American Journal of Pathology, Vol. 167, No. 4, October 2005

Vascular Biology, Atherosclerosis and Endothelium Biology

C-Reactive Protein in Atherosclerotic Lesions

Its Origin and Pathophysiological Significance

Huijun Sun,*† Tomonari Koike,* Tomonaga Ichikawa,* Kinta Hatakeyama,‡ Masashi Shiomi,§ Bo Zhang,¶ Shuji Kitajima,‖ Masatoshi Morimoto,‖ Teruo Watanabe,** Yujiro Asada,‡ Yuqing E. Chen,†† and Jianglin Fan*

From the Department of Pathology, Cardiovascular Disease Laboratory, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan; the First Department of Pathology,‡ Faculty of Medicine, University of Miyazaki, Miyazaki, Japan; Institute for Experimental Animals,§ Kobe University School of Medicine, Kobe, Japan; the Department of Cardiology,¶ Fukuoka University School of Medicine, Fukuoka, Japan; Analytical Research Center for Experimental Sciences‖ and President Office,** Saga University, Saga, Japan; Cardiovascular Research Institute,†† Morehouse School of Medicine, Atlanta, Georgia; and the Department of Pharmacology,† Dalian Medical University, Dalian, China*

C-reactive protein (CRP) is frequently deposited in the lesions of the arterial intima; however, the origin and pathological significance of CRP in these lesions are not completely understood. In this study, we measured CRP levels in the plasma of hypercholesterolemic rabbits and investigated CRP expression at both the mRNA and protein levels using rabbit and human atherosclerotic specimens. CRP levels were significantly elevated in both cholesterol-fed and Watanabe heritable hyperlipidemic rabbits, and CRP levels were clearly correlated with aortic atherosclerotic lesion size. Immunohistochemical staining coupled with Western blotting analysis revealed that CRP-immunoreactive proteins were found at all stages of atherosclerosis from the early to advanced lesions. CRP was present extracellularly and co-localized with apolipoprotein B but was rarely associated with the cytoplasm of macrophages and foam cells. Real-time reverse transcriptase-polymerase chain reaction analysis revealed that CRP mRNA in atherosclerotic lesions was barely detectable, and isolated macrophages did not express CRP mRNA, suggesting that CRP proteins found in the lesions were essentially derived from the circulation rather than synthesized *de novo* by vascular cells. These results suggest that there is a link between plasma CRP and the degree of atherosclerosis and that inhibition of plasma CRP may represent a therapeutic modality for the treatment of cardiovascular disease. *(Am J Pathol 2005, 167:1139–1148)*

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 on 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 recent studies failed to prove the risk of CRP compared to other risk factors.[10] Regardless of this controversy, emerging evidence indicates that high levels of CRP may be potentially atherogenic;[3,11] therefore, it is essential to clarify the functional roles of CRP in the arterial wall. Although it has been reported for a long time that CRP is present in human atherosclerotic lesions,[12,13] it is still not unequivocal whether CRP in the arterial wall is completely derived from the circulation or

Supported in part by grants-in-aid for scientific research from Ministry of Education, Culture, Sports and Technology, Japan (KAKENHI.16390089 and 16659099 to J.F.), the Takeda Science Foundation, the Ono Medical Research Foundation, and the Novartis Foundation for the Promotion of Science (to J.F.); and a grant from the Center for Tsukuba Advanced Research Alliance at the University of Tsukuba (to J.F.).

H.S. and T.K. contributed equally to this work.

Accepted for publication June 27, 2005.

Address reprint requests to Jianglin Fan M.D., Ph.D., Department of Pathology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, 305-8575 Japan. E-mail: j-lfan@md.tsukuba.ac.jp.

is locally synthesized by the arterial cells.[14,15] Several studies even suggested that CRP may possibly be produced by macrophages[16] and smooth muscle cells (SMCs).[17] The hypothesis of arterial wall-synthesized CRP is so intriguing and attractive that it led to a number of investigations attempting to determine the potential pathophysiological significance of the local production of CRP in terms of its atherogenicity *in vitro*. For example, CRP has been shown to modulate endothelial cell functions (NO and ET-1 production)[18–21] and increase SMC migration and mediate the uptake of oxLDL by macrophages.[22,23] CRP itself is a chemoattractant factor for monocytes[24] and induces the production of MCP-1 and the expression of adhesion molecules on endothelial cells *in vitro.*[25,26] If this hypothesis really represents the situation *in vivo*, CRP, along with hypercholesterolemia, may play a pivotal role in the initiation and progression of atherosclerosis, and therapeutic strategies to block CRP functions may be useful for treating atherosclerosis. Unfortunately, this hypothesis has not been tested or verified *in vivo* using appropriate experimental animals. Several recent studies have argued that biological effects of CRP on vascular cells reported previously were possible artifacts caused by the presence of sodium azide in the commercial CRP.[27–29] Furthermore, it has not been determined whether the level of CRP is associated with the degree of the lesions of atherosclerosis and whether CRP is involved in the initiation and progression of the lesions or with plaque rupture. These issues may be difficult to resolve by merely using human advanced lesions or *in vitro* models. Finally, there have been no experimental animal studies examining the relationship between CRP levels and degree of atherosclerosis. To address these issues, we performed the current study using rabbit atherosclerosis models as well as specimens of human coronary arterial plaques. Rabbits are excellent models for atherosclerosis because they are sensitive to cholesterol diet and rapidly develop atherosclerosis.[30] In addition, rabbit CRP has 70% homology with human CRP[1] and rabbit CRP levels are highly inducible and responsive during the inflammatory reaction.[31] We used both cholesterol-fed and Watanabe heritable hyperlipidemic (WHHL) rabbits because these two kinds of rabbit models exhibited different atherogenic lipoprotein profiles (remnant-rich hypercholesterolemia in cholesterol-fed rabbits versus low-density lipoprotein (LDL)-rich hypercholesterolemia in WHHL rabbits). Using these models, we were particularly interested in clarifying: 1) whether plasma CRP levels are correlated with aortic atherosclerosis; 2) whether CRP deposition patterns are different in early-stage lesions from the advanced lesions; and 3) whether vascular wall cells, especially macrophages, can synthesize CRP. In addition, we investigated the CRP deposition and expression in unstable and ruptured coronary arteries from human myocardial infarction patients.

Materials and Methods

Atherosclerotic Specimens

In this study, we used both rabbit and human specimens to examine CRP deposition and the relationship of CRP with other cellular components in atherosclerotic lesions. For rabbit experiments, we used two kinds of rabbits with atherosclerosis: 45 cholesterol-diet fed rabbits[32] (fed a diet containing 0.3% cholesterol and 3% soybean oil for 16 weeks) and 31 WHHL rabbits[33] (fed a chow diet, aged from 8 to 12 months) and 62 normolipidemic rabbits (fed a chow diet, aged from 4 to 10 months) as a control group. All rabbits were raised in a specific pathogen free-equivalent barrier facility at either the University of Tsukuba or Saga University and given food and water *ad libitum*. Human coronary arteries and aortas were obtained from patients who died of myocardial infarction within 6 hours after death. Autopsy was performed at the Department of Pathology, Faculty of Medicine, University of Miyazaki. All specimens were fixed in formalin for histological examinations or snap-frozen in liquid nitrogen and stored at −80°C for protein isolation and RNA extraction. All protocols for animal studies and use of human specimens were performed according to the guidelines and with the approval of the ethics review committee of the University of Tsukuba.

Histology and Immunohistochemical Staining

All specimens were embedded in paraffin and cut into 3-μm-thick serial sections. The specimens were mounted on slides and deparaffinized with graded concentrations of xylene and ethanol, and then stained with hematoxylin and eosin (H&E). For immunohistochemical staining, all slides were immersed in 3% H_2O_2 in methanol for 45 minutes at room temperature to block endogenous peroxidase activity. To stain human macrophages and complement 3 (C3), the slides

Table 1. Antibodies Used for the Current Study

Antibodies	Working dilution	Species	Manufacturers
hCRP	×500	Mouse	Sigma, St. Louis, MO
rCRP	×200	Chicken	Immunology Consultants Laboratory Inc., Newberg, OR
KP1	×50	Mouse	DakoCytomation Denmark A/S, Glostrup, Denmark
RAM11	×400	Mouse	Dako Japan Inc., Tokyo, Japan
HHF35	×400	Mouse	Enzo Biochemicals, NY
apoB	×200	Goat	Rockland Inc., Gilbertsville, PA
C3	×100	Mouse	Santa Cruz Biotechnology Inc., Santa Cruz, CA

hCRP, human C-reactive protein; rCRP, rabbit C-reactive protein; KP1, human CD68 macrophage; RAM11, rabbit monocyte/macrophages; HHF35, smooth muscle α-actin; C3, recombinant protein corresponding to amino acids 541 to 840 of C3 precursor of human origin.

Table 2. Primers Used for Real-Time RT-PCR

Genes		Primers
Rabbit	Forward	TCAAAGCCTTCACTGTGTGC
CRP	Reverse	AGGTGAGTTGGATCCACAGG
GAPDH	Forward	GCTGAACGGGAAACTCACTG
	Reverse	CCAGCATCGAAGGTAGAGGA
Human	Forward	GTGTTTCCCAAAGAGTCGGATACT
CRP	Reverse	CCACGGGTCGAGGACAGTT
GAPDH	Forward	CAATGACCCCTTCATTGACCTC
	Reverse	AGCATCGCCCCACTTGATT

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

were predigested with 0.4% proteinase K for 5 minutes at 37°C. The sections were then incubated with 10% normal goat serum for 60 minutes to reduce nonspecific background staining and reacted with each appropriately diluted antibody (Ab) at 4°C overnight. The panel of Abs used for examining rabbit and human CRP, apoB, C3, and macrophages and SMCs in the lesions is listed in Table 1. CRP antibodies were solid phase absorbed and then immunoaffinity purified of CRP proteins. Affinity purified CRP antibodies give a single band by immunoelectrophoresis and gel diffusion against fluids containing CRP. The specificity of CRP antibodies has been confirmed by Western blots. This antibody does not cross-react with serum amyloid P component, haptoglobin, α-1-acid glycoprotein and IgG. It can detect both native and denatured-reduced CRP proteins. After rinsing with phosphate-buffered saline (pH 7.4), the sections were incubated with peroxidase-conjugated secondary Abs at room temperature for 60 minutes and then the staining was visualized by reaction with 3-amino-9-ethylcarbazole (Nichirei, Tokyo) as described previously.[34] For negative controls, primary Abs were either omitted or replaced by nonspecific IgG.

Western Blot Analysis

To examine the expression of CRP proteins in the atherosclerotic lesions, aortic specimens were divided into lesions and nonlesions based on the gross observation. The aortic lesions were further separated into the intima, media, and adventitia under a dissecting microscope. In addition, alveolar macrophages, thioglycollate-elicited peritoneal macrophages, and liver were used. One hundred mg of each tissue were homogenized in ice-cold suspension buffer (0.02 mol/L Tris-HCl, pH 7.5) supplemented with phenylmethyl sulfonyl fluoride (1 μg/ml), aprotinin (1 μg/ml), and ethylenediamine tetraacetic acid (1 mmol/L). Ten-μg aliquots of the crude proteins from each sample were separated by 4 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient gel electrophoresis for Western blotting and probed with a mouse monoclonal Ab against human CRP.[35]

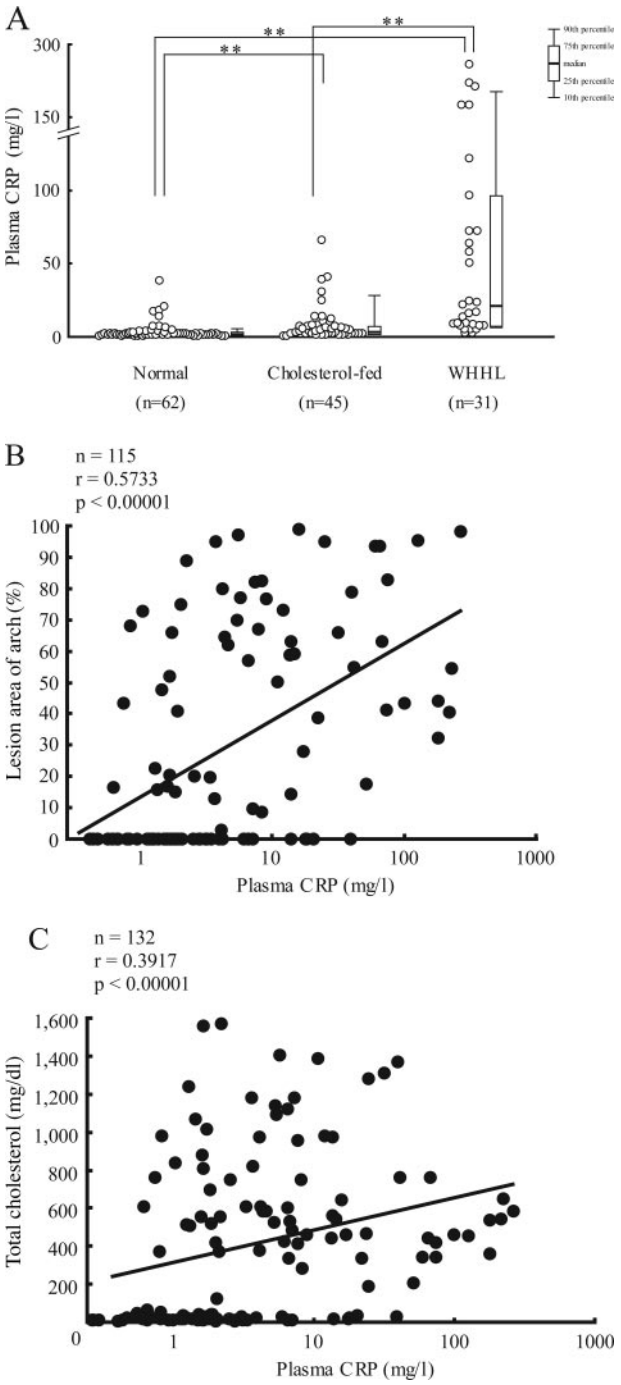

Figure 1. A: Increased plasma CRP levels in cholesterol-fed and WHHL rabbits. The values are expressed as mean ± SE. $^{**}P < 0.01$ versus normal rabbits or cholesterol-fed rabbits versus WHHL rabbits. Average levels of plasma total cholesterol are 30 ± 9 mg/dl in normal rabbits, 860 ± 53 mg/dl in cholesterol-fed rabbits, and 459 ± 21 mg/dl in WHHL rabbits. Total *en face* lesion area of the aorta is 14 ± 3.3% in cholesterol-fed rabbits and 42.3 ± 5.9% in WHHL rabbits. **B** and **C:** Correlations of plasma CRP and aortic atherosclerosis gross size (**B**) and plasma cholesterol levels (**C**).

Northern Blot and Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from various tissues of both normal and cholesterol-fed and WHHL rabbits using

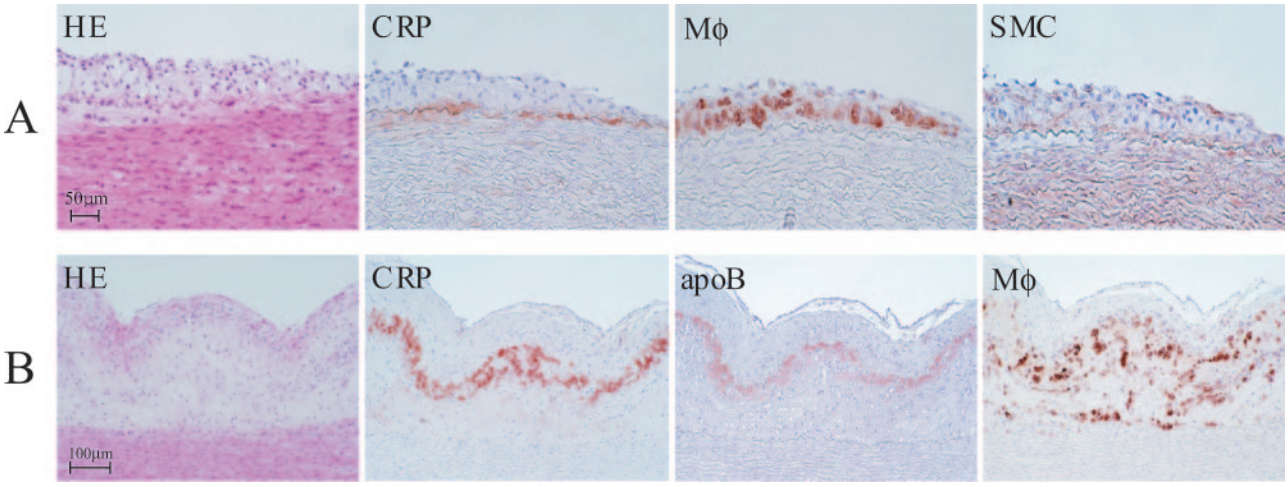

Figure 2. Demonstration of CRP deposition in atherosclerotic lesions of cholesterol-fed rabbits. Two representative lesions were selected from cholesterol-fed rabbits and stained with H&E or mAbs against rabbit CRP, macrophages (Mϕ), SMCs, and apoB. **A:** Early-stage lesion, which is composed of a single layer of macrophage-derived foam cells. **B:** Advanced lesion, which is covered by a fibrotic cap. CRP is co-localized with apoB staining. See supplemental data for sections stained with CRP Abs shown in higher magnification at *http://ajp.amjpathol.org*.

Trizol reagent (Invitrogen, Carlsbad, CA). Thioglycollate-elicited peritoneal and alveolar macrophages were collected as described.[35] Human specimens (aorta and liver) were obtained at autopsy. Ten μg of RNA was then subjected to electrophoresis in a 1.2% agarose gel and transferred to a Nytran nylon membrane. The membrane was hybridized in turn with the ^{32}P-labeled human CRP cDNA probe. The membrane was rehybridized with human β-actin cDNA probe as an internal standard. Total RNA was reverse-transcribed into cDNA by using Invitrogen reverse transcription reagents. Expression of CRP and a housekeeping gene (GAPDH) in the liver, aortic lesions, nonlesion regions of aortas, and macrophages by real-time RT-PCR (DNA Engine Option; MJ Research, Tokyo, Japan) were performed using DyNAmo SYBR Green qPCR kits (Finnzymes) according to the manufacturers' instructions.[35] The primers for rabbit and human CRP and GAPDH are listed in Table 2.

Measurement of Plasma CRP Levels in Rabbits by Enzyme-Linked Immunosorbent Assay (ELISA)

To determine whether CRP was elevated in cholesterol-fed or WHHL rabbits, levels of rabbit CRP in the plasma were quantified using two rabbit high-sensitive CRP ELISA kits from Kamiya Biomedical (Seattle, WA) and Shibayagi Co. (Gunma, Japan), and compared with normal rabbits. All measurements were performed independently by two researchers using different ELISA kits. The plasma specimens were mixed thoroughly before assays.

In Vitro *Investigation of CRP Expression*

To examine the effects of cytokines on the regulation of CRP expression in hepatocytes and possibly in macrophages, we used the Huh7 cell line (derived from human hepatoma) and U937 monocytic cells. Huh7 cells were cultured in serum-free Dulbecco's modified Eagle's medium and incubated with various concentrations of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α for 48 hours. U937 cells were cultured in RPMI 1640 medium and treated with phorbol 12-myristate 13-acetate (100 ng/ml) for 3 days before incubation with cytokines.[36] After that, these cells were collected for the extraction of total RNA using Trizol agent and CRP expression was evaluated using real-time RT-PCR as described above.

Statistical Analysis

All values are expressed as mean ± SE. Plasma CRP, total cholesterol, and aortic *en face* lesion area were compared with Student's *t*-test, Welch's *t*-test, or Mann-Whitney's *U*-test for nonparametric analysis. Correlations were analyzed using Spearman's correlation coefficient by rank test.

Results

Correlation between Plasma CRP and Cholesterol and Aortic Atherosclerosis

In normal rabbits, the range of plasma CRP levels was narrow with an average of 3.15 ± 0.8 mg/L. It seems that rabbit CRP levels are close to the mean values of healthy middle-aged humans (2.82 mg/L) reported in the literature.[37] Rabbits with hypercholesterolemia, either induced by cholesterol diet feeding or caused by LDL receptor deficiency, had significantly higher plasma CRP levels than normal rabbits: 2.5-fold higher in cholesterol-fed rabbits and 28-fold higher in WHHL rabbits on average (Figure 1A). We also examined the relationship between plasma CRP and aortic athero-

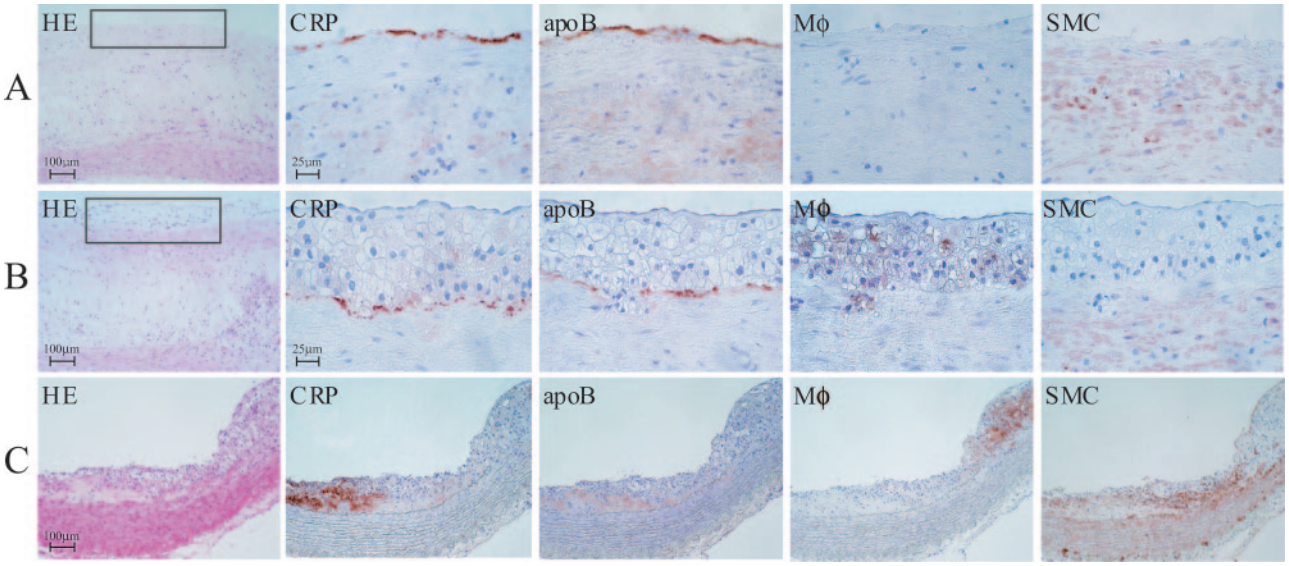

Figure 3. Demonstration of CRP deposition and apoB in the early-stage lesions of WHHL rabbits. Three representative lesions with different features were selected and stained with H&E or mAbs against CRP, apoB, macrophages (Mϕ), and SMCs. CRP is deposited on the surface (**A**) or under foam cells (**B**) and co-localized with apoB and does not overlap with macrophage-rich area (**C**). See supplemental data for sections stained with CRP Abs shown in higher magnification at *http://ajp.amjpathol.org*.

sclerosis *en face* size or plasma total cholesterol levels in hyperlipidemic rabbits. As shown in Figure 1B and C, the CRP level in plasma was well correlated with the lesion size in the aorta, and weakly associated with the plasma total cholesterol level.

Demonstration of CRP in Atherosclerotic Lesions of Rabbits

Because a large range of lesions were obtained from a sufficient number of rabbits in this study, we were able to select both early stage lesions (referred to as foam cell-rich lesions) as well as relatively advanced lesions (including fibrous plaques and complicated lesions). Immunohistochemical staining revealed that in the early-stage lesions, where a few layers of macrophages were present, CRP-immunoreactive proteins were found either on the surface or beneath the foam cells (Figure 2A). In advanced lesions, CRP was primarily localized beneath the fibrous cap and closely associated with apoB deposition (Figure 2B). In either case, CRP did not apparently overlap with the cytoplasm of macrophages and foam cells (Figure 4). CRP tended to be localized around the extracellular matrix and was seldom, if ever, associated with macrophages or SMCs (also see supplemental data for high magnification at *http://ajp.amjpathol.org*).

Atherosclerotic lesions of WHHL rabbits were more advanced than those of cholesterol-fed rabbits because these rabbits have higher levels of LDL-cholesterol rather than the levels of remnant lipoproteins present in cholesterol-fed rabbits. In WHHL rabbits, various kinds of atherosclerotic lesions were seen, from foam cell-rich lesions to complicated lesions in which calcification and necrotic cores were prominent. Similar to those of cholesterol-fed rabbits, CRP-immunoreactive proteins were detected on the surface of the lesions, beneath foam cells, or around the lipid or necrotic cores (Figures 3 and 4). CRP was co-localized with apoB but seemed not to be

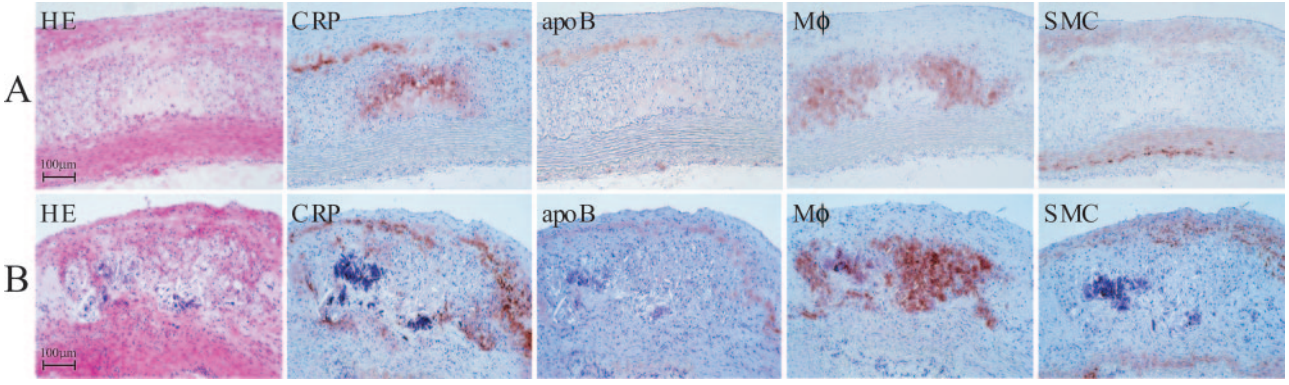

Figure 4. Demonstration of CRP deposition and apoB in advanced lesions of WHHL rabbits. Two representative lesions with a necrotic core (**A**) or calcification (**B**) were selected and both lesions show CRP deposition overlapping with apoB. See supplemental data for sections stained with CRP Abs shown in higher magnification at *http://ajp.amjpathol.org*.

associated with the cytoplasm of macrophages and foam cells. In these lesions, CRP was not deposited in any particular areas such as the cap or core. We also performed Western blot analysis. For this purpose, we separated the lesion from nonlesion areas (based on gross observation) and furthermore, we isolated the intima, media, and adventitia of the aorta from WHHL rabbits under a dissecting microscope. We were especially interested in examining whether macrophages could express CRP *per se*, and therefore we used macrophages in parallel. As shown in Figure 5, CRP was detected in the lesional intima but not in the media or adventitia. Isolated macrophages contained smaller amounts of CRP but the immunoreactive bands from macrophages showed faster mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, presumably they were degraded CRP proteins or taken up by macrophages.

Demonstration of CRP in Human Coronary Plaques

Next, we investigated CRP deposition in human coronary plaques. CRP was detected in all types of the lesions, intimal thickening, unstable plaques, and ruptured plaques, and especially in fragile or ruptured caps. In the vicinity of these lesions, strong CRP staining was seen around or concentrated on the surface, but did not overlap with the cytoplasm of macrophages (Figure 6). In these CRP-positive areas, C3 was also detected (Figure 6B), which was consistent with a previous report.[38] In stable plaques, CRP was also detected, either on the surface or in the deep area of thickened intima (Figure 7). The immunohistochemical findings were further confirmed by Western blot analysis and lesions of aortic atherosclerosis contained substantial amounts of CRP proteins regardless of the lesion characteristics (Figure 8).

CRP mRNA Expression

The fact that CRP-immunoreactive proteins were invariably present in atherosclerotic lesions raised the question of whether CRP was exclusively derived from the circulation or/and produced locally. To address this issue, we initially screened the CRP mRNA expression of multiple tissues from both chow- and cholesterol-fed rabbits using Northern blotting. As shown in Figure 9A, the liver was the only organ that expressed detectable mRNA by Northern blot hybridization. There was no expression of CRP mRNA in either atherosclerotic lesions or isolated macrophages. This result suggests that the liver is the exclusive organ for CRP production, while the arterial wall and macrophages do not express CRP mRNA or the levels of CRP mRNA expressed by the arterial wall and macrophages are too low to be detected. To further explore this possibility, we applied a real-time RT-PCR analysis, which is considered to be more sensitive than Northern blotting analysis and more specific than traditional quantitative RT-PCR.[39] In these experiments, we analyzed the CRP expression of the liver (n = 22 rabbits), lesions (n = 16 rabbits), nonlesion regions of the aorta (n = 12 rabbits), and macrophages (n = 28 rabbits). Using the liver expression of CRP as a standard, we calculated the CRP expression level using the $2^{-\Delta\Delta_{CT}}$ method by Livak and Schmittgen.[40] We found that isolated macrophages did not express CRP mRNA (90% of sample values were actually 0). The average relative CRP expression levels (arbitrary units) of lesions or nonlesions were less than 1/100th of the hepatic expression of CRP (Figure 9B), suggesting that lesional CRP expression was indeed extremely low and not comparable with the hepatic expression. This conclusion was further strengthened by examining human aortic atherosclerotic lesions. Analysis of eight freshly isolated human aortic lesions showed no mRNA expression (5 sample values were 0 and 3 were <0.02) by real-time RT-PCR (average value of livers of the same patients: 121 $\pm$ 48 arbitrary units). We also compared CRP mRNA expression using human Huh7 hepatoma cells and U937 macrophages. Huh7 hepatocytes expressed a much lower level of CRP under basal conditions (compared to normal human liver tissue) but the level was discernibly up-regulated by incubation with IL-1β, IL-6, and tumor necrosis factor-α (data not shown). U937 macrophages did not express CRP mRNA in either normal state or when stimulated by various cytokines at different concentrations (all sample values were 0). Therefore, these results demonstrate that macrophages from both rabbits and humans did not express CRP mRNA.

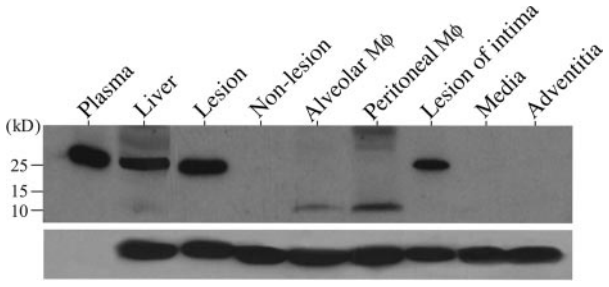

Figure 5. Immunoblotting analysis of tissue CRP from WHHL rabbits. Aortic lesions along with isolated alveolar and peritoneal macrophages were analyzed for the presence of CRP protein as described in Materials and Methods. Liver and plasma were used as positive controls. Note that CRP bands in macrophages are smaller than other CRP bands.

Discussion

In this study, we demonstrated for the first time that the CRP level was significantly elevated in hypercholesterolemic rabbits compared to normal rabbits. Although it is currently unknown whether the elevation of CRP in plasma is a cause or consequence or both of atherosclerosis, it is clear that elevated CRP levels in plasma were correlated with the severity of atherosclerosis and hypercholesterolemia in rabbits. It was reported that CRP can selectively bind to apoB-containing atherogenic lipoproteins, such as β-VLDL and LDL, in hypercholesterolemic rabbits as well as in humans[41] and thus increased levels of apoB-containing particles may facilitate the formation of CRP-lipoprotein complexes in plasma. Formation of CRP-apoB-containing lipoprotein complexes does not af-

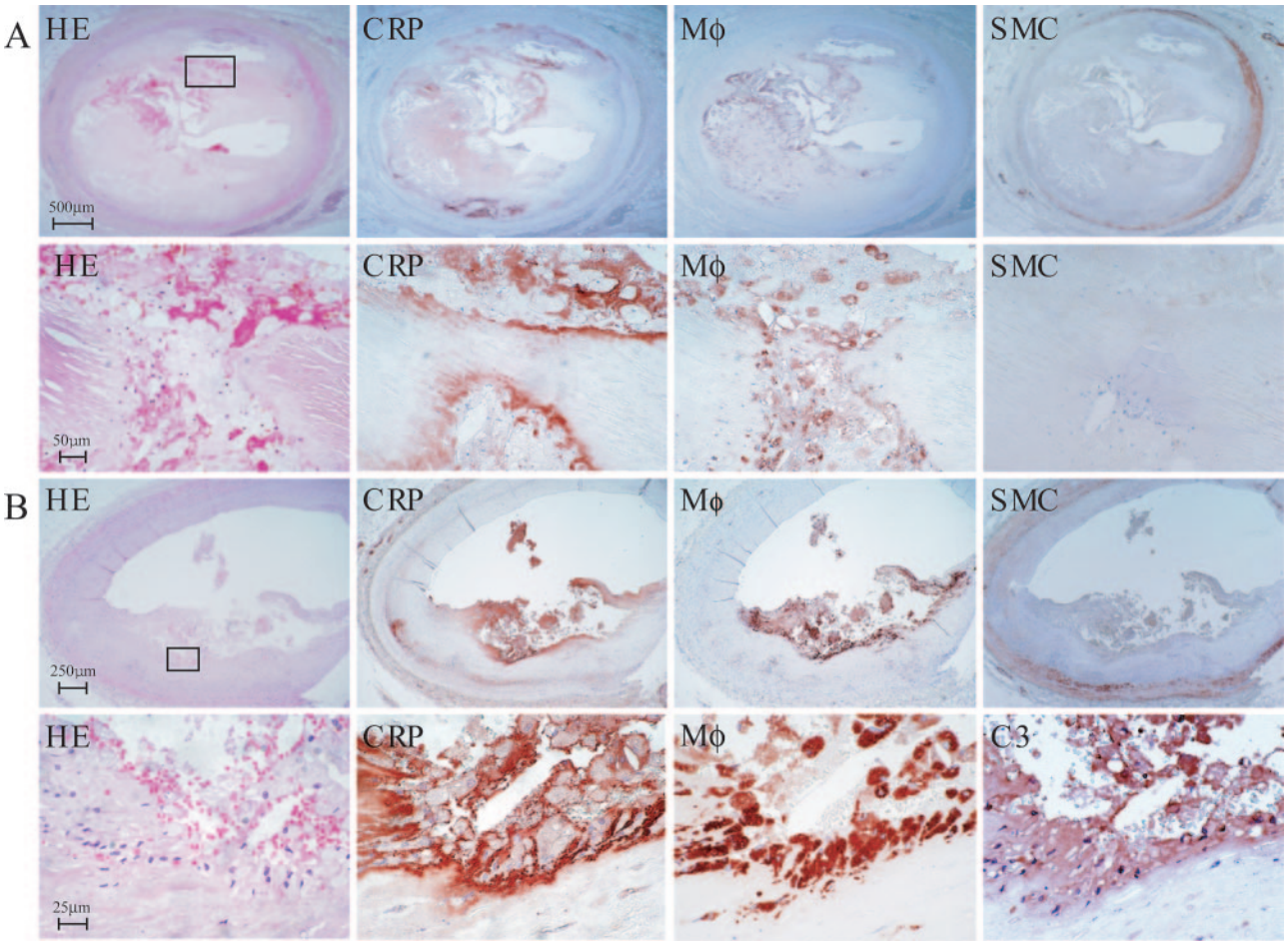

Figure 6. CRP was closely associated with unstable vulnerable plaque (**A**) or ruptured plaques (**B**) in human coronary arteries. Human coronary arteries, obtained from patients who died of MI, were used for the detection of CRP immunoreactive proteins. Macrophage-rich areas (stained by CRP, Mϕ, SMCs, or C3), **boxed area**, were shown in higher magnification (**B**). Note that CRP is concentrated on the surface of macrophages of both lesions. See supplemental data for sections stained with CRP Abs shown in higher magnification at *http://ajp.amjpathol.org*.

fect the clearance rate of ^{125}I-labeled rabbit CRP;[42] therefore, we speculate that the elevated CRP level in plasma is mainly caused by enhanced hepatic production rather than by reduced catabolism of CRP in rabbits.

Atherosclerosis is an inflammatory process[7] and many inflammatory cells, especially macrophages and foam cells can produce a variety of cytokines that may stimulate the hepatic expression of the CRP gene and up-

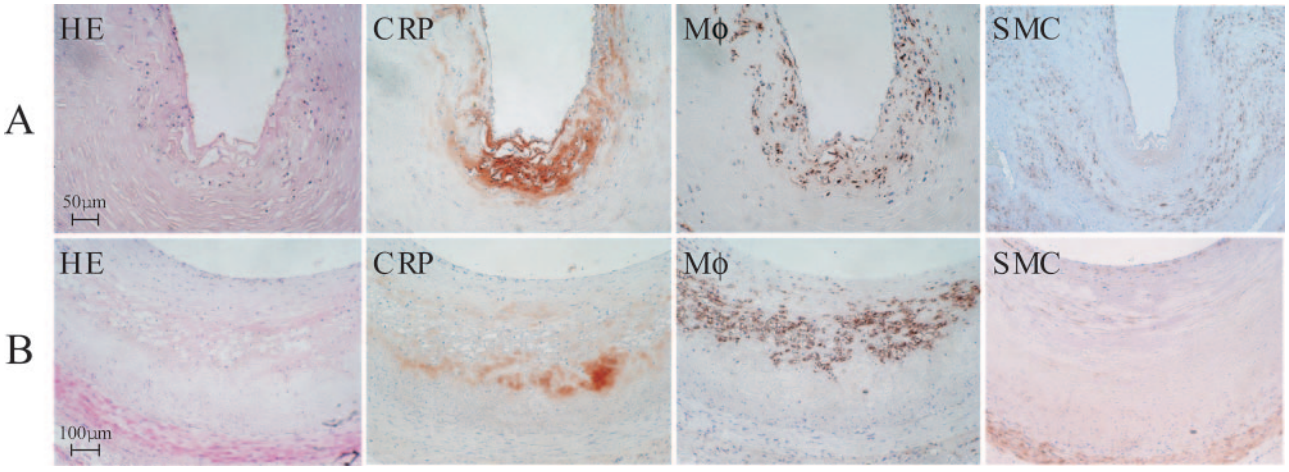

Figure 7. Detection of CRP deposition in stable plaques of human coronary arteries. Two macrophage-rich lesions were selected and both show CRP deposition. CRP was either located on the top (**A**) or the bottom (**B**) of the lesions. See supplemental data for sections stained with CRP Abs shown in higher magnification at *http://ajp.amjpathol.org*.

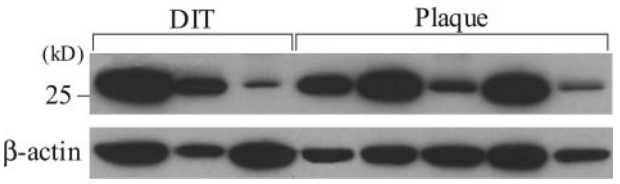

Figure 8. Demonstration of CRP protein in human aorta by Western blotting. The samples were from eight autopsy cases and all of them contained CRP proteins detected by Western blotting. The lesions were divided grossly: DIC, diffuse intimal thickening and plaques.

regulate CRP production in the liver. The pathophysiological significance of the elevated CRP in hyperlipidemic rabbits is still unknown; however, it may reflect an inflammatory state related to atherosclerosis or amount or/and activity of circulating proinflammatory cytokines or both. It seems unlikely that the increased CRP level in plasma was caused by *de novo* extrahepatic synthesis in atherosclerotic lesions because we failed to detect significant CRP mRNA expression in the lesions or other tissues by either Northern blot hybridization or real-time RT-PCR.

In accordance with previous reports,[12–14,24] our study clearly demonstrated that CRP protein was invariably present in atherosclerotic lesions, regardless of lesion type (early versus advanced) or nature (foam cell-rich versus extracellular matrix-rich) and was closely co-localized with apoB, suggesting that CRP may interact with apoB-containing particles in the intima, thereby facilitating their binding to extracellular matrix, increasing retention time and oxidation in the intima, and enhancing foam cell formation.[43,44] As reviewed in the Introduction, these premises or assumptions regarding the proatherogenic effects of CRP, however, should be considered cautiously, and need to be verified in the future. Despite this, CRP deposition rarely overlapped with the cytoplasm of macrophages and foam cells in our studies of both rabbit and human specimens. Our findings showed that although some macrophages could be positively stained by CRP antibodies, this was not a frequent and universal phenomenon and may occur only when a particular ligand is exposed on these cells. Three methods of analysis (immunohistochemical staining, Western blotting, and real-time RT-PCR) provided consistent observations, and therefore, these results rebut the contention that macrophages[16,17,38] are the major sources of CRP in the arterial wall or that macrophages are responsible for the increased plasma CRP levels. This notion is also consistent with a recent report using CRP transgenic mice showing that no CRP mRNA was detected in apoE knockout mouse lesions.[45] Our results are not in agreement with the report by Yasojima and colleagues,[38] who showed that human atherosclerotic plaques (10 human postmortem samples) contained higher (7.2-fold) amounts of CRP mRNA than liver and proposed that macrophages and SMCs are the main sources of plasma CRP. The discrepancy between our study and previous studies regarding the macrophage-produced CRP in the lesions is clearly due to the differences in the samples and methods applied. In sharp contrast to the previous reports, we used a large number and variety of atherosclerotic samples from two kinds of rabbits along with human coronary arteries and aortas as well as macrophages and livers. More importantly, we analyzed CRP mRNA expression using the most sensitive method available, real-time RT-PCR, whereas the previous studies performed only conventional semiquantitative RT-PCR,[38] which has been criticized as being unreliable.[39] It should be emphasized that none of the previous studies that concluded that CRP was expressed by vascular macrophages or SMCs hitherto were performed using real-time RT-PCR. In addition, our refutation was also supported by studies on cultured human Hu7h hepatocytes and U937 macrophages. It is noteworthy that CRP-immunoreactive proteins were also detected in unstable plaques or ruptured plaques, particularly on the fibrotic cap. CRP proteins were concentrated on the surface of macrophages and foam cells in these areas and co-localized with C3. Whether CRP in this particular region is directly involved in or triggers the plaque rupture remains to be determined.

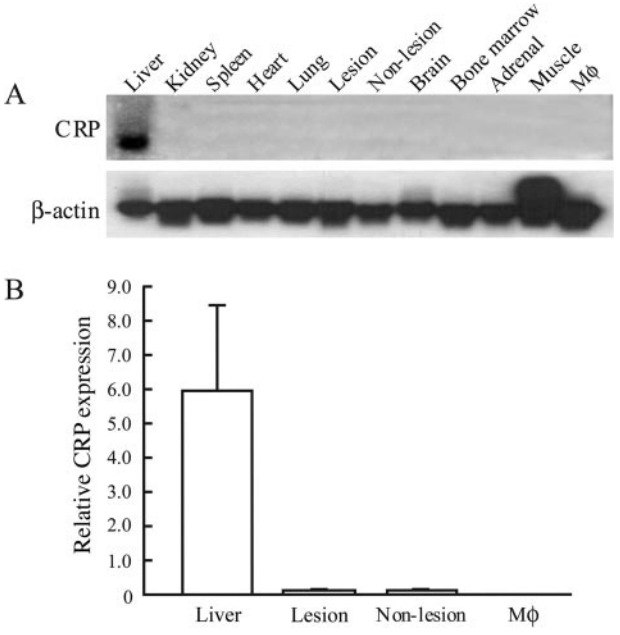

Figure 9. Northern blot (**A**) and real-time RT-PCR (**B**) analysis of CRP expression in rabbit tissues. **A:** The liver was the only organ that expresses detectable CRP by Northern blotting. **B:** Real-time RT-PCR analysis showed that compared to the liver, aortic lesions expressed very small amounts (~1/100th of the liver) of CRP mRNA whereas macrophages did not produce detectable CRP mRNA.

It should be pointed out that there are several differences between human CRP and rabbit CRP in terms of their structures and functional properties. For example, native rabbit CRP has higher affinity ability to apoB-containing lipoproteins and formed a complex whereas human CRP can only bind to apoB-containing lipoproteins when they are aggregated. Human CRP can activate human complement pathway whereas it is completely unknown whether rabbit CRP can activate rabbit complement pathway (M. Pepys, personal communication). It remains unclear whether these differences (rabbit versus human, bound CRP versus free CRP, native versus aggregated CRP) may affect their physiological functions such as in atherosclerosis. Perhaps a precise elucidation

of CRP pathological roles in atherogenesis awaits the generation of appropriate transgenic animals that can express high levels of inducible CRP in plasma or high levels of CRP specifically in lesions in the future. Recently, four studies using transgenic mice expressing either human[45–47] or rabbit[48] (K. Reifenberg, personal communication) CRP did not lead to a consistent conclusion regarding the atherogenic effect of CRP. One study showed that increased CRP moderately accelerated atherosclerosis in male apoE knockout mice[45] whereas other studies failed to demonstrate this effect. In mice, endogenous plasma CRP is not inducible and never increases more than 2 to 3 mg/L[49] and also apoE knockout mice have some defects in their complement system that cannot be activated by transgenic CRP.[48] In this regard, rabbits are apparently superior to mice because rabbit CRP levels are highly inducible and responsive during the inflammatory reaction.[31] Our separate study also showed that, as in human studies,[6,50] administration of statin in rabbits could effectively normalize plasma CRP levels and reduce the CRP levels in both liver and aortic lesions (J.F., unpublished data). This result suggests that the rabbit may be an alternative animal model to evaluate therapeutic effects of decreasing the CRP level.[51] Further studies will be required to clarify whether decreasing CRP alone without changing the plasma cholesterol level can be beneficial for the treatment of atherosclerosis. Recently, our laboratory has successfully created a line of human CRP transgenic rabbits (S.K. and J.F., unpublished data). In view of the multiple and complicated biological functions of CRP in many cardiovascular diseases and other inflammatory processes, CRP transgenic rabbits may be extremely valuable for elucidating both the pathophysiological functional roles and mechanisms of this protein in atherosclerosis, and for testing the efficacy of CRP-lowering drugs in the future.

In conclusion, our studies reported here demonstrate that there is a close relationship between the CRP plasma level and atherosclerosis in hypercholesterolemic rabbits. CRP immunoproteins were frequently present in the lesions of atherosclerosis regardless of the severity and degree of the lesions but this CRP was basically derived from the circulation rather than synthesized by vascular cells such as macrophages. In human coronary plaques, CRP was also frequently present in the ruptured fibrotic caps. In future studies, we are expecting to investigate whether elevated CRP levels are directly atherogenic or whether CRP is involved in the progression of atherosclerosis *in vivo*.

Acknowledgment

We thank T. Hachisu (Shibayagi Co.) for providing rabbit CRP ELISA kits and analyzing rabbit plasma CRP.

References

1. Pepys MB, Baltz ML: Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A protein. Adv Immunol 1983, 34:141–212
2. Black S, Kushner I, Samols D: C-reactive protein. J Biol Chem 2004, 279:48487–48490
3. Pepys MB, Hirschfield GM: C-reactive protein: a critical update. J Clin Invest 2003, 111:1805–1812
4. Taylor AW, Ku NO, Mortensen RF: Regulation of cytokine-induced human C-reactive protein production by transforming growth factor-beta. J Immunol 1990, 145:2507–2513
5. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR: Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. N Engl J Med 2002, 347:1557–1565
6. Ridker PM, Rifai N, Pfeffer MA, Sacks F, Braunwald E: Long-term effects of pravastatin on plasma concentration of C-reactive protein. The Cholesterol and Recurrent Events (CARE) Investigators. Circulation 1999, 100:230–235
7. Libby P, Ridker PM: Inflammation and atherosclerosis: role of C-reactive protein in risk assessment. Am J Med 2004, 116(Suppl 6A):9S–16S
8. Nissen SE, Tuzcu EM, Schoenhagen P, Crowe T, Sasiela WJ, Tsai J, Orazem J, Magorien RD, O'Shaughnessy C, Ganz P: Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. N Engl J Med 2005, 352:29–38
9. Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, Pfeffer MA, Braunwald E: C-reactive protein levels and outcomes after statin therapy. N Engl J Med 2005, 352:20–28
10. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, Lowe GD, Pepys MB, Gudnason V: C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. N Engl J Med 2004, 350:1387–1397
11. Labarrere CA, Zaloga GP: C-reactive protein: from innocent bystander to pivotal mediator of atherosclerosis. Am J Med 2004, 117:499–507
12. Hatanaka K, Li XA, Masuda K, Yutani C, Yamamoto A: Immunohistochemical localization of C-reactive protein-binding sites in human atherosclerotic aortic lesions by a modified streptavidin-biotin-staining method. Pathol Int 1995, 45:635–641
13. Reynolds GD, Vance RP: C-reactive protein immunohistochemical localization in normal and atherosclerotic human aortas. Arch Pathol Lab Med 1987, 111:265–269
14. Zhang YX, Cliff WJ, Schoefl GI, Higgins G: Coronary C-reactive protein distribution: its relation to development of atherosclerosis. Atherosclerosis 1999, 145:375–379
15. Ishikawa T, Imamura T, Hatakeyama K, Date H, Nagoshi T, Kawamoto R, Matsuyama A, Asada Y, Eto T: Possible contribution of C-reactive protein within coronary plaque to increasing its own plasma levels across coronary circulation. Am J Cardiol 2004, 93:611–614
16. Dong Q, Wright JR: Expression of C-reactive protein by alveolar macrophages. J Immunol 1996, 156:4815–4820
17. Kobayashi S, Inoue N, Ohashi Y, Terashima M, Matsui K, Mori T, Fujita H, Awano K, Kobayashi K, Azumi H, Ejiri J, Hirata K, Kawashima S, Hayashi Y, Yokozaki H, Itoh H, Yokoyama M: Interaction of oxidative stress and inflammatory response in coronary plaque instability: important role of C-reactive protein. Arterioscler Thromb Vasc Biol 2003, 23:1398–1404
18. Verma S, Wang CH, Li SH, Dumont AS, Fedak PW, Badiwala MV, Dhillon B, Weisel RD, Li RK, Mickle DA, Stewart DJ: A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. Circulation 2002, 106:913–919
19. Wang CH, Li SH, Weisel RD, Fedak PW, Dumont AS, Szmitko P, Li RK, Mickle DA, Verma S: C-reactive protein upregulates angiotensin type 1 receptors in vascular smooth muscle. Circulation 2003, 107:1783–1790
20. Venugopal SK, Devaraj S, Yuhanna I, Shaul P, Jialal I: Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. Circulation 2002, 106:1439–1441
21. Venugopal SK, Devaraj S, Jialal I: C-reactive protein decreases prostacyclin release from human aortic endothelial cells. Circulation 2003, 108:1676–1678
22. Zwaka TP, Hombach V, Torzewski J: C-reactive protein-mediated low density lipoprotein uptake by macrophages: implications for atherosclerosis. Circulation 2001, 103:1194–1197
23. Chang MK, Binder CJ, Torzewski M, Witztum JL: C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of

a common ligand: phosphorylcholine of oxidized phospholipids. Proc Natl Acad Sci USA 2002, 99:13043–13048
24. Torzewski M, Rist C, Mortensen RF, Zwaka TP, Bienek M, Waltenberger J, Koenig W, Schmitz G, Hombach V, Torzewski J: C-reactive protein in the arterial intima: role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis. Arterioscler Thromb Vasc Biol 2000, 20:2094–2099
25. Pasceri V, Willerson JT, Yeh ET: Direct proinflammatory effect of C-reactive protein on human endothelial cells. Circulation 2000, 102:2165–2168
26. Pasceri V, Cheng JS, Willerson JT, Yeh ET, Chang J: Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs. Circulation 2001, 103:2531–2534
27. Lafuente N, Azcutia V, Matesanz N, Cercas E, Rodriguez-Manas L, Sanchez-Ferrer CF, Peiro C: Evidence for sodium azide as an artifact mediating the modulation of inducible nitric oxide synthase by C-reactive protein. J Cardiovasc Pharmacol 2005, 45:193–196
28. Swafford Jr AN, Bratz IN, Knudson JD, Rogers PA, Timmerman JM, Tune JD, Dick GM: C-reactive protein does not relax vascular smooth muscle: effects mediated by sodium azide in commercially available preparations. Am J Physiol 2005, 288:H1786–H1795
29. van den Berg CW, Taylor KE, Lang D: C-reactive protein-induced in vitro vasorelaxation is an artefact caused by the presence of sodium azide in commercial preparations. Arterioscler Thromb Vasc Biol 2004, 24:168–171
30. Fan J, Watanabe T: Transgenic rabbits as therapeutic protein bioreactors and human disease models. Pharmacol Ther 2003, 99:261–282
31. Kushner I, Feldmann G: Control of the acute phase response. Demonstration of C-reactive protein synthesis and secretion by hepatocytes during acute inflammation in the rabbit. J Exp Med 1978, 148:466–477
32. Fan J, Shimoyamada H, Sun H, Marcovina S, Honda K, Watanabe T: Transgenic rabbits expressing human apolipoprotein(a) develop more extensive atherosclerotic lesions in response to a cholesterol-rich diet. Arterioscler Thromb Vasc Biol 2001, 21:88–94
33. Sun H, Unoki H, Wang X, Liang J, Ichikawa T, Arai Y, Shiomi M, Marcovina S, Watanabe T, Fan J: Lipoprotein(a) enhances advanced atherosclerosis and vascular calcification in WHHL transgenic rabbits expressing human apolipoprotein(a). J Biol Chem 2002, 277:47486–47492
34. Ichikawa T, Unoki H, Sun H, Shimoyamada H, Marcovina S, Shikama H, Watanabe T, Fan J: Lipoprotein(a) promotes smooth muscle cell proliferation and dedifferentiation in atherosclerotic lesions of human apo(a) transgenic rabbits. Am J Pathol 2002, 160:227–236
35. Wang X, Liang J, Koike T, Sun H, Ichikawa T, Kitajima S, Morimoto M, Shikama H, Watanabe T, Sasaguri Y, Fan J: Overexpression of human matrix metalloproteinase-12 enhances the development of inflammatory arthritis in transgenic rabbits. Am J Pathol 2004, 165:1375–1383
36. Wu L, Fan J, Matsumoto S, Watanabe T: Induction and regulation of matrix metalloproteinase-12 by cytokines and CD40 signaling in monocyte/macrophages. Biochem Biophys Res Commun 2000, 269:808–815
37. Ockene IS, Matthews CE, Rifai N, Ridker PM, Reed G, Stanek E: Variability and classification accuracy of serial high-sensitivity C-reactive protein measurements in healthy adults. Clin Chem 2001, 47:444–450
38. Yasojima K, Schwab C, McGeer EG, McGeer PL: Generation of C-reactive protein and complement components in atherosclerotic plaques. Am J Pathol 2001, 158:1039–1051
39. Bustin SA: Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000, 25:169–193
40. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 2001, 25:402–408
41. Rowe IF, Soutar AK, Trayner IM, Baltz ML, de Beer FC, Walker L, Bowyer D, Herbert J, Feinstein A, Pepys MB: Rabbit and rat C-reactive proteins bind apolipoprotein B-containing lipoproteins. J Exp Med 1984, 159:604–616
42. Rowe I, Baltz M, Soutar A, Pepys M: In vivo turnover studies of C-reactive protein. Clin Exp Immunol 1984, 58:245–252
43. Torzewski J, Torzewski M, Bowyer DE, Frohlich M, Koenig W, Waltenberger J, Fitzsimmons C, Hombach V: C-reactive protein frequently colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. Arterioscler Thromb Vasc Biol 1998, 18:1386–1392
44. Bhakdi S, Torzewski M, Klouche M, Hemmes M: Complement and atherogenesis: binding of CRP to degraded, nonoxidized LDL enhances complement activation. Arterioscler Thromb Vasc Biol 1999, 19:2348–2354
45. Paul A, Ko KW, Li L, Yechoor V, McCrory MA, Szalai AJ, Chan L: C-reactive protein accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. Circulation 2004, 109:647–655
46. Hirschfield GM, Gallimore JR, Kahan MC, Hutchinson WL, Sabin CA, Benson GM, Dhillon AP, Tennent GA, Pepys MB: Transgenic human C-reactive protein is not proatherogenic in apolipoprotein E-deficient mice, Proc Natl Acad Sci USA 2005, 102:8309–8314
47. Trion A, de Maat MP, Jukema JW, van der Laarse A, Maas MC, Offerman EH, Havekes LM, Szalai AJ, Princen HM, Emeis JJ: No effect of C-reactive protein on early atherosclerosis development in apolipoprotein E*3-leiden/human C-reactive protein transgenic mice, Arterioscler Thromb Vasc Biol 2005, 25:1635–1640
48. Reifenberg K, Lehr HA, Baskal D, Wiese E, Schaefer SC, Black S, Samols D, Torzewski M, Lackner KJ, Husmann M, Blettner M, Bhakdi S: Role of C-reactive protein in atherogenesis: can the apolipoprotein E knockout mouse provide the answer? Arterioscler Thromb Vasc Biol 2005, 25:1641–1646
49. Pepys MB: Isolation of serum amyloid P-component (protein SAP) in the mouse. Immunology 1979, 37:637–641
50. Plenge JK, Hernandez TL, Weil KM, Poirier P, Grunwald GK, Marcovina SM, Eckel RH: Simvastatin lowers C-reactive protein within 14 days: an effect independent of low-density lipoprotein cholesterol reduction. Circulation 2002, 106:1447–1452
51. Ivashchenko Y, Kramer F, Schafer S, Bucher A, Veit K, Hombach V, Busch A, Ritzeler O, Dedio J, Torzewski J: Protein kinase C pathway is involved in transcriptional regulation of C-reactive protein synthesis in human hepatocytes. Arterioscler Thromb Vasc Biol 2005, 25:186–192