

Functional Polymorphism in the Regulatory Region of Gelatinase B Gene in Relation to Severity of Coronary Atherosclerosis

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Background—Gelatinase B, a matrix metalloproteinase that has proteolytic activity against connective tissue proteins, has been suggested to be important in the connective tissue remodeling processes associated with atherogenesis and plaque rupture. This study tested the hypothesis that sequence variation in the promoter region of the gelatinase B gene influences its expression, predisposing individuals carrying certain genetic variants to more severe atherosclerosis.

Methods and Results—Single-strand conformation polymorphism analysis was carried out to search the promoter region of the gene encoding gelatinase B for naturally occurring genetic variation. As a result, an unreported common polymorphism was detected, which arose from a cytosine (C) to thymidine (T) transition at position –1562 relative to the start of transcription. Transient transfection experiments and DNA-protein interaction assays indicated that the T allele had a higher promoter activity than the C allele, which appeared to be due to preferential binding of a putative transcription repressor protein to the C allelic promoter. A sample of 584 male patients with myocardial infarction and 645 age-matched male healthy control subjects were genotyped. The allele frequencies were not significantly different between the cases and control subjects. However, in 374 patients with available angiographic data, 26% of those carrying 1 or 2 copies of the T allele had >50% stenosis in 3 coronary arteries, whereas only 15% of C/C homozygotes had triple-vessel disease.

Conclusions—These data suggest that this functional genetic variation influences gelatinase B gene promoter activity in an allele-specific manner and has an effect on atherosclerotic phenotype. (*Circulation*. 1999;99:1788-1794.)

Key Words: metalloproteinases ■ atherosclerosis ■ genetics ■ molecular biology

Atherosclerotic lesions are classified by the American Heart Association into 6 types, broadly representing different stages in lesion development.¹ It is also suggested that growth of early and intermediate atherosclerotic lesions (types I to IV, characterized by intracellular lipid accumulation and small extracellular lipid pools) is mainly due to lipid deposition, whereas progression of more advanced lesions (type V, typically consisting of a lipid core and a fibrous-muscular capsule) is largely the result of smooth muscle cell proliferation and connective tissue accumulation.¹ In addition to these mechanisms, repetitive minor plaque disruption with recurrent mural thrombosis and subsequent fibrotic organization of the thrombi is thought to contribute significantly to progression of complicated lesions (type IV).^{2,3}

The matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes with proteolytic activity against connective tissue proteins such as collagens, proteoglycans, and

elastin, appear to play important roles in the development and progression of the atherosclerotic lesion. First, the MMPs have been shown to be involved in vascular smooth muscle cell migration and proliferation in that the matrix-degrading activity of these enzymes confers on these cells the ability to break down the surrounding connective tissue barriers during such maneuvers.^{4,5} Moreover, there is also evidence indicating a role played by the MMPs in the weakening of atherosclerotic plaque that predisposes to lesion disruption. The most common locations where disruption occurs are the lateral regions of the plaque where there is macrophage accumulation accompanied by a local loss of connective tissue.^{6,7} In these disruption-prone areas, several MMPs have been found to be expressed abundantly.^{8–11} Over-expression of MMP activity is likely to lead to local destruction of the supporting connective tissue matrix, rendering the plaque liable to disruption.^{6–11} As mentioned above, plaque disruption

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tion with subsequent repair of the ensuing thrombus contributes to lesion growth.

Gelatinase B (also known as 92-kDa type IV collagenase, and MMP9) is one of the MMPs found to be highly expressed in the disruption-prone regions of atherosclerotic plaques.⁹⁻¹¹ It has a broad substrate specificity, being particularly active against gelatins (denatured collagens that have lost the typical triple helix) and type IV collagen (a major component of the basement membrane underlining the endothelium and surrounding each vascular smooth muscle cell).¹² It also possesses proteolytic activity against proteoglycan core protein and elastin, which are resistant to degradation by some other MMPs.¹² Expression of gelatinase B is regulated primarily at the level of transcription, where the promoter of the gene responds to different regulators such as interleukin-1, platelet-derived growth factor, tumor necrosis factor- α , and epidermal growth factor.¹³⁻¹⁵

Because sequence variation in the promoter region of the gelatinase B gene might give rise to a difference in gelatinase B level, which would be expected to influence connective tissue degradation during atherogenesis and the course of lesion progression, such variants would be particularly promising candidates for genetic susceptibility of coronary heart disease (CHD). Variants in known susceptibility genes do not account for all of the genetically determined risk of CHD and thus many investigators are searching for novel candidate genes. However, not all reported genetic associations with CHD are reproducible, and the frequent reason for this appears to be that functionally unimportant polymorphisms are being tested. Therefore, in this study, we performed a search for naturally occurring genetic variation in the promoter region of the gelatinase B gene and analyzed the effects of promoter variation on gene expression. Having identified a functionally important variant, we proceeded to study genotype-phenotype relations in patients with coronary atherosclerosis.

Methods

Subjects

The selection of subjects in the ECTIM (Etude Cas-Temoins de l'Infarctus du Myocarde) study has been described in detail previously.¹⁶ Briefly, male patients 25 to 64 years of age with a myocardial infarction were recruited from the WHO MONICA (MONItoring trends and determinants in CARdiovascular disease) centers in Northern Ireland (Belfast) and France (Strasbourg, Toulouse, and Lille). Age-matched male control subjects were randomly sampled from the general population in the 4 respective centers. All participants were Caucasian, descended from families resident in the region for at least 2 generations, and with all 4 of their grandparents born in Europe. Coronary angiographic data were available for 93% of the French cases and 18% of the Northern Irish cases. Angiograms were analyzed in each recruitment center, and the numbers of arteries with >50% stenosis were used as an assessment for severity of CHD.¹⁷ The relations between gelatinase B genotypes and coronary angiographic results presented here are only for the French samples, but the results were very similar if the Northern Irish cases were included in the analyses.

Promoter Variation Screening

The promoter region (2.2 kb) of the gelatinase B gene was amplified sequentially in \approx 450 base pair (bp) fragments by polymerase chain reaction (PCR). The PCR products were digested with appropriate

restriction endonucleases so that each amplicon was cleaved into 2 fragments of between 150 and 250 bp. The digests were subjected to single-strand conformation polymorphism analysis.

Identification of the C-1562T polymorphism was as follows: The sequence from -1809 to -1374 in the gelatinase B promoter was PCR amplified with primers A (5'-GCCTGGCACATAGT-AGGCC-3') and B (5'-CTTCCTAGCCAGCCGGCATC-3'). The 435-bp amplicon was cleaved into 244-bp and 191-bp fragments with restriction endonuclease *Mse*I. The digests were mixed with formamide loading buffer, denatured by heating, and subjected to native polyacrylamide gel electrophoresis (7.5%, acrylamide:bis-acrylamide=49:1). Samples showing different mobility patterns were subjected to DNA sequencing to determine the nucleotide differences with the use of a commercially available manual sequencing kit (Thermo Sequence cycle sequencing kit, Amersham).

Electrophoretic Mobility Shift Assay

Double-stranded 26-mer oligonucleotides (C: 5'-CAGGCGT-GGTGGCGCACGCCTATAAT-3' and T: 5'-CAGGCGTGGTGG-CGCATGCCTATAAT-3') corresponding to the sequences from -1578 to -1552 in the gelatinase B promoter were 5'-end-labeled with γ -³²P ATP. Three microliters of nuclear extracts prepared from murine lung macrophages (MALU), human fetal foreskin fibroblasts (HFFF2), or hepatoma cells (HepG2), with the use of a method by Alksnis et al,¹⁸ was incubated for 10 minutes on ice in a solution composed of 1 μ L of 10 mmol/L dithiothreitol, 1 μ L of 10 mmol/L EDTA-Na₂, 3 μ L of 0.1 mol/L HEPES (pH 7.9), 3.2 μ L of 50% Ficoll, and 2 μ L of 1 mg/mL poly(dI-dC). Radiolabeled C or T probes (30 000 cpm per reaction), with or without unlabeled oligonucleotide, were then added and the mixture incubated at room temperature for 20 minutes before nondenaturing polyacrylamide gel (7%, acrylamide:bis-acrylamide=80:1) electrophoresis and autoradiography.

Methylation Interference Assay

A double-stranded 55-mer oligonucleotide corresponding to the gelatinase B promoter sequence from -1586 to -1532 (5'-AAT-TTAGCCAGGCGTGGTGGCGCACGCCTATAATACCAGCTAC-TCGGGAGGCTGA-3') was 5'-end-labeled with ³²P on either the top or the bottom strand. Methylation interference was conducted as described previously.¹⁹ The radiolabeled probe was partially methylated with dimethyl sulfate and then incubated with nuclear extracts from HFFF2 under conditions identical to electrophoretic mobility shift assays (EMSAs) described above. Thereafter, polyacrylamide gel electrophoresis as described for EMSA was carried out to separate DNA-protein complexes from free probes, which were subsequently electroblotted onto a DEAE membrane. The probe, previously bound or unbound by nuclear proteins, was eluted and cleaved at the methylated guanine residues by piperidine, followed by denaturing polyacrylamide gel (12%, acrylamide:bis-acrylamide=19:1) electrophoresis and autoradiography.

Transient Reporter Gene Expression Experiment

Two reporter gene constructs, designated respectively as pC-CAT and pT-CAT, were created by cloning 3 concatenated copies of the oligonucleotides used in above EMSAs into the pCAT3-promoter vector (Promega) and individually transfected into MALU cells by electroporation. The pSV- β -galactosidase vector (Promega) was cotransfected into the cells to serve as a control for transfection efficiency. Twenty-four hours after transfection, cells were lysed, and the levels of CAT and β -galactosidase were measured with the use of commercial ELISA kits (Boehringer-Mannheim). Ratios of CAT level to β -galactosidase level from cells transfected with pC-CAT were compared with those from pT-CAT transfectants.

Two more reporter gene constructs, designated as pMMP9-C-Luc and pMMP9-T-Luc, respectively, were generated by cloning an \approx 2-kb promoter sequence (-2181 to +11) of the gelatinase B gene, with a C or a T, respectively, at the -1562 polymorphic site, into a promoterless plasmid vector (pGL3-basic vector, Promega) containing the coding sequence for firefly luciferase and individually

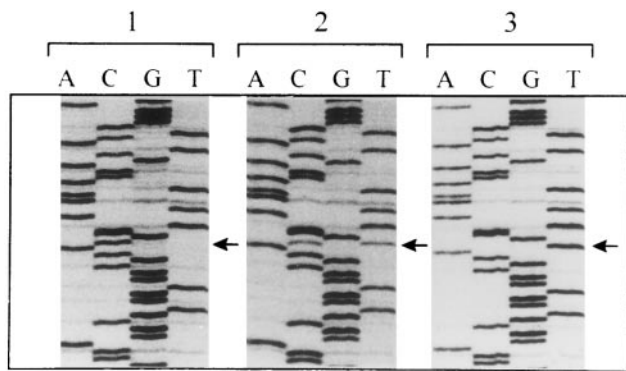


Figure 1. Autoradiograph of DNA sequencing. Sample 1, Homozygous for C allele; sample 2, heterozygous; sample 3, homozygous for T allele. Arrows indicate position of C-to-T transition at -1562 .

transfected into MALU cells by electroporation. A plasmid (pRL-TK, Promega) containing the thymidine kinase promoter upstream of a cDNA coding for *Renilla* luciferase was cotransfected into the cells to serve as a control for transfection efficiency. Twenty-four hours after transfection, cells were lysed, and the levels of firefly luciferase and *Renilla* luciferase were measured with the use of a Dual-luciferase Assay System (Promega). The ratio of firefly luciferase level to *Renilla* luciferase level was used as a measurement of gelatinase B promoter activity.

Determination of C-1562T Genotypes of the ECTIM Samples

PCR was carried out with primers A and B described above. The PCR products were digested with *Bbu*I and fractionated on a 1.5% agarose gel. Genotypes were scored according to the patterns of DNA bands. Samples of known genotypes (verified by sequencing) were used as references and run alongside the samples being analyzed.

Statistical Analysis

Data were analyzed with the use of SAS statistical software. χ^2 analysis was used to test for deviation of genotype distribution from Hardy Weinberg equilibrium and to determine whether there was any significant difference in allele or genotype frequencies between cases and control subjects. Logistic regression (SAS-PROC LOGISTIC) was carried out to test for difference in genotype distribution among patients with single-, double-, and triple-vessel disease, adjusting for age and referral centers. A value of $P < 0.05$ was taken to be statistically significant.

Results

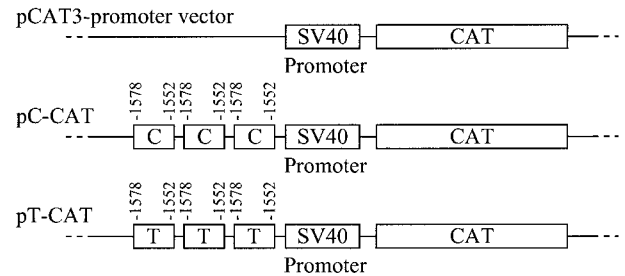
Identification of a Polymorphism in the Promoter Region of the Gelatinase B Gene

Single-strand conformation polymorphism analyses were carried out to search the promoter region of the gelatinase B gene for naturally occurring variation in 40 unrelated individuals. As a result, a common, biallelic polymorphism (referred to as C-1562T polymorphism below) was detected, which arose from a cytosine (C) to thymidine (T) transition at position 1562 bp upstream from the start of transcription (Figure 1).

Functional Effect of the Gelatinase B (C-1562T) Polymorphism

To investigate whether the C-to-T substitution had an effect on gene expression, mammalian cell transient transfection

(a)



(b)

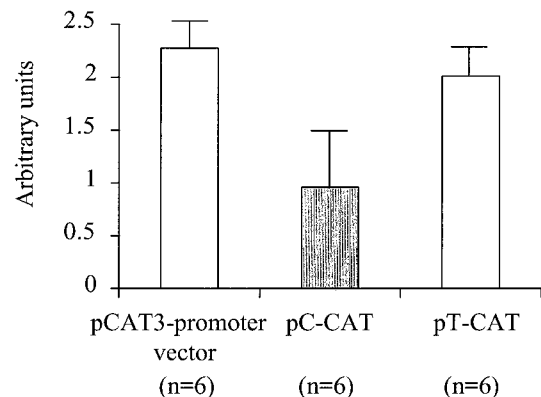


Figure 2. Transient reporter gene expression assays with constructs containing concatenated oligonucleotides corresponding to C-1562T polymorphic site. a, Schematic of reporter gene constructs, each containing 3 concatenated 26-bp DNA sequences corresponding to C or T allele; b, CAT levels in macrophages transfected with different reporter gene constructs. Data shown are mean CAT levels (standardized against β -galactosidase) from 3 transfection experiments in duplicate. $P < 0.003$ in *t* test.

experiments with allelic promoter-reporter gene constructs were carried out. In these experiments, 2 plasmid constructs were prepared in which 3 concatenated copies of the DNA sequence from -1578 to -1552 in the gelatinase B promoter, with either a C or T at the polymorphic site (-1562), were cloned immediately upstream of a SV40 minimal promoter and the gene encoding chloramphenicol acetyl transferase (CAT) in the pCAT3-promoter vector. The resultant constructs, designated as pC-CAT and pT-CAT, respectively, were individually transfected into cultured macrophages (MALU) by electroporation. The levels of CAT in the 2 different transfectants were compared with each other and with the CAT levels in cells transfected with the pCAT3-promoter vector without inserts. Figure 2 shows the results of these experiments: The level of CAT produced by pT-CAT was comparable with that produced by pCAT3-promoter vector without inserts but was 2-fold higher than the level of CAT produced by pC-CAT.

This allele-specific effect on promoter activity was also seen in transient transfection experiments with reporter gene constructs containing an ≈ 2 -kb promoter sequence (-2181 to $+11$) of the gelatinase B gene. As shown in Figure 3, reporter gene expression driven by the T allelic gelatinase B

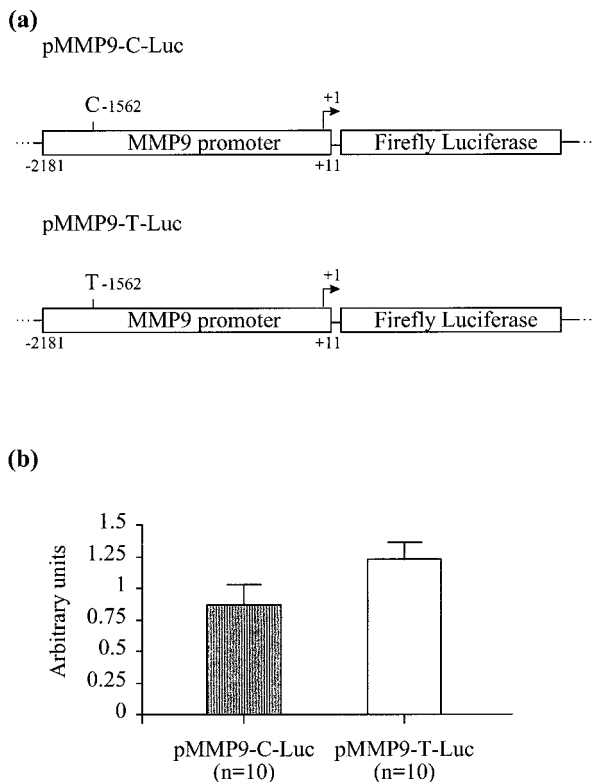


Figure 3. Transient reporter gene expression assays with constructs containing full-length gelatinase B promoter. a, Schematic presentation of reporter gene constructs containing a 2-kb gelatinase B (MMP9) gene promoter with the only difference between the 2 constructs being with either C or T at the -1562 polymorphic site; b, firefly luciferase levels (standardized against *Renilla* luciferase levels) in macrophages transfected with reporter gene constructs containing C or T allelic gelatinase B gene promoter. Data shown are mean luciferase levels from 3 transfection experiments (2 in triplicate and 1e in quadruplicate). $P < 0.001$ in *t* test.

promoter was ≈ 1.5 -fold greater than reporter gene expression directed by the C allelic promoter.

To investigate whether there were transcriptional regulatory proteins binding to the DNA sequence at the polymorphic site, electrophoretic mobility shift assays were carried out. In these assays, 2 oligonucleotide probes corresponding to the sequence from -1578 to -1552 in the gelatinase B promoter, with either a C or T at the -1562 polymorphic site, were labeled with ^{32}P and allowed to interact with crude nuclear extracts prepared from different cell lines including murine macrophages (MALU), human fibroblasts (HFFF2), and hepatoma cells (HepG2). Three groups of DNA-protein complexes (labeled as A, B, and C) were consistently detected in these assays irrespective of cell type. While the intensity of group C was comparable between assays with the C probe and those with the T probe, groups A and B were more readily detectable with the C probe than with the T probe (Figure 4). Methylation interference assay confirmed the DNA-protein interaction and indicated that the actual DNA sequence bound by the putative transcription factor in DNA-protein complex A was between positions -1567 and -1559 , relative to the start of transcription of the gelatinase B gene (Figure 5).

Association of the Gelatinase B (C-1562T) Polymorphism With Severity of Coronary Atherosclerosis

A sample of 584 male patients (mean [SD], age 54.0[8.1] years) with myocardial infarction and 645 age-matched male healthy control subjects (mean [SD], 53.2 [8.4] years) who participated in the ECTIM study were genotyped for the gelatinase B (C-1562T) polymorphism by restriction enzyme digestion (Figure 6). The distributions of genotypes were consistent with Hardy-Weinberg equilibrium. There was no significant difference in the frequencies of genotypes or alleles between patients and control subjects, with the frequency of the T allele being 0.14 in patients and 0.13 in control subjects.

However, in 374 French patients in whom coronary angiographic data were available, there was a significant association between the gelatinase B (C-1562T) polymorphism and severity of coronary atherosclerosis as assessed by a standard analysis of the numbers of coronary arteries that had a stenosis $>50\%$.^{17,20} Thus the percentage of patients with triple-vessel disease was higher in the *C/T* and *T/T* genotype class (26%) than in the *C/C* class (15%) ($P < 0.04$, $P < 0.02$ after adjusting for age and referral centers, Table).

Discussion

The expression of gelatinase B is regulated primarily at the transcription level. Several *cis* elements in the gelatinase B gene promoter have been shown to be important in the regulation of its transcription. These include 2 AP-1 sites (-533 , and -79 , bound by transcription factors *c-Fos* and *c-Jun*), a PEA3 motif (-540 , recognized by transcription factor Ets), and a consensus sequence (-600) for binding of nuclear factor- κB .^{13,21} The results of the present study suggest that a 9-bp sequence (GCGCAC/TGCC, -1567 to -1559) containing the C-1562T polymorphic site is also an important regulatory element that appears to be a binding site for a transcription repressor protein. In addition, the data also suggest that this DNA-protein interaction is abolished by the C-to-T substitution at the polymorphic site (-1562), resulting in a higher promoter activity of the T-allelic promoter, as shown by the transient expression experiments. This is also supported by our preliminary results of serum gelatinase B assays, in that individuals with the *T/T* genotype tend to have a higher level [$8(\pm 10)$ ng/mL for *T/T* genotype ($n=11$); $6(\pm 8)$ ng/mL for *C/T* genotype ($n=12$); and $6(\pm 8)$ ng/mL for *C/C* genotype ($n=16$)]. These findings do not preclude the possibility that polymorphisms elsewhere in the gelatinase B gene might also have an influence on the regulation of expression; however, the functional effect of the C-1562T polymorphism on promoter activity is supported regardless.

Gelatinase B possesses proteolytic activity against type IV collagen, a major component of the basement membrane, and has been shown to facilitate vascular smooth muscle cell migration.^{4,5} It has also been found that gelatinase B is highly expressed in the shoulder regions of advanced atherosclerotic lesions and therefore it is suggested that this potent matrix-degrading enzyme also contributes to plaque instability.⁹⁻¹¹ In this study, we observed an association between the gelatinase B gene promoter C-1562T polymorphism, which

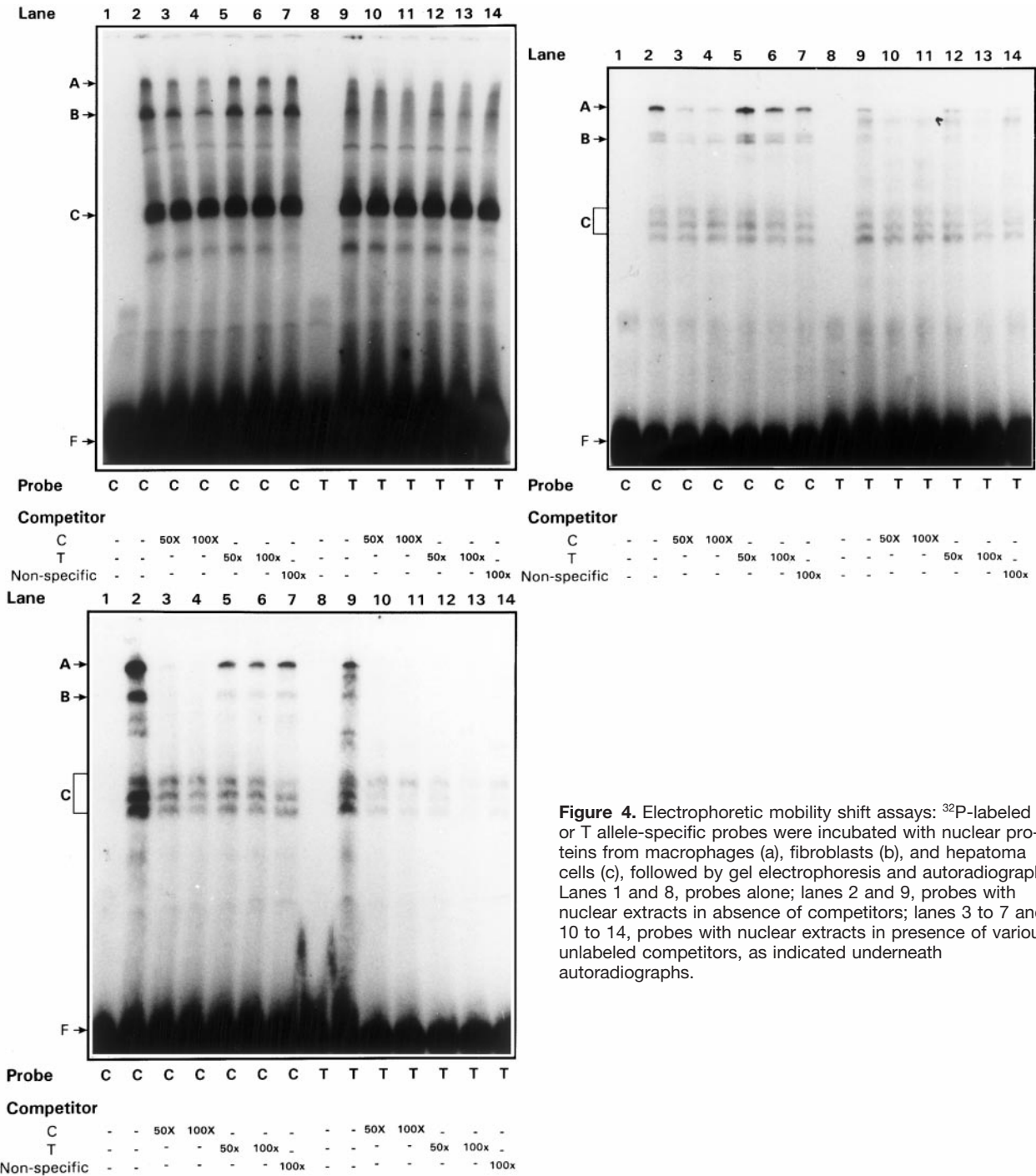


Figure 4. Electrophoretic mobility shift assays: ³²P-labeled C or T allele-specific probes were incubated with nuclear proteins from macrophages (a), fibroblasts (b), and hepatoma cells (c), followed by gel electrophoresis and autoradiography. Lanes 1 and 8, probes alone; lanes 2 and 9, probes with nuclear extracts in absence of competitors; lanes 3 to 7 and 10 to 14, probes with nuclear extracts in presence of various unlabeled competitors, as indicated underneath autoradiographs.

appears to regulate gene expression in an allele-specific manner, and severity of coronary atherosclerosis in a cohort of patients with CHD. Although the precise underlying mechanisms are unclear, it is likely that the effect is mediated through one or both of these pathways. It is plausible that higher gelatinase B expression associated with the T allele will enhance smooth muscle cell migration during atherogenesis. Individuals carrying the T allele may also be predisposed to increased plaque instability through matrix degradation and have a greater likelihood of developing advanced complicated lesions as a result of fibrotic organization of mural thrombi after recurrent lesion disruption. Fibrotic repair of

thrombus is an important mechanism for plaque growth. The process is analogous to that occurring after angioplasty and begins with migration of smooth muscle cells from the intima into the thrombus, where they synthesize and lay down connective tissue matrix, followed by growth of endothelial cells over the luminal surface.² Although this reparative process is a very important defensive mechanism in maintaining vascular integrity, it is paradoxically also a mechanism that contributes to lesion growth and postevent stenosis. It has been estimated by reconstruction of coronary lesions at autopsy that 70% of high-grade stenoses (angiographic >50% diameter) have had an episode of healed disruption,²²

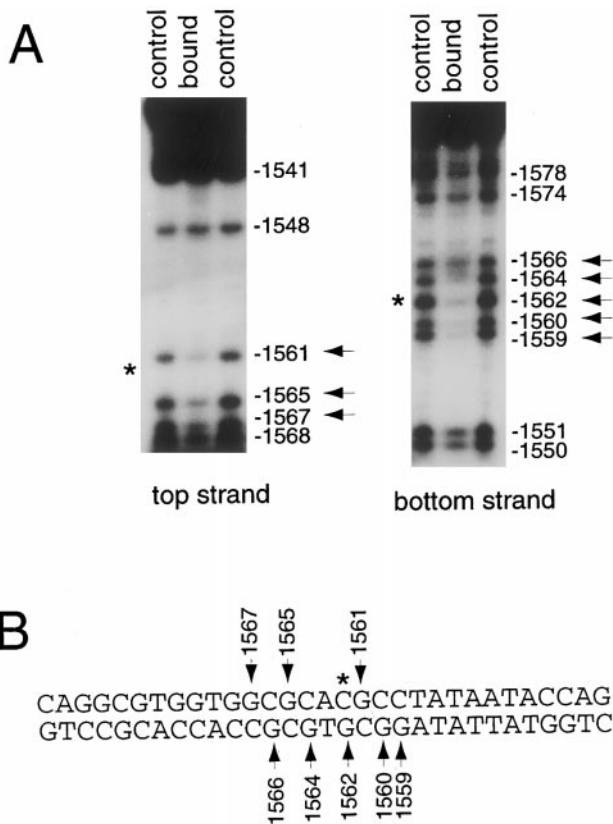


Figure 5. Dimethyl sulfate methylation footprinting. A, Methylation interference assay of protein complex A with double-stranded oligonucleotide corresponding to sequence from -1586 to -1532 of the gelatinase B promoter. Numbers denote position of guanine residues in relation to start of transcription. Arrows indicate interfering guanine residues. Stars denote site of polymorphism. B, Summary of footprinting data indicating interfering guanine residues by arrows. Star denotes site of polymorphism (-1562).

which highlights the role of recurrent disruption and thrombosis in the generation of advanced stenotic lesions.

This study did not detect an association with myocardial infarction events, which are believed to arise commonly from

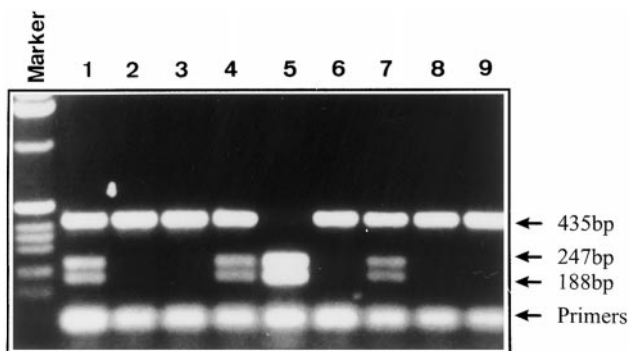


Figure 6. Ethidium bromide-stained agarose gel used for genotyping. Sequence (435 bp) from -1809 to -1374 in the gelatinase B gene promoter was PCR amplified and digested with *Bbu*I, which cleaved the T allele at the -1562 polymorphic site, generating 2 fragments (247 bp and 188 bp, respectively), but did not cut the C allele. Inferred genotypes are, respectively, C/T in lanes 1, 4, and 7; C/C in lanes 2, 3, 6, 8, and 9; and T/T in lane 5.

Numbers (Percentages) of Patients With Zero, 1, 2, and 3 Stenotic (>50%) Coronary Arteries According to Gelatinase B Genotype

Geno-type	Patients, n			Total No. of Patients
	With 1 Stenotic Vessel	With 2 Stenotic Vessels	With 3 Stenotic Vessels	
C/C	150 (54.74%)	84 (30.66%)	40 (14.59%)	274
C/T+T/T	49 (49.00%)	25 (25.00%)	26 (26.00%)	100

Logistic regression: $P < 0.04$; $P < 0.02$, adjusted for age and referral centers. Cases (n=12) with negative angiographic findings were excluded.

plaque rupture. This may indicate that the susceptibility conferred by the gelatinase B variant has a different effect on the pathways that lead to minor plaque rupture and progressive stenosis as opposed to deep rupture leading to myocardial infarction. Deep rupture is also probably dependent on a greater number of risk factors, including the size of lipid core that affects the overall distribution of circumferential stress on the plaque during systole²³ and the levels of local inflammatory stimuli influencing the repertoire of proteinase expression by macrophages,²⁴⁻²⁶ whereas occlusion and the consequent risk of ischemic events will also depend on the coagulant status of the individual.²⁷

In conclusion, we have identified an unreported promoter polymorphism in the gelatinase B gene that appears to impose an allele-specific effect on gene expression and have shown an association between this regulatory polymorphism and severity of coronary atherosclerosis. These findings support the hypothesis that connective tissue remodeling, mediated by MMPs, plays an important role in atherogenesis and that genetic variation influencing MMP expression/activity might contribute to the genetic variance of disease phenotype. Further studies are now indicated to validate these findings.

Acknowledgments

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