

Increased Thrombosis After Arterial Injury in Human C-Reactive Protein–Transgenic Mice

Haim D. Danenberg, MD; Alexander J. Szalai, PhD; Rajesh V. Swaminathan, BSc; Lin Peng, MD; Zhiping Chen, MS; Philip Seifert, MS; William P. Fay, MD; Daniel I. Simon, MD; Elazer R. Edelman, MD, PhD

Background—C-reactive protein (CRP), an acute-phase reactant long considered merely an innocent bystander in the inflammatory process, is now recognized as a powerful predictor of cardiovascular events. Emerging in vitro evidence suggests that CRP may have direct proinflammatory and prothrombotic effects on monocytes and endothelial cells. To determine whether CRP directly modulates vascular cell function in vivo, we subjected wild-type mice, which do not express CRP, and human CRP–transgenic (CRPtg) mice to 2 models of arterial injury.

Methods and Results—Baseline serum CRP levels in CRPtg mice were 18 ± 6 mg/L. CRP levels were undetectable in wild-type mice. Transluminal wire injury led to complete thrombotic occlusion of the femoral artery at 28 days in 75% of CRPtg arteries (6 of 8) compared with 17% (2 of 12) in wild-type mice ($P < 0.05$). In a model of arterial photochemical injury, clot formation time was shortened in CRPtg mice; mean time to occlusion was 33 ± 19 minutes compared with 59 ± 19 minutes in wild-type mice ($n = 10$; $P < 0.05$).

Conclusions—Arterial injury in CRPtg mice results in an expedited and higher rate of thrombotic occlusion. This is the first report of a prothrombotic phenotype directly attributable to the presence of human CRP in vivo. Investigation of the inflammatory-thrombotic axis in CRPtg mice may elucidate the prothrombotic actions of CRP in unstable arterial diseases and may pave the way for novel therapeutic interventions for preventing cardiovascular events. (*Circulation*. 2003;108:512-515.)

Key Words: inflammation ■ risk factors ■ thrombosis ■ heart disease

Inflammation plays a pivotal role in vascular injury and repair. Accordingly, the inflammatory marker C-reactive protein (CRP) has emerged as a powerful predictor for cardiovascular disease, associated with future cardiovascular events in seemingly healthy subjects and with worse prognosis in acute coronary patients.^{1–3} Long considered an innocent bystander in the atherosclerotic process, new evidence suggests that CRP might have direct proinflammatory effects on cardiovascular cells.^{4–6} However, direct evidence for a biological contribution of CRP to atherosclerosis in particular and the pathogenesis of cardiovascular disease in general remains elusive.

Mouse CRP circulates only in trace amounts, and its blood level does not change appreciably during inflammation.⁷ Transgenic mice that express human CRP^{7,8} might then serve as a model in which to examine the impact of human CRP on vascular repair in vivo. The response to injury of CRP-transgenic (CRPtg) mice was quantitatively and qualitatively different from that of wild-type mice. CRPtg mice experienced much faster and higher rates of complete thrombotic

occlusion than their wild-type counterparts. These findings support the notion that the role of CRP in vascular injury and repair is active and direct, and they might explain the association between inflammation and thrombosis in unstable coronary syndromes.

Methods

CRPtg Mice

The C57BL/6 congenic CRPtg mice used in this study have been described previously.^{7,8} CRPtg mice carry a 31-kb *Clal* fragment of human genomic DNA consisting of the *CRP* gene, 17 kb of 5'-flanking sequence, and 11.3 kb of 3'-flanking sequence.⁷

Wire Injury Model

Animal care and surgical procedures were in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and the National Institutes of Health. Male CRPtg mice and age-matched congenic wild-type mice underwent bilateral wire injury of the femoral artery as described previously.⁹ A longitudinal groin incision exposed the femoral vessels, and under surgical microscopic visualization (Carl Zeiss) the distal portion of the femoral artery was encircled with an 8-0 nylon suture. A vascular

Received January 21, 2003; de novo received May 30, 2003; accepted June 16, 2003.

From the Harvard–MIT Division of Health Sciences and Technology, Cambridge, Mass (H.D.D., R.V.S., P.S., D.I.S., E.R.E.); Department of Cardiology, Hadassah University Hospital, Jerusalem, Israel (H.D.D.); Cardiovascular Division, Brigham and Women's Hospital, Boston, Mass (D.I.S., Z.C., E.R.E.); Division of Clinical Immunology and Rheumatology, The University of Alabama at Birmingham (A.J.S.); and Department of Internal Medicine, University of Michigan Medical School, Ann Arbor (W.P.F., L.P.).

Correspondence to Haim Danenberg, MIT, Bldg 56-322, 77 Massachusetts Ave, Cambridge, MA 02139. E-mail danen@mit.edu

© 2003 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000085568.13915.1E

Blood Counts and CRP Levels in Wild-Type and CRPtg mice

	Wild-Type Mice (n=4)	CRPtg Mice (n=4)	P
Hemoglobin, g/dL	13.8±1.8	14.7±0.3	NS
White blood cell count, ×10 ³ /μL	5.9±1.1	8.4±1.8	0.05
Platelet count, ×10 ³ /μL	850±90	972±80	NS
CRP at baseline, μg/mL	ND	18±6	<0.0001
CRP at 24 h, μg/mL	ND	56±5	<0.0001

ND indicates not detectable; NS, not significant.

clamp was placed proximally at the level of the inguinal ligament, and an angioplasty guidewire (0.010 inch in diameter; Advanced Cardiovascular Systems) was introduced into the arterial lumen through an arteriotomy made just distal to the suture. After release of the clamp, the guidewire was advanced to the level of the aortic bifurcation and pulled back. Guidewire advance and retraction was repeated thrice. The guidewire was then removed and the arteriotomy site ligated. Femoral arteries were harvested for morphometry 28 days after injury, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 3 segments. Sections (5 μm thick) of each segment were obtained for staining (Verhoeff) or immunohistochemistry. Standard avidin-biotin procedures for smooth muscle cell (SMC) α-actin (DAKO Co) were used for immunohistochemistry. For morphometric analysis, microscopic images were digitized, and the amounts of media and neointima were quantified using the Adobe Photoshop 5.0 software.

Photochemical Thrombosis Model

CRPtg and wild-type mice were subjected to arterial injury by photochemical reaction, performed by an operator blinded to the genotype of mouse, as described previously.¹⁰ The left common carotid artery was isolated, and a vascular flow probe (Transonic Systems) was applied to monitor blood flow. Rose bengal (Fisher Scientific Co) at a concentration of 10 mg/mL in phosphate-buffered saline was injected into the tail vein to administer a dose of 50 mg/kg. The mid portion of the common carotid artery was then illuminated with a 1.5-mW green light laser (540 nm; Melles Griot Inc) until an occlusive thrombus was formed. The time required to form an occlusive thrombus, defined as absence of blood flow for 3 minutes or more, was recorded.

Measurement of Human CRP Levels

Mice were bled at 24 hours after wire injury. Serum human CRP levels were measured in duplicate by a commercial ELISA kit with a lower detection level of 1 μg/mL (Immuno-Biological Laboratories).

Statistics

Data are expressed as mean±SD. Fisher’s exact test was used to determine statistical significance of dichotomous variables (binary arterial occlusion). Comparisons of occlusion time and histological findings were done using the unpaired 2-tailed Student’s *t* test. Differences were considered statistically significant at *P*<0.05.

Results

Wire Injury Model

Serum CRP levels in CRPtg mice were 18±6 μg/mL and increased to 56±5 μg/mL 24 hours after surgery. Serum CRP levels were undetectable in wild-type mice throughout the experiment (Table). Hemoglobin levels and platelet counts of CRPtg mice were not significantly different from those of wild-type mice (Table). The leukocyte counts of CRPtg mice remained within the normal range but were slightly elevated and statistically significantly different from counts in control

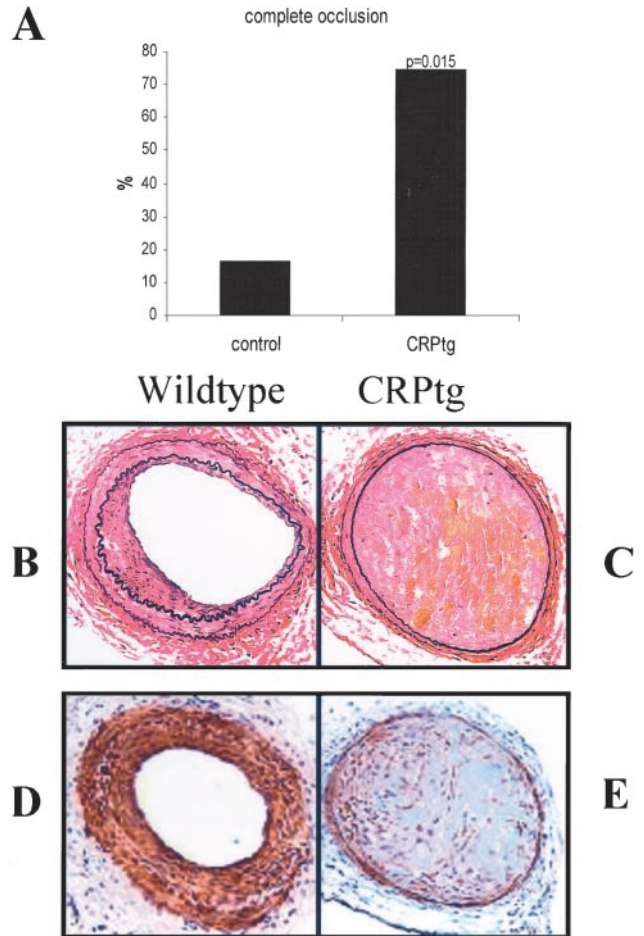


Figure 1. A, Complete arterial occlusion at 28 days after femoral wire injury in CRPtg (n=8) and wild-type mice (n=12). B through E, Photomicrographs of wild-type (left) and CRPtg (right) femoral arteries stained by Verhoeff elastin stain (B and C) and α-actin (D and E). Note the reduced SMC content in the intraluminal lesion of the CRPtg mouse.

animals. The rate of complete arterial occlusion observed after injury was significantly higher in CRPtg mice (75%; 6 of 8 arteries) compared with wild-type mice (17%; 2 of 12 arteries) (*P*=0.015) (Figure 1A). Occlusive lesions in CRPtg arteries (Figure 1C) were typically less cellular than those of wild-type arteries (Figure 1B). Cell number within the internal elastic lamina (per 10 000 μm²) was 54.6±13 and 33.8±16 in the wild-type and CRPtg arteries, respectively (*P*=0.01). Neointima/media ratio at 4 weeks in wild-type mice was 1.32±0.69 (n=10) but could not be analyzed in CRPtg mice given their high rate of complete thrombotic occlusion. Immunostaining for α-actin revealed 3-fold lower SMC content in the intraluminal lesions of CRPtg mice (22±19% of intraluminal lesion area; Figure 1E) than in wild-type mice (58±19%; Figure 1D).

Photochemical Thrombosis Model

To elucidate the effect of CRP on the development of arterial thrombosis in real time, carotid arteries of CRPtg mice and their wild-type littermates were subjected to photochemical injury, and blood flow was monitored. Mean time to occlu-

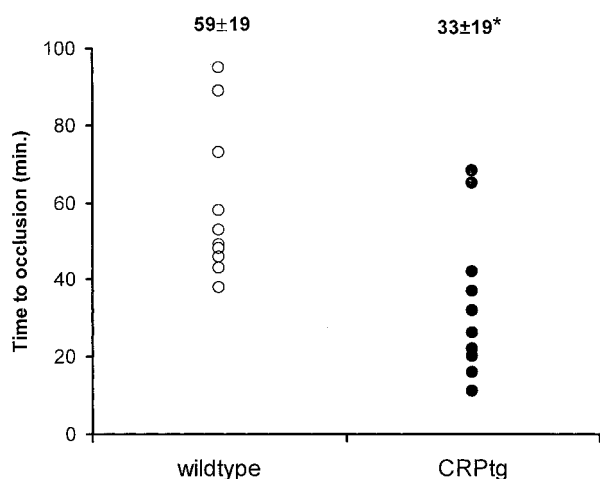


Figure 2. Time to cessation of arterial blood flow (occlusion) after photochemical injury in CRPtg and wild-type mice (n=10). * $P<0.05$.

sive thrombus formation in wild-type mice was 59 ± 19 minutes, compared with 33 ± 19 minutes in CRPtg mice (n=10 per group; $P<0.05$) (Figure 2). This increase confirms and validates the facilitation of thrombosis in CRPtg mice.

Discussion

High serum CRP levels are predictive of acute cardiovascular events in seemingly healthy individuals. However, the pathogenic importance of CRP remains undetermined. The present study describes the first in vivo experiments of vascular injury in CRPtg mice and clearly demonstrates a significantly faster and higher rate of arterial thrombosis in mice producing human CRP. These findings, which were verified here in 2 different injury models, indicate a cause-and-effect relationship for CRP in vascular thrombosis and occlusion.

Unlike human CRP, mouse CRP is not an acute-phase reactant, and it is synthesized in only trace amounts.⁷ However, male CRPtg mice constitutively produce human CRP, with serum levels ranging between 10 and 20 $\mu\text{g}/\text{mL}$ —levels that are comparable to or eclipse those considered to indicate high risk in humans.¹¹ Consequently, CRPtg mice provide an ideal model for studying the biological activities of human CRP in vivo.

The transluminal femoral wire injury model serves as a reliable model for studying the molecular mechanisms involved in the arterial wall response to injury.^{9,12,13} Complete thrombosis of the artery is normally infrequent, occurring in only 10% to 15% of cases.¹² In the present study, the thrombosis rate in wild-type arteries was 17%, and the neointima/media ratio at 4 weeks was 1.32 ± 0.69 , similar to results reported previously using wild-type C57BL/6J mice.¹³ The finding of organized thrombi in 75% of the injured arteries in CRPtg mice indicates an increased thrombotic response to injury mediated by human CRP. The facilitated thrombosis in the rose bengal photochemical thrombosis model further supports this interpretation. The high rate of complete occlusion in the wire injury model and consequent low rate of patent vessels masked neointimal formation in CRPtg mice. Modified experimental protocols that would

incorporate antithrombotic and anticoagulant agents are thus warranted to study the effect of human CRP on neointimal formation in the wire injury model.

Large epidemiological studies have correlated CRP levels with an increased risk of cardiovascular events in both healthy and acute cardiac patients. This correlation was much stronger than the association of high CRP and the burden of atherosclerosis or the extent of vascular disease.¹⁴ CRP-facilitated thrombotic occlusion fits into this scheme of events; vascular repair is impaired by increased thrombosis, which leads to a higher rate of complications after plaque rupture and vascular trauma. Although only a mediocre marker for atherosclerosis, CRP is a useful marker and an effector in the pathogenesis of cardiovascular events. Thus, it complements lipoprotein analysis in predicting atherosclerosis and its acute event risk.

Why does human CRP evoke rapid and frequent thrombosis? The interplay between inflammation and thrombosis is becoming increasingly well appreciated.¹⁵ CRP directly acts as a proinflammatory stimulus inducing expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and monocyte chemoattractant protein-1 by human endothelial cells^{4,5} and interleukin-1 β and tumor necrosis factor- α release by monocytes.¹⁶ In turn, IL-1 β and TNF- α stimulate tissue factor expression from monocytes. Furthermore, CRP was reported to directly increase tissue factor from peripheral blood monocytes¹⁷ and endothelial cells.¹⁸ Thus, CRP is capable of recruiting and activating monocytes and increasing expression of tissue factor, the key initiator for thrombosis. Recently, CRP was shown to induce plasminogen activator inhibitor-1 expression and activity in human endothelial cells, further supporting CRP association and atherothrombosis.¹⁹

The present report is the first to show that CRP is a risk factor and possible causal agent, rather than merely a risk marker, for increased rate of arterial thrombosis in vivo and worsened outcome after controlled vascular injury. These findings might help us understand the recent observation that, whereas lipoproteins predict burden of atherosclerotic disease, CRP may be more strongly associated with risk of acute cardiovascular events. Further research is now warranted to elucidate the inflammatory-thrombotic relationship and the exact role of CRP in thrombotic sequelae and to develop therapeutic strategies to treat patients deemed at high risk because of elevated CRP.

References

- Ridker PM, Rifai N, Rose L, et al. 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.
- Ridker PM. Inflammatory biomarkers, statins, and the risk of stroke: cracking a clinical conundrum. *Circulation*. 2002;105:2583–2585.
- Ridker PM, Hennekens CH, Buring JE, et al. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med*. 2000;342:836–843.
- Pasceri V, Willerson JT, Yeh ET. Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation*. 2000;102:2165–2168.
- Pasceri V, Cheng JS, Willerson JT, et al. Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs. *Circulation*. 2001;103:2531–2534.

6. Verma S, Wang CH, Li SH, et al. A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. *Circulation*. 2002;106:913–919.
7. Szalai AJ, McCrory MA. Varied biologic functions of C-reactive protein: lessons learned from transgenic mice. *Immunol Res*. 2002;26:279–287.
8. Szalai AJ, Briles DE, Volanakis JE. Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in transgenic mice. *J Immunol*. 1995;155:2557–2563.
9. Reis E, Smyth S, Collier B. Mouse model of transluminal femoral artery injury. In: Simon DI, Rogers C, eds. *Contemporary Cardiology: Vascular Disease and Injury: Preclinical Research*. Totowa, NJ: Humana Press; 2001:103–113.
10. Nagashima M, Yin Z-F, Zhao L, et al. Thrombin-activatable fibrinolysis inhibitor (TAFI) deficiency is compatible with murine life. *J Clin Invest*. 2002;109:101–110.
11. Ridker PM, Cushman M, Stampfer MJ, et al. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med*. 1997;336:973–979.
12. Roque M, Fallon JT, Badimon JJ, et al. Mouse model of femoral artery denudation injury associated with the rapid accumulation of adhesion molecules on the luminal surface and recruitment of neutrophils. *Arterioscler Thromb Vasc Biol*. 2000;20:335–342.
13. Reis ED, Roque M, Dansky H, et al. Sulindac inhibits neointimal formation after arterial injury in wildtype and apolipoprotein E-deficient mice. *Proc Natl Acad Sci U S A*. 2000;97:12764–12769.
14. Folsom A, Pankow J, Tracy R, et al. Association of C-reactive protein with markers of prevalent atherosclerotic disease. *Am J Cardiol*. 2001;88:112–117.
15. Libby P, Simon DI. Inflammation and thrombosis: the clot thickens. *Circulation*. 2001;103:1718–1720.
16. Ballou S, Lozanski G. Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein. *Cytokine*. 1992;4:361–368.
17. Cermak J, Key N, Bach R, et al. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. *Blood*. 1993;82:513–520.
18. Napoleone E, Di Santo A, Bastone A, et al. Long pentraxin PTX3 upregulates tissue factor expression in human endothelial cells: a novel link between vascular inflammation and clotting activation. *Arterioscler Thromb Vasc Biol*. 2002;22:782–787.
19. Devaraj S, Xu DY, Jialal I. C-reactive protein increases plasminogen activator inhibitor-1 expression and activity in human aortic endothelial cells: implications for the metabolic syndrome and atherothrombosis. *Circulation*. 2003;107:397–403.