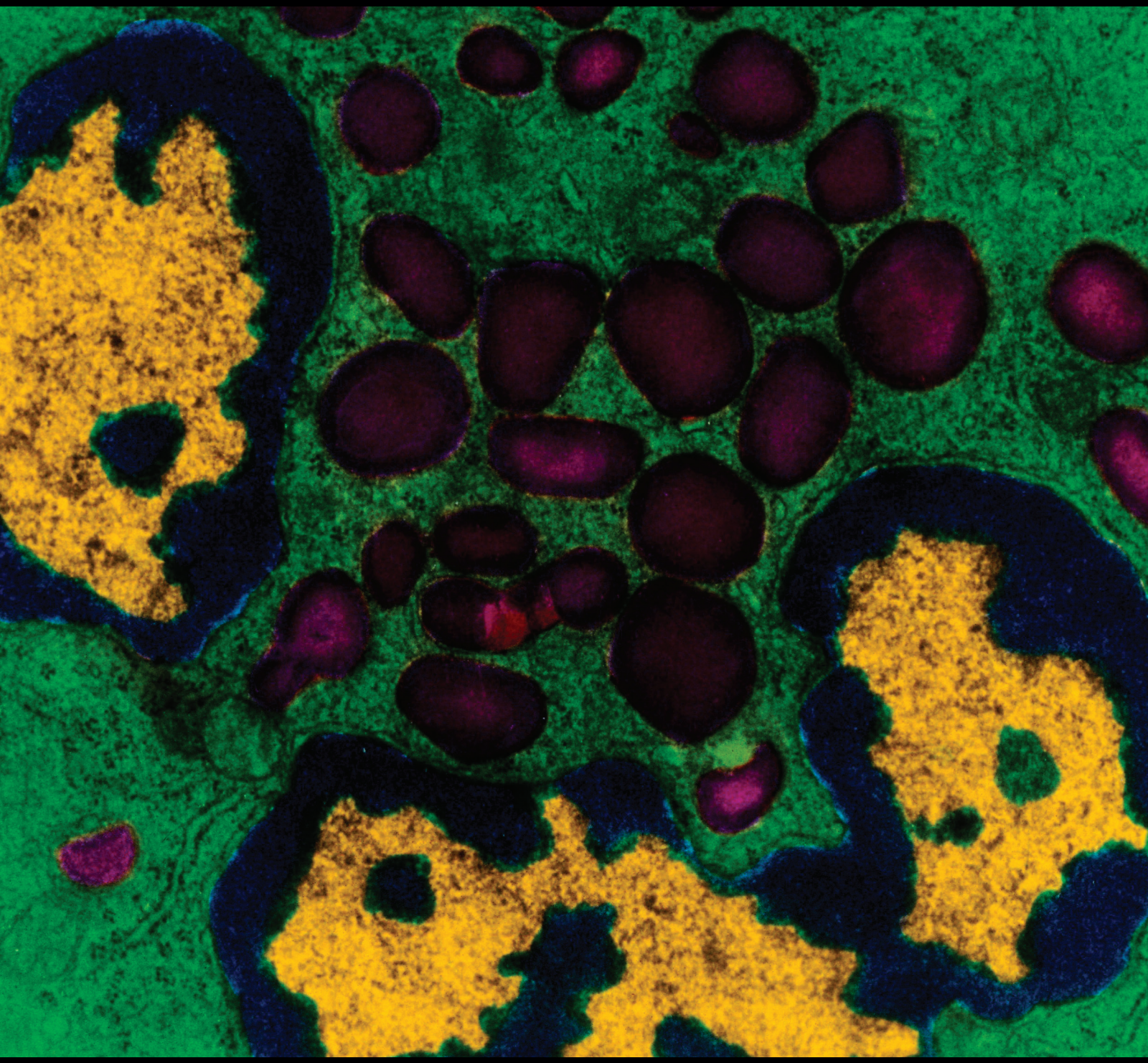
Mediators of Inflammation

C-Reactive Protein and Arteriosclerosis

Guest Editors: Jan Torzewski, Jianglin Fan, Heribert Schunkert, Alexander Szalai, and Michael Torzewski



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Editorial

C-Reactive Protein and Arteriosclerosis

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Although extensively studied and indeed emotionally discussed for more than two decades the role of C-reactive protein (CRP) in cardiovascular disease remains controversial. Three major questions are still not yet resolved. (1) Is CRP a clinically relevant marker of cardiovascular risk? (2) Is CRP even more than a risk marker, that is, a risk factor in cardiovascular disease? (3) Finally, is CRP a cardiovascular drug target?

This special issue comprises four review articles and three research articles that reflect the ongoing controversy over the subject. The review articles discuss CRP as a cardiovascular risk marker, CRP in animal models, CRP in its native and nonnative conformation, and CRP in human arteriosclerosis. The research articles deal with CRP as a cardiovascular risk marker in non-ST elevation acute coronary syndrome and with CRP as a drug target. The discussion climaxes in two research articles describing the use of CRP specific antisense oligonucleotides (ASOs) for the treatment of cardiovascular disease in animal models. The conclusions are indeed contradictory. When looking at the two articles, three general points may have to be taken into consideration. (1) Is the statistical power of the animal experiments adequate to draw definitive conclusions? (2) Are there off-target effects of the ASOs that may confound the results? And (3) is it necessary to design studies incorporating multiple on-target ASOs?

Specific CRP inhibition followed by use of CRP inhibitors in controlled clinical trials may be the only way to prove or disprove a causative role for CRP in cardiovascular disease.

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Review Article

Recognition Functions of Pentameric C-Reactive Protein in Cardiovascular Disease

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C-reactive protein (CRP) performs two recognition functions that are relevant to cardiovascular disease. First, in its native pentameric conformation, CRP recognizes molecules and cells with exposed phosphocholine (PCh) groups, such as microbial pathogens and damaged cells. PCh-containing ligand-bound CRP activates the complement system to destroy the ligand. Thus, the PCh-binding function of CRP is defensive if it occurs on foreign pathogens because it results in the killing of the pathogen via complement activation. On the other hand, the PCh-binding function of CRP is detrimental if it occurs on injured host cells because it causes more damage to the tissue via complement activation; this is how CRP worsens acute myocardial infarction and ischemia/reperfusion injury. Second, in its nonnative pentameric conformation, CRP also recognizes atherogenic low-density lipoprotein (LDL). Recent data suggest that the LDL-binding function of CRP is beneficial because it prevents formation of macrophage foam cells, attenuates inflammatory effects of LDL, inhibits LDL oxidation, and reduces proatherogenic effects of macrophages, raising the possibility that nonnative CRP may show atheroprotective effects in experimental animals. In conclusion, temporarily inhibiting the PCh-binding function of CRP along with facilitating localized presence of nonnative pentameric CRP could be a promising approach to treat atherosclerosis and myocardial infarction. There is no need to stop the biosynthesis of CRP.

1. Introduction

C-reactive protein (CRP) is a multifunctional and evolutionarily conserved plasma protein (reviewed in [1–8]). Through the circulation, CRP reaches tissues and is seen deposited at sites of inflammation. Human CRP is comprised of five identical subunits arranged in a cyclic pentamer [9]. In this paper, we review two recognition functions of pentameric CRP which are relevant to cardiovascular disease: the phosphocholine- (PCh-) binding function of native pentameric CRP that has been implicated in acute myocardial infarction and ischemia/reperfusion (I/R) injury and the atherogenic low-density lipoprotein- (LDL-) binding function of nonnative pentameric CRP that has been implicated in atherosclerosis.

2. PCh-Binding Function of Native Pentameric CRP, Myocardial Infarction, and I/R Injury

A major function of CRP in its native pentameric form is to bind, in a Ca^{2+}-dependent manner, to molecules and cells bearing exposed PCh groups, such as the cell wall of pneumococci and cell membrane of damaged cells [10, 11]. Once CRP is bound to a PCh-containing ligand, it activates the complement system to destroy the ligand [12, 13]. When CRP binds to foreign pathogens, it helps in the killing of the pathogen via complement activation. In mouse models of pneumococcal infection, CRP has been shown to be protective; that is, CRP decreases bacteremia and increases survival of infected mice ([14] reviewed in [15, 16]). Experiments performed *in vitro* using necrotic and apoptotic cells reveal that the binding of CRP to necrotic and apoptotic cells can facilitate the removal of such cells [17–21]. However, experiments performed *in vivo* using animal models of I/R injury reveal that the binding of CRP to damaged cells is detrimental to the tissue [22–25]. Combined data suggest that the consequences of the binding of CRP to damaged cells depend on the tissue. In many places in the body (skin and subcutaneous tissue, e.g.,), it does no harm to bind complement and hasten death of dead tissue. The situation for the organs which are working all the time and do not have the ability to regenerate their tissue (heart, e.g.,) is different

and hastening removal of dead tissue will be harmful. During myocardial infarction, the necrotic part of the myocardium will be removed by CRP. However, the ischemic part of the tissue where the damage can be reversed may also be removed by CRP, as described previously [26]. Thus, the PCh-binding function of CRP is defensive for the host because it leads to protection against pneumococcal infection and removal of necrotic tissue. On the other hand, the PCh-binding function of CRP is detrimental for the host when CRP binds to reversibly damaged myocardial cells, because it causes more damage to the tissue via complement activation.

Studies in animals (mice, rats, and rabbits) and human specimens have shown that both CRP and components of the activated complement system are deposited and colocalized in myocardial infarcts and that complement activation is due to the presence of CRP [27–32]. CRP has been shown to exacerbate left ventricular dysfunction and promote adverse left ventricular remodeling after myocardial infarction [33]. Mostly by employing animal models of I/R injury, it has been shown that CRP enhances the size of myocardial infarcts and also contributes to ischemic tissue damage in intestine, lung, kidney, and brain [22–25, 32–34]. In a mesenteric I/R model, CRP deposition correlated with complement deposition, suggesting a role of CRP in complement activation [23]; in these studies, inhibition of complement activation by using C1 inhibitor reduced the effects of CRP on intestinal injury. Similarly, inhibition of complement activation by decay-accelerating factor also prevented CRP-mediated intestinal injury and remote lung damages following mesenteric I/R [24]. In mice transgenic for human CRP, arterial injury resulted in an expedited and higher rate of thrombotic occlusion compared to that in nontransgenic mice [35]. CRP-mediated exacerbation of vascular injury involves complement since lowering the biosynthesis of CRP prevented complement consumption [36]. These findings indicated that an intact complement system is required for the damaging effects of CRP on myocardial injury because lowering of CRP level, depleting complement, or blocking CRP-mediated complement activation abrogated the effects of CRP. Thus, CRP- and CRP-mediated complement activation both contribute to myocardial injury.

Each subunit of CRP has a PCh-binding site. The three-dimensional structure of the PCh-binding site reveals that Glu81 in the PCh-binding hydrophobic pocket of CRP interacts with the nitrogen atom of choline in PCh, that Phe66 interacts with the three methyl groups of choline, and that Thr76 is critical for creating the appropriately sized pocket on CRP to accommodate PCh. The phosphate group of PCh directly coordinates with the two calcium ions bound to CRP [9, 37]. We generated a CRP triple mutant, F66A/T76Y/E81A, that does not bind to PCh and was therefore unable to form complexes capable of activating complement [14]. Such a mutant is suitable for use in experiments aimed at defining the contribution of the PCh-binding site of CRP in deteriorating tissue injury. In another approach, pharmacological inhibition of CRP using a PCh-based compound reduced the deposition of CRP at myocardial infarcts and inhibited complement activation, indicating that the PCh-binding site of CRP participates in worsening the infarct size and that the inhibition of the PCh-binding site is a useful strategy to prevent tissue-damaging conditions [38]. Similarly, pharmacological inhibition of biosynthesis of CRP also resulted in a reduction of CRP-mediated exacerbation of vascular injury [36].

3. LDL-Binding Function of Nonnative Pentameric CRP and Atherosclerosis

Native CRP does not bind to native LDL under normal physiological conditions [39–42]. Native CRP and native LDL interact with each other only when either one is immobilized, modified, or aggregated [39–44], raising the possibility that CRP and LDL may interact with each other under pathological conditions. The native pentameric structure of CRP can be modified *in vitro* and we have shown that the recognition functions of nonnative pentameric CRP are different from those of native CRP: one function of CRP in its nonnative pentameric conformation is to bind to atherogenic LDL [45–49]. Two types of LDL are used as models of atherogenic LDL: enzymatically modified LDL (E-LDL) and oxidized LDL (ox-LDL) [50–53]. To E-LDL, even native CRP binds and the binding is inhibited by free PCh [40, 45, 54]. Data obtained from PCh-inhibition experiments suggest that CRP binds to E-LDL through the PCh groups in E-LDL and that the binding is mediated by the PCh-binding site of CRP. However, the amino acids in CRP that contact PCh are not critical for the binding of CRP to E-LDL, indicating that the PCh groups present in E-LDL are not the only components in E-LDL through which CRP binds to E-LDL [45]. It has been shown that CRP binds to E-LDL through cholesterol also and that this binding was also PCh-inhibitable [55, 56]. Nonnative CRP binds to E-LDL more avidly than native CRP through an as-yet-undefined mechanism [45–47].

Several investigators have reported that native CRP can also bind to ox-LDL through the PCh moiety in ox-LDL [41, 54, 57] and several investigators have reported that native CRP does not bind to ox-LDL [42, 47, 48, 55, 58]. CRP has also been shown to bind to ox-LDL *in vivo* in diabetes mellitus patients with atherosclerosis and when ox-LDL is complexed with β2 glycoprotein I [59, 60]. We reported that a modification of the native pentameric structure of CRP was required for binding to ox-LDL and that CRP, in its nonnative pentameric conformation, binds efficiently to ox-LDL [46–48]. Taken together, it seems that the binding of CRP to ox-LDL depends on the stringency of the method used to prepare ox-LDL. If the PCh groups are exposed to ox-LDL, then native CRP would bind, if the PCh groups are not exposed to ox-LDL, then native CRP would not bind, and nonnative CRP would bind to ox-LDL regardless of the extent and nature of oxidation. The mechanism of interaction between nonnative CRP and atherogenic LDL, however, has not been elucidated yet. On the LDL molecules, the moieties that could interact with CRP include PCh, cholesterol, apoB, and phosphoethanolamine [40, 41, 43, 45, 51, 52, 55, 56, 61, 62]. In addition, the amyloid-like structures which are induced in LDL by oxidation could also be recognized by nonnative CRP [63]. We hypothesize that the LDL-binding site is buried (or absent)

in native CRP and is exposed (or formed) in nonnative CRP by the loosening up of the pentamer [47, 48]. CRP has been found deposited and colocalized with LDL in atherosclerotic lesions in humans and experimental animals, indicating the presence of nonnative CRP at the lesions [64–70].

The recognition function of CRP to bind to atherogenic LDL should have an effect on the formation of LDL-loaded macrophage foam cells and also on the proinflammatory effects of foam cells and LDL. The formation of foam cells represents an early step in atherosclerosis and begins when macrophages bind and take up LDL [71–73]. Using immunohistochemical staining of atherosclerotic lesions with antibodies to CRP and LDL, the outcome of the interactions among native or aggregated CRP, LDL, and macrophages with regard to the formation of macrophage foam cells has been investigated extensively [44, 57, 65, 74–76]; however, a review of the published literature does not provide a clear-cut overall conclusion [1, 77]. Similarly, it is also unclear whether both Fcγ receptor CD32 and LDL receptor CD36 on macrophages participate if there is an effect of CRP on the uptake of LDL by macrophages [44, 57, 76, 78]. We investigated the effect of CRP on the accumulation of lipid droplets made up of cholesteryl esters in E-LDL-treated macrophages and found that, in contrast to E-LDL alone, CRP-bound E-LDL was inactive for the formation of foam cells [45]. Other consequences of CRP-LDL interactions have also been reported [74, 79–81]. CRP causes charge modification of LDL [74]. CRP reduces the susceptibility of copper-induced oxidation of LDL [58, 79]. CRP attenuates adhesion and activation of monocytes via the prevention of binding of minimally modified LDL to monocytes; this effect was mediated by the binding of CRP to monocytes [80]. CRP also suppresses the proatherogenic effects of macrophages when bound to lysophosphatidylcholine, a moiety present in oxLDL [81]. Collectively, these findings suggest that CRP, under defined conditions, prevents foam cell formation and reduces proinflammatory effects of LDL and foam cells.

To determine the role of CRP in the development of atherosclerosis, human native CRP has been introduced into three different murine models of atherosclerosis: $ApoE^{-/-}$ mice, $LDLr^{-/-}$ mice, and $ApoB^{100/100}Ldlr^{-/-}$ mice (reviewed in [1, 77]). CRP was found to be neither proatherogenic nor atheroprotective in $ApoE^{-/-}$ mice [82–85]. Both passively administered CRP and transgenically expressed CRP had no effect on the development, progression, or severity of spontaneous atherosclerosis in $ApoE^{-/-}$ mice. In $LDLr^{-/-}$ mice also, there was no effect of CRP on the development of atherosclerosis [86]. In rabbits transgenic for human CRP also, CRP did not affect aortic or coronary atherosclerosis lesion formation [87]. However, two recent studies indicated atheroprotective effects of CRP [88, 89]. In $ApoB^{100/100}Ldlr^{-/-}$ mice, CRP slowed the development of atherosclerosis [88]. In $ApoE^{-/-}CRP^{-/-}$ and $LDLr^{-/-}CRP^{-/-}$ mice, the size of atherosclerotic lesions was either equivalent or increased when compared to that of $ApoE^{-/-}$ and $LDLr^{-/-}$ mice, suggesting that even mouse CRP may mediate atheroprotective effects. These data raise hopes that nonnative CRP may be more atheroprotective than native CRP considering the difference between the LDL-binding recognition functions of nonnative and native CRP.

Although much more experimentation needs to be done, there are already several lines of evidence to indicate that the LDL-binding function of CRP is beneficial and may contribute to atheroprotection. First, CRP reduces further oxidation of LDL. Second, CRP attenuates monocyte adhesion and activation via the prevention of binding of atherogenic LDL to monocytes. Third, CRP suppresses the proatherogenic effects of macrophages. Fourth, CRP prevents foam cell formation. Fifth, at least in two *in vivo* studies, both human and mouse CRP showed some atheroprotective effects.

4. Conclusions

There is no data to suggest that CRP causes a disease. CRP infused in healthy human adults does not result in any significant clinical, hematologic, coagulative, or biochemical changes or any increase in proinflammatory cytokines or acute phase proteins [90]. In case of acute myocardial infarction in model animals, CRP worsens an already existing disease; CRP does what it is programmed to do, that is, to bind to PCh and activate complement, and just in this case CRP does harm. We conclude that CRP is an atheroprotective molecule and, therefore, a host defense protein. CRP mutants (nonnative CRP) capable of efficiently binding to all forms of atherogenic LDL can be evaluated for their effects on the development of atherosclerosis in available animal models to test our conclusion. Administration of exogenously prepared CRP mutant may be useful to capture atherogenic LDL to prevent atherosclerosis. If it turns out that nonnative CRP is indeed atheroprotective, a long-term goal could be to focus on the discovery and design of small-molecule compounds to target CRP (a compound that can change the structure of endogenous CRP) for capturing atherogenic LDL. The purpose of administering a PCh-based compound to target CRP is to inhibit binding of CRP to damaged cells to prevent further damage to myocardial infarcts. As of now, we do not see any need to lower the circulating level of native CRP, as we have suggested previously [91].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effects of Antisense Oligonucleotides against C-Reactive Protein on the Development of Atherosclerosis in WHHL Rabbits

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Increased plasma levels of C-reactive protein (CRP) are closely associated with cardiovascular diseases, but whether CRP is directly involved in the pathogenesis of atherosclerosis is still under debate. Many controversial and contradictory results using transgenic mice and rabbits have been published but it is also unclear whether CRP lowering can be used for the treatment of atherosclerosis. In the current study, we examined the effects of the rabbit CRP antisense oligonucleotides (ASO) on the development of atherosclerosis in WHHL rabbits. CRP ASO treatment led to a significant reduction of plasma CRP levels; however, both aortic and coronary atherosclerotic lesions were not significantly changed compared to those of control WHHL rabbits. These results suggest that inhibition of plasma CRP does not affect the development of atherosclerosis in WHHL rabbits.

1. Introduction

C-reactive protein (CRP) is a classical plasma protein marker that is markedly elevated in the acute phase of inflammation, infection, and tissue damage and thus has been broadly used for monitoring and differential diagnosis [1, 2]. The major functions of CRP include its ability to bind to various ligands exposed to damaged tissue or bacteria (opsonization) for the enhancement of phagocytosis and activation of the complement pathway, thereby enabling it to exert both anti- and proinflammatory functions [2, 3]. CRP is mainly expressed by hepatocytes, and its synthesis is regulated at the posttranscriptional level by cytokines [4]. Ample data from both clinical and experimental studies have shown that a high level of plasma CRP is a risk factor as well as marker for cardiovascular diseases [5–9], although some studies failed to prove the risk of CRP compared to other risk factors. The JUPITER trial (Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin) showed that a lipid-lowering drug, rosuvastatin (Crestor), can significantly reduce the incidence of major cardiovascular events, even in apparently healthy subjects not exhibiting established risk factors such as hyperlipidemia, but with elevated high-sensitive CRP levels [10].

Regardless of this controversy, emerging evidence indicates that high levels of CRP may be potentially atherogenic [11, 12]. However, this hypothesis is under debate. Studies of transgenic mice (expressing either human or rabbit CRP) along with human CRP transgenic rabbits and CRP-deficient mice failed to provide a clear conclusion regarding whether CRP is atherogenic [13–23]. The major concerns about these animal studies are as follows: (1) mouse endogenous CRP is not physiologically active *in vivo* and (2) transgenic proteins are exogenous to animals, which may complicate the evaluation of CRP pathophysiological functions in these models [23]. In our previous study, we found that WHHL rabbits are an excellent model for the study of CRP and its relationship with atherosclerosis because they have higher levels of plasma CRP and immunoreactive CRP proteins are present in lesions of atherosclerosis [24]. In addition, rabbit CRP has 74% homology with human CRP [1] and rabbit CRP levels are highly inducible and responsive during the inflammatory reaction [25]. To examine whether CRP is involved in the development of atherosclerosis and whether therapeutic strategies to lower CRP levels are useful for treating atherosclerosis, we intravenously injected the rabbit CRP antisense oligonucleotides (ASOs) into WHHL rabbits. Using two different-aged WHHL models, we examined (1) whether CRP ASOs could reduce the plasma levels of CRP and (2) whether CRP lowering would affect the initiation and progression of aortic atherosclerosis and coronary atherosclerosis. However, we did not identity antiatherogenic effects of CRP antisense, suggesting that CRP is not an atherogenic factor or a therapeutic target for the treatment of atherosclerosis.

2. Materials and Methods

Watanabe heritable hyperlipidemic (WHHL) rabbits [26] were bred in a closed colony at Kobe University and housed in the animal facility of University of Yamanashi with a 12 h light/dark cycle at 23°C and 55% humidity. They were fed with a standard chow diet (CR-3), containing 17.6% protein, 4.1% fat derived from soybean oil, and 10.1% fiber (CLEA Japan, Inc., Tokyo, Japan) and had free access to water. All animal experiments were performed with the approval of the Animal Care Committee of the University of Yamanashi and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Rabbit CRP antisense oligonucleotides (ASO, 5′ATAAGCAAGCAAACACCC3′, no. 280290) and mismatched control oligonucleotides (5′CCTTCCCTGAAGGTTCCTCC3′, no. 141923) were designed and synthesized by ISIS Pharmaceuticals Inc. (Carlsbad, CA) [27]. ASO 280290 was selected among 100 candidate oligonucleotides and doses aimed at obtaining maximally inhibitory efficacy were screened using cultured rabbit hepatocytes. For *in vivo* studies, CRP ASOs were dissolved in saline solution and intravenously injected into WHHL rabbits through ear veins (60 mg/Kg BW/week) twice a week for 16 weeks. Control mismatched oligonucleotides were injected in the same way as CRP ASOs.

3. Experimental Design and Analysis

To examine whether rabbit CRP ASO administration could affect the development of atherosclerosis, we designed and performed two experiments. For the first experiment, we used young WHHL rabbits aged 3~4 months ($n = 9$ for each group, containing 5 males and 4 females). These WHHL rabbits started to develop aortic atherosclerosis, which represents the so-called early-stage lesions, such as fatty streaks in humans [28]. Therefore, we could examine whether CRP ASOs had any effects on the initiation and prevention of atherosclerosis. For the second experiment, relatively old WHHL rabbits (8–11 months old, $n = 10$ for each group) were used. These old WHHL rabbits developed advanced lesions, which are similar to human atherosclerotic plaques (including lipid cores, fibrous caps, and shoulders) [28]. In this way, we could clarify whether CRP ASO had any influence on the progression of the advanced lesions of atherosclerosis.

Plasma total cholesterol (TC), triglycerides (TG), and HDL-cholesterol (HDL-C) were determined using Wako assay kits (Wako Pure Chemical Industries, Osaka, Japan). For the analysis of lipoprotein profiles, plasma was resolved by electrophoresis in 1% agarose universal gels (Helena Laboratories, Saitama, Japan), and then the gels were stained with Fat Red 7B [29]. Plasma lipoproteins were also analyzed using high-performance liquid chromatography (HPLC) by Skylight Biotech, Inc. (Tokyo, Japan), at the end of the experiment. Plasma CRP levels were quantified using CRP enzyme-linked immunosorbent assay (ELISA) kits (Shibayagi Co., Ltd., Gunma, Japan) [30]. Liver mRNA expression levels of CRP were analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR), as described previously [30]. All rabbits were sacrificed after 16 weeks and their aortas and hearts were dissected for examination of atherosclerotic lesions using the standard method established in our laboratory [23, 31].

3.1. Statistical Analysis. All values are expressed as the mean ± SD and statistical significance was determined using Student's t-test. In all cases, statistical significance was set at $P < 0.05$.

4. Results

4.1. CRP ASO Effects on Young WHHL Rabbits. We first measured the plasma levels of CRP after CRP ASO treatment. As reported in our previous study, WHHL rabbits at 4 months exhibited higher plasma levels of CRP than JW wild-type rabbits [30]. As shown in Figure 1 (left panels), WHHL rabbits had about 10-fold (in males) and 20-fold higher CRP (in females) levels at 4 months than JW rabbits (3.15 ± 0.8 mg/L) [30]. 16 weeks later, plasma levels of CRP were further increased by 143% (on average in males and females), suggesting that these increased CRP levels of WHHL rabbits were accompanied by (or correlated with) the development of aortic lesions induced by hypercholesterolemia. Treatment with CRP ASOs for 16 weeks led to the reduction of plasma CRP by 61% in males and 56% in females compared with that

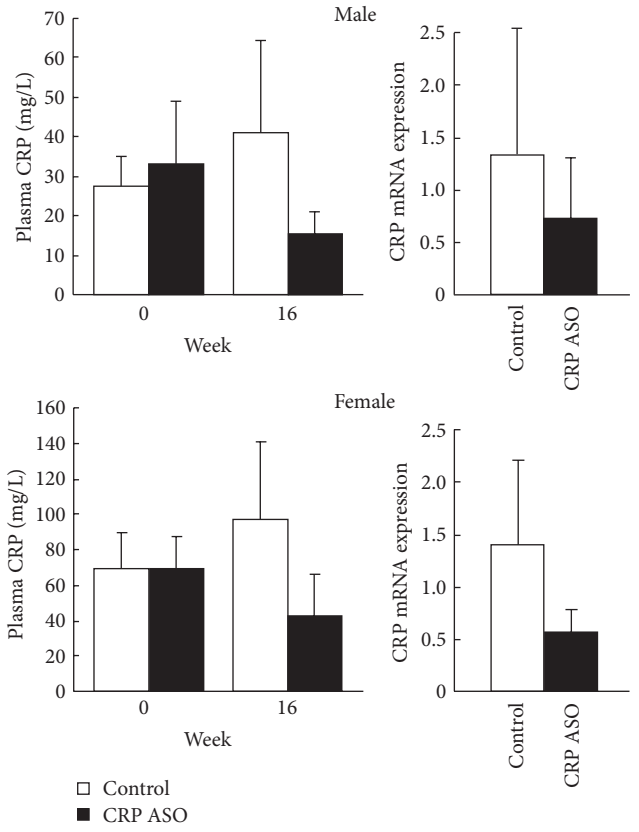

Figure 1: Plasma levels of CRP in WHHL rabbits and real-time RT-PCR analysis of hepatic mRNA of CRP expression. Plasma CRP levels were measured before and after treatment with CRP ASOs for 16 weeks. CRP mRNA expression in the liver was quantified at 16 weeks. The values are expressed as the mean ± SD. n = 4-5 for each group.

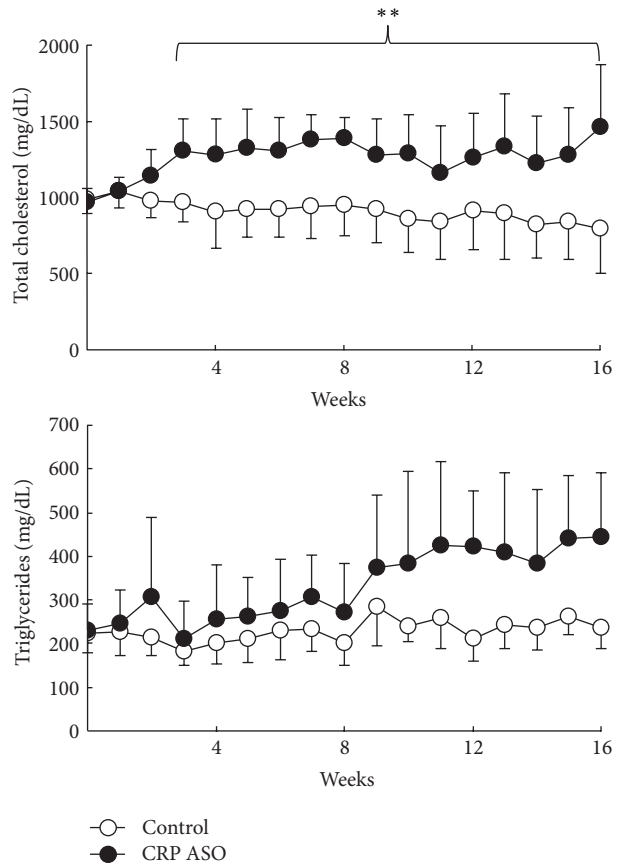

Figure 2: Analysis of plasma levels of lipids. CRP ASO treatment increased plasma lipids (TC and TG). Data are combined from male and female rabbits and expressed as the mean ± SD. n = 4-5 for each group.

of the controls, although the differences were not significant ($P = 0.06$). Reduced plasma levels of CRP were consistent with lower hepatic expression of CRP mRNA, as shown by RT-PCR analysis (Figure 1, right panels).

In spite of this, we found that CRP ASO-treated WHHL rabbits had somewhat higher plasma lipids including TC (starting from 3 weeks, $P < 0.05$) and TG (starting from 10 weeks, $P < 0.05$) than controls, (Figure 2) while HDL-C levels were unchanged (data not shown). Analysis of lipoprotein profiles revealed that increased plasma lipids in CRP ASO-treated WHHL rabbits were mainly caused by significantly increased very low density lipoproteins (VLDLs) ($P < 0.01$) and chylomicron remnant contents ($P < 0.05$) (Figure 3). To elucidate the possible mechanisms, we measured the rates of VLDL secretion in fasting animals *in vivo* using Triton WR-1339 to block hydrolysis of TG-rich lipoproteins by lipoprotein lipase [32]. The base line levels of VLDL-TG of ASO-treated WHHL rabbits were higher than those of controls; however, VLDL synthesis rate afterwards was similar to that in controls (Figure 3).

After rabbits were sacrificed, we compared the aortic lesions and examined the histological features under a light microscope. As shown in Figure 4, ASO treatment did not change the aortic *en face* lesion areas in all parts compared to those of controls. We further compared the microscopic lesion size and histological features. Quantitative analysis of the microscopic lesions and macrophage and smooth muscle cell contents along with CRP immunoreactive protein deposition did not reveal any significant differences between CRP ASO-treated WHHL rabbits and controls (Figure 5).

4.2. CRP ASO Effects on Old WHHl Rabbits. In the second experiment, we treated WHHL rabbits aged 8~11 months with CRP ASOs. These old rabbits showed extensive atherosclerotic lesions in both aortas and coronary arteries [28] accompanied by high levels of CRP [30]. After treatment with CRP ASOs for 16 weeks, plasma CRP levels were consistently lower than in controls (Figure 6).

Similar to the first experiment, plasma levels of TC were higher in the CRP ASO-treated group than that in controls (Figure 7), while TG and HDL-C levels were unchanged (data not shown). Regardless of prominently lower CRP levels, we did not find any differences between ASO-treated and control groups in both aortic gross and microscopic lesion areas (Figures 8 and 9). Histological examination revealed that CRP immunoreactive protein contents were slightly

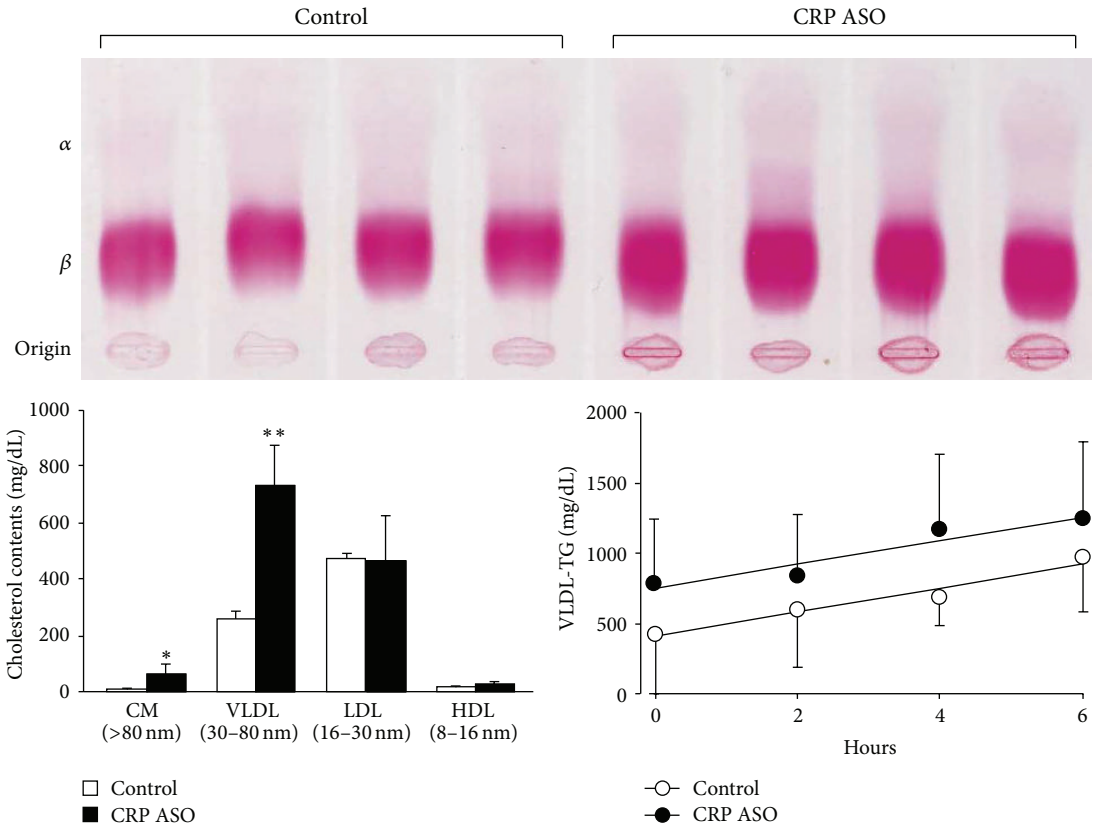

FIGURE 3: Analysis of lipoprotein profiles. Lipoprotein profiles were analyzed by agarose gel electrophoresis (top) and HPLC (bottom left). Post-Triton VLDL production rate in fasting rabbits was measured. Blood was drawn at 0 minutes (before administration of Triton WR-1339) and 2, 4, and 6 hours after Triton WR-1339 injection. VLDL-TG ($d < 1.006$ g/mL) contents were quantified (bottom right). ASO-treated rabbits showed increased levels of β-migrating particles (both VLDL and chylomicron remnants) at the origin. VLDL synthesis rate (expressed by the slope of each line) was the same in ASO-treated rabbits as in the control. Data are expressed as the mean ± SD * or $^{**}P < 0.05$ or 0.01 versus control. n = 4-5 for each group.

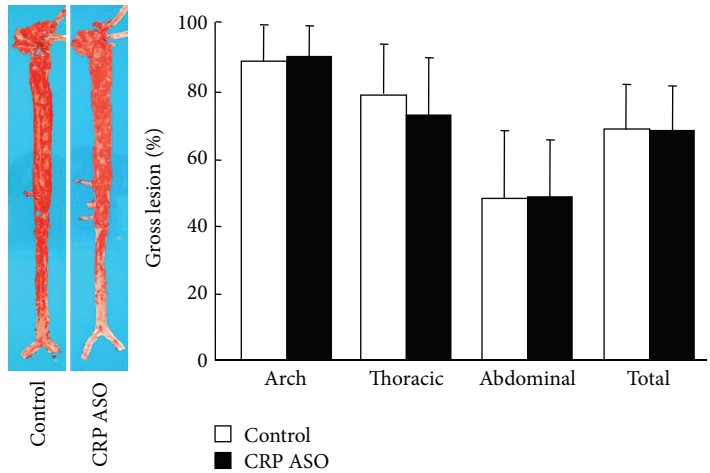

FIGURE 4: Analysis of aortic lesions. Representative photographs of pinned-out aortic trees stained with Sudan IV from CRP ASO-treated and control rabbits are shown (left), and aortic atherosclerotic lesions (defined by sudanophilic area) on the surface were quantified with an image analysis system (right). n = 9 for each group.

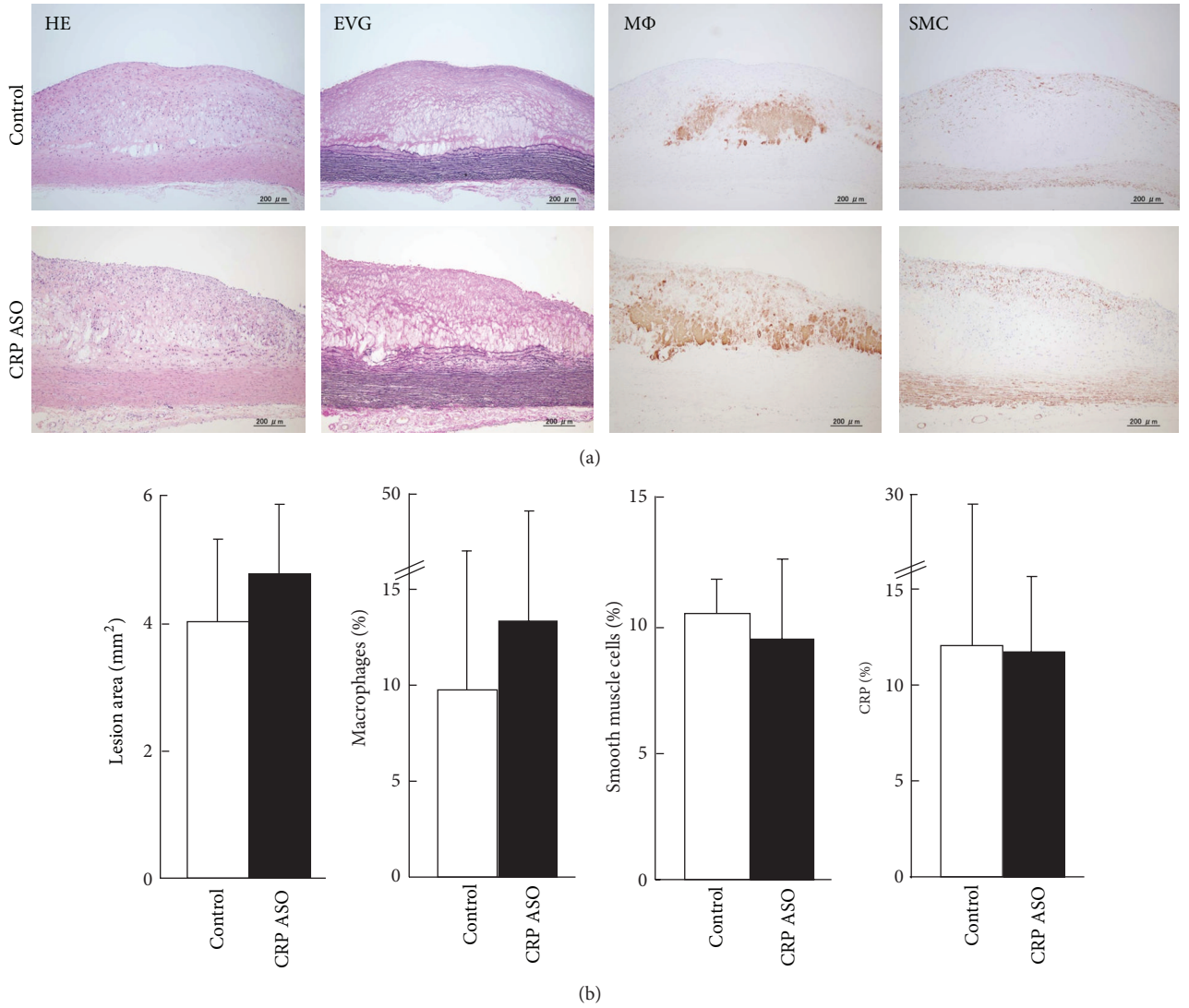

Figure 5: Microscopic analysis of the aortic lesions. Representative micrographs of the aortic lesions from CRP ASO-treated and control rabbits (a). Serial paraffin sections were stained with hematoxylin-eosin (HE) and elastica van Gieson (EVG) or immunohistochemically stained with monoclonal antibodies (mAbs) against either macrophages (Mφ) or α-smooth muscle actin for smooth muscle cells (SMC) or rabbit CRP. Intimal lesions on EVG-stained sections and positively immunostained areas of macrophages; SMC and CRP were quantified with an image analysis system (b). $n = 9$ for each group.

reduced in the lesions of CRP ASO-treated WHHL rabbits, but without statistical significance (Figure 9). Because old WHHL rabbits developed coronary atherosclerotic lesions, we further compared the coronary lesions (expressed as stenosis percentage) and found that left coronary stenosis was slightly less in CRP ASO-treated WHHL rabbits, although the difference was not statistically significant (Figure 10).

5. Discussion

C-reactive protein (CRP) is not only a predictor but also a potential risk factor of cardiovascular events [33]. Several lines of evidence showed that CRP may modulate the vascular functions and thereby influence the initiation and progression of atherosclerosis [11, 34]. On the other hand, many controversial and contradictory results from both human and experimental animals have been published on the effects of CRP on atherosclerosis [18, 23, 35]. In the current study, we first developed rabbit CRP antisense oligonucleotides and then evaluated their effects on WHHL rabbits, a well-established model for the investigation of atherosclerosis. Although CRP ASOs could reduce the plasma levels of CRP through inhibiting hepatic CRP synthesis, we failed to demonstrate any beneficial (antiatherogenic) effects caused by CRP lowering: CRP ASO treatment did not change the aortic and coronary atherosclerosis in two groups of WHHL rabbits compared with that of controls. In spite of this, CRP ASO did not affect the lesion cellular components as well.

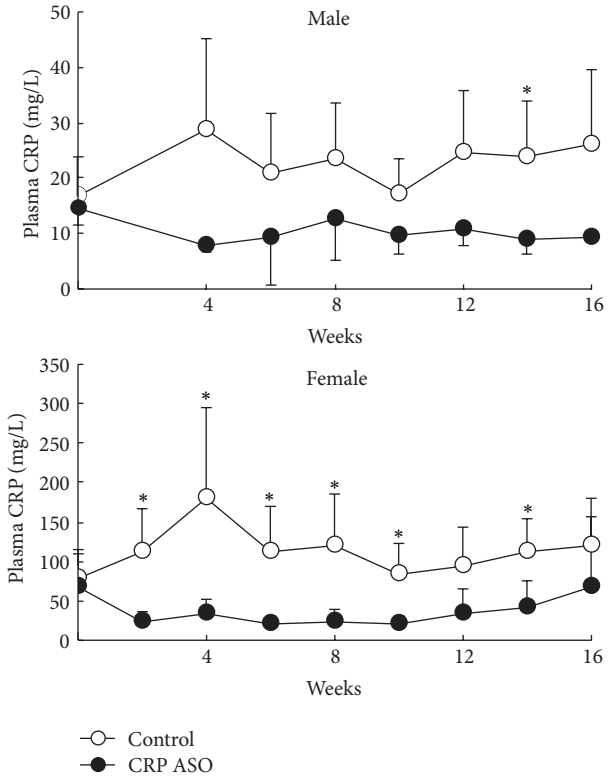

Figure 6: Plasma levels of CRP in WHHL rabbits. Plasma CRP levels were measured every two weeks after CRP ASO treatment. The values are expressed as the mean ± SD. $n = 5$ for each group. $^{*}P < 0.05$ versus control.

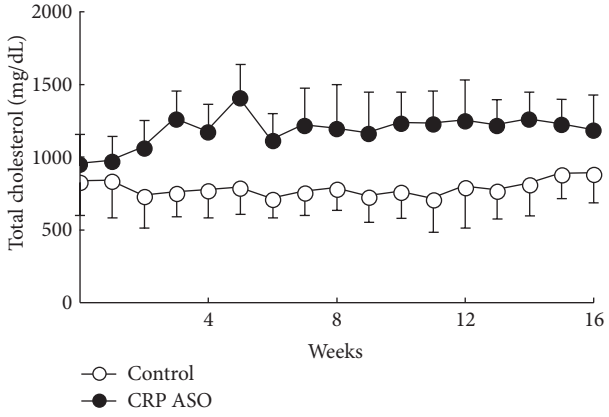

Figure 7: Plasma levels of total cholesterol. Data are combined from male and female rabbits and expressed as the mean ± SD. $n = 10$ for each group.

Therefore, these results are consistent with our previous study using cholesterol-fed human CRP transgenic rabbits [23] and further strengthen the notion that CRP is not an atherogenic factor but rather an inflammatory marker [36]. It is also unlikely that CRP can be a therapeutic target for the treatment of atherosclerosis. These observations are consistent with the

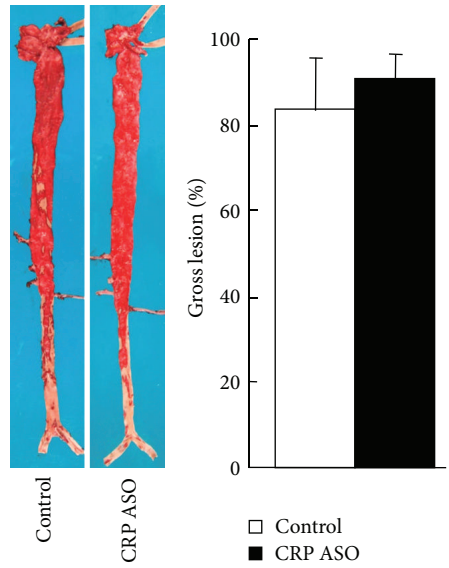

Figure 8: Analysis of aortic atherosclerosis. Representative photographs of pinned-out aortic trees stained with Sudan IV from CRP ASO-treated and control rabbits are shown (left), and aortic atherosclerotic lesions (defined by sudanophilic area) on the surface were quantified with an image analysis system (right). Data are combined from male and female rabbits and expressed as the mean ± SD. $n = 10$ for each group.

current clinical trial results showing that CRP inhibitors can reduce plasma CRP levels by ~80% in normal subjects, as well as, endotoxin challenged and atrial fibrillation patients while other key cytokines, signs, and symptoms remained entirely unchanged in the endotoxin challenged subjects (Graham, M. personal communications).

It should be pointed out, however, that the current results cannot rule out the possibility that CRP may be involved in other inflammatory diseases.

Unexpectedly, we found that CRP ASO treatment elevated plasma lipids in WHHL rabbits due to enhancement of apoB-containing particle production. CRP ASO-induced lipid raising effect was not found in human clinical trials using CRP ASOs. It is currently unknown whether elevated plasma lipids are caused by CRP inhibition or CRP ASOs themselves (such as off-targeting effects).

In conclusion, we found that CRP lowering does not have significant influence on the initiation and progression of atherosclerosis in WHHL rabbits; thus, CRP may not be a therapeutic target for the treatment of atherosclerosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Qi Yu and Zhengcao Liu contributed equally to this work.

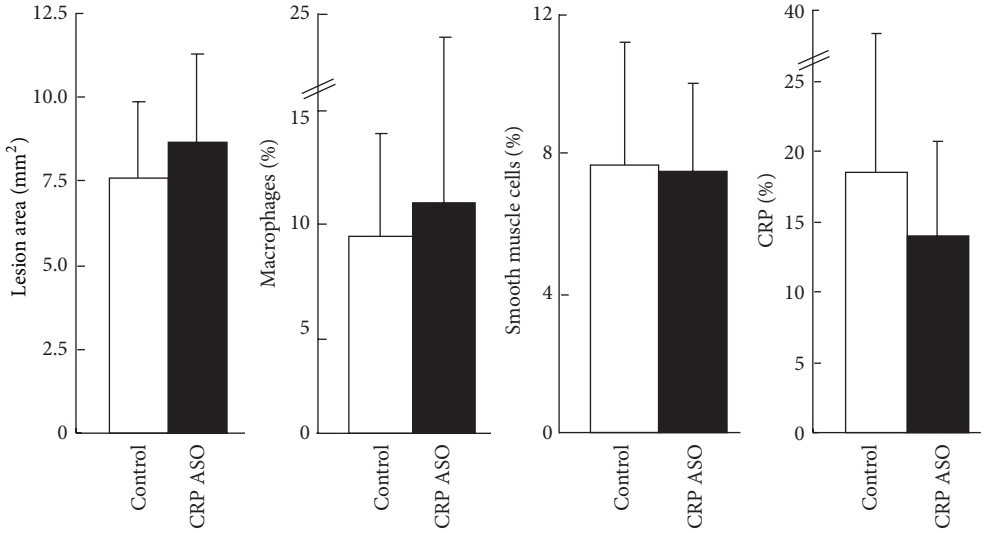

Figure 9: Microscopic analysis of the aortic lesions. Serial paraffin sections were stained with hematoxylin-eosin (HE) and elastica van Gieson (EVG) or immunohistochemically stained with monoclonal antibodies (mAbs) against either macrophages (Mφ) or α-smooth muscle actin for smooth muscle cells (SMC) or rabbit CRP. Intimal lesions on EVG-stained sections and positively immunostained areas of macrophages; SMC and CRP were quantified with an image analysis system. Data are combined from male and female rabbits and expressed as the mean ± SD. $n = 10$ for each group.

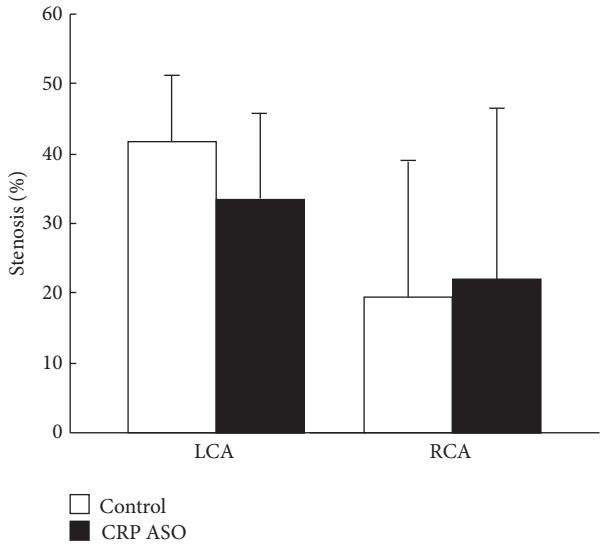

Figure 10: Analysis of coronary atherosclerosis. The heart was cut into 7 blocks, and blocks I and II containing left and right coronary trunks were sectioned in 500 μm intervals (3 sections from each block) and stained with EVG. Coronary stenosis (lesion area/total lumen area × 100(%) was measured and is expressed as percentage. LCA indicates left coronary artery trunks; and RCA, right coronary artery trunks.

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Review Article

Animal Models of C-Reactive Protein

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As the main theme of this special issue, CRP not only is an inflammatory marker but also has diverse biological functions associated with different diseases. To investigate CRP's physiologies and their relationship with human pathological significance, it is essential to use appropriate animal models for translational research. The most popular models for the study of CRP are transgenic mice. However, researchers should be careful when extrapolating the findings derived from these animal models. This review will discuss the current concerns on CRP transgenic mice and rabbits.

1. CRP Mouse Models

More than 30 epidemiological studies have demonstrated a significant association between elevated serum or plasma CRP concentration and the prevalence of atherosclerotic vascular disease, the risk of recurrent cardiovascular events among those with established disease, or the incidence of first cardiovascular events among those at risk [1]. This strong base of epidemiological evidence has led to the hypothesis that CRP is both a marker of and a causal mediator for the development of atherosclerosis.

The question regarding the role of CRP in human atherogenesis is potentially clinically relevant. If CRP as a proatherogenic factor was documented, therapeutic approaches aimed at inhibiting CRP's effects in patients with atherosclerosis would obviously be of interest. Formally, experimental approaches to investigate the role of CRP in mouse models necessitated the introduction of transgenes overexpressing human or rabbit CRP to murine strains or the generation of CRP-deficient mice that have been rendered prone to atherosclerosis. The human gene, when transferred into mice, behaves as it does in man: its expression is highly inducible and tissue-specific [2]. Male CRP transgenic mice constitutively produce human CRP, with serum levels ranging between 10 and 20 μg/mL levels [3] that are comparable to those considered to indicate high risk in humans [4]. Furthermore, as human CRP produced endogenously in transgenic mouse completely avoids any possible contamination or other problems associated with administration of an extraneous CRP preparation, CRP transgenic mice were considered to provide an ideal model for studying the biological activities of human CRP *in vivo*.

Consequently, during the past ten years, a plethora of mouse studies attempted to demonstrate an atherogenic effect of CRP in genetically modified mice. Unfortunately, no clear conclusion could be drawn because these studies gave controversial and contradictory results (Table 1). Rather than answering the question of whether CRP is pro- or antiatherogenic, the following key issues and problems challenging the validity of the mouse model in general were raised as discussed.

1.1. Is CRP an Acute-Phase Protein in Mice? It is a widespread belief that, unlike human CRP, mouse CRP is not an acute-phase reactant, and it is synthesized in only trace amounts [3]. However, owing to methodological shortcomings, serum levels of mouse CRP might be vastly underestimated and

Table 1: CRP in transgenic mouse models.

	Genotype	Sex	Diet	Duration	Treatment	CRP (μg/mL)	Cholesterol, triglycerides (mg/dL)	Morphometry	Significance
Atherosclerotic lesion development									
Paul et al., 2004 [5]	huCRPtg^{+}/ApoE$^{-/-}$	♂, ♀	SD	15 wks, 29 wks	Turpentine	>100	400–800, 60–120	Aortic sinus, en face	$P < 0.02$ (♂): proatherogenic
Reifenberg et al., 2005 [6]	rbCRPtg^{+}/ApoE$^{-/-}$	♂, ♀	Protein-rich	20 wks, 52 wks	—	<30	400–600, 100–200	Aorta, brachiocephalic arteries	n.s.
Trion et al., 2005 [7]	huCRPtg^{+}/E3L	♂, ♀	Cholesterol-rich	25 wks, 30 wks	—	<30	510–670, 100–150	Aortic sinus	n.s.
Hirschfield et al., 2005 [8]	huCRPtg^{+}/ApoE$^{-/-}$	♂	SD	12 wks, 20 wks, 56 wks	—	<30	70–820, 40–440	Aortic sinus	n.s.
Tennent et al., 2007 [9]	huCRPtg^{+}/ApoE$^{-/-}$	♂	SD	77 wks	—	1.51–15.91	101–685, 57–159	Brachiocephalic arteries	n.s.
Kovacs et al., 2007 [10]	huCRPtg^{+}/LDLR$^{-/-}$	♂	SD	15 wks, 30 wks, 40 wks, 50 wks	—	2.4–5.18	308–377, 124–199	Aortic sinus, en face	$P < 0.05$: antiatherogenic
Torzewski et al., 2008 [11]	huCRPtg^{+}/LDLR$^{-/-}$	♂	WTD	4 wks, 8 wks, 12 wks.	—	~10	1668–2555, 741–1424	Aortic sinus, en face	n.s
Teupser et al., 2011 [12]	CRP$^{-/-}$/ApoE$^{-/-}$, CRP$^{-/-}$/LDLR$^{-/-}$	♂, ♀	SD, low fat, semisynthetic diet	12 wks, 16 wks, 20 wks, 36 wks	—	~7.5	414–615, 143–327	Aortic sinus, brachiocephalic arteries, en face	n.s.
Thrombosis and neointima formation									
Danenberg et al., 2003 [13]	huCRPtg^{+}/C57BL/6	♂	SD	12 wks	Femoral wire injury, photochemical injury	18–56	—	Femoral arteries, LCCA	$P < 0.02$, $P < 0.05$: prothrombotic
Wang et al., 2005 [14]	huCRPtg^{+}/C57BL/6	♀	SD	28 days	Carotid artery injury	<30	—	Carotid arteries	$P < 0.05$: vascular injury (neointima formation)
Hage et al., 2010 [15]	huCRPtg^{+}/C3$^{-/-}$	♀	SD	28 days	Carotid artery injury	<30	—	Carotid arteries	$P < 0.05$: vascular injury (neointima formation)

Note: hu: human; rb: rabbit; SD: standard diet; WTD: Western-type diet; wks: weeks; nCRP: native CRP; mCRP: modified CRP; LCCA: left common carotid artery; n.s.: not significant.

comparable to those in humans (see below) [12]. In any case, to overcome the alleged problem of insufficient CRP synthesis in mice, a transgenic mouse that overexpresses human CRP was generated, and this model is widely used to study the role of CRP in cardiovascular disease. The very first report by Paul et al. suggested that the expression of human CRP in mice accelerates aortic atherosclerotic lesion progression, thus asserting that CRP is indeed a risk factor and an active player in atherogenesis *in vivo* [5]. However, this interpretation has been criticised vehemently [6, 8], as differences in lesion size were observed only in males and only at one time point at the end of the study. Moreover, the reported difference "was of marginal statistical significance that could be abolished by elimination of a single outlier" [8], and the use of turpentine to boost circulating CRP levels periodically, which itself might induce active inflammatory pathology in the animals studied, was poorly controlled. Accordingly, the baseline and acute-phase human CRP concentrations were extraordinarily high (100–500 μg/mL), suggesting the presence of active intercurrent inflammatory pathology in the animals studied, for which no controls were reported. Taking these issues together, the difference in the turpentine-treated group cannot be ascribed specifically to human CRP because turpentine is a major nonspecific inflammatory stimulus.

1.2. What Is the Gender Association of Human Transgene CRP Expression? It was demonstrated several years ago that both constitutive and IL-6-dependent acute-phase expression of the CRP transgene in mice requires testosterone [16, 17], restricting meaningful experimental analyses of the role of CRP in cardiovascular disease to male CRP transgenic mice. Surprisingly, despite the widespread knowledge that the expression of transgenic human CRP is under strict testosterone control, female mice were repeatedly included in the respective animal studies [5, 7]. It is no wonder that female mice were not those that provided positive results if any were obtained.

1.3. Which Atherosclerosis-Prone Mouse Model Mimics Human-Like Hypercholesterolemia? Knockout mice with a defect in either apolipoprotein E ($ApoE^{-/-}$) [18] or low-density-lipoprotein receptor ($LDLR^{-/-}$) [19] develop atherosclerotic lesions and are currently widely used as models for investigating the pathomechanisms underlying atherosclerosis. The conflicting results obtained partly suggest that the effects of CRP on atherosclerosis are dependent on the mouse model used. First of all, $ApoE^{-/-}$ mice have far more severe hypercholesterolemia than humans, and most of their cholesterol is contained in very low-density lipoproteins (VLDL) rather than in low-density lipoproteins (LDLs) as in humans. This issue is of high relevance as the effects of CRP on lesion development may be influenced by differences in the degree and type of hyperlipoproteinemia in the mouse models and the apoE protein *per se* can alter immune responses [20]. Immune responses that may directly involve apoE include phagocytosis of apoptotic bodies, altered macrophage dynamics [21] and altered antigen presentation efficiency [22]. Thus, the effects of human CRP on mouse atherosclerotic lesion development may be masked in $ApoE^{-/-}$ mice because they are VLDL animals and have altered immune functions.

Consequently, the $ApoE^{-/-}$ mouse model may not be ideal for studies of human CRP in atherosclerosis. In contrast, the $LDLR^{-/-}$ model may be superior for a number of reasons. First, atherosclerotic lesions do not develop spontaneously as in $ApoE^{-/-}$ animals but are inducible under a Western-type diet (WTD). Second, the serum lipoprotein pattern of $LDLR^{-/-}$ animals is characterized by high-level LDL rather than chylomicrons and VLDL (as in $ApoE^{-/-}$ mice) and thus more closely mimics the situation in humans [6, 10, 11].

1.4. What Is the Functional Role of Human CRP as a Foreign Protein in Mouse? It is unsurprising that there are no experimental animal models of CRP function that completely replicate the human situation. Human CRP is a foreign protein in the mouse, with many uncertainties concerning its functional role in the immune system of these animals. The situation becomes even more complicated when these mice are crossed with ApoE-deficient mice that lack a fully functional complement system. The study by Reifenberg et al. uncovered the disturbing facts that the interactions among CRP, complement, and LDL, as have been delineated in humans, may not exist similarly in mice [6]. It cannot be ruled out that mouse CRP might be active, but the inability of transgenic CRP to execute one of its primary functions (complement activation) places obvious constraints on the validity of this animal model. Moreover, transgenic human CRP operating in a xenogeneic murine environment might also fail to interact with further important mouse effector molecules, such as cellular receptors, the extracellular matrix, and lipoproteins, as pointed out by a number of studies [6, 10, 12, 23]. Thus, it is difficult to determine which of the reports are valid, because the model itself encounters several problems.

To help to overcome the above-mentioned issues, Reifenberg et al. crossbred mice expressing rabbit CRP (rbCRP) onto apoE knockout animals and studied the effect on atherogenesis [6]. Expression of the rbCRP transgene is independent of gender, and an additional inflammatory stimulus is also not required. rbCRP and human CRP are similar in structure and function. Both bind phosphocholine, C-polysaccharide, polycations, chromatin, and histones, activate complement and protect mice from lethal challenges with pneumococci [24–30]. However, no marked effect on the formation of moderately advanced atherosclerotic lesions could be discerned, either in male or in female apoE knockout mice.

1.5. What about the CRP-Deficient Mouse? Owing to doubts about the physiological role of genuine mouse CRP, overexpression rather than deletion of CRP was regarded as the only meaningful way to investigate the impact of CRP on murine atherogenesis. As already mentioned above, however, serum levels of mouse CRP might be vastly underestimated and comparable to those of humans, challenging the

dogma of insufficient CRP synthesis in mice. Recently, a complementary approach was chosen, generating mice with targeted deletion of the CRP gene on B6.ApoE$^{-/-}$ as well as B6.LDLR$^{-/-}$ genetic backgrounds [12]. This approach avoids the above-mentioned xenogeneic complications of CRP overexpression. Reliable commercial reagents along with serum CRP knockout mice as a stringent negative control indicated sufficient expression of mouse CRP even in the noninduced state. On the basis of quantitative analysis of atherosclerotic lesions, the data suggested that mouse CRP may even mediate atheroprotective effects rather than having a proatherogenic role in the two most widely used mouse models of atherosclerosis. These results together with the results by Torzewski [11] add a cautionary note to the idea of targeting CRP as therapeutic intervention against progressive cardiovascular disease and point out that CRP might actually serve a physiological and primarily nonharmful function, as first proposed in 2004 [31].

1.6. Is There an Association of CRP with Atherothrombosis rather than Just Atheroma? One has to bear in mind that most of the epidemiological studies demonstrating a significant association between elevated serum or plasma CRP concentration and the prevalence of human atherosclerotic vascular disease refer not to atherosclerotic lesion development but rather to its clinical sequelae caused by plaque rupture and thrombosis. It is therefore justified to ask whether there is an association of CRP with atherothrombosis rather than just atheroma in animal models. The respective studies, however, are far from being as numerous as those on atherosclerotic lesion development. A first report showed that occlusive thrombosis was more pronounced in CRP transgenic mice than in wild-type mice [13]. More recently, it was demonstrated that there is a more exaggerated response to vascular injury in human CRP transgenic mice [14] and that this response requires complement [15]. Caution on the interpretation of these data is warranted, however, because the vascular remodeling process associated with carotid artery ligation versus atherogenesis is not the same.

1.7. Conclusions. In conclusion, it is evident that each one of the above-mentioned genetically engineered mouse models addresses some of the discussed key issues and problems but leaves enough unresolved problems to call the respective mouse model into question. This can be extended to any "key issue" in atherosclerosis research that mouse models have claimed to address. They have led to very little true advance and, yet more importantly, they have generated many false and confusing concepts. Thus, caution should be exercised when extrapolating observations in genetically engineered mouse models with incompletely characterized physiological alterations to the situation in human disease. Finally, it may be appropriate to say that it was worth generating these mouse model systems, but they hardly enable us to answer definitively whether or not CRP actively contributes to human atherogenesis.

Table 2: Comparison of CRP in different species.

	Mouse CRP	Rabbit CRP	Human CRP
M.W. (Kd)	19.5	22	21
Plasma levels	<2 mg/L	<3 mg/L >100 mg/L*	<1 mg/L >10,000 mg/L*
Activation of complement	No	Yes	Yes
Binding to plasma LDLs	**	Yes	Yes
Deposition in the lesions of atherosclerosis	No	Yes	Yes

*In acute inflammatory state.
**Wild-type mice do not have LDLs as in humans and rabbits.
Also see [6, 32–38] for details.

2. Rabbits as an Alternative CRP Model for Studying Human CRP

As described above, there are many problems of using mouse models for the study of CRP's physiological functions and controversies have risen in regard to CRP's roles in atherosclerosis. To overcome these problems, alternative animals are needed; here, we focus on using rabbit models as another means of investigating CRP biology.

2.1. Rabbit CRP versus Mouse CRP: Which One Is Closest to Human CRP? The molecular and physiological features of rabbit CRP more closely resemble those of human CRP compared with mouse CRP in several aspects (Table 2) [32, 33]. First, like human CRP but unlike mouse CRP, rabbit CRP acts as a major acute-phase reactant (inflammatory marker) in the plasma and thus CRP levels are increased up to ~100 mg/L upon inflammatory stimulation [34]. It is well known that, in mouse plasma, the major inflammatory marker is serum amyloid protein (SAP) rather than CRP; plasma CRP levels in mouse are normally markedly lower than those in rabbits and humans and do not fluctuate regardless of the presence of inflammation [35]. Secondly, rabbit CRP can strongly bind with plasma atherogenic lipoproteins [36], like human CRP [37]. Thirdly, CRP immunoreactive proteins are present in all types of lesion of both rabbit and human atherosclerosis [38], but no CRP was detected in the lesions of mouse. These features of rabbit CRP lead to the notion that rabbits may be an ideal model (or a better model than mouse) for examining the physiological and pathophysiological roles of human CRP [23].

2.2. Rabbit Atherosclerosis Models for CRP. Rabbits have been used as an excellent model for the study of human atherosclerosis because their lipoprotein metabolism and cardiovascular system are similar to those of humans [39]. Unlike mice, but like humans, rabbits have abundant plasma cholesteryl ester transfer protein, an important regulator of cholesterol transfer, and exhibit hepatic apoB100 and intestinal apoB48 synthesis, and their lipoprotein profiles are rich in low-density lipoproteins (LDL) whereas mice are deficient

in cholesteryl ester transfer protein and their plasma lipoproteins are dominated by high-density lipoproteins (HDL). Rabbits are sensitive to a cholesterol-rich diet and develop atherosclerosis rapidly, whereas most strains of wild-type mice are resistant to a cholesterol-induced atherosclerosis. WHHL rabbits provide another means of studying human familial hypercholesterolemia and atherosclerosis because these rabbits are deficient in LDL receptors [40]. A decade ago, we used both cholesterol-fed rabbits and WHHL rabbits and revealed several important features of CRP and their relationship with atherosclerosis [38]. We first found that plasma CRP levels are increased in hypercholesterolemic rabbits and correlated with the severity of aortic lesion size. Secondly, we found that CRP immunoreactive proteins are present in the lesions of atherosclerosis of rabbits regardless of the lesion types. Basically, CRP is associated mainly with extracellular matrix and seldom with macrophages. CRP is also closely colocalized with apoB and the terminal complement complex, suggesting that possible interactions between CRP-apoB-complement are present in the lesions [31, 38, 41]. Thirdly, CRP is basically synthesized by the liver rather than the vascular wall (such as macrophages). The consensus is that it is hepatically synthesized CRP that regulates plasma levels of CRP [42]. Despite this, the presence of CRP deposition in the lesions of atherosclerosis sustained efforts in the cardiovascular field during the last decade to elucidate whether CRP truly constitutes another risk factor for alongside hypercholesterolemia and is indeed involved in the initiation and progression of atherosclerotic disease. If CRP is proinflammatory and atherogenic, can we target CRP for the prevention and treatment of atherosclerosis [23]? These findings obtained from rabbit studies further strengthened the notion that rabbits are an excellent model for illustrating the relationship between CRP and atherosclerosis. Nevertheless, this study using hypercholesterolemic rabbits still cannot answer the question of whether CRP is a mediator or a marker of atherosclerosis [43].

2.3. Human CRP Transgenic Rabbit Model. To elucidate whether high levels of plasma CRP participate in the development of atherosclerosis, our laboratory generated 2 lines of transgenic (Tg) rabbits expressing human CRP (hCRP) transgene in the liver [44]. Plasma levels of hCRP were 0.4 ± 0.13 mg/L and 57.8 ± 20.6 mg/L in these two lines of Tg rabbits, respectively. The expression of hCRP does not cause any health disorders or phenotypes (such as spontaneous atherosclerosis) in Tg rabbits. hCRP isolated from Tg rabbit plasma exhibited the ability to activate rabbit complement, suggesting that human CRP is indeed functional in Tg rabbits [44]. Using this powerful model, we compared the susceptibility of Tg rabbits to cholesterol-rich diet-induced aortic and coronary atherosclerosis with that of non-Tg rabbits. To our surprise, neither high nor low plasma concentrations of hCRP affected aortic or coronary atherosclerotic lesion formation in Tg rabbits, even though a massive amount of hCRP was detected in the lesions of atherosclerosis [44]. Therefore, high levels of plasma and lesional CRP in Tg rabbits do not enhance the development of atherosclerosis. While these results are disappointing to CRP believer, this study cannot exclude the possibility that CRP participates in other pathological processes such as thrombosis, myocardial infarction, and arthritis. On these issues, we performed a series of experiments using hCRP Tg rabbits.

Using double balloon-injury models of the femoral arteries in Tg rabbits, we demonstrated that high expression of hCRP led to enhanced thrombosis formation on the injured smooth muscle cell-rich neointima by upregulating tissue factor expression [45], suggesting that CRP mediates thrombosis. In addition to atherosclerosis, CRP along with complement activation has been shown to accelerate myocardial infarction in rats and targeting CRP can prevent CRP-induced myocardial injury [46, 47]. However, this hypothesis has not been tested in other models, including both mouse and rabbit. Using the coronary ligation method, we generated acute myocardial infarction models in Tg rabbits. In preliminary experiments, we did not find any significant roles of CRP on myocardial infarction size or plasma cardiac markers (Waqar et al. unpublished data).

2.4. Effects of CRP Antisense Oligonucleotides on WHHL Rabbits. The major concern about using cholesterol-fed Tg animals is that transgenic proteins (namely, human CRP) are exogenous to animals and the atherogenic lipoproteins are remnant lipoproteins, so-called β-VLDLs, which may complicate the evaluation of hCRP pathophysiological functions in these models [44]. To overcome this drawback, we attempted to use a therapeutic approach to inhibit endogenous CRP and then examined the CRP-lowering effect. Towards this goal, in collaboration with ISIS Pharmaceuticals, Inc., we designed and injected robust rabbit CRP antisense oligonucleotides into WHHL rabbits, which have elevated plasma CRP levels (10~20-fold higher than in wild-type rabbits) in addition to having atherosclerosis. While tremendous efforts were made in this regard, we failed to demonstrate any therapeutic effects on atherosclerosis in WHHL rabbits (see accompanied paper by Yu et al. in this special issue).

2.5. Conclusions. In conclusion, rabbits are a suitable model for the investigation of CRP physiology because they resemble humans in many aspects, compared with mice. Cholesterol-fed rabbits along with WHHL rabbits and hCRP transgenic rabbits offer another opportunity to elucidate CRP functions that cannot be conducted in mice. After a decade's effort using these unique models, it is time to draw a conclusion regarding the true role of CRP in atherosclerosis. Is CRP a marker or mediator of atherosclerosis? Do we still need to continue the debate? Should we treat CVD patients who have a high level of CRP? The answers are becoming clearer and clearer: without doubt, plasma CRP levels are indeed increased and CRP is intimately present in the lesions of atherosclerosis. However, the net effect exerted by CRP is not proatherogenic, while we cannot rule out the possibility that CRP is antiatherogenic or participates in other diseases. These experimental observations are also in support of the human studies reported recently [48–51]. These studies also

told us that it is unlikely that CRP can be a therapeutic target for the prevention and treatment of cardiovascular diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Change in Growth Differentiation Factor 15, but Not C-Reactive Protein, Independently Predicts Major Cardiac Events in Patients with Non-ST Elevation Acute Coronary Syndrome

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Among the numerous emerging biomarkers, high-sensitivity C-reactive protein (hsCRP) and growth-differentiation factor-15 (GDF-15) have received widespread interest, with their potential role as predictors of cardiovascular risk. The concentrations of inflammatory biomarkers, however, are influenced, among others, by physiological variations, which are the natural, within-individual variation occurring over time. The aims of our study are: (a) to describe the changes in hsCRP and GDF-15 levels over a period of time and after an episode of non-ST-segment elevation acute coronary syndrome (NSTE-ACS) and (b) to examine whether the rate of change in hsCRP and GDF-15 after the acute event is associated with long-term major cardiovascular adverse events (MACE). Two hundred and Fifty five NSTE-ACS patients were included in the study. We measured hsCRP and GDF-15 concentrations, at admission and again 36 months after admission (end of the follow-up period). The present study shows that the change of hsCRP levels, measured after 36 months, does not predict MACE in NSTEACS-patients. However, the level of GDF-15 measured, after 36 months, was a stronger predictor of MACE, in comparison to the acute unstable phase.

1. Introduction

Increasingly, cardiac biomarkers have provided important information in predicting short-term and long-term risk profiles in patients with acute coronary syndromes (ACS), particularly when they are used in combination [1]. Among the numerous biomarkers, high-sensitivity C-reactive protein (hsCRP) has received widespread interest and a large database has been accumulated on their potential role as predictor of cardiovascular events [2, 3]. Growth-differentiation factor-15 (GDF-15) is one of more than 40 members of the transforming growth factor-β superfamily and it was originally identified in activated macrophages [4]. Accumulating evidence indicates that circulating levels of GDF-15 are associated with the risk of death and myocardial infarction, independent of clinical variables and other biomarkers, including hsCRP and cardiac troponins [5, 6].

The inflammatory response triggered in the ACS setting is the cumulative result of preexisting, low-grade inflammation in vulnerable atherosclerotic plaques and the ongoing myocardial ischemic damage during the progression of an acute coronary event [7]. Consistently, the magnitude of the inflammatory response as reflected by the peripheral levels of inflammatory markers is largely determined by the

temporal interval between symptom onset and the time point of biochemical measurement. Optimal interpretation of the elevated circulating levels of biomarkers would, therefore, require knowledge of their release curves and consideration of the time point of blood sampling [8, 9]. However, the long-term temporal changes of hsCRP levels and the relation between the changes of GDF-15 levels have not been examined, after an episode of non-ST-segment elevation ACS (NSTE-ACS).

Consequently, the aims of our study were to

(1) describe changes of hsCRP and GDF-15 levels over time after an episode of NSTE-ACS,

(2) examine whether changes of hsCRP and GDF-15 levels are associated with long-term major cardiovascular adverse events (MACE).

2. Methods

2.1. Study Population. Three hundred and eighty consecutive ACS patients were admitted to the coronary care unit of a university hospital. One hundred and twenty-five patients were excluded from analysis for the following reasons: patients with a history of systemic inflammatory diseases, such as infections or autoimmune disorders, neoplastic or haematological disease, administration of anti-inflammatory or immune-suppressive drugs, and surgical procedures or trauma in the preceding 3 months, patients with an equivocal or uninterpretable electrocardiogram, including left bundle branch block or persistent ST-segment elevation due to a myocardial infarction and patients with significant changes in medical therapy during followup. Thus, 255 NSTE-ACS patients were included in the study.

Patients were followed up for three years regarding the occurrence of MACE (death, myocardial infarction, and unstable angina (Class IIIb)). Therapeutic management during hospitalisation and in the outpatient clinic was left to the discretion of the attending cardiologist, according to the patients' clinical course, standard institutional protocols, and current guidelines [10]. The Ethics Committee of our institution approved the research protocol, and all patients gave written, informed consent for inclusion in the study.

2.2. Biochemical Analysis. Serial venous blood samples were obtained on admission from 8 am to 3 pm, to avoid the diurnal variation of inflammatory biomarkers reported by our group [11]. Blood samples were also obtained on a follow-up evaluation 36 months after admission. Serum samples were obtained by centrifugation, after the formation of the clot of blood, and stored at −70°C for subsequent analyses.

Concentrations of the serum hsCRP were measured, by an ultrasensitive, enzyme-linked, immunosorbent assay kit (DRG Instruments GmbH, Germany). In this enzyme-linked immunosorbent assay, the lowest detection limit of hsCRP was 0.010 mg/L. Coefficients of variation were 5.12% and 11.6% for intra- and interassay variabilities, respectively. Serum GDF-15 concentrations were measured using a commercially, enzyme-linked immunosorbent assay (BioVendor GmB, Heidelberg, Germany). In this assay, the lowest detection limit of GFD-15 is 30.2 pg/mL. Coefficients of variation were 4.3% and 7.8% for intra- and interassay variability, respectively. Troponin I was determined immune enzymatically, using a technique based on sandwich ELISA (Boehringer Mannheim, Germany). Coefficients of variation were 2.2% and 5.9% for intra- and interassay variabilities, respectively.

All other biochemical measurements were performed in the biochemistry laboratory of our hospital from the samples obtained at baseline, using standard methods. Personnel, blinded to patient's baseline characteristics and clinical outcomes, carried out all measurements.

2.3. Statistical Analyses. Results for normally distributed continuous variables are expressed as mean ± SD; nonnormally distributed continuous variables are presented as median and interquartile range. Categorical data is expressed as a percentage. Analysis of normality of the continuous variables was performed with the Kolmogorov-Smirnov test. Unpaired 2-tailed t-tests and the Mann-Whitney U-test assessed differences between the groups for continuous variables, as appropriate. Categorical data and proportions were analysed by use of χ^2 or Fisher's exact test when required. GDF-15 and hsCRP levels had a nonnormal distribution and were, therefore, logarithmically transformed before regression analysis to fulfill the conditions required for this type of analysis.

The information regarding the appearance of the endpoint, combined at a 36-month followup, was available for all patients included in the study. In patients who died during the 36-month followup period, the blood sample was not available, so we evaluated independent predictors of unstable angina (class IIIb) and myocardial infarction (combined primary endpoint). We defined the value delta, as a value that represents the difference between the concentrations of inflammatory markers at admission and at 36-month followup.

Independent predictors of changes were identified by multiple linear regression analysis and multivariable regression analysis, as appropriate. Tested covariates included sex, age, current smoking, diabetes mellitus, hypertension, dyslipidemia, coronary revascularisation, left ventricular ejection fraction, and troponin I. Delta GDF-15 concentrations were introduced into the multivariate model as a binary variable, considering the median as the cut-off value. Backward stepwise selection was used in multivariate analysis to derive the final model for which significance levels of 0.1 and 0.05 were chosen to exclude and include terms, respectively. Differences were considered to be statistically significant if the null hypothesis could be rejected with >95% confidence. The SPSS 15.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for all calculations.

Table 1: Clinical variables of non-ST-segment elevation acute coronary syndrome patients with and without MACE at 36-month followup.

Variable	MACE		P value
	Yes ($n = 45$)	No ($n = 210$)	
Age (years)	68 ± 11	66 ± 11	0.24
Men	25 (55.6%)	138 (65.7%)	0.23
Hypertension (>140/90 mmHg)	32 (71.1%)	133 (63.3%)	0.39
Hypercholesterolemia (>5.17 mmol/L)	19 (42.2%)	99 (47.1%)	0.62
Smokers	31 (68.9%)	121 (57.6%)	0.18
Diabetes	14 (31.1%)	75 (35.7%)	0.61
TIMI risk score			0.47
2	9 (20%)	35 (16.7%)	
3	9 (20%)	74 (35.2%)	
4	16 (35.6%)	58 (27.6%)	
5	10 (22.2%)	38 (18.1%)	
6	1 (2.2%)	5 (2.4%)	
Coronary artery disease			0.35
1 vessel	22 (4.9%)	115 (54.8%)	
2 vessel	13 (28.9%)	40 (19%)	
3 vessel	9 (20%)	45 (21.4%)	
LVEF (%)	54 ± 10	56 ± 12	0.22
Treatment at admission			
Aspirin	45 (100%)	207 (98.6%)	0.9
Clopidogrel	39 (86.7%)	161 (76.7%)	0.16
Nitrates	44 (97.8%)	202 (96.2%)	0.9
Statins	45 (100%)	207 (98.6%)	0.9
Angiotensin-converting enzyme inhibitors	18 (40%)	68 (32.4%)	0.9
β-Blockers	42 (93.3%)	191 (91%)	0.77
Biochemistry			
Creatinine (mg/dL)	1.17 ± 0.25	1 ± 0.87	0.25
Total cholesterol (mmol/L)	4.05 ± 1.04	4.00 ± 1.18	0.48
Peak troponin I (ng/mL)	4.85 ± 0.16	4.39 ± 0.17	0.09

Data is expressed as mean ± standard deviation and number of patients (%) for categorical variables.
MACE: major adverse cardiovascular events; LVEF: left ventricular ejection fraction.

3. Results

Demographic and clinical data of patients with and without MACE are presented in Table 1. After 36 months of followup, the combined endpoint ((cardiac death (7 patients), myocardial infarction (3 patients), and unstable angina class IIIB (35 patients)) appeared in 45 patients (17.6%). There were no significant differences in age, sex, cardiovascular risk factors, TIMI risk score, severity of coronary artery disease, treatment, and standard biochemical results between the two groups.

Regarding inflammatory biomarkers, we found no differences between both groups in levels of hsCRP at admission and after a three-year followup (Table 2). However, delta hsCRP concentrations were higher in patients who developed MACE compared to patients who did not ($P = 0.01$) (Table 2). We found significant differences in the GDF15 levels after the three-year followup between both groups ($P < 0.001$) (Table 2). Moreover, delta GDF-15 concentrations were higher in patients who developed MACE ($P < 0.001$) (Table 2).

Multivariate analysis showed that delta GDF-15 (OR = 52.3, CI 95% 7-388.5, $P < 0.001$) was the unique independent predictor of the combined endpoint (class IIIB unstable angina and myocardial infarction) at 36-month followup. Variables such as age ($P = 0.15$), sex ($P = 0.23$), smoking ($P = 0.33$), diabetes mellitus ($P = 0.24$), hypertension ($P = 0.55$), dyslipidemia ($P = 0.57$), revascularization ($P = 0.70$), left ventricular ejection fraction ($P = 0.50$), and troponin I ($P = 0.15$) were not independent predictors of MACE.

4. Discussion

The results from our study demonstrate different patterns of release of the hsCRP and GDF-15 with, over time, between

Table 2: Inflammatory markers of non-ST-segment elevation acute coronary syndrome patients with and without MACE at 36-month followup.

Variable	MACE		P value
	Yes ($n = 45$)	No ($n = 210$)	
hsCRP at admission (mg/L)	7.4 [2.4–10.5]	7.4 [2.8–18.2]	0.79
hsCRP at 36-month followup (mg/L)	23 [10.4–34.7]	18.3 [7.3–26.6]	0.07
Delta hsCRP (mg/L)	15.5 [−28.7–73.3]	7.5 [−57.9–65.0]	0.01
GDF-15 at admission (pg/mL)	1639 [833–3151]	2190 [1333–3484]	0.09
GDF-15 at 36 months (pg/mL)	9105 [8071–9766]	3203 [2064–4572]	<0.001
Delta GDF-15 (pg/mL)	7605 [4831–8155]	602 [−405–2278]	<0.001

Data are expressed as median [interquartile range].
MACE: major adverse cardiovascular events; hsCRP: high-sensitivity C-reactive protein and GDF-15: growth-differentiation factor-15.

patients with NSTE-ACS. Moreover, this is the first study to show that the changes in GDF-15, over time, are of prognostic relevance in NSTE-ACS patients.

GDF-15 is emerging as a prognostic biomarker in patients with ACS. The predictive value of GDF-15 measured on admission has been investigated in the two large NSTE-ACS populations: the Global Utilisation of Strategies to Open Occluded Arteries IV (GUSTO-IV) and Fast Revascularisation during Instability in Coronary Artery Disease II (FRISC II) cohorts [5, 12]. In another study, Damman et al. have evaluated the long-term prognostic value of GDF-15, regarding death or myocardial infarction in NSTE-ACS patients. They have shown that the Kaplan-Meier curves diverged early and continued to diverge up to five years [13].

In a recent study, the circulating concentration of GDF-15 was measured at baseline ($n = 1734$) and at 12 months ($n = 1517$) in patients randomised in the Valsartan Heart Failure Trial (Val-HeFT) [14]. They demonstrated increases in GDF-15 over 12 months, which were independently associated with the risks of future mortality and first morbid event also, after adjustment for clinical prognostic variables, B-type natriuretic peptide, hsCRP, and high-sensitivity troponin T and their changes.

In another study recent, Eggers and colleagues analysed GDF-15 concentrations in participants from the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study. Measurements were performed at 70 and 75 years of age. They demonstrated that the GDF-15 concentrations and their changes over time are powerful predictors of mortality in elderly community-dwelling individuals [15].

In the results of our study, we found that NSTE-ACS patients, who developed MACE, displayed higher levels of GDF-15 at the 36-month followup than at admission. Furthermore, we were able to show that delta GDF-15 is associated with adverse outcomes, independently of established clinical and biochemical risk markers. Our results support the notion that GDF-15 integrates information on several relevant aspects and pathways in cardiovascular disease. The prominent antiapoptotic, antihypertrophic, and anti-inflammatory actions of GDF-15 in cardiovascular disease models indicate that this cytokine exerts protective effects in the context of acute cardiovascular injury [16]. Whether chronic increases in GDF-15 concentrations in NSTE-ACS patients play an adaptive or maladaptive role remains to be investigated.

In relation with hsCRP in our study, we measured hsCRP in two points, at admission and at 36 months. The delta value or rate of change of the hsCRP was useful to differentiate the group of patients with worse clinical outcome during 36 months of followup. However, after adjusting by different confounders, we have not demonstrated that delta hsCRP can predict MACE in NSTE-ACS patients. Recently, in a study by Karakas et al., they serially measured hsCRP concentrations in up to 6 blood samples, taken at monthly intervals from 200 postmyocardial infarction patients, who participated in the AIRGENE study. The results demonstrate considerable stability and good reproducibility for serial hsCRP measurements [17].

The implementation of hsCRP measurement into clinical practice requires sound data on the reliability of such measurement [11]. Data is still scarce for the long-term analytical variation of hsCRP measurement in patients with cardiovascular disease.

5. Conclusion

The present study shows that the rate of change of hsCRP measured at the 36-month followup was not predicting long-term MACE in NSTE-ACS patients. However, the delta GDF-15 at the 36-month followup seems to be a stronger predictor of MACE than during an acute unstable phase.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Review Article

C-Reactive Protein and Coronary Heart Disease: All Said—Is Not It?

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C-reactive protein (CRP) and coronary heart disease (CHD) have been the subject of intensive investigations over the last decades. Epidemiological studies have shown an association between moderately elevated CRP levels and incident CHD whereas genetic studies have shown that polymorphisms associated with elevated CRP levels do not increase the risk of ischemic vascular disease, suggesting that CRP might be a bystander rather than a causal factor in the progress of atherosclerosis. Beside all those epidemiological and genetic studies, the experimental investigations also try to reveal the role of CRP in the progress of atherosclerosis. This review will highlight the complex results of genomic, epidemiological, and experimental studies on CRP and will show why further studies investigating the relationship between CRP and atherosclerosis might be needed.

1. Introduction

C-reactive protein (CRP) circulates as a disc-shaped pentamer consisting of five identical subunits arranged around a pore in the middle (Figure 1). Measurements of its serum concentrations are used clinically as unspecific marker for inflammation. As the exact function of CRP is not fully understood yet, it is believed that it functions as part of the innate immune system [1]. It is also known that CRP rises in severe unstable angina and has a prognostic value [2]. Specifically high CRP levels following myocardial infarction are associated with adverse outcomes, including left ventricular failure [3], and increased rates in cardiac death and ventricle rupture [4, 5]. Massive data collected over the past decades showed an association between moderately elevated CRP levels and incident coronary heart disease (CHD) [6–9]. In 2008 a genetics study investigated the question of whether polymorphisms in the CRP gene are associated with increased levels of CRP, thereby offering an instrument for studying the causality of CRP in the risk of coronary heart disease [10] trying to answer the chicken or egg question [11]. This study came to the conclusion that genetically elevated CRP levels do not increase the risk of ischemic vascular disease, suggesting that CRP might be a bystander rather than a causal factor in the progress of atherosclerosis. In parallel to this disappointing data biochemical studies opened the opportunity that monomeric CRP—rather than the pentamer—may play a functional role in CHD. This review will highlight the complex results of genomic, epidemiological, and experimental studies on CRP and will show why further studies investigating the relationship between CRP and atherosclerosis might be needed.

2. C-Reactive Protein

2.1. The Structure of Human CRP. CRP circulates in the human serum as a noncovalently bound disc-shaped pentamer consisting of five identical subunits [12]. It presents two faces: a binding side where it binds calcium-dependent to its widely recognized specific ligands and an effector side. Each subunit consists of 206 amino acids with a molecular

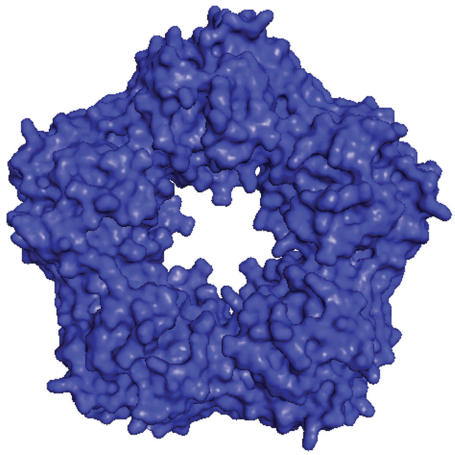

Figure 1: The picture shows the C-reactive protein as it circulates in the human blood stream after response to inflammatory stimuli. The five subunits are forming a disc-shaped pentamer around a central pore. (Picture was generated with information from the RCSB Protein Data Bank by using PyMOL software.)

weight of 23 kDa and carries 2 calcium ions essential for the pentameric isoform [13]. Under physiological circumstances, that is, calcium present in the extracellular environment and a physiological pH, it remains quite stable unless it binds to one of its specific ligands [14]. It has a particularly high affinity for phospholipids, especially lysophospholipids [15] found on the surface of damaged or apoptotic cells [16]. Upon binding to one of its ligands it dissociates into monomers [17, 18].

2.2. The Function of Human CRP. Since neither a deficiency of CRP is known nor a therapeutical inhibitor has yet been tested *in vivo,* the role of CRP in physiological or disease settings remains elusive. As it binds to phospholipids, especially lysophospholipids, and recognizes bacterial lipids, it has been suggested that it functions as part of the innate immune system. Once CRP has bound to one of its ligands and dissociated into its monomers, it presents properties not shared with the circulating pentameric CRP. The pentameric CRP appears to have no interaction with complement or the regulatory complement factor H [19] whereas the monomeric CRP can directly activate the complement cascade through C1q fixation [20] and induce platelet [21] and monocyte activation [22].

2.3. The Synthesis of Human CRP. CRP is mostly synthesised in the liver—although extrahepatic transcription of CRP has been described [23–25]—upon inflammatory stimuli as interleukin-1, interleukin-6, and tumor necrosis factor α [12]. It can rise from baseline to its 10,000-fold upon bacterial inflammation. Following myocardial infarction, an increase in CRP levels is also observed [26]. This response is very quick and happens within 12 hours reaching its peak at about 50 hours after stimuli [27]. Actually, it seems that most forms of adverse stress are associated with an increase in CRP levels [28] (see also review [12]).

3. C-Reactive Protein and Coronary Heart Disease

3.1. Epidemiological Studies. Since the first epidemiological study describing an association between elevated CRP levels and an increased risk for CHD events was published, more than 50 studies have followed [29]. In a meta-analysis published in the year 2004 by Danesh et al. 22 of those studies were included [30]. Those 22 studies involved 7068 cases of coronary heart disease with a mean followup of 12 years and presented an overall odds ratio of 1.58 (95 percent confidence interval, 1.48 to 1.68) among patients with values from the top third compared with the bottom third of baseline C-reactive protein concentrations. These results were quite similar to a subanalysis of the four biggest studies on 4107 cases of coronary heart disease, which provided an odds ratio of 1.49 (95 percent confidence interval, 1.37 to 1.62). Five years later another meta-analysis was published involving 23 studies with 8 more recent articles presenting an overall odds ratio of 1.60 (95 percent confidence interval, 1.43 to 1.78) by comparing CRP levels of <1.0 mL/dL versus >3.0 mL/dL showing no major difference to the studies published before [31].

Studies like those mentioned before implied that CRP levels can be used to reclassify subjects who fall into the intermediate-risk category of CHD in 2008, introducing the Reynolds Risk Score for man and woman [32, 33]. The scores improved accuracy of clinical algorithms for global cardiovascular risk prediction that reclassified subjects at intermediate-risk into higher- or lower-risk categories. Another score based on serum levels of CRP, fibrin degradation products, and heat shock protein 70 as predictors of future risk of death and myocardial infarction in patients with suspected or known CHD followed in 2013 [34].

In 2005 a statin therapy trial comparing moderate statin therapy (40 mg pravastatin daily) and intensive statin therapy (80 mg atorvastatin daily) for patients with CHD showed that a decrease in CRP levels during statin treatment independently and significantly correlates with progression of atherosclerosis [35]. Another study followed in 2008: the JUPITER trial [36]. JUPITER enrolled 17,802 individuals without manifest cardiovascular disease. All participants had low-density lipoprotein cholesterol (LDLC) levels below 130 mg/dL, but CRP levels greater than 2 mg/dL. The subjects were randomly designated to rosuvastatin 20 mg daily or placebo. The trial was stopped early because the interim results met the study's predefined stopping criteria by showing a 44 percent reduction in the trial primary endpoint of all vascular events. The placebo event rate in this study indicates that elevated CRP levels have high vascular risk even when LDLC levels lie within the range of current guidelines, being consistent with meta-analyses of CRP and CHD mentioned before.

Neither the meta-analyses nor the JUPITER trial were able to answer the question if CRP is a causal factor in coronary heart disease or just an innocent bystander in inflammation, a well- accepted pathomechanism in atherosclerosis [8, 37]. But several recent studies showed a significant contribution of CRP to coronary risk prediction independent

of the traditional Framingham Risk Score [38] and therefore it found its way into clinical guidelines for guidance of measures in primary prevention [39].

3.2. Experimental Studies

3.2.1. Pentameric CRP. CRP has been an object of experimental studies over the last three decades. There have been reports suggesting prothrombotic, proinflammatory, and proatherogenic properties *in vivo* for native CRP. Particularly since it was shown that CRP can activate the classical complement cascade, it was most likely that it would have proinflammatory properties in general [40, 41]. As CRP has been found in atherosclerotic plaques [42], it would have been a perfect explanation for the results from the epidemiological studies. But further studies were not always able to reproduce the above-mentioned properties, so that the initial results were most likely due to the commercial preparation of CRP with remaining toxic sodium acid or presence of bacterial endotoxin (lipopolysaccharide) in CRP produced by recombinant *Escherichia coli* [12, 43–45].

Not much less data exists for animal models trying to answer the question of a prothrombotic property of CRP [46–49]. With a single exception, these studies found no association between human CRP and a progression of atherosclerosis and it seemed more likely that CRP might act just as a bystander rather than a causal factor. But almost all of those studies used mice as an animal model for atherosclerosis which might have been the pitfall. The differences of CRP between species are enormous regarding ligand binding, secondary binding effects, and complement activation and behaviour as an acute phase reactant [29]. This is why Pepys et al. used a rat reperfusion model in 2006 to study tissue damage induced by human CRP following myocardial infarction and the ability to block the damage by a CRP inhibitor [50].

Even though it was shown that human CRP activates the classical complement cascade in humans and rats, it is not possible to explore those results of injecting human CRP into another species to the pathomechanism of the common clinical observation of increased CRP levels following myocardial infarction and the adverse outcomes.

3.2.2. Monomeric CRP. With these controversial results an elegant solution to the conflicting CRP data was the introduction of the concept that monomeric forms of CRP might occur following the binding of circulating pentameric CRP to one of its ligands [14, 51], resulting in subsequent dissociation and functional activation. The existence of monomeric CRP is known over decades [52] and seemed to be a nonsoluble tissue-based protein rather than a soluble plasma-based protein, with antigenicity-expressing neoepitopes differing from native CRP epitopes [53]. This monomeric CRP has proinflammatory properties not shared with pentameric CRP like C1q fixation [14], to promote neutrophil-endothelial cell adhesion [54], platelet activation [55], thrombus formation [55, 56], and monocyte chemotaxis [17]—to name just a few. Thus the controversial results can be either the product of contaminated CRP (with toxic sodium acid or bacterial endotoxin) or the product of dissociated CRP.

Particularly, studies showed that the monomeric form of CRP—and not native CRP—colocalizes with complement in infarcted regions [57–60]. Since Molins et al. showed that monomeric but not pentameric CRP displays a prothrombotic phenotype enhancing not only platelet deposition, but also thrombus growth under arterial flow conditions, a possible role for monomeric CRP in the pathogenesis of "active" CHD needs to be considered [55]. Interestingly, about 1 year later Eisenhardt et al. showed the deposition of monomeric CRP in human aortic and carotid atherosclerotic plaques but not in healthy vessels [17]. The pentameric isoform was found neither in healthy nor in diseased vessels. In a study from 2012 Habersberger et al. showed that the nonsoluble monomeric CRP can be detected on microparticles from patients with acute myocardial infarction, whereas significantly less monomeric CRP was detectable on microparticles from healthy controls and stable CHD patients [51].

But unless clinical studies with a direct inhibitor of (monomeric) CRP can be conducted in human beings, the complex function of CRP can only be speculated.

3.3. Genetic Studies. Epidemiological studies showing an association between an exposure (in this case CRP) and a disease (here CHD) are sometimes confounded even with the most carefully study design. Genetic studies using a Mendelian randomization design utilize a method to estimate the causal nature of exposures and to avoid reverse association bias [61]. As quite a few studies have shown that multiple single nucleotide polymorphisms in the CRP gene (or the promoter region of the CRP gene [62, 63]) are associated with an increase in CRP baseline levels [64–66], it was just a matter of time for large genetic studies to follow estimating the association of elevated CRP levels and an increased risk for CHD [10, 67–69].

A first hint gave the population-based Rotterdam Study published in 2006. Kardys et al. analysed 5231 men and women for the association between CRP-related haplotypes and CHD. In contrast to epidemiological studies, such genetically elevated CRP levels were not found to be an independent marker of increased risk for CHD in patients without a history of CHD. Three haplotypes were identified as being associated with CRP levels, but the CRP haplotypes themselves were not associated with CHD.

Then a few more epidemiological studies followed involving over 28,000 CHD cases and 100,000 controls with none of them showing an association between elevated CRP levels and CHD [10, 68].

As a designated number of patients and controls are needed for Mendelian randomization studies the CRP, CHD Genetics Collaboration was founded in 2008. About three years later in 2011 the results were published including over 46,000 patients with prevalent or incident CHD and almost 150,000 controls. CRP variants were associated with up to 30% difference in CRP concentration per allele. Like all the genetic studies before, there was no association between single nucleotide polymorphisms associated with raised CRP

levels and CHD so that most likely CRP is not even a modest causal factor in CHD.

The strong data from the genetic studies analysing an association between elevated CRP concentrations and the risk of CHD make it most doubtful that circulating pentameric CRP has a direct pathological role in the progression of CHD even though Mendelian randomizations also have limitations [70].

4. Conclusion

The data on CRP is massive and seems most controversial by trying to harmonize the results from genetic studies, epidemiological studies, and experimental investigations. The strong data from the Mendelian randomizations made it most unlikely that elevated CRP play a direct causal role in CHD. But that is consistent with experimental data as no prothrombotic or proinflammatory properties have been established for circulating CRP. The results showing proinflammatory and prothrombotic effects were most likely due to contamination of commercial CRP with either bacterial endotoxin, toxic sodium acid, or monomeric CRP. The probable tissue damage by CRP following myocardial infarction mediated by complement is most likely due to binding of CRP to apoptotic cells and subsequent dissociation to monomeric CRP which may have proinflammatory qualities like classical complement activation by complement fixation.

The epidemiological studies demonstrated an association between elevated CRP concentrations and an increased risk for CHD events in the first place. Meanwhile, with the results from the Mendelian randomization studies and the experimental data, elevated CRP in the epidemiological setting must be seen as a bystander rather than a causal factor of CHD. Nevertheless, it is undoubted that a prediction model that incorporates high-sensitivity CRP improves global cardiovascular risk prediction. The reduction of CHD events in patients with moderately elevated CRP levels and an intensive statin therapy can be the result of unknown anti-inflammatory property of statins and the subsequent decrease of CRP levels.

Thus, we should not stop our investigations at the "marker versus maker" debate on CRP but try to understand the inflammatory process associated with atherosclerosis. As pointed out before CRP is a downstream biomarker of elevated interleukin-1, interleukin-6, and tumor necrosis factor α. Interestingly, two Mendelian randomization studies showed that a genetic polymorphism in the interleukin-6 receptor signalling pathway associates with lower levels of CRP and a reduction of cardiovascular events [71, 72]. This data supports a causal association between inflammatory activation and atherosclerosis. With these data in mind we are looking forward to the results from the CANTOS and CRIT trials. In both studies the investigators are targeting inflammatory upstream pathways. In the Canakinumab Anti-inflammatory Thrombosis Outcomes Trial (CANTOS), a human monoclonal antibody (Canakinumab) that specifically inhibits IL-1β is tested to reduce recurrent vascular events whereas in the Cardiovascular Inflammation Reduction Trial (CIRT) methotrexate as a tumor necrosis factor α and interleukin-6 inhibitor is applied to postmyocardial infarction patients to examine the promising animal data showing a slowdown in atherosclerotic lesion progression in cholesterol-fed rabbits [73].

5. Perspective

As monomeric CRP has an effect on thrombus formation, the question would be if moderately elevated CRP levels are associated with an increased risk for CHD events in "active" CHD. Do instable plaques expose binding ligands to circulating pentameric CRP which can lead to CRP dissociation and induction of local inflammation? This question is answered neither by current data of genetic analyzes nor by current data of experimental approaches nor by current data of epidemiological studies.

An elegant way to evaluate a functional role of CRP in CHD would be randomized trial with a direct CRP inhibitor. With 1,6-bis-phosphocholine such compound was first tested in animal models. Another interesting approach is the antisense oligonucleotide ISIS-CRP_{Rx}, which reduces the CRP production in the liver and is currently tested in a phase 2 study on patients with rheumatoid arthritis, according to the manufacturer's website with promising success. Noteworthy, in 2011 Wang et al. showed an aptamer binding specific to monomeric but not to pentameric CRP [74]. With an additional blocking quality we would have a new approach to directly distinguish between monomeric and pentameric effects [74]. We are most excited about the first results from *in vivo* applications from all of those approaches.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Inhibiting C-Reactive Protein for the Treatment of Cardiovascular Disease: Promising Evidence from Rodent Models

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Raised blood C-reactive protein (CRP) level is a predictor of cardiovascular events, but whether blood CRP is causal in the disease process is unknown. The latter would best be defined by pharmacological inhibition of the protein in the context of a randomized case-control study. However, no CRP specific drug is currently available so such a prospective study cannot be performed. Blood CRP is synthesized primarily in the liver and the liver is an organ where antisense oligonucleotide (ASO) drugs accumulate. Taking advantage of this we evaluated the efficacy of CRP specific ASOs in rodents with experimentally induced cardiovascular damage. Treating rats for 4 weeks with a rat CRP-specific ASO achieved >60% reduction of blood CRP. Notably, this effect was associated with improved heart function and pathology following myocardial infarction (induced by ligation of the left anterior descending artery). Likewise in human CRP transgenic mice treated for 2 weeks with a human CRP-specific ASO, blood human CRP was reduced by >70% and carotid artery patency was improved (2 weeks after surgical ligation). CRP specific ASOs might pave the way towards a placebo-controlled trial that could clarify the role of CRP in cardiovascular disease.

1. Introduction

C-reactive protein (CRP), the prototypic acute phase reactant, is produced primarily by the liver as part of the body's mechanism to restrict injury and promote repair after an inflammation evoking injury [1–3]. CRP is a member of the phylogenetically ancient and evolutionarily conserved pentraxin family of proteins and consists of five noncovalently bound subunits, each of 206 amino acids, arranged symmetrically around a central pore [4]. The molecule has a ligand recognition face that contains a Ca^{2+}-dependent binding site, and an effector molecule binding face that is capable of initiating fluid phase pathways of host defence (by activating the complement system) and cell-mediated ones (by activating complement or binding to Fc receptors) [4]. Regulation of CRP expression occurs mostly at the transcriptional level, with interleukin 6 (IL-6) being its major inducer and interleukin 1 (IL-1) synergistically enhancing the IL-6 effect [4, 5]. The rise in blood CRP after tissue injury is rapid, with levels increasing by as much as 1000-fold above baseline within 24 hours. This plasticity makes blood CRP an ideal clinical marker of a patient's general health status, a purpose for which it has been used for half a century [1–6].

Since the early 1980s, largely because of increasingly widespread use of automated high sensitivity CRP assays, clinicians and physician scientists have been able to reproducibly and accurately measure the low levels of blood CRP (≤3 mg/L) routinely seen in ostensibly healthy people. This

capacity has led to accumulation of extensive observational data linking CRP to various kinds of disease [6–10]. The relationship of CRP to the inflammatory aspects of cardiovascular disease (CVD) has been an area of keen interest. Indeed, based upon multiple prospective epidemiological studies, CRP is now recognized as an independent marker and powerful predictor for future risks of myocardial infarction (MI), stroke, and death from coronary heart disease (CHD) in individuals apparently free of known CVD. Further, data from at least four clinical trials (PROVE IT-TIMI, REVERSAL, JUPITER, and SATURN) suggest a role for CRP in the atherogenic process [11–14]. In these studies, the indirect reduction of blood CRP levels that accompanied treatment with statins was found to be independently and significantly related to event-free survival and/or decreased progression of documented coronary disease and/or major cardiovascular events. In patients where low density lipoprotein cholesterol (LDL-C) alone was reduced, disease progression was slowed by statin therapy, but in patients where LDL-C and CRP were both reduced, atheroma progression was halted. Furthermore in at-risk patients given maximally intensive statin therapy, lowering of CRP was associated with atheroma regression [14].

Based on the known biology of CRP it would not be a surprise if the protein was ultimately found to contribute to the pathophysiological processes leading to CVD. For example various studies demonstrate that CRP can activate complement and endothelial cells and promote their dysfunction [15–17]. Others show that CRP is detected in early atherosclerotic lesions [17, 18] and that it is colocalized with activated complement components and enzymatically degraded LDL in human vascular lesions isolated by atherectomy [19–21]. In addition there is compelling direct evidence from multiple transgenic models indicating that human CRP has a pathogenic role in vascular disease [22–25]. Despite these data, generated independently by many different groups, the exact biological role of CRP in CVD in humans and the overall importance of its contribution therein remains equivocal [26] because there is no way to selectively reduce CRP in patients. Towards solving this nagging problem a small molecule inhibitor of human CRP, 1,6-bis(phosphocholine)-hexane, was synthesized and tested in a preclinical rodent model *in vivo* [27]. The compound is designed to crosslink two CRP molecules, thereby increasing the protein's clearance and blocking its ability to bind endogenous ligands. It was shown that in rats that were administered human CRP by injection, administration of this compound ameliorated human CRP associated exacerbation of MI caused by ligation of the coronary artery [27]. Despite the fact this was a xenogenic system, the study demonstrated for the first time that therapeutic inhibition of CRP might be a promising new approach for cardioprotection in acute MI. Still, to definitively address the question of causality of CRP in the pathogenesis of CVD and whether reduction of CRP would result in a meaningful decrease in adverse CVD outcomes, the use of a specific pharmacological inhibitor of endogenously expressed CRP is preferred.

Antisense oligonucleotides (ASOs) are highly specific agents that can be used therapeutically to prevent the translation of disease-associated proteins, an effect achieved via selective degradation of targeted mRNAs [28]. Because of their specificity and propensity to distribute to the liver, where CRP is synthesized and secreted [3–5], ASOs provide an efficient means of reducing CRP expression. Using CRP transgenic mice (CRPtg) [29, 30] we previously established that human CRP targeting ASOs are effective for reduction of human CRP and efficacious against collagen-induced arthritis [31]. Herein we employed additional species-specific CRP targeting ASOs and tested their efficacy in two different animal models of CVD wherein a role for CRP has already been established, namely, rats subjected to experimentally induced MI [27] and CRPtg mice subjected to carotid artery ligation [24, 25]. We provide new evidence that ASO-mediated reduction of endogenously expressed rat CRP (in rats) and human CRP (in CRPtg) is efficacious in both diseases. CRP specific ASOs have the potential to be therapeutically beneficial in humans at risk for CVD.

2. Materials and Methods

2.1. Animals. Eleven-week-old Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, fed a standard rat pellet diet *ad libitum*, and acclimated to local conditions for 1 week before use in any experiments. Wild-type mice (C57BL/6 strain) and littermate CRPtg were from our own colonies. Details of the human CRP transgene and its human-like expression in CRPtg have been described elsewhere [29, 30]. In CRPtg human CRP is present in the blood at concentrations relevant to human disease, that is, low levels under steady-state conditions (<3 μg/mL) and high levels during an acute phase response (>500 μg/mL). All mice were fed a standard mouse pellet diet *ad libitum* and they were 8–12 weeks old when used in experiments. Only male rats and male mice were subjected to experimentation and all were maintained at constant humidity (60 ± 5%) and temperature (24 ± 1°C) with a 12 hour light cycle (6 AM to 6 PM). All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham and were consistent with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health "Public Health Service Policy on Humane Care and Use of Animals, DHEW Publication number 96-01, PHS Policy revised in 2002."

2.2. Antisense Oligonucleotides. ASOs designed to specifically hybridize to either rat or human CRP mRNA were synthesized and purified as described previously [28, 31, 32]. Each ASO was 20 nucleotides in length and comprised a central unmodified core consisting of 10 or 14 nucleotides, flanked by phosphorothioate linkages and three or five 2′-O-methoxyethyl (2′-MOE) modifications on the 3′ and 5′ flanking ends. The ASOs thus had a "3-14-3" or a "5-10-5" configuration. Candidate ASOs were evaluated for their ability to reduce IL-6 stimulated CRP mRNA expression in cultures of rat or human hepatoma cells (data not shown).

In these experiments several ASOs significantly reduced CRP mRNA levels, with IC_{50} values for the lead compounds in the 5 nM range (data not shown). Based on their potency in these assays, a rat CRP specific ASO (ISIS 197178) and a human CRP specific ASO (ISIS 353512) were chosen for evaluation *in vivo*. A third ASO (ISIS 141923), which is a scrambled ASO not complementary to any known rat or human gene sequence, served as a control.

2.3. Rat Myocardial Infarction Model. After acclimatization, rats were randomly assigned to 3 treatment groups and received for 4 wks the rat CRP-specific ASO ISIS 197178 or the control ASO ISIS 141923 (both at 150 mg/kg/wk i.p.) or vehicle (0.9% NaCl). At the beginning and end of the 4-week treatment phase blood was collected to assess CRP, IL-6, and alanine transaminase (ALT) levels. The next day echocardiography was performed and then the rats were subjected to left anterior descending coronary artery (LADCA) ligation as described [33]. Briefly, rats were anesthetized with ketamine-xylazine (80–15 mg/kg i.p.), intubated, ventilated with a rodent respirator, and laid on a heating pad warmed to 37°C. The heart was exposed via a left intercostal thoracotomy through the 4th intercostal space, and the pericardium removed for identification of the LADCA. The LADCA was ligated 2 mm below the left atrium using a tapered needle and a 5-0 polypropylene ligature. Occlusion was confirmed by a sudden pallor of the anterior wall of the left ventricle (LV). The chest cavity was closed and the rats allowed to recover, with analgesic (0.05 mg/kg buprenorphine s.c.) given twice daily over the next 3 days. One week after LADCA echocardiography was repeated and the rats euthanized with an overdose of ketamine-xylazine. Hearts were then removed, weighed, and cut into 2 mm slices (average of 5 transverse slices/heart at the level below the LADCA) perpendicular to the apex-base axis [33]. Tissue slices were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 5 μm sections, and stained with picrosirius red (0.1%) for assessment of collagen area (an index of replacement fibrosis and infarct size) [33]. Morphometric analysis of tissue sections was carried out by light microscopy with a Qimaging QiCam digital camera (Qimaging) interfaced with a computer system running Metamorph 6.2v4 software (Universal Imaging). For infarct size estimation, the ratio of the picrosirius red-stained area (or the perimeter of that area) divided by the total area of the ventricle (or the perimeter of the ventricle) was calculated for six 5 μm sections taken from 0–2, 2–4, 4–6, 6–8, and 8–10 mm distal from the heart apex.

2.4. Echocardiography. Echocardiography was performed on isoflurane anesthetized rats using a Philips Sonos 5500 ultrasound system equipped with a 15 MHz transducer as described previously [33]. After recording heart rate (HR), LV end-systolic dimension (LVESD) and LV end-diastolic dimension (LVEDD) were measured by two-dimensional-guided M-mode imaging from the parasternal short-axis view below the mitral valve. LV end-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF), and fractional shortening (FS) were calculated as follows: $EDV = 7 \times LVEDD^3/(2.4 + LVEDD)$, $ESV = 7 \times LVESD^3/(2.4 + LVESD)$, $FS = (LVEDD - LVESD)/LVEDD \times 100$, and $EF = (EDV - ESV)/EDV \times 100$. The mean velocity of circumferential shortening (VCFR) was also calculated. A single examiner blinded to treatment performed and interpreted all studies.

2.5. Mouse Vascular Injury Model. Wild-type versus CRPtg mice were randomly assigned to treatment groups to receive for 2 wks the human CRP-specific ASO 353512 (10, 25, or 50 mg/kg i.p.), the control ASO ISIS 141923 (20 mg/kg/wk i.p.), or an equal volume vehicle (0.9% NaCl). At the beginning and end of the 2 wk treatment phase blood was collected to assess human CRP and IL-6. Following the run-in phase the right common carotid artery (RCCA) was ligated to stimulate neointima generation as described previously [24, 25]. Briefly, the RCCA was exposed through a midline cervical incision and ligated with an 8-0 silk suture just proximal to the bifurcation. The left common carotid artery was surgically exposed but not ligated and served as an internal control. Treatments continued postsurgically for 2 more weeks and then the mice were anesthetized and euthanized with an overdose of pentobarbital. The vasculature was immediately flushed with 0.01 M sodium phosphate buffer (pH 7.4) and perfused with 10% formalin and both carotid arteries were excised, fixed in 10% formalin, embedded in paraffin, and sectioned. Representative serial sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope to locate the ligature site; then additional sections of the artery taken 200, 350, 500, and 700 μm proximal to the ligation site were identified and treated with Verhoeff's elastin stain to enhance the elastic laminae. Sections of the unligated contralateral vessels were obtained and processed in the same fashion (data not shown). Computer-assisted morphometric analysis of digitized images captured from each arterial section was performed with image analysis software (Scion Image). To calculate vessel patency, the cross-sectional area of the vessel lumen was divided by the cross-sectional area of the area bounded by the internal elastic lamina. All measurements were performed by a single examiner blinded to the genotype and treatment of the mice.

2.6. Measurement of Serum CRP, IL-6, and ALT. Rat CRP was measured using a commercially available enzyme-linked immunoassay kit and the manufacturer's instructions (EMD Millipore, Darmstadt, Germany) and human CRP was measured by an ELISA as described previously [24, 25]. ALT was determined using INFINITY ALT reagents (Sigma-Aldrich Corp., St. Louis, MO).

2.7. Statistical Analysis. Results are expressed as the mean ± SEM without transformation. Pairwise comparisons were done using Student's *t*-tests, and comparisons among multiple experimental groups were performed with one-way ANOVA followed by pairwise multiple comparisons using the protected least-squares difference test. Differences were considered significant when the associated *P* value was <0.05. All statistical analyses were performed using the SigmaStat software package (SigmaStat, Jandel Scientific).

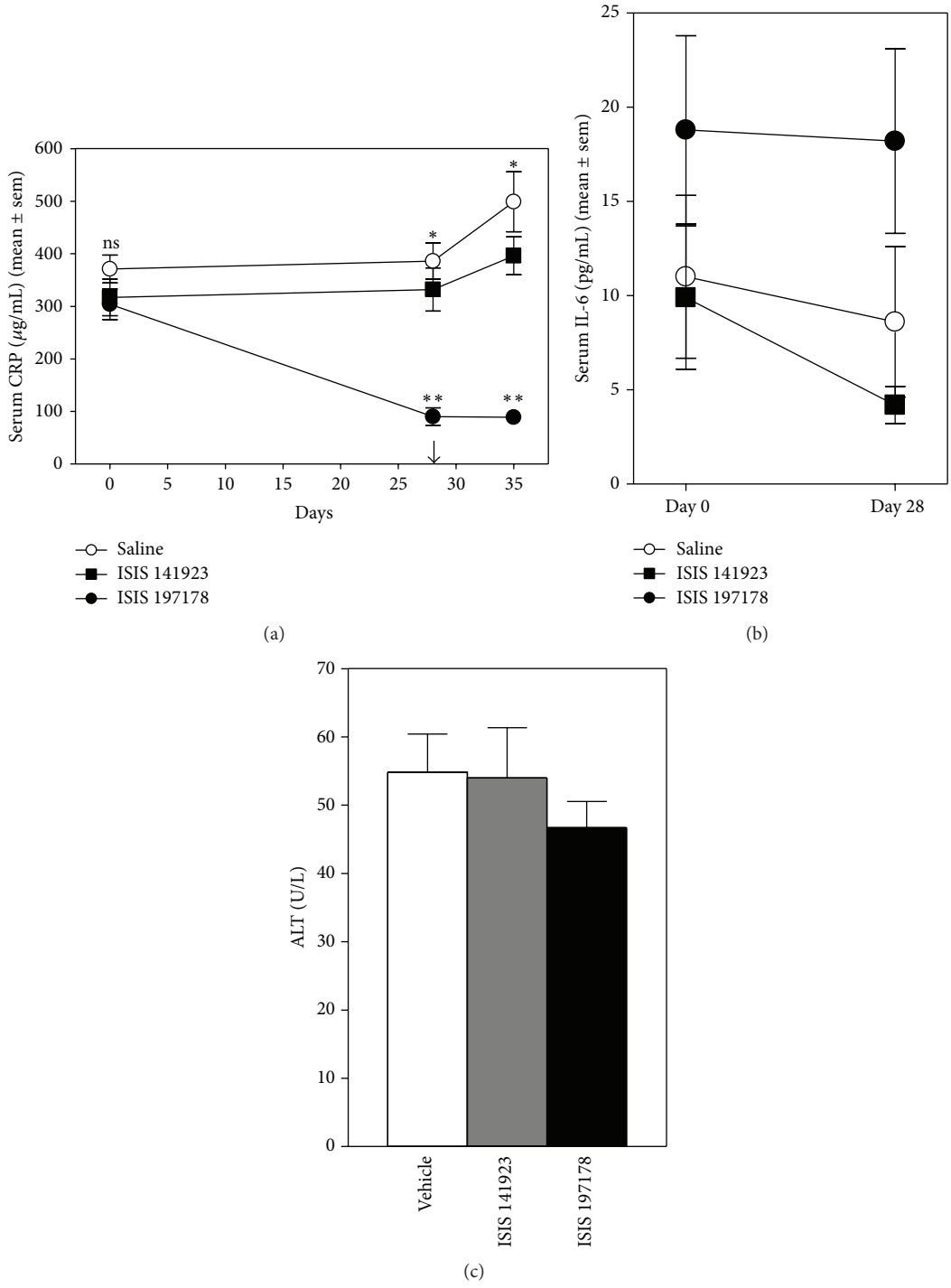

Figure 1: ASO-mediated lowering of CRP in rats subjected to LADCA ligation. (a) Serum CRP values determined by ELISA for rats injected i.p. with a CRP targeting ASO (ISIS 197178; 150 mg/kg/wk, $n = 19$) (■), a control ASO (ISIS 141923; 150 mg/kg/wk, $n = 9$) (□), or an equivalent volume of 0.9% saline (vehicle, $n = 8$ rats) (○). The arrow indicates the day LADCA ligation surgery was performed. The single asterisks and "ns" above the curves indicate $P < 0.0001$ or not significant ($P > 0.05$), respectively, for ANOVAs comparing the three treatment groups on each day. The double asterisks below the lines indicate $P < 0.0001$ for protected least-squares difference tests comparing the ISIS 197178 treated group to both other groups. (b) Serum IL-6 determined by ELISA for the rats shown in (a). (c) Serum alanine transaminase (ALT) levels determined by ELISA on day 35.

3. Results

3.1. ASO-Mediated Lowering of Rat CRP Is Beneficial in a Rat Model of Acute MI. Blood CRP level in rats ($n = 36$) was 321.9 ± 18.9 μg/mL at baseline, with no significant difference among the three randomly assigned treatment groups (ANOVA). Administration of the rat CRP specific drug ISIS 197178 ($n = 19$ rats) reduced blood CRP by an average 65% - from 303.8 ± 29.5 μg/mL at baseline to 90.4 ± 17.2 μg/mL on day 28 (Figure 1(a)). In comparison, rats treated with saline ($n = 8$) or the control ASO ISIS 141923 ($n = 9$) showed no significant deviation of blood CRP from baseline levels on day 28 (Figure 1(a)). Indeed during this time period CRP levels increased, rather than decreased, in rats receiving saline (10% increase from baseline) and ISIS 141923 (24% increase). IL-6 level at baseline and on day 28 were highly variable with no significant differences among the three treatment groups (ANOVAs) (Figure 1(b)). Importantly, there was no reduction of IL-6 (the major inducer of rat CRP) [34] in rats treated with ISIS 197178 (Figure 1(b)). Both the control and the CRP-specific ASOs were well tolerated as judged by absence of elevation of blood ALT (Figure 1(c)). LADCA ligation on day 28 caused blood CRP to increase by 33% by day 35 for saline-treated rats and by 13% for ISIS 141923-treated rats, but the elevation of CRP was completely blocked for rats receiving ISIS 197178 (Figure 1(a)).

After eliminating rats that died during surgery or within hours after LADCA ligation due to lethal MI (3/19 ISIS 197178 treated rats, 3/9 ISIS 141923 treated rats, and 2/8 saline treated rats) and after inspecting post-MI echocardiograms and stained heart sections to eliminate rats in which the LADCA was not properly ligated so no MI was achieved (4/16 ISIS 197178 treated rats, 1/6 ISIS 141923 treated rats, and 2/6 saline treated rats), 21 rats remained for further study. For statistical purposes the remaining saline-treated and ISIS 141923-treated animals ($n = 9$ in total) were pooled and compared to the remaining ISIS 197178-treated rats ($n = 12$). Echocardiographic indices in this subset were consistent with a subtle but significant improvement in post-MI cardiac function for rats treated with ISIS 197178, which showed a 14–23% improvement in EF, FS, and VCFR (Figure 2). Consistent with the echocardiography findings, infarct size was reduced in rats treated with ISIS 197178 compared to controls (Figure 3), with a small but significant zone of protection observed proximal to the site of LADCA ligation (i.e., 6–10 mm from the apex of the heart) (Figure 3).

These results show that for rats treated with ISIS 197178, an ASO that targets rat CRP mRNA and thereby lowers circulating CRP protein level effectively without causing toxicity or inflammation, cardiac dysfunction resulting from experimentally induced acute MI was reduced.

3.2. ASO-Mediated Lowering of Human CRP Is Beneficial in a CRPtg Mouse Model of Acute Vascular Injury. In these experiments CRPtg mice were treated for 2 weeks with different doses of a human CRP specific ASO (ISIS 353512; doses of 10, 25, or 50 mg/kg/week i.p.) versus a control ASO (ISIS 141923 at 20 mg/kg/week i.p.) or vehicle (0.9% saline). Following this 2-week run-in phase the RCCA was ligated to stimulate neointima generation. Therapy continued for 2 more weeks and the ligated vessels were harvested to quantitate the effect of human CRP lowering on the blood vessel injury response.

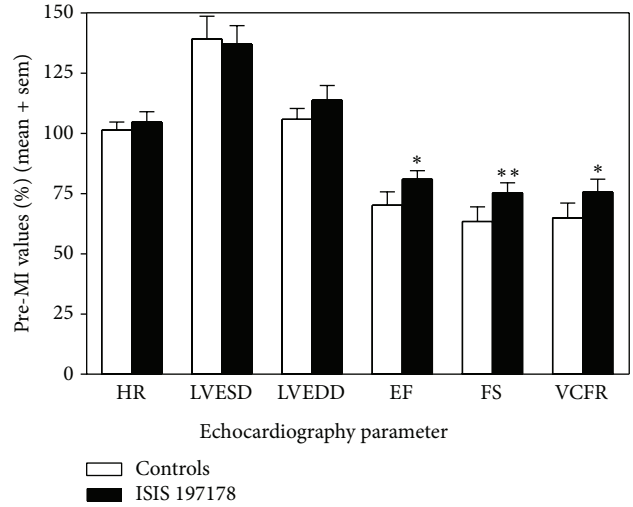

Figure 2: ASO-mediated lowering of CRP in rats subjected to coronary artery ligation improves myocardial infarction (MI) induced cardiac dysfunction. LV function was measured by echocardiographic analysis in rats at baseline and at 1 wk after MI. Heart rate (HR), LV end-systolic dimension (LVESD), LV end-diastolic dimension (LVEDD), ejection fraction (EF), fractional shortening (FS), and the mean velocity of circumferential shortening (VCFR) were calculated as described in the *Materials and Methods*. The results shown are for $n = 12$ rats treated with ISIS 197178 versus $n = 9$ controls (see the *Results* section for details). Single and double asterisks indicate $P < 0.05$ and $P < 0.005$ for unpaired t-tests.

Serum human CRP level in CRPtg mice ($n = 69$) was 12.7 ± 1.03 μg/mL at baseline with no statistically significant difference (ANOVA) among the various treatment groups. For both saline-treated and ISIS 141923-treated CRPtg mice, serum human CRP level increased above baseline by ~30% by day 14 and by ~40–85% by day 28 (two weeks after RCCA ligation) (Figure 4). In contrast for mice receiving ISIS 353512, the increase in human CRP level was prevented at the 10 mk/kg/wk dose and reversed in a dose-dependent fashion at the higher doses (Figure 4). Like the ASO we tested in rats, the human CRP specific ASO ISIS 353512 neither reduced serum IL-6 nor raised serum ALT levels in CRPtg mice (data not shown). Furthermore, ISIS 353512 did not reduce mouse CRP levels (data not shown) [31].

For CRPtg mice subjected to surgery, patency of the ligated RCCA was significantly improved by treatment with the human CRP lowering ASO ISIS 353512 (Figure 5(a)). The protective effect was most pronounced distal to the ligature. In stark contrast ISIS 353512 had no effect on ligated RCCA patency in mice that did not express human CRP (Figure 5(b)).

These results show that for CRPtg mice treated with ISIS 353512, an ASO that targets human CRP mRNA and thereby

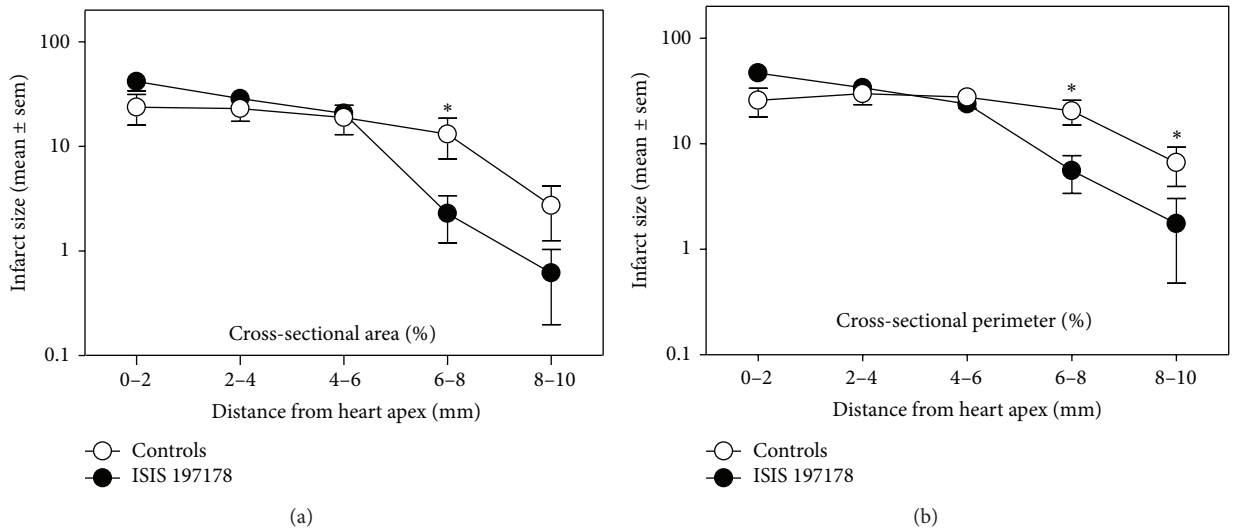

Figure 3: ASO-mediated lowering of CRP in rats subjected to coronary artery ligation reduces infarct size. For hearts from the animals shown in Figure 2, percent infarcted region (area (a) or perimeter (b)) was calculated from picrosirius red-stained LV sections (see Materials and Methods). Rats were injected i.p. with either 0.9% saline or ASO 141923 at 150 mg/kg/wk) (controls, $n = 9$), or an equivalent dose of the CRP targeting ASO ISIS 197178 ($n = 12$). The asterisks indicate $^{*}P < 0.05$ for unpaired t-tests.

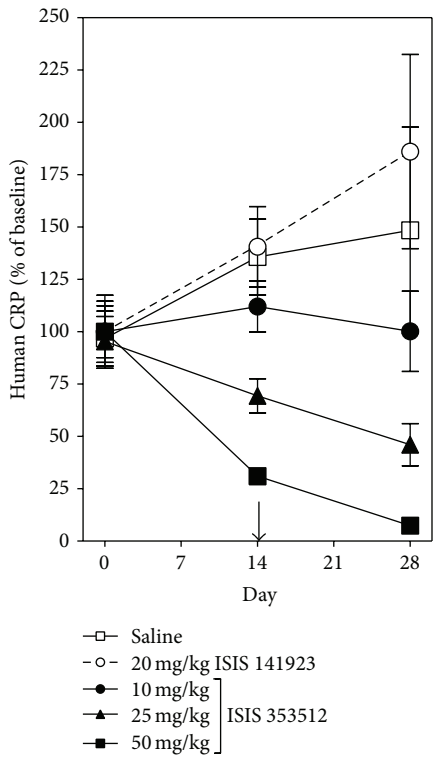

Figure 4: ASO-mediated lowering of human CRP in CRPtg mice subjected to RCCA ligation. Serum human CRP values were determined by ELISA for rats injected i.p. with (i) the CRP targeting ASO ISIS 353512 (10 mg/kg/wk, $n = 9$; 25 mg/kg/wk, $n = 30$; 50 mg/kg/wk, $n = 5$), (ii) the control ASO ISIS 141923 (20 mg/kg/wk, $n = 12$), or (iii) an equivalent volume of 0.9% saline ($n = 13$). The arrow indicates the day RCCA ligation surgery was performed.

lowers circulating human CRP protein level effectively without causing toxicity or inflammation, carotid artery stenosis resulting from blood vessel ligation is reduced.

4. Discussion

The association of elevated baseline CRP to increased risk of CHD is widely recognized [35–37], but it is not known if this association is causal. Furthermore if the association is causal it is not known which of the many biological actions of CRP might support the effect. Notwithstanding the many informative studies of CRP biology performed *in vitro* and in animal models, a clinically approved and specific inhibitor of human CRP will be needed before a true understanding of the physiologic role of CRP in humans at risk of CHD can be ascertained. If treatment of at-risk patients with a CRP lowering drug is found to lower the risk, then that would certainly settle the ongoing debate about whether CRP plays a role in cardiovascular disease. On the other hand even if lowering CRP has no protective effect, the predictive association would still remain.

A small molecule inhibitor of CRP [1,6-bis(phosphocholine)-hexane] that occludes the ligand-binding "B" face of CRP and thereby reportedly blocks its ability to activate complement was tested preclinically [27]. Since (i) complement activation is known to occur in concert with cardiovascular disease [20, 21], (ii) complement activation products are found in vascular lesions associated with cardiovascular disease [19], and (iii) CRP is known to activate complement and colocalize with complement fragments deposited in vascular lesions [19], then administration of 1,6-bis(phosphocholine)-hexane should be of

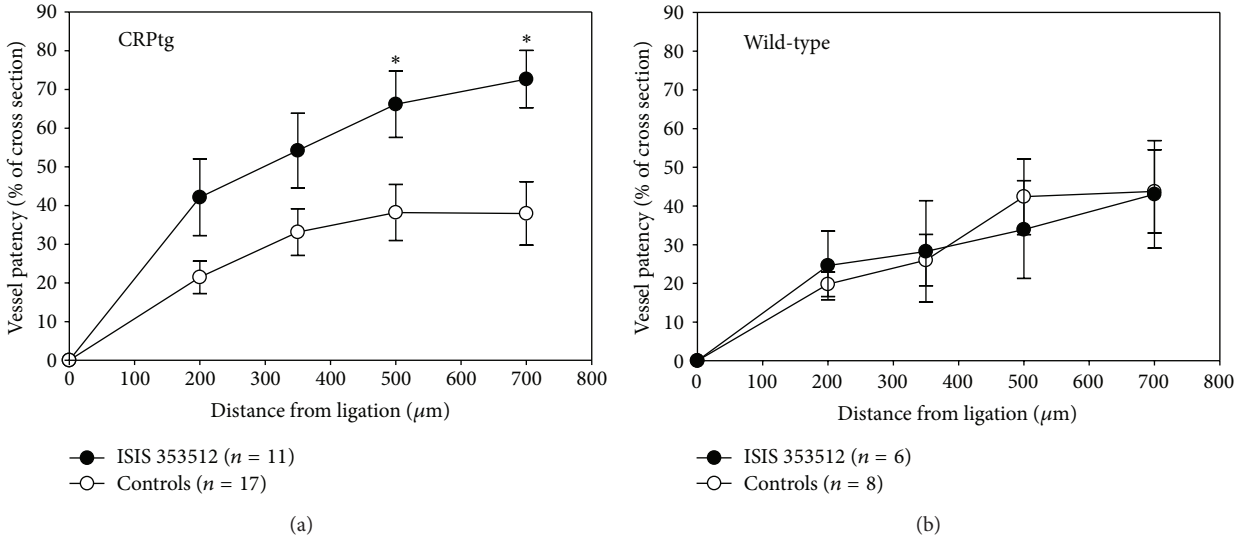

Figure 5: Treatment with the human CRP-specific ASO ISIS 353512 improves blood vessel patency following RCCA ligation in CRPtg mice (a) but not in wild-type mice (b). Blood vessel patency was calculated as described in the *Materials and Methods*. The asterisks indicate $^{*}P < 0.05$ for unpaired t-tests.

benefit in CVD. Indeed in rats, this compound was shown to prevent the exacerbating effect of exogenously administered human CRP on experimentally induced MI [27]. However, since CRP also interacts with FcγRs [4], treatment with 1,6-bis(phosphocholine)-hexane may not completely block all of the potentially detrimental functions of the protein. Also it is not clear what consequences circulating CRP decamers (two pentamers crosslinked by 1,6-bis(phosphocholine)-hexane) might have on the vasculature, since presumably these complexes could be deposited and potentially cause autoimmune or inflammatory side effects. Therefore, despite the fact that this drug improved infarct size and improved cardiac function in rats receiving human CRP, these shortcomings could ultimately limit its therapeutic potential in man.

Our CRP lowering tactic was different. Rather than depleting the circulating protein from the blood, the ASOs we used inhibit CRP production by specifically and selectively preventing the translation of CRP mRNAs. The ASO approach has been successfully used to target proteins not readily amenable to small molecule or antibody based therapeutic interventions. For example Kynamro, an antisense inhibitor of apolipoprotein B [32], the principal apoprotein present on all atherogenic lipids [38], was approved by the FDA in January 2013 for use as an adjunct to first-line lipid-lowering therapies in homozygous familial hypercholesterolemia [39–41]. Because ASOs have much longer half-lives compared to small molecule inhibitors [41, 42], ASOs can be administered more infrequently to patients. Furthermore, CRP is well suited for inhibition using ASO technology because the protein is synthesized primarily by hepatocytes [3, 4, 29, 34], cells that readily accumulate antisense drugs and are sensitive to ASO pharmacology [42–44]. ASOs also accumulate in extrahepatic cells and tissues known to make CRP, such as the kidney, alveolar macrophages, and adipocytes, [19, 37, 42].

The ASOs we tested here were designed to target rat CRP and human CRP (in CRPtg mice). Each ASO was well tolerated at all doses and in both species tested, and both CRP targeting inhibitors specifically reduced their respective CRP serum levels after only short-term administration with a modest dosing regimen. We did not test complement levels in the current study, but we have shown previously that ASO mediated lowering of human CRP is accompanied by reduced complement activation in CRPtg mice [45], an effect like that of 1,6-bis(phosphocholine)-hexane in rats [27]. Notably, ASO-mediated reduction of CRP in both species was not due to reduction of IL-6 and each of the tested ASOs blunted (or reversed) the rise in serum CRP caused by surgery. The proven ability of antisense inhibitors to reduce baseline expression of rat CRP in rats and human CRP in CRPtg mice and to block CRP upregulation after surgery, after treatment for a short duration with only low doses of antisense drugs, suggests that these agents should be useful for intervention in both chronic and acute disease processes. Indeed, we have previously shown that another human CRP specific ASO (ISIS 329993) is efficacious in a CRPtg mouse model of inflammatory arthritis [31]. We have shown here for the first time that, in both rats and mice, ASO-mediated lowering of CRP results in improved outcomes following cardiovascular insult.

Despite a body of evidence that CRP level is a fairly strong predictor of CVD, there is still no agreement on how to integrate CRP measurement into clinical practice or indeed whether it should even routinely be evaluated [46–48]. The main reason is that it is still unknown whether CRP plays a pathophysiologic role in CVD in humans. Without a human specific-CRP drug it has not been possible to conduct clinical trials to test the CRP-CVD hypothesis. The CRP-specific ASO inhibitors that we describe here could fill this gap and provide the impetus for future *in vivo* pharmacological, toxicological,

and ultimately clinical studies that will help clearly delineate the role of CRP in CVD in humans. If future studies do confirm a role for CRP in CVD, then perhaps a reasonable ASO treatment group would be patients whose admission values of CRP are 10 mg/L or greater, as these are the patients at highest risk for death and MI [48].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

C-Reactive Protein in Human Atherogenesis: Facts and Fiction

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The role of C-reactive protein (CRP) in atherosclerosis is controversially discussed. Whereas initial experimental studies suggested a pathogenic role for CRP in atherogenesis, more recent genetic data from Mendelian randomization trials failed to provide evidence for a causative role of CRP in cardiovascular disease. Also, experimental results from laboratories all over the world were indeed contradictory, partly because of species differences in CRP biology and partly because data were not accurately evaluated. Here we summarize the published data from experimental work with mainly human material in order to avoid confusion based on species differences in CRP biology. Experimental work needs to be reevaluated after reconsideration of some traditional rules in research: (1) in order to understand a molecule's role in disease it may be helpful to be aware of its role in physiology; (2) it is necessary to define the disease entity that experimental CRP research deals with; (3) the scientific consensus is as follows: do not try to prove your hypothesis. Specific CRP inhibition followed by use of CRP inhibitors in controlled clinical trials may be the only way to prove or disprove a causative role for CRP in cardiovascular disease.

1. CRP and Its Role in Physiology

C-reactive protein (CRP), the prototype human acute phase protein, is a pentameric molecule consisting of 5 identical subunits of 23 kD each [1, 2]. CRP has been first identified and described by Tillet and Francis in 1930 via its ability to bind to the C-fragment of *Streptococcus pneumoniae* [3]. In acute phase response, CRP plasma concentrations, within a few hours, can raise up to 1000-fold compared to normal [1, 2]. Because of its role as the prototype acute phase protein, CRP is one of the most frequently quantified molecules in clinical medicine. It is widely used by clinicians to monitor acute phase, for example, in pneumonia, sepsis, skin and soft tissue infections, and trauma and also in controlling the patient's response to antibiotic therapy. Furthermore, CRP is an indicator of activity in autoimmune diseases [4].

Paradoxically, in spite of this wide-spread clinical use relatively little is known about the molecule's biological functions. It is interesting to note that even in well-recognized and careful reviews summarizing the current evidence on CRP and cardiovascular disease, the basic science which has been accumulated over more than 80 years and has unraveled very few major functions of CRP in the human immune system seems to be almost forgotten [5, 6].

C-reactive protein is synthesized mainly in the liver in response to interleukin-6 (and interleukin-1) [1, 2]. The two prominent CRP functions are (1) activation of the classical complement pathway via C1q binding [7]. Each 23 kD subunit contains a Ca^{2+}-dependent phosphorylcholine binding site as well as a complement C1q binding site [8]. Phosphorylcholine binding induces a conformational change on the opposite site of the molecule with consecutive C1q binding and activation of the classical complement pathway (reviewed in [9]); (2) binding to human immunoglobulin Fcγ receptors and, hereby, opsonization of biological particles for macrophages [10–14]. Notably, these functions are also antibody functions. Therefore, it is not unlikely that CRP has been the first antibody-like molecule in the evolution of the mammalian immune system. As CRP functions have been taken over by

Table 1: Colocalization of CRP, complement components, and FcγR/macrophages as a general feature in histopathology of human disease.

Disease	CRP	Complement components	FcγR/macrophages
Atherosclerosis	+	+	+
Myocardial infarction	+	+	+
Dilated cardiomyopathy	+	+	+
Alzheimer's disease	+	+	+
Ischemic stroke	+	?	?
Rheumatology	+	+	+

antibodies with time, CRP may well be an atavism in the human immune system.

CRP is highly conserved in the evolution as evidenced by the atlantic horseshoe crab "*Limulus polyphemus*," which evolved in Paleozoic Era and expresses a CRP-like molecule in its immune system [15]. Nonetheless, there are surprising species differences in CRP biology [1, 2]. For example, CRP is not an acute phase reactant in the most broadly available experimental animal model, that is, the mouse [1, 2, 16, 17]. Any attempt to overcome this problem by overexpressing human CRP in mice [17] finally causes expression of a foreign antigen in these organisms with unforeseeable consequences for the animal's immune system [17]. The latter holds true for any other animal species and also for injection of human CRP into such animals. Further problems are known for the rat model with very high basic CRP plasma levels in these animals [18]. The only smaller animal model that seems to be useful for studying CRP biology and its role in cardiovascular disease is the rabbit model. Here, CRP is an acute phase reactant and activates complement [19, 20]. With regard to this animal model three major points have to be taken into consideration. First, the cholesterol hypothesis in atherosclerosis originates from the Anichkov rabbit model [21]; secondly, cholesterol feeding induces very high CRP levels in the plasma as well as CRP deposition in atherosclerotic plaques in the rabbit model [19]; and thirdly, complement C6 deficiency in rabbits protects these animals from lesion development [22, 23]. Here, an almost ideal animal model [19, 24] (with no need to overexpress human CRP in these animals [25]) seems to exist, although the latter has not yet been fully recognized [26]. However, interesting new data from this issue presented by Szalai and colleagues tell us that it may nonetheless be wrong to dismiss any animal model from the cardiovascular disease portfolio.

2. CRP and Its Role in Pathology

In contrast to the large number of epidemiological studies that correlate plasma levels of high sensitivity CRP (hsCRP: CRP measured by highly sensitive assays) with various diseases, for example acute coronary syndrome, heart failure, stroke, chronic obstructive pulmonary disease, peripheral artery disease, hemodialysis, cancer, hypertension, atrial fibrillation or coronary heart disease (F. Strang, and H. Schunkert, "C-reactive-protein and coronary heart disease: All said—isn't it?," Mediators of Inflammation, 2014), there is much less information about its molecular interactions within the affected tissues. Again, we would like to focus mainly on human material and again, the two prominent biological CRP functions, that is, complement activation and binding to human immunoglobulin Fcgamma receptors (Table 1), will primarily be discussed.

2.1. Atherosclerosis. Based on the identification of CRP as a cardiovascular risk marker in humans, the vast amount of experimental research with human material has been performed on atherosclerosis as the underlying cause for many cardiovascular disease entities [27–42].

(A) Histopathology of Human Atherosclerotic Lesions. Valid histopathology with its simple phenomenological approach may be the observational basis for any hypothesis on atherosclerotic lesion development. Although initially denied [27], CRP indeed deposits in all stages of human atherogenesis [28–31]. Notably, CRP in the lesion colocalizes with activated complement fragments [29, 31], and notably, with regard to cell types, CRP colocalizes almost exclusively with macrophages [30]. The conclusions to draw from these histopathological findings are as follows. First, CRP may be an important complement activating molecule in human atherogenesis and may thereby sustain a chronic autotoxic mechanism operating in the diseased arterial wall; second, macrophages (which strongly express Fcγ receptors [32]) are likely the target cells for CRP action in the arterial wall.

(B) In Vitro Experiments. Many *in vitro* studies have proposed diverse CRP effects on vascular cells. In these studies, some of the above mentioned rules have not been taken into account. The known biological CRP functions were not the underlying basis for the *in vitro* experiments. Consequently, CRP effects on smooth muscle cells and endothelial cells have been reported that were caused by contaminants of the applied CRP preparations, rather than by the CRP itself [33, 34]. Such publications have, unnecessarily, shed a dark light on the research topic in general [35, 36].

(C) CRP-Mediated Opsonization of LDL for Macrophages via Fcγ Receptors. Compelling evidence from *in vitro* studies on CRP-mediated opsonization of biological particles reports that (1) CRP binds to various unmodified and modified forms of LDL [37–39], (2) CRP binds to and signals via Fcγ receptors [11–14], and (3) LDL-bound CRP is taken up by macrophages via FcγR dependent and FcγR independent pathways [40].

In brief, CRP colocalization with macrophages in the lesion, high expression levels of FcγRs on macrophages, and CRP-mediated LDL uptake into macrophages suggest that CRP opsonizes LDL for macrophages. Thereby, CRP may be deeply involved in foam cell formation in atherogenesis [40].

(D) CRP Mediates Complement Activation in Atherogenesis. Compelling evidence from *in vitro* studies on CRP and

complement activation in atherogenesis suggests that (1) CRP conformation, either pentameric or monomeric, regulates complement activation in the vessel wall [41] and (2) CRP may also have protective effects by haltering modified lipoprotein-mediated complement activation before detrimental terminal sequence [42].

In brief, colocalization of CRP with activated complement fragments in atheroslerotic lesions and complement activation by CRP/LDL complexes *in vitro* strongly suggest that lipoprotein-bound CRP is intimately involved in complement activation in atherogenesis.

(E) Genetic Studies on CRP and Cardiovascular Disease. Concerning genetic studies on CRP and cardiovascular disease we refer to another article in this issue (Strang et al.). It is, however, important to note that results from Mendelian randomization trials do not support the concept that CRP is causally involved in atherogenesis and its sequelae [43–45]. Still, the limitations of Mendelian randomization need to be taken into account [46, 47], especially in case of a molecule like CRP whose synthesis in the liver is very complexly regulated on the transcriptional level [48–50].

2.2. CRP and Nonvascular Disease

2.2.1. Cardiology. A contribution of CRP to pathogenesis has been suggested for two other cardiovascular disease entities, that is, myocardial infarction and dilated cardiomyopathy.

(A) Myocardial Infarction. CRP plasma levels significantly rise after myocardial infarction indicating the human body's acute phase response [51, 52]. CRP deposits in human myocardial scars following coronary occlusion [51, 52]. In this location, CRP again colocalizes with activated complement fragments suggesting that CRP-mediated complement activation in necrotic tissue is a more general phenomenon. While in rats CRP-mediated complement activation seems to contribute to myocardial damage and while inhibition of CRP by a CRP-cross-linker may be protective [53], complement inhibition in human myocardial infarction has never been conclusively demonstrated to be beneficial [54]. The therapeutical focus in myocardial infarction should certainly be laid on reopening of the clotted coronary artery.

(B) Dilated Cardiomyopathy. Presence and distribution pattern of myocardial CRP in patients suffering from nonischemic chronic cardiomyopathy have been investigated [55]. Myocardial biopsies from dilated cardiomyopathy (DCM) patients either with or without accompanying chronic myocardial inflammation or virus were immunohistochemically studied for CRP and C5b-9 [55]. Myocardial CRP was detected in almost one-third of the patients. CRP again colocalized with macrophages and the terminal complement complex C5b-9. As there was no correlation with hsCRP plasma levels and as spacial distribution of myocardial C5b-9 was much broader than CRP distribution, CRP may not be the only myocardial complement activator in DCM.

2.2.2. Neurology. CRP and complement proteins were also detected in cerebral lesions in Alzheimer's disease [56]. Compared with normal brains, CRP mRNA levels were elevated in brains of Alzheimer patients, and thus it was concluded that CRP might be produced within the brain rather than being derived from the plasma [57]. CRP, again, seems to activate the complement system in brains and may lead to chronic neuroinflammation which then may cause neuronal death in Alzheimer's disease. CRP was also detected in brain lesions of patients who died of acute ischemic stroke or spontaneous intracerebral hemorrhage [58]. This observation was associated with a significant increase of hsCRP plasma level indicating the organism's acute phase response.

2.2.3. Rheumatology. CRP was also detected in synovial biopsies from patients with rheumatoid arthritis [59]. The nuclei of synoviocytes and histiocytes in the rheumatoid synovial membrane were found to bind CRP. Synovial-bound CRP was not of local origin and colocalized with antibodies and complement C3. Nonarthritis patients did not have CRP within their synovial membranes. Again this observation may indicate an active or causative role for CRP in rheumatoid arthritis [59]. Other studies showed CRP deposition in the synovial fluid of different joint diseases [60, 61]. There is evidence that CRP is either being selectively bound in synovium or specifically consumed in synovial fluid. Thus, CPR may play an important role also in the inflammatory process of this disease [61].

3. Targeting C-Reactive Protein for the Treatment of Cardiovascular Disease

In spite of huge effort to develop CRP inhibitors, up to the present day no laboratory or pharmaceutical company worldwide has succeeded in developing a specific anti-CRP agent that is readily applicable in humans. As a consequence, less specific anti-inflammatory drugs like IL-1β antibodies or methotrexate are currently tested in clinical trials to prevent patients from the progression of cardiovascular disease [62, 63]. In our view, these trials will very unlikely be successful because the side effects of these approaches may eliminate the potential therapeutic benefits. The trials, however, are ongoing and therefore we do not know what the outcome of the studies will be.

Assumed that CRP is an atavism in the human immune system (see above), specific CRP inhibition may be less immunosuppressive and thus the only way to proceed. Specific inhibition, however, is demanding and will probably depend on future development of novel pharmaceutical strategies to target biomolecules. Principle strategies include (1) cross-linking of CRP subunits, (2) antisense strategies, (3) blockage of CRP-mediated complement activation, (4) blockage of CRP receptors, and (5) inhibition of CRP synthesis.

Cross-linking of CRP subunits has been attempted in the past [53]. Although originally touted as a major breakthrough, the molecule that resulted from these attempts is a more or less ubiquitous cross-linker and has never found its way into human application. Use of *antisense*

strategies obtained promising results in two animal models and antisense molecules have also successfully lowered CRP plasma levels in healthy volunteers [64], [1, in this issue]. Although one of the challenges of this approach is how to apply such molecules continuously over years, the approach seems promising. *Inhibition of CRP-mediated complement activation* by competitive blockage of the C1q binding site has been tried but was not successful due to steric reasons. In view of the identification of Fcγ receptors as CRP receptors and the expectable side effects, *blockage of CRP receptors* seems not reasonable. Finally, high throughput screening (HTS) with a hepatoma cell line stably transfected with the CRP promoter surprisingly resulted in the identification of cardiac glycosides as potent *transcriptional CRP inhibitors* [65]. This *in vitro* result is difficult to interpret. Whether the *in vitro* effect also applies *in vivo* in humans is currently under investigation. The latter can easily be done and is ethically justified in heart failure patients, because cardiac glycosides have been used in cardiac insufficiency for 230 years [66] and, according to heart failure guidelines, still provide an additive treatment option in NYHA classes III and IV [67].

Since generation of induced pluripotent stem (IPS) cells has recently facilitated HTS with primary human cells [68] it may be worthwhile to repeat HTS for CRP synthesis inhibitors with hepatocytes derived from IPS cells.

In summary it can be stated that, as CRP is also involved in the pathogenesis of other diseases (see above), the molecule may be a rewarding drug target. Future technologies in drug development may facilitate achievement of this demanding goal.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Oliver Zimmermann and Kefei Li contributed equally to this work.

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