Transcription Factor Sp1 Phosphorylation Induced by Shear Stress Inhibits Membrane Type 1-Matrix Metalloproteinase Expression in Endothelium*

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Membrane type 1-matrix metalloproteinase (MT1-MMP) plays a key role in endothelial cell migration, matrix remodeling, and angiogenesis. Previous studies demonstrated that a mechanical force, cyclic strain, increases MT1-MMP expression by displacing Sp1 with increased Egr-1 expression and binding to the promoter site. However, the effect of shear stress (SS) on MT1-MMP expression is poorly understood. Although Egr-1 mRNA transcription and protein was induced (7.6-fold) in response to SS (n = 5, 0–8 h, p < 0.05), SS decreased MT1-MMP mRNA transcription and protein levels in a time-dependent fashion (10, 50, and 90% reduction at 1, 4, and 8 h, respectively; n = 5, p < 0.05). Egr-1 protein was increased after SS and cyclic strain, but Sp1 was serine-phosphorylated only after SS. SS increased Sp1 DNA binding (3.8-, 5.8-, and 2.4-fold increase at 1, 4, and 8 h, respectively; n = 5, p < 0.05) that was inhibitable by calf intestinal phosphatase. Thus, SS inhibits MT1-MMP expression despite Egr-1 up-regulation by inducing the serine phosphorylation of Sp1, which in turn increases its binding affinity for its site on the MT1-MMP promoter, reducing the ability of Egr-1 to displace it. These data illustrate the complex control of microvascular endothelial cell MT1-MMP expression in response to distinct environmental stimuli (cyclic strain versus shear stress), consisting of both the modulation of specific transcription factor expression (Egr-1) as well as transcription factor post-translational modification (serine phosphorylation of Sp1).

Angiogenesis is critical for different physiologic and pathologic processes including wound healing, tissue remodeling, chronic inflammatory disease, and tumorigenesis (1–3). Angiogenesis requires endothelial cells (EC)† to coordinate multiple functions including reduction of intercellular adhesion, degradation of subendothelial matrix, migration, proliferation, and formation of new capillary tubes (4). The matrix metalloproteinases (MMPs) are a family of structurally related zinc endopeptidases capable of proteolysis of numerous components of the extracellular matrix (5). Production and activation of MMPs correlate strongly with migration and invasion in endothelium (6, 7). Membrane type-1 matrix metalloproteinase (MT1-MMP, MMP-14), one of the members of the family of matrix-bound MMPs by a C-terminal transmembrane domain, is identified as a physiological activator of pro-MMP-2 on the cell surface as well as a functional enzyme that degrades a number of extracellular matrix components (8, 9). It is not surprising that MT1-MMP has been shown to be a key regulator of angiogenesis (10, 11).

MT1-MMP mRNA and protein levels are expressed at low levels in most noninvasive cells but are up-regulated during EC migration and matrix remodeling, and this up-regulation correlates with MMP-2 activation (11). The up-regulation of MT1-MMP mRNA and protein occurs in microvascular EC cultured in a three-dimensional collagen type-I matrix as a consequence of increased production and promoter binding of the transcription factor early growth response (Egr-1) (12, 13). Egr-1 is a key regulator of MT1-MMP activation and is involved in the differentiation to a migratory and invasive endothelial phenotype (13). However, the regulation of MT1-MMP expression and the mechanism of its activation induced by environmental forces are not well understood. We demonstrated previously (14) that cyclic strain induced up-regulation of MT1-MMP mRNA and protein in microvascular EC. We also demonstrated that a GC-rich sequence between −288 and −275 in the promoter contains overlapping consensus binding sites for Sp1 and Egr-1, and increasing levels of Egr-1 displace the basal transcription factor Sp1 from the MT1-MMP promoter, resulting in a significantly greater rate of transcription (15). Other studies have shown that fluid shear stress increases Egr-1 expression but decreases MT1-MMP mRNA and protein expression in vascular smooth muscle cells (15).

In this study, we investigate the regulation of MT1-MMP expression in microvessel EC by shear stress and the roles of the transcription factors Egr-1 and Sp1 in this process.

MATERIALS AND METHODS

Cell Culture—Rat microvascular EC were harvested from the epidermal fat pads of Sprague-Dawley rats and cultured as described previously (16). Twelve rats were used per preparation, and experiments were performed on two separate isolations of cells. Briefly, cells were grown on gelatin-coated tissue culture plates (1.5% gelatin in phosphate-buffered saline) and maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 25% sterile-filtered conditioned bovine aortic EC medium and 10% fetal bovine serum. For all experiments, EC were cultured in Dulbecco's modified Eagle's medium (Invitrogen) con-

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‡The abbreviations used are: EC, endothelial cells; MT1-MMP, membrane type 1-matrix metalloproteinase; SS, shear stress; MMPs, matrix metalloproteinases; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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taining 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT) and antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml, and amphotericin B 250 ng/ml (Invitrogen)) in a humidified atmosphere of 5% CO₂ on type I collagen-coated 35-mm well culture dishes at 37 °C. Serum-containing medium was replaced by serum-free medium 24 h prior to each experiment. Cells were synchronized by serum-starving for 24 h.

**Application of Shear Stress**—Shear stress was applied to confluent cultures with an orbital shaker (Lab-Line, Melrose Park, IL) (17–19). Although this technique does not result in uniform application of laminar shear stress across the entire monolayer, the majority of the cells are exposed to near-maximal shear stress ($\tau_{max}$), which can be calculated as shown in Equation 1,

$$\tau = \frac{a \cdot v}{\eta \sqrt{2f}} \quad \text{(Eq. 1)}$$

where $a$ is the radius of orbital rotation (1.4 cm); $v$ is the density of the culture medium (1.0 g/ml); $\eta$ is the viscosity of the medium (0.0075 poise measured by viscometer); and $f$ is the frequency of rotation (rotation/s).

A shaking frequency of 270 rpm results in a shear stress of 14 dynes/cm², which is a normal level in arteries.

**Application of Cyclic Strain**—EC were seeded on flexible silicon membranes coated with type I collagen (Flex-Cell International Corp., McKeesport, PA) and synchronized as described above. EC were exposed to a range creating a Flexi-Strain Unit (Flexcell FX-9000 system; Flex-Cell International Corp.), which consists of a vacuum manifold with recessed ports controlled by computer program. The apparatus has been extensively characterized and utilized. The cyclic strain regimen was carried out utilizing 150 mm Hg vacuum deformation at 60 cycles/min, resulting in 24% maximum strain.

**Northern Blot Analysis**—EC were lysed, and total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. RNA concentration and relative purity were quantified by measuring the absorbance at 260 nm and the ratio of absorbance at 260 nm relative to that at 280 nm. Twenty five μg of RNA were denatured by heating and then loaded and separated on a 1% agarose gel containing 6% formaldehyde, prior to transfer to a positively charged nylon membrane (Roche Molecular Biochemicals) by capillary blotting using 10X standard saline citrate (SSC: 1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0). Hybridization was performed with probes constructed from the Egr-1 expression vector and from human MT1-MMP cDNA (12, 13), labeled with the random priming Oligolabeling Kit (Amersham Biosciences) using [α-32P]dCTP (PerkinElmer Life Sciences). Blots were then washed twice in 1× SSC, 0.1% SDS for 15 min at room temperature and then once in 0.1× SSC, 0.1% SDS for 15 min. The blots were visualized by standard autoradiography techniques with X-Omat AR Film (Eastman Kodak Co.). Blots were stripped and reprobed with a GAPDH probe (ATCC, Manassas, VA) to normalize for RNA loading.

**Western Blot Analysis**—Whole cell extracts prepared from EC were lysed in 50 mM HEPES, 150 mM NaCl, 10 mM Tris (pH 7.5), 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10% glycerol, 1 μM sodium fluoride, and 1 μM sodium pyrophosphate. The extracts were heat-denatured in Laemmli sample buffer and boiled for 5 min. The samples were separated by 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 5% nonfat dry milk and incubated with primary antibodies to MT1-MMP (Chemicon International, Inc., Temecula, CA), Egr-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin antibody (Sigma–Aldrich Co.). After washing three times for 30 min in TBS-T, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies. The protein concentration of the extract was determined using the Bio-Rad reagent (Bio-Rad), and extracts were used immediately in EMSA reactions or stored at −80 °C.

**Electrophoretic Mobility Shift Assays (EMSA)**—Consensus Sp1 and Egr-1 binding double-stranded oligonucleotides, and mutated oligonucleotides were end-labeled by the Synthesis Facility, Yale University) of the MT1-MMP promoter were annealed and end-labeled using [γ-32P]ATP (PerkinElmer Life Sciences). The probe was purified using QuickSpin™ columns (Roche Molecular Biochemicals). Ten micromoles of nuclear extract were incubated in a final volume of 20 μl containing 10 mM Tris-Cl, 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, 0.5 μg of poly(dI-dC) in a buffer containing 10 mM Tris-HCl, pH 7.5, 10% glycerol, 10 μM dithiothreitol, 10 μM sodium fluoride, and 1 μM sodium pyrophosphate competitors at 25 °C. For supershift analysis, 2 μl of the Sp1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the reaction and incubated for 15 min prior to gel loading. Nuclear extracts were pretreated with 10 milliliters of calf intestinal phosphatase (CIP) (Promega, Madison, WI) for 30 min at 25 °C in EMSA buffer prior to adding the probe. Reactions were electrophoresed on a 4% non-denaturing polyacrylamide gel containing 45 mM Tris base, 45 mM boric acid, and 1 mM EDTA followed by autoradiography.

**Immunoprecipitation**—EC were washed three times with cold PBS and scraped into 500 μl of lysis buffer (50 mM Tris-Cl, pH 8.0, at 4 °C, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μg of leupeptin per ml). The cells were incubated at 4 °C for 30 min with rotation. After centrifugation for 15 min, the supernatant was transferred to a fresh microcentrifuge tube, and the extract was pre-cleared with 20 μl of protein G PLUS-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h with rotation. The beads were pelleted, and the supernatant was transferred to a new tube, and 20 μl of protein G PLUS-agarose beads conjugated to Sp1-specific antibody (Santa Cruz Biotechnology) was added. Immunoprecipitation was performed overnight at 4 °C with rotation, after which the immunoprecipitated proteins were washed four times with lysis buffer. After the last wash, the beads were resuspended in 12.5 μl of 2× Laemmli sample buffer and boiled for 5 min. The samples were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane and probed with anti-phosphoserine-specific antibody (Zymed Laboratories Inc.). Serine-phosphorylated Sp1 was detected using an enhanced chemiluminescent system as described above.

**Statistical Analysis**—Data are presented as mean ± S.E. The Student’s t test was used for analyzing densitometric data for each experiment compared with the respective static control (time, 0 min). p values of less than 0.05 were considered significant.

**RESULTS**

**Shear Stress Down-regulates MT1-MMP mRNA and Protein in Rat Microvascular EC**—We determined whether shear stress induced changes in MT1-MMP mRNA transcription or protein expression. Shear stress decreased MT1-MMP mRNA transcription (10, 50, and 90% reduction at 1, 4, and 8 h, respectively; p < 0.05. Fig. 1A), and this effect was associated with a diminution in MT1-MMP protein (10, 53, and 74% reduction at 1, 4, and 8 h, respectively; p < 0.05). The inhibition by shear stress was in a time-dependent fashion (Fig. 1B).

**Shear Stress Up-regulates Egr-1 mRNA and Protein in Rat Microvascular EC**—The primary response transcription factor, Egr-1, was rapidly activated by a variety of extracellular stimuli. Egr-1 binds to a sequence found in the proximal promoter of the MT1-MMP gene in EC. As shown in Fig. 2A, shear stress induced the expression of Egr-1 mRNA transiently and rapidly. The densitometric data indicated that shear stress for 1 h induced Egr-1 mRNA transcription 7.6-fold greater than that of static control. Egr-1 protein was also induced by shear stress, which peaked at 4 h after exposure to shear stress and was 5.2-fold greater than that under static conditions (Fig. 2B). Egr-1 mRNA and protein levels have been reported to be low or undetectable in quiescent EC but are dramatically increased upon exposure to shear stress (20, 21).

**Sp1 Protein Expression Is Not Changed after Shear Stress in Rat Microvascular EC**—The ability of Sp1 to bind to nuclear elements is critical for basal expression of MT1-MMP in static
The Sp1-binding sites in the MT1-MMP promoter overlap with a number of consensus elements for the structurally related transcription factor, Egr-1 (13, 20, 22). As shown in Fig 3, Sp1 protein levels were not affected in EC exposed to shear stress.

Sp1 Is Not Displaced by Egr-1 in the MT1-MMP Promoter after Shear Stress—EMSA analyses, using oligonucleotides containing wild-type MT1-MMP promoter sequences (Fig. 4A), were performed to assess Egr-1 and Sp1 binding. Nuclear extracts from EC exposed to shear stress displayed a shifted complex that was more prominent compared with extracts from cells grown under no flow conditions (Fig. 4B). Bands super-shifted by an anti-Sp1 antibody were also detected in shear stress-stimulated EC. These findings indicate that increased Egr-1 protein induced by shear stress cannot displace pre-bound Sp1 protein from the MT1-MMP promoter site and, therefore, cannot up-regulate MT1-MMP expression as seen in EC exposed to cyclic strain (14).

Shear Stress Increases the Affinity of Sp1 to MT1-MMP Promoter—To examine Sp1 protein binding further, we used mutant oligonucleotides containing a GG to TA mutation within the Egr-1-binding site. This mutation blocks Egr-1 but not Sp1 binding (Fig. 5A). As shown in Fig. 5B, EMSAs with nuclear extracts exposed to shear stress displayed a shifted complex that was more prominent compared with extracts from cells grown under no flow conditions (Fig. 4B). Bands super-shifted by an anti-Sp1 antibody were also detected in shear stress-stimulated EC. These findings indicate that increased Egr-1 protein induced by shear stress cannot displace pre-bound Sp1 protein from the MT1-MMP promoter site and, therefore, cannot up-regulate MT1-MMP expression as seen in EC exposed to cyclic strain (14).

Shear stress induced a rapid and transient up-regulation of Egr-1 mRNA levels in rat microvascular EC which was maximal (7.6-fold) after 1 h shear stress (p < 0.05). B, cell lysates were resolved by SDS-PAGE, before transfer to nitrocellulose membrane and Western blotting with a polyclonal antibody against Egr-1, using vimentin to normalize for protein loading. After enhanced chemiluminescence detection, bands were quantitated by scanning densitometry. Shear stress induced Egr-1 protein expression which peaked after 4 h of exposure to shear stress and was 5.2-fold greater than the static controls (p < 0.05). A and B blots are representative of 5 independent experiments.

FIG. 1. Northern and Western blotting for MT1-MMP in microvascular endothelial cell exposed to 14 dynes/cm² shear stress. A, total RNA (10 µg) from rat microvascular EC exposed to 14 dynes/cm² shear stress for 0, 1, 4, and 8 h was resolved on 1% agarose gels containing 6% formaldehyde and transferred to nylon membranes before hybridization with a 3²P-labeled cDNA probe encoding human MT1-MMP. Blots were stripped and reprobed with a GAPDH probe to normalize for RNA loading. Shear stress decreased MT1-MMP mRNA transcription in a time-dependent fashion (10, 50, and 90% reduction at 1, 4, and 8 h, respectively; n = 5, p < 0.05). B, MT1-MMP protein levels were assessed in rat microvascular EC exposed to 0, 1, 4, and 8 h, using vimentin to normalize for protein loading. Shear stress down-regulated MT1-MMP protein in a time-dependent fashion (10, 53, and 74% reduction at 1, 4 and 8 h, respectively; p < 0.05). A and B blots are representative of 5 independent experiments.

FIG. 2. Northern and Western blotting for Egr-1 in microvascular endothelial cell exposed to 14 dynes/cm² shear stress. Cells were exposed to shear stress for 1, 4, and 8 h. Control cells were grown under static condition at 37 °C. A, samples were normalized for total RNA (10 µg) prior to electrophoresis and transfer to a nylon membrane. The blots were hybridized with a 3²P-labeled cDNA probe encoding human Egr-1, and binding was assessed by autoradiography. After autoradiographic detection, bands were quantitated by scanning densitometry. GAPDH was used to normalize loading for total RNA loaded. Shear stress induced a rapid and transient up-regulation of Egr-1 mRNA levels in rat microvascular EC which was maximal (7.6-fold) after 1 h shear stress (p < 0.05). B, cell lysates were resolved by SDS-PAGE, before transfer to nitrocellulose membrane and Western blotting with a polyclonal antibody against Egr-1, using vimentin to normalize for protein loading. After enhanced chemiluminescence detection, bands were quantitated by scanning densitometry. Shear stress induced Egr-1 protein expression which peaked after 4 h of exposure to shear stress and was 5.2-fold greater than the static controls (p < 0.05). A and B blots are representative of 5 independent experiments.

FIG. 3. Western blotting for Sp1 in microvascular endothelial cells exposed to 14 dynes/cm² shear stress. Confluent EC were exposed to 14 dynes/cm² shear stress for 0, 1, 4 and 8 h, and lysates were resolved by SDS-PAGE. Western blotting analysis was performed using a polyclonal antibody against Sp1. After enhanced chemiluminescence detection, bands were quantitated by scanning densitometry. Sp1 protein levels were not affected by shear stress in microvascular EC. Blots are representative of 5 independent experiments.

Shear stress increased Sp1 binding to the MT1-MMP promoter despite an increase in Egr-1 levels and no change in total Sp1 levels.
Nogalamycin Increases the Affinity of Sp1 to MT1-MMP Promoter—Nogalamycin, an anthracycline isolated from *Streptomyces nogalator* var. (23), prevents Egr-1 nucleoprotein complex formation and stimulates Sp1 phosphorylation (24). We assessed the gel-shift profiles of nuclear extracts from EC treated with 10 μM nogalamycin for 0, 1, 4, or 8 h. Our results confirm that nogalamycin stimulates Sp1 binding to the MT1-MMP promoter site (Fig. 6).

Cyclic Strain Decreases the Affinity of Sp1 to MT1-MMP Promoter—We assessed Sp1 protein binding to MT1-MMP in EC exposed to cyclic strain using the mutant oligonucleotide (Fig. 5A) and anti-Sp1 polyclonal antibody. Exposure to cyclic strain decreased the Sp1 band, 53 and 77% at 1 and 4 h, respectively, compared with that of static cells (p < 0.05) (Fig. 7). This suggests that cyclic strain decreases Sp1 binding to the MT1-MMP promoter with Egr-1 up-regulation and without a change of total Sp1 levels.

Shear Stress and Nogalamycin Stimulate Sp1 Phosphorylation—We next investigated potential mechanism(s) underlying the change in affinity of Sp1 to DNA elicited by shear stress and nogalamycin. Regulation of Sp1-dependent transcription could be affected by changes in Sp1 abundance, DNA binding activity, and/or transactivation activity (25). Interestingly, phosphorylation has been implicated in changes in Sp1 binding and transcriptional activity (26, 27). Although tyrosine residues are present, the vast majority of Sp1 phosphorylation in rat EC is on serine residues (25, 28, 29).

We investigated the effects of shear stress and Sp1 phosphorylation on EC. Lysates were prepared from EC exposed to shear stress, nogalamycin, and cyclic strain and immunoprecipitated with an anti-phosphoserine antibody. Total Sp1 levels remained relatively unchanged with shear stress, but phosphorylation of Sp1 was induced by shear stress in a time-dependent fashion (Fig. 8A) similar to the treatment of EC with nogalamycin. Phosphorylation of Sp1 modulates MT1-MMP expression (34811).
nogalamycin (Fig. 8B). Our previous studies showed that increasing levels of Egr-1, induced by cyclic strain, displaced the basal transcription factor Sp1 from the promoter, resulting in a significant greater rate of MT1-MMP transcription in microvascular EC (14). In contrast to the shear stress and nogalamycin groups, however, there was no change in the phosphorylation state of Sp1 with cyclic strain exposure (Fig. 8C).

To confirm that these bands represent the phosphorylated form of Sp1, protein extracts were exposed to calf intestinal phosphatase (CIP) for 30 min prior to Western blot analysis. As shown in Fig. 9, the density of bands was partially diminished by CIP treatment.

**Phosphatase Inhibits Sp1 DNA Binding after Shear Stress and Nogalamycin Exposure**—To demonstrate that phosphorylation of Sp1 regulates the affinity of Sp1 for the MT1-MMP promoter site, EMSAs were performed on Sp1 pretreated with or without CIP. We demonstrate a significant difference between the DNA affinity of Sp1 treated with CIP, compared with Sp1 after shear stress and nogalamycin exposure without CIP (Fig. 10). Moreover, this result demonstrates that dephosphorylation of Sp1 does not abolish the binding complex completely. These results suggest that phosphorylation of Sp1 enhances the basal level of Sp1 binding to MT1-MMP promoter and transcriptional regulation of MT1-MMP.

**DISCUSSION**

Matrix metalloproteinases constitute a family of structurally related zinc endopeptidases capable of proteolysis of numerous components of the extracellular matrix (6, 30). The wide substrate specificity suggests that MMPs play an important role in mediating diverse fundamental cellular programs that depend on stromal remodeling such as cell migration, angiogenesis, and wound healing (5–7, 31, 32). Whereas most MMPs are secreted, MT1-MMP, also known as MMP-14, has a single transmembrane domain and an extracellular catalytic domain that has been suggested to be a key enzyme in tumor metastasis and angiogenesis. The membrane localization makes MT1-MMP suited to function in pericellular proteolysis (33); MT1-MMP also activates MMP-2 (gelatinase A) and possibly other secreted MMPs (34–36).

MT1-MMP is activated during migration of EC, by culture of EC within three-dimensional collagen lattices (12), and in cancer cells (37, 38). However, despite the correlation between MT1-MMP levels and invasive phenotype, the mechanism of MT1-MMP activation is not clear. For example in EC, the effects of mechanical stresses, such as cyclic strain or shear stress, on MT1-MMP expression have not been well demonstrated. Previously, we reported that cyclic strain increased MT1-MMP expression in rat microvascular EC (14). Interestingly, others (15) have demonstrated that shear stress decreases MT1-MMP expression in bovine smooth muscle cells.

The enzymatic activity of MT1-MMP is regulated at several levels, including transcription, but little is known about the mechanisms underlying transcriptional regulation of MT1-MMP mRNA. Previously, we assessed the transcriptional activity of the MT1-MMP promoter region in microvessel EC.
Sp1 Phosphorylation Modulates MT1-MMP Expression

using full-length and truncated promoter sequences coupled to the reporter gene encoding luciferase, and the region between −300 and −220 bp was sufficient to provide enhanced transcriptional activity (13). The GC-rich sequence (−288 to −275) within this region contains overlapping consensus binding sites for Sp1 and Egr-1. Sp1 and Egr-1 transcription factors compete for binding to such regions, with higher levels of transcription occurring when increasing levels of Egr-1 displace the basal transcription factor Sp1 from the promoter sequence. Thus enhanced MT1-MMP expression in EC upon cyclic strain stimulation appears to depend on binding of Egr-1 to the MT1-MMP promoter. Egr-1 mRNA and protein levels are typically low or undetectable in EC but rapidly and transiently increase when induced by any of a large number of factors (21, 39). Inducible expression of Egr-1-dependent genes involves displacement of prebound Sp1 by Egr-1 from their overlapping binding sites. Once bound to DNA, Egr-1 stimulates the expression of several endothelial genes such as platelet-derived growth factor-A, platelet-derived growth factor-B, transforming growth factor-β, urokinase-type plasminogen activator, and tissue factor, which are typically involved in the vascular response to injury (39). Several previous reports have shown that shear stress induces Egr-1 activation (39, 40). However, Sp1 levels are unchanged in EC exposed to shear stress (20). Thus, Sp1 could be displaced from the proximal promoters of each of these induced genes by Egr-1 (21, 39).

In this study, we report that the expression of Egr-1 is induced by 14 dynes/cm² shear stress in EC (Fig. 2), but shear stress inhibits MT1-MMP expression despite Egr-1 up-regulation (Fig. 1). To elucidate possible mechanisms underlying the MT1-MMP inhibition of shear stress, we have examined the binding profile of Sp1 and Egr-1 in the MT1-MMP promoter site. We detected a faint Sp1-DNA complex in the static cells and a more prominent Sp1-DNA complex in the extract from cells exposed to shear stress (Fig. 5). These observations imply that increasing levels of Egr-1 by shear stress cannot displace Sp1 from the promoter, and shear stress may increase the binding affinity of Sp1 to DNA, resulting in a significantly lower rate of transcription of MT1-MMP. Thus, the increased affinity of Sp1 to DNA by shear stress, rather than the Sp1 to Egr-1 ratio or the absolute amount of Egr-1, can result in the change of MT1-MMP expression in EC.

Sp1 is a ubiquitous, 778-amino acid transcription factor that recognizes GC-rich sequences present in many promoters including the MT1-MMP promoter (25, 41). Regulation of Sp1-dependent transcription could be affected by changes in Sp1 abundance, DNA binding activity, and/or transactivation activity. Sp1 is O-glycosylated and phosphorylated, and both of these modifications are likely to be important in its regulation. Phosphorylation of Sp1 has been implicated in changes in Sp1 binding affinity and transcriptional activation, and changes in O-glycosylation alter the stability of Sp1 in vivo (42). Although not examined here, O-glycosylation is another potential mechanism for regulation of Sp1, which has been shown to play role in the association of Sp1 with other factors and in regulation of its degradation.

Recent studies (29) have demonstrated that serum increases Sp1 phosphorylation due to serine phosphorylation. Sp1 can be phosphorylated in vitro by protein kinase CK2 (43), a DNA-dependent protein kinase (44), protein kinase A (27), and by cell cycle-regulated, Sp1-associated kinase activity (25). Sp1 can be phosphorylated at multiple sites by these kinases, which are likely to be important in regulation of its activity in response to diverse signals. DNA-dependent protein kinase has been reported to increase Sp1 activity, whereas phosphorylation of the C terminus of Sp1 by casein kinase II decreases its DNA binding property (43, 44). In order to demonstrate that shear stress is involved in the phosphorylation of Sp1, we performed immunoprecipitations with anti-tyrosine and anti-serine antibodies, and our results suggest that the vast majority of Sp1 phosphorylation induced by shear stress is on serine in rat microvascular EC (Fig. 7). Low levels of phosphorylation were detected only when the cells were incubated under static conditions, and serine phosphorylation was greatly increased when the cells were exposed to shear stress. Furthermore, we could demonstrate that Sp1 was not phosphorylated by cyclic strain (Fig. 7C), and the level of DNA binding was decreased by displacement with increased Egr-1. These findings suggest
that dephosphorylated Sp1 has a low affinity to bind DNA and can be displaced easily with increased Egr-1. This is supported by the findings that the amount of Sp1 and DNA complex in the nuclear extracts treated with CIP was lower than that of untreated extracts. These findings are consistent with the recent studies that show that Sp1 phosphorylation induces increased levels of DNA binding that involve alterations in the association of Sp1 with other proteins such as Egr-1 (24, 25, 28, 29, 45, 46).

Sp1 is a ubiquitous transcription factor that is capable of inducible regulation by several diverse mechanisms. Changes in the level of Sp1 phosphorylation are important for displacement by Egr-1 in the common binding site of the MT1-MMP promoter. Shear stress induces Sp1 phosphorylation in EC and increases its DNA binding affinity. These findings suggest that increased levels of Egr-1 cannot displace prebound Sp1 and that MT1-MMP can be inhibited by shear stress in EC. The results of this study further support a role for Sp1, not only in the regulation of basal transcription of the MT1-MMP gene but also in the modulation of the expression of this gene in response to various hemodynamic stimuli.

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