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C-Reactive Protein Induces Matrix Metalloproteinase-1 and -10 in Human Endothelial Cells

Implications for Clinical and Subclinical Atherosclerosis

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OBJECTIVES We examined the effect of C-reactive protein (CRP) on matrix metalloproteinase (MMP) and inhibitor expression in endothelial cells and in patients with clinical and subclinical atherosclerosis.

BACKGROUND In addition to predicting atherosclerotic vascular disease, CRP may directly promote a proinflammatory/proatherosclerotic phenotype.

METHODS Human umbilical vein endothelial cells (HUVECs) and aortic endothelial cells (HAECs) were incubated in the presence or absence of CRP (50 μg/ml). Microarray analysis, real-time polymerase chain reaction, immunological and activity assays for MMPs were performed. Specific inhibitors of mitogen-activated protein kinase pathway were used. The MMP-1 and -10 plasma levels were measured in apparently healthy subjects (n = 70). Immunolocalization of CRP, MMP-1, and MMP-10 was performed in human mammary arteries and carotid endarterectomy specimens.

RESULTS C-reactive protein augmented MMP-1 and -10 messenger ribonucleic acid expression in HUVEC ($p < 0.05$) and HAEC ($p < 0.01$). C-reactive protein stimulation also increased MMP-1 and -10 protein in conditioned culture medium ($p < 0.001$), as well as MMP activity ($p = 0.001$). Specific inhibition of p38 or MEK abolished the CRP induction of the MMP-1, whereas MMP-10 induction blockade required the simultaneous inhibition of p38 and Jun N-terminal kinase pathways. Subjects with CRP values >3 mg/l (n = 37) had increased plasma MMP-1 and -10 ($p < 0.05$), the association being significant after adjustment for confounding variables ($p = 0.04$ and $p = 0.008$, respectively). The MMP-10 levels were elevated in subjects with higher carotid intima-media thickness ($p = 0.009$). Increased CRP and MMP-10 colocalized in endothelial layer and macrophage-rich areas in advanced atherosclerotic plaques.

CONCLUSIONS Increased local and systemic CRP-related MMP activation might provide a link between inflammation and plaque vulnerability. (J Am Coll Cardiol 2006;47:1369–78) © 2006 by the American College of Cardiology Foundation

Vascular inflammation plays a key role in all stages of atherosclerosis, from the initiation and progression of the atherosclerotic lesion to plaque rupture, and eventually to the occurrence of cardiovascular events (1). Inflammation heightens production of biomarkers, such as C-reactive protein (CRP), which provide information about the risk of developing cardiovascular disease and may furnish new criteria for treatment (2).

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C-reactive protein powerfully and independently predicts myocardial infarction, stroke, and vascular death in a variety of clinical settings (3,4). In addition, recent studies suggest that CRP has direct proatherosclerotic effects on cellular functions implicated in atherosclerotic lesion formation: it promotes endothelial cell (EC) activation, uptake of low-density lipoprotein by macrophages, and expression of angiotensin II type 1 receptor by vascular smooth muscle cells (5–9). However, a possible protective role of CRP in atherogenesis has also been suggested (10).

Atherosclerotic plaques that have ruptured characteristically contain numerous macrophages that produce matrix metalloproteinases (MMPs) capable of degradation of extracellular matrix molecules. Plaques with a weakened fibrous cap may rupture and provoke thrombosis (11–13). It has been previously reported that macrovascular and microvascular EC in human plaques also overexpress MMPs (11).

We hypothesized that CRP induces MMP expression in ECs. Furthermore, we analyzed different mitogen-activated protein kinases (MAPK) cascades in regulation of MMPs expression. In addition, we examined the levels of candidate proteins in plasma of subjects in relation to systemic CRP

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Abbreviations and Acronyms

BMI	= body mass index
CCA	= common carotid arteries
CRP	= C-reactive protein
EC	= endothelial cell
ERK	= extracellular signal-regulated kinase
HAEC	= human aortic endothelial cell
HUVEC	= human umbilical vein endothelial cell
IMT	= intima-media thickness
JNK	= Jun N-terminal kinase
MAPK	= mitogen-activated protein kinases
MMP	= matrix metalloproteinase
RT-PCR	= reverse transcription-polymerase chain reaction
TIMP	= tissue inhibitor of metalloproteinase
vWf	= von Willebrand factor

levels, and the expression of CRP and MMPs in human atherosclerotic lesions.

Our findings suggest that CRP can increase both endothelial and systemic MMP expression, and may thus function as both marker and mediator of plaque inflammation.

METHODS

CRP stimulation in human ECs. Human umbilical vein endothelial cells (HUVECs) were isolated as previously described (14) and grown in MEM 199 (Invitrogen, Carlsbad, California) supplemented with 10% fetal calf serum and 5% human serum (both PAA Laboratories, Pasching, Austria). Cryopreserved human aortic endothelial cells (HAEC) (Cambrex, East Rutherford, New Jersey) were obtained at passage 3. Human umbilical vein ECs and HAEC (n = 4) were incubated for 12 h in presence or absence of 50 μg/ml human recombinant CRP (Calbiochem, San Diego, California), as previously reported based on previous observations in endothelial cultures (5,6). C-reactive protein purity was checked by SDS-PAGE followed by Coomasie staining and endotoxin-contamination-excluded by polymyxin-B (50 μg/ml, Sigma, St. Louis, Missouri) and Limulus assay (endotoxin <0.125 EU/ml, Chromogenix AB, Mölndal, Sweden). To assess a possible effect of sodium azide in the commercial preparation, cells were incubated with 0.025% sodium azide, equivalent to that found in 50 μg/ml CRP. Specific inhibitors for MEK (PD98059, 50 μM, Sigma, St. Louis, Missouri), p38 (SB203580, 1 μM, Sigma), and Jun N-terminal kinase (JNK) (SP600125, 1 μM, Calbiochem) were added 30 min before CRP incubation. Trypan blue staining assessed cell viability.

Affymetrix microarray analysis. A total of 10 μg of total ribonucleic acid from HUVEC, isolated with TRIPURE (Roche, Burlington, North Carolina) and purified by RNeasy kit (Qiagen, Hilden, Germany), were used to generate and label cRNA according to the manufacturer's instructions. Target quality was ensured by hybridization with a Test3 Array (Affymetrix, Santa Clara, California). Each target was hybridized to the GeneChip Human Genome U133A (Affymetrix). Arrays were washed and stained with streptavidin-phycoerythrin on Affymetrix Fluidic Station and scanned on Affymetrix Genechip Scanner. Initial analysis was performed using Microarray Suite (MAS5.0, Affymetrix) and subsequent analysis carried out with TMev (Stanford University, Stanford, California) (15). Each probe set on the experimental array was compared to its counterpart by the Wilcoxon rank sum test, and the "change p value" calculated indicating increase (I), decrease (D), or no change (NC) in gene expression. The resulting p values are "two-tailed" spanning the range from 0 to 1, with values associated with an increase near 0 and values associated with a decrease near 1.

Real-time reverse transcription-polymerase chain reaction (RT-PCR). Genes differentially expressed between CRP-stimulated HUVEC and controls were confirmed by RT-PCR using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, California), western blotting, and enzyme immunoassay (ELISA). Reverse transcription was performed by converting 2.5 μg of total ribonucleic acid to complementary deoxyribonucleic acid using random priming and MMLV reverse transcriptase (Invitrogen).

Complementary deoxyribonucleic acid (50 ng) was amplified with SYBR Green PCR Master Mix (Applied Biosystems) using specific primers for MMP-1 (5′-ACGCCAGATTTGCCAAGAG-3′ and 5′-TTGACCCT-CAGAGACCTTGGT-3′) and β-actin (5′-AGCCTCGCC-TTTGCCGA-3′ and 5′-CTGGTGCCTGGGGCG-3′), designed by Primer Express software. Matrix metalloproteinase-10, MMP-9, and tissue inhibitor of metalloproteinase (TIMP)-1 amplifications were performed with specific Taqman assays-on-demand (Applied Biosystems). Beta-actin was used to normalize messenger ribonucleic acid levels.

Global MMP activity. A total of 30 μM fluorogenic peptide (MCA-Arg-Pro-Lys-Pro-Val-Glu-Nval-Trp-Arg-Lys-[DNP]-NH_2) (R&D Systems, Minneapolis, Minnesota) was used to assess global MMP activity. Fluorescence was kinetically assessed on SpectraMAX GeminiXS (Molecular Devices, Sunnyvale, California) (320 nm excitation and 405 nm emission). EDTA (10 mmol/l) was used to inhibit calcium-dependent activation of MMPs.

Western blotting. Western blotting evaluated the zymogens of MMP-1 and -10 (52 and 54 kDa, respectively) and active forms (42 and 44 kDa, respectively). Conditioned media were separated by SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose membrane. Membranes were probed with murine anti-MMP-1 (1 μg/ml, Oncogene) or anti-MMP-10 (1 μg/ml, Lab Vision/Neomarkers, Freemont, California) followed by a peroxidase-linked rabbit anti-mouse IgG (1:2000, Zymed, Camarillo, California). Blots were developed with chemoluminescence (ECL, Amersham Bioscience, Piscataway, New Jersey) on ChemiDoc system

and quantified by densitometry (QuantityOne, Bio-Rad, Hercules, California).

Study population. The study population consisted of 70 apparently healthy subjects (57 men, 50 ± 9 years) presenting to the internal medicine department for global risk assessment. Subjects lacked clinical atherosclerotic disease based on absence of history or physical signs of coronary disease, stroke or peripheral arterial disease, and normal electrocardiogram. Exclusion criteria were severely impaired renal function, arteritis, connective tissue diseases, acute infection, and administration of anti-inflammatory, antithrombotic, or hormonal therapy in the previous two weeks.

Cardiovascular risk factors included arterial hypertension (systolic blood pressure >139 mm Hg, and/or diastolic blood pressure >89 mm Hg, and/or use of antihypertensive drugs); dyslipidemia (total cholesterol ≥200 mg/dl, and/or high-density lipoprotein cholesterol ≤40 mg/dl, and/or low-density lipoprotein cholesterol ≥130 mg/dl, and/or use of cholesterol lowering drugs), obesity (body mass index [BMI] ≥30 kg/m^2); smoking (≥1 cigarette a day); and diabetes (fasting glucose ≥126 mg/dl and/or use of pharmacological treatment).

Subjects were grouped according to serum CRP concentrations used to denote low and high risk for primary prevention (2).

Carotid intima-media thickness (IMT). All subjects underwent ultrasonography (ATL 5000 HDI, Philips, Bothell, Washington) of common carotid arteries (CCA) to assess the carotid IMT as a surrogate marker of subclinical atherosclerosis (16). Carotid IMT was measured 1 cm proximal to the carotid bulb of each CCA at plaque-free sites and determined as the average of near- and far-wall measurements. Intra- and interobserver coefficients of variation were 5% and 10%, respectively.

The local committee on human research approved the study, performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent.

Protein immunolocalization in human atherosclerotic lesions. Specimens from 15 patients undergoing carotid endarterectomy (>75% stenosis) and 20 mammary arteries sections as controls were fixed with 4% paraformaldehyde and paraffin embedded. Serial sections were analyzed by immunohistochemistry as described (13), using antibodies anti-MMP-1 (2 μg/ml, Oncogene, Merck-Biosciences, Nottingham, United Kingdom), anti-MMP-10 (10 μg/ml, Neomarkers), anti-CRP (80 μg/ml, Sigma), anti-CD68 (0.2 μg/ml, Dako, Carpinteria, California), and anti-von Willebrand factor (vWf) (3.3 μg/ml, Dako).

Samples for confocal microscopy (n = 5) were frozen and cut with a sliding microtome. After incubating with primary antibodies (overnight, 4°C), 30-μm sections were washed and incubated (2 h, room temperature) with 10 μg/ml Alexa Fluor 568 and 488 anti-IgG antibodies (Molecular Probes, Eugene, Oregon). TO-PRO-3 (1.25 μM) was used for nuclear counterstaining and slides mounted in SlowFade Antifade reagent (Molecular Probes) and visualized under confocal laser microscope (Zeiss LSM-510 Meta, Carl Zeiss, Jena, Germany). The specificity of the signal was checked in samples processed without primary antibodies.

Laboratory analysis. Fasting serum and plasma samples were collected by venipuncture, centrifuged (20 min, 1200 *g*), and stored at −80°C until analysis. Plasma fibrinogen activity was measured by clotting assay (Clauss), high-sensitive CRP by immunoassay (Immulyte, Diagnostic Product Corporation, Los Angeles, California), and vWf by enzyme immunoassay (Asserachrom, Diagnostica Stago, Asnières, France).

Matrix metalloproteinase-1, TIMP-1 (Biotrak, Amersham Biosciences), MMP-10, and interleukin-6 (Quantikine, R&D Systems) levels were assayed in conditioned medium and plasma by ELISA. Matrix metalloproteinase assays recognize the zymogens and active forms. Inter and intra-assay coefficients of variation for all ELISAs were <6%.

Serum total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, and glucose were measured by standard laboratory techniques.

Statistical analysis. Differences between controls and stimulated cultures were assessed by Mann-Whitney *U* test. Normality was assessed by the Shapiro-Wilk test. Normally

Table 1. Transcriptional Profiling of MMP and TIMP Genes in CRP-Stimulated HUVEC

Gene	Fold Change vs. Controls	"Change p Value"	
MMP-1	2.30	0.000020	I
MMP-2	0.93	0.500000	NC
MMP-3	0.93	0.500000	NC
MMP-7	1.00	0.500000	NC
MMP-8	1.07	0.500000	NC
MMP-9	1.07	0.500000	NC
MMP-10	4.92	0.000020	I
MMP-11	0.81	0.725952	NC
MMP-12	0.87	0.969033	NC
MMP-13	0.44	0.500000	NC
MMP-14 (MT1-MMP)	0.47	0.088938	NC
MMP-15 (MT2-MMP)	0.81	0.822588	NC
MMP-16 (MT3-MMP)	0.41	0.500000	NC
MMP-17 (MT4-MMP)	0.38	0.057676	NC
MMP-19	1.00	0.500000	NC
MMP-20	0.25	0.681091	NC
MMP-23A	1.23	0.366593	NC
MMP-24 (MT5-MMP)	1.23	0.118009	NC
MMP-25 (MT6-MMP)	1.00	0.454766	NC
MMP-26	1.52	0.194801	NC
MMP-27	1.07	0.274048	NC
MMP-28	0.54	0.596188	NC
TIMP-1	1.07	0.500000	NC
TIMP-2	0.93	0.500000	NC
TIMP-3	0.57	0.767451	NC
TIMP-4	1.52	0.500000	NC

The values close to 0 indicate increase in transcript expression level, and close to 1 indicate decrease in transcript expression level.

CRP = C-reactive protein; HUVEC = human umbilical vein endothelial cells; I = increase; MMP = matrix metalloproteinase; MT = membrane type; NC = no change; TIMP = tissue inhibitor of metalloproteinase.

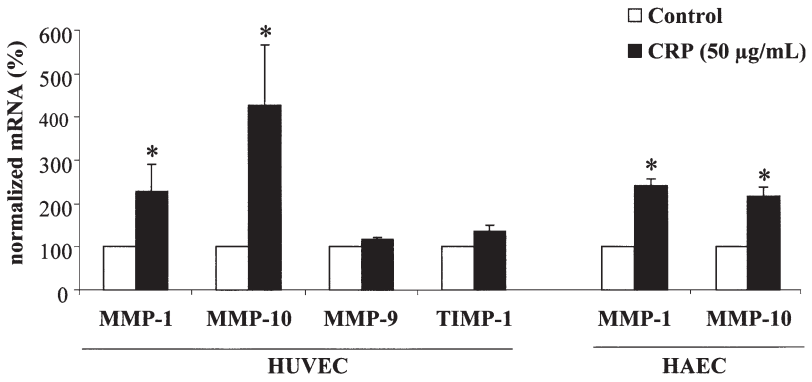

Figure 1. C-reactive protein (CRP) increases matrix metalloproteinase (MMP)-1 and MMP-10 messenger ribonucleic acid (mRNA) expression in human endothelial cell. Reverse transcription-polymerase chain reaction for MMP-1, -9, -10, and tissue inhibitor of metalloproteinases (TIMP)-1 mRNA was performed in CRP-stimulated human umbilical vein endothelial cells (HUVEC) (12 h, 50 μg/ml) and mRNA normalized values (target gene/β-actin mRNA copies) obtained. C-reactive protein-induced MMP-1 and -10 expression in human aortic endothelial cell (HAEC) is also shown. Data are presented (mean ± SEM, n = 4) as percentage from controls set as 100%. *$p < 0.05$ compared with control.

distributed continuous variables in patients were expressed as mean ± SD and non-normally distributed as median (interquartile range). Differences in the baseline characteristics between study groups were evaluated by the Student *t* test for normally or Mann-Whitney *U* test for non-normally distributed variables, and chi-square statistic for categorical variables. Pearson's correlation test assessed univariate correlations between MMPs and normally or loga-

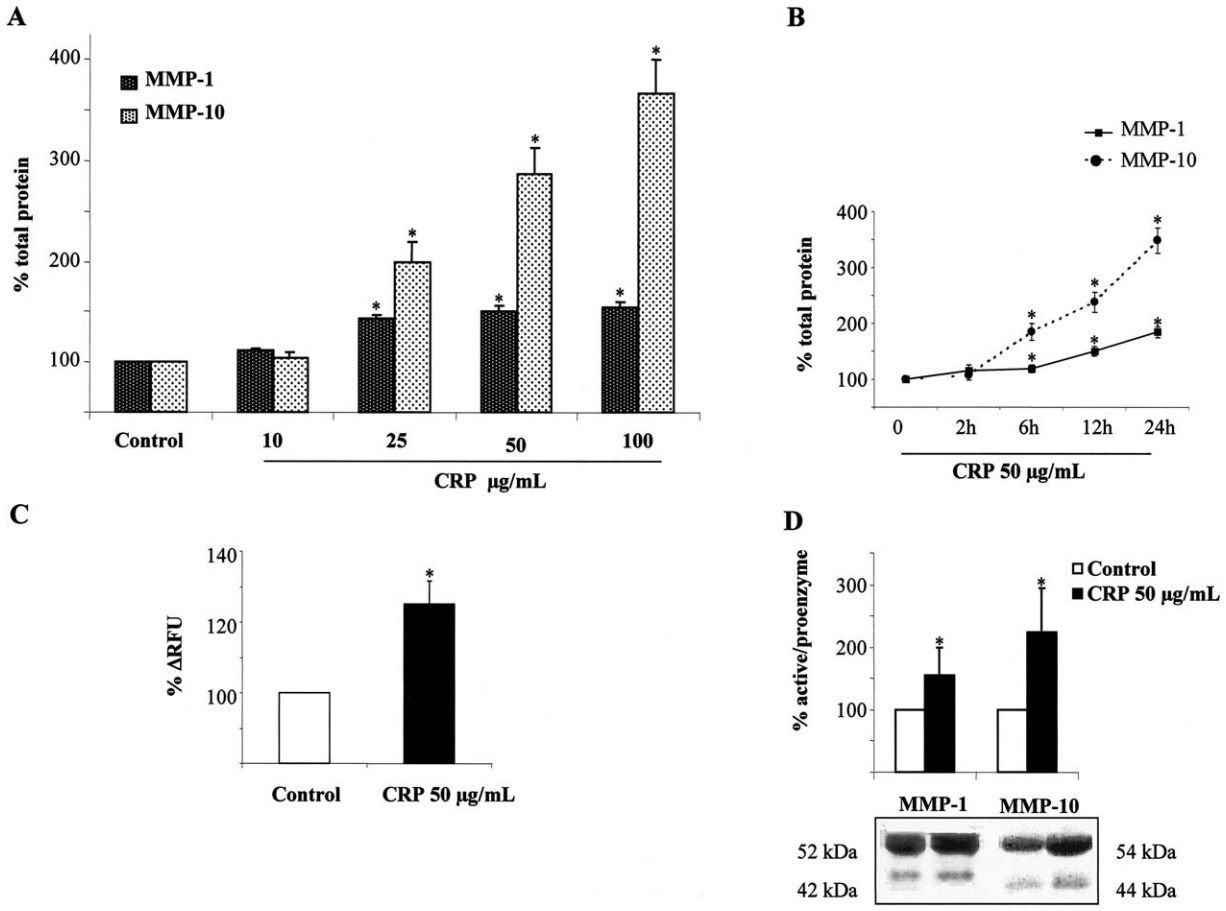

Figure 2. C-reactive protein (CRP) increases endothelial matrix metalloproteinase (MMP)-1 and -10 protein in a concentration and time-dependent manner. **(A)** Matrix metalloproteinase-1 and -10 dose response after incubation with CRP (10 to 100 μg/ml) for 12 h. **(B)** Time course of MMP-1 and -10 secretion in conditioned medium after incubation with CRP (50 μg/ml) for 24 h. **(C)** C-reactive protein-induced (50 μg/ml) global MMP activity assessed by fluorogenic substrates. **(D)** Representative Western blot of MMP-1 and -10 and densitometric analysis corresponding to increased active form/zymogen ratio of MMP-1 and -10 after CRP (50 μg/ml) stimulation. Data are presented as mean ± SEM (n = 4). *$p < 0.05$ compared with control. RFU = relative fluorescent unit.

rithmically transformed non-normally distributed variables (CRP, triglycerides, glucose, vWf, and carotid IMT). Multivariable linear regression analysis was conducted with MMP-1 or -10 as dependent variables including in the model the traditional risk factors and those variables that were significant in the univariate analysis. Statistical significance was established as $p < 0.05$ (version 11.0, SPSS Inc., Chicago, Illinois).

RESULTS

Transcriptional profiling. The concentration dependence of EC activation by CRP (10 to 100 μg/ml) was assessed by measuring interleukin-6 release as described (7). Increased interleukin-6 production was observed at 25 μg/ml, being significant ($p < 0.05$) at 50 μg/ml (two-fold increase in relation to unstimulated cultures, data not shown), the level chosen for further analysis.

Microarray analysis of stimulated and control HUVEC showed at least two-fold increase in the expression of 181 genes while 79 genes decreased by two-fold in CRP-treated versus untreated HUVEC (data not shown). We focus on regulation of genes involved in vascular proteolysis, namely MMPs and TIMPs. C-reactive protein stimulated MMP-1 and -10 messenger ribonucleic acid expression (2.3- and 4.9-fold, respectively; both $p = 0.001$), whereas variations in TIMPs and other MMPs gene expression showed no significant differences (Table 1).

CRP augments MMP-1 and -10 expression in human EC. C-reactive protein increased MMP-1 and -10 expression in HUVEC, assessed by RT-PCR (2.3 ± 0.6-fold and 4.2 ± 1.4-fold change vs. control, respectively, both $p = 0.014$), but did not change MMP-9 or TIMP-1 expression significantly, in accordance with microarray data. C-reactive protein also increased significantly MMP-1 and -10 expression in HAEC ($p < 0.01$) (Fig. 1).

Addition of CRP (10 to 100 μg/ml) increased MMP-1 and -10 secretion in HUVEC-conditioned medium in a dose-dependent manner (Fig. 2A). The baseline levels of MMP-1 and -10 (17.8 ± 0.7 ng/ml and 15.8 ± 1.0 ng/ml, respectively) were significantly increased (1.5 ± 0.1-fold and 2.8 ± 0.2-fold, respectively, both $p < 0.001$) after CRP stimulation (50 μg/ml), whereas TIMP-1 levels did not change significantly (data not shown). To exclude any effect of azide (17,18), 0.025% sodium azide was added to EC without changes on MMP-10 ribonucleic acid expression (1.3 ± 0.1-fold increase as compared with control cultures) or protein secretion (10.5 ± 0.3 ng/ml vs. 14.4 ± 1.6 ng/ml in control cultures), indicating that its presence was irrelevant.

As shown in Figure 2B, CRP induced a time-dependent increase of MMP-1 and -10, starting at 6 h until 24 h ($p < 0.001$). Global MMP activity also assessed in conditioned medium by a kinetic fluorescence assay was ~25% higher in CRP-treated than in control cells ($p = 0.001$) (Fig. 2C). Addition of EDTA (10 mmol/l) to the medium eliminated any detectable fluorescence.

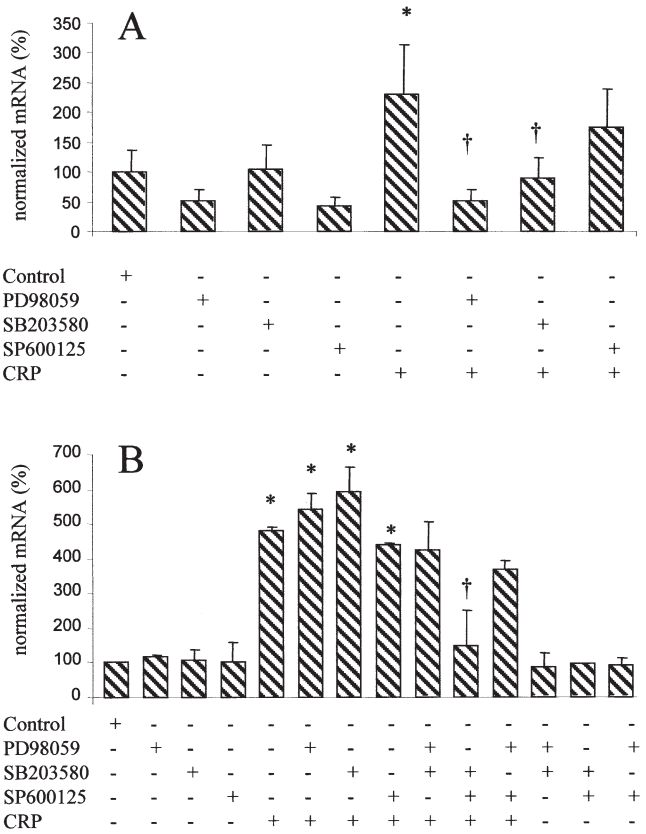

Figure 3. Mitogen-activated protein kinase-mediated C-reactive protein (CRP)-induced matrix metalloproteinase (MMP)-1 **(A)** and MMP-10 **(B)** up-regulation. **(A)** An MEK inhibitor (PD98059) abolished the CRP-induced MMP-1 expression, whereas a p38 inhibitor (SB203580) partially reduced it. **(B)** Both SB203580 and SP600125 (a JNK inhibitor) blocked the CRP-induced MMP-10 up-regulation. *$p < 0.05$ vs. control; †$p < 0.05$ vs. CRP.

Interestingly, significant increases of the proportion of active protein/zymogen of MMP-1 and -10 (1.5 ± 0.5-fold and 2.2 ± 0.7-fold change vs. control, respectively; both $p = 0.014$) were observed by western blotting in CRP-stimulated HUVEC compared to controls (Fig. 2D).

Overall, these results suggest that CRP directly induces endothelial proteolysis by stimulating expression and activity of MMP-1 and -10.

Role of MAPK pathways in CRP-mediated MMP-1 and -10 induction. The specific roles of extracellular signal-regulated kinase (ERK)1/2, p38, and Junk in the regulation of MMP-1 and -10 expression were examined in HUVEC treated with CRP alone, or in combination with MEK1/2 inhibitor PD98059, p38 MAPK-specific inhibitor SB203580, or JNK inhibitor SP600125. The enhancement of MMP-1 by CRP was suppressed by PD98059 and SB203580, whereas SP600125 had no effect (Fig. 3A). The CRP-induced MMP-10 up-regulation was blocked by simultaneous inhibition of p38 and JNK pathways, but not by SB203580 or SP600125 alone (Fig. 3B), suggesting that MAPK signal transduction is involved in MMP induction.

Table 2. Baseline Characteristics and Biochemical Profile of Subjects Stratified According to CRP Levels

	CRP <3 mg/l (n = 33)	CRP >3 mg/l (n = 37)	p Value
Age, yrs	48.4 ± 8.9	51.8 ± 9.4	0.12
Gender, M/F	27/6	30/7	0.94
Body mass index, kg/m^2	26.1 ± 3.4	29.0 ± 3.3	0.001
Glucose, mg/dl*	92 (88–103)	99 (92–110)	0.04
Total cholesterol, mg/dl	220.6 ± 43.3	239.0 ± 38.7	0.06
HDL-C, mg/dl	51.3 ± 12.0	49.8 ± 14.7	0.62
LDL-C, mg/dl	148.2 ± 41.8	164.1 ± 37.0	0.09
Triglycerides, mg/dl*	85 (55–116)	112 (84–153)	0.03
Smoking, N/Y	21/12	23/14	0.89
Systolic blood pressure, mm Hg*	120 (110–130)	135 (113–150)	0.67
Diastolic blood pressure, mm Hg*	80 (78–85)	80 (80–90)	0.95
CRP, mg/l*	1 (0.95–1.25)	6 (5.10–8.75)	0.001
Fibrinogen, mg/dl	282.4 ± 66.2	337.1 ± 78.3	0.001
von Willebrand factor, %*	91 (70–119)	123 (90–160)	0.001
MMP-1, ng/ml	4.0 ± 0.5	4.6 ± 1.1	0.03
MMP-10, pg/ml	558.0 ± 30.8	737.8 ± 71.2	0.02
TIMP-1, ng/ml	92.8 ± 13.0	101.9 ± 17.3	0.06
Carotid IMT, mm*	0.67 (0.60–0.74)	0.70 (0.65–0.85)	0.02

Values are expressed as mean ± SD, except for skewed variables*, which are shown as median (interquartile range).
HDL-C = high-density lipoprotein-cholesterol; IMT = intima-media thickness; LDL-C = low-density lipoprotein; other abbreviations as in Table 1.

Circulating levels of MMPs and TIMP-1 in relation to CRP. Seventy apparently healthy subjects were stratified according to their serum CRP (Table 2). Subjects with high-sensitive CRP levels >3 mg/l had significantly higher glucose ($p = 0.04$), triglycerides ($p = 0.03$), fibrinogen ($p = 0.001$), vWf ($p = 0.001$), BMI ($p = 0.001$), and carotid IMT ($p = 0.02$) than subjects with CRP <3 mg/l. A significant increase of plasma MMP-1 and -10 ($p = 0.03$ and $p = 0.02$, respectively) was observed in this group, without differences in TIMP-1 levels.

C-reactive protein significantly correlated with MMP-1 and -10 ($r = 0.36$, $p = 0.001$; $r = 0.46$, $p = 0.001$, respectively) in the univariate analysis (Table 3). Matrix metalloproteinase-1 also correlated with BMI ($r = 0.34$, $p = 0.001$) and triglycerides ($r = 0.32$, $p = 0.001$), whereas MMP-10 positively correlated with age ($r = 0.28$, $p = 0.02$), fibrinogen ($r = 0.36$, $p = 0.001$), vWf ($r = 0.30$, $p = 0.02$), and TIMP-1 ($r = 0.47$, $p = 0.001$), and inversely with high-density lipoprotein cholesterol ($r = -0.32$, $p = 0.001$).

Interestingly, the association between CRP and both MMP-1 ($p = 0.040$) and MMP-10 ($p = 0.008$) remained significant after adjusting for traditional risk factors and inflammatory markers (Table 4).

Considering wall thickness (values higher or lower than 75th percentile of carotid IMT) as a surrogate of atherosclerosis, subjects in the highest IMT quartile (>0.79 mm) had significantly higher plasma MMP-10 levels than those in the lowest quartile (670.4 ± 181.4 pg/ml vs. 532.2 ± 156.6 pg/ml, $p = 0.009$).

Immunolocalization of CRP, MMP-1, and -10 in human arteries. Immunohistochemical staining for CRP, MMP-1, and MMP-10 was performed in vascular sections from carotid and mammary artery specimens (Fig. 4). An intense positive signal for these proteins was observed in all carotid sections analyzed (Figs. 4A to 4C), whereas a weaker signal was found in mammary arteries (Figs. 4G to 4I). The strongest immunostaining corresponds to macrophage-rich areas within endarterectomy specimens (Fig. 4D). Interestingly, a positive signal for both MMP-10 and CRP was observed within the endothelial layer (Figs. 4E and 4F). Confocal microscopy confirmed the colocalization of MMP-10 and CRP in macrophage-rich areas and in the endothelial layer (Fig. 5).

Table 3. Correlation Coefficients Between MMP-1, MMP-10, and Cardiovascular Risk Factors

	MMP-1		MMP-10	
	(r)	p	(r)	p
Age, yrs	−0.092	0.49	0.28	0.02
BMI, kg/m^2	0.34	0.001	0.16	0.17
Glucose, mg/dl*	0.22	0.09	0.16	0.19
Total cholesterol, mg/dl	0.11	0.41	−0.06	0.63
HDL-C, mg/dl	−0.054	0.709	−0.32	0.001
LDL-C, mg/dl	0.13	0.33	0.03	0.80
Triglycerides, mg/dl*	0.32	0.001	−0.07	0.58
Systolic blood pressure, mm Hg	0.23	0.08	0.19	0.12
Diastolic blood pressure, mm Hg	0.09	0.5	0.17	0.15
CRP, mg/l*	0.36	0.001	0.46	0.001
Fibrinogen, mg/dl	−0.20	0.13	0.36	0.001
von Willebrand factor, %*	0.22	0.10	0.30	0.02
MMP-1, ng/ml	—	—	0.08	0.569
TIMP-1, ng/ml	0.23	0.12	0.47	0.001
Carotid IMT, mm*	−0.06	0.71	0.24	0.053

*Skewed variables that were logarithmically transformed.
BMI = body mass index; other abbreviations as in Tables 1 and 2.

Table 4. Correlation of CRP With MMP-1 and -10 in Multiple Linear Regression Analysis With MMP-1 and -10 as Dependent Variables

	B	SE(B)	p	Partial R^2 (%)
MMP-1*				
CRP, mg/l	3.11	1.6	0.040	30.0
BMI, kg/m^2	0.098	0.039	0.017	34.8
MMP-10†				
CRP, mg/l	12.49	4.568	0.008	34.6
Gender	−193.56	93.30	0.043	−26.9
Smoking	197.23	70.71	0.007	35.2
Fibrinogen, mg/dl	1.43	0.479	0.004	37.4

*R^2 = 19.8% for the total population (p = 0.015) (adjusted for age, gender, smoking, body mass index (BMI), glucose, total cholesterol, systolic blood pressure, triglycerides, and CRP); †R^2 = 47.6% for the total population (p = 0.001) (adjusted for age, gender, smoking, BMI, glucose, total cholesterol, systolic blood pressure, HDL-C, CRP, fibrinogen, von Willebrand factor, TIMP-1, and carotid IMT).
Abbreviations as in Tables 1 and 2.

DISCUSSION

Matrix metalloproteinases may contribute to the development of unstable plaques. This study demonstrates that CRP induces endothelial MMP-1 and -10 expression, and elevated CRP levels are associated with increased MMP-1 and -10 expression in vivo, providing a link between inflammation and atherosclerotic plaque destabilization.

Expression of MMPs by ECs in response to CRP. Despite reports showing proatherogenic properties of CRP on EC (5–8,19), there is scarce evidence of its role on vascular proteolytic potential (20). A novel finding was that CRP significantly increased messenger ribonucleic acid and protein expression of MMP-1 and -10 in HUVEC and HAEC, without significantly affecting the expression of other MMPs or TIMPs, as assessed by transcriptional profiling and verified by RT-PCR and immunological methods. A previous analysis demonstrated increased expression of both MMPs by EC upon stimulation with oxidized high-density lipoprotein (21).

C-reactive protein also increased global MMP activity and the proportion active protein/zymogen of MMP-1 and -10, suggesting increased endothelial proteolytic activation, not explained by increased chymase/tryptase, urokinase-type plasminogen activator, or thrombin, because their transcriptional profile was not altered (data not shown). Matrix metalloproteinase-1 and MMP-10, by degrading major extracellular matrix components in atherosclerotic plaques (12), might promote superficial erosion of the intima, an important process leading to coronary thrombosis (22,23).

The mechanisms of CRP-mediated stimulation of MMP-1 and -10 in HUVEC are not fully characterized. C-reactive protein stimulates MMP-1 and annexin A5 expression by U937 cells via Fcγ receptors and ERK pathway (20,24). It has also been suggested that conformational rearrangement of CRP via FcγRIII (CD16) may be a prerequisite for activation of EC independently of CD32 (25). In contrast to mature EC, HUVEC do not express CD32 nor CD16 receptors, therefore suggesting an Fcγ receptor-independent induction of proteinase expression.

In this study, we also demonstrated that the MAPK signaling cascade constitutes one of the possible pathways that mediates the effect of CRP on endothelial MMP production; ERK1/2, p38, and JNK pathways have been reported to be involved in the regulation of MMP-1 and -10 in different cell types (20,26,27), and there is evidence that CRP could activate the MEK/ERK signaling pathway in HUVEC (28).

In addition, CRP can contribute to the extent of vascular damage via complement activation (29). Atherogenic factors, such as cytokines and oxidized low-density lipoprotein, known to increase CRP, may also stimulate MMP-1 activity by cells present within atheroma (30,31).

Systemic levels of MMP-1 and -10 in relation to serum CRP. Significant associations of CRP with MMP-1 and -9 have been found in patients with cardiovascular disease (32) and rheumatoid arthritis (33). To further explore in vitro findings, systemic levels of MMP-1 and -10 were measured in asymptomatic subjects stratified according to CRP values. Patients at high cardiovascular risk (2) (CRP >3 mg/l) showed higher plasma MMP-1 and -10 and increased carotid IMT, a surrogate marker of atherosclerosis (34). The association between CRP and both MMPs remained significant after adjusting for cardiovascular risk factors. These observations suggest a close relationship between ongoing inflammatory markers and systemic proteolytic activation with subclinical atherosclerosis, and extend previous data showing that elevated CRP also predicts recurrent instability and mortality in patients with coronary disease (35,36).

Immunohistochemistry for CRP, MMP-1, and -10 in human arteries. C-reactive protein can accumulate in atherosclerotic lesions and may be expressed by cells in advanced atherosclerotic plaques (37,38). In addition, elevation of both MMP-1 and -10 has been reported in these plaques (11,12,39).

Our present novel observations demonstrate increased CRP, MMP-1, and MMP-10 in macrophage-rich regions from endarterectomy specimens as compared with mammary arteries. C-reactive protein and MMP-10 also showed a strong signal within the endothelial layer of advanced human atherosclerotic plaques. This novel finding could be related to the presence of CRP adsorbed and transported into the arterial wall as previously described (5,25), also in agreement with a recent in vitro study showing CRP expression in HAEC (40).

Interestingly, CRP and MMP-10 colocalized within regions previously described as rupture prone. Given the importance of MMPs in weakening atherosclerotic plaques (22,30,41), increased local and systemic MMP activation may contribute to the association of CRP with cardiovascular events caused by plaque complication.

Study limitations. This study did not assess the effect of CRP on collagen synthesis; however, previous studies showed no increase in collagen synthesis in advanced atherosclerotic lesions (13). Our in vivo and in vitro data are

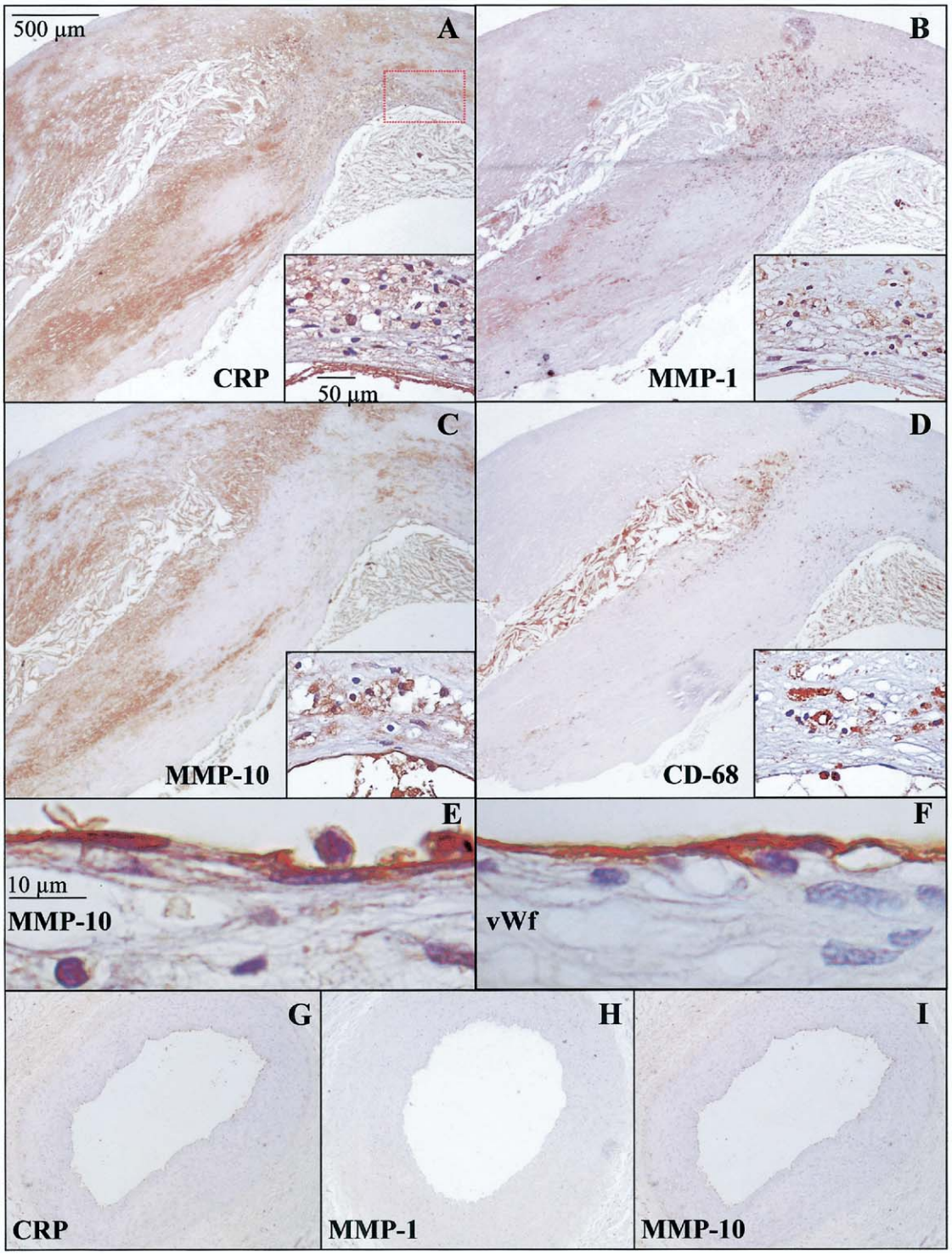

Figure 4. (A to C) Representative immunohistochemical analysis of C-reactive protein (CRP), matrix metalloproteinases (MMP)-1, and -10 in adjacent sections of human atherosclerotic plaques. Area squared in **red** corresponds to magnified details. Higher magnification revealed intense positive signal for CRP, MMP-1, and MMP-10 within macrophage-rich areas (CD-68 positive) **(D)**. Magnification detail of endothelial layer (×100) showed that endothelial cell, positive for von Willebrand factor (vWf), also expressed MMP-10 **(E and F)**. **(G to I)** Inmmunolocalization of CRP, MMP-1, and MMP-10 in control mammary arteries.

consistent with a causal role for CRP in atherosclerosis although a cause/effect relationship cannot be assumed. The CRP concentrations employed in vitro were higher than those observed in the plasma of patients at high cardiovascular risk. However, because the CRP mRNA levels in atherosclerotic plaques are 10-fold higher than in normal arteries (42), it is likely that local CRP levels in atherosclerotic lesion are sufficient to stimulate MMP secretion by

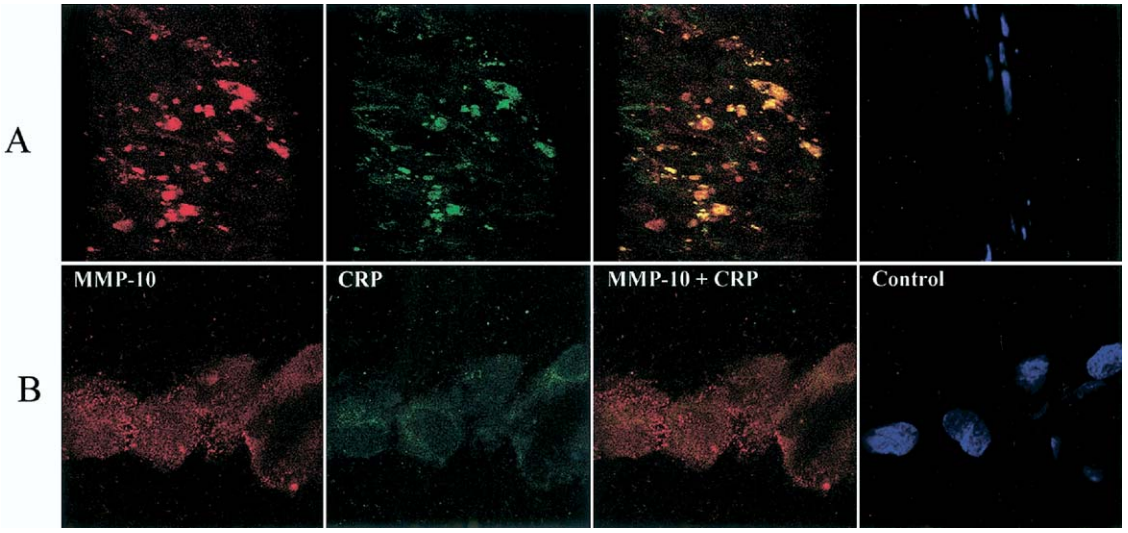

Figure 5. Confocal microscopy in advanced human atherosclerotic plaques (n = 5). Matrix metalloproteinases (MMP)-10 **(red)** and C-reactive protein (CRP) **(green)** colocalized **(yellow to orange)** in macrophage-rich areas **(A)** and endothelial layer **(B)**. No signal was detected in the absence of the primary antibodies (control). Nuclei were counterstained with TOPRO-3 **(blue)**.

EC. Finally, another report found no effect of CRP on HUVEC, likely due to different experimental conditions (different culture medium and supplement growth factors) (20).

The present study adds to evidence that CRP may not merely serve as a marker but also as a proatherogenic factor and regulator of plaque biology through endothelial proteolysis, thus providing a link between inflammation and plaque instability.

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