Vascular Medicine

Human C-Reactive Protein Does Not Promote Atherosclerosis in Transgenic Rabbits

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Background—Although there is a statistically significant association between modestly raised baseline plasma C-reactive protein (CRP) values and future cardiovascular events, the debate is still unsettled in regard to whether CRP plays a causal role in the pathogenesis of atherosclerosis.

Methods and Results—We generated 2 lines of transgenic (Tg) rabbits expressing human CRP (hCRP). The plasma levels of hCRP in hCRP-Tg-1 and hCRP-Tg-2 rabbits were 0.4±0.13 (n=14) and 57.8±20.6 mg/L (n=12), respectively. In addition, hCRP isolated from Tg rabbit plasma exhibited the ability to activate the rabbit complement. To define the role of hCRP in atherosclerosis, we compared the susceptibility of hCRP-Tg rabbits to cholesterol-rich diet-induced aortic and coronary atherosclerosis with that of non-Tg rabbits. After being fed with a cholesterol-rich diet for 16 weeks, Tg and non-Tg rabbits developed similar hypercholesterolemia and lesion sizes in both aortic and coronary arteries. Immunohistochemical staining and Western blotting revealed that hCRP was indeed present in the lesions but did not affect macrophage accumulation and smooth muscle cell proliferation of the lesions.

Conclusions—Neither high nor low plasma concentrations of hCRP affected aortic or coronary atherosclerosis lesion formation in hCRP-Tg rabbits. (Circulation. 2009;120:2088-2094.)

Key Words: atherosclerosis ■ cardiovascular disease ■ coronary disease ■ pathology ■ risk factors

There have been many controversial and contradictory results published on the effects of C-reactive protein (CRP), and a very active debate continues about its role in the pathogenesis of cardiovascular disease (CVD). Despite the clinical importance of CRP as a potential marker of increased risk of CVD, the lack of an appropriate animal model has made it difficult to determine whether CRP is merely a marker or is an active mediator in the progression of CVD. Several lines of evidence have revealed that CRP may modulate vascular function, thereby directly participating in the pathogenesis of atherosclerosis. This notion has been suggested by the pathological demonstration of CRP in atherosclerotic lesions and the finding that CRP causes a number of biological changes in endothelial cells, smooth muscle cells, and macrophages in vitro that are considered to promote lesion progression. In addition, the recent 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 relatively high baseline plasma CRP levels (≥2 mg/L). These studies thus far have raised concerns in regard to whether we should develop CRP-lowering therapies for reducing CVD or whether we should aggressively treat those CVD patients with high levels of CRP in both primary and secondary prevention stages in the same way used to treat hyperlipidemia.

Unfortunately, the critical issue of whether high levels of CRP are indeed atherogenic remains unresolved. Many
researchers have attempted to address this issue using transgenic (Tg) mice expressing either human CRP (hCRP) or rabbit CRP, but the results thus far are quite controversial and contradictory: CRP is either proatherogenic,13,14 has no effect on atherosclerosis,15–19 or is even atheroprotective.20 Although the cause of these discrepancies is unclear, it appears that the mouse is not an appropriate model for evaluation of CRP because plasma levels of CRP in mice, even in the presence of inflammatory stimuli, are extremely low compared with humans and rabbits.21 Furthermore, hCRP and rabbit CRP cannot activate complement in the mouse.17 Given the limitations of the CRP Tg mouse models, it is imperative to develop CRP Tg rabbits as an alternative model for the study of CRP in vivo. Rabbits have been used as an excellent model for human atherosclerosis because their lipoprotein metabolism and cardiovascular system are similar to those of humans.22 In addition, the acute-phase reactant CRP response of rabbits resembles that of humans,23 and rabbit CRP and hCRP have similar characteristics in structure and function.24 Furthermore, we have demonstrated that the severity of atherosclerosis is also closely associated with plasma CRP levels in cholesterol-fed and Watanabe heritable hyperlipidemic rabbits.9 In the present study, we have successfully generated 2 lines of hCRP Tg rabbits and compared the susceptibility of Tg rabbits to cholesterol-rich diet-induced aortic and coronary atherosclerosis with that of non-Tg rabbits.

**Methods**

**Generation of Tg Rabbits**

Tg rabbits were generated by the methods described previously.22 In this study, Japanese White rabbits (std:JW/CSK) were purchased from SLC, Inc (Shizuoka, Japan), and zygotes were microinjected with a DNA construct consisting of 1.13 kb hCRP complementary DNA under the control of liver-specific expression elements from the human apolipoprotein E gene25 with 4 copies of the chicken β-globin insulator (INS). Northern blotting was performed to examine the hCRP transgene expression in hCRP-Tg-2 rabbits (B). Hematoxylin-eosin (HE) staining (left) and immunohistochemical staining of the liver with the use of mAb against hCRP (right) are shown in C.

**Western Blotting and Complement Consumption Assay**

hCRP concentrations in the plasma of Tg rabbits were measured by latex agglutination with the use of an automatic analyzer (JCA-BM2250, JEOL, Tokyo, Japan). Two founder Tg rabbits expressed hCRP in the plasma at levels of 0.8 mg/L (designated as Tg-1) and 50 mg/L (designated as Tg-2). To analyze hCRP proteins in Tg rabbit plasma, we subjected the plasma to electrophoresis on a 4% to 12% nondenaturing polyacrylamide gradient gel without sodium dodecyl sulfate (SDS)28 and also on 10% SDS–polyacrylamide gels (SDS-PAGE), followed by immunoblotting with hCRP monoclonal antibody (mAb). To investigate whether hCRP produced by Tg rabbits was physiologically functional, we isolated hCRP from Tg-2 rabbit plasma using an affinity column with rabbit mAb against hCRP (Epitomics Inc, Burlingame, Calif) and 0.1 mol/L glycine-HCl (pH 2.5) as elution buffer. As described previously,17 a complement consumption assay was conducted with the use of enzymatically modified human low-density lipoprotein (E-LDL) as a CRP ligand. E-LDL concentrations (400 to 800 μg/mL) were adjusted in accor-
dance with the rabbit sera so that a background consumption of ≈50% was achieved without CRP. The complement consumption induced by E-LDL alone was used as a control and expressed as 100%. Total consumption of CRP from different sources (normal rabbit, human, and Tg-2 rabbit) was compared with that of the controls.

Analysis of Blood and Plasma Biochemistry
To exclude the possibility that expression of hCRP may have any adverse effects on rabbit health, blood cells were analyzed with the use of an automated hematology analyzer (Sysmex XE-2100, Sysmex Co, Kobe, Japan), and plasma biochemistry was measured with the use of an autoanalyzer (JCA-BM2250, JEOL, Tokyo, Japan).

Cholesterol-Rich Diet Experiments
To investigate the effect of hCRP on the development of atherosclerosis, male Tg rabbits (4 to 5 months) and sex- and age-matched non-Tg littermates were fed a diet containing 0.3% cholesterol and 3% soybean oil for 16 weeks. To minimize the variations of plasma cholesterol concentrations in cholesterol-fed rabbits, we measured plasma lipids biweekly and adjusted the cholesterol content of the diet according to the changes in plasma cholesterol of each animal. Hypercholesterolemia of both Tg and non-Tg rabbits was induced and maintained at “atherogenic levels” (600 to 1200 mg/dL) throughout the experiment (see below). The animals were fed ad libitum, and plasma levels of total cholesterol, triglycerides, and high-density lipoprotein cholesterol were measured with the use of Wako assay kits (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Plasma levels of hCRP were measured before and after cholesterol diet feeding for 16 weeks. For the analysis of lipoprotein profiles and apolipoproteins, plasma lipoproteins from rabbits at 8 and 16 weeks of cholesterol diet feeding were isolated by sequential ultracentrifugation and analyzed as described previously.29

Quantification of Aortic and Coronary Atherosclerosis
At the end of the cholesterol diet feeding, all rabbits were euthanized by injection of an overdose of sodium pentobarbital solution. The aortas were en face stained with Sudan IV for evaluation of gross atherosclerotic lesions as described previously.30 For microscopic quantification of lesion areas, each portion of the aorta was dehy-

Figure 3. Western blotting analysis and complement activation assay. Tg hCRP was isolated from Tg-2 rabbit plasma as described in Methods and analyzed by either nondenaturing gel (top) or SDS-PAGE (bottom) and immunoblotted with hCRP mAb (left). Isolated Tg hCRP from Tg rabbits exhibited the same ability to augment activation of rabbit serum complement by E-LDL as native rabbit and hCRP (right).

Figure 4. hCRP-Tg rabbits developed hypercholesterolemia similar to that of non-Tg rabbits during cholesterol diet feeding (A, left). The values are expressed as mean±SE. HDL-C indicates high-density lipoprotein cholesterol. Representative photographs of pinned-out aortic trees stained with Sudan IV from each group are shown (B, left), and aortic atherosclerotic lesions (defined by sudanophilic area) on the surface were quantified by an image analysis system (B, right). The values are expressed as mean±SD. P<0.05, P=0.36, P=0.52, and P=0.49 in arch, thoracic, abdominal, and whole aorta, respectively, by ANOVA. Because P<0.05 by ANOVA was noted in arch, we further analyzed these data by Scheffe F test and found that P=0.38 (non-Tg vs Tg-1) and P=0.35 (non-Tg vs Tg-2). Therefore, there was no statistical difference between Tg and non-Tg rabbits in all parts of the aorta.
drated in ethanol and embedded in paraffin (10 segments for the aortic arch and abdominal aorta and 20 for the thoracic aorta). All specimens were cut into 3-μm-thick sections and stained with hematoxylin and eosin and elastica van Gieson. For microscopic evaluation of the cellular components of the lesions, serial paraffin-embedded sections of the aorta were immunohistochemically stained with mAbs against macrophages (RAM11) and α-smooth muscle actin (HHF35), as shown in Table I in the online-only Data Supplement, and visualized with Histofine Simple Stain MAX-PO kits (Nichirei Biosciences Inc, Tokyo, Japan). To assess coronary atherosclerosis, rabbit hearts were sectioned into 7 blocks, and the lesions of the left and right coronary arteries were quantified under a light microscope and expressed as the stenosis (%) of the lumen area [(lesion area/(total lumen area)) × 100%] by the method described previously. All measurements were performed blindly and independently by 2 separate researchers. To detect CRPs in lesions, immunohistochemical staining was performed with the use of Abs against hCRP and rabbit CRP. We first evaluated the reactivity of 2 Abs against denatured proteins by SDS-PAGE followed by Western blotting and found that hCRP mAb showed slight cross-reactivity with rabbit plasma CRP, whereas rabbit CRP polyclonal Ab cross-reacted with hCRP (Figure I in the online-only Data Supplement). For native CRP (though fixed) in the lesions, hCRP mAb showed slight cross-reactivity with rabbit CRP (see below). Because this cross-reactivity was faint and not often present compared with the reactivity of rabbit CRP Ab in the same section, we could evaluate the hCRP deposition in the lesions by immunohistochemical staining. For negative controls, primary Abs were replaced by either nonspecific mouse immunoglobulin G or chicken immunoglobulin Y. In addition, the lesions of aortas were homogenized, and proteins (10 μg) were run on SDS-PAGE followed by immunoblotting with hCRP mAb.

### Statistical Analysis
ANOVA was used to assess differences between 3 groups of gross aortic lesions and plasma biochemistry. Two-factor repeated-measures ANOVA was used for the time-course data of plasma lipids after a cholesterol-rich diet. One-way ANOVA with the Scheffé F test or Kruskal-Wallis test was used for parametric and nonparametric analysis. Microscopic analyses of aortic lesions, coronary arterial lesions, and plasma lipoproteins between 2 groups were compared by Student’s t test or Mann–Whitney U test depending on the data distribution. In all cases, statistical significance was set at \( P < 0.05 \).

### Results

#### Characterization of Tg Rabbits
We generated 2 lines of Tg rabbits expressing different levels of plasma hCRP. Average plasma levels of hCRP in F1 Tg-1 and Tg-2 rabbits at 3 to 4 months were 0.4 ± 0.13 (\( n = 14 \)) and 57.8 ± 20.6 mg/L (\( n = 12 \)), respectively (Figure II in the online-only Data Supplement). The hCRP transcripts were expressed almost exclusively in the liver of Tg rabbits (Figure II). Histological examination revealed no abnormalities in the liver of Tg rabbits, and hCRP-immunoreactive proteins were immunohistochemically detected only in hepatocytes but not in blood vessels or bile ducts (Figure 1C).
Western blotting analysis revealed that hCRP in the plasma of Tg rabbits was present as a complex with a high molecular weight (pentamer) on non-denaturing gels and a monomer on SDS-PAGE (with an approximate molecular weight of 26 kDa) (Figure 2). To investigate whether hCRP produced by Tg rabbits was physiologically functional, we conducted a complement consumption assay using E-LDL as a CRP ligand. We found that isolated hCRP from Tg-2 rabbits exhibited the same ability to augment activation of rabbit serum complement in the presence of E-LDL as native rabbit and hCRP (Figure 3).

Cholesterol-Rich Diet Experiments
To investigate the effect of hCRP on the development of atherosclerosis, male Tg rabbits and non-Tg littermates were fed a cholesterol-rich diet for 16 weeks. Both Tg and non-Tg rabbits developed similar hypercholesterolemia during the experimental period, and lipoprotein profiles were identical (Figure 4A and Figure III in the online-only Data Supplement). Plasma hCRP levels of Tg-2 rabbits remained as “high” as those of a normal chow diet–fed rabbits during the cholesterol diet, whereas plasma hCRP levels of Tg-1 rabbits were constantly “low” (0.4 to 5 mg/L) (Figure II in the online-only Data Supplement). Expression of hCRP did not lead to obvious changes in the variables of blood and plasma in both Tg rabbits and non-Tg rabbits during the experiment (Table II in the online-only Data Supplement).

Quantification of Aortic and Coronary Atherosclerosis
At the end of the experiment, all rabbits were euthanized, and the severity of aortic and coronary atherosclerosis was examined and quantified with the use of an image analysis system. Compared with non-Tg control rabbits, neither of the Tg rabbit lines showed any statistical differences (P=0.5 versus non-Tg by ANOVA) in aortic atherosclerotic lesions defined by Sudan IV staining (Figure 4B). Because plasma levels of hCRP in both lines of Tg rabbits were quite variable at 16 weeks (Figure II in the online-only Data Supplement), we also evaluated the correlation between plasma hCRP and the extent of aortic lesions of each Tg rabbit. However, we did not find any correlations between plasma hCRP and aortic lesions in all Tg rabbits (data not shown). We further examined sections of the lesions under a light microscope and quantified the microscopic lesion areas. However, we did not find any differences in lesion sizes or cellular components (macrophages and smooth muscle cells) between Tg-2 and non-Tg rabbits (Figure 5). To confirm the presence of CRP in lesions, we performed immunohistochemical staining using Abs against either human or rabbit CRP and showed that hCRP-immunoreactive proteins were regularly detected in atherosclerotic lesions of Tg rabbits, whereas rabbit CRP was present in both Tg and non-Tg rabbit lesions (Figure 6, top, and Figure IV in the online-only Data Supplement). Western blotting analysis of the aortic lesions confirmed that the CRP contents were markedly increased in the lesions of Tg rabbits compared with non-Tg rabbits (Figure 6, bottom). Finally, we examined the effect of hCRP on coronary arterial lesions. As shown in Figure 7, the coronary stenosis of Tg-2 rabbits was not statistically different from that of non-Tg rabbits (P=0.33 in left and P=0.64 in right coronary artery versus non-Tg) even though CRP-immunoreactive proteins were detected in the lesions. Taken together, hCRP does not affect the development of atherosclerosis in Tg rabbits, which is supported by 2 recent human genetic studies.

Discussion
For the first time, we have successfully generated 2 lines of hCRP Tg rabbits to define the role of CRP in atherosclerosis. CRP is a highly controversial marker of CVD. The rabbit model was selected for this undertaking because of its usefulness in studying both the development of atherosclerosis and CRP pathophysiological functions.

We found that hCRP isolated from Tg rabbit plasma exhibited the ability to activate the rabbit complement in the presence of E-LDL, confirming that hCRP of Tg rabbits is functional in vivo. Expression of hCRP in Tg rabbits did not lead to any health problems because we did not find any pathological abnormalities, and hematologic and biochemical data of blood were unchanged compared with those of non-Tg rabbits. Spontaneously atherosclerotic lesions were not detected in both lines of hCRP-Tg rabbits on a chow diet for up to 1 year (data not shown). Therefore, we administered a cholesterol-rich diet for 16 weeks, a method that has been used in many studies for investigating the interactions between different genes and the development of atherosclerosis in rabbits. Plasma total choles-
terol levels and lipoprotein profiles of Tg rabbits were basically similar to those of non-Tg rabbits. Taken together, we have established hCRP-Tg rabbits that allow us to investigate the direct effects of plasma hCRP on the development of atherosclerosis.

As illustrated by our analysis, average plasma levels of hCRP-Tg-2 rabbits are above the risk levels (3 to 10 mg/L) generally proposed in humans. Plasma hCRP levels of Tg-1 rabbits were initially <1 mg/L but increased to 4.97±4.63 mg/L at 16 weeks of the cholesterol diet. Regardless of hCRP expression in Tg rabbits, both lines of Tg rabbits did not show any enhancement of either lesion size or any changes in the cellular components (macrophages and smooth muscle cells) of lesions. Immunohistochemical staining coupled with Western blotting revealed that hCRP-immunoreactive proteins were indeed present in lesions. Because hCRP, like endogenous rabbit CRP, is expressed exclusively by the liver but not by aorta or macrophages in Tg rabbits, we considered that CRP in the lesions was essentially derived from the circulation rather than synthesized de novo by vascular cells. Despite this observation, both aortic and coronary atherosclerotic lesions were not significantly changed in Tg rabbits compared with non-Tg rabbits, suggesting that hCRP at these levels is not proatherogenic in Tg rabbits. In past years, many studies attempted to demonstrate the atherogenic effect of CRP in genetically modified mice, but the results thus far are controversial. It is apparent that our results obtained from 2 lines of Tg rabbits expressing different plasma levels of hCRP rebut the notion that CRP is proatherogenic. Our data are also in support of the recent study showing that genetically elevated CRP does not play a causal role in ischemic vascular disease. Nevertheless, we cannot exclude the possibility that hCRP may have a pathophysiological role in aspects of CVD that are not modeled in the present study, such as myocardial infarction and thrombosis. It also remains to be determined whether local CRP present in the arterial wall is involved in plaque rupture. Cross-breeding hCRP Tg rabbits with Watanabe heritable hyperlipidemic myocardial infarction rabbits that develop spontaneous atherosclerosis in both aorta and coronary arteries as well as myocardial infarction will certainly provide a powerful model to examine these hypotheses in the future.

In summary, the present study does not support a direct role of hCRP in the pathogenesis of atherosclerosis in hCRP-Tg rabbits, suggesting that CRP is a marker rather than a maker in the development of atherosclerosis.

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Disclosures
None.

References
Despite the clinical importance of C-reactive protein (CRP) as a potential marker for cardiovascular diseases, the lack of an appropriate animal model has made it difficult to determine whether CRP is merely a marker or is an active mediator in the pathogenesis of atherosclerosis. In past years, studies with the use of transgenic mice expressing either human or rabbit CRP have generated quite controversial and contradictory results. In fact, mice are not appropriate for evaluation of CRP pathophysiology because CRP in mice is not functional in terms of complement activation. In the present study, we have generated novel transgenic rabbits expressing human CRP and documented that human CRP does not affect aortic or coronary atherosclerosis lesion formation in apolipoprotein E-deficient mice. Taken together, our data suggest that CRP may not be a contributor of human atherosclerosis.
## Supplemental Material

### Supplemental Tables

**S-Table 1. Antibodies used for the immunohistochemical staining**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Working dilution</th>
<th>Species</th>
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<td>Human CRP</td>
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<td>Chicken</td>
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<td>RAM11</td>
<td>x 400</td>
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<td>Dako Japan Inc., Tokyo, Japan</td>
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<td>HHF35</td>
<td>x 300</td>
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<td>Enzo Biochemicals, NY</td>
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### S-Table 2. Blood and biochemical analysis of cholesterol-fed Tg and non-Tg rabbits

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<th>Variables examined</th>
<th>non-Tg (n=13)</th>
<th>hCRP-Tg-1 (n=13)</th>
<th>hCRP-Tg-2 (n=5)</th>
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<td>WBC (10^3/µL)</td>
<td>113 ± 11</td>
<td>102 ± 5</td>
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<td>RBC (10^4/µL)</td>
<td>447 ± 44</td>
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<td>HGB (g/dL)</td>
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<td>PLT (10^3/µL)</td>
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<td>281 ± 27</td>
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<td>NEUT (%)</td>
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<td>Alb (g/dL)</td>
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<td>ALP (IU/L)</td>
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<td>LAP (IU/L)</td>
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<td>CK (IU/L)</td>
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<td>545 ± 60</td>
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<td>Amylase (IU/L)</td>
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<td>Glu (mg/dL)</td>
<td>99 ± 2</td>
<td>102 ± 2</td>
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WBC, white blood cells, RBC, red blood cells, HGB, hemoglobin, HCT, hematocrit, PLT, platelets, NEUT, neutrophils, LYMPH, lymphocytes, MONO, monocytes, EO, eosinophils, BASO, basophils, TP, total protein, Alb, albumin, ALP, alkaline phosphatase, LAP, leucine aminopeptidase, γ-GT, γ-glutamyl transferase, LDH, Lactate dehydrogenase, AST, aspartate aminotransferase, ALT, alanine aminotransferase, Cre, creatinine, Glu, glucose. The blood was collected from rabbits fed a cholesterol diet for 16 weeks. Rabbits were fasted for 16h before bleeding. Data are expressed as means ± SE. **P<0.01, *P<0.05 vs. non-Tg by ANOVA with Sheffe’s F test.
Supplemental Figures

S-Fig. 1
S-Fig. 2
S-Fig. 3
S-Fig. 4
Legends for Supplemental Figures

S-Figure 1. Comparison of antibodies against human and rabbit CRP by immunoblotting analysis
Plasma (1 µl) obtained from a healthy volunteer human, non-Tg, hCRP-Tg-1 and Tg-2 rabbits were electrophoresed by 10% SDS-PAGE and immunoblotted with hCRP mAb and rCRP polyclonal Ab as described in Methods. Note that human CRP mAb cross-reacted slightly with rabbit plasma CRP whereas rabbit polyclonal Ab showed cross-reactivity with human plasma CRP.

S-Figure 2. Plasma levels of hCRP of two lines of Tg rabbits either fed a chow diet or cholesterol (CHO) diet for 16 weeks.

S-Figure 3. Lipoprotein profiles and apolipoprotein distribution of Tg and non-Tg rabbits at 16 weeks of cholesterol diet feeding. Density gradient fractions were isolated from fasting rabbit plasma by ultracentrifugation, and total cholesterol and triglyceride levels were measured as described previously1 (top). The combined recovery of cholesterol from each animal averaged ~80% of the total plasma level. Data are expressed as mean ± SE (n=4 for each group). Lipoproteins were resolved by electrophoresis in a 1% agarose gel and visualized with Fat Red 7B staining, and apolipoproteins were detected by immunoblotting with specific Abs against apo-B, apo-E, and apo-AI (bottom).

S-Figure 4. Detection of CRP immnoreactive proteins in the aortic lesions
Micrographs taken at lower magnification x4 show the both human and rabbit CRP immunoreactive proteins by immunohistochemical staining. Human CRP mAb showed slight cross-reactivity with rabbit endogenous CRP.

Supplemental Reference