Allele-Specific Regulation of Matrix Metalloproteinase-7 Promoter Activity Is Associated With Coronary Artery Luminal Dimensions Among Hypercholesterolemic Patients

Sofia Jormsjö, Carl Whatling, Dirk H. Walter, Andreas M. Zeiher, Anders Hamsten, Per Eriksson

Abstract—An enhanced expression of matrix metalloproteinase (MMP)-7 has previously been demonstrated in atherosclerotic and aneurysmal tissue. Because perturbed regulation of MMP-7 may influence the development of these diseases, we searched the MMP-7 promoter for functional polymorphisms. An A to G substitution at position −181 (−181 A/G) and a C to T substitution at position −153 (−153 C/T) with frequencies of 0.50 and 0.10, respectively, were identified. Allele-specific associations were studied in 350 patients undergoing percutaneous transluminal coronary angioplasty. Hypercholesterolemic patients carrying the −181G allele or the −153T allele had smaller reference luminal diameters before percutaneous transluminal coronary angioplasty. Reverse transcription–polymerase chain reaction demonstrated that expression of MMP-7 was confined to differentiated U937 cells. Northern blot analysis could not detect an effect of native or oxidatively modified low density lipoprotein on MMP-7 expression. Thus, the limitation of allele-specific effects on vessel wall remodeling to hypercholesterolemic patients may be secondary to lipid-mediated accumulation of MMP-7–expressing monocyte-derived macrophages within the vessel wall. Both polymorphisms influenced the binding of nuclear proteins. Furthermore, in transient transfection studies, the combination of the 2 rare alleles conferred an increased promoter activity. In conclusion, the present study identified and characterized 2 common polymorphisms in the promoter region of the MMP-7 gene that are functional in vitro and seem to influence coronary arterial dimensions in hypercholesterolemic patients with manifest coronary artery disease. (Arterioscler Thromb Vasc Biol. 2001;21:1834-1839.)

Key Words: matrix metalloproteinase-7 ■ matrilysin ■ polymorphisms ■ LDL ■ gene regulation

In atherosclerosis, matrix remodeling is believed to influence the migration and proliferation of cells within the plaque. Several groups of proteolytic enzymes are able to degrade components of the extracellular matrix. Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that have the ability to degrade all components of the extracellular matrix. Therefore, MMPs have been implicated in atherogenesis, aneurysm formation, and plaque rupture.1 The MMP family includes collagenases, gelatinases, elastases, and stromelysins. Of the cells present in the atherosclerotic plaque, smooth muscle cells, endothelial cells, and macrophages have the capacity to synthesize and secrete a wide range of MMPs. Therefore, perturbed regulation of MMP activity would be envisaged to influence the risk of cardiovascular disease.

MMP-7 (also known as matrilysin or PUMP-1) is a protease with broad substrate specificity, being able to degrade elastin, proteoglycans, fibronectin, and type IV collagen. MMP-7 is among the smallest members of the MMP family described so far.2 It lacks the COOH-terminal hemopexin-like domain contained in all other members of the MMP-family. The function of this domain is unknown, but it may be involved in determining substrate specificity, or it may represent a region that recognizes a cell surface receptor. MMP-7 was first characterized from a human rectal carcinoma cell line, and overexpression of MMP-7 has been linked to colorectal cancer.3 In addition, it has been suggested that increased MMP-7 activity can play an important role in coronary artery disease (CAD). First, MMP-7 is expressed by lipid-laden macrophages at sites of potential plaque rupture in atherosclerotic lesions.4 By use of in situ hybridization and immunohistochemistry on carotid endarterectomy specimens, a prominent expression of MMP-7 was demonstrated at the border between the acellular lipid core and the overlying fibrous area. Second, MMP-7 mRNA and protein are overexpressed in abdominal aortic aneurysms.5 In aneurysmal tissue, increased activity of matrix-degrading enzymes has been linked to the presence of disease, causing destruction of the vessel wall. Therefore, studies on the regulation of matrix-degrading enzymes are important in the understanding of different manifestations of vascular disease, such as the.
atherosclerotic process, aneurysm formation, and events leading to plaque destabilization and plaque rupture.

There is growing evidence that dysregulation of gene expression may play an important role in determining the risk of vascular disease. However, the genetic component remains obscure. The increased proteolytic activity within the coronary arterial wall that is associated with coronary heart disease suggests that functional polymorphisms in genes involved in matrix remodeling may account for part of the genetic predisposition to atherosclerosis and its clinical manifestations.6 In the present work, we searched the promoter region of the MMP-7 gene for common variations that could influence MMP-7 expression and thus be of importance for vascular remodeling and clinical CAD. Furthermore, we analyzed the effects of LDL on the expression of MMP-7.

Methods

Single-Strand Conformation Polymorphism Analysis

Polymerase chain reaction (PCR) was carried out with 0.3 μCi (α-32P)dATP per reaction. The promoter was amplified from genomic DNA from 30 healthy individuals by using 3 sets of primer pairs covering the region from a sequence 1200 to a sequence 2000 mapping (a) position 1, forward primer 5’-AATTGAGGAGTGTGGCTCTAGT and reverse primer 5’-GGGAACTGCTCTCATTGC; fragment 2, forward primer 5’-GTGAGATTTTGTTGGCTTGG and reverse primer 5’-TCAGATTGCTGCTCTCGAACT and fragment 3, forward primer 5’-TGTTACCATAGTCTGCTTGAATG and reverse primer 5’-TTATATAGCTTCTCAGCTGTC. PCR fragments 1 and 2 were digested with restriction endonucleases NglI and BstYI, respectively. The digests, mixed with an equal volume of stop solution (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), were denatured at 95°C for 3 minutes and loaded onto a 4% nondenaturing polyacrylamide gel (49:1) with or without 10% glycerol. Electrophoresis was performed at room temperature for 12 to 16 hours or at 4°C for 3 to 6 hours. The exact location of polymorphisms was established by direct sequencing with the use of the ABI PRISMA dye terminator cycle sequencing core kit (Perkin-Elmer).

Genotyping

Genotyping for the –181 A/G and –153 C/T polymorphisms was performed by PCR-based restriction fragment length polymorphism analysis and digestion with restriction enzymes EcoRI and NlaIII, respectively. The forward primer for the –181 G/A polymorphism was 5’-TGGTACATATGGCTCTGATG and the reverse mismatch primer was 5’-TCAGTTGCTGCTCTCGAACT. The forward primer for the –153 C/T polymorphism was 5’-ACGAAATACATGTGCTCCTGCGAATCA and the reverse primer was 5’-TTTATAGCTCTCAGGCGTCC.

Cell Culture

The human monocyte/macrophage cell line U937 was maintained in 7.5% FCS–RPMI 1640 medium. Cell density was kept between 0.2 to 1 million cells per milliliter. For differentiation experiments, cells (10 mL) were seeded in 10-cm2 dishes at 2 to 1 million cells per milliliter. For differentiation experiments, cells were kept at 37°C with 10% CO2 and 95% air.

Electrophoretic Mobility Shift Assay

Nuclear extract preparations and electrophoretic mobility shift assay (EMSA) were accomplished as described.7 The sequences of the probes were CTTTGAAGAGCAGAACATACTTGTTCTGC and CTTTGAAGAGCAGAACATACTTGTTCTGC for the –181 G/A polymorphism and TCTGGCCAATGAGATGTAATAC and TCCTGGCCAATGAGATGTAATAC for the –153 C/T polymorphism. Incubation for EMSA was conducted essentially as described.7 The sequences of the EMSA probes used for analysis of both polymorphisms simultaneously were CCTTGGAAAGAAGATACATTGTGTGTGCTTC and CCTTTGAAAGAAGATACATTGTGTGTGCTTC for the –181 A/G–153 C and –181 G/A–153 T haplotypes, respectively. Polymorphic sites are underlined.

Transfection and Transient Gene Expression Assay

Four DNA constructs covering the region from –298 to 41 of the MMP-7 promoter and thereby harboring both of the polymorphic sites were designed by PCR and ligated into a TA cloning vector (Invitrogen). Three combinations could be picked up from genomic DNA, and the fourth variant was constructed by using the Quick Change Site-Directed Mutagenesis kit (Stratagene). The constructs were then cut and ligated into pKpn- and XhoI-restricted PGL-3 Basic Vector (Promega). Transient transfection was as described.7 Luciferase levels were expressed as arbitrary units after standardization against Renilla luciferase levels.

RNA Isolation and RT-PCR

Total RNA (1 μg) extracted from U937 cells was reverse-transcribed using oligo dT(15) and Superscript II Reverse Transcriptase (Life Technologies), according to the manufacturer’s instructions. First-strand cDNA was made up to 200 μL with 10% glycerol, and 1 μL was used as template in each reverse transcription (RT)-PCR. The RT-PCR reactions were performed in 25-μL reactions containing 1.5 mmol/L Mg2+ or 2 mmol/L Mg2+ (MMP-7 and Mac 1), 5 pmol each primer, and 1 μL Taq DNA polymerase (Promega). Amplification conditions were as follows: 94°C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, 56°C for 20 seconds, 72°C for 1 minute, and a final 8-minute extension at 72°C. Aliquots (5 μL) of each PCR were analyzed.

Lipoprotein Preparation

Lipoprotein preparations and UV oxidation were performed as described.9 Lipid peroxidation was measured by quantification of thiobarbituric acid-reactive substance specified as nanomoles malondialdehyde per milligram of protein.

Northern Blot

Electrophoretic separation of 10 μg of total RNA of 0.5 μg of mRNA and Northern transfers were performed according to standard procedures.10 cDNA probes were labeled by using a Megaprime DNA labeling system (Amersham Pharmacia Biotech). Hybridizations were performed overnight by using a prehybridization buffer containing 5×106 cpm of denatured 32P-labeled probe per milliliter. Membranes were washed twice in 2× SSPE and once in 0.1× SSPE; each wash was at room temperature for 15 minutes. Washed membranes were exposed to x-ray film (Kodak) at –80°C for between 1 and 7 days.

Human DNA Samples

Three cohorts were genotyped for the MMP-7 polymorphisms. First, allele frequencies were determined in 97 healthy men of northern European descent, aged 23 to 45 years (40.3±3.4 years, mean±SD), who had been recruited at random from the general population living in Stockholm County, Sweden. Second, a total of 96 white men11 with a first myocardial infarction before the age of 45 years (age range 21 to 44 years) who belonged to a consecutive series of 131 male patients13 were selected for genotyping. Third, genotype relationships to the vascular response after percutaneous transluminal coronary angioplasty (PTCA) with stent implantation were investigated in 350 consecutive white patients (age range 38 to 88 years, 89% men) undergoing this procedure between January 1996 and March 1997 in the Department of Internal Medicine of the University Hospital, Johann Wolfgang Goethe University, Frankfurt, Germany.13 Indications for stenting were coronary dissection after prior PTCA of the same segment. Patients undergoing stent implant-
The MMP-7 promoter was screened for polymorphisms by single-strand conformation polymorphism analysis. The result of the PCR-based genotyping was in total agreement with that obtained by single-strand conformation polymorphism analysis and DNA sequencing. Both genotype associations with that obtained by single-strand conformation polymorphism analysis and DNA sequencing. Both genotype and allele frequencies were 0.57/0.43 and 0.95/0.05 for the −181 A/G and −153 C/T polymorphisms, respectively.

Genotype Associations With Coronary Artery Luminal Dimensions

To explore potential genotype-phenotype associations of the −181 A/G and −153 C/T polymorphisms, genotype groups were compared with respect to QCA measurements of the coronary segment subjected to intervention in the patients undergoing PTCA with stent implantation. The QCA analyses were performed on coronary angiograms taken immediately before and after PTCA and at follow-up 4 to 6 months later. No allele-specific differences were found when the entire patient group was examined (n = 350). However, hypercholesterolemic individuals (152 men and 24 women with cholesterol >220 mg/dL) carrying the −181G allele had a smaller reference diameter before PTCA (P < 0.01), as shown in the Table. Furthermore, the −153T allele was associated with a smaller reference diameter before PTCA (P = 0.05), as shown in the Table. There were no associations between the 2 polymorphisms and minimal luminal diameter measurements (data not shown), nor were there any associations in the subset of patients with diabetes.

Role of Hypercholesterolemia

Because the influence of the −181 A/G and −153 C/T polymorphisms on coronary artery dimensions was confined to hypercholesterolemic patients, there arises the question of whether a cholesterol-induced MMP-7 transcription is needed for the polymorphisms to have an impact on MMP-7 transcription. Alternatively, increased MMP-7 expression could be a result of enhanced monocyte differentiation within the vessel wall that is due to hypercholesterolemia. Therefore, the endogenous MMP-7 mRNA production during the differentiation of U937 cells was analyzed by using RT-PCR. Undifferentiated U937 cells produced only small amounts of MMP-7 mRNA (Figure 2). However, there was a strong induction of MMP-7 mRNA on differentiation with the use of PMA for 24 hours. In contrast, 50 μg/mL native LDL did not influence MMP-7 mRNA levels in either differentiated U937 or THP-1 cells (Figure 3). Three different levels of LDL oxidation were then used to study the effect of oxidized LDL oxidation on MMP-7 mRNA production.

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<tr>
<th>Reference Luminal Diameter in Hypercholesterolemic Patients Undergoing PTCA With Stent Implantation</th>
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<td>Diameter</td>
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<td>Before PTCA, mm</td>
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<td>After PTCA, mm</td>
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Values are mean±SE. One-way ANOVA was performed to test whether genetic variation in the MMP-7 promoter was associated with differences in reference luminal diameter.

*Comparing carriers of the T allele and individuals homozygous for the C allele.
In the present study, we identified and characterized 2 common polymorphisms in the promoter region of the MMP-7 gene that are functional in vitro and seem to influence coronary arterial dimensions in a preliminary study.

Discussion

In the present study, we identified and characterized 2 common polymorphisms in the promoter region of the MMP-7 gene that are functional in vitro and seem to influence coronary arterial dimensions in a preliminary study.
of hypercholesterolemic patients with manifest CAD. Hypercholesterolemic patients carrying the G allele at position −181 had smaller reference luminal diameters before PTCA than did patients homozygous for the A allele. Furthermore, carriers of the T allele at position −153 had smaller reference diameters before PTCA than did patients homozygous for the C allele. In vitro in the human monocyte/macrophage cell line U937, the −81 A/G and the −153 C/T polymorphisms influenced the binding of nuclear proteins. Also, basal promoter activity was higher in promoter constructs harboring the combination of the 2 rare alleles in transient transfection studies.

The finding that the G allele of the −181 A/G and the T allele of the −153 T/C polymorphisms, both of which are associated with higher basal transcriptional activity in vitro, influence coronary artery dimensions only within the subgroup of hypercholesterolemic patients suggests either that there is an allele-specific effect of cholesterol on MMP-7 expression or that MMP-7 is expressed under only hypercholesterolemic conditions. Our results favor the latter interpretation, inasmuch as we could not detect any significant effect of native LDL or oxLDL on MMP-7 mRNA expression. However, allele-specific effects of oxLDL on MMP-7 mRNA expression cannot be excluded because only 1 allelic type of U937 cells was used. Furthermore, MMP-7 was expressed only in differentiated U937 cells. Possibly, hypercholesterolemia may result in accumulation of activated monocyte-derived macrophages that express increased amounts of MMP-7 within the affected vessel wall. Indeed, previous studies have demonstrated that entrapment of LDL particles induces production of proinflammatory molecules (such as monocyte-specific chemotraactants) that accelerate the atherosclerotic process.16,17 Furthermore, oxidatively modified LDL has been demonstrated to enhance monocyte adhesion to endothelial cells in vitro through the activation of CD11b.18,19 However, we cannot rule out that there is a direct effect of lipids contained in LDL particles on MMP-7 expression in vascular foam cells and that this effect is allele specific. Interestingly, in the Regression Growth Evaluation Statin (REGRESS) study, a clinical trial assessing the effect of the potent cholesterol-lowering compound pravastatin on progression of coronary atherosclerosis, an allele-specific effect of a common polymorphism within the promoter of the MMP-3 gene on the number of clinical events was observed mainly within the placebo group, with persisting mild to moderate hypercholesterolemia, and not within the pravastatin group, in which substantially lower plasma cholesterol concentrations were attained.20

There were no associations between the 2 polymorphisms and minimal luminal diameter at the different time points. In addition, there was no allele-specific effect on the change in minimal luminal dimension during the 6-month period after PTCA. This suggests that the polymorphisms did not influence the restenotic process occurring at the site of intervention after PTCA. In contrast, both polymorphisms were associated with the reference luminal diameter. There are both clinical and experimental data suggesting that arterial remodeling at the reference site may occur. For example, this has been demonstrated in New Zealand White rabbits.21 At a 4-week follow-up, the minimal lumen diameter and the reference diameter differed significantly between restenotic and nonrestenotic segments. In addition, in the Multicenter European Research Trial With Cilazapril After Angioplasty to Prevent Transluminal Coronary Obstruction and Restenosis (MERCATOR) study, it was concluded that the restenotic process also involves the apparently normal vessel wall adjacent to the actual lesion.22 However, in these 2 examples, the remodeling at the reference site was analyzed after PTCA and occurred in association with ongoing restenosis. In the present study, we detected allele-specific effects of the MMP-7 polymorphisms before PTCA, and there were no effects on the remodeling process at the site of intervention. It may be speculated that the allele-specific differences in reference diameter are a result of MMP-7–mediated effects on diffuse atherosclerosis. The process of remodeling appears to be most prominent during the early phases of atherosclerosis,23 which may explain why there were no allele-specific effects on the minimal luminal diameter before PTCA. The lack of association between the 2 polymorphisms and minimal luminal diameter after PTCA, on the other hand, could be secondary to stent implantation. It should also be emphasized in this context that 42% of the patients had undergone prior PTCA of the same segment. The fact that a significant proportion of study segments had thus been subjected to secondary to stent implantation. It should also be emphasized in this context that 42% of the patients had undergone prior PTCA of the same segment. The fact that a significant proportion of study segments had thus been subjected to remodeling at the reference site was analyzed after PTCA and occurred in association with ongoing restenosis. In the present study, we detected allele-specific effects of the MMP-7 polymorphisms before PTCA, and there were no effects on the remodeling process at the site of intervention. It may be speculated that the allele-specific differences in reference diameter are a result of MMP-7–mediated effects on diffuse atherosclerosis. The process of remodeling appears to be most prominent during the early phases of atherosclerosis, which may explain why there were no allele-specific effects on the minimal luminal diameter before PTCA. The lack of association between the 2 polymorphisms and minimal luminal diameter after PTCA, on the other hand, could be secondary to stent implantation. It should also be emphasized in this context that 42% of the patients had undergone prior PTCA of the same segment. The fact that a significant proportion of study segments had thus been subjected to recent remodeling may explain why the MMP-7 genotype effect was most evident before PTCA.

The results of the EMSA showing increased affinity of a nuclear protein to the −181G allele along with the increased promoter activity conferred by the −181G allele in combination with the −153T allele in transient transfection experiments suggest that the allele-specific regulation of the −181 A/G polymorphism could be a result of enhanced binding of a transcriptional activator to the −181G allele (complex I, Figure 4A). The interpretation of the functionality of the −153 C/T polymorphism is more complex. Three factors show allele-specific binding. The experiment using unlabeled probes as competitors in the EMSA reaction (Figure 4B, lanes 7 to 14) demonstrates that the upper and more slowly
migrating protein-DNA complex (complex II) is formed only with the T allele, whereas the proteins of the lower complexes (complexes III and IV) bind with higher affinity to the C allele. The lower affinity of protein complex III and IV to the T allele could be secondary to competition between the factor binding to the T allele (complex II) and the common factors binding to both alleles (complex III and complex IV). Whether the increased promoter activity of the T allele is a result of complex II acting as a transcriptional activator or results from the lower affinity of complexes III and IV to the T allele, which thus act as transcriptional repressors could not be determined in the present study. Furthermore, the interaction between the 2 polymorphic sites makes the evaluation of the underlying mechanisms of the increased promoter activity of the −181G/−153T haplotype even more difficult.

The functional synergy between the two polymorphic sites demonstrated in vitro suggests that there may also be synergistic or cooperative effects of the 2 polymorphisms on the reference diameter in vivo. Unfortunately, the limited patient sample precluded haplotype analysis. However, the finding that the −181G/−153T haplotype is associated with the highest transcription activity in vitro would be in agreement with the observation that the smallest luminal diameter (2.50 mm before PTCA) was found in a hypercholesterolemic patient with this allelic combination. Needless to say, it is impossible to draw any conclusions from observations made in only 1 patient, and replication of this finding is required in a much larger cohort.

In summary, the present work demonstrates the existence of functional polymorphisms influencing the transcriptional activity of the MMP-7 gene and suggests a role of MMP-7 in the matrix remodeling associated with CAD. However, firm conclusions regarding the clinical significance of the MMP-7 promoter polymorphisms cannot be drawn until much larger cohorts have been analyzed.

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References

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