Transcriptional Up-regulation of Endothelial Cell Matrix Metalloproteinase-2 in Response to Extracellular Cues Involves GATA-2*

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Matrix metalloproteinase-2 (MMP-2) plays a critical role in endothelial cells during the processes of angiogenesis and vascular remodeling. Endothelial cell production of MMP-2 is greatly enhanced when cells are cultured within a three-dimensional type I collagen matrix coinciding with the increased invasive and migratory phenotype of the cells. To define the transcriptional regulation of MMP-2 in rat microvascular endothelial cells, we performed promoter-reporter assays with a series of promoter truncations. Activity of the full promoter was significantly greater in cells cultured within three-dimensional type I collagen compared with cells cultured as a monolayer (two-dimensional) on type I collagen. Truncation of the region encompassing base pairs -1562 to -1375 (relative to the start codon) of the MMP-2 promoter resulted in loss of this differential activity of the MMP-2 promoter. Analysis of this region indicated two putative GATA-2 binding domains between -1437 and -1387. Southwestern blot analysis and electrophoretic mobility shift assays confirmed the binding of GATA-2 to this region of the MMP-2 promoter. Overexpression of GATA-2 in COS-7 cells significantly increased the activity of the full-length MMP-2 promoter-luciferase construct. Endothelial cells expressed greater levels of GATA-2 protein in three-dimensional compared with two-dimensional cultures, and activity of the -1437/-1387 region of the MMP-2 promoter was significantly greater in three-dimensional cultured endothelial cells. Together, these results indicate GATA-2 regulation of the MMP-2 promoter in endothelial cells and that the GATA-2 binding domain is sufficient to drive increased activity of the MMP-2 promoter in response to an extracellular matrix stimulus.

The matrix metalloproteinases (MMPs) consist of a family of zinc- and calcium-dependent endopeptidases that cleave specific subsets of extracellular matrix proteins, growth factors, and cell surface receptors (1,2). Endothelial cell production and activation of MMPs, including MMP-2, are critical for the process of angiogenesis (3,4). Angiogenesis in skeletal muscle may be initiated by growth factors, by hemodynamic forces, or by mechanical stretching forces (5). The angiogenic process requires that endothelial cells proteolyze their basement membrane and then migrate through the interstitial matrix to form a new capillary. Inhibition of MMP activity prevents basement membrane degradation and the process of capillary sprouting in activity-stimulated skeletal muscle (6). When cultured within a three-dimensional type I collagen matrix, endothelial cells gain an invasive and migratory phenotype including increased production of matrix metalloproteinases and enhanced cell motility similar to what is seen in vivo during capillary sprouting (7, 8). Regulated transcription of MMP-2 is important in determining this invasive phenotype in endothelial cells.

Regulation of MMP-2 occurs at both transcriptional and post-translational levels. Although the rate of MMP-2 protein production is controlled largely through alterations in mRNA transcription, proteolytic activity is regulated through secretion, cell surface binding, and activation of the latent enzyme. In contrast to the large amount of information available regarding the regulation of MMP-2 activity at the protein level, transcriptional regulation of MMP-2 has not been well characterized.

MMP-2 promoter analysis has been carried out in several cell types of both rat and human origin. The MMP-2 promoter has no TATA or CAAT boxes and is considered to be under constitutive control via GC-rich promoter regions. An enhancer region, known as RE1, has been identified at base pairs -1322 to -1282 of the rat promoter sequence (9). Binding proteins within the RE1 domain are well characterized and include Y-box protein-1 (YB-1), p53, and AP-2 (10, 11), as well as the transcriptional suppressor, nm23-β (12). These transcription factors contribute greatly to the constitutive expression of MMP-2 in rat mesangial cells. Characterization of the promoter in rat endothelial cells identified an additional enhancer region (RE2) at base pairs -1435/-1375 (13). These authors demonstrated greater binding activity to the enhancer region in endothelial cells derived from spontaneously hypertensive (SHR) versus normotensive (WKY) rats, but the binding protein(s) was never identified. The human MMP-2 promoter sequence differs from the rat sequence, but several enhancer domains appear to be common between the two species. For instance, the human MMP-2 promoter is activated by p53 (14) and AP-2 (15) in a region designated enhancer region 2 (r2) that is located at -1635/-1593 relative to the transcription initiation start.

We have shown that MMP-2 production in rat microvascular endothelial cells is sensitive to the extracellular matrix environment and is largely unresponsive to growth factor stimula-
MMP-2 Promoter Regulation Involves GATA-2

MATERIALS AND METHODS

Cell Culture—Microvascular endothelial cells were isolated from rat extensor digitorum longus muscles using a protocol adapted from Madri and Williams (16). Briefly, the muscles were isolated, minced, and digested with 0.5% collagenase type A (Invitrogen) in sterile HEPES-saline buffer (140 mM NaCl, 10 mM HEPES, pH 7.4, 10 mM KCl, 0.1 mM CaCl₂, 0.2 mM MgCl₂) in a shaking water bath (37 °C for 30 min). The tissue was then centrifuged, resuspended twice in HEPES-saline buffer containing 5% bovine serum albumin, then resuspended in 1 ml of HEPES-saline with 1% bovine serum albumin and layered over a Percoll gradient and centrifuged at 13,000 × g for 20 min. The layer corresponding to the microvascular fragments was removed, washed with HEPES-saline, and plated on 25-cm² tissue culture dishes. Gels were fixed and stained with Coomassie Blue R-250 as documented system, and densitometry was performed using AlphaEase software. For Southwestern blots, nuclear extracts (15 µg) were separated using 12% polyacrylamide gels under reducing conditions, which were then transferred to nitrocellulose membranes. After renaturing and blocking overnight at 4 °C in TNE buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, and 1 mM dithiothreitol) containing 5% skim milk powder, blots were washed with TNE buffer and then hybridized for 5 h at room temperature using TNE buffer containing 10 µg/ml poly(dI·dC) and 5 µg of pBR322 or relaxed double-stranded probe corresponding to base pairs 1345 to 1387 of the MMP-2 promoter (synthesized by Sigma). Blots were washed twice for 15 min with TNE and then exposed to x-ray film at −80 °C using an enhancing screen.

Nuclear Extracts—Nuclear extracts of endothelial cells cultured for 24 h were isolated by nuclei lysis buffer (100 mM NaCl, 5 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM glycerol, 1 µg of poly(dI·dC), 500 ng of salmon sperm DNA, 1 µl of T4 DNA polymerase, or 5–10 µg of nuclear extract. For supershift assays, 1–4 µg of antibody (anti-GATA-2 from Santa Cruz Biotechnology) or YB-1 (kindly provided by Dr. Stuart Orkin) was added to the reaction and incubated for 15 min prior to gel loading. Reactions were electrophoresed through a 4% nondenaturing polyacrylamide gel followed by autoradiography.

Northern Blotting—Total cellular RNA was extracted from two-dimensional or three-dimensional cultures using TRIzol (Invitrogen) according to the manufacturer’s directions. The RNA concentration was determined by spectrophotometer. 10 µg of total RNA was electrophoresed through a 1% agarose-formaldehyde gel as described previously (8). Gels were stained with 0.5 µg/ml ethidium bromide and photographed with a UV transilluminator (UVP). Images were captured using digital camera (Sony). Blots were hybridized overnight with a radiolabeled probe and analyzed by phosphoimager analysis (Molecular Dynamics). Oligonucleotides were synthesized by Sigma.

RESULTS

Rat skeletal muscle microvascular endothelial cells cultured for 24 h within a three-dimensional collagen type I matrix produced significantly greater MMP-2 mRNA and protein when compared with cells cultured on a thin monolayer coating of collagen type I (Fig. 1, A and B). Likewise, the MMP-2 promoter analysis demonstrated greater transcriptional activity of the full-length promoter in endothelial cells cultured in three-dimensional compared with two-dimensional conditions (Fig. 1C). Truncation of the promoter region (from −1562 to −1375) removed the responsiveness to the three-dimensional collagen culture condition. Further deletion of −1365 to −510 again reduced promoter activity but did not have a differential effect on cells in two-dimensional versus three-dimensional culture (Fig. 1C).

Electrophoretic Mobility Shift Assays—Consensus GATA-2 binding double-stranded oligonucleotides (5′–CCTGGGTTATTCCTCGGCTGC–3′; Panomics) and complementary oligonucleotides corresponding to base pairs −1345 to −1387 or −1329 to −1293 of the MMP-2 promoter were annealed and end-labeled using γ[32P]ATP. An enriched source of human GATA-2 in vitro transcribed and translated from pMT2-GATA2 (kindly provided by Dr. Stuart Orkin) with the Promega Tnt system was used to define the binding pattern of this transcription factor to consensus GATA-2 binding domains and to the MMP-2 promoter region. Gel shift reactions consisted of a 5 × 10⁶ cpm oligonucleotide probe, binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol), 1 µg of poly(dI·dC), 500 ng of salmon sperm DNA, 1 µl of T4 RNA polymerase, or 5–10 µg of nuclear extract. For supershift assays, 1–4 µg of antibody (anti-GATA-2 from Santa Cruz Biotechnology) or YB-1 (kindly provided by Dr. Stuart Orkin) was added to the reaction and incubated for 15 min prior to gel loading. Reactions were electrophoresed through a 4% nondenaturing polyacrylamide gel followed by autoradiography.

Western and Southwestern Blots—For Western blots, nuclear extracts (10 µg) were separated using 12% polyacrylamide gels under reducing conditions and then transferred to polyvinylidene difluoride membranes. After blocking, blots were incubated overnight with anti–bodies for AP-2 or p53 (Santa Cruz Biotechnology) or YB-1 (kindly provided by Dr. David Lovett, San Francisco Veterans Affairs Medical Center, University of California). After secondary antibody incubation, bands were detected using the SuperSignal West Pico (Pierce) and FluorChem gel documentation systems (AlphaInnotech). Densitometry was performed using AlphaEase software. For Southwestern blots, nuclear extracts (15 µg) were separated using 12% polyacrylamide gels under reducing conditions, which were then transferred to nitrocellulose membranes. After renaturing and blocking overnight at 4 °C in TNE buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, and 1 mM dithiothreitol) containing 5% skim milk powder, blots were washed with TNE buffer and then hybridized for 5 h at room temperature using TNE buffer containing 10 µg/ml poly(dI·dC) and 5 µg of pBR322 or relaxed double-stranded probe corresponding to base pairs −1345 to −1387 of the MMP-2 promoter (synthesized by Sigma). Blots were washed twice for 15 min with TNE and then exposed to x-ray film at −80 °C using an enhancing screen.

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Transient Transfections—The rat MMP-2 promoter (72-PGL2) was provided kindly by Dr. David Lovett. The full-length promoter (−1686 to +47786) was inserted into the pGL3 Basic plasmid (Promega). Truncations were constructed using restriction enzyme double digests (KpnI-AflII, KpnI-AIwN1, KpnI-PstI, and KpnI-Smal) followed by gel purification and ligation of the plasmid, transformation into bacteria, and purification of selected colonies using Qiafilter Midi Preps (Qiagen). LipofectAMINE-based transient transfections of MMP-2 promoter sequences and Renilla luciferase were performed on COS-7 cells or on primary cultures of rat microvascular endothelial cells (passages 6–10) according to the manufacturer’s directions (Invitrogen). For COS-7 transfections, cells were lysed 48 h after transfection. For endothelial cell transfections, 24 h after transfection, cells were trypsinized and divided into two-dimensional and three-dimensional conditions. 48 h after transfection, cells were harvested and assayed for luciferase activity according to the Promega Dual Luciferase Reporter System. Luminescence was detected with a Berthold 9501 tube luminometer. Data were normalized for activity of Renilla luciferase to account for transfection efficiency and then expressed as fold increase in comparison with the light output of the pGL3 Basic or pGL3 promoter constructs. Results from four independent experiments, each with duplicate wells, were averaged and presented as mean ± S.E.

Statistics—Statistical analyses of two-dimensional and three-dimensional conditions were performed using Student’s paired t tests with significance established as p < 0.05.
The region between −1322 and −1282 has previously been designated as RE1 by Lovett and colleagues (9). This region contains binding sites for transcription factors YB-1, AP-2, and p53 (11). We tested whether these transcription factors were induced in three-dimensional culture by Western blotting of nuclear extracts. There was no change in p53 (0.91 ± 0.32), a slight decrease in AP-2 (0.80 ± 0.07), and a strong decrease in YB-1 (0.11 ± 0.1, p < 0.05), in three-dimensional culture compared with two-dimensional levels (Fig. 2, A–C). Gel shift assays using double-stranded oligonucleotides corresponding to base pairs −1327 to −1293 of the MMP-2 promoter showed two shifted complexes; the faster mobility complex was of similar intensity in nuclear extracts of cells in two-dimensional compared with three-dimensional cultures, whereas the slower mobility complex was greatly reduced in three-dimensional compared with two-dimensional nuclear extracts (Fig. 2D).

We thus focused on the region of the MMP-2 promoter that lies between −1562 and −1375. A transcription factor search on this region using the TRANSFAC data base (18) identified two sites located at −1431/−1422 and −1399/−1390 that shared considerable homology to the nominal binding sequence for the transcription factor GATA-2 (Fig. 3A). This region corresponds to a portion of the enhancer region described by Bottles et al. (13). We then examined protein binding to this sequence first through Southwestern blotting. Using endothelial cell nuclear extract as a protein source, this technique...
identified a weak band (of ~53 kDa) that interacted with the labeled MMP-2 promoter sequence (Fig. 3B). This size is consistent with GATA-2, as has been demonstrated by other researchers using Western blotting (19). We tested specifically for GATA-2 protein binding ability to the MMP-2 promoter sequence using gel shift assay with \textit{in vitro} transcribed and translated GATA-2 protein. Two shifted bands were detected when binding to the MMP-2 promoter sequence, whereas one band was detected when binding to the consensus GATA-2 oligonucleotide (Fig. 3C). Incubation of the protein-DNA complex with anti-GATA-2 antibody resulted in a supershifted complex, a decreased intensity of the slower mobility protein-DNA complex (\textit{solid arrow}), and increased intensity of the faster mobility protein-DNA complex (\textit{open arrowhead}) (Fig. 3D). It is likely that both bands correspond to GATA-2 binding and arise from variable levels of protein interaction to the two GATA-2 binding sites present in the MMP-2 promoter sequence (compared with the single site on the consensus oligonucleotide).

We tested for direct evidence that GATA-2 \textit{trans}-activates the MMP-2 promoter by cotransfecting COS-7 cells with GATA-2 or empty expression vector and the full-length MMP-2 promoter-luciferase construct. Western blotting confirmed the effectiveness of the overexpression procedure in elevating levels of GATA-2 protein in the COS cells (Fig. 4A). MMP-2 promoter activity was enhanced 3.3-fold ($p < 0.05$) in the presence of GATA-2 overexpression compared with the empty vector (Fig. 4B).

To assess the role of GATA-2 in the transcriptional control of MMP-2 in endothelial cells, we first examined endogenous levels of GATA-2 in cells cultured in two-dimensional or three-dimensional type I collagen. By Western blotting, GATA-2 protein was increased in the nuclear extracts of endothelial cells cultured in three-dimensional collagen compared with two-dimensional collagen (Fig. 5A). Similarly, gel shift assays showed formation of GATA-2 complexes in the presence of both two-dimensional and three-dimensional nuclear extracts, but these complexes were more intense in the presence of the three-dimensional cultured endothelial cell nuclear extracts (Fig. 5B). These complexes were identical to those seen using the \textit{in vitro} transcribed and translated GATA-2 (Fig. 3, C and D) and were competed with 10–50-fold excess cold oligonucleotide and supershifted with anti-GATA-2 antibody (data not shown). We confirmed the differential activity of the GATA-2 binding region in response to extracellular matrix cues through transient transfection of microvascular endothelial cells with the GATA-2 binding sequence of the MMP-2 promoter (~1435/1387) coupled to the pGL3 promoter followed by culturing the transfected cells in two-dimensional or three-dimensional culture conditions for 48 h. Extracts from cells in the three-
two-dimensional culture condition had significantly greater transactivation of the GATA-2 binding sequence compared with those from two-dimensional culture conditions (1.8-fold above two-dimensional conditions, \( p < 0.05 \); Fig. 5C).

**DISCUSSION**

This study shows that the transcription factor, GATA-2, enhances the transcription of MMP-2 in microvascular endothelial cells cultured within a three-dimensional type I collagen matrix. These results provide the first mechanistic evidence of extracellular matrix-driven transcriptional regulation of the MMP-2 gene.

Despite considerable evidence for regulation of the MMP-2 promoter by YB-1, AP-2, and p53 interactions in rat mesangial cells (10,11), we failed to detect a role for this region in conferring extracellular matrix responsiveness to the MMP-2 promoter. These transcription factors may contribute to overall positive transcriptional responses. However, this remains to be clarified through additional experimentation as we have not specifically examined the effects of YB-1 overexpression on the MMP-2 promoter.

Rather, we defined a role for GATA-2 direct interaction with the MMP-2 promoter region \(-1435/-1387\) utilizing analyses of *in vitro* DNA-protein complex formation (Figs. 3 and 5) and confirmed the trans-activation capability of GATA-2 through measurements of MMP-2 transcriptional activity in COS-7 and microvascular endothelial cells (Figs. 4 and 5). Together, these experiments provide clear evidence that GATA-2 interacts with the MMP-2 promoter and that this interaction enhances promoter activity. Increased levels of GATA-2 protein and enhanced binding of GATA-2 to the MMP-2 promoter occur in response to culture within a three-dimensional collagen matrix, implying that this domain acts as a response element for extracellular matrix-driven signaling events.

Although we have not tested for GATA-2 binding to the human MMP-2 promoter sequence, it is notable that the dual GATA-2 binding sites with 21 nucleotides separating them are highly conserved between human and rat promoters. The equivalent binding domains are located at base pairs \(-1434/-1387\).
expression vector compared with the empty pMT2 vector (B/H11006 are presented as mean transcription factors are known to play critical roles as cofactors. Several studies have shown cooperative interactions between GATA-2 and additional transcription factors. erativity between GATA-2 and additional transcription factors. However, GATA-2 of endothelial cells is linked to reduced expression of several endothelial cell-specific, and, in fact, cellular dedifferentiation of endothelial cells is linked to reduced expression of several transcription factors in both erythroid (33) and cardiac muscle (34,35). It is possible that similar cooperative interactions can modulate the effectiveness of GATA-2 transactivation of the MMP-2 promoter. Utilizing gel shift analysis, we observed two shifted bands corresponding to GATA-2 interactions with the MMP-2 promoter sequence (~1435/~1387). We think that both bands show GATA-2 binding site activity in endothelial cells. The sequence ~1435/~1387 of the double stranded MMP-2 promoter was subcloned into the pGL3 promoter and transiently transfected into microvascular endothelial cells. Luciferase activities of the ~1435/~1387 promoter construct were measured after 2 days of culture as a monolayer (2D) or within three-dimensional type I collagen matrix (3D) and normalized to the activity of the enhancerless pGL3 promoter. Data are presented as mean ± S.E. for 3 independent experiments. The asterisk denotes significance (p < 0.05) compared with the two-dimensional culture condition.

-1425 and -1403/1394 of the human MMP-2 sequence, as published by Bim and Sun (14).

GATA-2 is a member of the GATA family of DNA binding proteins that interacts with a DNA sequence motif (WGATAR) through highly conserved C2C2 zinc finger domains (23,24). This family of transcription factors initially was recognized to play an important role in regulating expression of multiple genes in cells of erythroid origin, although it is now known that these transcription factors are also expressed in cells of other origin such as fibroblasts, embryonic brain, cardiac muscle, liver, and kidney (25). Human GATA-2 was cloned from endothelial cells following discovery of its critical role in regulating expression of multiple endothelial cell genes including endothelial cell nitric oxide synthase (27), vascular cell adhesion molecule-1 (28), platelet/endothelial cell adhesion molecule-1 (29), and the vascular endothelial growth factor receptor flk1/KDR (30).

Interestingly, many of the genes regulated by GATA-2 are endothelial cell-specific, and, in fact, cellular dedifferentiation of endothelial cells is linked to reduced expression of several transcription factors including GATA-2 (31). However, GATA-2 itself cannot provide cell type specificity because it is found in a number of cell types. Rather, it is thought that endothelial cell-specific gene expression in these cases is achieved by cooperativity between GATA-2 and additional transcription factors. Several studies have shown cooperative interactions between GATA-2 and other transcription factors, including Sp1 (27) and c-fos and c-jun (32). In addition, a family of proteins that include “friend of gata,” or FOG, FOG-2, and U-shaped (Ush) transcription factors are known to play critical roles as cofactors for GATA transcription factors in both erythroid (33) and cardiac muscle (34,35).
migrating protein-DNA complex and an increase in intensity of the faster migrating protein-DNA complex (Fig. 3D or 2), when a greater amount of antibody was added, in the complete competition of both bands (data not shown). Despite this evidence, it is possible that the multiple gel shift bands result from related GATA protein interactions with the DNA probe, or from GATA-2 interactions with other proteins. Although GATA-2 was thought to be the predominant GATA factor found in endothelial cells (25), a more recent study has identified the mRNA for GATA-3 and GATA-6 in human umbilical vein endothelial cells and demonstrated that these factors can bind to and trans-activate the vascular cell adhesion molecule-1 promoter (36). We have shown that GATA-2 protein, by itself, is sufficient to trans-activate the MMP-2 promoter (Fig. 4B), but the possibility of enhanced MMP-2 transcription in the presence of additional GATA factors bears further investigation.

We previously found that the early growth response-1 (egr-1) transcription factor trans-activates the membrane type 1 (MT1)-MMP promoter sequence in response to three-dimensional type I collagen stimulation (17). Because MT1-MMP and MMP-2 gene products frequently appear to be co-regulated, we originally were surprised that the MMP-2 promoter lacks consensus binding sites for egr-1. The current study provides evidence for GATA-2 as a critical transcription factor in the regulation of the MMP-2 promoter under the pro-angiogenic condition of three-dimensional type I collagen stimulation. We have recently demonstrated a critical role for extracellular signal-regulated kinase-1/2 activation in the regulation of both MMP-2 and MT1-MMP mRNA in three-dimensional collagen-stimulated endothelial cells.2 Notably, both egr-1 and GATA-2 are downstream effectors of the extracellular signal-regulated kinase-1/2 signal pathway (19, 37, 38), implying that extracellular signal-regulated kinase-1/2 signal pathway (19, 37, 38), implying that extracellular signal-regulated kinase-1/2 may be a critical component of a common signal pathway responsible for recruitment of both transcription factors. Further studies will elucidate the mechanisms by which extracellular matrix-driven signaling results in coordinated recruitment of diverse transcription factors and activation of the transcription of multiple genes critical for the process of angiogenesis.

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