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NOTE

Endothelial cell TIMP-1 is upregulated by shear stress via Sp-1 and the TGFβ1 signaling pathways

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Abstract: Laminar shear stress promotes vascular integrity by inhibiting proteolysis of the extracellular matrix (ECM) surrounding the microvasculature. We hypothesized that the matrix metalloproteinase inhibitor TIMP-1 would be upregulated in endothelial cells exposed to shear stress. Microvascular endothelial cells isolated from rat or mouse skeletal muscles were exposed to laminar shear stress for 2, 4, or 24 h. A biphasic increase in TIMP-1 protein was observed at 2 and 24 h of shear stress exposure. Sp-1 siRNA prevented the increase in TIMP-1 after 2, but not 24, hours of shear exposure. TGFβ production and Smad2/3 phosphorylation are increased by shear stress. Inhibition of TGFβ signaling, either by use of the TGFβ receptor 1 inhibitor SB-431542 or with Smad 2/3 siRNA, abrogated the shear stress-induced increase in TIMP-1 mRNA after 24 h of shear stress exposure. These results suggest that both acute and chronic elevated laminar shear stress act to maintain vessel integrity through increasing TIMP-1 production, but that the TGFβ signaling pathway is essential to maintain TIMP-1 expression during chronic shear stress.

Key words: microvasculature, protease inhibitor, Smad.

Résumé : La force de cisaillement laminaire favorise l'intégrité vasculaire en inhibant la protéolyse de la matrice extracellulaire avoisinant la microvasculature. Nous avons examiné l'hypothèse que l'inhibiteur des métalloprotéases de la matrice, TIMP-1, pourrait être régulé à la hausse dans les cellules endothéliales exposées à une force de cisaillement. Des cellules endothéliales microvasculaires isolées du muscle squelettique de rat ou de souris ont été exposées à une force de cisaillement pendant 2, 4 ou 24 heures. Une augmentation bi-phasique de TIMP-1 a été observée à 2 et 24 heures d'exposition à la force de cisaillement. La production de TGFβ et la phosphorylation de Smad2/3 étaient accrues par la force de cisaillement. L'inhibition de la signalisation du TGFβ, soit par l'utilisation d'un inhibiteur du récepteur du TGFβ, le SB-431542, soit par celle d'un siARN Smad2/3, abrogeait l'augmentation de l'ARNm de TIMP-1 induite par la force de cisaillement après 24 heures d'exposition au cisaillement. Ces résultats suggèrent que tant la force de cisaillement aigue que chronique agit afin de maintenir l'intégrité vasculaire au moyen d'une production accrue de TIMP-1, mais que la voie de signalisation du TGFβ est essentielle au maintien de l'expression de TIMP-1 lors d'une force de cisaillement chronique. [Traduit par la Rédaction]

Mots-clés : microvasculature, inhibiteurs de protéases, Smad.

Introduction

Endothelial cell function is established by exposure to physiological levels of shear stress. In arteries, sustained laminar shear stress promotes expression of anti-oxidant and anti-proliferative genes, and downregulates expression of atherogenic genes (Chien 2007). In the microvasculature, laminar shear stress also regulates endothelial cell homeostasis through initiating processes of vascular remodeling, including angiogenesis (Cunningham and Gotlieb 2005; Skalak and Price 1996; Zhou et al. 1998). Shear stress-induced signaling is recognized to promote nitric oxide production, to inhibit matrix metalloproteinase (MMP) production, and to maintain basement membrane integrity (Chen and Wang 2004; Milkiewicz et al. 2006; Traub and Berk 1998; Zhou et al. 1998).

Tissue inhibitor of metalloproteinase (TIMP)-1 is an endogenous inhibitor of MMPs that functions by binding to the active site of MMPs, as well as by preventing cleavage of the pro-peptide domain (thus preventing formation of active MMPs) (Blavier et al. 1999; Lambert et al. 2004; Murphy and Willenbrock 1995). TIMP-1 has been shown to maintain endothelial barrier integrity in the presence of pro-inflammatory stimuli (Forster et al. 2007). Impairment of TIMP-1 production in pathological conditions is associated with excessive basement membrane degradation (Haorah et al. 2008).

Considering that TIMP-1 may be an important contributor to maintenance of endothelial basement membrane integrity, we hypothesized that TIMP-1 is upregulated in response to laminar shear stress. Previously, our lab found that mRNA levels of TIMP-1 are increased by shear stress both in vivo and in vitro. The increase in TIMP-1 mRNA in response to short term (2 h) shear stress exposure relied on activation of the transcription factor Ets-1 (Milkiewicz et al. 2008). However, the signaling pathway(s) underlying the sustained increase in TIMP-1 mRNA production has(have) not been elucidated.

Sp-1 is one of several transcription factors that binds to shear stress response elements (Silberman et al. 2009; Urbich et al. 2003). In endothelial cells, Sp-1 acts as a co-activator to modulate transcription of several genes, including VEGFR2 and MT1-MMP, in response to shear stress (Abumiya et al. 2002; Lin et al. 1997; Yun et al. 2002). Additionally, Sp-1 and Ets-1 were reported to co-activate the protease inhibitor PAI-1 in response to shear stress in hepatocytes (Nakatsuka et al. 2006).

Transforming growth factor-β (TGFβ) is a cytokine that regulates proliferation, migration, and extracellular matrix (ECM)

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production by endothelial cells through autocrine signaling (Goumans et al. 2002; Ignotz et al. 1989). Activation of the TGFβ receptor I phosphorylates Smad 2/3, causing the nuclear translocation and transcriptional activity of these factors (Miyazono et al. 1993). TGFβ signaling promotes fibronectin and laminin production (Usui et al. 1998) and contributes to vascular barrier function and homeostasis in the adult retinal microvasculature (Walshe et al. 2009). TGFβ has been shown to regulate basal TIMP-1 expression in human microvascular endothelial cells (Ito et al. 1995).

TGFβ is upregulated by shear stress in arteries and endothelial cells (Cucina et al. 1998; Negishi et al. 2001). The contribution of TGFβ signaling to shear stress regulation of TIMP-1 expression in microvascular endothelial cells is unknown. The purpose of this study was to determine whether Sp-1 and (or) TGFβ signaling mediate the shear stress-dependent upregulation of TIMP-1 in microvascular endothelial cells.

Materials and methods

Chemicals were purchased from Sigma-Aldrich Canada Co. (Oakville, Ontario, Canada) unless otherwise stated.

Cell culture

Skeletal muscle endothelial cells were isolated from extensor digitorum longus muscles of male Sprague–Dawley rats or C57BL/6 mice and cultured as previously described (Han et al. 2003). Surgical procedures involved in cell isolation were carried out in accordance with Animal Care Procedures at York University and in conformity with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Shear stress

Endothelial cells were plated on gelatin-coated glass coverslips and cells were exposed to 12 dyn/cm^2 laminar shear stress using a parallel plate closed chamber (FCS2, Bioptechs, Butler, Pa., USA) as described previously (Milkiewicz et al. 2006). Control cells were maintained under static conditions for an equivalent amount of time. Cells were lysed for protein (100 mmol/L Tris HCl, pH 8.7, 0.1% Triton X-100, 5% glycerol supplemented with 10% protease inhibitor cocktail (#P8340, Sigma) and 1.1 μmol/L sodium orthovanadate) or for mRNA (Cells to cDNA lysis buffer; Ambion, Burlington, Ontario, Canada) analyses. For assessment of protein secretion, OptiMem (Invitrogen) was utilized instead of DMEM-10% FBS. OptiMem was collected and concentrated by centrifugal filtration (UFC901024 Millipore, Billerica, Mass., USA). In some cases, cells were pretreated with 10 μmol/L TGFβRI inhibitor (SB-431542; Tocris, Minneapolis, Minn., USA) for 1 h prior to shear stress exposure.

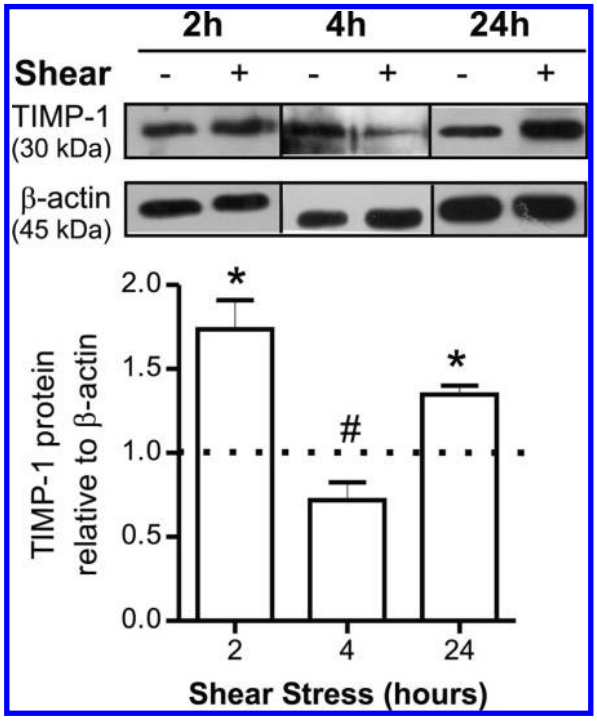
Shear conditioned media

Endothelial cell monolayers were treated for 30 min. with conditioned media collected from 2 h shear stress-exposed endothelial cells, or from corresponding static controls, then lysed for Western blot analysis.

Immunostaining

Endothelial cells were fixed briefly with 3.7% paraformaldehyde, then blocked (5% normal goat serum and 0.1% Triton X-100 in PBS). Smad 2/3 primary antibody (1:200, sc-6032, Santa Cruz) was incubated for 1.5 h followed by secondary antibody (1:400; Alexa 568-conjugated donkey anti-goat secondary antibody, Molecular Probes (Burlington, Ontario, Canada)). Cells were counterstained with DAPI (1:1500 in PBS), then mounted onto slides with Immunofluor mounting medium (Vector Labs, Burlington, Ontario, Canada) and viewed with a Zeiss Axiovert 200M light microscope equipped with a cooled digital CCD camera (Quantix Photometrics A01F6020, Roper Scientific Inc., USA). Nuclear pixel intensities were quantified using MetaMorph imaging software (Universal Imaging Corp., Bedford Hills, NY, USA). Nuclei were outlined by identifying DAPI-stained areas, and average pixel intensities within these regions were measured.

Fig. 1. TIMP-1 protein is increased by shear stress. Cultured endothelial cells were stimulated with 12 dyn/cm^2 shear stress (+) and compared to time-matched static (–) controls. TIMP-1 protein was measured via Western blot. Protein levels in sheared cells were compared to the relevant time-matched static controls, which were set to 1, and indicated by the dotted line (*p < 0.05 vs time-matched control, #p < 0.05 vs 2 and 24 h SS; n = 3).



TGFβ1 treatment

Endothelial cell monolayers were treated with 10 ng/ml recombinant TGFβ1 (T7039, Sigma) for 2 or 6 h and analyzed by qPCR or Western blot.

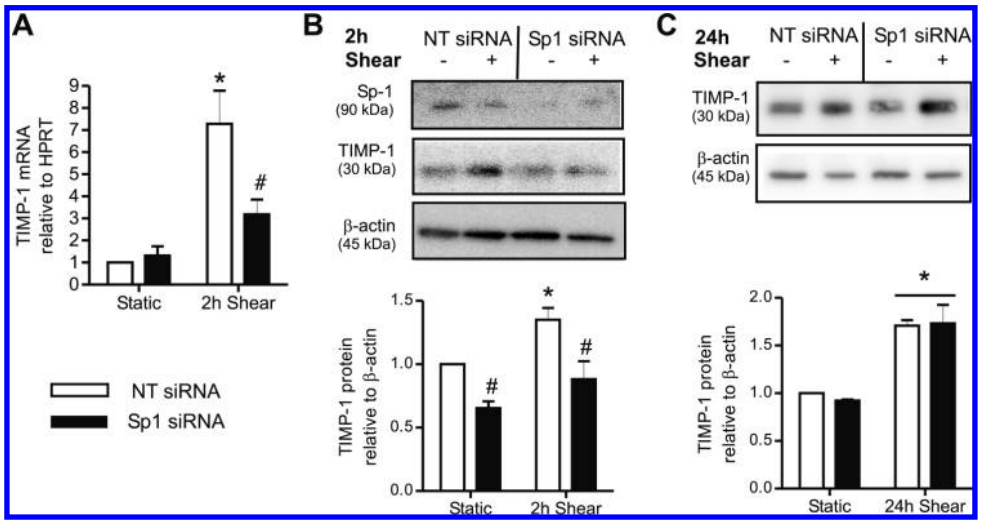
siRNA

250 nmol/L murine Sp-1 (L-040633-01), Smad2 (J-040707-08), Smad3 (J-040706-05), and non-targeting siRNA (D-001810-01-05) (Thermo Scientific, Pittsburgh, Pa., USA) were transfected into mouse endothelial cells using the Amaxa nucleofector kit (VPI-1001, Lonza, Germany). Cells were transfected according to manufacturer's instructions, using program #T-023 (Lonza). Cells were plated on gelatin coated glass coverslips and allowed to recover for 48 h prior to shear stress exposure.

Western blot

Protein was assessed by Western blot, as described previously (Milkiewicz et al. 2008). The primary antibodies used were TIMP-1 [sc-5538, Santa Cruz (Dallas, Tex., USA) or MAB9801, R&D Systems (Burlington, Ontario, Canada)], Smad 2/3 (#3102, Cell Signaling), Sp-1 (c-59, Santa Cruz), phospho-Smad 2/3 (#3101, Cell Signaling (Danvers, Mass., USA), TGFβ (#3709, Cell Signaling), and β-actin (#4967, Cell Signaling or sc-47778, Santa Cruz). HRP-conjugated secondary antibodies used were α-rabbit (111-035-003, Jackson ImmunoResearch Laboratories, West Grove, Pa., USA), α-mouse (115-035-003 Jackson ImmunoResearch Laboratories), and α-rat (112-035-003 Jackson ImmunoResearch Laboratories). Signals were detected by using enhanced chemiluminescent reagents (SuperSignal® West Pico Chemiluminescent Substrate (34080, Thermo

Fig. 2. An acute bout of shear stress increases TIMP-1 production via Sp-1. (A) Endothelial cells were exposed to 2 h of shear stress in the presence of Sp-1 siRNA or non-targeting (NT) siRNA as a control. TIMP-1 mRNA was measured via quantitative real-time PCR. Two-way ANOVA indicated main effects of shear and of siRNA treatment ($p < 0.05$). Post hoc testing showed a significant effect of shear in the cells transfected with NT siRNA (*$p < 0.05$ vs static NT) and a significant difference between NT and Sp-1 siRNA-treated cells in the shear condition (#$p < 0.05$, $n = 4$). (B) Cells were treated as in (A) for 2 h, and TIMP-1 protein was measured via Western blot. Two-way ANOVA indicated main effects of shear and of siRNA treatment ($p < 0.05$). Post hoc testing showed a significant effect of shear in the cells transfected with NT siRNA (*$p < 0.05$ vs static NT) and a significant difference between NT and Sp-1 siRNA-treated cells in the static and shear conditions (#$p < 0.05$, $n = 4$). (C) Cells were treated as in (A) for 24 h, and TIMP-1 protein was measured via Western blot. Two-way ANOVA identified a main effect of shear (*$p < 0.05$, $n = 4$).



Scientific) or Immobilon™ Western Chemiluminescent HRP Substrate (WBKLS0100, Millipore)), and imaged using a Kodak MM4000Pro digital imager. Densitometry analysis was performed (Carestream Molecular Imaging System), and signal intensities were normalized to the β-actin signal intensities from the same membrane.

Quantitative real time PCR

Quantitative real-time PCR (qPCR) analysis for TIMP-1 was performed using TaqMan® FAM-labeled probes (Applied Biosystems Assays on Demand: Rn00587558_m1 and Mm00441818_m1, Burlington, Ontario, Canada), and housekeeping genes GAPDH (Rn99999916_s1) or HPRT (Mm00446968_m1), using the 7500 Fast Real Time PCR system (Applied Biosystems), as described previously (Milkiewicz et al. 2011). The comparative Ct method was used to determine relative mRNA expression, normalizing to levels of GAPDH or HPRT.

Data analysis

Data are presented as means ± SEM. Paired two-tailed *t*-tests, one-way ANOVA, or two-way ANOVA, with Tukey or Bonferroni post hoc tests, were applied to determine statistical significance ($p < 0.05$) for each data set.

Results

Intracellular TIMP-1 is increased by shear stress

Exposure of endothelial cells to 12 dyn/cm^2 shear stress caused a biphasic increase in cellular levels of TIMP-1 protein at 2 and 24 h of shear, while at 4 h of shear stress TIMP-1 protein returned to control levels (Fig. 1). We previously reported significant increases in TIMP-1 mRNA in response to 2 and 24 h of shear stress (Milkiewicz et al. 2008). Now, we assessed TIMP-1 mRNA after 4 h and found no difference between shear and static cells (1.0 vs. 0.97 ± 0.17, $p > 0.05$, $n = 3$), indicating that the TIMP–1 mRNA response to shear stress also is biphasic.

Sp-1 regulates TIMP-1 expression at rest and in response to an acute bout of shear stress

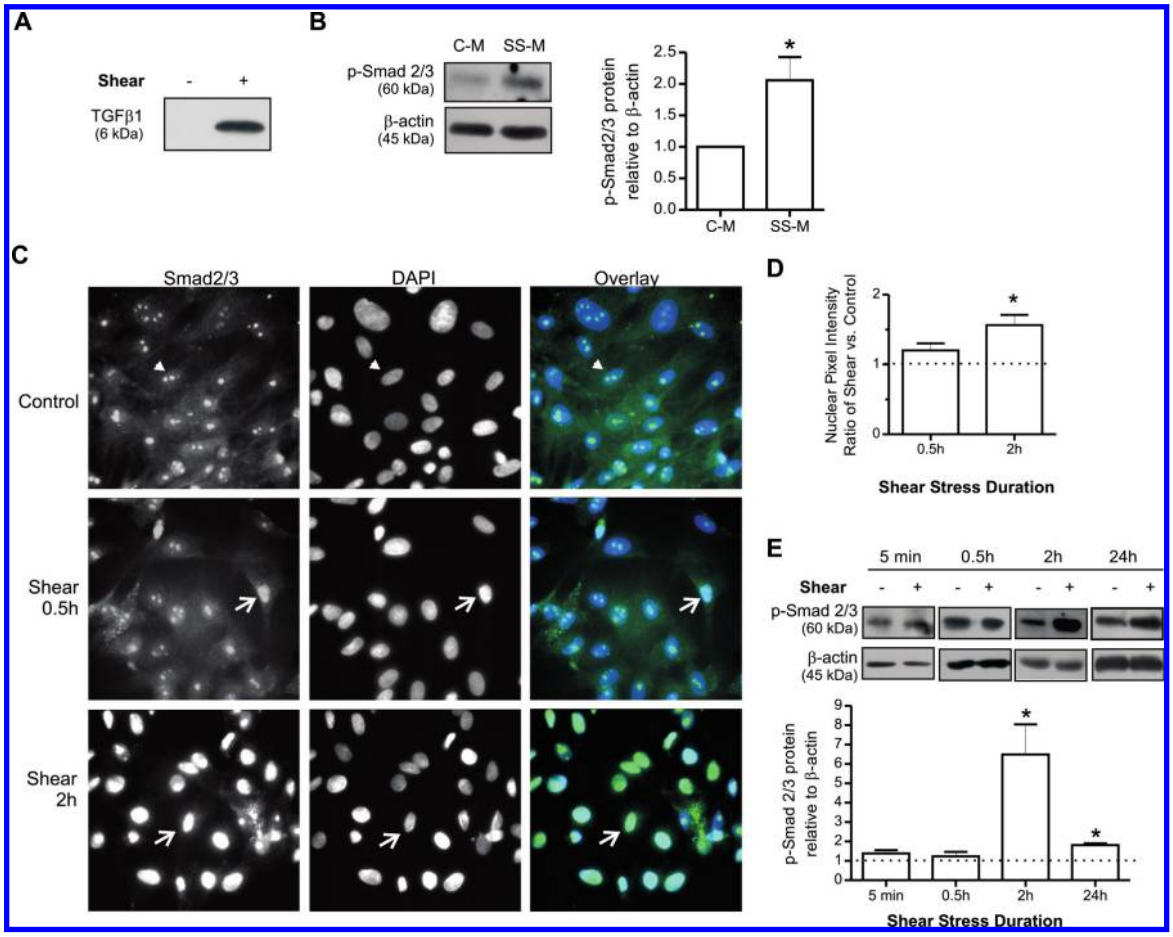
Sp-1 is a co-activator of transcriptional activity during shear stress in endothelial cells (Lin et al. 1997) and may be a regulator of TIMP-1 transcription (Aicher et al. 2003). We tested this by silencing Sp-1 expression with siRNA prior to exposing endothelial cells to shear stress, which resulted in a reduction of cellular levels of Sp-1 protein by ~78% (Fig. 2B). Sp-1 siRNA inhibited the increases in both TIMP-1 mRNA and protein observed after 2 h of shear stress exposure (Figs. 2A and 2B). However, Sp-1 siRNA did not have an effect on TIMP-1 expression in cells exposed to shear stress for 24 h (Fig. 2C).

The TGFβ signaling pathway is activated by shear stress and regulates TIMP-1 expression

The TGFβ pathway is known to modulate TIMP-1 expression (Akool et al. 2005). As a first step in establishing whether the TGFβ pathway may contribute to the regulation of TIMP-1 expression in response to shear stress, we determined whether microvascular endothelial cells release TGFβ1 upon exposure to shear stress. By Western blot, TGFβ1 was detectable only within the media of cells stimulated with shear stress and not in media from static control cells (Fig. 3A). We collected media from shear stress-exposed endothelial cells and subsequently added it to static control endothelial cells. Cells treated with the shear-conditioned media had increased levels of Smad 2/3 phosphorylation, providing further evidence that an activator of the TGFβ/Smad 2/3 signal pathway was released from the sheared cells (Fig. 3B). We next assessed if the TGFβ pathway is activated in endothelial cells in response to shear stress. Nuclear-localized Smad 2/3 immunostaining was increased significantly after 2 h of shear (Figs. 3C and 3D). A similar increase in Smad 2/3 phosphorylation was observed after 2 h of shear stress, which remained significantly elevated above control levels after 24 h of shear (Fig. 3E).

Having established that the TGFβ/Smad2/3 signal pathway is promoted in microvascular endothelial cells in response to elevated shear stress, we tested its involvement in the regulation of TIMP-1 expression. Consistent with other reports, treatment of

Fig. 3. Shear stress activates Smad 2/3 in endothelial cells. (A) Cultured endothelial cells were stimulated with 12 dyn/cm² shear stress for 2 h in OptiMem. Media was collected and concentrated for analysis. Equal volumes of OptiMem were analyzed for TGFβ1 protein via Western blot. Blot is representative of 3 independent experiments. (B) Endothelial cells were exposed to 12 dyn/cm² shear stress for 2 h or remained under static conditions. Media was collected from both conditions and applied to static cultured cells for 30 min. Phosphorylated Smad 2/3 protein was measured via Western blot (*$p < 0.05$ vs control, $n = 6$). C-M represents cells that were incubated with media from static cultures. SS-M represents cells treated with shear-conditioned media. (C) Endothelial cells were exposed to 12 dyn/cm² shear stress for the indicated time periods. Cells were fixed and stained for Smad 2/3 (green) and DAPI (blue). Arrowheads indicate a nucleus with a few small areas of Smad 2/3 staining in the control cells, while in the sheared cells Smad 2/3 staining fills the entire nucleus (arrows). (D) Pixel intensities in the nuclei of each field of view were quantified using MetaMorph software. Sheared cells were compared to time-matched static controls, as indicated by the dotted line (*$p < 0.05$ vs control, $n = 3$). (E) Phosphorylated Smad 2/3 protein was measured via Western blot. Sheared cells were compared to time-matched static controls, as indicated by the dotted line (*$p < 0.05$ vs time-matched control, $n = 4$).



skeletal muscle microvascular endothelial cells with 10 ng/ml recombinant TGFβ1 resulted in a significant increase in both TIMP-1 mRNA (Fig. 4A) and protein (Fig. 4B). Endothelial cells were treated with Smad2 and 3 siRNA prior to shear stress exposure. Smad2/3 siRNA effectively reduced Smad2/3 protein levels by ~65% (Fig. 4C). Smad2/3 siRNA prevented the increase in TIMP-1 mRNA that occurred in response to 2 h of shear stress (Fig. 4D). The shear-induced increase in TIMP-1 mRNA also was prevented in cells exposed to 24 h of shear stress (Fig. 4E).

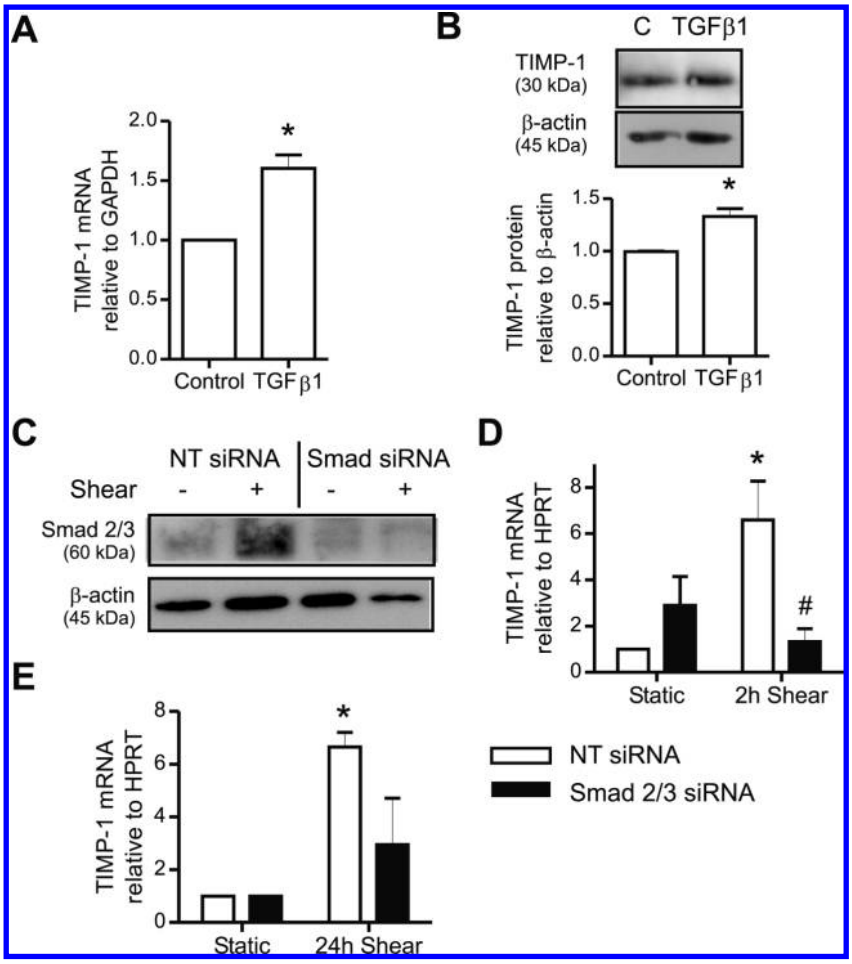
As an alternate means of establishing the role of the TGFβ signal pathway in regulating TIMP-1 expression, TGFβ signaling was blocked with the TGFβ receptor I inhibitor SB-431542 (10 μmol/L) prior to cell exposure to shear stress. Basal levels of TIMP-1 were unaffected by SB-431542 treatment. After 2 h of shear stress, TIMP-1 mRNA and protein levels were elevated both in SB-431542-treated and vehicle-treated cells, indicating no effect of the receptor inhibitor (Figs. 5A and 5C). However, the TGFβ inhibitor prevented the shear stress-induced increase in TIMP-1 mRNA and protein observed after 24 h of shear stress (Figs. 5B and 5D).

Discussion

Elevated shear stress increases TIMP-1 mRNA and protein in microvascular endothelial cells. This increase is evident after 2 and 24 h, but not 4 h, of shear stress exposure. This temporal biphasic pattern suggests transcriptional regulation by two separate signaling pathways, which is supported by our analysis of Sp-1 and Smad2/3 transcription factors. Sp-1 and Smad2/3 regulated TIMP-1 mRNA levels in response to an acute exposure to shear stress. Conversely, after 24 h of exposure to shear stress, TIMP-1 upregulation occurred independently of Sp-1 and instead required activation of TGFβRI and recruitment of Smad 2/3.

Our lab has demonstrated previously that the transcription factor Ets-1 is involved in the regulation of the shear stress-induced increase in TIMP-1 mRNA at 2 h of shear stress (Milkiewicz et al. 2008). We add to these data by showing that Sp-1, a known co-activator of Ets-1, also regulates TIMP-1 mRNA and protein expression in response to a short duration exposure to elevated shear stress. Sp-1 activation via phosphorylation has been observed after as little as 1 h of shear stress in endothelial cells (Yun et al. 2002).

Fig. 4. Smad 2/3 signaling is required for the shear stress-induced increase in TIMP-1. (A) Cultured endothelial cells were treated with 10 ng/ml recombinant TGFβ1 for 2 h. Quantitative real time PCR was performed and TIMP-1 mRNA measured (*$p < 0.05$ vs control, $n = 3$). (B) Cultured endothelial cells were treated with 10 ng/ml recombinant TGFβ1 for 6 h. TIMP-1 protein was measured via Western blot (*$p < 0.05$ vs control, $n = 3$). (C) Endothelial cells were stimulated with 12 dyn/cm^2 shear stress for 2 h in the presence of Smad 2/3 siRNA or non-targeting (NT) siRNA as a control. A 65% knockdown of Smad 2/3 was achieved, as measured by Western blot. (D) TIMP-1 mRNA was measured via quantitative real time PCR. Two-way ANOVA indicated a significant interaction ($p < 0.05$). Post hoc testing showed a significant effect of shear in the cells transfected with NT siRNA (*$p < 0.05$ vs static NT) and a significant difference between NT and Smad 2/3 siRNA-treated cells in the shear condition (#$p < 0.05$, $n = 4$). (E) Cells were treated as in (A) for 24 h, and TIMP-1 mRNA was measured via pPCR. Two-way ANOVA identified a main effect of shear ($p < 0.05$). Post hoc testing showed a significant effect of shear in the cells transfected with NT siRNA (*$p < 0.05$ vs static NT, $n = 4$).



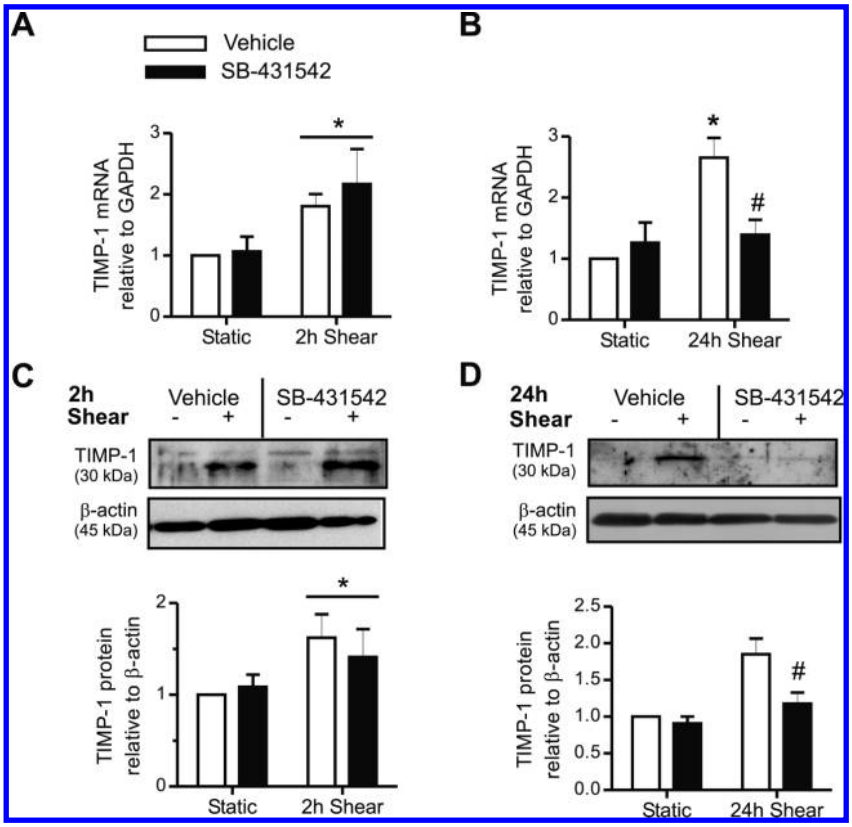
An Sp-1/Ets-1 complex also has been shown to increase PAI-1 transcription in hepatocytes in response to an acute bout of shear stress (Nakatsuka et al. 2006). Our findings add to these data and implicate Sp-1 as a general regulator of protease inhibition during shear stress.

Extending this knowledge, we demonstrate that recruitment of the TGFβ signaling pathway via Smad2/3 contributes to the increase in TIMP-1 mRNA observed after 2 and 24 h of shear. While TGFβ regulation of TIMP-1 expression was shown in endothelial cells (Ito et al. 1995), it has not been demonstrated previously within the context of a shear stress-dependent signal pathway. Interestingly, Smad2/3 siRNA treatment inhibited the 2 h shear stress-induced increase in TIMP-1 mRNA, whereas SB-431542 treatment did not. This difference may indicate that disruption of Smad protein levels exerts a broader cellular influence due to their functions as transcriptional co-activators. Another possibility is that temporal differences between the siRNA and pharmacological treatments altered the observed response. SB-421542 was given 1 h prior to shear stress exposure, while the siRNA was transfected 48 h before shear stress. Additionally, SB-431542 is less specific than the siRNA treatment, because it inhibits not only TGFβRI but also ALK4 and ALK7.

Endothelial cell deletion of Smad2/3 results in loss of integrity of the vasculature, with reduced cell-cell junctions and fatal embryonic hemorrhaging observed (Itoh et al. 2012; Jakobsson and van Meeteren 2013). Our data are consistent with these observations, as TIMP-1 is a known regulator of vascular integrity (Forster et al. 2007; Haorah et al. 2008). Laminar shear stress also is known to promote vascular integrity (Traub and Berk 1998), and our data suggest that the Smad2/3-TIMP-1 pathway is involved in this process.

Recently, Sp-1/Smad2 complexes were shown to regulate the transcription of MMP-11, and this interaction was dependent on the acetylation status of Smad2 rather than its phosphorylation status (Barrasa et al. 2012). Similarly, Sp-1/Smad interactions have been demonstrated to promote the transcription of α1(I) and α2(I) collagens and p21 (Pardali et al. 2000; Poncelet and Schnaper 2001; Sysa et al. 2009). Thus, it is possible that Sp-1/Smad co-operation may occur in response to shear stress and co-ordinate the transcription of genes such as TIMP-1.

Fig. 5. TGFβRI signaling is required for the chronic shear stress-induced increase in TIMP-1. (A) Endothelial cells were stimulated with 12 dyn/cm^2 shear stress for 2 h in the presence or absence of 10 μmol/L of the TGFβ receptor I inhibitor SB-431542. TIMP-1 mRNA was measured via quantitative real time PCR. Two-way ANOVA identified a main effect of shear (*$p < 0.05$, $n = 6$). (B) Cells were treated as in (A) for 24 h, and TIMP-1 mRNA was measured via quantitative real time PCR. Two-way ANOVA indicated a main effect of shear and a significant interaction ($p < 0.05$). Post hoc testing showed a significant effect of shear in the treated with vehicle (*$p < 0.05$ vs static vehicle) and a significant difference between vehicle and SB-431542-treated cells in the shear condition (#$p < 0.05$, $n = 3$). (C) Cells were treated as in (A), and TIMP-1 protein was measured via Western blot. Two-way ANOVA indicated a main effect of shear (*$p < 0.05$, $n = 6$). (D) Cells were treated as in (A) for 24 h, and TIMP-1 protein was measured via Western blot. Two-way ANOVA indicated main effects of shear and of drug treatment, as well as a significant interaction ($p < 0.05$). Post hoc testing showed a significant difference between vehicle and SB-431542-treated cells in the shear condition (#$p < 0.05$ vs shear vehicle, $n = 6$).



It is interesting to note that we found multiple signaling pathways contribute to the early upregulation of TIMP-1 in response to shear stress, but only one that appears to modulate TIMP-1 after long term exposure to shear stress. This is consistent with other research and our own previous observations that a broad range of signal pathways are activated within endothelial cells upon acute shear stimulation but do not remain activated upon continual exposure to shear stress (Butler et al. 2000; Gee et al. 2010; Geiger et al. 1992; Helmke et al. 2000). The presence of redundant signaling pathways also suggests the importance of increased TIMP-1 in response to an acute increase in shear stress.

In conclusion, we have demonstrated that shear stress increases TIMP-1 production in skeletal muscle microvascular endothelial cells via multiple signaling pathways after an acute exposure and by the TGFβ-Smad2/3 pathway in response to a chronic increase in shear stress. This expands our knowledge of the mechanisms by which laminar shear stress maintains integrity of the endothelial cell layer and suggests a novel signaling pathway to study in vascular dysfunction caused by a chronic alteration in shear stress.

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