Progression of Coronary Atherosclerosis Is Associated with a Common Genetic Variant of the Human Stromelysin-1 Promoter Which Results in Reduced Gene Expression*

(Received for publication, November 7, 1995, and in revised form, January 25, 1996)

Shu Ye†, Per Eriksson‡, Anders Hamsten§, Markku Kurkinen†, Steve E. Humphries‡, and Adriano M. Henney†

From the Division of Cardiovascular Genetics, Department of Medicine, University College London Medical School, London WC1E 6JJ, United Kingdom; the Atherosclerosis Research Unit, King Gustaf V Research Institute, Karolinska Hospital, Stockholm S-171 76, Sweden, and the Center for Molecular Medicine and Genetics and Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48202.

*This work was supported by Grants PG/92021, FS/95011, and RG16 from the British Heart Foundation, Grant 8691, from the Swedish Medical Research Council, and by the Swedish Heart-Lung Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed. Present address: The Welcome Trust Centre for Human Genetics, University of Oxford, Windmill Road, Oxford OX3 2BN, United Kingdom. Tel.: 44-01865-742441; Fax: 44-01865-742196; E-mail: adriano.henney@well.ox.ac.uk.

‡This support was provided by the Medical Research Council, and by the Swedish Heart-Lung Foundation.

§Address correspondence and reprints to Steve E. Humphries, Department of Medicine, University College Hospital, London WC1E 6JJ, United Kingdom.

1 The abbreviations used are: MMP, matrix metalloproteinase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; PAI-1, plasminogen activator inhibitor-1; VSMC, vascular smooth muscle cell; TIMP, tissue inhibitor of metalloproteinases.

Stromelysin-1 is a key member of the matrix metalloproteinase (MMP) family, with a broad substrate specificity. It can degrade types II, IV, and IX collagen, proteoglycans, laminin, fibronectin, gelatins, and elastin (1–3). In addition, stromelysin-1 can also activate other MMPs such as collagenase, matrix metalloelastase, and gelatinase B, rendering stromelysin-1 crucial in connective tissue remodeling (4–6). Expression of stromelysin-1 is primarily regulated at the level of transcription, where the promoter of the gene responds to various stimuli, including growth factors, cytokines, tumor promoters, and oncogene products (7–10). The regulatory effects of such stimuli are mediated through a number of cis-elements located in the stromelysin-1 promoter. For instance, the activator protein-1 binding site at positions −63 to −70 is necessary for the basal expression of the gene and is also involved in interleukin-1 induction (11–13). A promoter element located between −1218 and −1202 is responsible for the induction of stromelysin-1 expression by platelet-derived growth factor B/B (14, 15), whereas three sequences that share strong homology with the glucocorticoid-responsive consensus element are likely to be involved in the dexamethasone suppression (16).

Over the last few years, MMPs have been implicated in the connective tissue remodeling during atherogenesis (17–22). By in situ mRNA hybridization, we originally demonstrated the presence of stromelysin-1 in coronary atherosclerotic plaques (18). Extensive expression of the stromelysin-1 gene was localized particularly to the regions considered prone to rupture, such as the cap and its adjacent tissues. These observations have been supported subsequently by the studies of Galis et al. (19, 20), who demonstrated the gelatinolytic and caseinolytic activity in atherosclerotic but not in the uninvolved arterial tissues, using in situ zymography, a method that allows direct detection and microscopic localization of MMP activity in tissue sections. Additional reports of MMP expression in atheroma have also appeared (21, 22).

Using single strand conformation polymorphism analysis, we recently identified a common variant in the promoter of the stromelysin-1 gene (23). This variant gives rise to one allele having a run of six adenosines (6A) and another having five adenosines (5A) at position −1171 according to the sequence published by Quinones et al. (16). The frequency of the two alleles (5A/6A) was found to be 0.51/0.49 in a sample of 354 healthy individuals from the United Kingdom. However, in a study of 72 patients with coronary heart disease defined by angiography, the 5A/6A polymorphism was associated with progression of coronary atherosclerosis, with those who were homozygous for the 6A allele showing more rapid progression of both global and focal atherosclerotic stenoses over the 3-year study period (17). This observation supports the findings by others that the MMPs are involved in the connective tissue remodeling during atherogenesis (18–22).

It was, however, unclear whether the 5A/6A polymorphism plays a role in the regulation of stromelysin-1 expression, or if...
it is in linkage disequilibrium with variants elsewhere at the gene locus that are functional. Therefore, we carried out experiments to address this question and report here the results of these studies.

**EXPERIMENTAL PROCEDURES**

Cell Culture — Human fetal foreskin fibroblasts (HFFF2) were purchased from ECACC. Primary human vascular smooth muscle cells (VSMCs) were kindly provided by Dr. J. Nilsson, Karolinska Hospital, Stockholm, Sweden. A rat vascular smooth muscle cell line (A10) was a kind gift from Dr. O. C. Yalkinoglu, Bayer AG, Wuppertal, Germany. Cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 5% (v/v) fetal calf serum (Life Technologies, Inc.). Cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal calf serum (Life Technologies, Inc.) and antibiotics. The Lipofectin-DNA mixture was incubated at room temperature. The unbound free probes were separated from the DNA-protein complexes by electrophoresis through a 7% nondenaturing polyacrylamide gel (the ratio of acrylamide/bisacrylamide = 80:1) in 0.25 × TBE buffer for 2 h at 4°C, 200 V. The gels were then exposed to Hyperfilm-MP (Amersham) at room temperature without intensifying screen for 1 week.

DNAse I Footprinting Assays — Two double-stranded 35/36-mer oligonucleotides (CTTTGATGGGGGGAAA(A)ACATGCTGTGCTGGA) were ligated into the top or bottom strand. The 32P-labeled probes were then incubated with nuclear extracts from HFFF2 in conditions identical to those for the EMSA described above. The protein-DNA complexes were fractionated on a 7% polyacrylamide gel, and the retarded bands were electroblotted onto a DEAE membrane (Schleicher & Schuell). Then the DNAs were eluted into a high salt buffer (1 M NaCl, 0.1 M EDTA, and 2 mM Tris, pH 8.0), followed by phenol/chloroform extraction and ethanol precipitation. Finally the precipitates, with equal amount of radioactivity loaded onto each lane, were electrophoresed through a 12% denaturing polyacrylamide gel (19.1).

**RESULTS**

The 6A Allele Has a Lower Promoter Activity Than the 5A Allele — Transient gene expression experiments were carried out to investigate whether the 5A/6A polymorphism has an effect on the strength of stromelysin-1 promoter. For these experiments, two reporter gene constructs were made, in which the stromelysin-1 promoter with either 5As or 6As at the polymorphic site was placed upstream of the reporter gene CAT. The resultant plasmids, 5A-CAT and 6A-CAT, respectively, were transfected into cultured human fetal foreskin fibroblasts (HFFF2), together with a β-galactosidase transfection control. Levels of CAT expression driven by the allelic constructs were compared after standardization for β-galactosidase activity. The results of these experiments demonstrated that 5A-CAT transfectants expressed approximately 2-fold higher CAT activity than the cells transfected with 6A-CAT: 275 ± 31 versus 162 ± 23 (n = 7, p = 0.013). The stromelysin-1 promoter in the 5A-CAT and 6A-CAT constructs was derived from the stromelysin-1 gene cloned by Quinones et al. (16). However, a discrepancy with this promoter sequence was reported recently (27), showing that the XbaI-SacI fragment between −479 and −1303 is in the reverse orientation in the human genome. Therefore, two other constructs (5T-CAT and 6T-CAT) were made in which the −479 to −1303 fragment was inverted as compared to 5A-CAT and 6A-CAT. Levels of CAT expression driven by the allelic constructs were compared after standardization for β-galactosidase activity. The results of these experiments demonstrated that 5A-CAT transfectants expressed approximately 2-fold higher CAT activity than the cells transfected with 6A-CAT: 275 ± 31 versus 162 ± 23 (n = 7, p = 0.013). The stromelysin-1 promoter in the 5A-CAT and 6A-CAT constructs was derived from the stromelysin-1 gene cloned by Quinones et al. (16). However, a discrepancy with this promoter sequence was reported recently (27), showing that the XbaI-SacI fragment between −479 and −1303 is in the reverse orientation in the human genome. Therefore, two other constructs (5T-CAT and 6T-CAT) were made in which the −479 to −1303 fragment was inverted as compared to 5A-CAT and 6A-CAT, to restore it to the correct genomic orientation. In these constructs the polymorphic site (6T/5T) is located at −601. 5T-CAT and 6T-CAT were then transfected into HFFF2 cells, and the transiently expressed CAT activities were compared. Irrespective of the orientation of the −479 to −1303 fragment, very similar CAT activities were observed: 275 ± 31 and 333 ± 15 (5A and 5T, respectively), and 162 ± 23 and 137 ± 12 (6A and 6T, respectively). The activity of the 5T-CAT construct was again approximately 2-fold higher than that of the 6T-CAT construct: 333 ± 15 versus 137 ± 12 (n = 10, p < 0.001).
Takentogether,theseresultssuggestthat,infibroblasts,thethe5A/6Apolymorphismmayinfluencestromelysin-1promoteractivityinanallelespecificmannerandthattheeffectisindependentoftheorientationandpositionoftheregulatorysequence.Suchallelespecificregulationwasalsoobservedinthetransientexpressionexperimentsonarattissueculturesmoothmusclecellline(A10),inwhichthe5T-CATtransfectantspressed1.5-foldmoreCATactivitythanthe6T-CATtransfectantcellline(A10),inwhichthe5T-CATtransfectantsexpressedthepromotersequenceinatransientexpressionexperimentonarattissueculturesmoothmusclecellline.A10,indifferentoftheorientationandpositionoftheregulatorysequences.5A/6APolymorphicSite—TwoDNAelementsboundbynuclearelements1and2mayoverlapinthesameregion(bothfactorsbindtothe26/27-meroligonucleotidedeep probing assays in EMSAs, conventional DNase I footprinting techniques would not be suitable for this study. Therefore, a modified footprinting approach was used. Two oligonucleotide probes corresponding to the sequences of the 5A or 6A alleles were 5'-end-labeled on either the top or bottom strands. The probes were incubated with nuclear extract and then subjected to partial DNase I digestion. The nucleoprotein-DNA complexes were then separated by polyacrylamide gel electrophoresis and electroblotted to a DEAE membrane. The shifted bands 1 and 2, as well as the bands of the free probes, were then isolated from the membrane and electrophoresed in a DNA sequencing gel. Comparing the DNA sequence ladders from bands 1 and 2 with those from the free probes revealed the regions protected by the nuclear protein(s).

Fig. 1. Electrophoretic mobility shift assays: nucleoprotein factor 1 binds preferentially to the 6A allele. Two synthetic oligonucleotides corresponding to the sequence from 1116 to 1189 in the stromelysin-1 promoter with 5As or 6As at the polymorphic site were 5'-end-labeled and used as probes in the EMSAs. The 5A (lanes -1-7) or 6A (lanes 8-14) probes were incubated with nuclear extracts from human fetal foreskin fibroblasts (A) or vascular smooth muscle cells (B) prior to polyacrylamide gel electrophoresis. Lanes 1 and 8, probes alone; lanes 2 and 9, probes with nuclear extracts in the absence of competitor; lanes 3 and 10-14, probes with nuclear extracts in the presence of various unlabeled competitors, i.e. 50-fold (lanes 3 and 10) to 100-fold (lanes 4 and 11) excess of the 5A oligonucleotide, 50-fold (lanes 5 and 12) to 100-fold (lanes 6 and 13) excess of the 6A oligonucleotide, or nonspecific DNA (lanes 7 and 14).
Infection experiments were then carried out to test whether the abolition of protein binding would increase the strength of the stromelysin-1 promoter. The M-CAT construct was introduced into HFFF2 and A10 cells, and CAT activities in these cells were compared with those in the transfectants of 6T-CAT and 5T-CAT. The results of these experiments showed that in HFFF2, the CAT activity was 2-fold higher in the M-CAT transfectants than the cells transfected with 6T-CAT (M-CAT 275 ± 13 versus 6T-CAT 137 ± 12; n = 10, p < 0.001), but there was no difference between the M-CAT and 5T-CAT transfectants. In A10 cells, however, M-CAT expressed 2.7-fold more CAT activity than 6T-CAT (M-CAT 220 ± 12 versus 6T-CAT 8 ± 16; n = 4, p < 0.001), and 1.8-fold more CAT activity than 5T-CAT (M-CAT 220 ± 12 versus 5T-CAT 125 ± 4; n = 4, p < 0.001).

**DISCUSSION**

The MMPs play important roles in connective tissue remodeling during tissue repair, cell migration, angiogenesis, tissue morphogenesis, and growth (2, 3). The normal operation of such physiological processes requires a tightly controlled balance between the MMPs and specific tissue inhibitors of metalloproteinases (TIMPs). Disruption of the balance, however, could lead to various pathological states. For instance, unregulated expression of MMPs is partially the cause of the accelerated breakdown of extracellular matrix in arthritic disease, tumor invasion, and metastasis (1, 28–30), while on the other hand, inadequate production of these enzymes is associated with the excessive accumulation of connective tissue in systemic sclerosis (31). Clear evidence supporting the importance of maintaining the balance between active enzyme and inhibitor is provided by the recent discovery of mutations in the TIMP-3 gene, which cause abnormal connective tissue remodeling in the eye (32, 33).

In this study, we investigated the possible function of the stromelysin-1 promoter 5A/6A polymorphism in regulating the expression of this metalloproteinase gene. In transient expression experiments, cultured fibroblasts and vascular smooth muscle cells transfected with the constructs containing 6As expressed a roughly 2-fold lower amount of reporter gene product as compared with the transfectants of the constructs containing 5As. DNA-protein interaction assays showed the binding of one or more nuclear protein(s) to the sequence surrounding the polymorphic site. One of the nucleoprotein factors bound preferentially to the 6A allele (the allele associated with lower promoter strength), suggesting that it may be a transcriptional repressor. Replacing the 5A/6A cassette with a random DNA sequence abolished the binding of the nucleoprotein(s) and diminished the repressive effects. In vascular smooth muscle cells, the construct with a random DNA sequence replacing the 5A/6A cassette (M-CAT) expressed more CAT activity than the constructs containing the 5A or 6A allelic promoters (5T-CAT and 6T-CAT, respectively), suggesting the effective removal of the repressive effect. In fibroblasts, however, although CAT activity was higher in the M-CAT transfectants than the cells transfected with 6T-CAT, no difference was observed in CAT activity between the M-CAT and 5T-CAT.
transfectants. The reason for this is not clear, but it may reflect a difference in the repertoire of transacting factors that are present in SMC and fibroblasts under these conditions, such that additional repressive effects are being exerted elsewhere in the 1.3 kilobase pairs of promoter by fibroblast-specific nuclear proteins.

It has been demonstrated that variation in the promoters of a number of genes can affect the expression of gene products directly. For example, the 4G/5G polymorphism in the plasminogen activator inhibitor-1 (PAI-1) promoter regulates the transcription of the PAI-1 gene in an allele-specific manner, and is associated with inter-individual differences in plasma PAI-1 levels (26, 34, 35). The promoter activity of the 4G allele is 2-fold higher than that of the 5G allele in transiently transfected HepG2 cells (26). Similar findings have also been reported on the genes encoding apolipoprotein A1 and β-fibrinogen (36–38). The results in the present study suggest that analogous mechanisms may be involved in the regulation of stromelysin-1 gene expression.

In a study of 72 patients with coronary heart disease, we reported previously that the 6A allele of the stromelysin 5A/6A polymorphism was associated with a more rapid progression of coronary stenosis due to atherosclerosis (23). In the present study, the results suggest a lower promoter activity with the 6A allele. Thus, reduced stromelysin-1 expression seems to be associated with more rapid progression of atherosclerosis. But what are the mechanisms?

One of the characteristics of atherosclerosis is the alteration in the content of extracellular matrix in the arterial wall (39–41). Thus, it is suggested that, during atherosclerosis, there is continuous connective tissue remodeling, which involves synthesis and degradation of the extracellular matrix proteins including interstitial collagens, proteoglycans, and elastin (42, 43). Recently, stromelysin-1 and several other MMPs have been implicated in this remodeling process (18–22, 44). A number of studies have demonstrated an increase in MMP expression in certain lesional areas such as the plaque shoulders, and it is suggested that MMP activity may contribute to the weakening of atherosclerotic lesions and consequently to plaque rupture (18–22). However, in many atherosclerotic lesions, there is a net increase in the content of extracellular matrix, suggesting that the overall matrix turnover may have favored deposition rather than degradation. This is in agreement with the observations by R. Tyagi et al. (44), who recently studied the MMP levels in relation to the extracellular matrix contents in normal and atherosclerotic arteries, and showed that weight for weight atherosclerotic vessels contained more collagen and proteoglycans but lower collagenolytic activity than normal vascular tissues.

Based on the results in this study, we speculate that, compared with other genotypes, individuals homozygous for the 6A allele could have lower stromelysin-1 levels in their arterial walls because of reduced transcription of the gene. We have previously reported augmented expression of stromelysin-1 in advanced human atherosclerotic plaques (18). These data, supported by subsequent work by others (19–22), demonstrate that there is a qualitative difference in expression of MMPs in atherosclerotic tissues compared with normal vessels, where active remodeling is not taking place. The majority of people in Western populations will develop atherosclerosis to some extent, and these enzymes will have a role in the matrix remodeling events associated with atherogenesis. The genetic studies identify inter-individual differences in the regulation of expression of these genes that may result in a quantitative difference between individuals in the level of enzyme activity, and which may therefore have an impact on the extent to which the matrix is degraded. It is emphasized that these changes are likely to be small, consistent with the contribution expected from a single gene operating in a complex multifactorial disorder. Our data support the concept that such differences may exist, possibly affecting the amount of enzyme in the vessel wall. In the model presented here, reduced levels of stromelysin-1 in the 6A homozygotes compared with other genotypes might affect the balance between synthesis and degradation during matrix turnover to favor increased deposition of matrix, leading to more rapid chronic growth of the atherosclerotic plaque, consistent with our previous findings (23).

In conclusion, the data presented here suggest that the 5A/6A polymorphism may play a functional role in regulating the expression of the stromelysin-1 gene. The genetic variant appears to influence stromelysin-1 expression in an allele-specific manner, a mechanism that could underlie its association with the progression of atherosclerosis in patients with coronary heart disease, as reported previously (23). As this is a common variant, with over a quarter of the population having the 6A6A genotype (23), its effect on stromelysin-1 expression with a 2-fold difference between the two alleles is likely to be biologically important. Although this common variant has been studied in relation to atherosclerosis, it is possible that it may also be relevant in the progression of other common chronic connective tissue disorders.

Acknowledgment—We are grateful to Dr. Gillian Murphy for helpful discussion.

REFERENCES
1. Murphy, G., and Reynolds, J. J. (1993) in Connective Tissue and Its Heritable Disorders (Royce, P. M., and Steinman, B., eds) pp. 287–316, Wiley-Liss, New York
2. Woesener, J. F., Jr. (1991) FASEBJ 5, 2145–2154
3. Matrisian, L. M. (1990) Trends Genet. 6, 121–125
4. Shapiro, S. D., Fliszar, C., J., Broekmann, T., J., Mehan, R. P., Senior, R. M., and Welgus, H. G. (1995) J. Biol. Chem. 270, 6351–6356
5. Murphy, G., Ward, R., Gavrilovic, J., and Atkinson, S. (1992) Matrix 1, Suppl. 1, 224–230
6. Inai, K., Yokohama, Y., Nakanishi, I., Ohuchi, E., Fujii, Y., Nakai, N., and Okada, Y. (1995) J. Biol. Chem. 270, 6691–6697
Differential Regulation of the Human Stromelysin-1 Gene

1. Frish, S. M., and Ruley, H. E. (1987) J. Biol. Chem. 262, 16300–16304
2. Kerr, L. D., Holt, J. T., and Matrisian, L. M. (1988) Science 242, 1424–1427
3. Kerr, L. D., Miller, D. B., and Matrisian, L. M. (1990) Cell 63, 267–278
4. Hanemaaijer, R. (1993) Biochem. J. 296, 803–809
5. Giovanna, B., Quinones, S., and Kurkinen, M. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 8154–8158
6. Galis, Z. S., Sukhova, G. K., Lark, M. W., and Libby, P. (1994) J. Clin. Invest. 94, 2493–2503
7. Brown, D. L., Hibbs, M. S., Kearney, M., Loushin, C., and Isner, J. M. (1995) Circulation 91, 2125-2131
8. Stary, H. C., Chandler, B., Dinsmore, R. E., Fuster, V., Glagov, S., Insull, W., Jr., Rosenfeld, M. E., Schwartz, C. J., Wagner, W. D., and Wissler, R. W. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 1512–1531
9. Libby, P. (1995) Circulation 91, 2844–2850
10. Newby, A. C, Southgate, K. M., and Davies, M. (1994) Basic Res. Cardiol. 89, 59–70
11. Dollery, C. M., McEwan, J. R., and Henney, A. M. (1995) Circ. Res. 77, 863–868
12. Tyagi, S. C., Meyer, L., Schmaltz, R. A., Reddy, H. K., and Voelker, D. J. (1995) Atherosclerosis 116, 43-57
13. Ye, S., Green, F. R., Scarabin, P. Y., Nicaud, V., Bara, L., Humphries, S. E., Evans, A., Luc, G., Cambou, J. P., Arveiler, D., Henney, A. M., and Cambien, F. (1995) Thromb. Haemost. 74, 837–841
14. Angotti, E., Mele, E., Costanzo, F., and Awedimento, E. V. (1994) J. Biol. Chem. 269, 17371–17374
15. Anderson, G. M., Shaw, A. R., and Shafer, A. (1993) J. Biol. Chem. 268, 22650–22655
16. Humphries, S., Ye, S., Talmud, P., Bara, L., Wilhelmsen, L., and Tipret, L. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 96–104
17. Stary, H. C., Chandler, B., Dinsmore, R. E., Fuster, V., Glagov, S., Insull, W., Jr., Rosenfeld, M. E., Schwartz, C. J., Wagner, W. D., and Wissler, R. W. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 1512–1531
18. Davies, M. J. (1990) Circulation 82, Suppl. II, 38–III–46
19. Libby, P. (1995) Circulation 91, 2844–2850
20. Newby, A. C, Southgate, K. M., and Davies, M. (1994) Basic Res. Cardiol. 89, 59–70
21. Dollery, C. M., McEwan, J. R., and Henney, A. M. (1995) Circ. Res. 77, 863–868
22. Tyagi, S. C., Meyer, L., Schmaltz, R. A., Reddy, H. K., and Voelker, D. J. (1995) Atherosclerosis 116, 43-57

Correction for accession no. U56422
Progression of Coronary Atherosclerosis Is Associated with a Common Genetic Variant of the Human Stromelysin-1 Promoter Which Results in Reduced Gene Expression

Shu Ye, Per Eriksson, Anders Hamsten, Markku Kurkinen, Steve E. Humphries and Adriano M. Henney

J. Biol. Chem. 1996, 271:13055-13060.
doi: 10.1074/jbc.271.22.13055

Access the most updated version of this article at http://www.jbc.org/content/271/22/13055

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 22 of which can be accessed free at http://www.jbc.org/content/271/22/13055.full.html#ref-list-1