Association of a Decreased Number of d(CA) Repeats in the Matrix Metalloproteinase-9 Promoter with Glomerulosclerosis Susceptibility in Mice

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Abstract. The genetic background plays an important role in the development of progressive glomerulosclerosis. However, no marker is available for the reliable prediction of genetic susceptibility to glomerulosclerosis. Because matrix metalloproteinase-9 (MMP-9) levels are decreased in models of glomerulosclerosis and MMP-9 promoter polymorphism has been observed among patients with diabetic nephropathy, MMP-9 could be one such marker. The object of this study was to determine whether MMP-9 promoter polymorphism was associated with altered MMP-9 expression in mesangial cells (MC) from two mouse strains, i.e., ROP (glomerulosclerosis prone) and B6SJL (glomerulosclerosis resistant). ROP MC expressed 12-fold less MMP-9 mRNA. The MMP-9 promoter in ROP MC contained fewer d(CA) repeats, which was associated with lower MMP-9 expression and activity. Phorbol-12-myristate-13-acetate (3 to 60 ng/ml) increased MMP-9 expression in both MC types (3- to 4.5-fold), but the level in ROP MC never reached that in B6SJL MC. Although reciprocal transfection of ROP and B6SJL MMP-9 promoter constructs into B6SJL and ROP cells revealed that the promoters were functional in both cell types, the B6SJL promoter was less responsive to phorbol-12-myristate-13-acetate stimulation when transfected into ROP MC, suggesting a role for other factors. In conclusion, the MMP-9 promoter exhibits a decreased number of d(CA) repeats in the sclerosis-prone strain. Because fewer d(CA) repeats associated with decreased MMP-9 expression in MC, it might be a genetic marker for glomerulosclerosis.

Glomerulosclerosis is the leading cause of end-stage renal disease in the United States. Glomerulosclerosis results from an imbalance between glomerular extracellular matrix synthesis and degradation, leading to the accumulation of matrix collagens, including type IV collagen (1). Type IV collagen and laminin act as in vitro substrates for matrix metalloproteinase-9 (MMP-9) (2,3) and accumulate in glomerulosclerosis (4,5). Therefore, reduced MMP-9 activity could be an important contributor to the pathogenesis of glomerulosclerosis (1). The recently reported association between MMP-9 promoter polymorphism and diabetic glomerulosclerosis (6) provides additional evidence that this enzyme may play a role in the development of glomerulosclerosis.

Several disease processes are associated with changes in MMP-9 expression. In wound healing, for example, the accumulation of collagen is inversely correlated with the amount of MMP-9 protein produced locally (7). In addition, MMP-9 plays a role in autoimmune encephalomyelitis (8), myocardial infarctions (9), angiogenesis (10), and aortic aneurysm development (11).

Regulation of MMP-9 transcription occurs in the promoter region, where Sp-1, polyoma virus enhancer A-binding protein-3 (PEA-3), activator protein-2 (AP-2), nuclear factor-κB, and AP-1 domains have been identified (12,13). The AP-1 site plays a major role in the regulation of MMP-9 expression (14–16), and phorbol-12-myristate-13-acetate (PMA) is known to activate MMP-9 transcription through the AP-1 site, via protein kinase C (PKC) activation (16). Two types of alterations in the MMP-9 promoter have been demonstrated to be correlated with sclerosing diseases, i.e., point mutations and d(CA) repeat polymorphisms. Point mutations are correlated with coronary atherosclerosis (17), whereas the number of d(CA) repeats is correlated with the development of intracranial aneurysms (18) and diabetic nephropathy (6). Therefore, we hypothesized that structural abnormalities in the MMP-9 promoter might be associated with alterations in MMP-9 transcription in mesangial cells (MC).

MC have been postulated to be major contributors to glomerulosclerotic lesions (19) and have been demonstrated to express MMP-9 in certain species and experimental conditions (20–22). The behavior of MC in vitro might be different from that in vivo, and behavior is influenced by the substrate and cell culture conditions (23). However, we previously demonstrated that MC isolated from both normal and glomerulosclerotic mice retain many of their in vivo phenotypic characteristics (24). An altered phenotype has been observed for MC from
diabetic mice and rats (25,26) and for skin fibroblasts from diabetic mice and human subjects (27,28). Similarly, vascular smooth muscle cells from hypertensive patients (29) and skin fibroblasts from patients with scleroderma (30) retain their phenotype in vitro.

We reported that MMP-9 levels were reduced in MC from mice with progressive glomerulosclerosis, diabetic NOD mice (24), and mice transgenic for bovine growth hormone (21). Marked decreases in MMP-9 mRNA expression and enzymatic activity were also noted in the glomeruli of diabetic obese Zucker rats (31). We observed that baseline MMP-9 expression and activity were markedly decreased in MC after long-term exposure to high ambient glucose levels (32,33). MMP-9-knockout mice with a heterogeneous background (129/Sv ES cells injected into blastocysts of C57BL/6J mice backcrossed to female Swiss Black mice) did not develop spontaneous glomerulosclerosis as adults (34). However, there are no data comparing MMP-9 expression levels, different genetic backgrounds, and susceptibility to glomerulosclerosis. We identified both glomerulosclerosis-resistant (C57BL/6 and B6SJL/F1) and glomerulosclerosis-prone (ROP/Le) mouse strains (35–37). MC from ROP mice express very low levels of MMP-9, compared with B6SJL MC (33,38). The aims of this study were to investigate whether (1) there are genetic variations in the MMP-9 promoter between glomerulosclerosis-prone (ROP) and glomerulosclerosis-resistant (B6SJL) mice, (2) variations in the MMP-9 promoter account for the differences in promoter transcriptional activation observed in MC isolated from ROP or B6SJL mice, and (3) variations in the MMP-9 promoter affect the response to PKC stimulation by PMA.

Materials and Methods

Cell Culture

Three independently isolated lines of MC from glomeruli from B6SJLF1/J (B6SJL) and ROP/Le+ Eslb/ES1a (ROP) mice were studied. Both B6SJL and ROP cells were previously characterized in our laboratory (38). MC were maintained in Dulbecco’s modified Eagle’s medium/F-12 medium (3:1) (Life Technologies BRL, Rockville, MD) supplemented with 20% fetal bovine serum (FBS) (Life Technologies BRL), 1 mM glutamine (Biofluids, Rockville, MD) supplemented with 20% FBS, which was replaced with medium containing 0.1% FBS at 12 h after seeding. Supernatants were collected after 48 h, and cell numbers were determined. MMP-9 activity was examined at different cell densities (ranging from 20,000 to 100,000 cells/cm²) for all MC lines. Supernatants were centrifuged to remove cellular debris and were appropriately diluted with regular medium (0.1% FBS) to normalize cell numbers (10,000 cells). Zymography was performed as described previously (39). The amount of supernatant loaded onto the gel was determined before each experiment with the development of standard curves. For each data point, conditions were chosen so that the digestion was performed in the linear range of enzymatic activity. We were thus able to compare ROP and B6SJL MC. This principle was also applied to assessments of the effect of PMA stimulation. For each cell line, we chose a supernatant amount that was in the linear range of enzymatic activity after PMA stimulation. Protein extraction from kidney cortices and microdissected glomeruli was performed in cold Hanks’ balanced salt solution, after tissue sonication. Either 10 μg of protein from kidney cortex or total protein from 30 microdissected glomeruli was loaded onto zymographic gels. The gels were analyzed by using the public domain NIH Image program (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

DNA Sequencing

For DNA extraction, 100 microdissected glomeruli, 5-mm tail snips, or 200,000 cells were incubated in a protease K solution (500 μg/ml protease K, 10 mM Tris, pH 8.0, 100 mM NaCl, 100 mM ethylenediaminetetraacetate, 0.5% sodium dodecyl sulfate) at 60°C for 2 h and were then centrifuged at 4°C. Phenol/chloroform (Sigma) was added, and the aqueous phase was precipitated with ethanol and sodium acetate. Direct PCR of the distal MMP-9 promoter region (positions −1187 to −1157) was performed with four overlapping sets of primers (Table 1). PCR was performed as previously described for reverse transcription-PCR, but a high-fidelity Taq polymerase (Roche,

Reverse Transcription-PCR

MC were plated into fibronectin-coated, 25-cm² flasks. The medium was changed 12 h after seeding. A cell density of 100,000 cells/cm² was consistently used. Total mRNA was extracted after 48 h by using Tri-Reagent (MRC Inc., Cincinnati, OH), and reverse transcription-PCR was performed, as described previously (39), with cultured MC, total kidney cortex, and microdissected glomeruli. The primer sequences for MMP-9 (414 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (561 bp; used as a housekeeping gene) were reported previously (21).

MMP-9 Activity

MC were plated in six-well plates in medium supplemented with 20% FBS, which was replaced with medium containing 0.1% FBS at 12 h after seeding. Supernatants were collected after 48 h, and cell numbers were determined. MMP-9 activity was examined at different cell densities (ranging from 20,000 to 100,000 cells/cm²) for all MC lines. Supernatants were centrifuged to remove cellular debris and were appropriately diluted with regular medium (0.1% FBS) to normalize cell numbers (10,000 cells). Zymography was performed as described previously (39). The amount of supernatant loaded onto the gel was determined before each experiment with the development of standard curves. For each data point, conditions were chosen so that the digestion was performed in the linear range of enzymatic activity. We were thus able to compare ROP and B6SJL MC. This principle was also applied to assessments of the effect of PMA stimulation. For each cell line, we chose a supernatant amount that was in the linear range of enzymatic activity after PMA stimulation. Protein extraction from kidney cortices and microdissected glomeruli was performed in cold Hanks’ balanced salt solution, after tissue sonication. Either 10 μg of protein from kidney cortex or total protein from 30 microdissected glomeruli was loaded onto zymographic gels. The gels were analyzed by using the public domain NIH Image program (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

Table 1. Primers used for MMP-9 promoter sequence analysis

<table>
<thead>
<tr>
<th>Primer Position</th>
<th>Primer Sequence</th>
</tr>
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<tbody>
<tr>
<td>−1187 to −1157</td>
<td>agaagcatactaagaagct</td>
</tr>
<tr>
<td>−1122 to −1102</td>
<td>gttgggaacacatgaaggt</td>
</tr>
<tr>
<td>−817 to −797</td>
<td>tggagtctgtaaacactgc</td>
</tr>
<tr>
<td>−612 to −592</td>
<td>tctttcccctcccaagagg</td>
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<tr>
<td>−411 to −391</td>
<td>ttctcagagcccaagaga</td>
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<td>−254 to −274</td>
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<td>+11 to −9</td>
<td>caggactcgtggagac</td>
</tr>
<tr>
<td>+70 to +50</td>
<td>cgctgtggagggggcagaga</td>
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a MMP-9, matrix metalloproteinase-9.
Indianapolis, IN) was used. PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA). The purity of the PCR products was verified by agarose gel electrophoresis. PCR products were quantified by spectrophotometric analysis, and DNA was subjected to cycle sequencing with a Sequencer 3100 (Applied Biosystems, Foster City, CA), using BigDye terminator. Consensus sequences were constructed by using the public domain Multiple Sequence Alignments program provided by the Human Genome Sequencing Service (Baylor College of Medicine) (available at http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) and were compared with the published sequence (National Center for Biotechnology Information accession number X72794).

Simple Sequence Length Polymorphism Analysis

DNA derived from the same DNA extraction as that used for sequence analysis was used for simple sequence length polymorphism (SSLP) analysis of the d(CA) repeats (positions -161 to -109). PCR was performed with a set of primers that amplified a 128-bp fragment containing the dinucleotide repeats of the MMP-9 promoter. The following primer sequences (5' to 3') were used for PCR amplification: CTGCCGACTGCGGATG and TCCAGGGTATGCTGACT. In addition, SSLP analysis was performed for the tissue inhibitor of metalloproteinase-3 (TIMP-3) and regulated upon activation, normal T cell expressed and secreted (RANTES) promoters, which are also characterized by the presence of d(CA) repeats (40,41). Primers that amplified a 269-bp fragment of the RANTES promoter and a 189-bp fragment of the TIMP-3 promoter were used for PCR. The following primer sequences (5' to 3') were used: RANTES promoter, TCCTGTGCCCACACTTA and GGATCTGATAGGGCATAC; TIMP-3 promoter, TGCCCTACATAGTAAGAC and AAGCATTACGTGAG. Samples were separated on 2% agarose gels, as well as on 15% polyacrylamide gels.

Cloning

PCR was performed as described for reverse transcription-PCR, with the high-fidelity thermostable DNA polymerase (Roche) and 50 ng of genomic DNA from both B6SJL and B6SJL MC. Primers were designated to incorporate a KpnI or BglII restriction enzyme recognition sequence at either end of the resulting PCR product. The following primer sequences (5' to 3') were used for PCR amplification: TATAACCAGCTGCGGATG and TCCAGGGTATGCTGACT. In addition, SSLP analysis was performed for the tissue inhibitor of metalloproteinase-3 (TIMP-3) and regulated upon activation, normal T cell expressed and secreted (RANTES) promoters, which are also characterized by the presence of d(CA) repeats (40,41). Primers that amplified a 269-bp fragment of the RANTES promoter and a 189-bp fragment of the TIMP-3 promoter were used for PCR. The following primer sequences (5' to 3') were used: RANTES promoter, TCCTGTGCCCACACTTA and GGATCTGATAGGGCATAC; TIMP-3 promoter, TGCCCTACATAGTAAGAC and AAGCATTACGTGAG. Samples were separated on 2% agarose gels, as well as on 15% polyacrylamide gels.

Transient Transfection Experiments

Cells grown at a density of 40,000 cells/cm², in 24-well plates, were transfected by using TransFast transfection reagent (Promega), with 0.25 µg of MMP-9 promoter-pGL3 construct and 0.25 µg of a Rous sarcoma virus-β-galactosidase reporter construct (a gift from Dr. J. Seegars, United States Uniformed Health Services, Bethesda, MD), according to the suggestions of the manufacturer. In particular, MC from both ROP and B6SJL MC were transfected with a pGL3 vector containing the promoter from either B6SJL MC or ROP MC. Transfections with an empty pGL3 vector were performed as an internal control. Briefly, cells were washed with phosphate-buffered saline, and medium was replaced with 200 µl of Dulbecco’s modified Eagle’s medium/F-12 medium (3:1) containing TransFast transfection reagent and the constructs. After a 1-h incubation at 37°C, medium supplemented with 0.1% FBS was added to a total volume of 1 ml. MC were collected 24 h later with 100 µl of cell culture lysis reagent (Promega) and were assayed for luciferase activity by using a luciferase assay system (Promega), as described by the manufacturer. Transfections with either B6SJL or ROP promoters were also performed with COS-7 cells (an independent, transfectable, mammalian cell line). To establish whether the ROP and B6SJL MMP-9 promoters were equally responsive to PKC stimulation, transfection experiments were performed in the presence of increasing concentrations of PMA (3 to 60 ng/ml).

Data Analyses

Results represent the mean of four or five independent experiments performed in duplicate or triplicate. Results are expressed as mean ± SD. Results were compared by using an unpaired t test or one-way ANOVA. When ANOVA demonstrated a statistically significant difference, a group-by-group comparison was performed by using a t test with Tukey’s correction for multiple comparisons. Statistical significance was set at P < 0.05.

Results

MMP-9 mRNA Expression and Activity

MMP-9 mRNA expression was lower in the kidney cortex and in microdissected glomeruli from three ROP mice, compared with three B6SJL mice (Figure 2a). In particular, MMP-9/GAPDH ratios in microdissected glomeruli were 0.4 ± 0.02 for ROP mice and 2.1 ± 0.07 for B6SJL mice (P < 0.01). The MMP-9 activity of 30 microdissected glomeruli was higher for B6SJL mice than for ROP mice (P < 0.05) (Figure 2b), but no significant difference in the MMP-9 enzymatic activity of protein from total kidney cortex was observed for B6SJL versus ROP mice.

MMP-9 Promoter Studies

A schematic representation of the murine MMP-9 promoter, with delineation of key transcription factor binding sites and the d(CA) repeats, is presented in Figure 3. SSLP analysis was performed by analyzing 128-bp fragments, involving the microsatellite regions, that had been amplified by PCR. PCR products obtained with the ROP MMP-9 promoter were smaller than those obtained from B6SJL mice (Figure 4a). DNA isolated from tail snips and microdissected glomeruli from three ROP and three B6SJL mice was examined to
determine whether \textit{in vitro} artifacts could account for the differences in promoter length between ROP and B6SJL MC. The length of the amplified promoter region from both the tail snips and the glomeruli was identical to that from the corresponding MC. Sequencing data confirmed the SSLP results. The MMP-9 promoter regions (positions $-1187$ to $+70$) from B6SJL and ROP MC were also sequenced. They differed only in the number of d(CA) repeats proximal to the transcription initiation site; whereas the MMP-9 promoter from ROP MC was characterized by the presence of 20 d(CA) repeats, the MMP-9 promoter from B6SJL MC contained 24 d(CA) repeats (Figure 4b). The passage number (passages 2 to 20) did not affect the number of d(CA) repeats in either cell line (data not shown). In addition, the lengths of the dinucleotide repeats in the MMP-9 promoter did not change with cell density.

\section*{Effects of MMP-9 Promoter Length on Gene Transcription}

A 1257-bp fragment of the MMP-9 promoters from both ROP and B6SJL MC was cloned into the luciferase reporter-pGL3 construct. These constructs were transfected into MC for determination of whether alterations in the number of d(CA) repeats affected gene transcription. Compared with the B6SJL promoter, the ROP MMP-9 promoter exhibited reduced transcriptional activity (Figure 5). In particular, the transcriptional activity of the ROP promoter was independent of the cell type used for transfection experiments (ROP, B6SJL, or COS-7 cells). There was no significant activation of the luciferase reporter gene when transfections were performed with an empty pGL3 vector (data not shown).

\section*{Regulation of MMP-9 Promoter Activity by PMA}

We tested MMP-9 promoter transcriptional activation by using the PKC activator PMA, as described previously (16). We transfected B6SJL and ROP MC with the two different MMP-9 promoter vectors in the presence of increasing concentrations of PMA (3 to 60 ng/ml). We observed that both
promoters were responsive to PMA (Figure 6). ROP promoter activation was equal when the ROP promoter construct was transfected into ROP or B6SJL MC. However, activation of the B6SJL promoter required higher doses of PMA when the B6SJL promoter construct was transfected into ROP MC, compared with B6SJL MC. The levels of MMP-9 mRNA and active enzyme were increased after the addition of PMA in both ROP and B6SJL MC. MMP-9 mRNA expression and enzymatic activity were 3- to 4.5-fold higher in both MC types after PMA (60 ng/ml) treatment (Figure 7). However, even after PMA stimulation, MMP-9 mRNA expression and enzymatic activity in ROP MC were still far below those present in unstimulated B6SJL MC.

SSLP Analysis of Other Promoters with Similar Structures

To determine whether the differences in the number of dinucleotide repeats in ROP and B6SJL MC were restricted to the MMP-9 promoter, we examined the lengths of the promoter regions containing d(CA) repeats from ROP and B6SJL mice. The d(CA) repeat is underlined. +1 indicates the start codon.

Figure 3. Murine MMP-9 promoter structure. The MMP-9 promoter region studied herein (positions −1187 to +70) contains four activating protein-1 (AP-1) sites, four PEA-3 sites, three Sp-1 sites, one AP-2 site, and one nuclear factor-κB (NF-κB) site. The dinucleotide repeat region is localized proximal to one of the AP-1 sites (positions −161 to −109). The promoter structure derived from the published sequence (National Center for Biotechnology Information accession number X72794) is shown.

Figure 4. Dinucleotide repeat region sequencing analysis and simple sequence length polymorphism (SSLP) analysis. (a) Representative SSLP analysis of the d(CA) repeat region from three ROP and three B6SJL cell lines. (b) Comparison of the sequences of the MMP-9 promoter region containing d(CA) repeats from ROP and B6SJL mice. Arrow indicates d(Ca) length difference between the ROP and B6SJL MMP-9 promoter. The d(Ca) repeat is underlined. +1 indicates the start codon.

Figure 5. Transfection of B6SJL, ROP, or COS-7 MC with either ROP or B6SJL promoter-reporter constructs. B6SJL MMP-9 promoter (prom) activity (expressed as luciferase activity/β-galactosidase activity) was higher in all cell types, compared with that of the ROP MMP-9 promoter. This result was independent of the cell line into which the reporter construct was transfected (B6SJL, ROP, or COS-7 cells). The differences were larger in both B6SJL MC and COS-7 cells (***P < 0.001), compared with ROP MC (**P < 0.01).

Discussion

Associations between decreased numbers of d(CA) repeats in the MMP-9 promoter, MMP-9 expression, and the propensity to develop glomerulosclerosis have not been previously reported. In this study, we observed a reduction in the number of d(CA) repeats in the MMP-9 promoter of the ROP strain (prone to glomerulosclerosis), compared with the B6SJL strain (resistant to glomerulosclerosis). Although we do not know the role that MMP-9 promoter length might play in the susceptibility to glomerulosclerosis in the ROP strain, our data dem-
onstrate that the shortened MMP-9 promoter in ROP MC is associated with decreased MMP-9 gene expression and enzymatic activity. This may be one factor accounting for the increased amount of extracellular matrix that accumulates in vivo and in vitro in the glomerulosclerosis-prone strain (ROP) (35–37).

MMP-9 promoter polymorphism has recently been observed in diabetic nephropathy (6), suggesting that MMP-9 may be an important marker of susceptibility to glomerulosclerosis. We demonstrated that susceptibility to glomerulosclerosis is determined by eight to 10 loci, but we did not identify a specific quantitative trait locus associated with glomerulosclerosis (42).

Interestingly, linkage analysis with the MODY-linked marker D20S197 provided evidence for linkage to non-insulin-dependent diabetes mellitus among patients with diabetic nephropathy (43), and this marker has been mapped to the same region as the MMP-9 gene (20q12.2–13.1) (44). Therefore, the MMP-9 locus may be an important marker associated with diabetic nephropathy. Whether the effect of the MMP-9 promoter on gene transcription plays a role in the pathogenesis of glomerulosclerosis remains to be established. Our identification of MMP-9 d(CA) polymorphism in murine models of glomerulosclerosis might provide an experimental tool for determination of whether this polymorphism plays a direct role in the pathogenesis of this disease.

We confirmed and extended our previous findings that MMP-9 mRNA expression and enzymatic activity were lower in MC isolated from ROP (glomerulosclerosis-prone) mice, compared with those from B6SJL (glomerulosclerosis-resistant) mice (38). These findings were independent of the cell density and passage number. MMP-9 mRNA expression and enzymatic activity were also lower in microdissected glomeruli from three ROP mice, compared with three B6SJL mice. However, whereas the MMP-9 mRNA expression from total cortex was lower in ROP mice than in B6SJL mice, the enzymatic activity was not significantly different, suggesting that compensatory mechanisms might contribute to MMP-9 activation in kidney cortex from ROP mice. Examination of the MMP-9 promoter in ROP and B6SJL MC with SSLP analysis and automated sequencing revealed that the MMP-9 promoter region studied (positions −1187 to +70) was identical in the two strains, except for the number of d(CA) repeats.

Figure 6. Stimulation of MMP-9 promoter activity by phorbol-12-myristate-13-acetate (PMA). PMA treatment induced dose-dependent increases in both ROP and B6SJL MMP-9 promoter activity. However, the source of the MC used for transfection affected the amplitude of the response for B6SJL MMP-9 promoter activation by PMA. Activation of the B6SJL MMP-9 promoter by PMA occurred at a dose of 30 ng/ml in B6SJL MC but was observed only at a dose of 60 ng/ml in ROP MC. Activation of the ROP MMP-9 promoter by PMA occurred at a dose of 30 ng/ml in both B6SJL and ROP MC. *P < 0.05, **P < 0.01, ***P < 0.001, comparing PMA-treated ROP and B6SJL MC with untreated MC.

Figure 7. Stimulation of MMP-9 gene expression and enzymatic activity by PMA. (a) Graphs indicating that PMA treatment induced three- to fourfold increases in MMP-9 mRNA expression in both ROP and B6SJL MC at the dose of 60 ng/ml. However, the baseline and stimulated levels of MMP-9 mRNA expression in B6SJL MC were eight- to 10-fold higher than those in ROP MC. ***P < 0.001, PMA versus control (C). (b) Representative zymographic analysis, demonstrating that MMP-9 activity was induced by PMA (60 ng/ml) in both ROP and B6SJL cells. The lower band of activity represents bovine MMP-9 activity, resulting from medium supplemented with 0.1% fetal bovine serum.
of these observations, we concluded that alterations in the MMP-9 promoter were associated with differences in MMP-9 mRNA expression and activity.

We compared the responses of the B6SJL and ROP MMP-9 promoters to a pharmacologic stimulus (16). We chose PMA because it is known to activate MMP-9 transcription via the AP-1 site, which is in close proximity to the d(CA) repeats; the number of dinucleotide repeats might thus affect its activation. We observed approximately threefold increases in the responses of the B6SJL and ROP MMP-9 promoters to PMA in both cell types. Interestingly, as was observed under baseline conditions, higher doses of PMA were required to activate the B6SJL MMP-9 promoter when it was transfected into ROP cells. This observation supports the possibility that ROP MC might be deficient in specific cellular factors involved in the pathway activated by PMA. PKC stimulation by 60 ng/ml PMA was associated with 3- to 4.5-fold increases in MMP-9 mRNA expression and enzymatic activity in both ROP and B6SJL MC. This observation suggested that PMA induced MMP-9 promoter activation, MMP-9 mRNA expression, and the release of active enzyme in MC isolated from both strains. However, the levels of MMP-9 expression and enzymatic activity observed in ROP MC after PMA stimulation were always much lower than those observed in B6SJL MC. Although both promoters were responsive to PMA, the effects on MMP-9 expression and activity were much less in ROP MC, compared with B6SJL MC.

We hypothesized that differences in MMP-9 mRNA expression and activity in MC were associated with a difference in the number of d(CA) repeats. To establish whether transcriptional activation of the MMP-9 promoter in MC was affected by the number of d(CA) repeats, we developed MMP-9 promoter constructs from both ROP and B6SJL MC and transfected each construct into either ROP or B6SJL MC. We observed that the transcriptional activity of the B6SJL MMP-9 promoter was approximately twofold higher than that of the ROP MMP-9 promoter, in cell lines from both ROP and B6SJL mice. The transcriptional activity of the B6SJL MMP-9 promoter was also higher in an independent cell line (COS-7 cells). Therefore, the difference in the number of d(CA) repeats had a significant effect on MMP-9 promoter activation, irrespective of the cell type in which the promoter was present. On the basis of these observations, we concluded that alterations in the MMP-9 promoter were associated with differences in MMP-9 mRNA expression and activity.

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We compared the responses of the B6SJL and ROP MMP-9 promoters to a pharmacologic stimulus (16). We chose PMA because it is known to activate MMP-9 transcription via the AP-1 site, which is in close proximity to the d(CA) repeats; the number of dinucleotide repeats might thus affect its activation. We observed approximately threefold increases in the responses of the B6SJL and ROP MMP-9 promoters to PMA in both cell types. Interestingly, as was observed under baseline conditions, higher doses of PMA were required to activate the B6SJL MMP-9 promoter when it was transfected into ROP cells. This observation supports the possibility that ROP MC might be deficient in specific cellular factors involved in the pathway activated by PMA. PKC stimulation by 60 ng/ml PMA was associated with 3- to 4.5-fold increases in MMP-9 mRNA expression and enzymatic activity in both ROP and B6SJL MC. This observation suggested that PMA induced MMP-9 promoter activation, MMP-9 mRNA expression, and the release of active enzyme in MC isolated from both strains. However, the levels of MMP-9 expression and enzymatic activity observed in ROP MC after PMA stimulation were always much lower than those observed in B6SJL MC. Although both promoters were responsive to PMA, the effects on MMP-9 expression and activity were much less in ROP MC, compared with B6SJL MC.

We examined promoters with similar structures, i.e., RANTES and TIMP-3 (40,41), to determine whether the strain-specific differences in the number of d(CA) repeats were restricted to the MMP-9 promoter. We did not detect differences in the lengths of the dinucleotide repeats in either the RANTES or TIMP-3 promoter, but we cannot conclude that our findings are restricted to MMP-9, because of the small number of genes we sampled. However, we can conclude that strain-specific differences in the number of d(CA) repeats do not seem to represent a generalized phenomenon among promoters with similar structures.

In summary, we observed that the MMP-9 promoter in the ROP strain was characterized by a decreased number of d(CA) repeats, compared with the B6SJL strain. This decrease was associated with marked reductions in MMP-9 expression and enzymatic activity in MC and glomeruli from ROP mice. We also observed that PKC activation did not increase MMP-9 activity in ROP MC supernatants to the level noted in B6SJL MC supernatants. Our data, together with a recent description of MMP-9 promoter polymorphism among patients with diabetic nephropathy, suggest that alterations in the MMP-9 promoter might represent a genetic marker for glomerulosclerosis. Furthermore, the data suggest that altered mesangial MMP-9 expression attributable to promoter abnormalities might be one factor contributing to the development of glomerulosclerosis.

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References


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In “Association of a Decreased Number of d(CA) Repeats in the Matrix Metalloproteinase-9 Promoter with Glomerulosclerosis Susceptibility in Mice” by Fornoni et al., which appeared on pages 2068 to 2076 of the August 2002 issue of JASN, an early draft of Figure 4 was mistakenly printed. The correct version appears below.

**Figure 4.** Dinucleotide repeat region sequencing analysis and simple sequencing length polymorphism (SSLP) analysis. (a) Representative SSLP analysis of the d(CA) repeat region from three ROP and three B6SJL cell lines. (b) Comparison of the sequences of the MMP-9 promoter region containing d(CA) repeats from ROP and B6SJL mice. Arrow indicates d(CA) length differences between the ROP and B6SJL MMP-9 promotor. The d(CA) repeat is underlined. +1 indicates the start codon.

In “Cumulative Risk for Developing End-Stage Renal Disease in the US Population” by Kiberd et al., which appeared in the June 2002 issue of JASN, Figures 1 and 2 were transposed.