

## C-Reactive Protein Accelerates the Progression of Atherosclerosis in Apolipoprotein E-Deficient Mice

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**Background**—Plasma C-reactive protein (CRP) concentration is a strong predictor of atherosclerosis. However, to date, there is no *in vivo* evidence that CRP is proatherogenic.

**Methods and Results**—We studied the effect of human CRP transgene (tg) expression, under basal and turpentine-stimulated conditions, on atherosclerosis in apolipoprotein (apo) E<sup>-/-</sup> mice. Aortic atherosclerotic lesions in 29-week-old male mice were 48% larger ( $P < 0.02$ ) in turpentine-treated mice and 34% larger ( $P < 0.05$ ) in untreated CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> mice. Turpentine treatment per se did not affect the extent of atherosclerosis in CRP transgenic or nontransgenic apoE<sup>-/-</sup> mice. Transgenic mice exhibited lower plasma complement C3 but increased deposition of CRP and C3 in the lesions, which suggests that CRP stimulated activation of complement within the lesion. There was more intense and widespread vascular cell adhesion molecule-1 and collagen staining in the lesions of CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> mice than in CRPtg<sup>0/0</sup>/apoE<sup>-/-</sup> littermates. Lesions of CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> mice contained increased angiotensin type 1 receptor (AT1-R) transcripts and displayed increased AT1-R immunostaining compared with those of CRPtg<sup>0/0</sup>/apoE<sup>-/-</sup> mice. There was no difference in blood pressure in the 2 types of mice, which indicates that the proatherogenic effect of CRP-associated AT1-R overexpression is local and not mediated by its hypertensive properties.

**Conclusions**—Human CRP transgene expression causes accelerated aortic atherosclerosis in apoE<sup>-/-</sup> mice. CRP was detected in the lesion, which was associated with increased C3 deposition and increased AT1-R, vascular cell adhesion molecule-1, and collagen expression. These data document a proatherogenic role for CRP *in vivo*. (*Circulation*. 2004; 109:647-655.)

**Key Words:** C-reactive protein ■ angiotensin ■ atherosclerosis

Atherosclerosis is an important underlying pathology of cardiovascular disease (CVD), the leading cause of morbidity and mortality in developed countries.<sup>1</sup> Clinical markers for atherosclerosis are a useful tool for identifying individuals at high risk for CVD development. C-reactive protein (CRP) has been recommended as the marker of choice to monitor cardiovascular risk, being a stronger predictor of atherosclerosis than even plasma LDL concentration.<sup>2,3</sup> Inflammation is a prominent feature of atherosclerosis,<sup>1,4</sup> and it is postulated that as an acute-phase protein, elevation of plasma CRP may signal the underlying atherosclerotic process. Although numerous epidemiological studies have shown that plasma CRP level is an excellent independent predictor of CVD in both men and women<sup>3,5</sup> and an excellent marker of the rate of progression of atherosclerosis,<sup>6,7</sup> to date there is no proof that CRP is an active participant in the

atherogenic process *in vivo*. Because atherosclerosis is an inflammatory condition, it is possible that plasma CRP levels rise as a result of the atherosclerotic process. However, the lack of association between CRP levels and extent of atherosclerosis quantified by imaging is evidence against CRP elevation being purely a consequence of atherosclerosis development.<sup>2</sup>

There is substantial circumstantial evidence that CRP may be directly involved in atherogenesis, ie, that CRP is a risk factor. CRP binds to a large number of autologous and extrinsic ligands, including native and modified plasma lipoproteins, phospholipids, and apoptotic cells, which are present in the atherosclerotic lesions.<sup>8</sup> When bound to ligands, CRP activates the classic pathway of complement, a major player in the immune and inflammatory response, and reacts with Fc $\gamma$  receptors on phagocytic cells.<sup>8-10</sup> Both CRP

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**TABLE 1. Distribution of Mice in Different Treatment Groups**

	Male				Female			
	No Treatment		Turpentine Treated		No Treatment		Turpentine Treated	
	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>
Short term	12 (11)	13 (13)	13 (10)	12 (10)	14 (14)	10 (8)	14 (11)	14 (14)
Long term	12 (10)	12 (11)	17 (14)	13 (10)	12 (11)	11 (7)	15 (14)	15 (14)

Numbers in parentheses indicate number of animals that survived to the end of the study that were used for atherosclerosis measurements.

and complement are known to colocalize in human atherosclerotic lesions, which suggests that CRP, by activating the complement, may be an active participant in atherosclerosis development.<sup>11</sup>

In vitro experiments showed that CRP modulates the activity and expression of multiple factors implicated in atherogenesis. It downregulates endothelial nitric oxide synthase (eNOS)<sup>12,13</sup> and stimulates the production of endothelin-1 (ET-1) in endothelial cells in vitro.<sup>14</sup> CRP may also facilitate leukocyte adhesion and internalization into the arterial wall by stimulating the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1, E-selectin, and monocyte chemoattractant protein-1.<sup>15,16</sup> Moreover, CRP itself was found to be chemotactic for monocytes<sup>17</sup>; it also binds to oxidized LDL<sup>18</sup> and facilitates the uptake of LDL by macrophages.<sup>10,19</sup> CRP increases smooth muscle cell (SMC) migration and neointimal formation after carotid angioplasty in rats.<sup>20</sup> It may also activate nuclear factor- $\kappa$ B and activator protein-1 (AP-1) and upregulate angiotensin type 1 receptors (AT1-R) in human vascular SMCs, which could mediate many of the proinflammatory effects of angiotensin (Ang) II.<sup>20,21</sup>

The numerous reported potential proatherogenic properties of CRP notwithstanding, there is no direct evidence that CRP plays an active role in atherosclerosis in vivo. Therefore, to date, it is not known if CRP is a risk marker or an actual risk factor for atherosclerosis.<sup>2</sup> In fact, there are properties associated with CRP that can be interpreted as having a vasculoprotective or an antiatherogenic function, eg, CRP was shown to relax human vessels in vitro<sup>22</sup> and to inhibit basal and vascular endothelial growth factor-stimulated angiogenesis,<sup>12</sup> which would in turn inhibit plaque neovascularization,<sup>23</sup> macrophage accumulation, and lesion progression.<sup>23,24</sup> To determine whether CRP is solely a risk marker for atherosclerosis or is also an active player in vivo, we have undertaken this study to examine the effect of transgenic expression of human CRP on atherosclerosis development in apolipoprotein (apo) E<sup>-/-</sup> mice.<sup>25</sup> The data show that expression of human CRP in mice accelerates aortic atherosclerotic lesion progression, thus providing the first evidence that CRP is indeed a risk factor and an active player in atherogenesis in vivo.

## Methods

### Mice

The protocol for this study was approved by the Institutional Animal Care and Use Committee from Baylor College of Medicine. CRP transgenic mice, congenic to the C57BL/6J strain, were derived from

mice originally described by Ciliberto et al.<sup>26</sup> Homozygous CRP transgenic mice were crossed twice with apoE<sup>-/-</sup> (B6.129P2-Apoe<sup>tm1Unc</sup>, Jackson Laboratories, Bar Harbor, Me) to generate hemizygous transgenic CRP (CRPtg<sup>+0</sup>) mice on an apoE<sup>-/-</sup> background. Mice had free access to water and standard laboratory chow.

CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> mice were crossed with CRPtg<sup>0/0</sup>/apoE<sup>-/-</sup> mice to generate the mice used for the present study. Mice in 2 paired groups were killed at 15 weeks of age (short-term study), when the atherosclerotic lesions would consist mainly of fatty streaks.<sup>27</sup> Mice in another 2 paired groups were killed at 29 weeks of age (long-term study), when apoE<sup>-/-</sup> mice would be expected to possess advanced atherosclerotic lesions.<sup>27</sup> In both experiments, each group of mice was further divided into 2 subgroups. Half of these mice were treated with turpentine injections to induce inflammation, and the other half received no treatment. The number of mice, distribution of CRP genotypes, and treatments received among the different groups of apoE<sup>-/-</sup> mice are shown in Table 1.

### Induction of Inflammation

We induced inflammation in some of the mice using turpentine injections as described previously.<sup>28</sup> Briefly, once every 14 days, we anesthetized the mice by intraperitoneal administration of Avertin (2,2,2-tribromoethanol, 0.4 mL of a 25 mg/mL solution [Aldrich]) and injected subcutaneously 0.1 mL of a 1:1 mixture of turpentine (Fisher Scientific) and peanut oil (Acros Organics).

### Blood and Plasma Parameters and Blood Pressure Measurements

Plasma cholesterol and triglyceride levels were measured at 8 weeks (baseline) and at 15 and 29 weeks by microtiter assays with kits from WAKO Chemicals USA, Inc. We fractionated plasma into lipoprotein fractions by fast-performance liquid chromatography gel filtration using Superose 6B columns (Pharmacia LKB Biotechnology).<sup>29</sup> Plasma human CRP concentrations were measured by an ELISA kit (Anogen). For quantifying the relative concentration of complement C3, we collected 0.25  $\mu$ L of plasma from 5 pairs of CRPtg<sup>0/0</sup> and CRPtg<sup>+0</sup> littermates and fractionated them on a 15% polyacrylamide gel, which was subsequently used for Western blot analysis<sup>29</sup> with goat anti-mouse C3 antibody (ICN Biochemicals).

We measured the systolic and diastolic blood pressures (BP) and resting heart rate using an automated tail-cuff system (BP-2000, Visitech Systems).<sup>30</sup> Briefly, the mice were submitted to 3 training sessions in which the measurements were made but not recorded. We then put them through 3 sessions of measurements that were recorded, each including 2 sets of 10 measurements, during 3 consecutive days.

### Quantitative Morphometry of Atherosclerotic Lesion Involvement

In 15- and 29-week-old mice, the aortic arch area of apoE<sup>-/-</sup> mice was the principal part of the aorta that exhibited atherosclerotic lesion formation, which we measured by morphometry of cross sections of the aortic sinus area by a computer-assisted technique, as described previously.<sup>31,32</sup> Lesions were essentially absent outside the arch for the 15-week-old group; for 29-week-old animals that had small, demonstrable lesions distal to the aortic arch, we additionally

TABLE 2. Body Weights, Plasma Lipids, and Basal CRP Levels in Different Treatment Groups

	Male				Female			
	No Treatment		Turpentine Treated		No Treatment		Turpentine Treated	
	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>
Short-term study								
Final body weight, g	22.6±1.4	23.1±1.5	21.6±1.5	21.8±1.2*	18.8±0.9	17.5±0.4	17.2±0.8*	16.7±1.3
Cholesterol, mg/dL	507±82	511±108	368±61†	391±69*	435±115	426±102	367±61	361±56
Triglycerides, mg/dL	95±18	100±16	76±18*	89±22	78±21	84±26	69±16	62±13
CRP, mg/L	0	120±77	0	156±55	0	0.5±0.70	0	3.52±2.45†
Long-term study								
Final body weight, g	26.2±2.3	24.9±2.4	24.5±2.2	23.0±2.3	19.9±1.4	20.1±1.9	20.1±1.3	20.1±1.5
Cholesterol, mg/dL								
15 weeks	451±71	427±81	433±86	425±92	408±29	382±109	391±55	413±74
29 weeks	542±88	653±134	740±236*	638±111	607±102	489±143	532±89	583±125
Triglycerides, mg/dL								
15 weeks	98±18	81±22‡	96±24	80±12‡	68±19	66±27	72±13	69±16
29 weeks	96±23	115±23	94±23	82±33*	78±22	91±23	67±18	69±14
CRP, mg/L								
15 weeks	0	105±22	0	123±33	0	0.40±0.28	0	4.29±4.56*
29 weeks	0	86±38	0	69±26	0	0.31±0.21	0	1.56±3.03

\* $P < 0.05$  with respect to untreated mice of the same gender and genotype.

† $P < 0.01$  with respect to untreated mice of the same gender and genotype.

‡ $P < 0.05$  with respect to mice of the same gender and with same treatment.

measured the extent of lesion involvement of the rest of the descending aorta by an en face technique using computer-assisted quantitative morphometry.<sup>33</sup> We used a digitizing morphometry image-analysis system (AxioVision, Carl Zeiss Vision, GmbH and SigmaScan Pro 5, SPSS Inc) to analyze lesion areas and specifically stained regions.

### Immunohistochemistry and Special Stains

We performed immunohistochemistry using primary antibodies specific for CRP (Sigma), Mac-3 (Santa Cruz Biotechnology),  $\alpha$ -actin (Spring Bioscience), VCAM-1 (Santa Cruz Biotechnology), complement C3 (ICN Pharmaceuticals), and AT1-R (Santa Cruz Biotechnology). Briefly, slides holding 5- $\mu$ m cross sections were immersed in cold acetone, rehydrated in PBS (pH 7.4) and blocked with 3% H<sub>2</sub>O<sub>2</sub> and 3% normal serum from the same animal species in which the secondary antibody was raised. We incubated tissue sections sequentially with primary antibodies and appropriate biotinylated secondary antibodies. Avidin-biotin horseradish peroxidase (Vectastain ABC reagent, Vector Laboratories) and NovaRED substrate kits for peroxidase (Vector Laboratories) were used to visualize the primary antibody. To increase the sensitivity of the immunostain for AT1-R, we used the Dako Envision<sup>+</sup> System, Peroxidase (AEC). We used hematoxylin as counterstain and Masson's trichrome stain to visualize collagen deposits according to standard protocols. For quantification by image analysis, we set a threshold to automatically compute the areas positive for each antibody or histochemical stain and then computed the ratio of positively stained area to the total lesion area studied.

### Analysis of mRNA Expression by Real-Time Reverse Transcription–Polymerase Chain Reaction

We isolated RNA from whole aortas of 29-week-old male mice using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene) and performed real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) with the LightCycler RNA Master SYBR Green I kit (Roche Diagnostics) according to the manufacturer's instructions. The RT-PCR mixture contained 50 ng of RNA and the

following primers: for CRP, 5' primer, 5'-AGC CTC TCT CAT GCT TTT GG-3'; 3' primer, 5'-TGT CTC TTG GTG GCA TAC GA-3'. For AT1-R, 5' primer, 5'-CTG CTC ACG TGT CTC AGC AT-3'; 3' primer, 5'-CGT AAT GAA AAG CGC AAA CA-3'. For eNOS, 5' primer, 5'-GAC CCT CAC CGC TAC AAC AT-3'; 3' primer, 5'-GCT CAT TTT CCA GGT GCT TC-3'. Mouse transferrin was used for RNA template normalization: 5' primer, 5'-GGT GTG CAC TGA GTC ACC TG-3'; 3' primer, 5'-CTC TGC CAT GAC AGG CAC TA-3'. Amplification and detection of specific products were performed with the Opticon System (MJ Research).<sup>34</sup>

### Statistical Analysis

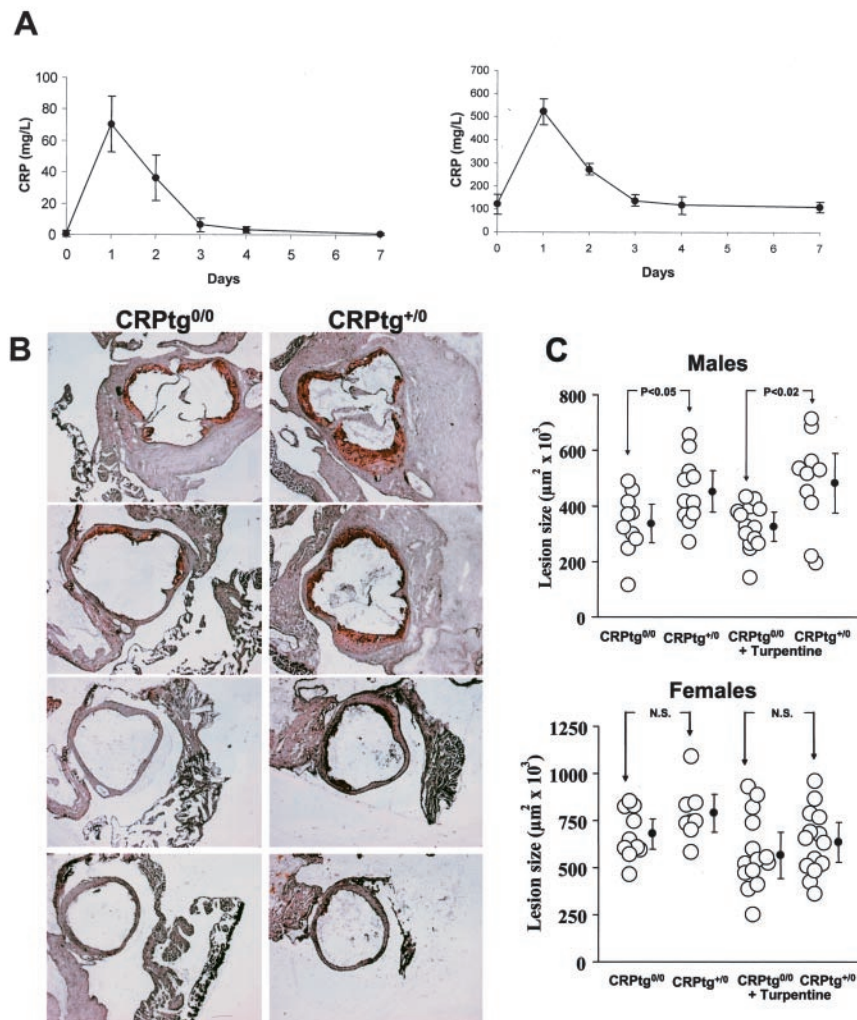
Data were analyzed with SPSS 11.0 for Windows. Most of the outcome parameters used in this study did not follow a normal distribution as judged by Shapiro-Wilk tests, so statistical analyses were performed with Mann-Whitney *U* tests. Differences were considered significant when  $P < 0.05$ . In all tables and figures, the results are expressed as mean  $\pm$  SD.

## Results

### Body Weights, Plasma Lipids, and Hematological Parameters

ApoE<sup>-/-</sup> mice with and without the CRP transgene had similar body weights and displayed no difference in activity or behavior. Their body weight curves were the same whether CRP was present or not. Body weight was slightly lower (<10% difference) in mice treated with turpentine, but the reduction was the same for turpentine-treated wild-type (CRPtg<sup>0/0</sup>) and CRPtg<sup>+0</sup> mice (Table 2). Likewise, there was slight variation in plasma cholesterol level, but importantly, there was no difference in plasma cholesterol concentration between CRPtg<sup>+0</sup> and CRPtg<sup>0/0</sup> mice in any of the paired groups. Minor but inconsistent differences in plasma triglyceride concentrations were noted. We note that all paired





**Figure 1.** A, Plasma levels of CRP in female (left) and male (right) CRP<sup>+0</sup> mice after turpentine injection. B, Cross sections of aortas from male CRP<sup>0/0</sup> and CRP<sup>+0</sup> mice at 29 weeks of age. Oil red O-stained sections were taken from level where complete aortic valve cusps were attached to aorta (top row), moving distally away from heart at level where valve attachments are still visible (middle 2 rows), moving up to level immediately distal to attachment (bottom row). C, Computer-assisted morphometric measurement of aortic atherosclerotic lesion areas at 29 weeks from male (upper panel) and female (lower panel) CRP<sup>0/0</sup> and CRP<sup>+0</sup> mice. Values are mean ± SD.

groups were littermates, which probably accounts for the very similar within-treatment lipid profiles (Table 2). We further fractionated the plasma lipoproteins by fast-performance liquid chromatography and found no appreciable difference in the lipoprotein profile between treatment groups (data not shown).

Turpentine injections produced a decrease in white blood cell count during the first 24 hours after treatment, which we attribute to migration of the leukocytes to the site of inflammation, followed by a subsequent increase until day 4, when the values returned to the baseline level (data not shown). We observed no effect of the treatment on the number of red blood cells and hematocrit level or on the total platelet count. There was no significant difference between apoE<sup>-/-</sup> mice, with or without the CRP transgene, in these hematological parameters (data not shown).

### CRP Levels in Transgenic Mice

Human CRP was detected in the plasma of CRP<sup>+0</sup>/apoE<sup>-/-</sup> but not CRP<sup>0/0</sup>/apoE<sup>-/-</sup> mice. Consistent with previous observations,<sup>35</sup> basal CRP concentration was higher in male than in female CRP transgenic mice (Table 2). On turpentine treatment, the plasma human CRP level of male mice increased from ≈100 mg/L to >500 mg/L and gradually

decreased toward baseline values by day 4. In females, plasma CRP level increased from ≈1 to ≈70 mg/L, again returning toward baseline by day 4 (Figure 1A). Persistently mildly elevated CRP levels were noted in the turpentine-treated female mice throughout the short-term and long-term studies (Table 2).

### Quantitative Morphometry of Aortic Atherosclerosis Involvement

We had 2 data collection time points, 15 weeks and 29 weeks. A comprehensive analysis of multiple subgroups of animals (8 groups of animals for each time point, or 16 groups for the 2 time points; Table 1) allowed us to discern the effect of gender, stage of maturity of the atherosclerotic lesions, presence and absence of CRP, and presence and absence of overt inflammation and the acute-phase response that may modulate atherosclerosis development. Atherosclerotic lesion involvement in apoE<sup>-/-</sup> mice was confined mainly to the aortic arch throughout the entire experiment, irrespective of the presence or absence of CRP expression. Morphometric analysis of en face lesions distal to the aortic arch at the 29-week time point revealed very minimal lesion involvement and no significant differences between treatment-matched groups. At the 15-week time point, the degree of

TABLE 3. Quantitative Morphometry of Atherosclerotic Lesions

	Male				Female			
	No Treatment		Inflammation		No Treatment		Inflammation	
	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>	CRPtg <sup>0/0</sup>	CRPtg <sup>+0</sup>
Short term (cross sections, $\mu\text{m}^2 \times 10^3$ )	32 ± 15	49 ± 40	48 ± 20	43 ± 23	140 ± 50§	98 ± 38*	82 ± 54	93 ± 45‡
Long term (cross sections, $\mu\text{m}^2 \times 10^3$ )	337 ± 109	452 ± 118†	325 ± 80	482 ± 172†	679 ± 126§	790 ± 159§	565 ± 193§	634 ± 172
Long term (en face, $\text{mm}^2$ )	0.42 ± 0.048	0.55 ± 0.45	0.37 ± 0.40	0.23 ± 0.21	0.44 ± 0.60	0.40 ± 0.43	0.22 ± 0.23	0.34 ± 0.28

\* $P < 0.05$  with respect to males with same treatment and genotype.

† $P < 0.05$  with respect to CRPtg<sup>0/0</sup> mice.

‡ $P < 0.01$  with respect to males with same treatment and genotype.

§ $P < 0.001$  with respect to males with same treatment and genotype.

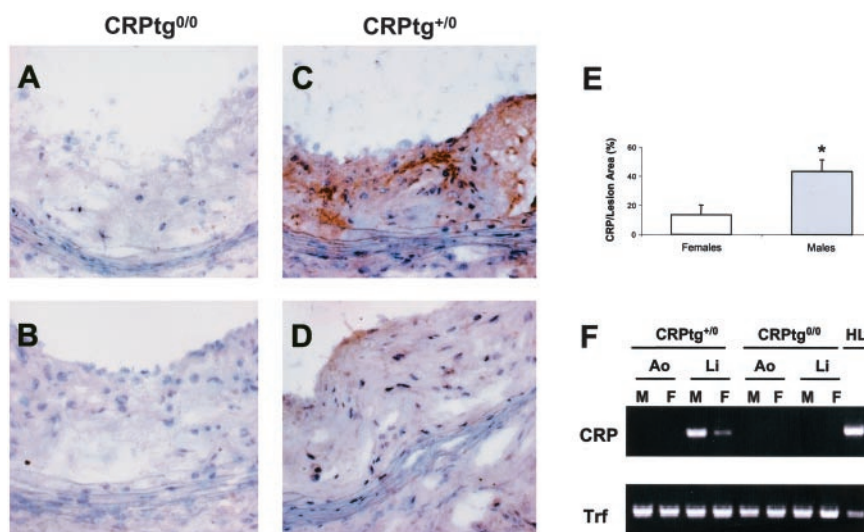
aortic arch involvement by cross-section analysis was also relatively mild, and there was no consistent difference between mice with and without CRP (Table 3). However, in 29-week-old mice, the total lesion area was significantly greater in female apoE<sup>-/-</sup> mice than in treatment-matched male apoE<sup>-/-</sup> mice, whether or not they carried the CRP transgene. Importantly, at this time point, we also found significantly larger lesion size by cross-sectional analysis of the aortic sinus area in male apoE<sup>-/-</sup> mice expressing CRP than in age- and treatment-matched males that did not express the protein. The difference in lesion size was large ( $\approx 48\%$  larger in CRPtg<sup>+0</sup> than in CRPtg<sup>0/0</sup> mice) and highly significant ( $P < 0.02$ ) when we compared male mice that were treated with turpentine; the difference was not as large, but still substantial ( $\approx 34\%$ ) and significant ( $P < 0.05$ ), when we compared male CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> and CRPtg<sup>0/0</sup>/apoE<sup>-/-</sup> mice that had not received turpentine (Table 3; Figures 1B and 1C). In female apoE<sup>-/-</sup> mice with and without the CRP transgene, we observed a similar trend in both turpentine-treated ( $\approx 12\%$  larger in CRPtg<sup>+0</sup>) and untreated mice ( $\approx 16\%$  larger in CRPtg<sup>+0</sup>), but in neither instance did the difference reach statistical significance (Table 3; Figure 1C). Interestingly, proinflammatory (turpentine) treatment did not significantly affect the size of atherosclerotic lesions in either male or female CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> or CRPtg<sup>0/0</sup>/apoE<sup>-/-</sup> mice.

### CRP and Complement C3 Deposition in Atherosclerotic Lesions

By immunohistochemical analysis, we detected immunoreactive CRP in the lesions of CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> mice and not in those of CRPtg<sup>0/0</sup>/apoE<sup>-/-</sup> mice (Figure 2; compare panels A [male] and B [female] CRPtg<sup>0/0</sup> versus panels C [male] and D [female] CRPtg<sup>+0</sup> mice). Furthermore, in CRPtg<sup>+0</sup> mice, there was much more intense (compare Figure 2C versus 2D) and widespread ( $43.74 \pm 7.7\%$  in males versus  $13.44 \pm 7.1\%$  in females, Figure 2E) CRP immunostaining in lesions from male animals compared with those from female animals, which likely reflects the much higher plasma CRP concentration in male versus female CRP transgenic mice (Table 2).

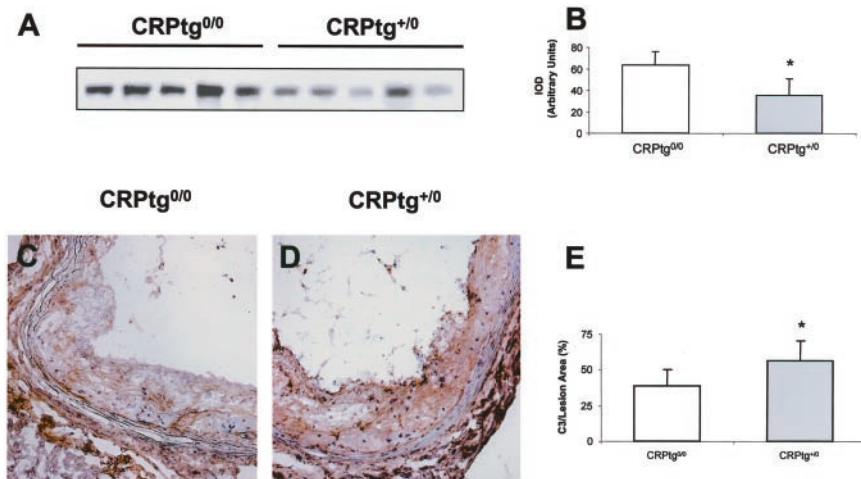
Human atherosclerotic lesions have been found to harbor CRP,<sup>11</sup> as well as CRP mRNA.<sup>36</sup> The possible expression of CRP mRNA in arterial tissue of CRP transgenic mice had not been examined previously. We analyzed for the presence of CRP mRNA by RT-PCR and found that unlike humans, CRP mRNA was detected only in the liver and not the aorta of CRP transgenic mice (Figure 2F). Therefore, the CRP deposited in the atherosclerotic lesion of these animals was most likely derived from the circulation.

Human CRP has been reported to bind to many different autologous and extrinsic ligands.<sup>8</sup> When it is bound to macromolecular ligands, CRP activates the classic com-



**Figure 2.** Immunoreactive human CRP in aortic atherosclerotic lesion of 29-week-old apoE<sup>-/-</sup> mice. CRP was undetectable in lesions in male (A) or female (B) CRPtg<sup>0/0</sup> mice. Immunoreactive CRP staining was evident in lesions of male (C) and female (D) CRPtg<sup>+0</sup> mice. E, Computer-assisted quantification of CRP immunostaining in 5 pairs of transgenic male and female (CRPtg<sup>+0</sup>) mice ( $*P < 0.005$ ). F, Liver- but not aorta-specific expression of human CRP mRNA as detected by RT-PCR in CRPtg<sup>+0</sup> mice. Positive band was identified in liver (Li) but not aorta (Ao) of male (M) and female (F) CRPtg<sup>+0</sup> mice, but not in nontransgenic (CRPtg<sup>0/0</sup>) controls. Human liver RNA (HL) was used as positive control (last band on extreme right). Transferrin RT-PCR product (Trf) was used as internal positive control. Values are mean  $\pm$  SD.





**Figure 3.** Plasma concentration and immunoreactive complement C3 in atherosclerotic lesion. Relative concentration of circulating C3 was determined by Western blotting (A and B) in 5 pairs of CRPtg<sup>0/0</sup> and CRPtg<sup>+0</sup> littermates. Integrated optical density (IOD), arbitrary units (\**P*<0.05). C and D, Immunoreactive C3 staining (brown) in representative sections of aortic lesion from male CRPtg<sup>0/0</sup> (C) and CRPtg<sup>+0</sup> (D) mice at 29 weeks of age. E, Computer-assisted analysis of mean area of C3 immunostaining in lesions from 5 pairs of CRPtg<sup>0/0</sup> and CRPtg<sup>+0</sup> littermates (\**P*<0.05). Values are mean±SD.

plement pathway, thereby engaging C3. We determined the relative concentration of plasma C3 by Western blotting and found it to be significantly (44%) lower in CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> than in CRPtg<sup>0/0</sup>/apoE<sup>-/-</sup> mice (Figures 3A and 3B). C3 has been reported to be present in human atherosclerotic lesions.<sup>37,38</sup> In agreement with the studies in humans and similar studies in mice,<sup>39</sup> we found the presence of C3 in the lesion of both CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> and CRPtg<sup>0/0</sup>/apoE<sup>-/-</sup> mice (Figures 3C and 3D), which indicates that complement activation or deposition could have occurred in the lesion in both types of animals. The amount of positive immunoreactive C3 staining in the lesion was significantly higher in CRPtg<sup>+0</sup> than in CRPtg<sup>0/0</sup> mice lesions (56.3±14.5% in CRPtg<sup>+0</sup> versus 39.1±11.0% in CRPtg<sup>0/0</sup>, Figure 3E).

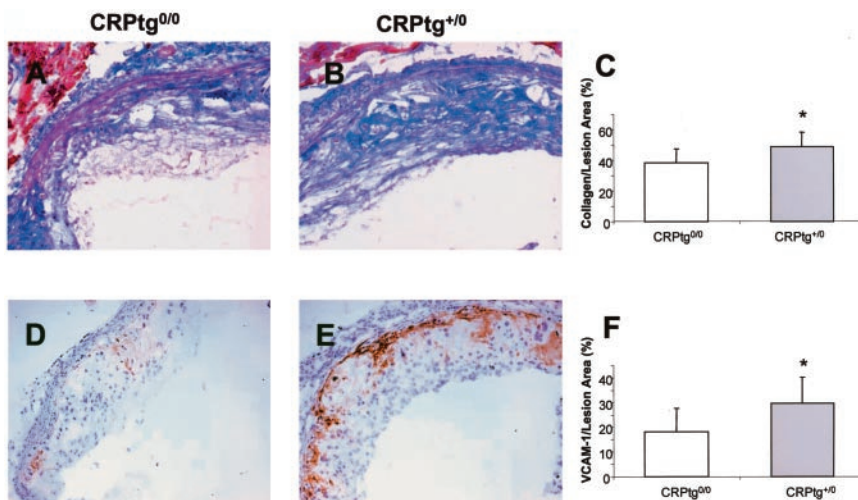
#### Phenotypic Analysis of Atherosclerotic Lesions

Phenotypic analysis revealed no obvious difference in terms of atherosclerotic lesion maturity between the 2 groups of mice at 29 weeks; most of the lesions presented with evident fibrous caps and, in some cases, necrotic cores. The areas that stained positive for macrophages were similar in CRPtg<sup>+0</sup> and CRPtg<sup>0/0</sup> mice (52.29±11.28% for CRPtg<sup>+0</sup> and 47.12±16.92% for CRPtg<sup>0/0</sup>). However, we detected a signif-

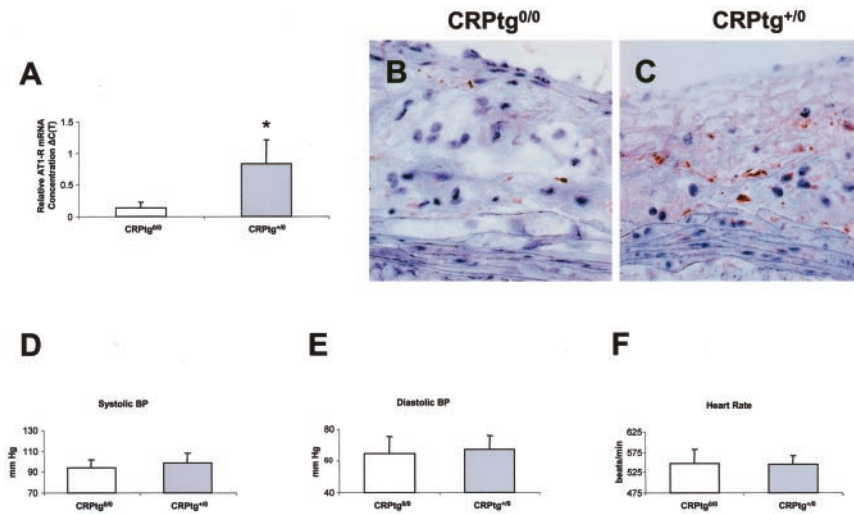
icant increase in the amount of collagen deposited in the lesions in CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> mice compared with CRPtg<sup>0/0</sup>/apoE<sup>-/-</sup> mice (48.6±9.75% versus 38.13±9.25%; Figures 4A, 4B, and 4C). Similarly, the amount of immunoreactive VCAM-1 was also increased in the lesion of the CRPtg<sup>+0</sup> animals (29.7±10.5% versus 18.1±9.6%; Figures 4D, 4E, and 4F).

#### Expression of eNOS and AT1-R in CRP Transgenic Mice

CRP has been reported to decrease eNOS expression in cultured human endothelial cells<sup>12,13</sup> and to increase AT1-R in cultured human vascular SMC and in vivo in a rat model of carotid angioplasty.<sup>20</sup> We quantified the amount of eNOS and AT1-R mRNA by RT-PCR of total RNA extracted from the aorta of CRPtg<sup>+0</sup>/apoE<sup>-/-</sup> and CRPtg<sup>0/0</sup>/apoE<sup>-/-</sup> mice. CRP transgene expression did not affect the expression of eNOS [ $\Delta C(T)$  relative to transferrin mRNA 0.21±0.13 in CRPtg<sup>+0</sup> versus 0.22±0.15 in CRPtg<sup>0/0</sup>], but, in turn, it was associated with a 6-fold increase in AT1-R mRNA expression [ $\Delta C(T)$  relative to transferrin mRNA 0.84±0.37 in CRPtg<sup>+0</sup> versus 0.13±0.09 in CRPtg<sup>0/0</sup>; Figure 5A]. We observed the presence of immunoreactive AT1-R in lesions from both groups of mice; however, consistent with the increased



**Figure 4.** Collagen and immunoreactive VCAM-1 staining in aortic lesions. A and B, Sections from CRPtg<sup>0/0</sup> (A) and CRPtg<sup>+0</sup> (B) males killed at 29 weeks of age stained with Masson's trichrome, which stains collagen blue. C, Quantification of positively stained areas in 2 types of lesion from 5 pairs of mice (\**P*<0.05). D and E, VCAM-1 immunostaining (brown) of lesions from male CRPtg<sup>0/0</sup> (D) and CRPtg<sup>+0</sup> (E) littermates. F, Quantification of VCAM-1 immunoreaction positive areas in lesions of 5 pairs of CRPtg<sup>0/0</sup> and CRPtg<sup>+0</sup> littermates (\**P*<0.01). Values are mean±SD.



**Figure 5.** Effect of CRPtg on AT1-R protein expression and BP in 29-week-old male CRPtg<sup>0/0</sup> and CRPtg<sup>+/0</sup> mice. A, Relative AT1-R mRNA concentration [ $\Delta$ C(T) relative transferrin (Trf) mRNA] as determined by real-time RT-PCR in 4 CRPtg<sup>0/0</sup> and 6 CRPtg<sup>+/0</sup> littermates ( $*P < 0.02$ ). B and C, AT1-R immunostaining (brown) in aortic lesion of male CRPtg<sup>0/0</sup> (B) and CRPtg<sup>+/0</sup> mice (C) at 29 weeks of age. Systolic BP (D), diastolic BP (E), and basal heart rate (F) in 7 pairs of male CRPtg<sup>0/0</sup> and CRPtg<sup>+/0</sup> littermates. Values are mean  $\pm$  SD.

amounts of AT1-R transcript level, we detected much more intense immunostaining for the protein in CRPtg<sup>+/0</sup> than in CRPtg<sup>0/0</sup> mice (Figures 5B and 5C).

The upregulated AT1-R protein expression in CRPtg<sup>+/0</sup>/apoE<sup>-/-</sup> mice could explain some of the differences in atherosclerotic lesions in CRPtg<sup>+/0</sup> versus CRPtg<sup>0/0</sup> mice, because AT1-R is a proinflammatory molecule.<sup>40,41</sup> To examine whether AT1-R could have led to accelerated atherosclerosis by elevating the BP of CRPtg<sup>+/0</sup> mice, we measured the systolic and diastolic BP and the resting heart rate of mice with and without the CRP transgene. We found no significant difference in systolic BP ( $99.21 \pm 7.91$  mm Hg in CRPtg<sup>+/0</sup> versus  $94.42 \pm 7.91$  mm Hg in CRPtg<sup>0/0</sup>; Figure 5D) or diastolic BP ( $67.63 \pm 8.83$  mm Hg in CRPtg<sup>+/0</sup> versus  $64.58 \pm 10.95$  mm Hg in CRPtg<sup>0/0</sup>; Figure 5E) or in the resting heart rate ( $546 \pm 20$  bpm in CRPtg<sup>+/0</sup> versus  $547 \pm 35$  bpm in CRPtg<sup>0/0</sup>; Figure 5F) in these 2 types of animals. Therefore, the proatherogenic effect of CRP-stimulated AT1-R overexpression appears not to be mediated by a change in BP.

## Discussion

Despite the fact that plasma CRP level has been recommended as a risk marker of choice in predicting atherosclerosis development,<sup>2</sup> and numerous reports based on in vitro experiments have strongly suggested a proatherogenic role for CRP, until the present study, there was no evidence to support an active proatherogenic action of CRP in vivo. In this investigation, we show that in male apoE<sup>-/-</sup> mice, transgenic expression of human CRP accelerates aortic atherosclerosis development. We observed a very substantial and significant effect, ie, a 34% to 48% increase in atherosclerotic lesion area in male mice that express human CRP compared with those that do not (Figures 1B and C; Table 3). A similar but not significant trend was observed in female mice. Interestingly, the proatherogenic effect of CRP was evident in male mice with well-established lesions at 29 weeks but not at 15 weeks, when lesions were mainly composed of fatty streaks, which suggests that CRP affects progression rather than initiation of atherosclerosis. We also saw little difference in lesion size when turpentine injections were used to periodically boost the circulating CRP; hence, the amount of

CRP present in male mice under basal conditions appears to be enough to exhibit the proatherogenic effect.

As reviewed in the introduction, on the basis of in vitro experiments, a host of proatherogenic actions of CRP have been reported.<sup>10,12,14–21</sup> Among the phenotypic consequences associated with CRP expression that we examined, there are some that warrant additional comment. We detected the presence of CRP in atheromatous lesions (Figure 2), which suggests that part or all of the proatherogenic action of CRP could have been mediated via its local action in the vascular wall where the protein is deposited. Contrary to a report in human lesions,<sup>36</sup> we did not detect CRP mRNA expression in the aorta of mice by RT-PCR. Interestingly, we detected increased amounts of C3 deposition in the lesions of CRP transgenic mice, associated with a significantly lower circulating C3 level in these mice (Figure 3). This combination of in vivo findings is consistent with the notion that activation or deposition of complement might play a role in atherogenesis modulated by the presence of CRP.

Another phenotypic feature of atherosclerotic lesions in CRP transgenic mice that may be of pathogenic significance is the increased expression of the AT1-R, both at the mRNA (Figure 5A) and at the protein (immunostaining) level (Figures 5B and 5C). The very impressive (>600%) increase in AT1-R mRNA and the stronger AT1-R immunostaining is consistent with a previous report documenting that CRP upregulates AT1-R in vascular SMCs.<sup>20</sup> AT1-R is a major receptor for Ang II that mediates many of the proinflammatory effect of this bioactive molecule. In apoE<sup>-/-</sup> mice, Ang II infusion was shown to promote atherosclerotic lesions and aneurysm formation in 1 study<sup>42</sup> and to accelerate atherosclerosis development by induced hypertension in another.<sup>43</sup> In the present study, we found that CRP expression has no significant effect on the blood pressure of apoE<sup>-/-</sup> mice (Figure 5), which indicates that hypertension is not a factor in the accelerated atherosclerosis of these animals. Therefore, in agreement with previous reports,<sup>42,43</sup> Ang II has proatherogenic effects above and beyond its action on BP. Previously reported Ang II effects include reactive oxygen species production,

vascular SMC migration, proliferation, extracellular matrix production, and vascular remodeling.<sup>44–47</sup> Ang II also induces monocyte chemoattractant protein-1 and VCAM-1 expression.<sup>48–50</sup> The much higher AT1-R expression in the presence of the CRP transgene might explain the increased amounts of VCAM-1 (Figures 4D, 4E, and 4F) and collagen deposition (Figures 4A, 4B, and 4C) in the CRPtg<sup>+0</sup> lesions; Ang II has been reported previously to be associated with increased expression of both VCAM-1<sup>49,50</sup> and collagen in animal models.<sup>47</sup>

In conclusion, we have shown that CRP expression causes accelerated atherosclerosis progression in apoE<sup>−/−</sup> mice. A single recent report showed that occlusive thrombosis was more pronounced in CRP transgenic mice than in wild-type mice.<sup>51</sup> The present study is the first to confirm a role of CRP in atherosclerosis in vivo, supporting and extending previous observations of potentially proatherogenic actions of CRP in vitro. We conclude that CRP is not merely a risk marker for but also an active participant of atherogenesis in vivo.

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## C-Reactive Protein Accelerates the Progression of Atherosclerosis in Apolipoprotein E-Deficient Mice

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