Coregulation of vascular tube stabilization by endothelial cell TIMP-2 and pericyte TIMP-3

W. Brian Saunders,1 Brenda L. Bohnsack,4 Jennifer B. Faske,1 Nicholas J. Anthis,1 Kayla J. Bayless,1 Karen K. Hirschi,4 and George E. Davis1,2,3

1Department of Pathology and Laboratory Medicine, Texas A&M University System Health Science Center, College Station, TX 77843
2Department of Medical Pharmacology and Physiology and 3Department of Pathology and Anatomical Sciences, School of Medicine, Christopher S. Bond Life Sciences Center and Dalton Cardiovascular Research Center, University of Missouri-Columbia, Columbia, MO 65212
4Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX 77030

The online version of this article contains supplemental material.

Correspondence to George E. Davis: davisgeo@health.missouri.edu

Abbreviations used in this paper: ADAM, a disintegrin and metalloproteinase; BRP, bovine retinal pericyte; CASMC, coronary artery smooth muscle cell; DN, downstream primer; EC, endothelial cell; HUVEC, human umbilical vein EC; MMP, matrix metalloproteinase; MT, membrane type; Plg, plasminogen; S1P, sphingosine-1 phosphate; SDF-1α, stromal-derived factor-1α; TIMP, tissue inhibitor of metalloproteinase; UP, upstream primer; VE, vascular endothelial; VEGFR-2, VEGF receptor-2.

The version of this article contains supplemental material.

Supplemental Material can be found at:
/content/suppl/2006/10/09/jcb.200603176.DC1.html

Published October 9, 2006

The Journal of Cell Biology, Vol. 175, No. 1, October 9, 2006 179–191
http://www.jcb.org/cgi/doi/10.1083/jcb.200603176

Introduction

During angiogenesis, a complex coordination of cues from cytokines, growth factors, proteinases, and integrins mediate cellular changes to control the processes of sprouting, lumen formation, and proliferation (Davis et al., 2002; Carmeliet, 2005; Davis and Senger, 2005). Once networks of endothelial cell (EC)–lined tubes are formed, the stabilization of these structures is regulated by support cells such as pericytes (Orlidge and D’Amore, 1987; Jain, 2003; von Tell et al., 2006). In PDGF-B and -β receptor knockout mice, the lack of pericyte recruitment results in vascular instability and embryonic lethality (Lindahl et al., 1997; Hirschi et al., 1998; Hellstrom et al., 1999, 2001; Jain, 2003). A molecular understanding of how pericyte–EC interactions lead to capillary tube regression in these cocultures is a matrix metalloproteinase-1 (MMP-1)–, MMP-10–, and ADAM-15 (a disintegrin and metalloproteinase-15)–dependent manner. Furthermore, we show that EC tube morphogenesis (lumen formation and invasion) is primarily controlled by the TIMP-2 and -3 target membrane type (MT) 1 MMP. Additional targets of these inhibitors include MT2-MMP and ADAM-15, which also regulate EC invasion. Mutagenesis experiments reveal that TIMP-3 requires its proteinase inhibitory function to induce tube stabilization. Overall, these data reveal a novel role for both TIMP-2 and -3 in the pericyte-induced stabilization of newly formed vascular networks that are predisposed to undergo regression and reveal specific molecular targets of the inhibitors regulating these events.
contribute to tissue regression in the mammary gland (Green and Lund, 2005), vasculature (Davis et al., 2001; Saunders et al., 2005; Davis and Saunders, 2006), and during the menstrual cycle (Curry and Osteen, 2003). In this study, we present the novel concept that EC-derived TIMP-2 and pericyte-derived TIMP-3 coregulate capillary tube stabilization by the inhibition of key EC targets such as MT1-MMP, ADAM-15 (a disintegrin and metalloproteinase-15), MMP-1, and MMP-10, which normally control EC tube formation and/or regression.

**Results**

**TIMP-2 and -3 markedly inhibit EC invasion and tubular morphogenesis events in 3D collagen matrices**

Using an in vitro model of angiogenic sprouting, human ECs invade ~500 μm into 3D collagen matrices over a 48-h period (Fig. 1 A). This invasion response is completely inhibited by TIMP-2 and -3 (Fig. 1, A and B) but not by TIMP-1. Although control and TIMP-1–treated invading ECs form lumenal structures, no lumen formation is seen from TIMP-2– or -3–treated invading ECs (Fig. 1 B). Similar results using ECs transfected with lentiviral vectors expressing control GFP, TIMP-1, or TIMP-3 were observed (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200603176/DC1).

Time-lapse videos were performed with ECs suspended as individual cells within collagen matrices (vasculogenesis-like assay; Davis et al., 2002) over a 48-h period and treated with TIMP-1, -2, or -3 versus the control (Videos 1–4, available at http://www.jcb.org/cgi/content/full/jcb.200603176/DC1). Photographs from these videos are shown at 48 h of culture (Fig. 1 C). ECs treated with TIMP-2 and -3 fail to form tubes (i.e., no lumen structures observed) and instead send out fine processes over this time course (Fig. 1 C, arrowheads; and Videos 1–4). Addition of the chemical MMP inhibitor GM6001 shows essentially identical results, suggesting that these effects are likely the result of proteinase inhibition (Video 5). In contrast, obvious luminal and tube structures are observed in the control and TIMP-1–treated cultures (Fig. 1 C). Quantitation of the sprouting assay as well as quantitation of the vasculogenesis assay reveals marked blockade of invasion and lumen formation by both TIMP-2 and -3 (Fig. 1 D). These results demonstrate that both TIMP-2 and -3 completely inhibit EC invasion and lumen formation (perhaps indicating a common molecular target), whereas TIMP-1 has no effect.

---

**Figure 1.** EC invasion and tubular morphogenesis in 3D collagen matrices are inhibited by TIMP-2 and -3. (A) ECs were seeded onto collagen matrices and stimulated to invade for 48 h in response to 1 μM S1P in the absence (control) or presence of 5 μg/ml TIMP-1, -2, or -3. Arrows indicate the EC monolayer; arrowheads point to the invading EC sprouts. Bar, 100 μm. (B) Plastic sections of these cultures are shown to illustrate the presence (control; TIMP-1) or absence (TIMP-2 and -3) of EC luminal structures (arrowheads). Arrows indicate the EC monolayer; arrowheads point to EC lumens. Bar, 40 μm. (C) ECs were suspended within collagen matrices and allowed to undergo morphogenesis and tube network formation for 48 h in the absence (control) or presence of 5 μg/ml TIMP-1, -2, or -3 using time-lapse microscopy. Arrows point to multicellular EC tube networks; arrowheads indicate fine processes. Bar, 50 μm. (D) Graphs show the mean invasion distance in micrometers (left) or the total number of ECs forming lumens (right) ± SD (error bars) using time-lapse images at 48 h. *, P < 0.01 for TIMP-2– or -3–treated ECs compared with controls. HPF, high-powered field.
TIMP-3 markedly blocks vasculogenesis and angiogenesis in mouse embryo cultures

To determine the ability of TIMP-3 to affect vessel formation in vivo, mouse embryo cultures were established from embryonic day (E) 7.5 to E9.5 in the presence of TIMP-1, TIMP-3, GM6001, and DMSO control (Fig. 2 A). Untreated, DMSO, and TIMP-1–treated cultures were photographed live at 48 h and exhibited the remodeling of capillary networks, large vitelline vessels at the origin in the embryo, and large vessels leading to capillaries. The large vitelline vessels (Fig. 2 A, insets) along with capillary networks stained positively for vascular endothelial (VE) cadherin. TIMP-3– and GM6001–treated embryos lacked vitelline vessels, exhibited an immature capillary plexus, and failed to turn. No large vessel structures were formed in GM6001– (unpublished data) or TIMP-3–treated explants as compared with the untreated or TIMP-1–treated explants. These data indicate that TIMP-3 treatment blocked the assembly of vascular structures in vivo, which directly correlated with our in vitro findings.

TIMP-3 is expressed by perivascular mesenchymal cells and the visceral endoderm during early vascular development

RT-PCR analysis of embryonic region (E6.5 and E7.5), extraembryonic region (6.5× and 7.5×), and yolk sac (8.5 and 9.5 d) tissues during vascular development revealed that TIMP-3 was expressed abundantly in both embryonic and extraembryonic tissue as well as by the yolk sac at the time points indicated (Fig. 2 B). TIMP-3 showed increased expression up to E8.5, whereas TIMP-1 expression decreased over this time period. Immunofluorescence imaging of visceral endoderm and mesoderm sections of yolk sac at E8.5 and E9.5 revealed the colocalization of TIMP-3 with angiopoietin-1 in perivascular mesenchymal cells (pericyte and vascular smooth muscle precursors), which surround the early vascular tubes (Fig. 2 C). Also, strong staining for TIMP-3 was observed in the visceral endoderm, an important supporting structure that controls development of the early vasculature (Bohnsack et al., 2004). These staining distributions were confirmed by in situ hybridization (unpublished data). Thus, the visceral endoderm and mesenchyme surrounding EC tubes (stained with VE-cadherin; Fig. 2 C) in the mesoderm may serve to control and stabilize EC tubular morphogenesis by delivering TIMP-3 to newly formed vascular tubes.

TIMP-3 stabilizes preexisting capillary tube networks by blocking tube formation and MMP-1–dependent tube regression

Because TIMP-3 completely inhibits EC tube morphogenesis, we asked whether TIMP-3 could induce the collapse and regression of preexisting networks of capillary tubes. A previous study revealed that α2-integrin–blocking antibodies interfere with the formation of tubes but also cause the rapid collapse (within several hours) of previously formed tube networks (Davis et al., 2001). EC cultures were treated at different times with TIMP-1, -3, or blocking antibodies directed to the α2- and α5-integrin subunits (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200603176/DC1). The treatment of EC cultures from 0–8 or 0–24 h with α2-integrin–blocking

Figure 2. MT metalloproteinases are required for the remodeling of vascular networks in vivo during mouse vascular development, and TIMP-3 is expressed by perivascular mesenchyme.

(A) E7.5 cultures were established as described previously (Bohnsack et al., 2004) and, in addition, were incubated in the presence of 5 μg/ml TIMP-1 or -3. Cultures were photographed live (left two panels) or were immunostained (right) after 48 h for VE-cadherin expression. Magnified live images from the boxed areas are shown in the adjacent panels for each treatment condition. (B) RT-PCR analysis was performed on extraembryonic (X) and embryonic tissue (E) at the days indicated using primers specific for TIMP-1, -3, and control. (C) Tissue sections at the indicated times were stained with antibodies directed to TIMP-3, VE-cadherin, and angiopoietin-1 (Ang-1). Bars, 100 μm.
antibodies or TIMP-3 strongly inhibited intracellular vacuole and lumen formation (Fig. S2, A and B). TIMP-1 addition or blocking antibodies directed to the α5-integrin subunit had no effect and were identical to the control. The addition of these agents to preestablished EC networks (48 h) revealed that although α2-integrin–blocking antibodies completely collapsed lumenal structures and tubular networks, TIMP-3 did not have this effect (Fig. S2 C). From these data, we hypothesized that TIMP-3 may serve as a stabilizing signal by preventing further morphogenic events and blocking the natural tendency of EC tubes to regress. A time-lapse experiment shown in Fig. S3 supports this conclusion in that TIMP-3 addition to preformed tubes does not cause collapse but retards further morphogenic progression, whereas TIMP-1 addition had no effect on either process.

We also tested the ability of TIMP-3 to block proteinase-mediated regression of endothelial networks. We used a previously established model for EC network regression and collagen gel contraction (Davis et al., 2001; Saunders et al., 2005) in which ECs were allowed to assemble into tube networks for 24 h before plasminogen (Plg) addition to activate pro–MMP-1 and -10. Cultures were either left untreated (control) or were treated with TIMP-1 or -3. After 48 h, cultures were fixed, and the percentage of collagen gel contraction was quantitated (collagen gel contraction represents the end stage of capillary tubular network collapse after proteolysis of the collagen gel matrix scaffold). These data are shown in Fig. S2 D, and they support the concept that TIMP-3 functions to stabilize and prevent the regression of EC networks. TIMP-1 also inhibits regression events that are regulated by MMP-1 and -10, which is consistent with our previous observations (Davis et al., 2001; Saunders et al., 2005). TIMP-2 and GM6001 show essentially identical effects compared with TIMP-3 in these latter assays (unpublished data).

Marked production of TIMP-3 by pericytes and regulation of pericyte TIMP-3 production by ECs in 3D collagen matrices

Pericytes are known to stabilize the developing or wound vascularization (von Tell et al., 2006). We hypothesized that perhaps pericytes deliver TIMP-3 to EC tubes to stabilize them. To evaluate TIMP-3 production by vascular cells, including EC, bovine retinal pericytes (BRPs), and coronary artery smooth muscle cells (CASMCs), cells were cultured on plastic substrates and treated with or without TGF-β for 24 h (Fig. S1 B). Western blot analyses of cell lysates revealed that TIMP-3 was not produced by ECs. However, marked production of TIMP-3 was observed from pericytes and to a lesser degree by CASMCs.

Because pericytes appear to be a strong source of TIMP-3, 3D cocultures were established with ECs and pericytes at varying pericyte/EC ratios using constant levels of ECs (Fig. 3 A). In addition, we performed the reverse experiment whereby pericytes were added at a constant level and ECs were added at varying levels (Fig. 3 B). As shown in Fig. 3 (A and B), increasing the levels of pericytes in the presence of constant ECs or increasing the levels of ECs with constant pericytes leads to a strong increase in TIMP-3 production. Interestingly, TIMP-3 is strongly produced by pericytes on plastic dishes (Fig. S1 B), but this production markedly decreases when they are cultured alone in 3D collagen matrices (Fig. 3 B). Importantly, the coculture of ECs with pericytes strongly induces TIMP-3 production by pericytes (Fig. 3, A and B). In contrast, it is clear that ECs are by far the predominant source of TIMP-2, TIMP-1, and PAI-1 in this 3D model. Little production of these factors is seen from pericytes alone, whereas the strong production of each of these factors is observed with ECs alone. To evaluate this conclusion further, siRNA suppression experiments were performed using both ECs and pericytes in 3D culture to assess which cell type is producing each proteinase inhibitor. As shown in Fig. 3,
siRNAs directed to these proteins show that ECs are the predominant source of TIMP-2, TIMP-1, and PAI-1, whereas pericytes are the source of TIMP-3. Thus, it appears that TIMP-3 production is markedly regulated by EC–pericyte interactions, whereas the other proteinase inhibitors do not appear to be altered. Interestingly, pro–MMP-1 levels, which control capillary tube regression, are progressively decreased with increasing levels of pericytes in the cocultures (Fig. 3 A). In contrast, pro–MMP-10 levels do not change under the same conditions.

Pericytes completely block MMP-1- and -10-dependent capillary tube regression events leading to capillary tube stabilization
To address whether pericytes are able to contribute to capillary tube stabilization through the blockade of MMP-1– and -10–dependent tube regression, cocultures were prepared, and quantitative analysis of tube regression over time was examined. To illustrate the coculture system, GFP-labeled ECs were cocultured with mRFP-labeled (Campbell et al., 2002) pericytes, which clearly shows the ability of pericytes to associate with developing EC tubes (Fig. 4 A). As shown in Fig. 4 B, the addition of pericytes induced a strong delay in regression until the addition of 20 or 30% pericytes relative to ECs where tube regression is completely inhibited. To examine whether the tube regression event correlates with pro–MMP-1 or -10 activation, Western blots were performed (Fig. 4 B). The addition of 30% pericytes completely blocks MMP-1 and -10 activation in response to Plg addition, indicating that proteinase inhibition is the most likely mechanism by which pericytes interfere with the capillary tube regression response (Fig. 4 B). To address whether pericytes were unique in their ability to block EC tube regression, 30% human fibroblasts or CASMCs were added, and neither cell type was able to block the regression response like the pericytes at this cell/EC percentage (Fig. 3 E).

Figure 4. Pericytes markedly block EC tube regression via the inhibition of MMP-1 and -10. (A) EC or BRP stable cell lines expressing GFP or mRFP, respectively, were cocultured with 30% BRPs relative to ECs for 72 h before fluorescence photography. Pericytes (arrowheads) are shown associating with EC-labeled tubes (arrows). Bar, 50 μm. (B) ECs were suspended in 3D collagen matrices in the presence of an increasing concentration of BRPs (shown as the percentage relative to ECs). Cultures were supplied with standard media containing 2 μg/ml Plg. Cultures were monitored every 4 h for EC tubular network collapse and collagen gel contraction. Data are reported as the mean percent collagen gel contraction ± SD (error bars) at the indicated times (n = 8). (C) At 72 h, conditioned media were collected and analyzed for MMP-1 and -10 expression and activation via Western blotting. Arrows indicate proenzymes; arrowheads point to activated enzymes.

EC-derived TIMP-2 and pericyte-derived TIMP-3 prevent capillary tube regression events (leading to tube stabilization) in EC-pericyte cocultures
To address the role of the different TIMPs and PAI-1 in this coculture model of pericyte-induced capillary tube stabilization, siRNA suppression was used. As shown in Fig. 5, siRNA suppression of specific TIMPs in ECs versus pericytes was observed relative to an actin control. In this experiment, ECs were treated with luciferase control, TIMP-1, or -2 siRNAs, whereas pericytes were treated with luciferase control or TIMP-3 siRNA. The different cell types were mixed in different combinations and were cultured in 3D collagen matrices at two pericyte/EC ratios of 30 or 50% pericytes relative to ECs (which previously completely inhibited capillary tube regression; Fig. 4). As shown in Fig. 5 B, at two different time points (3 or 6 d), siRNA suppression of EC TIMP-2 and pericyte TIMP-3 resulted in tube regression responses of the EC–pericyte cocultures. At later times of culture at 6 d, the suppression of EC TIMP-2 alone resulted in a tube regression response in a coculture containing 30% pericytes. At 3 d, the combination of EC TIMP-2 and pericyte TIMP-3 strongly induced capillary tube regression. In 50% pericyte cocultures, the only combination of siRNAs that affected tube regression was EC TIMP-2 combined with pericyte TIMP-3. This data strongly argues that both EC-derived TIMP-2 and pericyte-derived TIMP-3 are required for EC–pericyte cocultures to stabilize capillary tubes.

Identification of molecular targets of TIMP-2 and -3 that control capillary tube stabilization: MT1-MMP is a key regulator of EC tube formation and invasion responses
A key question in our studies is what molecular targets are blocked by TIMP-2 and -3 that interfere with both tube formation (Fig. 1) as well as the tube regression response (Fig. 4). To address this issue, we performed experiments with a series of potential targets of these TIMPs using an siRNA suppression approach. As shown in Fig. 6, we have suppressed the protein expression of a variety of MT-MMPs as well as ADAM proteinases and...
Blocking influence (Fig. 7 A). In contrast, no other siRNA had a strong suppression in that only the indicated siRNA suppressed the expression of the appropriate protein and had no influence on any of the other proteinases using Western blot analysis. The MT1-MMP siRNA is shown to markedly block tube and lumen formation response. Collectively, these data show that MT1-MMP is the likely key target of TIMP-2 and -3 in vasculogenesis assays is MT1-MMP, which is known to be blocked by these two TIMPs (Baker et al., 2002). The inability of TIMP-1 to block in these assays also supports this conclusion because it has no ability to block MT1-MMP (Baker et al., 2002).

To further confirm this conclusion, we performed ELISA capture assays using GFP epitope-tagged MT1-MMP or TIMP-3 to demonstrate binding interactions between the two proteins. As shown in Fig. 6 C, the strong binding of TIMP-2 and -3 is detected to GFP epitope-tagged MT1-MMP by capturing the complex on anti-GFP–coated wells in HEK293 cell cotransfection experiments. The reverse experiment also shows the binding of MT1-MMP to TIMP-3 in ECs when a TIMP-3–GFP fusion protein is expressed in ECs using a recombinant adenovirus. TIMP-3–GFP is captured onto wells, and the wells were probed with anti–MT1-MMP antibodies to reveal the binding interaction (Fig. 6 C).

To further analyze the influence of these metalloproteinase siRNAs, we performed two additional assays that mimic the process of angiogenesis whereby ECs invade into collagen matrices from a matrix surface (Davis et al., 2002; Davis and Senger, 2005). For these assays, we used two distinct stimuli to induce EC invasion and tube morphogenesis in 3D collagen matrices. We previously reported that sphingosine-1-phosphate (S1P) induces EC invasion (Bayless and Davis, 2003), and, here, we report for the first time that the chemokine stromal-derived factor-1α (SDF-1α) strongly induces invasion and tube morphogenesis in our system when it is incorporated into a 3D collagen matrix. This latter chemokine is known to synergize with VEGF in both developmental and pathological vascular morphogenetic events (Ceradini et al., 2004; Kryczek et al., 2005). It may represent a more physiologically relevant stimulus than S1P in this type of assay system.

As shown in Fig. 7, MT1-MMP siRNA dramatically blocks the invasion stimulated by either S1P or SDF-1α. This is shown quantitatively in Fig. 7 (B and C) and through photographs in Figs. S4 and S5 (available at http://www.jcb.org/cgi/content/full/jcb.200603176/DC1). Interestingly, MT2-MMP siRNA also inhibits SDF-1α invasion while partially inhibiting S1P invasion. Interestingly, ADAM-15 siRNA suppresses invasion selectively with SDF-1α–induced responses. ADAM-9 siRNA stimulates invasion responses like the aforementioned vasculogenesis assay, whereas ADAM-17 siRNA partially stimulates the SDF-1α invasion response. Collectively, these data show that MT1-MMP is required for EC tube formation in vasculogenic assays as well as in invasion and tube morphogenesis in response to two strong invasion-promoting stimuli, SDF-1α and S1P. Both MT2-MMP and ADAM-15 appear to play a lesser but detectable role selectively in SDF-1α–induced EC invasion responses.

**Figure 5.** EC-derived TIMP-2 and BRP-derived TIMP-3 are required for EC–pericyte interactions to stabilize EC tube networks. ECs were transfected with siRNAs targeting TIMP-1, TIMP-2, PAI-1, or luciferase control (Luc), and BRPs were transfected in a similar manner with siRNAs targeting TIMP-3 or luciferase control. [A] Western blot analysis of EC-conditioned media and BRP cell lysates indicates the effectiveness of siRNA treatment on the expression of the indicated TIMPs as well as actin controls. (B and C) ECs were suspended in collagen matrices in the presence of 30 or 50% BRPs (relative to ECs) in the indicated combinations. EC tube network regression was induced with 1 μg/ml of activated human plasmin and was quantitated at 3 or 6 d (reported as the mean percentage of gel contraction ±SD [error bars]). *, P < 0.01 compared with luciferase control; †, P < 0.01 for EC TIMP-2 siRNA–treated ECs compared with other samples with 50% BRPs. PC, pericyte.

This page contains diagrams and statistical data related to the role of various metalloproteinases in the capillary tube formation and regression processes. The diagrams illustrate the interactions between endothelial cells (ECs) and pericytes (PCs) and the effects of specific siRNA treatments on these interactions. The text provides detailed descriptions of the experimental methods used, including Western blot analysis, ELISA capture assays, and vascular invasion assays using S1P and SDF-1α stimuli. The results indicate that MT1-MMP is a key target for TIMP-2 and TIMP-3, and that ADAM-15 and ADAM-17 also play roles in these processes. The data support the conclusion that these metalloproteinases are essential for stabilizing EC tube networks and for EC invasion and tube morphogenesis in 3D collagen matrices.
Marked delays in the regression response occurred with three siRNA treatments: MMP-1, MMP-10, and ADAM-15 (Fig. 8 A). Previously, we reported that MMP-1 and -10 both control capillary tube regression (Saunders et al., 2005). ADAM-15, a known TIMP-3 target, appears to play a strong role in this process as well. A partial delay occurred with MT1-MMP–treated cells, which may relate to the fact that these cells fail to undergo substantial tube formation. EC tube formation is not blocked with MMP-1–, MMP-10–, or ADAM-15–treated cells. Interestingly, ADAM-9– and -17–treated ECs show accelerated regression responses compared with control luciferase as well as ADAM-10 and -12 treatments.

To address how these treatments affect pro–MMP-1 or -10 activation, Western blots were performed (Fig. 8 B). MMP-1 and -10 siRNA knockdown shows marked suppression of the respective proteins, whereas ADAM-15 knockdown appears to suppress the overall levels of both pro–MMP-1 and -10 as well as the activated forms of these two enzymes, which appears to account for the regression delay. The mechanism by which ADAM-15 suppression affects MMP-1 or -10 levels and activation is unclear. Interestingly, ADAM-9 and -17 suppression appears to accelerate the regression response by affecting MMP-1 activation, whereby a greater proportion of activated pro–MMP-1 is observed in both cases. Overall, these data suggest that three primary enzymes appear to play a central, required role in the MMP-dependent regression response (MMP-1, MMP-10, and ADAM-15). These metalloproteinases are all blocked by TIMP-3, whereas MMP-1 and -10 are known to be blocked by TIMP-2.

The ability of TIMP-3 to stabilize tubes is dependent on its proteinase inhibitory activity

To further confirm that TIMP-3 acts as a stabilizing factor through blocking metalloproteinase targets, we performed site-directed mutagenesis of TIMP-3 using known mutations that interfere with its proteinase inhibitory activity (Langton et al., 1998; Brew et al., 2000). We mutated either cysteines 1, 3, or both and made lentiviral vectors that express these constructs in comparison with TIMP-1. Stable EC cell lines expressing these recombinant proteins were selected, and each of the respective recombinant proteins were produced at appropriate levels (Fig. 9 A). To address whether the recombinant proteins influence tube formation or regression, we performed various biologic assays (Fig. 9, B–D). As shown in Fig. 9, TIMP-3 wild type is able to block lumen formation (Fig. 9 B), invasion responses (Fig. 9 C), and capillary tube regression (Fig. 9 D). In contrast, increased TIMP-1 expression fails to block tube formation or invasion but completely inhibits tube regression through its known ability to inhibit MMP-1– and -10–dependent proteolytic events (Davis et al., 2001; Baker et al., 2002). The TIMP-3 mutants failed to block EC lumen formation or invasion, suggesting that the proteinase inhibitory function of TIMP-3 is required for the ability of TIMP-3 to stabilize tubes through interference with EC tube morphogenesis.
Surprisingly, the TIMP-3 C1S mutant retained its ability to block tube regression responses, whereas the TIMP-3 C3S mutant or C1SC3S double mutant did not. Overall, these data demonstrate that the proteinase inhibitory function of TIMP-3 is central to its ability to stabilize tubes through the blockade of tube morphogenesis and tube regression responses.

**Discussion**

**EC-derived TIMP-2 and pericyte-derived TIMP-3 coregulate human capillary tube stabilization in 3D collagen matrices**

There is considerable interest in the mechanism by which pericytes stabilize capillary tubes during development and other vascular morphogenic events (Orlidge and D’Amore, 1987; Jain, 2003; von Tell et al., 2006). Much data support the concept that pericytes stabilize EC-lined tubes as a result of observations that tubes without associated pericytes are more susceptible to regression (Jain, 2003; von Tell et al., 2006). However, the molecular mechanisms that regulate these phenomena remain unclear. The data presented here propose a new concept in that pericyte-induced capillary tube stabilization can be mediated, in part, by proteinase inhibition (Fig. 10). Our work suggests that proteinase inhibition of both EC tube morphogenesis (leading to the cessation of morphogenesis) and tube regression is necessary to control tube stabilization.

Previous data from our laboratory support the concept that ECs undergoing the lumen formation process and assembling into 3D networks induce a gene program to regulate tube formation but also establish the conditions necessary for tube regression (Bell et al., 2001; Davis et al., 2002; Davis and Saunders, 2006). In studies of EC network collapse of 3D collagen matrices, ECs secrete high levels of a latent collagenase, pro–MMP-1, as well as pro–MMP-10 during the assembly of 3D tubular networks (Davis et al., 2001; Saunders et al., 2005), which we have shown are proregression agents. In the presence of serine proteinases, MMP-1 and -10 zymogen activation occurs, and active MMP-1 controls collagen and ECM dissolution and collapse of capillary networks. This regression event is completely blocked by the presence of sufficient pericytes (i.e., to deliver TIMP-3; Figs. 3–5) or the addition of exogenous TIMP-3 or -2 (Fig. 1 and Fig. S2). These observations all support the concept that the regulation of proteinase activity during angiogenesis...
(e.g., during wound healing) must be tightly controlled to prevent the regression of newly formed vascular networks. A similar conclusion was reached in studies of a mouse knockout of the MMP inhibitor RECK. An embryonic lethal phenotype occurs at E10.5 secondary to excessive collagenase activity, vascular collapse, and hemorrhage (Oh et al., 2001). Marked destruction of the collagen scaffold was observed in vivo and was associated with defects in vascular tube networks in a manner that was remarkably similar to our previous studies (Davis et al., 2001; Saunders et al., 2005).

Our work here shows that distinct metalloproteinases, including members of the MMP and ADAM families, influence pro-morphogenic versus proregression events (Fig. 10). Furthermore, this study shows that EC-derived TIMP-2 and pericyte-derived TIMP-3 play direct roles in regulating these stabilizing effects by inhibiting specific MMPs and ADAMs. EC-derived molecular targets for TIMP-2 and -3 include MT1-MMP, which appears to be the major regulator of EC tube morphogenesis by controlling EC invasion and lumen formation. In addition to MT1-MMP, we show that MT2-MMP and ADAM-15 appear to contribute to tube morphogenesis by regulating the EC invasion of collagen matrices. Other targets include MMP-1, MMP-10, and ADAM-15, which act together to regulate capillary tube regression.

Pericyte-derived TIMP-3 inhibits EC tube formation and vascular assembly in vitro and in vivo and stabilizes preexisting EC tubular networks

TIMP-3 has previously been reported as an antiangiogenic agent and also inhibits the growth of solid tumors (Anand-Apte et al., 1997; Spurbeck et al., 2002; Qi et al., 2003). Interestingly, TIMP-3 is frequently down-regulated by various tumors through methylation of its promoter (Wild et al., 2003). In this study, we show that TIMP-3 prevents EC tubular morphogenesis in 3D matrices in vitro as well as vasculogenesis and vascular remodeling in vivo (Figs. 1 and 2). Importantly, TIMP-3 expression from pericytes was strongly induced by the presence of ECs (Fig. 3, A and B) in 3D collagen matrices. We show that siRNA suppression of TIMP-3 in pericytes markedly diminishes their ability to stabilize EC-lined tubes that are susceptible to...
and -3 can act as vascular stabilizing agents by blocking distinct chemokine and vascular activities (Jain, 2005). Thus, both TIMP-2 and -3 have been reported to inhibit VEGF receptor-2 (VEGFR-2) signaling (Qi et al., 2003). The inhibition of VEGFR-2 is a major therapeutic target in antiangiogenic protocols (Seo et al., 2005), and the metalloproteinase inhibitor GM6001 was obtained from Calbiochem. 5-erythro-S1P was obtained from Avanti Polar Lipids, Inc. A blocking antibody targeting α2 integrin (BHA2.1) was purchased from Chemicon, and a blocking antibody against α5 integrin (mAb 16) was purchased from Becton Dickinson. Antibodies targeting MMP-10 (mAb 9101), TIMP-1 (AF970), TIMP-2 (AF971), ADAM-9 (AF949), and ADAM-15 (AF945) were obtained from R&D Systems. Antibodies targeting ADAM-12 (AB 19133A), ADAM-17 (AB 19027), and TIMP-3 (mAb 3318) were obtained from Chemicon. A polyclonal antibody against human MT1-MMP (RP1–MMP-14) was purchased from Triple Point Biologics. The PAI-1 polyclonal antibody, MMP-1 mAb (IM35L), and actin mAb (CP01) were obtained from Calbiochem. Oligonucleotide primers were obtained from Sigma-Gensys. All other reagents were purchased from Sigma-Aldrich unless otherwise noted. The pAdEasy adenoviral system was obtained from B. Vogelstein (Johns Hopkins Medical School, Baltimore, MD), and the mRFP construct was obtained from R. Tsien (University of California, San Diego, La Jolla, CA).

Cell culture
Human umbilical vein ECs (HUVECs) were purchased from Cambrex and were used from passage 2–6. BRPs were cultured in DME containing 10% FBS in gelatin-coated flasks.

BRP isolation
Bovine eyes were obtained from the Rosenthal Center (Texas A&M University), transported on ice, and submerged in Betadine for 10 min before retinas were dissected and placed into sterile buffer. The isolation protocol was essentially as previously described (Nayak et al., 1988). At confluency, pericytes were screened for the expression of 3G5 antigen and α-smooth muscle actin.

EC invasion, lumen formation, and regression assays
ECs were placed in 3D invasion assays as described previously (Bayless and Davis, 2003), in which ECs invade into 3.75 mg/ml of collagen matrices containing either 1 μM S1P or 200 ng/ml SDF-1α. Alternatively, lumen formation assays were used where individual ECs were suspended within 3.75 mg/ml of collagen type I matrices and allowed to assemble into 3D networks over time (Davis et al., 2002). Cultures were fixed at indicated time points with 3% glutaraldehyde in PBS, pH 7.5, for at least 30 min before additional manipulation. In some cases, cultures were stained with 0.1% toluidine blue in 30% methanol and were dehydrated before visualization and photography. Conditioned media were collected to examine differential protein expression at various time points. Conditioned media were run on SDS-PAGE gels, transferred to polyvinylidene membranes, probed, and developed. 3D collagen gels were also extracted in some cases to examine protein expression.
Regression assays were performed as described previously (Saunders et al., 2005). For quantitative analysis of gel contraction, 15-μl mixtures of ECs and collagen matrix (n = 8 cultures per condition) were placed in 384-well tissue culture plates (VWR Scientific Products). Cultures were allowed to equilibrate for 30 min before the addition of media with or without the serine proteases Plg or plasmin. The addition of culture media denoted time zero, and cultures were monitored every 4 h for gel contraction. Upon initiation of gel contraction, gel area was recorded using an ocular grid, and percent collagen gel contraction was calculated as follows: [(original area - current gel area/original area) × 100]. When contraction was complete, cultures were fixed or extracted, and media was examined for MMP expression and activity. Data are reported as the mean percent gel contraction ± SD.

Coculture experiments
Confluent ECs were resuspended in 3.75 mg/ml of 3D collagen matrices at varying ratios as indicated. EC or EC/PC cocultures were allowed to equilibrate for 30 min before the addition of media with 175 Fls containing 80% confluent 293FTs in 5 ml of antibiotic-free DMEM/10% FCS was transfected in a similar manner except that LipofectAMINE plus 1 μg plasmid DNA/embryo was added to 0.5 ml of DME (Invitrogen). The following morning, media was removed, and 12 ml of antibiotic-free DMEM/10% FCS was supplied. 293FTs cells were allowed to produce lentivirus for 48 h. Media containing live lentivirus was collected and spun to remove cell debris before storage at −70°C. Low passage HUVECs or BrPs were allowed to grow to 80% confluency in 175 Flasks. Cells were infected with lentivirus at the following ratio: 1 ml of lentivirus:1 ml of cell culture media. Cells were incubated for 72 h before the aspiration of media and initiation of stable cell line selection using Blastidin (Invitrogen) at a final concentration of 5 μg/ml. Cells were cultured in the presence of Blastidin for 4-10 days to ensure stable cell line selection. The expression of respective proteins was verified using Western blot analysis.

Generation of TIMP-3 and MT1-MMP Gfp fusion constructs and TIMP-3/TIMP-3–GFP adenosviruses
TIMP-3–GFP was amplified from plasmid EX-M0327-M02 (Genecopoeia) containing the full-length cDNA for human TIMP-3 (MMP-14). Standard restriction digestion cloning was performed to clone MT1-MMP into pAdTrack-CMV using the primers MT1-MMP HindIII UP (5′-AGAAGCTTCGACATCGCCCAAGGC-3′) and TIMP-1 EcoR DN (5′-AGGATATGCAGCTCAGGTCCTCAGCTC-3′) into pAdTrack-CMV. TIMP-3–GFP was amplified from the pEGFP-N2 vector (Clontech Laboratories, Inc.) using the primers TIMP-3–GFP EcoR DN (5′-AGGATATGCAGCTCAGGTCCTCAGCTC-3′) and TIMP-3–GFP HindIII UP (5′-AGAAGCTTCGACATCGCCCAAGGC-3′). The amplified TIMP-3–GFP DNA was cloned into the pAdTrack-CMV vector using standard molecular cloning methods. The amplified TIMP-3–GFP DNA was verified by plasmid DNA cloning and sequence analysis. Furthermore, protein expression was verified via Western blot analysis.

Application of vectors and stable cell lines: cloning and preparation of lentiviral vectors
TIMP-1 was amplified from HUVEC cDNA (Bell et al., 2001) using the primers TIMP-1 HindIII UP (5′-AGAAGCTTCGACATCGCCCAAGGC-3′) and TIMP-1 EcoR DN (5′-AGGATATGCAGCTCAGGTCCTCAGCTC-3′) into pAdTrack-CMV. TIMP-3 was amplified from human placental cDNA (BD Biosciences and Clontech Laboratories, Inc.) using the primers TIMP-3 XhoI UP (5′-AGGATATGCAGCTCAGGTCCTCAGCTC-3′) and TIMP-3 XhoI DN (5′-AGGATATGCAGCTCAGGTCCTCAGCTC-3′) into pAdTrack-CMV. TIMP-3–GFP was amplified from the pEGFP-N2 vector (Clontech Laboratories, Inc.) using the primers TIMP-3–GFP EcoR DN (5′-AGGATATGCAGCTCAGGTCCTCAGCTC-3′) and TIMP-3–GFP HindIII UP (5′-AGAAGCTTCGACATCGCCCAAGGC-3′). The amplified TIMP-3–GFP DNA was cloned into the pAdTrack-CMV vector using standard molecular cloning methods. The amplified TIMP-3–GFP DNA was verified by plasmid DNA cloning and sequence analysis. Furthermore, protein expression was verified via Western blot analysis.

The production of recombinant lentivirus was accomplished using the pAdTrack-CMV vector with the primers TIMP-1 plenti UP (5′-CACCATGGGCGGCAAGCTCCTG-3′) and TIMP-1 plenti DN (5′-GAGGATATGCAGCTCAGGTCCTCAGCTC-3′) into pAdTrack-CMV. TIMP-3 was amplified from the pAdTrack-CMV vector with the primers TIMP-3 plenti UP (5′-CACCATGGGCGGCAAGCTCCTG-3′) and TIMP-3 plenti DN (5′-GAGGATATGCAGCTCAGGTCCTCAGCTC-3′) into pAdTrack-CMV. TIMP-3–GFP was amplified from the pEGFP-N2 vector (Clontech Laboratories, Inc.) using the primers TIMP-3–GFP EcoR DN (5′-CACCATGGGCGGCAAGCTCCTG-3′) and TIMP-3–GFP HindIII UP (5′-GAGGATATGCAGCTCAGGTCCTCAGCTC-3′). The amplified TIMP-3–GFP DNA was cloned into the pAdTrack-CMV vector using standard molecular cloning methods. The amplified TIMP-3–GFP DNA was verified by plasmid DNA cloning and sequence analysis. Furthermore, protein expression was verified via Western blot analysis.
were generated from expression plasmids TIMP-3 pAdTrack-CMV and TIMP-3-GFP pAdShuttle-CMV via recombination with the pAdEasy adenoviral vector as described previously (Bell et al., 2001).

**Cotransfection of TIMP-2, TIMP-3, and MT1-MMP constructs in HEK293 cells for ELISA**

HEK293 cells were cultured to 95% confluence in DME/10% FCS in 12-well plates pre-coated with 20 μg/ml of collagen type I. LipofectAMINE 2000 was used to cotransfect cells with 750 ng plasmid encoding TIMP-2 or -3 in combination with 750 ng of plasmids MT1-MMP pAdTrack or MT1-MMP-GFP pAdShuttle encoding MT1-MMP or MT1-MMP-GFP (1.5 μg of total DNA per well). Transfected HEK293 cells were allowed to recover for 24 h, and transfection efficiency was verified via fluorescent microscopy. Detergent lysates of cotransfected HEK293 cells were made using 4°C 2% Triton X-100 in 1 × TBS, pH 8.0, containing complete EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics). Before cell lysis, 12-well plates were washed once with 1.0 ml of warm, serum-free DME and placed on ice. Each well was lysed with 500 μl of cold lysis buffer. Lysates were transferred to 1.7-ml Eppendorf tubes and incubated on ice for 20 min before centrifugation at 20,000 rpm for 20 min at 4°C to pellet-insoluble cellular debris. Supernatants were removed and stored at −70°C until use.

A mAb (36E) against GFP used to capture GFP fusion proteins was obtained from Qbiogene. Polysynthetic microwell plates (VWR Scientific Products) were pre-coated with or without 2 μg/ml anti-GFP antibody in PBS overnight at 4°C. The following morning, plates were warmed to room temperature, aspirated to remove residual GFP antibody, and blocked in Tris-Tween 20 detergent solution for 20 min. HEK293 cell cotransfection lysates were diluted 1:2 with Tris-Tween 20 containing 1% BSA and incubated at 100 μl/well vol for 2 h at room temperature. Lysates were aspirated, and plates were washed four times in Tris-Tween 20 before incubation with polyclonal antibodies (50 μl/well) against TIMP-2 or -3 (R&D Systems) diluted at 1:200 and 1:100, respectively, in Tris-Tween 20 and 1% BSA. In separate wells, a control antibody targeting MT1-MMP (Triple Point Biologics) was used to ensure the proper capture of MT1-MMP-GFP and not MT1-MMP pAdTrack. Additionally, conditioned media and lysates were assessed via Western blotting to ensure the proper protein expression of each transfection condition. Primary antibodies were incubated for 1 h at room temperature, aspirated, and plates were washed four times before the addition of HRP-conjugated secondary antibodies (50 μl/well; DakoCytoChem) diluted 1:2,000 in Tris-Tween 20 and 1% BSA. Secondary antibodies were incubated for 30 min at room temperature before washing three times in Tris-Tween 20 and were washed twice in ddH2O. ELISA plates were developed using a typical O-Phenylenediamine substrate solution (100 μl/well) and stopped with 100 μl/well of 1 M sulfuric acid. Absorbance was read at 490 nm. The mean absorbance reported was the mean absorbance for duplicate wells and have been background corrected against control wells, which were not precoated with the capturing GFP mAb.

**Infection of HUVECs with TIMP-3 and TIMP-3-GFP adenoviruses for ELISA capture and detection of EC-derived MT1-MMP**

HUVECs were cultured to confluence in T25 flasks. Viral transformation of ECs with recombinant adenoviruses encoding TIMP-3 or TIMP-3-GFP fusion was performed as described previously (Bell et al., 2001). The following morning, transformation efficiency was assessed using fluorescent microscopy. Flask were washed twice with 3 ml of warm M199, and detergent lysates of TIMP-3- and TIMP-3-GFP-producing ECs were prepared using 1.5 ml of cold lysis buffer per T25 flask. The ELISA capture technique was performed as described above except that HUVEC lysates, TIMP-3-GFP was captured, and lysates were probed for the EC-derived target MT1-MMP. The MT1-MMP polyclonal antibody (Triple Point Biologics) was used at a 1:200 dilution. Data are reported as the mean absorbance for duplicate wells and have been background corrected against control wells, which were not precoated with the capturing GFP mAb.

**Microscopy and imaging**

A fluorescence inverted microscope (Eclipse TE2000-U; Nikon) was used to visualize EC invasion, vasculogenesis, and regression as well as immunofluorescence microscopy. Lenses used for time lapse included a CFI plan-Fluor 20× with an NA of 0.45, whereas for immunofluorescence, a CFI plan-Apo 60× oil objective with an NA of 1.4 was used. FITC and rhodamine were used for immunofluorescence, whereas GFP and mRFP were used for still photographs of EC-pericyte cocultures. Imaging media included Slow Fade (Invitrogen) for immunofluorescence and M199 culture media without phenol red for all time lapses of EC cultures and still photography of EC-pericycle cultures. Imaging of cocultures used an inverted microscope (CKX4; Olympus) and 20× NA 0.45 luc plan FLN or 40× NA 0.6 Luc Plan FL objectives. Images were acquired using a camera (DP70; Olympus) and DP manager software version 2.1.1.163 (Olympus). Time-lapse imaging of living cells used the Eclipse microscope and was performed with a temperature-controlled chamber (Solent Scientific) set to 37°C with continuous flow of 5% CO2. Time-lapse two-photon imaging was performed at the lowest possible excitation levels. Images were captured every 10 min in single z planes with a monochromatic camera (CoolSNAP HQ; Photometrics) and a 6.45 × 6.45-μm pixel pitch (Photometrics) using MetaMorph software (Molecular Devices). After image acquisition, stacks of each stage position were assembled using MetaMorph software.

**Statistical analysis**

Statistical analysis of selected EC invasion, vasculogenesis, and regression data was performed using SPSS 11.0 software (SPSS, Inc.). Analysis of variance was used to compare means when analyzing more than two groups. The Tukey’s post-hoc test was used to determine groups of similarity. Statistical significance was set at P < 0.01. Unpaired t tests were used when analyzing two groups within a single experiment.

**Online supplemental material**

Fig. S1 shows that EC invasion of 3D collagen matrices is markedly inhibited by ECs expressing TIMP-3 but not by ECs expressing TIMP-1 and that pericytes are an abundant source of TIMP-3. Fig. S2 shows that TIMP-3 stabilizes ECs in EC tubular networks in association with invasion or regression, whereas anti–α2-integrin-blocking antibodies cause the rapid collapse of EC tubular networks. Fig. S3 shows that TIMP-3 blocks further lumen development and network remodeling when added to preexisting EC tubular networks without causing the collapse of these structures. Fig. S4 shows that the suppression of membrane-associated metalloproteinases via siRNAs in ECs indicates a critical role for MT1-MMP in the S1P-mediated invasion of 3D collagen matrices. Fig. S5 shows that the suppression of membrane-associated metalloproteinases via siRNAs in ECs indicates a critical role for TIMP-3 in the S1P-mediated invasion of 3D collagen matrices. Videos 1 is a time-lapse video of control EC tube morphogenesis in 3D collagen matrices. Video 2 is a time-lapse video of normal EC tube morphogenesis in the presence of TIMP-1 in 3D collagen matrices. Videos 3 and 4 are time-lapse videos of the blockade of EC tube morphogenesis by TIMP-2 and -3, respectively, in 3D collagen matrices. Video 5 is a time-lapse video of the blockade of EC tube morphogenesis by the MMP inhibitor GM6001 in 3D collagen matrices. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200603176/DC1.

We would like to thank Dr. Bert Vogelstein for providing the pAdEasy adenoviral vector system, Dr. Roger Tsien for providing the mRFP vector, Dr. Gail Martin for help with lentiviral construction of an mRFP-containing construct, and Dr. Ann Ellis for expert technical assistance with plastic sections.

This work was supported by National Institutes of Health grants HL 59273 and HL 70960 to G. E. Davis.

Submitted: 31 March 2006
Accepted: 1 September 2006

**References**


Hirschi, K.K., S.A. Rohovsky, and P.A. D’Amore. 2001. PDGF, TGF- 


