Allele-Specific Regulation of Matrix Metalloproteinase-12 Gene Activity Is Associated With Coronary Artery Luminal Dimensions in Diabetic Patients With Manifest Coronary Artery Disease

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Abstract—Both the processes of atherosclerosis and plaque rupture are indicated to be influenced by matrix metalloproteinase (MMP) activity. We therefore searched for common functional variation in the matrix metalloelastase (MMP-12) gene locus that may be implicated in coronary artery disease. Single-strand conformation polymorphism analysis of DNA from healthy individuals detected a common polymorphism within the MMP-12 gene promoter (an A-to-G substitution at position –82). The frequency of the G allele was 0.19. The polymorphism influences the binding of the transcription factor activator protein-1 (AP-1) in electromobility shift assay. A higher binding affinity of AP-1 to the A allele was associated with higher MMP-12 promoter activity in vitro in transient transfection studies in U937 and murine lung macrophage (MALU) cells. Phorbol 12-myristate 13-acetate (PMA) and insulin, 2 known activators of AP-1, increased the binding of AP-1 to the MMP-12 promoter, with higher affinity for the A allele. In transfection experiments, both the A and the G alleles responded to insulin and PMA, the A allele showing higher promoter activity than the G allele. Furthermore, Western blot analysis demonstrated that insulin increased MMP-12 protein production. To analyze whether the –82 A/G polymorphism is associated with coronary artery disease, 367 consecutive patients who underwent percutaneous transluminal coronary angiography with stent implantation were genotyped. In patients (n = 71) with diabetes, the A allele was associated with a smaller luminal diameter. In conclusion, a common functional polymorphism within the MMP-12 promoter influences coronary artery luminal dimensions in diabetic patients with manifest coronary artery disease. (Circ Res. 2000;86:998-1003.)

Key Words: metalloproteinase-12 ■ matrix ■ polymorphism ■ insulin ■ activator protein-1

Matrix metalloproteinases (MMPs) are closely related proteinases that together are able to degrade all macromolecules present in the connective tissue matrix. MMPs are potentially implicated in both atherogenesis and the precipitation of acute coronary syndromes by regulating connective tissue remodeling, thus determining the volume expansion of the atherosclerotic plaque, its stability, and the potential for smooth muscle cell proliferation.1 Indeed, both smooth muscle cells and macrophages synthesize and secrete a range of MMPs, and MMP expression has been demonstrated in human atherosclerotic plaques.3-7 The regulation of MMP expression is complex and takes place at both the transcriptional and the posttranscriptional levels. Phorbol 12-myristate 13-acetate (PMA); insulin; and several cytokines and growth factors, such as interleukin-1, platelet-derived growth factor, and tumor necrosis factor-α induce MMPs in vitro, whereas other agents, such as transforming growth factor-β, heparin, and corticosteroids have an inhibitory effect.1,8,9

Elastin is the major component of elastic fibers that provide resilience to tissues exposed to dynamic stress. MMP-12 (macrophage elastase) was first identified as an elastolytic metalloproteinase secreted by activated macrophages.10,11 Besides elastase activity, MMP-12 displays a broad substrate specificity, including extracellular matrix (ECM) proteins such as fibronectin, laminin, vitronectin, type IV collagen, and heparan sulfate.12,13 Thus, MMP-12 not only digests elastin but also degrades the basement membrane, which enables macrophages to penetrate injured tissues during inflammation. It is notable that lipid-laden macrophages located in the boundary between the acellular lipid core and fibrous areas in atherosclerotic plaques express MMP-12.7

In the present study, we have identified a common polymorphism within the MMP-12 gene promoter (an adenosine
[A]→guanosine [G] substitution at position −82) that influences the binding of the transcription factor activator protein-1 (AP-1). There is also an allele-specific response of MMP-12 promoter activity to both insulin and PMA. Furthermore, the MMP-12 −82 A/G polymorphism is associated with coronary artery luminal dimensions in diabetic patients requiring percutaneous transluminal coronary angioplasty (PTCA) with stent implantation.

Materials and Methods

Single-Strand Conformation Polymorphism (SSCP) Analysis

SSCP analysis was performed as described. The promoter was amplified from genomic DNA obtained from 40 healthy individuals using 3 sets of primer pairs covering the region from −1064 to +145.

Genotyping

A polymerase chain reaction (PCR)–based restriction fragment length polymorphism (RFLP) introducing a PvuII restriction site using mismatch PCR primer was performed, and products were separated on a 3% Metaphor agarose gel (FMC BioProducts).

Cell Culture

U937 and MonoMac cells were maintained in RPMI 1640 with 7.5% or 10% FCS, respectively. Murine lung macrophage (MALU) cells were obtained from the Sir William Dunn School of Pathology Sciences Cell Bank and cultured in RPMI 1640 with 2 mmol/L L-glutamine and 10% FCS.

Electromobility Shift Assay (EMSA)

Double-stranded 26-mer oligonucleotides corresponding to the sequence from −91 to −66 in the MMP-12 promoter were used. Nuclear extract preparations and EMSA were as described.

Transfection and Transient Gene Expression Assay

Two DNA fragments covering the region from −155 to +28 harboring either the A or the G site were ligated into pGEL2-Basic Vector (Promega). Cells (2×10^6) were transfected with 50 μg of construct and 15 μg of β-galactosidase plasmid, and 100 nmol/L insulin or 1 μmol/L PMA was added. Cells were harvested after 24 hours. Luciferase levels were expressed in arbitrary units after standardization against β-galactosidase levels (U937 cells) or Renilla luciferase levels (MALU cells). Data are presented as percentage of unstimulated controls because of the difference in control vectors and because of variations in transfection efficiency between the different experiments.

Statistical Methods

Differences in continuous variables between groups were tested either by ANOVA with the Scheffé F test used as a post hoc test or by the Student paired t test. Values are given as mean±SE. A χ2 test was used to compare the observed numbers of each MMP-12 genotype with those expected for a population in Hardy-Weinberg equilibrium.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Identification of a Common Polymorphism in the Promoter Region of the MMP-12 Gene

PCR-SSCP analysis was performed to screen for common sequence variations within the MMP-12 promoter from positions −1064 to +145 (Figure 1A). Variations in electrophoretic mobility were detected in the fragment closest to the transcription start site in 14 of the 40 individuals (Figure 1B). Direct sequencing demonstrated that this was due to a single nucleotide substitution at position −82, where an A residue had been changed to a G (Figure 1C). Analysis of the remainder of the promoter fragment revealed no other sequence variants. To analyze the allele frequency of the −82 A/G polymorphism, 183 healthy middle-aged men were genotyped using a PCR-based RFLP. The results were consistent with those obtained by SSCP analysis and DNA sequencing. The frequency of the G allele was 0.19. The genotype distribution (117, 62, and 4 individuals belonging to the AA, AG, and GG genotype classes, respectively) was consistent with the population being in Hardy-Weinberg equilibrium.

The −82 A/G Polymorphic Site Influences the Binding of Transcription Factor AP-1

Database analysis of the sequence around the polymorphic site revealed a potential binding site for the transcription factor AP-1 (Figure 1C). An EMSA was therefore carried out using a probe corresponding to the sequence from −91 to
of the A allele, which was incubated with nuclear extracts prepared from U937 cells. As can be seen in Figure 2A, 2 major protein-DNA complexes were formed. Antibodies against c-Jun, a component of the AP-1 complex, resulted in a supershift (Figure 2A). Furthermore, when an excessive amount of an unlabeled oligonucleotide corresponding to a consensus AP-1 site as a competitor was included in the assay, the upper complex was abolished, whereas it was not when an oligonucleotide corresponding to a mutated AP-1 site was used (Figure 2A). Taken together, these results demonstrate that AP-1 (the upper complex indicated by an arrow in Figure 2A) binds to the polymorphic site.

To study the possibility of allele-specific differences in the binding of AP-1, increasing amounts of either unlabeled A or G sites were used as competitors in an EMSA. As demonstrated in Figure 2B, the A allele resulted in a stronger competition of the AP-1 complex than the G allele. Allele-specific differences in AP-1 binding were also studied in the presence of either PMA (Figure 3A) or insulin (Figure 3B), 2 known activators of AP-1 binding. Both PMA and insulin increased the binding of AP-1 to the MMP-12 promoter, with higher affinity for the A allele (mean ± SE, 57 ± 37% [n=4] and 47 ± 13% [n=3] higher binding affinity for the A probe than the G probe when stimulated with PMA or insulin, respectively) (Figures 3A and 3B). In accordance with the results obtained under basal conditions (Figure 2B), competition experiments using increasing amounts of either unlabeled A or G probe in assays with extracts from insulin-stimulated U937 cells demonstrated a stronger affinity of the AP-1 complex to the A allele (data not shown). EMSAs using nuclear extracts derived from MonoMac 6 cells showed a similar allele-specific difference in binding of AP-1 (data not shown).

**Figure 2.** The −82 A/G polymorphic site in the MMP-12 promoter influences the binding of transcription factor AP-1. A, EMSA of nuclear extract derived from U937 cells using a double-stranded 26-mer oligonucleotide corresponding to the sequence from positions −91 to −66 of the A allele of the MMP-12 promoter. Arrow indicates AP-1 complex; F, free DNA. Lane 1, without extract; lane 2, with 0.08 mg/mL extract; lanes 3 through 7, with extract in the presence of anti-fos antibodies (lane 3), anti-c-Jun antibodies (lane 4), anti-nuclear factor κB antibodies (lane 5), 100-fold excess of unlabeled AP-1 consensus site (lane 6), or 100-fold excess of unlabeled mutated AP-1 site (lane 7). B, EMSA of 0.08 mg/mL nuclear extract derived from U937 cells interacting with the A allele in the presence of increasing amounts of unlabeled double-stranded 26-mer oligonucleotide corresponding to the sequence from positions −91 to −66 of either the A allele (lanes 2 through 9) or the G allele (lanes 10 through 17) of the MMP-12 promoter. Amounts of competitors were as follows: lanes 3 and 11, 0.17 pmol; lanes 4 and 12, 0.34 pmol; lanes 5 and 13, 0.51 pmol; lanes 6 and 14, 0.69 pmol; lanes 7 and 15, 0.86 pmol; lanes 8 and 16, 1.03 pmol; and lanes 9 and 17, 1.20 pmol. Lane 1, no extract. Arrow indicates AP-1 complex; F, free DNA.

**Figure 3.** Allele-specific difference in binding of AP-1 to the −82 A/G polymorphic site of the MMP-12 promoter. A, EMSA of nuclear extract derived from U937 cells interacting with the A allele (lanes 1 through 4 and 9 through 11) or the G allele (lanes 5 through 8 and 12 through 14). Arrow indicates AP-1 complex; F, free DNA. Lanes 2 through 8, Extracts from nonstimulated cells; lanes 9 through 14, extracts from cells stimulated with 1 μmol/L PMA; lanes 1 and 6, no extract; lanes 2, 6, 9, and 12, 0.01 mg/mL extract; lanes 3, 7, 10, and 13, 0.03 mg/mL extract; lanes 4, 8, 11, and 14, 0.1 mg/mL extract. B, EMSA of nuclear extract derived from U937 cells and interacting with the A allele (lanes 1 through 3) or the G allele (lanes 4 and 5). Lane 1, no extract; lanes 2 through 5, 0.1 mg/mL of nonstimulated extract (lanes 2 and 4) and extract stimulated with 100 nmol/L insulin (lanes 3 and 5).
The MMP-12 mRNA (Figure 5B) and protein production were stimulated with 100 nmol/L insulin (143 \pm 16\% versus \(241 \pm 12\%\) [\(n = 9\)] of the luciferase activity of unstimulated controls, \(P < 0.01\)) (Figure 4). Transfection experiments were also performed using U937 cells with essentially the same results (275 \pm 64\% versus 234 \pm 43\% [\(n = 8\)] of the luciferase activity of unstimulated control for the A and G alleles, \(P = 0.07\)) (Figure 4). Furthermore, both the A and G alleles responded to insulin in transfection experiments using U937 cells. The promoter activity of the A allele tended to be slightly higher than that of the G allele when transfected cells were stimulated with 100 nmol/L insulin (143 \pm 16\% versus 123 \pm 8\% [\(n = 7\)] of unstimulated controls, \(P = 0.17\)) (Figure 4). RT-PCR and Western blot analysis were performed to study whether insulin also increased the endogenous production of MMP-12 mRNA and protein in U937 cells. As demonstrated in Figure 5, 100 nmol/L insulin resulted in an increase in both the MMP-12 mRNA (Figure 5B) and protein production in U937 cells. As demonstrated in Materials and Methods, Probability values are from a paired \(t\) test.

**Figure 4.** PMA- and insulin-mediated regulation of MMP-12 promoter activity. DNA constructs covering the region from positions −155 to +28 of either A or G genotype placed upstream of a luciferase reporter gene were transfected into MALU cells or U937 cells. Cells were stimulated with either 1 \(\mu\)mol/L PMA or 100 nmol/L insulin as described in Materials and Methods. Probability values are from a paired \(t\) test.

**Figure 5.** Insulin enhances endogenous MMP-12 expression in U937 cells. A, Western blot analysis of MMP-12 expression after insulin stimulation. Cytosolic extracts were derived from U937 cells after incubation with insulin for 48 hours. Lanes 1 and 3, Unstimulated cells; lanes 2 and 4, cells stimulated with 100 nmol/L insulin. B, RT-PCR analysis on MMP-12 (lanes 1 through 3) and GAPDH (lanes 4 through 6) mRNA levels after stimulation for 16 hours with 1 \(\mu\)mol/L PMA (lanes 2 and 5) or 100 nmol/L insulin (lanes 3 and 6).

**Discussion**

In the present study, we identified a common polymorphism within the promoter region of the MMP-12 gene locus that influences the binding of the transcription factor AP-1. The greater binding affinity of AP-1 to the A allele is associated with higher MMP-12 promoter activity in vitro. Furthermore, the A allele was associated with a smaller luminal diameter of the procedure-related coronary segment after dilation compared with the G allele in diabetic patients undergoing PTCA with stent implantation.

The transcription factor AP-1 appears to play an important and general role in regulating MMP expression. AP-1 consensus sequences have been demonstrated in the promoter regions of several MMP genes. Transfection studies have demonstrated that the AP-1 sites in the MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, and MMP-13 promoters are functional. The present work provides evidence for a functional AP-1 site also in the MMP-12 promoter. In a previous study, it was demonstrated that PMA, a well-known inducer of AP-1 activity, enhances MMP-12 mRNA levels in the murine tumor cell line P388D1. It was suggested that PMA modifies the MMP-12 mRNA level by acting on the transcriptional activity of the gene rather than on transcript stability. A putative AP-1 binding site in the MMP-12 promoter was described when the genomic clone for
MMP-12 was obtained.\textsuperscript{11} In the present study, using EMSA with antibodies against AP-1 and with competitor oligonucleotides harboring either a wild-type or a mutant AP-1 site, we show that this site is, indeed, a binding site for AP-1. Furthermore, the proximal promoter of the MMP-12 gene responds to PMA in transient transfection experiments using both U937 and MALU cell lines. This response is influenced by the – 82 A/G polymorphism flanking the AP-1 binding site and influencing the binding affinity of AP-1. The differences in response to PMA or insulin between the 2 alleles were rather small. However, this is not surprising, because the polymorphism influences the binding affinity of AP-1 instead of creating a new response element in the promoter. In addition, a small change in promoter activity may have a significant effect on a long-term lesion. Taken together, these results argue in favor of the existence of an AP-1 response element within the MMP-12 promoter.

The present work also demonstrates that insulin influences the binding of the AP-1 complex and that insulin has a stimulatory effect on MMP-12 promoter activity in vitro. The insulin receptor signal transduction cascade involves the activity of numerous cytoplasmic and nuclear effectors. When insulin binds to its receptor, cytoplasmic protein kinases are activated, some of which are contained in the mitogen-activated protein kinase pathway. A number of kinases are activated, some of which are contained in the mitogen-activated protein kinase pathway. When insulin binds to its receptor, cytoplasmic protein kinases are activated, some of which are contained in the mitogen-activated protein kinase pathway. A number of potential nuclear substrates for mitogen-activated protein kinase have been identified, such as c-Jun and c-Fos, components of the transcription factor AP-1. Accordingly, it has been demonstrated that insulin enhances the binding of AP-1 to its consensus binding site and that insulin activates transcription through an AP-1 response element.\textsuperscript{17,18}

Imbalance between synthesis and degradation of ECM proteins has been suggested to contribute to the atherosclerotic process and to restenosis after angioplasty.\textsuperscript{23} For example, matrix degradation is likely to occur when monocytes pass extracellular barriers, such as the basement membranes underlying the endothelium. Degradation of the dense network of proteoglycans will also influence the migration of smooth muscle cells. Furthermore, degradation of the ECM may also result in clinically silent plaque disruption, causing intraplaque thrombosis with a subsequent smooth muscle cell proliferative response that is analogous to that occurring after angioplasty.\textsuperscript{22–24} Another potential mechanism by which matrix turnover could influence vascular disease involves effects on the vascular tone, and it has been suggested that matrix degradation could influence vessel dilation through the NO synthase pathways.\textsuperscript{25} In the present study, we found that the A allele of the MMP-12 promoter, possessing increased transcriptional activity in vitro in monocytes/macrophages, is associated with a smaller coronary artery luminal diameter in vivo in patients with diabetes and manifest coronary artery disease requiring PTCA with stent implantation, as assessed by computer-based measurement of the reference diameter of the procedure-related coronary segment. The finding of an association between increased MMP-12 promoter activity and smaller, not larger, coronary luminal diameter is surprising. In studies of aneurysms, an increased elastolytic activity has been linked to widened luminal diameter. However, mechanisms leading to a smaller luminal diameter could also be envisioned. It has, for instance, been shown that some degradation products of elastin are chemoattractant for leukocytes and thereby potentially contribute to inflammation and the development of atherosclerosis.\textsuperscript{26,27} Alternatively, the elastase may facilitate leukocyte entry into the vessel wall by breaking down mechanical barriers. However, it cannot be excluded that the allele-specific effect on the luminal diameter is a result of decreased elasticity of the vessel wall due either to altered MMP-12 elastolytic activity or to an effect on vascular tone. Further studies with analysis of multiple sites of the coronary arteries and analysis of patients with aneurysms are needed to resolve this issue. An association between the – 82 A/G polymorphism and MLD was present after PTCA. This finding is difficult to interpret, because the stent implantation should prevent effects on the elasticity of the procedure-related coronary segment. A possible explanation is that allele-specific differences in vessel elasticity at the time of PTCA and stent implantation could be the cause of these results. The finding that there was no allele-specific difference in the change of MLD between the postangioplasty and follow-up angiographies suggests that luminal narrowing/restenosis after PTCA is not influenced by MMP-12 expression. Needless
to say, the present study cannot distinguish whether the relationship of the MMP-12 polymorphism to the reference diameter reflects differences in atherosclerosis in this area or is a result of differences in vascular elasticity. It should also be stressed in this context that we have no direct evidence that the increased transcriptional activity associated with the A allele is accompanied by increased protease activity in vivo. Nevertheless, the association between the −82 A/G polymorphism and the luminal dimensions in diabetic patients requiring angiography argue that this may, indeed, be the case.

The fact that AP-1 binding sites are present in several promoters of the MMP gene family implies that insulin may have a stimulatory effect also on other MMP genes. Recently, the AP-1 motif of the MMP-1 promoter was shown to be the target for insulin signaling in vitro. Because hyperinsulinemia is a common finding among patients with type II diabetes as well as in nondiabetic patients with manifest coronary artery disease, this could have profound consequences for the atherosclerotic process and the risk of restenosis and/or the regulation of vascular tone in diabetic patients as well as in patients with other causes of elevated AP-1 activation. However, increased MMP expression in vivo remains to be demonstrated in diabetic patients. Large-scale clinical studies are also needed to determine whether the −82 A/G polymorphic site influences the atherosclerotic process or the risk of restenosis, particularly in patients with type II diabetes and in nondiabetic individuals with hyperinsulinemia secondary to insulin resistance.

In summary, the major findings of the present work are that (1) there is a functional AP-1 response element in the MMP-12 promoter; (2) transcription factor AP-1 is involved in insulin-enhanced MMP-12 expression; (3) a common A/G polymorphism in the MMP-12 promoter influences the binding affinity of AP-1; and (4) in a preliminary study, the A/G polymorphism in the promoter region influences the binding affinity of AP-1.

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References


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