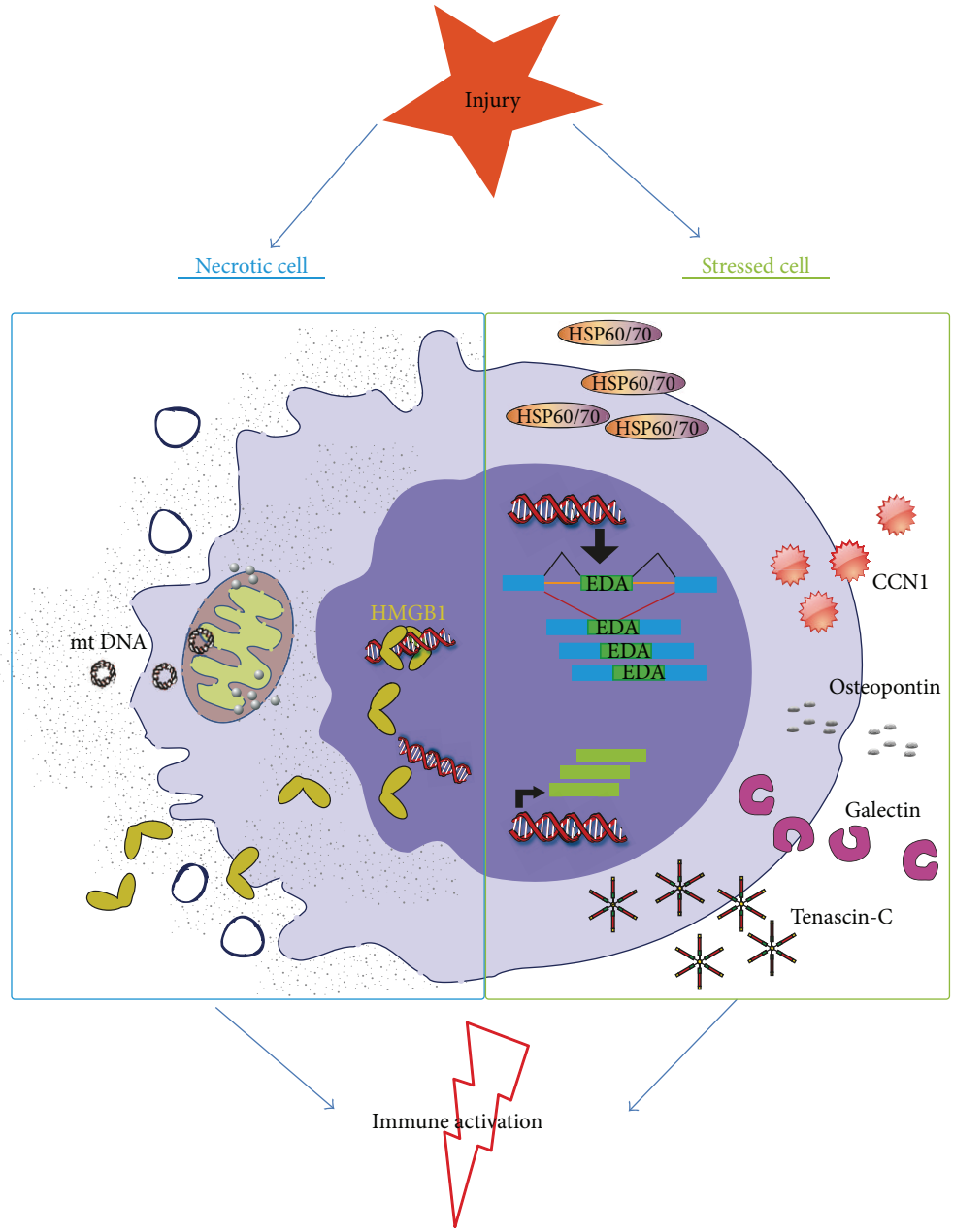

Figure 2: Proposed simplified mediators of danger signal release during myocardial infarction. Necrotic cells in the myocardium are leaky and release a subset of DAMPs, for instance, mtDNA and the DNA binding protein HMGB1. Furthermore, viable cells get stress signals from their surroundings and start to produce and secrete a range of proteins. These cells start the production of the EDA splice variant of fibronectin, HSPs and the matricellular proteins CCN1, osteopontin, galectins, and tenascin-C. Both the proteins released by necrotic cells and the produced proteins by stressed cells are able to activate or aggravate the immune response in the heart following MI.

lymphocytes in autoimmune myocarditis [150]. To explain the paradoxical role of CCN1, Löbel and colleagues showed a diphasic immune modulator response for CCN1; initial stimulation with CCN1 attracts and activates leukocytes; however, prolonged CCN1 stimulation and enhanced secretion of CCN1 by leukocytes immobilize systemic leukocytes [151]. It can be speculated that CCN1 may function as a danger signal after MI by attracting and activating leukocytes, however, in vivo studies are necessary to state this.

7.4. Galectins. Galectins are a family of proteins that have an affinity for binding β-galactosides sugars. So far, 15 different galectins have been described. Some galectins have been characterized as matricellular proteins [152], including galectin-1 and galectin-3. Both galectins have been shown to function as a DAMP [153] and found to be upregulated after MI in human [154, 155] and mice [155]. Galectin-3 can support neutrophil adhesion, migration, and activation. Furthermore, galectin-3 functions as a chemoattractant for

macrophages [156] and both galectin-1 and galectin-3 can alternatively activate macrophages. Importantly, galectin-1 is able to augment DC migration, and induce maturation [157]. Interestingly, Seropian and colleagues recently showed that galectin-1 prevents cardiac inflammation in a mouse model of acute MI [155]. These studies might suggest that different galectins have distinct functions in inflammation. Not many in vivo studies have been conducted yet to establish the role of galectins as a DAMP following MI. However, it can be hypothesized that both galectin-1 and galectin-3 play a role in the inflammatory reaction.

8. Clinical Implications

In the clinical context, understanding the role of danger signals could have important applications. Hypothetically, all danger signals that can be measured in blood may serve as biomarkers for diagnostic and/or prognostic purposes. For example, high plasma levels of HMGB1 are shown to be strongly associated with increased mortality in patients with STEMI independent of age, sex, troponin I, and CK-MB [158]. In addition, in patients with unstable angina or NSTEMI, high serum levels of HBGB1 are associated with higher mortality during 49 month followup [159]. Both of these studies demonstrate that HMGB1 levels can be used as a new prognostic biomarker in patients with acute coronary syndrome.

Also HSP70 might be a new biomarker for patients with heart failure. HSP70 is elevated in AMI patients and after 14 days HSP70 levels were higher in patients with heart failure compared to patients without heart failure [38]. In addition, Li et al. showed that elevated levels of HSP70 correlate with the progression of HF [110].

In critically ill patients, high levels of FN-EDA correlate with increased risk for progression to acute hypoxemic respiratory failure [160]. It would be interesting to study the prognostic value of FN-EDA levels in patients with acute coronary syndrome.

On a theoretical basis, danger signals are excellent therapeutic targets because they are only released or upregulated after injury. Nevertheless, DAMP-induced inflammation is also essential for proper healing of the infarcted area. Hence, it is of utmost importance to establish the exact time frame in which intervention is optimal. Injection of HMGB1 or an antibody against HMGB1 has extensively been studied in animal models. However, no uniform effects were observed. In some models HMGB1 seems to prevent cardiac remodeling, however, in other models HMGB1 appears to be detrimental. This is probably due to the two different MI models used to study cardiac remodeling: the permanent coronary ligation and ischemia-reperfusion. Before HMGB1 or anti-HMGB1 can be brought to the clinic, it is crucial that the mechanism of action and the therapeutic window are established. In addition to targeting a DAMP for therapeutic intervention, it is also an option to target receptors. It is challenging to inhibit a certain receptor in order to prevent DAMP-PRRs interaction, since the same PRRs are necessary for host defense. Nevertheless, a few examples can be given. An anti-TLR2 antibody reduced leukocyte influx and infarct size after MI in both mice and pigs [13]. Furthermore in a brain ischemic-reperfusion model a TLR4 inhibitor reduced injury [30] and because many DAMPs signal through TLR4 this is also an interesting candidate for the treatment of MI.

9. Concluding Remarks

The danger model has shown to be useful as a theoretical framework in cardiovascular science. Interesting new DAMPs are identified that might influence the deleterious and beneficial effects of the immune system in tissue healing and scar formation. Figure 2 shows how and which danger signals can be released following MI. However, for only a few danger signals a true causal relationship has been established in MI and for many danger signals research is still ongoing to establish their effects in MI. It will be interesting to use conditional KO and bone marrow chimera approaches to investigate which cells release and produce the danger signal of interest. Furthermore, ECM breakdown products and matricellular proteins are of main interest to study as potential danger signals. Danger signals, or DAMPs, may also be used as diagnostic and prognostic markers. Additional studies on correlation between specific danger signals and primary and/or secondary outcome are necessary before clinical application. Intracellular and inducible DAMPs, such as mtDNA and matricellular proteins, are interesting candidates for therapeutic interventions, considering that they are only present in the injured environment.

To conclude, extended research is necessary to define the role of specific danger signals in MI. Regardless, DAMPs may be of additive value in the clinic as diagnostic/prognostic markers and therapeutic targets.

Conflict of Interests

The author declare that they have no conflict of interests.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 261054, 6 pages
http://dx.doi.org/10.1155/2013/261054

Research Article

Actin Is a Target of T-Cell Reactivity in Patients with Advanced Carotid Atherosclerotic Plaques

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Received 30 July 2013; Accepted 7 October 2013

Academic Editor: Fatih Arslan

Atherosclerosis is a chronic inflammatory disease of the arterial wall associated with autoimmune reactions. In a previous study, we observed the presence of actin-specific antibodies in sera from patients with carotid atherosclerosis. To extend our previous results we evaluated the possible role of actin as antigenic target of cell-mediated immune reactions in carotid atherosclerosis. Peripheral blood mononuclear cells (PBMC) from 17 patients and 16 healthy subjects were tested by cell proliferation assay and by ELISA for cytokine production. Actin induced a proliferative response in 47% of patients' PBMC samples, with SI ranging from 2.6 to 21.1, and in none of the healthy subjects' samples (patients versus healthy subjects, $P = 0.02$). The presence of diabetes in patients was significantly associated with proliferative response to actin ($P = 0.04$). IFN-γ and TNF-α concentrations were higher in PBMC from patients than in those from healthy subjects and in PBMC proliferating to actin than in nonproliferating ones. Our data demonstrate for the first time a role of actin as a target autoantigen of cellular immune reactions in patients with carotid atherosclerosis. The preferential proinflammatory Th1 activation suggests that actin could contribute to endothelial dysfunction, tissue damage, and systemic inflammation in carotid atherosclerosis.

1. Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall in which immune responses play a crucial role. Atherosclerotic plaques are characterized by the presence of an inflammatory cell infiltrate mainly composed of macrophages and T lymphocytes that modulate the atherosclerotic process by secreting inflammatory mediators. Infiltrating T lymphocytes are activated T cells expressing CD25 on their surface [1] and predominantly expressing a Th1 phenotype in advanced lesions [2, 3]. In this context, identifying the antigens responsible for T lymphocyte activation in atherosclerosis may be relevant. Accelerated atherosclerosis has been reported in patients with various autoimmune diseases [4–6], suggesting an involvement of autoimmune mechanisms in atherogenesis [7]. Although infectious agents have been associated with the activation of immune mechanisms, several lines of evidence suggest that the main antigenic targets in atherosclerosis are modified endogenous structures [8]. Different self-antigens or modified self-molecules have been identified as target of humoral and cellular immune responses in patients with atherosclerotic disease thus behaving as dangerous signals able to activate proinflammatory responses. Oxidative stress, increasingly reported in these patients [9], is the major event causing structural modification of proteins [10].

Oxidized low density lipoproteins (LDL) are the best characterized autoantigen. In particular, it has been demonstrated that about 10% of T lymphocytes infiltrating human atherosclerotic plaques are specific for oxidized LDL [11]. In addition to LDL, other self-molecules modified by oxidative stress become target of autoimmune reaction in atherosclerosis [12–14]. Another two categories of autoantigens that have been implicated in atherosclerosis are the stress-induced

heat shock proteins and antigens expressed by dying cells [15, 16]. Cell death in the atherosclerotic plaque may occur by apoptosis or by necrosis [17, 18]. The uptake of apoptotic cells by macrophages and some subsets of dendritic cells may induce an anti-inflammatory response and play an important role in maintaining peripheral immune tolerance [19, 20]. Conversely, the uptake of necrotic cells or even a delayed uptake of apoptotic cells may result in immune activation and risk for the development of autoimmunity [21].

In a previous study, by the use of a molecular cloning strategy to identify endothelial autoantigens, we provided evidence of serum anti-actin antibodies in patients with carotid atherosclerosis and we suggested that actin is an autoantigenic molecule of potential clinical interest in carotid atherosclerosis [22].

We designed this study to confirm and extend our previous results on the possible role of actin as target antigen of immune reactions in carotid atherosclerosis. For this purpose, we evaluated the proliferative response of circulating T lymphocytes obtained from patients and healthy subjects, stimulated *in vitro* with actin.

We also investigated the ability of actin-specific circulating T lymphocytes to produce the pro-inflammatory cytokines IFN-γ and TNF-α and the anti-inflammatory cytokines IL-4 and IL-10.

2. Materials and Methods

2.1. Subjects. We enrolled 17 consecutive patients with asymptomatic severe or preocclusive carotid-artery stenosis ≥70% or with symptomatic stenosis undergoing endarterectomy (CEA) at the Sapienza University of Rome. Patients were grouped according to the histological type of their atherosclerotic plaques following the classification of Stary et al. [23]. Thirteen patients had type V plaques and 4 patients had type VI plaques. In brief, type V plaques are defined as lesions in which prominent new fibrous connective tissue has formed. Type VI plaques are defined as lesions in which disruption of the lesion surface, hematoma, or hemorrhage and thrombotic deposits have developed and may be referred to as complicated lesions. The baseline characteristics of patients are reported in Table 1. We also recruited 16 sex- and age-matched healthy subjects as controls. Exclusion criteria for patients were recent infections (<1 month), autoimmune diseases, malignancies, and inflammatory diseases before enrollment. The inclusion criteria for healthy subjects were no history of myocardial infarction, coronary bypass, coronary angiography with angioplasty or stenting or both, cerebrovascular accident, or peripheral vascular disease. None of them had ultrasonographically evident carotid or femoral artery atherosclerotic disease. All hematological variables including risk factors for atherosclerosis were in the range of "normal" values. The investigation conforms with the principles outlined in the Declaration of Helsinki. Informed consent was obtained before enrollment.

2.2. Blood Samples. Venous peripheral blood was drawn in heparin tubes from the 17 patients (before surgery) and from the 16 healthy subjects. Peripheral blood mononuclear cells (PBMC) were separated from plasma by density gradient centrifugation (Lympholyte, Cedarlane, ON, Canada) and were used in the proliferation assay. PBMC samples were stored at −80°C until use.

Table 1: Baseline characteristics of the 17 patients with carotid atherosclerosis.

Parameter	
N (%)	17 (100)
Age (years), median (range)	73 (62–84)
Male/female (*n*)	10/7
Diabetes*, *n* (%)	7 (41)
Smoking†, *n* (%)	10 (59)
Hypertension‡, *n* (%)	10 (59)
Family history‖, *n* (%)	8 (47)
Hypercholesterolemia§, *n* (%)	6 (35)
Body mass index, median (range)	27.7 (25–30.5)
Erythrocyte sedimentation rate, median (range)	15 (12–20)

*Diabetes is type 2, defined as fasting glucose levels ≥140 mg/dL or need for antidiabetic medications.
†Smoking is defined as current smokers.
‡Hypertension is defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or need for hypertension medication.
‖Family history is defined as having relatives with known heart or vascular disease, including myocardial infarction, heart failure, aneurysm, stroke, sudden death, arrhythmia, and rheumatic fever.
§Hypercholesterolemia is defined as total cholesterol >200 mg/dL or need for lipid-lowering therapy.

2.3. Actin Proliferation Assay. Triplicate cultures of PBMC (1×10^6 cells/mL) were stimulated for 7 days with rabbit muscle actin (Sigma-Aldrich, Milan, Italy, 20 μg/mL), phytohemagglutinin (PHA, Burroughs Wellcome Co., Beckenham, UK, 2 μg/mL) as a positive control of the assay, or human serum albumin (HSA, Sigma-Aldrich, 10 μg/mL) as a negative control, or left unstimulated. Endotoxin contamination in actin, as determined by the quantitative chromogenic Limulus amebocyte lysate assay (QCL-1000, BioWhittaker, Walkersville, MD), was <0.03 endotoxin units/μg of protein. To neutralize a possible endotoxin effect, all cells were cultured in the presence of polymyxin B (10 μg/mL, Sigma-Aldrich).

Cell proliferation was assessed by ^{3}H-methyl-thymidine incorporation assay as previously described [24]. The proliferative response was expressed as stimulation indices (SI, ratio between the mean cpm in stimulated cultures and that in unstimulated cultures). The mean stimulation index in healthy subjects + 3 standard deviations was taken as the threshold level for positivity.

2.4. Cytokine Determination. IFN-γ, TNF-α, IL-4, and IL-10 concentrations in culture supernatants of circulating T lymphocytes were quantified with commercially available enzyme-linked immunosorbent assay (ELISA) sets (OptEIA set, BD Biosciences, CA, USA) as recommended by the manufacturer. The limits of detection were 1 pg/mL for IFN-γ, 2 pg/mL for TNF-α, IL-4, and IL-10.

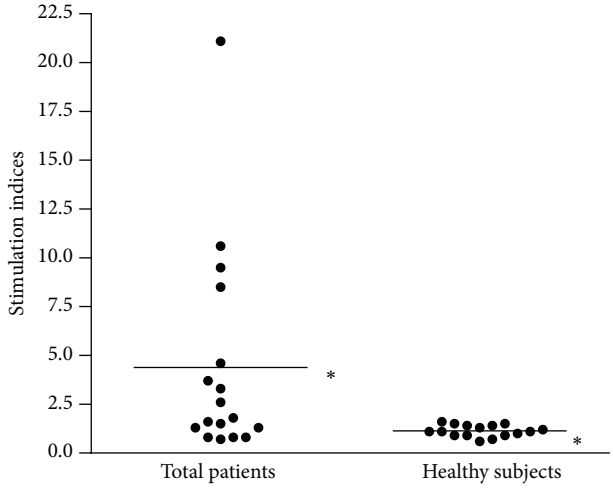

Figure 1: Proliferative response of peripheral blood mononuclear cell samples obtained from the 17 patients with carotid atherosclerosis and from the 16 healthy subjects. $^{*}P = 0.02$.

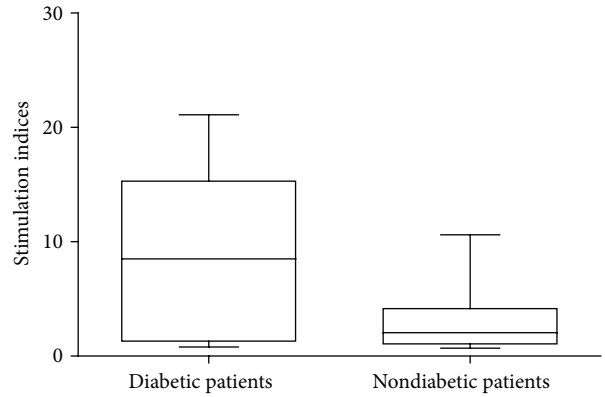

Figure 2: Proliferative response of peripheral blood mononuclear cell samples obtained from the 17 patients with carotid atherosclerosis divided according to the presence/absence of type 2 diabetes. $^{*}P = 0.04$.

2.5. Statistical Analysis. Results are expressed as arithmetic means or medians and interquartile ranges. Mann-Whitney U and Wilcoxon nonparametric tests were used to investigate the significance of unpaired and paired data. All the covariates were examined in univariate analyses as predictors for actin-specific cellular response. Fisher's exact test and Mann-Whitney U test were used to evaluate the differences in discrete and continuous clinical characteristics between patients' groups. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Proliferative Response of Circulating T Lymphocytes to Actin. In our selected healthy subject population, we determined a mean SI value of 1.13 and a SD of 0.29 and we calculated the value of 2.0 as the cutoff level for positivity. Actin induced a proliferative response in 8 of 17 (47%) patients' PBMC samples, with SI ranging from 2.6 to 21.1 (Figure 1). PBMC samples from healthy subjects did not proliferate in response to actin. The difference between the SI mean values in patients and healthy subjects was statistically significant (4.4 versus 1.1, $P = 0.02$ by Mann-Whitney U test). Univariate analysis showed that the presence of diabetes in patients was significantly associated with proliferative response to actin ($P = 0.04$, Figure 2).

3.2. Cytokine Production. PBMC samples from patients produced higher concentrations of IFN-γ and TNF-α than PBMC from healthy subjects (Figure 3). In patients, IFN-γ and TNF-α concentrations were higher in PBMC samples that proliferated in response to actin than in nonproliferating ones (Figure 3). No significant differences were observed for IL-4 and IL-10 production (Figure 3). We found the presence of a positive correlation between IFN-γ concentrations and SI ($P < 10^{-4}$; $r = 0.71$) (Figure 4).

4. Discussion

In this study, we demonstrated for the first time a role of actin as a target autoantigen of cellular immune reactions in patients with carotid atherosclerosis. As observed for other candidate autoantigens, actin induced a proinflammatory Th1 activation, characterized by high IFN-γ and TNF-α expression. Th1 response, not counteracted by an increase of anti-inflammatory IL-4 and IL-10 production, may contribute to tissue damage, endothelial dysfunction, and systemic inflammation [12]. Further characterization of these will establish whether they are a regulatory population able to counteract. Our results support previous findings indicating that inflammatory autoimmune reactions are not exclusively localized within atherosclerotic lesions but can also contribute to systemic inflammation in patients with atherosclerosis [12–14]. Inflammation in atherosclerosis is modulated by cytokines that differentially affect endothelial dysfunction. Distinct cytokines promote pro- as well as antiatherogenic processes, thus modulating plaque development and clinical outcome [14, 25, 26]. IFN-γ and TNF-α mediate proatherogenic processes by promoting monocyte activation and by influencing collagen synthesis and expression of adhesion molecules, tissue factor, and matrix metalloproteinases [27, 28].

Our finding on actin-specific T-cell activation is in line with a previous study where we identified actin as a candidate autoantigen of humoral immune response in patients with carotid atherosclerosis [22]. Actin is a globular protein quite abundant in eukaryotic cells. It can polymerize in the presence of ATP and its structure is remarkably conserved during evolution. Anti-actin antibodies have been associated with various autoimmune diseases including systemic lupus erythematosus [29], a disease in which endothelial damage plays a key role. Anti-filamentous actin antibodies characterize autoimmune hepatitis type 1 where the binding domain of α-actinin on actin was shown to be a predominant actin epitope [30]. Anti-actin antibodies were also found in 52–85% of patients with autoimmune hepatitis or chronic active hepatitis, in 22% of patients with primary biliary cirrhosis, and in

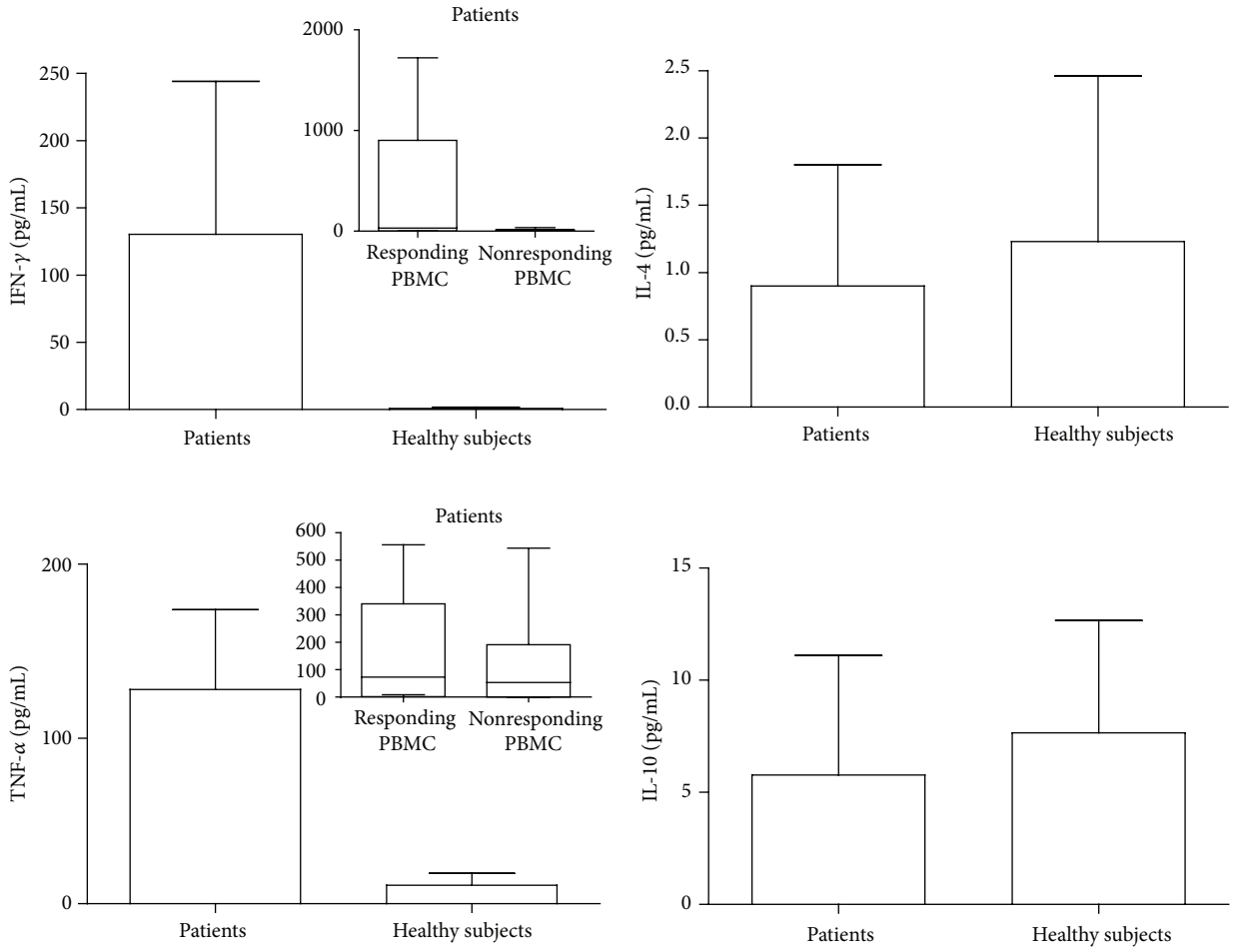

Figure 3: Cytokine secretion in culture supernatants from patients' and healthy subjects' peripheral blood mononuclear cell (PBMC) samples; IFN-γ and TNF-α production by patients' PBMC responding or not to actin in proliferation assay.

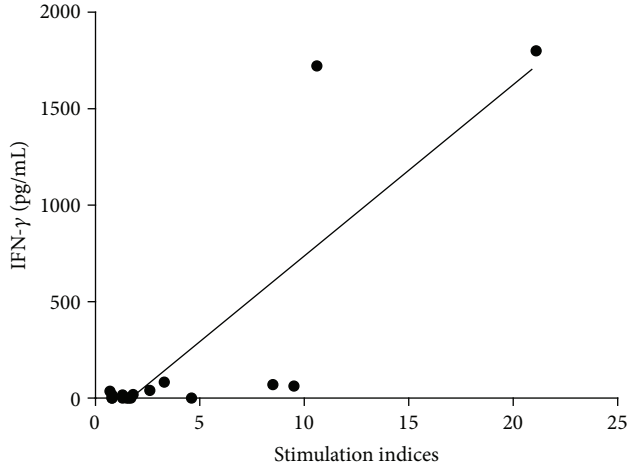

Figure 4: Positive correlation between IFN-γ concentrations and proliferative response to actin (stimulation indices) in patients with carotid atherosclerosis. $P < 10^{-4}$; $r = 0.71$.

patients with celiac disease and with autoimmune haemolytic anaemia [31–34]. Furthermore, nonmuscle α-actinin 4 and cytoplasmic β-actin were identified as immunodominant ovarian autoantigens involved in ovarian autoimmunity [35].

An interesting and extremely important aspect in autoimmune diseases is to understand how abundant and highly conserved self-proteins can become the antigenic target of autoimmune reactions. One of the mechanisms breaking tolerance to self could be apoptosis. It has been shown that apoptotic cancer cells may render actin immunogenic by exposing it on their surfaces [36, 37]. In addition, many autoantigens, and in particular actin, represent a substrate for the proapoptotic cysteine proteases. The polypeptides produced in this way can be released into the extracellular space or can be presented as neoantigens, thus generating an autoimmune response [38, 39]. Interestingly, several studies have shown the presence of apoptotic cells, particularly macrophages and smooth muscle cells, in all stages of atherosclerosis development [40, 41].

In our study, we observed a positive association between the presence of diabetes and the response to actin. Pancreatic beta-cell death by apoptosis, which can be induced by multiple stresses, contributes significantly to the pathogenesis of type 2 diabetes [42]. The possibility that diabetes, characterized by increasing oxidative stress and apoptosis, may trigger the autoimmune response to actin is interesting and needs further investigation.

A limitation of our study is that it does not provide a causal association between the T-cell response to actin and atherosclerosis in humans. *In vivo* experimental models are required to address this question.

5. Conclusions

Our study takes research into the involvement of autoimmune responses in the pathogenesis of atherosclerosis, a small step ahead indicating actin as a candidate autoantigen target of cell-mediated immune responses in a proportion of patients with carotid atherosclerosis. Our findings here call for further studies to identify epitopes on actin recognized by specific T lymphocytes. The identification of these epitopes might be useful to design novel preventive strategies.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 390890, 10 pages
http://dx.doi.org/10.1155/2013/390890

Research Article

Natural Antioxidant-Isoliquiritigenin Ameliorates Contractile Dysfunction of Hypoxic Cardiomyocytes via AMPK Signaling Pathway

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Received 16 July 2013; Accepted 14 August 2013

Academic Editor: Fatih Arslan

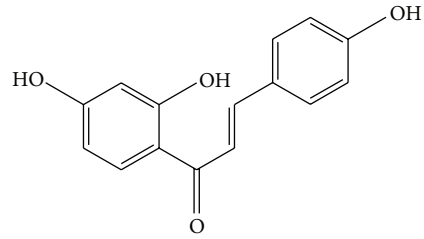
Isoliquiritigenin (ISL), a simple chalcone-type flavonoid, is derived from licorice compounds and is mainly present in foods, beverages, and tobacco. Reactive oxygen species (ROS) is a critical factor involved in modulating cardiac stress response signaling during ischemia and reperfusion. We hypothesize that ISL as a natural antioxidant may protect heart against ischemic injury via modulating cellular redox status and regulating cardioprotective signaling pathways. The fluorescent probe H_2DCFDA was used to measure the level of intracellular ROS. The glucose uptake was determined by 2-deoxy-D-glucose-^{3}H accumulation. The IonOptix System measured the contractile function of isolated cardiomyocytes. The results demonstrated that ISL treatment markedly ameliorated cardiomyocytes contractile dysfunction caused by hypoxia. ISL significantly stimulated cardioprotective signaling, AMP-activated protein kinase (AMPK), and extracellular signal-regulated kinase (ERK) signaling pathways. The ROS fluorescent probe H_2DCFDA determination indicated that ISL significantly reduced cardiac ROS level during hypoxia/reoxygenation. Moreover, ISL reduced the mitochondrial potential ($\Delta\psi$) of isolated mouse cardiomyocytes. Taken together, ISL as a natural antioxidant demonstrated the cardioprotection against ischemic injury that may attribute to the activation of AMPK and ERK signaling pathways and balance of cellular redox status.

1. Introduction

Myocardial infarction is one of the major causes of death in the world. Although restoration of blood flow is the only way to save the myocardium from necrosis, reperfusion-induced injury is at the background of a high mortality rate [1]. Extensive studies showed that myocardial ischemia-reperfusion (I/R) injury is associated with increased generation of reactive oxygen species (ROS). The ROS may result in depressed contractile function, arrhythmias, depletion of endogenous antioxidant network, and membrane permeability changes [2]. There is evidence that AMP-activated protein kinase (AMPK) signaling pathway is involved in cardiac redox regulation [3–6]. Our laboratory and others have provided clear evidence that AMPK plays a critical role in protection against ischemia/reperfusion injury in the heart [7–14].

Isoliquiritigenin (ISL), a simple chalcone-type flavonoid, is derived from licorice compounds, and present in foods, beverages, and tobacco [15]. It has been reported to possess a wide range of biological and pharmacological activities including antioxidative activity [16], antiplatelet aggregation effects [17, 18], antitumor activities [19], and estrogenic properties [20]. It has been reported that pretreatment with ISL markedly decreased the severity of reperfusion-induced

arrhythmias and myocardial infarct size and reduced the activities of lactate dehydrogenase (LDH) and creatinine phosphokinase (CPK) [18]. Increased JAK2/STAT3 phosphorylation in the heart by ISL appears to the mechanism by which ISL protects the heart against ischemia and reperfusion injury [18]. To further characterize the cardioprotective effects of ISL on cardiomyocytes under hypoxic stress, we isolated mouse cardiomyocytes to investigate the effects of ISL on cardiomyocytes contractile functions during hypoxia/reoxygenation and the signaling mechanism that mediated ISL action on cardiomyocytes.



Isoliquiritigenin

Scheme 1

2. Materials and Methods

2.1. Drugs and Chemicals. Isoliquiritigenin (ISL) was purchased from Sigma (St. Louis, MO) (Scheme 1). ISL was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution of 10 mmol/L, and the DMSO final concentration was less than 0.01% (v : v). Other chemicals were of analytical purity.

2.2. Animals and Cell Line. 8–12-week-old male FVB/NJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animals were kept in the institutional animal facility at the State University of New York (SUNY) at Buffalo and were fed *ad libitum*. All animal procedures used in this study were approved by the Institutional Animal Care and Use Committees at the State University of New York (SUNY) at Buffalo.

2.3. Isolation of Mouse Cardiomyocytes. Cardiomyocytes were enzymatically isolated as previously described [21]. Briefly, adult mouse hearts were removed and perfused with oxygenated (5% CO_2/95% O_2) Krebs-Henseleit bicarbonate (KHB) buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 HEPES, and 11.1 Glucose. All the chemicals were purchased from Sigma (St. Louis, MO, USA). Hearts were then perfused with a Ca^{2+}-free KHB containing Liberase Blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN, USA) for 15–20 min. After perfusion, left ventricles were removed and minced to disperse cardiomyocytes in Ca^{2+}-free KHB buffer. Extracellular Ca^{2+} was added incrementally back to 1.25 mM. Only rod-shaped myocytes with clear edges were selected for pharmacological test and cell contractility studies. The cardiomyocytes were treated with ISL (50, 100 μM) for AMPK signaling, ROS level, mitochondrial membrane potential, and glucose uptake measurements.

2.4. Hypoxia Treatment. Isolated mouse cardiomyocytes were subjected to two groups (normal and hypoxic groups). Hypoxic groups were kept at 37°C in a humidified sealed chamber under a humidified atmosphere of 5% CO_2 and 95% N_2 for 20 min. Normal groups were placed into a water-jacketed incubator at 37°C during the same period.

2.5. Cardiomyocytes Shortening/Relengthening Measurement. The mechanical properties of ventricular myocytes were assessed using a SoftEdge MyoCam system (IonOptix Corporation, Milton, MA, USA) [22]. In brief, left ventricular myocytes were placed in a chamber mounted on the stage of an inverted microscope (Olympus, IX-70, Center Valley, PA, USA) and incubated at 25°C with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Glucose, and 10 HEPES, at pH 7.4. The cells were stimulated with suprathreshold voltage at a frequency of 0.5 Hz, 3 msec. duration, using a pair of platinum wires placed on opposite sides of the chamber and connected to an electrical stimulator (FHC Inc, Brunswick, NE, USA). The myocytes being studied were displayed on a computer monitor using an IonOptix MyoCam camera. An IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS), the amplitude myocytes shortened on electrical stimulation, indicative of peak ventricular contractility; time-to-PS (TPS), the duration of myocyte shortening, an indicative of systolic duration; time-to-90% relengthening (TR90), the duration to reach 90% relengthening, an indicative of diastolic duration (90% rather 100% relengthening was used to avoid noisy signal at baseline concentration); and maximal velocities of shortening/relengthening, maximal slope (derivative) of shortening and relengthening phases, indicative of maximal velocities of ventricular pressure increase/decrease. In the case of altering stimulus frequency, the steady-state contraction of myocytes was achieved (usually after the first 5-6 beats) before PS amplitude was recorded.

2.6. Intracellular Ca^{2+} Transient Measurements. Isolated cardiomyocytes were loaded with fura-2/AM (0.5 μM) for 15 min, and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). Myocytes were placed on an Olympus IX-70 inverted microscope and imaged through a Fluor (St. Louis, MO, USA) ×40 oil objective. Cells were exposed to light emitted by a 75 W lamp and passed through either a 360 or a 380 nm filter, while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 sec. and then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol, and qualitative changes in intracellular Ca^{2+} concentration were

inferred from the ratio of fura-2 fluorescence intensity at two wavelengths (360/380). Fluorescence decay time was measured as an indication of the intracellular Ca^{2+} clearing rate. Both single and biexponential curve fit equations were applied to calculate the intracellular Ca^{2+} decay constant [21].

2.7. Determination of Reactive Oxygen Species (ROS). ROS were detected as previously described [23, 24]. Briefly, 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes, Eugene, OR, USA) is a cell-permeant indicator for ROS that is nonfluorescent until removal of the acetate groups by intracellular esterases, and oxidation occurs within the cells. It is intracellularly deesterified and turns into highly fluorescent DCF upon oxidation. Isolated cardiomyocytes were suspended in HEPES-saline buffer and preincubation with 10 μM H_2DCFDA at 37°C for 30 min in the darkness. After cells were washed twice, fluorescence intensity was read at excitation wavelength of 485 nm and emission wavelength of 530 nm in a fluorescence plate reader (Microplate fluorometer, Spectra GEMINIXS, Molecular Device, USA). The wells containing ISL, but not H_2DCFDA, were used as blanks. The production of ROS is expressed as fluorescence intensity in relative to untreated control cells.

2.8. Assessment of Mitochondrial Membrane Potential ($\Delta\psi$). The $\Delta\psi$ was measured using 5, 5′,6,6′-Tetrachloro-1, 1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1, Sigma, St. Louis, MO, USA). Briefly, JC-1 is a positively charged fluorescent compound which is taken up by mitochondria proportionally to the inner mitochondrial membrane potential [25]. When a critical concentration is exceeded, JC-1 monomer forms J-aggregates and becomes fluorescent red, altering the fluorescence properties of the compound. Thus, the ratio of red (J-aggregate) green (monomeric JC-1) emission is directly proportional to the mitochondrial membrane potential. Isolated cardiomyocytes were suspended in HEPES-saline buffer and preincubation with 10 μM JC-1 for 10 min at 37°C. After cells were washed twice, fluorescence of each sample was read at excitation wavelength of 490 nm and emission wavelength of 530 nm and 590 nm in a fluorescence plate reader (Microplate fluorometer, Spectra GEMINIXS, Molecular Device, USA). Results in fluorescence intensity were expressed as 590 to 530 nm emission ratio.

2.9. Immunoblotting Analysis and Antibodies. Immunoblotting was performed as previously described [14, 24, 26]. Isolated cardiomyocytes proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). For reprobing, membranes were stripped with 50 mM Tris-HCl, 2% SDS, and 0.1 M β-mercaptoethanol (pH 6.8). Membranes were blocked with 5% nonfat milk in TBS (pH 7.4) containing 0.1% Tween-20 for 1 h and subsequently incubated with primary antibodies (1 : 1000 dilution) at 4°C for overnight. Immunoreactive bands were detected using anti-rabbit horseradish peroxidase-conjugated secondary antibodies and visualized using chemiluminescent substrate (ECL). Rabbit polyclonal antibodies against phospho-AMPK (Thr^{172}), total AMPKα, phospho-ACC, phospho-Akt, phospho-ERK (Thr^{202}/Tyr^{204}), phospho-p38 MAPK (Thr^{180}/Tyr^{182}), phospho-STAT3, and GAPDH were purchased from Cell Signaling (Danvers, MA, USA). The densities of immunoblot bands were analyzed using a scanning densitometer (model GS-800; Bio-Rad) coupled with Bio-Rad personal computer analysis software [27–29].

2.10. Glucose Uptake. 2-Deoxy-d-[1-^{3}H] glucose accumulation in H9c2 cells was performed as previously described [30, 31]. H9c2 cells grown in 6-well plates were washed twice with serum-free DMEM and incubated with 2 mL of the same medium at 37°C for 2 h. The cells were washed 3 times with Krebs-Ringer-HEPES (KRH) buffer and incubated with 2 mL KRH buffer at 37°C for 30 min. Insulin (10 nM, Sigma, St. Louis, MO) and/or ISL (50, 100 μM) were then added to H9c2. Glucose uptake was initiated by the addition of 0.1 mL KRH buffer and 2-deoxy-d-[1-^{3}H] glucose (0.21 Ci/mL, GE Healthcare, Piscataway, NJ, USA) and 5 mM glucose as final concentrations. Glucose uptake was terminated by washing the cells three times with cold PBS. The cells were lysed overnight with 1 mL 0.5 M NaOH and 0.1% SDS (w/v). The radioactivity retained by the cell lysates was determined by a scintillation counter (Beckmann LC 6000IC) and normalized to protein amount measured with a Micro BCA Protein Assay Kit (Pierce Chemical, Rockford, IL, USA).

2.11. Statistical Analysis. For each experimental series, data are presented as mean ± SE. Statistical comparisons were made using analysis of variance (ANOVA). Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. ISL Ameliorated Cardiomyocyte Contractile Dysfunction Induced by Hypoxia. To determine whether ISL protects cardiomyocytes against hypoxic injury, we investigated the cardiomyocyte contractility when they were exposed to hypoxia atmosphere. The mechanical properties of cardiomyocyte contractility were obtained under extracellular Ca^{2+} of 1.0 mM and a stimulus frequency of 0.5 Hz. As shown in Figure 1, ISL (100 μM) treatment did not affect resting cardiomyocyte contractile function under the normal or hypoxic condition. However, during hypoxic conditions, the cardiomyocytes displayed severe impaired peak shortening (PS) (Figure 1(b)) and reduced maximal velocity of shortening/relengthening ($+dL/dt$, $-dL/dt$) (Figures 1(c) and 1(d)), while ISL treatment significantly ameliorates the contractile dysfunction of cardiomyocytes as reflected by both peak shortening and maximal velocity of shortening/relengthening (Figures 1(c) and 1(d)). Moreover, hypoxia caused the prolonged time-to-peak shortening (TPS) and time-to-90% relengthening (TR90) of cardiomyocytes (Figures 1(e) and 1(f)); however, ISL (100 μM) markedly inhibited the hypoxia-induced prolonged TPS and TR90 of cardiomyocytes (Figures 1(e) and 1(f)). These results suggest that ISL protects cardiomyocytes from hypoxia-induced contractile dysfunction.

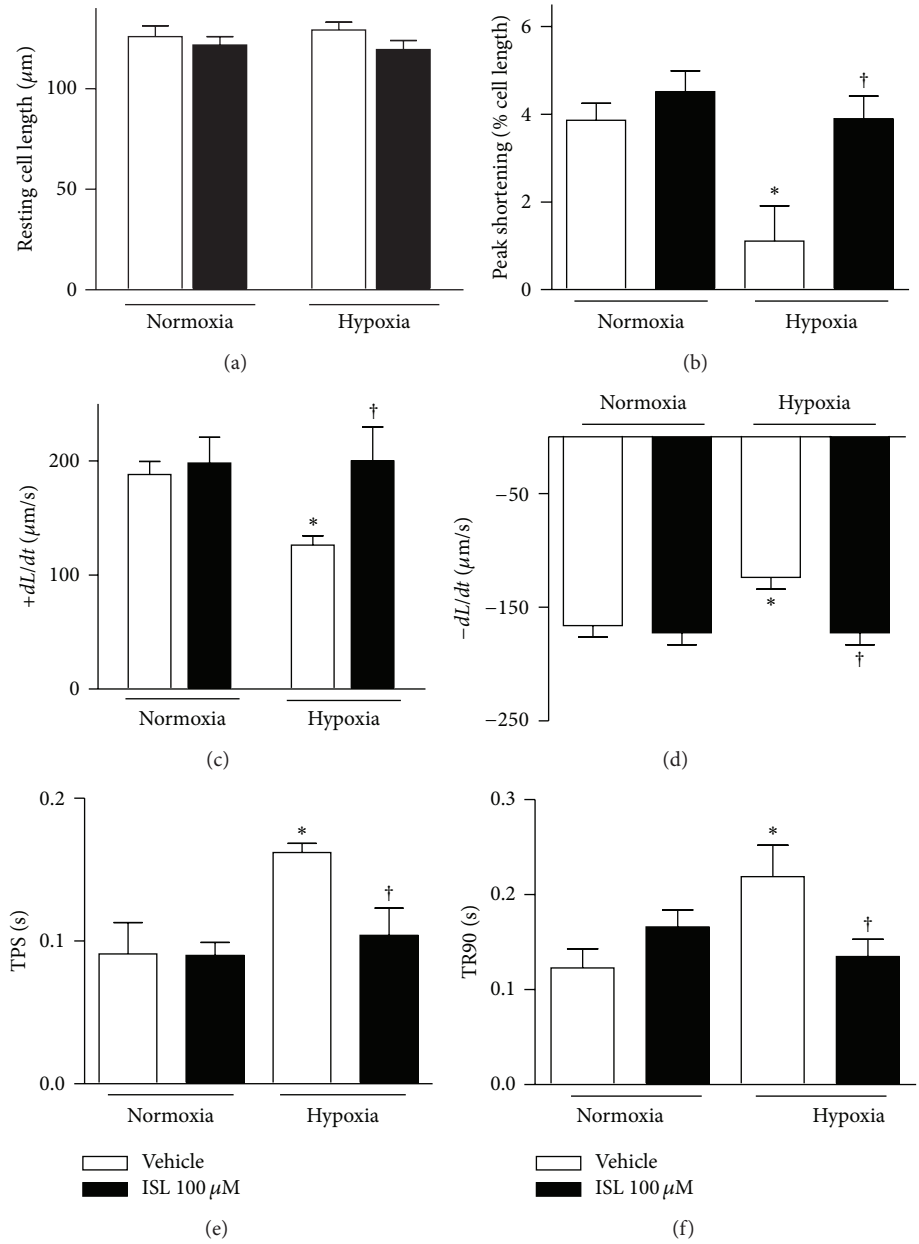


Figure 1: Contractile properties of cardiomyocytes from vehicle and ISL treatment after being exposed to hypoxia. (a) Resting cell length; (b) peak shortening (PS, normalized to cell length); (c) maximal velocity of shortening ($+dL/dt$); (d) relengthening ($-dL/dt$); (e) time-to-peak shortening (TPS); (f) time-to-90% relengthening (TR90). Values are means ± SE, $n = 50$–60 cells per group, $^{*}P < 0.05$ versus normoxia vehicle; $^{\dagger}P < 0.05$ versus hypoxia vehicle.

3.2. The Intracellular Ca^{2+} Properties of Cardiomyocytes. To explore the potential mechanisms involved in the protection of ISL against hypoxic cardiomyocyte contractile defect, intracellular Ca^{2+} homeostasis was evaluated using the fluorescence dye fura-2/AM [32]. The results revealed that hypoxia caused an elevation of the resting intracellular Ca^{2+} levels in isolated cardiomyocytes (Figure 2(a)) and reduced intracellular Ca^{2+} clearance with prolonging the fluorescence decay time (both single and biexponential decays, Figures 2(c) and 2(d)) as compared with cardiomyocytes under normoxia conditions. ISL (100 μM) did not elicit any overt effect on resting intracellular Ca^{2+} and fluorescence decay time in nonhypoxic conditions (Figures 2(a)–2(d)), but markedly recovered the elevated resting intracellular Ca^{2+} levels and reduced intracellular Ca^{2+} clearance in isolated cardiomyocytes under hypoxic conditions (Figure 2). However, hypoxia did not change electrically stimulated rise in intracellular Ca^{2+} levels (Figure 2(b)).

3.3. ISL Stimulated Cardioprotective Signaling Pathways. Our group and others provided evidence that AMP-activated protein kinase (AMPK) is a critical signaling in cardioprotection

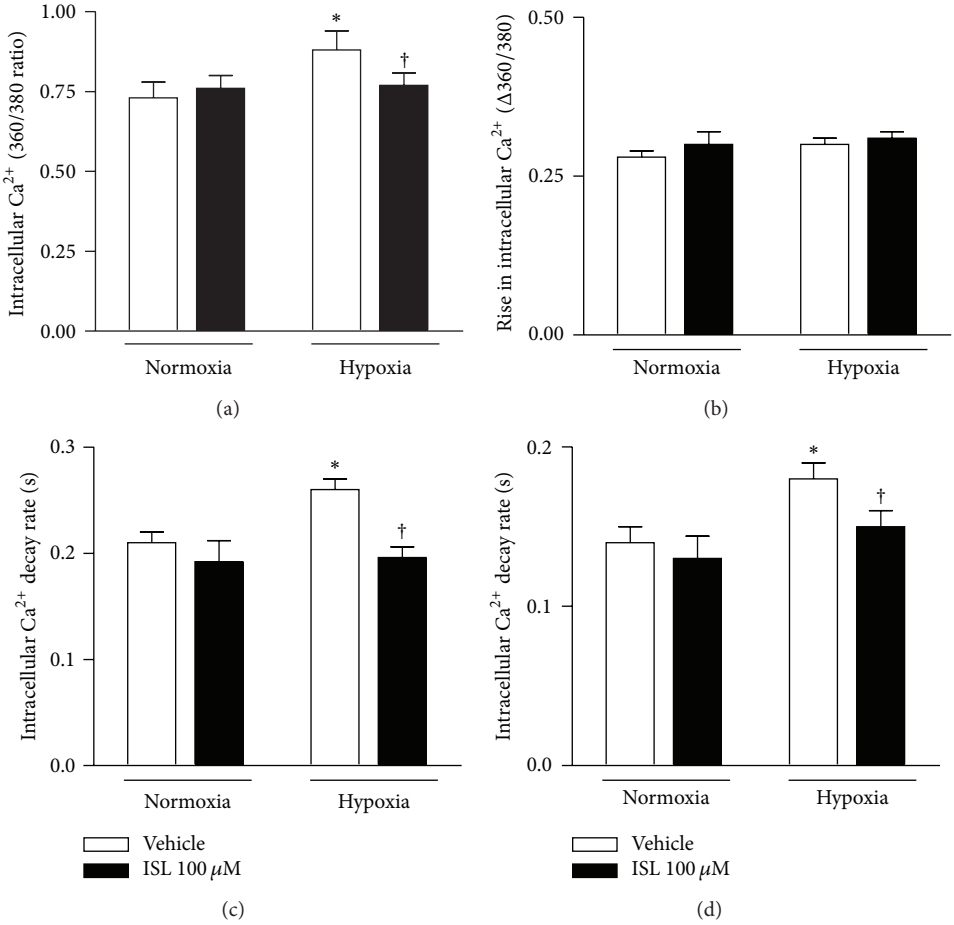


Figure 2: Intracellular Ca^{2+} properties of cardiomyocytes. (a) The intracellular Ca^{2+} levels; (b) the rise in intracellular Ca^{2+} levels in response to electrical stimulus; (c) the first exponential decay constant of intracellular Ca^{2+}; (d) the biexponential decay constant of intracellular Ca^{2+} in response to hypoxia (20 min). Values are means ± SE, $n = 60$–90 cells per group, $^{*}P < 0.05$ versus normoxia vehicle; $^{\dagger}P < 0.05$ versus hypoxia vehicle.

against ischemic injury [7, 11–13]. To define the mechanism involved in the cardioprotective effect of ISL, AMPK signaling pathways were detected in isolated cardiomyocytes in response to ISL treatment. The results showed that ISL significantly triggered AMPK Thr^{172} phosphorylation as compared with vehicle group (Figure 3(a)). In parallel with AMPK activation, the downstream targets of AMPK, the phosphorylation of acetyl CoA carboxylase (ACC) was induced by ISL treatment (Figure 3(b)). Intriguingly, ISL treatment also induced extracellular signal-regulated kinase (ERK) signaling pathway in the cardiomyocytes (Figure 3(c)). These data suggest that ISL treatment can induce phosphorylation of α catalytic subunit at Thr^{172} of AMPK and trigger a survival signaling ERK activation.

3.4. ISL Decreased the Intracellular ROS Level in Isolated Cardiomyocytes. Upon reperfusion of the myocardium after ischemia/hypoxia, there is a rapid increase in intracellular calcium that will induce the opening of the mitochondrial permeability transition pore (mPTP) [33]. Uncoupling of the electron transport chain within the mitochondria leads to the release of destructive reactive oxygen species (ROS) [34] this increase in ROS is a significant contributor to the cell death seen at the onset of reperfusion [33]. The fluorescent probe H_2DCFDA was used to measure the effect of ISL on the level of intracellular ROS in isolated cardiomyocytes under hypoxia/reoxygenation conditions. As shown in Figure 4(a), ROS level of cardiomyocytes under hypoxia/reoxygenation was much higher than that of vehicle normoxia group ($P < 0.01$ versus vehicle normoxia). ISL treatment significantly decreased the intracellular ROS levels of isolated cardiomyocytes during hypoxia/reoxygenation ($P < 0.05$ versus vehicle hypoxia). It is suggested that ISL demonstrated cardioprotection against the hypoxia-induced contractile dysfunction through modulating the cellular redox status of cardiomyocytes.

3.5. ISL Reduced the Mitochondrial Membrane Potential of Cardiomyocytes. There is evidence that AMPK signaling pathway is involved in regulation of mitochondrial membrane potential ($\Delta\psi$) [35]. To understand the mechanisms by which ISL activates cardiac AMPK signaling pathway,

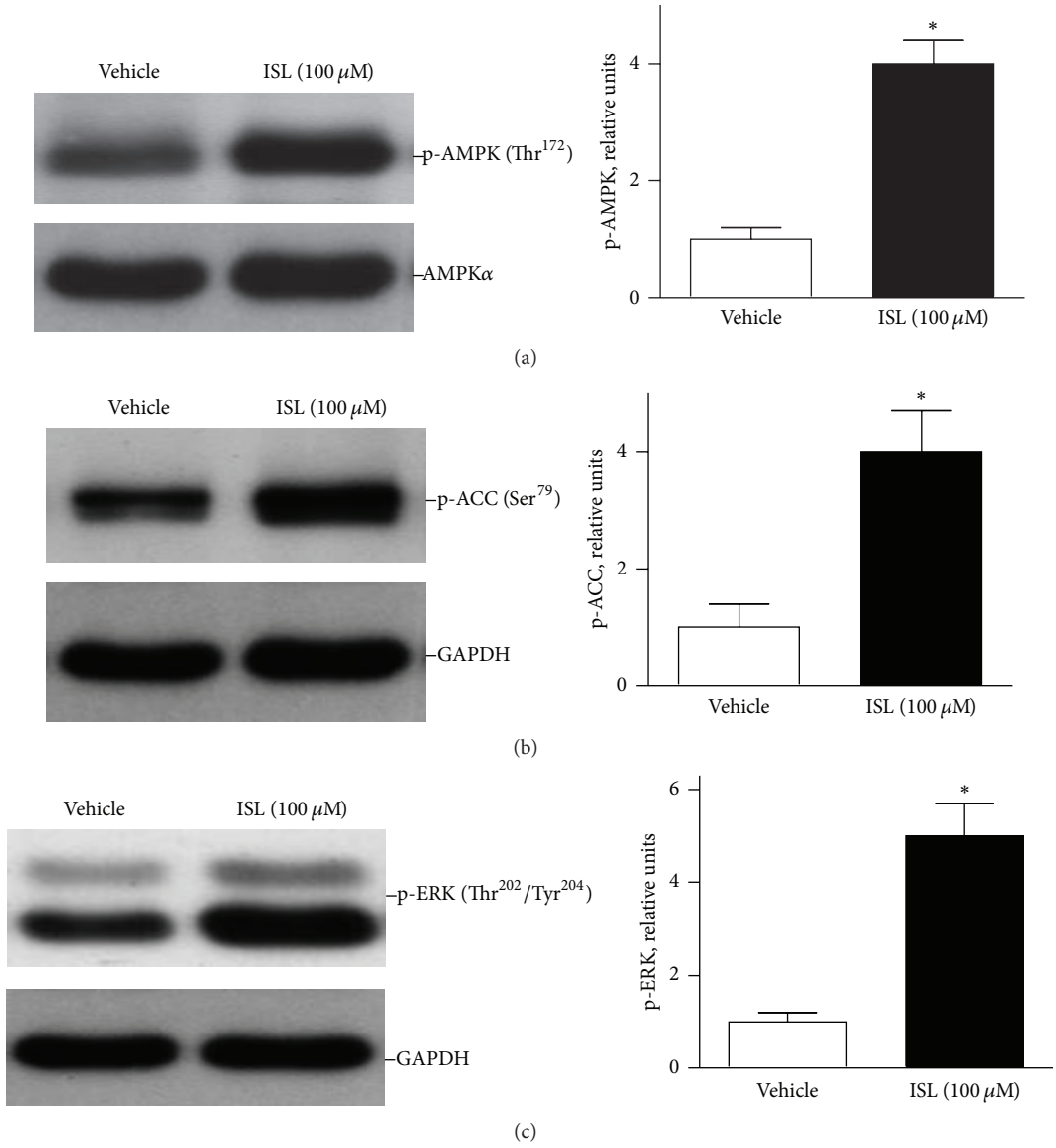


Figure 3: ISL treatment stimulated cardiac AMP-activated protein kinase (AMPK) and ERK signaling pathways. Representative immunoblots of isolated mouse cardiomyocytes showed phosphorylation of (a) AMPK at Thr^{172} (p-AMPK), (b) ACC (Ser^{79}), and (c) ERK. Phosphorylated AMPK was quantified relative to total AMPKα. Phosphorylated ACC and ERK were quantified relative to GAPDH. Values are expressed as means ± SE (n = 3–6), $^{*}P < 0.05$ versus vehicle.

the mitochondrial membrane potential ($\Delta\psi$) of cardiomyocytes was assessed using JC-1, a lipophilic fluorophore that forms J-aggregates in proportion to its intramitochondrial concentration. Isolated cardiomyocytes were preincubated for 20 min with 10 μM JC-1, rinsed thoroughly, and treated with ISL accordingly. Figure 4(b) represented the ratio of red/green fluorescence, corresponding to JC-1 in J-aggregate versus monomeric form. The results demonstrated that ISL treatment significantly reduced JC-1 dye accumulation and decreased J-aggregate formation in cardiomyocyte mitochondria in an independent manner, which indicated that ISL caused mitochondrial membrane depolarization. The $\Delta\psi$ reduction may contribute to the activation of AMPK induced by ISL.

3.6. ISL Stimulated Glucose Uptake in the Cardiomyocytes. To explore whether ISL-activated cardiac AMPK signaling modulates glucose metabolism in the cardiomyocytes, the effect of ISL on glucose uptake in cardiomyocytes was investigated using the 2-deoxy-D-1-^{3}H-glucose uptake assay [31]. As shown in Figure 4(c), ISL significantly stimulated glucose uptake in the isolated cardiomyocytes ($P < 0.01$ versus vehicle). Interestingly, ISL treatment can enhance insulin-induced glucose uptake of cardiomyocytes (Figure 4(c)).

4. Discussion

ROS have been implicated in the pathogenesis of stress-induced injury, including myocardial ischemia/reperfusion

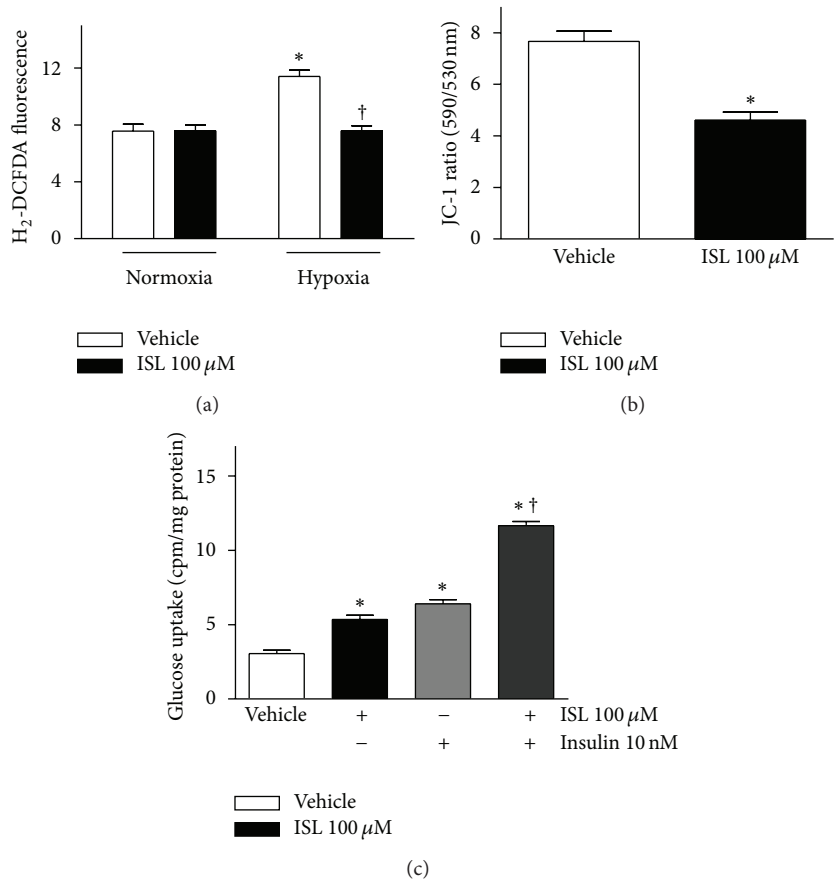


Figure 4: (a) ISL reduced the intracellular ROS levels in isolated mouse cardiomyocytes during hypoxia/reoxygenation. Intracellular ROS levels were measured by the fluorescent probe H_2DCFDA after treatment with ISL (100 μM) or DMSO (vehicle). ROS production was expressed as fluorescence intensity relative to untreated control cells. Data are presented as means ± SE (n = 4–6). $^*P < 0.05$ versus normoxia vehicle; $^\dagger P < 0.05$ versus hypoxia vehicle; (b) ISL reduced mitochondrial membrane potential ($\Delta\psi$) in isolated cardiomyocytes. Mitochondrial membrane potential ($\Delta\psi$) was measured by JC-1 fluorescence assay. The result was presented as the ratio of red/green fluorescence measured at 590 nm and 530 nm, respectively. Values are means ± SE (n = 6–10). $^*P < 0.01$ versus vehicle; (c) ISL treatment augmented glucose uptake of cardiomyocytes. The cardiomyocytes were preincubated for 30 min with or without ISL (100 μM) and/or insulin (10 nM), before addition of 2-deoxy-[1-^{3}H]glucose for additional 30 min to measure glucose uptake. Values are means ± SE for 5 experiments. $^*P < 0.05$ versus vehicle; $^\dagger P < 0.05$ versus insulin alone.

injury. ROS generation intracellularly contributes to contractile dysfunction and cell death during simulated ischemia/reperfusion in a perfused cardiomyocyte model [2, 36]. Extensive studies showed that some herbal extracts or active components of herbs exhibited antioxidant effects [18]. Although these studies have implicated antioxidative and cardioprotective effects of ISL, whether these actions of ISL can correlate with its cardiomyocyte contractile function is not well understood. In the present study, ISL as a natural antioxidant did not affect cardiomyocyte contractile function under the normal condition. However, cardiomyocytes displayed severe impaired contractile functions, while ISL markedly ameliorated the hypoxia-caused contractile dysfunction of cardiomyocytes. Additionally, ISL did not elicit any overt effect on resting intracellular Ca^{2+} and fluorescence decay time in nonhypoxic conditions, but it markedly elevated the electrically stimulated intracellular Ca^{2+} levels and recovered the elevated resting intracellular Ca^{2+} levels and reduced intracellular Ca^{2+} clearance in hypoxia-isolated cardiomyocytes. The explanation of mechanical defects observed in our study may be the impaired intracellular Ca^{2+} handling. The reduction of intracellular Ca^{2+} clearance is likely responsible for prolonged relaxation duration (TR90) and reduced PS in hypoxic cardiomyocytes. Meanwhile, the ROS level of hypoxic cardiomyocytes is much higher than that of normal cardiomyocytes, which may contribute to the contractile dysfunction of cardiomyocytes. When cardiomyocytes were exposed to hypoxia atmosphere, ISL treatment significantly decreased the intracellular ROS level and ameliorated the contractile dysfunction of cardiomyocytes. Generally, Hypoxia-induced ROS production may cause the membrane lipid peroxidation and protein denaturation that disturbed Ca^{2+} transportation and mitochondrial membrane potential. Therefore, the antioxidative activity of ISL could reduce the intracellular ROS levels and ameliorate the Ca^{2+} transportation and mitochondrial membrane potential; all of which

contribute to the improvement of contractile function of cardiomyocytes under hypoxic stress conditions.

Currently, AMP-activated protein kinase (AMPK) pathway was revealed to be one of the signaling pathways that protect against cardiac ischemia [7, 37–39]. AMPK is a stress-sensitive kinase that can be activated by ATP depletion such as hypoxia [5], ischemia [13], and exercise [40]. Activated AMPK can phosphorylate Acetyl-CoA carboxylase (ACC) to inhibit its activity involved in fatty acid synthesis [41]. Other downstream effects of AMPK pathways include glucose uptake [42, 43], glycolysis [44], and fatty acid oxidation [45], which favor the ATP production that supply enough energy for cell living under the stress conditions. AMPK promotes glucose transport, maintains ATP stores, and prevents injury and apoptosis during ischemia [39]. Our results showed that ISL stimulated AMPK Thr^{172} phosphorylation and activation in the isolated cardiomyocytes. ISL also significantly stimulated the AMPK downstream effector glucose uptake in the cardiomyocytes. These data strongly suggest that ISL may directly trigger cardiac AMPK signaling pathway that modulates glucose homeostasis to protect hypoxia-induced cardiomyocytes injury.

AMPK has several direct molecular targets on the heart but also may interact with other stress-signaling pathways.

On the other hand, ERK activation is antiapoptotic in most tissues [46]. Our results demonstrated that ISL as a natural antioxidant triggers ERK signaling in the isolated cardiomyocytes, even though the molecular mechanism by which ISL activates the cardiac ERK pathway needs to be characterized in future studies.

In conclusion, ISL demonstrated cardioprotection against contractile dysfunction caused by hypoxia/reoxygenation. The mechanisms of cardioprotection of ISL are associated with the activation of cardioprotective signaling pathways and modulation of intracellular redox status in the cardiomyocytes. Therefore, ISL is a potential small molecule for treatment of ischemic heart diseases in the future. In terms of our previous studies [10, 24, 27, 38, 47], regarding the clinical setting, it may be beneficial to phosphorylate AMPK during ischemic injury in patients suffering from acute myocardial infarction. For this reason, ISL could be administrated just prior to percutaneous coronary intervention (PCI) to reduce the ischemia/reperfusion injury.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contributions

Xiaoyu Zhang, Ping Zhu, and Xiuying Zhang are equally contributed to this work.

Acknowledgments

This study was supported by China Scholarship Council and Gansu Province Natural Science Foundation of China (no. 0710RJZA037, no. 1208RJZA231) to Xiaoyu Zhang, American Heart Association SDG 0835169N and 12GRNT 11620029 to Ji Li, American Diabetes Association Basic Sciences Grant 1-11-BS-92 to Ji Li, the Major International (Regional) Joint Research Project 2008DFA31140 and 2010DFA32660 to Ping Zhu, and Guangdong Natural Science Fund 10251008002000002 and S2011010005836 to Ping Zhu, and Chinese Science and Technology Support Program 2011BAI11B22 to Jian Zhuang.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 370715, 8 pages
http://dx.doi.org/10.1155/2013/370715

Review Article

Update on the Pathophysiological Activities of the Cardiac Molecule Cardiotrophin-1 in Obesity

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Received 14 February 2013; Revised 20 March 2013; Accepted 21 March 2013

Academic Editor: Fatih Arslan

Cardiotrophin-1 (CT-1) is a heart-targeting cytokine that has been reported to exert a variety of activities also in other organs such as the liver, adipose tissue, and atherosclerotic arteries. CT-1 has been shown to induce these effects via binding to a transmembrane receptor, comprising the leukaemia inhibitory factor receptor (LIFRβ) subunit and the glycoprotein 130 (gp130, a common signal transducer). Both local and systemic concentrations of CT-1 have been shown to potentially play a critical role in obesity. For instance, CT-1 plasma concentrations have been shown to be increased in metabolic syndrome (a cluster disease including obesity) probably due to adipose tissue overexpression. Interestingly, treatment with exogenous CT-1 has been shown to improve lipid and glucose metabolism in animal models of obesity. These benefits might suggest a potential therapeutic role for CT-1. However, beyond its beneficial properties, CT-1 has been also shown to induce some adverse effects, such as cardiac hypertrophy and adipose tissue inflammation. Although scientific evidence is still needed, CT-1 might be considered as a potential example of damage/danger-associated molecular pattern (DAMP) in obesity-related cardiovascular diseases. In this narrative review, we aimed at discussing and updating evidence from basic research on the pathophysiological and potential therapeutic roles of CT-1 in obesity.

1. Introduction

Since its recent recognition as a disease [1], the scientific community started to consider obesity as a disorder reaching epidemic proportions in developed countries and bearing an increased cardiovascular risk [2, 3]. Intensive investigations on the pathogenesis of obesity have been performed to better understand the molecular mechanisms underlying the disease development and its association with atherogenesis. Therefore, novel molecules have been identified as potential common mediators influencing obesity, atherosclerosis as well as postinfarction tissue injury. In particular, recent evidence suggests that endogenous molecules (also named damage/danger-associated molecular patterns (DAMPs)) might interfere with both innate and adaptive immunity as well as atherosclerotic inflammation in several phases of the disease [4]. For instance, some adipocytokines (such as adiponectin, leptin, or resistin), which have been shown to be dysregulated in obese subjects [5], have been suggested as critical cardiovascular risk biomarkers in atherosclerotic diseases [6, 7]. On the other hand, considering that gp130 ligands have been shown to modulate the energy balance in obesity [8], these molecules have been investigated as a potential therapeutic targets against insulin resistance [8, 9]. Cardiotrophin-1 (CT-1) is a gp130 ligand and a member of the interleukin- (IL-) 6 family, originally described as an active inducer of cardiac hypertrophy, atherosclerosis and, thus, a potential appropriate example of DAMP [10]. *In vivo* studies indicated that CT-1 induced hypertrophic properties within the myocardium [11]. In fact, chronic systemic administration of CT-1 (up to 2 microg twice a day for 14 days) dose dependently induced cardiac hypertrophy in mice (assessed as an increase in the ventricular weight without an increased number of cardiomyocytes) [12]. In addition to hypertrophy,

increased circulating plasma levels of CT-1 have been associated with the development of the metabolic syndrome [13]. Consistently, chronic exposure of adipocytes to CT-1 resulted in decreased insulin responsiveness [14]. Confirming previous studies that identified CT-1 as an highly protective molecule in many tissues (such as the kidney and the liver) [15–17], a recent study showed that acute and chronic treatments with recombinant CT-1 were able to correct insulin resistance in animal models of genetic and acquired obesity [18]. In this narrative review, we aimed at discussing the activities of CT-1 and its potential implications as a therapeutic molecule in obesity. In addition, the specific role of CT-1 as a DAMP directly triggering atherosclerotic and adipose tissue inflammation will be updated.

2. Potential Sources of Cardiotrophin-1

The expression of CT-1 has been originally identified on neonatal cardiomyocytes [10]. In addition, CT-1 transcripts have also been detected in many other tissues [19, 20]. Interestingly, a recent study revealed that during the first period of the embryonic development (day 8.5), CT-1 is confined in the primitive heart tube in mice [20]. During the heart development, CT-1 is expressed within the atriums and ventricles, but not in the endocardium. At a later developmental stage, CT-1 is expressed at relatively high level within the global heart, whereas most of the other organs (such as brain, liver, kidney, and lung) have been shown to express relatively low protein levels of CT-1 [20]. These studies indicate that CT-1 might play a fundamental role in the cardiac development. Indeed, this is supported by the study of Yoshida and coworkers showing that gp130 (a subunit of the CT-1 receptor) knockout mice develop myocardial ventricular hypoplasia, resulting in death in utero [21]. In adult animals, gp130 is ubiquitously expressed suggesting a physiological role of this receptor not only during embryogenesis but also during adult life. Consistently, the postnatal inactivation of gp130 leads to severe defects in both the heart and other systems in mice [22]. Mice lacking specifically cardiac functional gp130 during both embryonic and adult periods have been shown to develop normal cardiac structure and function [23]. However, in response to an aortic pressure overload, these conditional knockout mice have been observed to be rapidly affected by a dilated cardiomyopathy with increased cardiomyocyte apoptosis when compared to the control mice [23]. Although it is important to note that CT-1 is mainly synthesized within the heart by both cardio myocytes and noncardiac cells, the cardiac defects observed in gp130 knockout mice may be partially associated with disorders in the other animal organs with a final negative impact on the myocardium.

Once produced by the heart, CT-1 is secreted through the coronary sinus into the peripheral systemic circulation [24]. Similar to the myocardium, CT-1 has been shown to be expressed by many other tissues such as adipose tissue, liver, lung, and skeletal muscles [12]. For instance, adipocytes have also been shown as an important cellular source of CT-1 in both physiological and pathological conditions [13]. On the other hand, increased levels of CT-1 have been detected in a variety of pathological states, where this mediator might influence the tissue function and injury [13, 25, 26]. For instance, CT-1 has been found to be upregulated in the hypertrophic ventricles of genetically hypertensive rats [27]. Similarly, enhanced CT-1 expression has also been reported in the rat ventricle after myocardial infarction [28].

CT-1 expression has been shown to be regulated by various transcription factors that are activated only under pathophysiological conditions, such as hypoxia [29, 30]. Both systemic and local levels of CT-1 might be increased as a result of not only an upregulated production, but also a potential reduction in its degradation. However, no evidence is currently available on the potential catabolic pathways of CT-1. One of the major proteolytic structures for cytosolic proteins is represented by proteasome [31]. Since it has been shown that nonobese diabetic mice might be characterized by a defective proteasome activity [32], modifications in CT-1 circulating and tissue levels could be explained by a reduction of the proteasome-mediated catabolism. However, this point remains highly speculative and requires further investigations. In addition, other traditional catabolic pathways for CT-1 elimination have been never explored. This approach might represent an interesting research field to better understand the dynamics and activities of CT-1 in cardiovascular diseases and obesity.

3. Regulation of CT-1 Expression

Since CT-1 was originally identified as a cytokine inducing cardiac hypertrophy, several basic research studies (targeting the regulation of the CT-1 gene expression *in vitro* in cardiomyocytes and rodent cardiovascular disease models) have been published. Similar to the human gene [19], mouse CT-1 was shown to contain several consensus sequences, such as SP-1, CRE, NF-IL6, AP-1, AP-2, and GATA [20]. Since CT-1 has been proposed to prevent the cardiac ischemic injury, a recent study showed that the hypoxia-inducible factor 1 (HIF-1) markedly enhanced the expression of CT-1, resulting in an improved cardiomyocyte survival in response to ischemia [29]. Others found that 60-minute hypoxia upregulated the mRNA expression of CT-1 in primary cultured cardiomyocytes [33]. Furthermore, hypoxia concomitantly upregulated both CT-1 and HIF-1 mRNA and protein expression in embryonic stem-cell-derived cardiac cells, confirming a protective role with improvement of both cell survival and proliferation [34]. In addition, CT-1 expression might be regulated by endocrine factors such as norepinephrine [33], aldosterone [35], fibroblast growth factor-2 (FGF-2) [36], and urocortin [37]. For instance, FGF-2 has been shown to increase CT-1 mRNA levels within the heart. In this work, Jiang and coworkers demonstrated that intracardiac administration of FGF-2 (2 microg/heart) reduced the infarct size, and induced postinfarction hypertrophy in a rat model of acute myocardial infarction and irreversible chronic ischemia [36]. These effects were associated with the cardiac upregulation of the CT-1, suggesting that FGF-2 might directly modulate CT-1 expression.

On the other hand, aldosterone, which is a recognized inducer of cardiac hypertrophy [38], has also been shown to increase CT-1 expression in cultured HL-1 cardiomyocytes [35]. In this study, the authors demonstrated that aldosterone-mediated activation of mineralocorticoid receptors was associated with the upregulation of CT-1 expression via the phosphorylation of the cytosolic p38 mitogen activated protein kinase (MAPK) [35]. Since p38 MAPK activation was shown to regulate the expression of IL-24 by stabilization of the 3′UTR of IL-24 mRNA [39], we can speculate that a similar mechanism might also influence p38 MAPK-induced expression of CT-1.

High glucose and insulin levels have been demonstrated to promote cardiac hypertrophy [40, 41]. More recently, Liu and coworkers showed that the antidiabetic drug pioglitazone was able to reduce glucose and insulin levels in diabetes and concomitantly inhibit *in vitro* cardiomyocyte hypertrophy. Importantly, the authors showed that both glucose- and insulin-induced myocardial hypertrophy might be mediated by CT-1, suggesting that CT-1 expression could be directly increased by insulin and glucose stimulations [42]. These studies have not been confirmed by other research groups and require additional validations to support a crucial pathophysiological role for CT-1 in the cardiac microenvironment. The different sources of CT-1 as well as the molecular mechanisms influencing its production remain to be clarified.

4. CT-1-Triggered Signaling Pathways

CT-1-induced effects are mediated through the molecular binding to a transmembrane receptor gp130/leukaemia inhibitory factor receptor (LIFR) (Figure 1) [43]. This receptor is composed of two subunits (gp130 and LIFR) that are both necessary for an effective intracellular signal transduction [44]. Upon binding of CT-1 to its receptor, several signaling pathways have been shown to be activated. For instance, the antiapoptotic effects of CT-1 in cardiomyocytes are mediated by the activation of the p38 MAPK, protein kinase B (or Akt), and extracellular regulated kinases (ERKs) [45]. The downstream mechanisms involved in the cytoprotective role of these kinases remain controversial and are still under intensive investigation. Since several antiapoptotic signaling pathways mediate their effects through activation of the transcription factor NFκB, CT-1 has been proposed to potentially activate NFκB [45]. Confirming this hypothesis, CT-1-induced NFκB activation and associated cardiomyocyte protection against hypoxic stress were abrogated using selective inhibitors of p38 MAPK, ERKs, or Akt in cultured adult rat cardiomyocytes [45]. This study indicates that intracellular kinase activation is required for CT-1-mediated benefits in cultured cardiomyocytes [45]. MEK5-ERK5 pathway has been also shown to be activated by CT-1 in cardiac hypertrophy [46]. In addition to these pathways, it has been demonstrated that CT-1 might modulate *in vitro* the activity and expression of the suppressor of cytokine signaling (SOCS3) as well as the peroxisome proliferator-activated receptor (PPAR) within cardiomyocytes [14, 18, 19].

Similar to other members of the IL-6 family, the binding of CT-1 to its receptor induces a plethora of intracellular signaling pathways that transduce its effects [11, 47]. Tian and coworkers have shown that the selective inhibition of signal transducer and activation of transcription- (STAT-) 3 strongly reduced CT-1-induced hypertrophy in cultured rat cardiomyocytes [48]. Importantly, ERK1/2 phosphorylation in this model was associated with the inhibition of STAT3 pathway, thus negatively regulating CT-1-mediated benefits [48]. The controversial results shown in the articles cited above mainly confirm that further investigations are needed to identify the different CT-1-triggered pathways.

5. Specific Role of CT-1 in Obesity

Obesity is associated with a chronic low-grade inflammatory state, characterized by elevated circulating levels of cytokines, and the activation of proinflammatory signaling pathways [49, 50]. Wellen and Hotamisligil analyzed findings from several studies investigating metabolic and immunological disorders in obesity and related cardiovascular diseases [50]. The authors recommended considering systemic inflammation as a promoting factor for the metabolic and cardiovascular diseases [50]. Insulin resistance has been also described as strongly associated with abnormal accumulation of adipose tissue in obesity [51]. Therefore, adipocytes were investigated not only as an energy storing organ, but also as critical players in the regulation of glucose metabolism. More recently, it became evident that adipose tissue also represents a major source of hormones and cytokines (also called adipokines) [5]. In particular, Hotamisligil and coworkers confirmed that adipocytes display several immunomodulatory properties, including the secretion of inflammatory hormones and cytokines [52]. Importantly, these mediators have been shown as pivotal players in the modulation of inflammation, as well as glucose and lipid metabolism [50]. Among these molecules, the members of the interleukin-6 (IL-6) family (thus, potentially CT-1) have been directly correlated with development of insulin resistance in asymptomatic subjects [53] as well as in frankly diabetic patients [54]. In particular, CT-1 has been hypothesized to promote insulin resistance in cultured adipocytes [14]. The point that CT-1 might directly induce insulin resistance and it can be in turn upregulated by hyperglycemia or hyperinsulinemia represents a matter of debate. Chronic treatment with recombinant CT-1 has been shown to potentially downregulate food intake in mice [55]. In humans, intense physical exercise has been shown to be directly associated with plasma CT-1 levels [56]. This potential nutritional and stress-mediated rapid regulation of CT-1 levels is in partial contrast with the hypothesis of a causal activity of CT-1 in insulin resistance and potentially associated metabolic syndrome. Furthermore, it was found that the molecular pathways underlying such response involve an increased activation of STAT1, 3, 5A, and 5B, as well as the upregulated expression of SOCS3 mRNA in adipocytes [14]. Concomitantly, a transient decrease of the peroxisome proliferator-activated receptor γ (PPARγ) mRNA was also

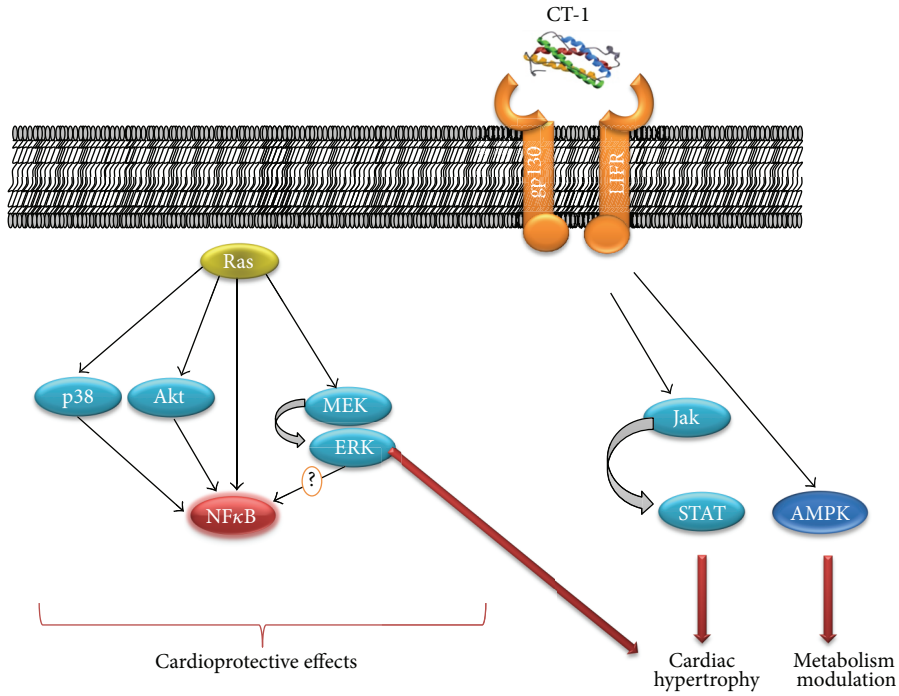

Figure 1: Activation of the Jak-Stat, MAP kinase signal transduction pathways, and AMPK, by CT-1 in cardiomyocytes. The mechanisms of Stat activation as well as a putative signaling cascade leading to NFκB (nuclear factor-kappa B) activation are shown. Downstream target genes linking the Jak-Stat, MAP kinase signal transduction pathways, and AMPK to the induction of cardiomyocyte hypertrophy, antiapoptotic and metabolic effects of CT-1 remain to be elucidated.

observed in these cells [14]. The chronic stimulation with CT-1 on 3T3-L1 adipocytes also resulted in a decrease of both fatty acid synthase and insulin receptor substrate-1 protein expression [14]. Taken together, these results revealed that evidence for a direct role of CT-1 as potential promoter of insulin resistance in adipocytes remains to be confirmed [14]. However, this study did not reveal whether adipocytes are a major source of CT-1 and whether circulating CT-1 is enhanced in patients with an increased adipose tissue mass. Accordingly to the known association of centripetal obesity with other cardiovascular risk factors (i.e., in metabolic syndrome) [57], increased circulating levels of CT-1 have been also confirmed in patients with obesity and hyperglycemia as compared to healthy controls [13]. Indeed, Natal and coworkers showed that adipocytes under hyperglycemic conditions were an important source of CT-1, which might in turn favor insulin resistance [13]. Different from adults, obesity in adolescents has not been confirmed as a condition characterized by increased plasma levels of CT-1 [58]. In particular, also body mass index and waist circumference did not correlate with CT-1 plasma levels in adolescents [58]. Importantly, in contrast to adults, systolic blood pressure at rest correlates inversely with CT-1 [58], suggesting that CT-1 might not be a pathophysiological link between obesity, insulin resistance and metabolic syndrome in young human beings. Considering these controversial results in humans and mice, it could not be concluded that CT-1 might directly promote insulin resistance, particularly in hyperglycemic and obese patients [13]. Evidence from studies in animal models is in partial contrast with these preliminary results *in vitro* and in human beings. Surprisingly, CT-1 knockout mice were shown to develop insulin resistance that could be prevented by administration of exogenous CT-1 [18]. Indeed, the same CT-1 knockout mice also developed dyslipidemia, hypercholesterolemia, type 2 diabetes, and adult onset obesity, thus mimicking the human metabolic syndrome. However, in contrast to humans where metabolic syndrome is often associated with an increase in food intake, in these animals, metabolic defects result from reduced energy expenditure [18]. Exogenous CT-1 administration in wild-type mice increased energy expenditure, fatty acid oxidation, and glucose cellular uptake [18]. In the same article, the authors showed that in mouse models of genetic and acquired obesity, chronic treatment with recombinant CT-1 lead to increased lipolysis, enhanced fatty acid oxidation, and stimulation of mitochondrial biogenesis as well as adipocytes shrinkage. Finally, treatment with CT-1 increased insulin-stimulated Akt and basal AMPK phosphorylation within the skeletal muscle, which might, respectively, explain the restored insulin responsiveness and increased fatty acid oxidation [18]. In line with the results of this important article, we have recently shown that high dose of CT-1 (10 nM) improved insulin responsiveness in cardiomyocytes through activation of AMPK and Akt [59]. Although controversies exist, short-term CT-1 administration might be considered as a potential treatment to prevent obesity, insulin resistance, and metabolic syndrome [55]. In fact, while acute administration of CT-1 might improve glucose metabolism and insulin resistance, chronic treatment

Table 1: Summary of the most relevant CT-1-mediated effects *in vitro*, *in vivo*, and in humans in adipose tissue and obesity.

Author [Ref.]	Year	Model	Exogenous treatment	Effects
			In vitro	
Natal et al. [13]	2008	Murine 3T3-L1 preadipocytes versus differentiated adipocytes	Not applicable	Upregulation of CT-1 levels in differentiated adipocytes and in response to proinflammatory molecules
Zvonic et al. [14]	2004	Murine 3T3-L1 preadipocytes and differentiated adipocytes	Recombinant human CT-1 (0.02–2 nM)	Dose- and time-dependent activation and nuclear translocation of STAT1, -3, -5A, and -5B as well as ERK1 and -2
			In vivo	
Zvonic et al. [14]	2004	7-week-old C57B1/6J mice	Recombinant human CT-1 at 0.5 microg/animal versus vehicle	Activation of MAPK, STAT-1, -3 in epididymal fat pads
López-Andrés et al. [55]	2012	Wistar rats	Treatment with rat recombinant CT-1 (20 μg/kg per day till 6 weeks) versus vehicle	Chronic treatment with CT-1 increases fibrosis within heart vessel and kidney as compared to controls
Moreno-Aliaga et al. [18]	2011	CT-1 knockout versus wild-type mice under normal diet, high-cholesterol diet, or streptozotocin- (STZ-) induced diabetes	Treatment with rat recombinant CT-1 (0.2 mg/kg per day for 6–10 days) versus vehicle	CT-1 knockout mice develop obesity, insulin resistance, and hypercholesterolemia despite a reduced caloric intake as compared to wild type. Acute treatment with CT-1 decreased blood glucose in an insulin-independent manner as compared to vehicle. Chronic treatment with CT-1 treatment reduced food intake, enhanced energy expenditure, and induced white adipose tissue remodeling as compared to vehicle
			Humans	
Natal et al. [13]	2008	Patients with metabolic syndrome ($n = 43$) versus healthy controls ($n = 94$)	Not applicable	Increased plasma levels of CT-1 in metabolic syndrome patients as compared to controls
Limongelli et al. [56]	2010	Triathletes versus matched controls ($n = 20$ per group)	Not applicable	During physical exercise, plasma levels of CT-1 were significantly increased as compared to levels at rest in triathletes
Jung et al. [58]	2008	White adolescents (aged 13 to 17 years) overweight ($n = 37$) versus normal weight controls ($n = 35$)	Not applicable	No increase in CT-1 plasma levels in overweight adolescents as compared to normal weight controls

might induce negative effects on the heart, arteries, and kidney [55]. Although a 10-day treatment with CT-1 did not cause any cardiac hypertrophy nor cardiac enlargement, a prolonged therapy with CT-1 (20 μg/kg per day for 6 weeks) has been shown to increase myocardial dilatation and fibrosis, renal glomerular and tubule-interstitial fibrosis, arterial stiffness, and collagen content independently of blood pressure levels in CT-1-treated Wistar rats as compared to control vehicle. Therefore, chronic CT-1 administration has been indicated as a potential profibrotic approach particularly for the heart, vessels, and kidney [55]. In contrast to these effects observed with high dose of CT-1 (10 nM), we found that low dose of CT-1 (1 nM) is detrimental for the glucose metabolism in cultured cardiomyocytes [59]. These opposite activities of CT-1 on glucose metabolism in different organs could be partly explained by a dose-dependent effect. Accordingly, Zolk and coworkers described that chronically increased synthesis and release of CT-1 could accelerate contractile dysfunction, whereas acute administration of CT-1 could preserve contractility [60]. Thus, if confirmed, adverse effects of CT-1 in the myocardium and other organs have to be considered before starting a first-in-men clinical trial, mainly for chronic treatments.

6. Conclusion

Although promising protective activities have been shown for CT-1 against liver apoptosis [15], hepatocyte ischemic injury [16], and renal toxicity of iodinated contrast media [17], evidence from basic research studies indicates CT-1 to be a controversial molecule with potential opposite activities in obesity, insulin resistance, and related increased cardiovascular risk. CT-1 has been shown to promote cardiac hypertrophy, but also potentially restore insulin responsiveness. Therefore, it remains unclear whether CT-1 is a beneficial or deleterious cytokine in obesity. Furthermore, its role as a DAMP-triggering atherosclerotic inflammation is still debatable. The most relevant studies on CT-1 mediated effects *in vitro*, *in vivo,* and in humans on adipose tissue and obesity have been

summarized in Table 1. Although CT-1 has been shown to activate several intracellular signaling pathways via its transmembrane gp130/LIFR receptor, further studies are needed to elucidate the different selectively triggered cellular functions and danger signals. Interestingly, very recently CT-1 has been described as key regulator of glucose and lipid metabolism in mice. However, it is also important to take into account the potential negative effects of CT-1 on the myocardium [42]. To clarify these controversial issues, the scientific community is waiting for the results of the first-in-men phase I, randomized, double blind clinical trial (NCT01334697) evaluating safety, tolerability, and early pharmacokinetics of the intravenous administration of recombinant human CT-1 versus placebo in healthy volunteers. Promising results in human beings might confer to this cytokine also a therapeutic potential.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This research was funded by EU FP7, Grant number 201668, AtheroRemo to F. Mach. This work was also supported by the Swiss National Science Foundation Grants to F. Mach (no. 310030-118245) and F. Montecucco (no. 32003B-134963/1). This work was funded by a Grant from Novartis Foundation to F. Montecucco.

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