A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2

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Tissue inhibitor of metalloproteinases-3 (TIMP3) is one of four members of a family of proteins that were originally classified according to their ability to inhibit matrix metalloproteinases (MMP). *TIMP3*, which encodes a potent angiogenesis inhibitor, is mutated in Sorsby fundus dystrophy, a macular degenerative disease with submacular choroidal neovascularization. In this study we demonstrate the ability of TIMP3 to inhibit vascular endothelial factor (VEGF)–mediated angiogenesis and identify the potential mechanism by which this occurs: TIMP3 blocks the binding of VEGF to VEGF receptor-2 and inhibits downstream signaling and angiogenesis. This property seems to be independent of its MMP-inhibitory activity, indicating a new function for this molecule.

Angiogenesis is the process by which new capillaries are formed by sprouting from pre-existing vessels. VEGF is a potent enhancer of angiogenesis and vascular permeability *in vivo* and has been shown to be crucial to vasculogenesis and angiogenesis. These biological effects are mediated by binding of one of the isoforms of VEGF to members of the VEGF receptor (VEGFR) family: FLT1 (also known as VEGFR1), KDR (also known as VEGFR2 and FLK-1) or neuropilin. Both FLT1 and KDR are essential for normal vascular development. Experiments with mice genetically engineered to lack either Kdr or Flt1 determined that Kdr is essential for the development of endothelial cells, whereas Flt1 is necessary for the organization of the embryonic vasculature[1–3]. The K_d for VEGF binding to KDR is 75–770 pM[4–7], which is 10–90 times higher than that for FLT1 (10 pM)[7,8]. Both receptors undergo distinct signal transduction pathways after binding VEGF, with KDR showing strong ligand-dependent tyrosine phosphorylation in comparison with FLT1[4,7–9].

Remodeling of the extracellular matrix (ECM) is an important regulator of neovascularization, vascular morphogenesis and vascular invasion. The initiating stage in angiogenesis is believed to be a proteolytic degradation of capillary basement membrane by MMPs and serine proteases[10]. Members of the MMP family have an important role in angiogenesis[11–13]. Mice deficient in MMP2 and MMP9 show reduced angiogenesis *in vivo*[14]. TIMP3, an ECM-bound protein that is expressed ubiquitously, is a potent inhibitor of angiogenesis and tumor growth[15–17]. TIMP3 is a member of a family of endogenous MMP inhibitors, of which there are currently four members (TIMP1 through TIMP4). By virtue of their MMP-inhibitory activity, TIMP family members have a potentially important function in regulating matrix composition and thereby affect a wide range of physiological processes including cell growth, invasion, migration, angiogenesis, transformation and apoptosis. Mutations in TIMP3 are associated with Sorsby fundus dystrophy, a macular degenerative disease manifested by sudden loss of visual acuity in the third to fourth decades of life due to choroidal (submacular) neovascularization[18–20]. We reasoned that because TIMP3 might contribute to the regulation of VEGF-mediated angiogenesis, an examination of its mechanism of action would be important in understanding its role in physiological and pathological conditions.

Effect of TIMP3 on VEGF-mediated angiogenesis

To determine the effect of TIMP3 on *in vivo* angiogenesis, we used the chick embryo chorioallantoic membrane (CAM) assay[21]. Recombinant human $VEGF_{165}$ (100 ng), incorporated into methylcellulose disks, induced branching and leakage of blood vessels, presumably due to the induction of vascular permeability. At doses of 0.75 μg/disk and 1.5 μg/disk, TIMP3 inhibited both these responses in 75% (n = 8) and 90% (n = 10) of CAMs, respectively (Fig. 1*a*). At similar doses, TIMP1 did not show any angiogenesis inhibition (0% of CAMs tested, n = 10). Quantitation of tertiary branchpoints of capillaries determined a decreased angiogenic index with VEGF plus TIMP3 (90 ± 1) as

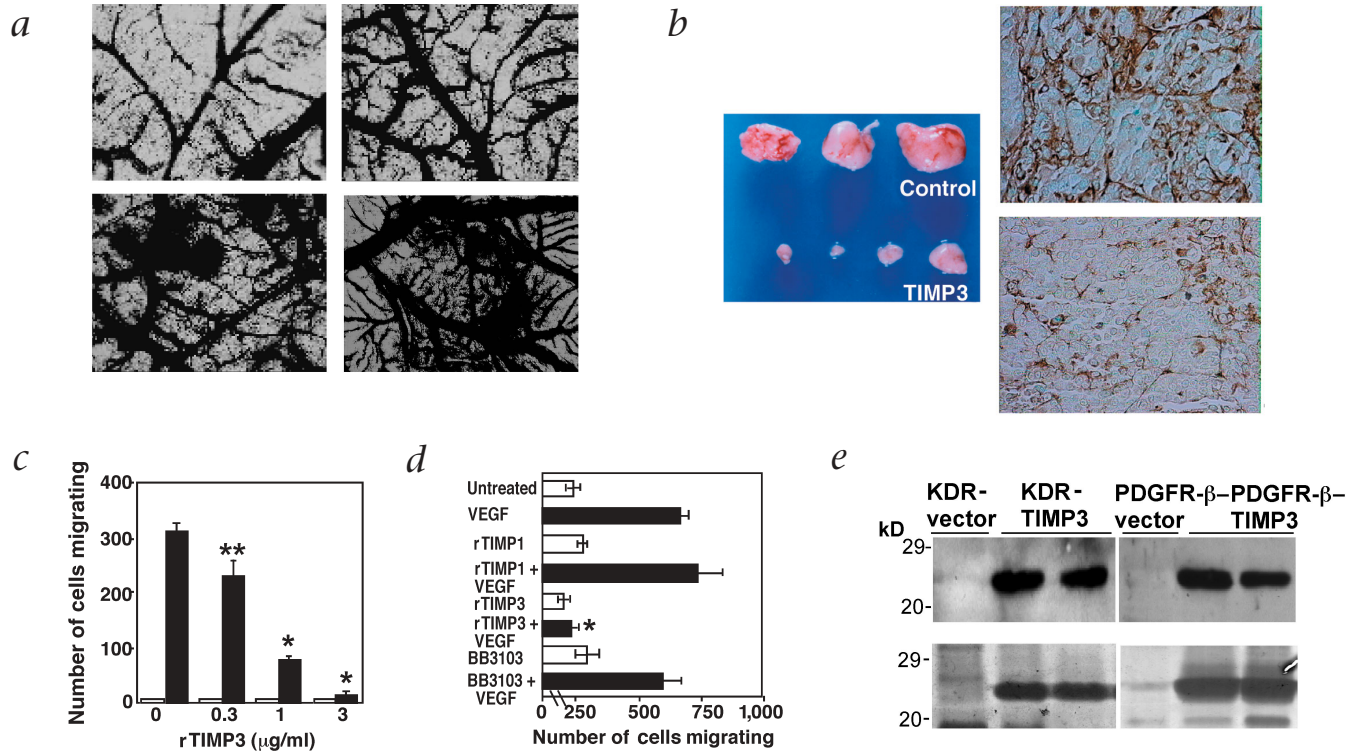
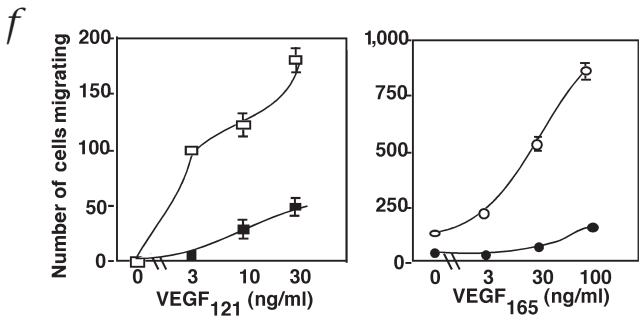


Fig. 1 TIMP3 inhibits VEGF-mediated angiogenesis. ***a***, Chicken CAMs (3 d) were exposed to control buffer (top left), 100 ng VEGF (bottom left), 100 ng VEGF plus 1.5 μg TIMP3 (top right) or 100 ng VEGF plus 1.5 μg TIMP1 (bottom right) for 48 h. ***b***, Tumors from mice injected subcutaneously with MDA-MB435 human breast carcinoma cells, transfected with empty pCEP4 vector (control) or pCEP4-TIMP3 (TIMP3; left panel). Representative photographs of immunohistochemical staining of 3-μm sections with antibodies to von Willebrand factor (right; control, top; TIMP3, bottom). Brown reaction product depicts endothelial cells lining the vessels. ***c***, Inhibition of $VEGF_{165}$ (10 ng/ml)-mediated migration of PAE-KDR cells by recombinant TIMP3 (rTIMP3). ***d***, Comparison of the effects of rTIMP3, rTIMP1 and BB3103 on $VEGF_{165}$-induced migration of PAE-KDR cells. *c* and *d*, ■, $VEGF_{165}$; □, unstimulated. ***e***, Analysis of endothelial cells expressing TIMP3. Expression of TIMP3 protein was confirmed by immunoblotting with TIMP3-specific antibody in PAE-KDR cells (top left) and PAE–PDGFR-β cells (top right). Ability of overexpressed TIMP3 to inhibit gelatinases was analyzed by reverse zymography in ECM preparations of PAE-KDR (bottom left) and PAE–PDGFR-β cells (bottom right). ***f***, Migration of PAE-KDR cells expressing TIMP3 (KDR-TIMP3) toward $VEGF_{121}$ and $VEGF_{165}$. In all migration experiments, migrating cell number is expressed as mean ± s.e.m. of quadruplicate samples. All experiments were done in triplicate. Open symbols, KDR-vector; closed symbols, KDR-TIMP3. *, $P < 0.01$; **, $P < 0.05$.



compared with VEGF (190 ± 5) and VEGF plus TIMP1 (172 ± 8). We previously showed inhibition of tumor growth by TIMP3 (ref. 15). Immunohistochemical staining of human breast tumors expressing TIMP3, with antibodies against von Willebrand factor, showed a decrease in neovascularization as compared with control tumors (Fig. 1*b*), similar to that recently described in a mouse tumor model[22]. These results suggest an important regulatory function for TIMP3 in tumor-induced neovascularization. Considering that VEGF is believed to induce angiogenesis by engaging KDR, we tested the ability of TIMP3 to inhibit VEGF-induced migration and proliferation of endothelial cells expressing KDR. VEGF can induce endothelial cell chemotaxis, an essential component of angiogenesis. When added exogenously to endothelial cells, recombinant TIMP3 inhibited their migration toward VEGF in a dose-dependent manner (Fig. 1*c*), with complete inhibition occurring at a dose of 3 μg/ml. The inhibition of migration was not observed with TIMP1 or with the synthetic MMP inhibitor BB3103 (Fig. 1*d*), indicating that TIMP3 is unique in this regard. We generated stable endothelial cell lines that expressed KDR or platelet-derived growth factor receptor-β (PDGFR-β) on their surface as well as functional TIMP3 in their ECM (Fig. 1*e*). The stable expression of TIMP3 was confirmed by western blot analysis using polyclonal antibodies against TIMP3 (Fig. 1*e*). Reverse zymography confirmed that TIMP3 was a functional MMP inhibitor in these cells (Fig. 1*e*). Stable expression of TIMP3 in the endothelial cells resulted in an attenuation of the chemotactic response to $VEGF_{121}$ and $VEGF_{165}$ (Fig. 1*f*).

Purified recombinant TIMP3, when added exogenously to endothelial cells at doses of 0.3–1 μg/ml, inhibited the proliferative response to $VEGF_{165}$ and $VEGF_{121}$ (Fig. 2*a*). Recombinant TIMP3 (1 μg/ml and 10 μg/ml), but not recombinant TIMP2 or synthetic MMP inhibitor, could also inhibit DNA synthesis of cells in response to $VEGF_{165}$, as measured by uptake of [^{3}H]thymidine

(Fig. 2*c*). As with exogenously added recombinant TIMP3, overexpression of TIMP3 in endothelial cells resulted in an attenuation of the proliferative response to $VEGF_{165}$ and $VEGF_{121}$ (Fig. 2*d*). Neither recombinant TIMP3 nor TIMP3 overexpression affected the proliferative response of endothelial cells to PDGF-BB or basic fibroblast growth factor (bFGF; Fig. 2*b* and *d*), suggesting the inhibitory effect to be specific for VEGF. None of the other TIMPs tested (Fig. 2*a*) could inhibit this response, indicating that this VEGF-inhibitory function is unique to TIMP3 and may be independent of its MMP-inhibitory activity. The N-terminal domain of TIMP3 contains the Cys 1 residue that is critical for metalloproteinase inhibition. To confirm that MMP inhibition is not required for the anti-angiogenic effect, we infected mouse endothelial cells (MS1) with an adenovirus (Rad66; see Methods) expressing a recombinant TIMP3 protein ($TIMP3_{Cys1\text{-}Ser}$) that has reduced metalloproteinase-inhibitory activity, as previously described[23]. Earlier studies showed that infection with the adenovirus expressing wild-type TIMP3 could promote apoptosis in some cell lines such as vascular smooth muscle cells, HeLa and HT1080 (refs. 24–26), but not in others such as COS-7 (ref. 27) and endothelial cells (A.B., unpublished observations). No apoptosis was observed in the MS1 cells after infection with wild-type or mutant virus. Whereas expression levels in cells infected with the recombinant adenovirus $Rad\text{-}TIMP3_{Cys1\text{-}Ser}$ were similar to those produced by the adenovirus expressing wild-type TIMP3 (Fig. 2*e*), reverse zymography of ECM extracts showed that recombinant $TIMP3_{Cys1\text{-}Ser}$ had reduced metalloproteinase-inhibitory activity (Fig. 2*e*). Endothelial cells infected with $Rad\text{-}TIMP3_{Cys1\text{-}Ser}$ were still inhibited in their proliferative response to VEGF, as measured by incorporation of [^{3}H]thymidine (Fig. 2*f*).

TIMP3 inhibits VEGF-induced KDR and MAPK phosphorylation

VEGF mediates its activity by binding to its receptor, leading to phosphorylation of the receptor and a series of downstream signaling events. We found that 1 μg/ml of recombinant TIMP3 strongly inhibited $VEGF_{165}$-induced phosphorylation of KDR in response to $VEGF_{165}$ (Fig. 3*a*) but did not affect the phosphorylation of PDGFR-β when stimulated with PDGF-BB. Similarly, overexpression of TIMP3 in endothelial cells also attenuated the

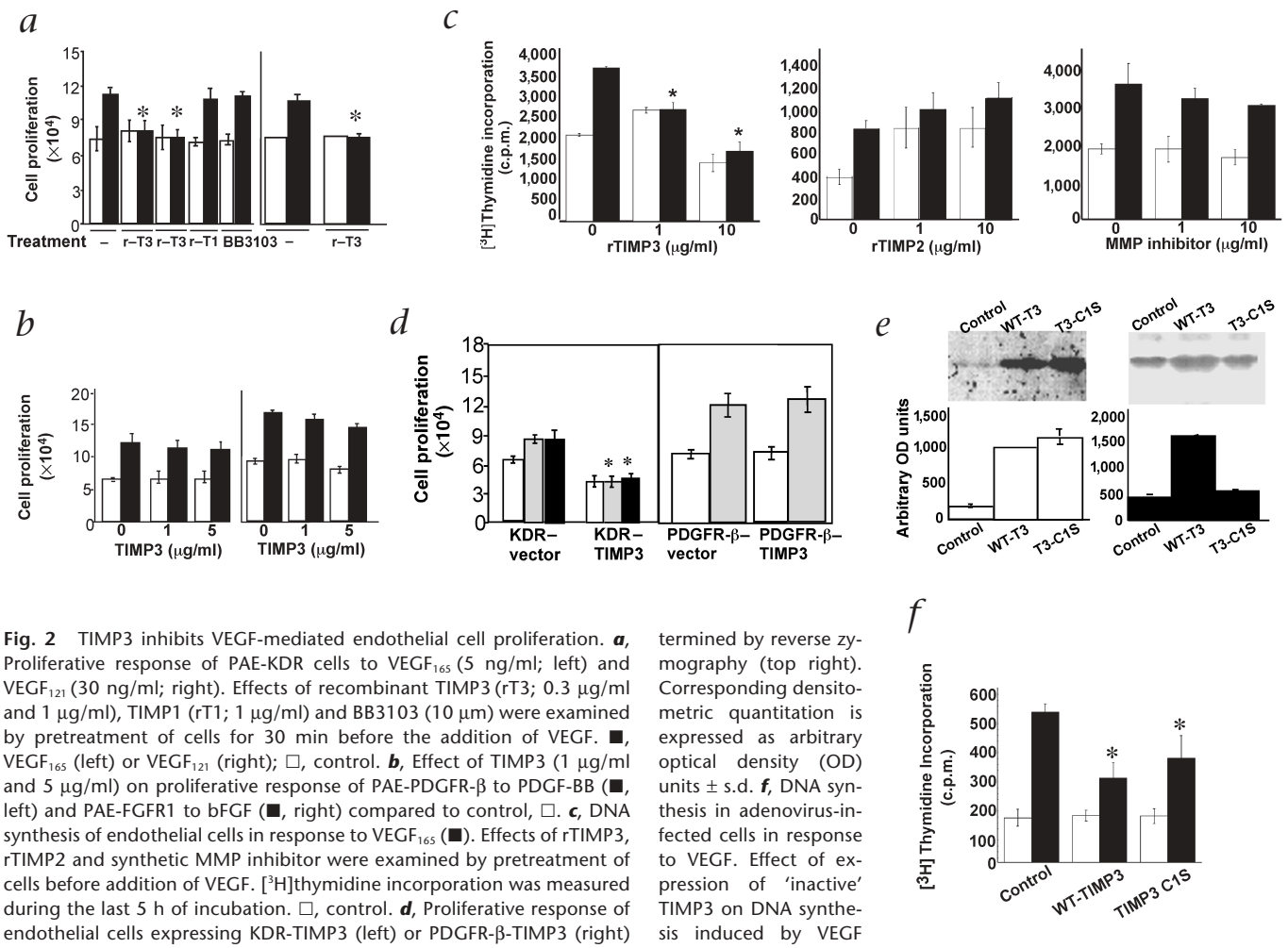


Fig. 2 TIMP3 inhibits VEGF-mediated endothelial cell proliferation. ***a***, Proliferative response of PAE-KDR cells to $VEGF_{165}$ (5 ng/ml; left) and $VEGF_{121}$ (30 ng/ml; right). Effects of recombinant TIMP3 (rT3; 0.3 μg/ml and 1 μg/ml), TIMP1 (rT1; 1 μg/ml) and BB3103 (10 μm) were examined by pretreatment of cells for 30 min before the addition of VEGF. ■, $VEGF_{165}$ (left) or $VEGF_{121}$ (right); □, control. ***b***, Effect of TIMP3 (1 μg/ml and 5 μg/ml) on proliferative response of PAE-PDGFR-β to PDGF-BB (■, left) and PAE-FGFR1 to bFGF (■, right) compared to control, □. ***c***, DNA synthesis of endothelial cells in response to $VEGF_{165}$ (■). Effects of rTIMP3, rTIMP2 and synthetic MMP inhibitor were examined by pretreatment of cells before addition of VEGF. [^{3}H]thymidine incorporation was measured during the last 5 h of incubation. □, control. ***d***, Proliferative response of endothelial cells expressing KDR-TIMP3 (left) or PDGFR-β-TIMP3 (right) to $VEGF_{121}$ (■) and $VEGF_{165}$ (■) was compared with response in cells lacking TIMP3 (KDR-vector and PDGFR-β–vector, respectively). PDGF-BB (■, right); □, unstimulated. ***e***, Analysis of mouse endothelial cells infected with adenovirus expressing WT-TIMP3 (WT-T3) and $TIMP3_{Cys1\text{-}Ser}$ (T3-C1S). Expression of TIMP3 protein was determined by immunoblotting with antibody against TIMP3 (top left). MMP-inhibitory activity was determined by reverse zymography (top right). Corresponding densitometric quantitation is expressed as arbitrary optical density (OD) units ± s.d. ***f***, DNA synthesis in adenovirus-infected cells in response to VEGF. Effect of expression of 'inactive' TIMP3 on DNA synthesis induced by VEGF was measured by incorporation of [^{3}H]thymidine. In all proliferation experiments, total number of cells was counted at 48 h. Proliferating cell number is expressed as means ± s.e.m. of quadruplicate samples. DNA synthesis data represent the means ± s.e.m. of triplicate samples; similar results were obtained in at least 2 different experiments. ■, $VEGF_{165}$; □, control. *, $P < 0.01$.

response to $VEGF_{165}$ but not to PDGF-BB (Fig. 3*b*). As expected, downstream phosphorylation of ERK1 and ERK2 was inhibited in response to VEGF, but not to PDGF-BB, by recombinant TIMP3 (Fig. 3*a*), as well as in cells overexpressing TIMP3 (Fig. 3*b*). Neither exogenous addition of TIMP3 nor overexpression of TIMP3 affected the protein expression of KDR, ERK1 or ERK2.

TIMP3 functions independently of its TIMP activity

We examined the effect of TIMP3 on the binding of VEGF to one of its receptors, KDR, on the surface of endothelial cells. Exogenous addition of recombinant TIMP3 (Fig. 4*a*) as well as overexpression of TIMP3 in endothelial cells (Fig. 4*b*) resulted in decreased binding of [^{125}I]VEGF to the surface of cells expressing KDR. Pretreatment of KDR-transduced porcine aortic endothelial (PAE-KDR) cells with recombinant TIMP3 inhibited the binding of [^{125}I]VEGF to PAE-KDR cells in a concentration-dependent manner, with a half-maximal inhibitory concentration (IC_{50}) of 3.3–4.5 μg/ml (Fig. 4*a*). In comparison, cold VEGF competed with labeled VEGF for binding to PAE-KDR cells with an IC_{50} of 8.5–15 ng/ml (Fig. 4*b*). Neither recombinant TIMP3 nor overexpression of TIMP3 could inhibit binding of PDGF-BB to PAE–PDGFR-β cells (Fig. 4*a* and *c*) in which PDGF-BB could inhibit binding with an IC_{50} of 19–23 ng/ml (Fig. 4*c*). The effect of TIMP3 on the affinity and binding capacity of VEGF for PAE-KDR cells was evaluated in a binding assay (Fig. 4*d*). Scatchard plot analysis revealed that untreated PAE-KDR cells possessed a large number of high-affinity VEGF-binding sites (3.3×10^6–6.9×10^6 sites/cell), which bound VEGF with a K_d of 50–100 pM. These results are consistent with those reported earlier for endothelial cells[4]. Treatment with recombinant TIMP3 resulted in reduced binding affinity of $VEGF_{165}$ with a K_d of 333 pM and expression of 1.9×10^6 binding sites per cell (Fig. 4*d*). Similar experiments in PAE-KDR-TIMP3 cells stably transfected with *TIMP3* cDNA showed a reduction in the binding affinity of VEGF (K_d = 250 pM) and the number of binding sites for VEGF (1.4×10^5 binding sites per cell), compared with cells transfected with an empty vector (K_d = 50–100 pM; Fig. 4*e*). These results suggest that the mechanism of inhibition of VEGF binding by TIMP3 is likely to be competitive. Overexpression of TIMP3 in PAE cells expressing PDGFR-β did not affect either the binding affinity of [^{125}I]PDGF (K_d = 1 nM) or the number of binding sites per cell (4.2×10^7 binding sites per cell), as compared with cells expressing PDGFR-β but not TIMP3 (K_d = 1.1 nM and 3.9×10^7 binding sites per cell; Fig. 4*c* and *f*). We also found that recombinant TIMP1, recombinant TIMP2, synthetic MMP inhibitor BB3103, and MMP-2 and MMP-9 inhibitor (2*R*)-[(4-biphenylylsulfonyl)amino]-*N*-hydroxy-3-phenylpropionamide could not inhibit binding of VEGF to PAE-KDR cells under the same conditions (Fig. 4*g*). Endothelial cells infected with adenovirus expressing TIMP3 with reduced MMP-inhibitory activity ($TIMP3_{Cys1\text{-}Ser}$) also showed reduced binding of VEGF to its cell surfaces (Fig. 4*h*). This indicated that the inhibition of VEGF binding by TIMP3 was likely to be independent of its MMP-inhibitory activity. Taken together, these results suggest that TIMP3 is capable of specifically and competitively inhibiting VEGF binding to KDR, thereby attenuating receptor activation and downstream signaling. To ensure that

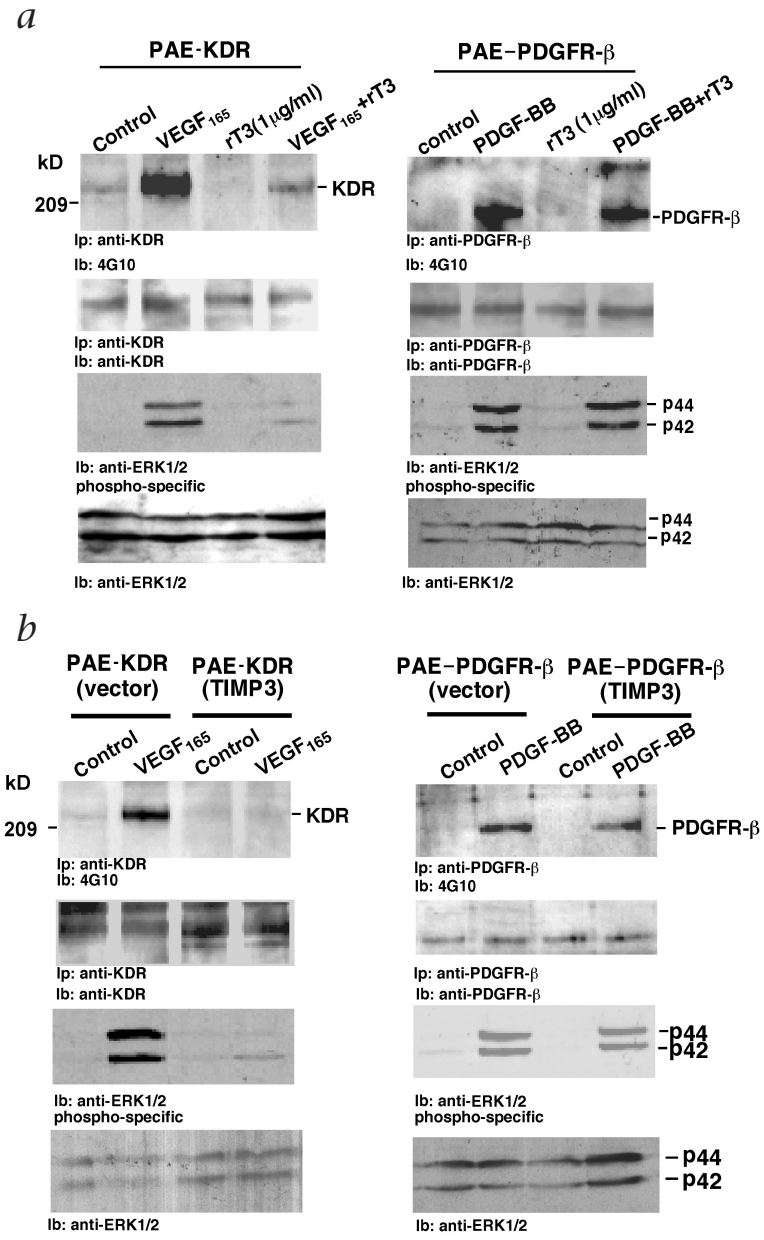


Fig. 3 TIMP3 inhibits KDR-mediated signaling. ***a***, Effect of recombinant TIMP3 on growth factor–mediated phosphorylation of the receptor was examined by immunoprecipitation (Ip) of cell lysates with antibody specific for KDR (anti-KDR) and immunoblotting (Ib) with phosphotyrosine-specific antibody 4G10 in PAE-KDR cells treated with 50 ng/ml of $VEGF_{165}$ (top left panel). PDGF-BB-mediated phosphorylation was examined similarly in PAE–PDGFR-β cells (anti-PDGFR-β; top right panel). ***b***, $VEGF_{165}$-mediated phosphorylation of KDR in PAE-KDR cells overexpressing TIMP3 (top left). PDGF-BB–mediated phosphorylation of PDGFR-β in PAE-PDGFR-β cells overexpressing TIMP3 (top right). KDR protein or PDGFR-β protein was analyzed by both immunoprecipitation and immunoblotting with antibodies specific for KDR (left) or for PDGFR-β (right; *a* and *b*, second panels from top). Phosphorylation of ERK1 and ERK2 in response to VEGF or PDGF-BB was detected by immunoblotting with phosphospecific ERK1/2 antibodies (anti-ERK1/2 phosphospecific; *a* and *b*, third panels from top). Total protein levels of ERK were determined by immunoblotting with antibodies specific for ERK1/2 (anti-ERK1/2; *a* and *b*, bottom).

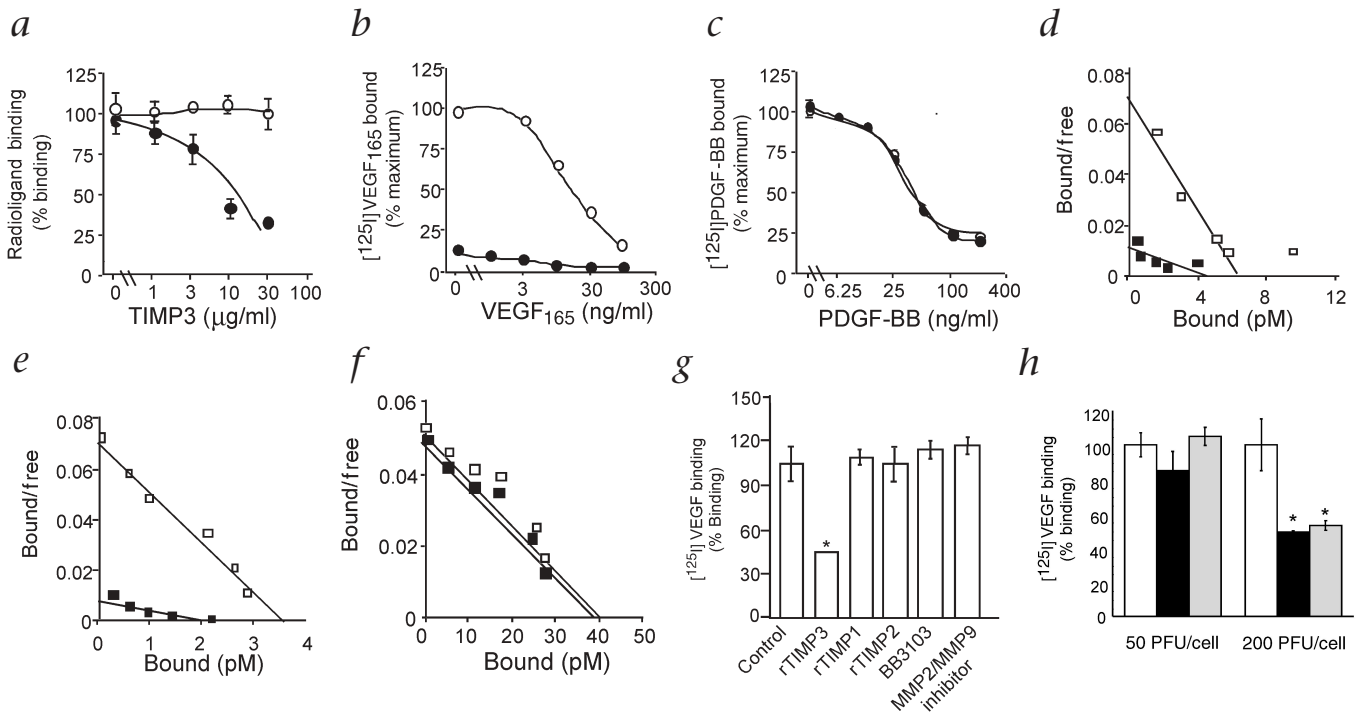


Fig. 4 TIMP3 inhibits binding of VEGF to its receptor, KDR. ***a***, Effect of TIMP3 on the binding of [^{125}I]VEGF (●) to the surface of PAE-KDR cells and [^{125}I]PDGF-BB (○) to the surface of PAE-PDGFR-β cells. The amount of bound radioligand in the absence of TIMP3 is taken as 100%. Radioligand binding is expressed as percent binding and is the mean ± s.e.m. of triplicate samples. ***b***, Confluent cultures of PAE-KDR cells (○) and PAE-KDR-TIMP3 cells (●) were incubated with 250 pM [^{125} I]$VEGF_{165}$ in the absence or presence of various concentrations of unlabeled VEGF. Results are expressed as percent maximum, with binding to PAE-KDR cells in the absence of unlabeled VEGF as 100%. All values are expressed as mean ± s.e.m. of triplicate samples. ***c***, [^{125}I]PDGF-BB binding to PAE-PDGFR-β (○) and PAE-PDGFR-β-TIMP3 (●). ***d***, Scatchard representation of specific binding of [^{125}I]$VEGF_{165}$ to PAE-KDR cells in the absence (□) or presence (■) of recombinant TIMP3. ***e***, Scatchard representation of specific binding of [^{125}I]$VEGF_{165}$ to PAE-KDR-vector (□) or PAE-KDR-TIMP3 (■) cells. ***f***, Scatchard representation of specific binding of [^{125}I]PDGF-BB to PAE-PDGFR-β-vector (□) or PAE-PDGFR-β-TIMP3 (■) cells. ***g***, Comparison of the effect of rTIMP1, rTIMP2, rTIMP3, BB3103 and MMP-2/MMP-9 synthetic inhibitor on the binding of [^{125}I]$VEGF_{165}$ to PAE-KDR cells. Binding is expressed as mean ± s.e.m. Data is shown as percent binding of triplicate samples, with binding to PAE-KDR cells in the absence of any inhibitor as 100%. *, $P < 0.01$. ***h***, Effect of expression of WT-TIMP3 (■) and TIMP3-C1S (■) on binding of ^{125}I-labeled $VEGF_{165}$ to MS1 endothelial cells. □, control adenovirus. *, $P < 0.01$.

TIMP3 was blocking the binding of VEGF to KDR specifically, we conducted cross-linking and immunoprecipitation experiments using KDR antisera. The expected cross-linked monomeric and dimeric KDR receptor forms were recovered from PAE-KDR cells (Fig. 5*a*), but not in the presence of excess unlabelled VEGF or from PAE-KDR-TIMP3 cells. We ruled out the possibility that TIMP3 induced internalization of surface KDR because the expression of KDR in plasma membrane and lysates of PAE-KDR and PAE-KDR-TIMP3 cells (Fig. 5*b*) was similar.

TIMP3 specifically blocks VEGF-KDR interaction

To determine whether TIMP3 could directly block the interaction between KDR and VEGF, we used an *in vitro* solid-phase binding assay with soluble KDR-IgG and [^{125}I]$VEGF_{165}$. Binding of VEGF was decreased in the presence of recombinant TIMP3 (Fig. 5*c*), in a concentration-dependent manner (Fig. 5*d*) with an apparent IC_{50} of 2.9 μg/ml, which is similar to what we observed in PAE-KDR cells. In contrast, similar doses of TIMP3 could not inhibit [^{125}I]VEGF binding to soluble FLT1-IgG (Fig. 5*d*), indicating the inhibitory effect of TIMP3 to be specific for KDR. These results suggested the possibility of TIMP3 binding to either VEGF or KDR to mediate this inhibition. Solid-phase binding assays using [^{125}I]VEGF and wells coated with KDR-IgG, TIMP1, TIMP2 or TIMP3 did not detect direct binding of VEGF to TIMP3 or any of the other members of the TIMP family (Fig. 5*e*).

TIMP3 binds directly to KDR

To determine whether TIMP3 could interact directly with KDR, we first tested whether TIMP3 and KDR formed a complex on the surface of PAE-KDR-TIMP3 cells. Co-immunoprecipitation experiments with lysates from PAE-KDR and PAE-KDR-TIMP3 cells confirmed this interaction. Immunoprecipitation with TIMP3 antisera could pull down KDR protein; reciprocal immunoprecipitation with KDR antisera could pull down TIMP3 protein only in PAE-KDR cells expressing TIMP3 or treated with exogenous recombinant TIMP3 (Fig. 6*a*). [^{125}I]TIMP3 could be covalently cross-linked to KDR-IgG by bis(sulfosuccinimidyl)suberate (Fig. 6*b*). In addition, both unlabeled VEGF and TIMP3 (Fig. 6c) could inhibit the binding of labeled TIMP3 to the surface of endothelial cells. To determine if this KDR-TIMP3 interaction occurs *in vivo*, we conducted immunoprecipitation experiments on extracts of human placenta that are known to be rich in TIMP3 protein. Co-immunoprecipitation experiments confirmed that immunoprecipitation with TIMP3 antisera could pull down KDR and that reciprocal immunoprecipitations with KDR antisera could pull down TIMP3 (Fig. 6*d*). These data

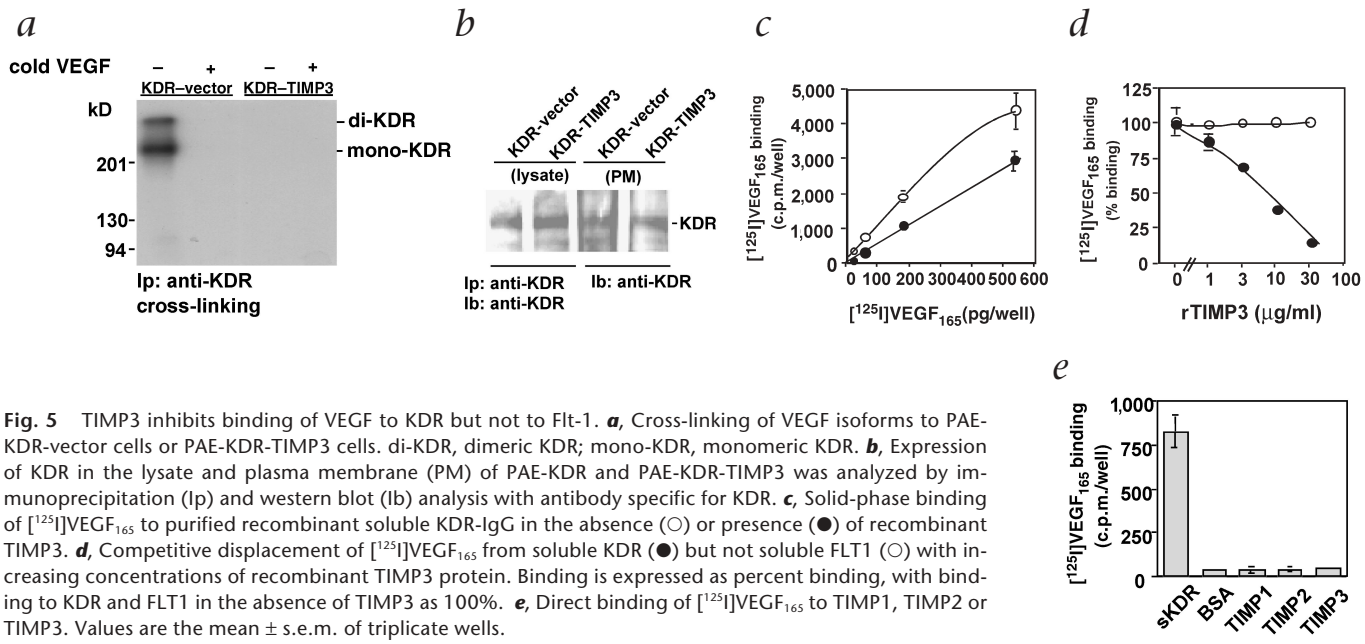


Fig. 5 TIMP3 inhibits binding of VEGF to KDR but not to Flt-1. ***a***, Cross-linking of VEGF isoforms to PAE-KDR-vector cells or PAE-KDR-TIMP3 cells. di-KDR, dimeric KDR; mono-KDR, monomeric KDR. ***b***, Expression of KDR in the lysate and plasma membrane (PM) of PAE-KDR and PAE-KDR-TIMP3 was analyzed by immunoprecipitation (Ip) and western blot (Ib) analysis with antibody specific for KDR. ***c***, Solid-phase binding of $[^{125}I]VEGF_{165}$ to purified recombinant soluble KDR-IgG in the absence (○) or presence (●) of recombinant TIMP3. ***d***, Competitive displacement of $[^{125}I]VEGF_{165}$ from soluble KDR (●) but not soluble FLT1 (○) with increasing concentrations of recombinant TIMP3 protein. Binding is expressed as percent binding, with binding to KDR and FLT1 in the absence of TIMP3 as 100%. ***e***, Direct binding of $[^{125}I]VEGF_{165}$ to TIMP1, TIMP2 or TIMP3. Values are the mean ± s.e.m. of triplicate wells.

strongly suggest that TIMP3 can bind to KDR *in vivo* and regulate its interaction with VEGF to inhibit angiogenesis.

Discussion

The stimulatory role of MMPs in the process of angiogenesis and subsequent tumor growth has been recently investigated[28,29]. Our previous report of the angio-inhibitory properties of TIMP3 suggested that this might be a consequence of simply inhibiting MMPs. We undertook the present study to examine the exact molecular mechanism by which TIMP3 inhibits VEGF-mediated angiogenesis. Much to our surprise, we discovered that TIMP3 can inhibit angiogenesis by attenuating the binding of VEGF to KDR, thereby inhibiting the downstream signaling pathways necessary to stimulate the cell. This property appeared to be independent of its MMP-inhibitory activity, because other TIMPs (TIMP1 and TIMP2) and synthetic MMP inhibitors could not inhibit the binding of VEGF to KDR in intact cells. TIMP3 could inhibit binding of VEGF to purified soluble KDR in an *in vitro* system in the absence of cell membranes. In addition, the overexpression of a TIMP3 with reduced activity could inhibit binding of VEGF, a finding that confirmed that MMP inhibition is not required for inhibition of angiogenesis. This inhibition appeared to be specific for VEGF, because signaling by PDGF and bFGF through their receptors was unaffected. A higher dose of TIMP3 (IC_{50} = 3.3–4.5 μg/ml) was required to block the binding of VEGF to KDR compared with the dose required to block the biological responses of *in vivo* angiogenesis, endothelial cell proliferation and migration (0.3–3 μg/ml). Although the difference in the amount of TIMP3 required to induce these effects is probably due to the difference in the sensitivity of readout of the different assays, there is a possibility that TIMP3 might act through two different mechanisms—one at low doses to block the biological responses and the other at a higher dose to compete for binding to KDR. In addition, the inhibition of VEGF binding was specific for KDR with no effect on FLT1. This suggests a unique function for TIMP3 that is independent of its MMP-inhibitory activity. It also suggests a direct specific interaction between TIMP3 and KDR rather than between TIMP3 and VEGF or other receptor-like molecules present on the surface of endothelial cells. We found no evidence of a direct binding of TIMP3 to VEGF, nor could it induce internalization of surface KDR in intact cells. However, *in vitro* cross-linking analysis showed that TIMP3 could directly bind soluble KDR (KDR-Fc). The affinity of KDR for TIMP3 is weaker than its affinity for VEGF because TIMP3 could inhibit VEGF binding to cells with an IC_{50} of 3.3–4.5 μg/ml, as compared with 8.5–15 ng/ml for VEGF. TIMP-2 can directly suppress the mitogenic response to tyrosine kinase-type epidermal growth factor (EGF), bFGF and PDGFR-β in an MMP-independent fashion[30]. TIMPs have been shown to alter *in vitro* cell growth and survival of a variety of cell types, independent of their MMP-inhibitory activity. Thus, TIMPs may be multifunctional, with specific effects based on their localization in tissues.

TIMP3 is a secreted 24-kD protein that distinguishes itself from other members of the TIMP family by its ability to bind to the ECM. In the outer retina, TIMP3 is synthesized by the retinal pigment epithelium (RPE) and deposited into Bruch's membrane[31–34]. The relationship between the choriocapillaris, Bruch's membrane and the RPE has been a focus of interest to both clinicians and basic scientists for a number of years. The specific localization of KDR at the inner choriocapillaris, facing the RPE, supports the notion that VEGF secreted by the RPE is involved in a physiological paracrine association with the choriocapillaris[35]. By virtue of its angio-inhibitory properties, as well as its presence in Bruch's membrane, a major function can be hypothesized for TIMP3 in the regulation of the angiogenic state of the choriocapillaris. Sorsby fundus dystrophy is an early-onset, inherited form of macular degeneration, characterized by thickening of Bruch's membrane and submacular neovascularization, which are also features of age-related macular degeneration. Mutations in the *TIMP3* gene have been associated with the disease[20,27], and increased amounts of the protein were observed in the large subretinal deposits in the eye of one patient[31]. TIMP3 levels are elevated in drusen and Bruch's membranes from the eyes of

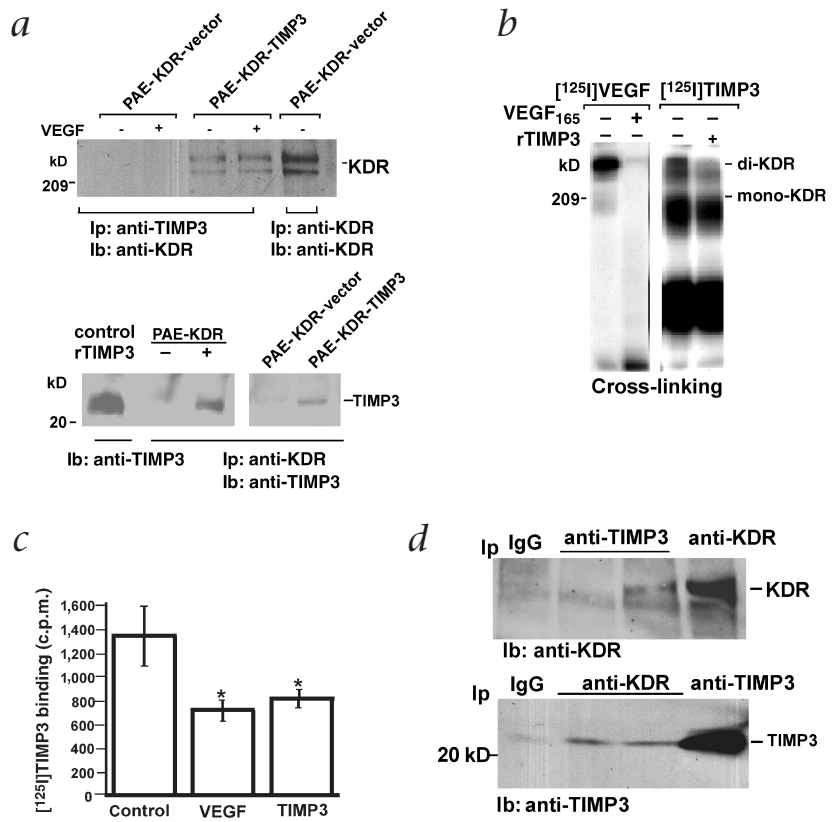


Fig. 6 TIMP3 interacts with KDR. ***a***, Immunoprecipitation (Ip) and western blot (Ib) analysis of TIMP3 and KDR from PAE-KDR-vector and PAE-KDR-TIMP3 cells. Immunoprecipitation of cell lysates with antibodies specific for TIMP3 was followed by immunoblotting with antibodies specific for KDR (top). As a control for KDR receptor, cell lysates were immunoprecipitated with antibodies specific for KDR and probed with antibodies specific for KDR. Immunoprecipitation with antibodies specific for KDR and immunoblotting with antibodies specific for TIMP3 (bottom) were carried out on PAE-KDR cells exposed to recombinant TIMP3 or PAE-KDR-vector and PAE-KDR cells overexpressing TIMP3, to confirm the interaction. ***b***, Direct binding of [^{125}I]TIMP3 to soluble KDR protein. Interaction of soluble KDR with [^{125}I]TIMP3 was analyzed after cross-linking, SDS-PAGE and autoradiography. Competition with unlabeled TIMP3 was carried out to ensure specificity. As a control for KDR, cross-linking of [^{125}I]VEGF$_{165}$ and KDR was carried out in the absence or presence of unlabeled VEGF. ***c***, Competitive displacement of [^{125}I]TIMP3 from the surface of endothelial cells by unlabeled recombinant VEGF (2 μg/ml) and TIMP3 (10 μg/ml) protein. *, $P < 0.01$. ***d***, *In vivo* interaction of KDR and TIMP3 in human placenta. Immunoprecipitation using antibodies specific TIMP3 (top) and KDR (bottom) was followed by immunoblotting with antibodies specific for KDR (top) and TIMP3 (bottom).

age-related macular degeneration patients[32]. In the same study, TIMP3 immunoreactivity was markedly reduced in areas where choroidal neovascularization was observed, and where the RPE was absent. It is possible that RPE atrophy leads to a decrease in TIMP3 expression, which in turns allows VEGF to bind its receptors on the endothelial cells of the choriocapillaris and promote choroidal neovascularization.

TIMP3 overexpression suppresses primary tumor growth and metastasis[15,16,22,36]. Adenovirally delivered TIMP3 has recently been shown to have potent antitumor activity as well as a bystander effect in an animal model of human melanoma[37]. Although decreased TIMP3 expression has been observed in a variety of tumor cell lines[15,36,38,39], its localization in tumor tissue is variable. The expression of TIMP3 in stroma adjacent to cancer cells, as well as a focal concentration around blood vessels in colorectal tumors[40–44], might be indicative of a host response to limit and restrict the extent of local tissue degradation, tumor invasion and angiogenesis. This warrants further exploration of the possible therapeutic use of TIMP3 in the regulation of VEGF-mediated pathological angiogenesis.

Methods

Cells and reagents. PAE cell lines expressing KDR, PDGFR-β and fibroblast growth factor receptor-1 (ref. 7) were cultured in Ham's F-12 DMEM supplemented with 10% FBS (Cambrex, East Rutherford, New Jersey). Recombinant VEGF$_{121}$ and bFGF was from R&D Systems (Minneapolis, Minnesota); recombinant human PDGF-BB was from Chemicon International (Temecula, California); monoclonal antibodies specific for KDR and PDGFR-β were from Sigma (St Louis, Missouri); MMP-9 and MMP-2 inhibitor (2*R*)-[(4-biphenylylsulfonyl)amino]-*N*-hydroxy-3-phenylpropionamide, MAPK-specific antibody and antibody against phosphorylated MAPK were from Calbiochem (San Diego, California). Recombinant human VEGF$_{165}$ was a gift from Genentech, B3103 was a gift from British Biotech (Oxford, UK) and TIMP3-specific polyclonal antibody was a gift from S. Apte (Cleveland, Ohio). Recombinant forms of human TIMP1, TIMP2 and TIMP3 were expressed in NSO myeloma cells and purified as described[45]. Monoclonal antibodies against phosphotyrosine (clone 4G10) were purchased from Upstate Biotechnology (Lake Placid, New York).

Generation of TIMP3–expressing endothelial cell lines. A 550-bp *TIMP3* insert from a human cDNA clone was fused in frame with the FLAG epitope DYKDDDK at its C-terminal end and cloned into expression vector pCEP4 (Invitrogen, Carlsbad, California) to be used for transfection.

Adenovirus infection. Recombinant adenovirus Rad66 containing the cytomegalovirus immediate-early promoter and polyadenylation signal and a wild-type *TIMP3* or *TIMP3*$_{Cys1\text{-}Ser}$ transgene has been described[23,25]. MS1 mouse endothelial cells (American Type Culture Collection, Manassas, Virginia) were cultured until 80% confluent and infected with adenovirus as described[23]. Cells were infected at 50 or 200 plaque-forming units per cell for 18 h. Medium was then replaced with fresh complete medium and left for 48 h until analysis.

Reverse zymography. Conditioned medium, cell lysates and ECM fractions from transfected cells were prepared. Equal amounts of protein were loaded onto a 12% gel with 1 mg/ml gelatin plus RPE cell-conditioned medium, as a source of MMPs, for reverse zymography and processed as described[46].

Immunoprecipitation and immunoblotting. Serum-deprived cells were pretreated for 30 min with or without 3 μg/ml recombinant TIMP3 and then stimulated for 10 min with the indicated concentrations of ligands. Cell fractions, prepared as described earlier, or immunoprecipitates of the lysates with the indicated antibodies, were subjected to SDS-PAGE. Proteins were detected with horseradish peroxidase–conjugated antibodies specific for either rabbit or mouse IgG (Amersham Pharmacia, Piscataway, New Jersey) followed by enhanced chemiluminescence.

Endothelial cell migration and proliferation assays. A modified Boyden chamber assay for migration and proliferation assays were carried out as

described[16]. [^{3}H]thymidine incorporation was measured in cells stimulated with 5 ng/ml VEGF for 24 h and subjected for 5 h to the addition of 1 mCi/ml of [^{3}H]thymidine.

CAM assays. The CAM assay was done as described[47] with slight modifications. Fertilized 3-day-old white Leghorn eggs (Sunnyside, Beaver Dam, Wisconsin) were cracked and embryos with the yolk intact were placed in glass-bottomed Petri dishes. Methylcellulose disks of 5 mm diameter containing VEGF with or without inhibitors were implanted on the CAM on day 3, injected with India ink and photographed after 3 d. Angiogenesis was reported as the angiogenic index (mean number of tertiary branchpoints from experimental conditions minus the mean number of branchpoints from buffer controls with no angiogenic stimulator).

Radio-iodination of $VEGF_{165}$ and TIMP3 and cross-linking experiments. Purified recombinant human $VEGF_{165}$ or TIMP3 was radiolabeled with $Na^{125}I$ by Iodobeads (Pierce, Rockford, Illinois) to a specific activity of 1.5×10^5 c.p.m. per ng VEGF or 1.0×10^5 c.p.m. per ng TIMP3. Ligand–receptor complexes on the cell surface were crosslinked with 0.5 mM disuccinimidyl suberate (Pierce) and analyzed by immunoprecipitation and SDS-PAGE followed by autoradiography. For *in vitro* assays, radioligand was crosslinked with 250 ng soluble KDR.

Ligand-binding experiments. Pre-incubation of PAE cells with unlabeled recombinant TIMPs for 1–2 h at 37 °C was followed by a 2-h incubation with 250–500 pM [^{125}I]$VEGF_{165}$ (specific activity 1,805 Ci/mmol) or [^{125}I]PDGF-BB (specific activity 1,325 Ci/mmol; Amersham) on ice. For measurement of M_{ax} (maximum number of binding sites) and the K_d value, increasing concentrations of radiolabeled ligand were added to cells that were pre-incubated with or without 30 μg/ml recombinant TIMP3. Data were analyzed by Scatchard plot analyses.

For [^{125}I]$VEGF_{165}$ binding to soluble KDR or FLT1 receptor (R&D Systems), the wells of 96-well enzyme- and radioimmunoassay strip plates were coated with 100 μl of Fc-specific IgG (8 μg/ml; Sigma). Soluble KDR-Fc or FLT1-Fc (50 ng) was added to each well. After incubation and blocking, the wells were incubated with either (i) 0.8 ng/ml of [^{125}I]$VEGF_{165}$ in the absence or presence of indicated concentrations of recombinant TIMP3, or (ii) increasing concentrations of [^{125}I]$VEGF_{165}$ in the absence or presence of 10 μg/ml recombinant TIMP3 for 2 h. After washing, the bound protein was solubilized with 1% SDS and 1 M NaOH and radioactivity measured in a γ counter.

Statistical analysis. The statistical significance of differential findings between experimental and control groups was determined by Student's *t*-test and considered significant if two-tailed *P* values were <0.05.

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Competing interests statement

The authors declare that they have no competing financial interests.

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1. Fong, G.H., Rossant, J., Gertsenstein, M. & Breitman, M.L. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376, 66–70 (1995).
2. Shalaby, F. *et al.* Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376, 62–66 (1995).
3. Shalaby, F. *et al.* A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* 89, 981–990 (1997).
4. Terman, B.I. *et al.* Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem. Biophys. Res. Commun.* 187, 1579–1586 (1992).
5. Millauer, B. *et al.* High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72, 835–846 (1993).
6. Quinn, T.P., Peters, K.G., De Vries, C., Ferrara, N. & Williams, L.T. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc. Natl. Acad. Sci. USA* 90, 7533–7537 (1993).
7. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. & Heldin, C.H. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J. Biol. Chem.* 269, 26988–26995 (1994).
8. de Vries, C. *et al.* The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255, 989–991 (1992).
9. Seetharam, L. *et al.* A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. *Oncogene* 10, 135–147 (1995).
10. Pepper, M.S., Montesano, R., Mandriota, S.J., Orci, L. & Vassalli, J.-D. Angiogenesis: a paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. *Enzyme Protein* 49, 138–162 (1996).
11. Hiraoka, N., Allen, E., Apel, I.J., Gyetko, M.R. & Weiss, S.J. Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. *Cell* 95, 365–377 (1998).
12. Stetler-Stevenson, W.G. The role of matrix metalloproteinases in tumor invasion, metastasis, and angiogenesis. *Surg. Oncol. Clin. N. Am.* 10, 383–392 (2001).
13. Werb, Z., Vu, T.H., Rinkenberger, J.L. & Coussens, L.M. Matrix-degrading proteases and angiogenesis during development and tumor formation. *APMIS* 107, 11–18 (1999).
14. Itoh, T. *et al.* Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res* 58, 1048–1051 (1998).
15. Anand-Apte, B. *et al.* A review of tissue inhibitor of metalloproteinases-3 (TIMP-3) and experimental analysis of its effect on primary tumor growth. *Biochem. Cell. Biol.* 74, 853–862 (1996).
16. Anand-Apte, B. *et al.* Inhibition of angiogenesis by tissue inhibitor of metalloproteinase-3. *Invest. Ophthalmol. Vis. Sci.* 38, 817–823 (1997).
17. Apte, S.S., Mattei, M.-G. & Olsen, B.R. Cloning of the cDNA encoding human tissue inhibitor of metalloproteinase-3 (TIMP-3) and mapping of the *TIMP-3* gene to chromosome 22. *Genomics* 19, 86–90 (1994).
18. Langton, K.P. *et al.* A novel tissue inhibitor of metalloproteinases-3 mutation reveals a common molecular phenotype in Sorsby's fundus dystrophy. *J. Biol. Chem.* 275, 27027–27031 (2000).
19. Tabata, Y., Isashiki, Y., Kamimura, K., Nakao, K. & Ohba, N. A novel splice site mutation in the tissue inhibitor of the metalloproteinases-3 gene in Sorsby's fundus dystrophy with unusual clinical features. *Hum. Genet.* 103, 179–182 (1998).
20. Weber, B.H., Vogt, G., Pruett, R.C., Stohr, H. & Felbor, U. Mutations in the tissue inhibitor of metalloproteinases-3 (*TIMP3*) in patients with Sorsby's fundus dystrophy. *Nat. Genet.* 8, 352–356 (1994).
21. Nguyen, M., Shing, Y. & Folkman, J. Quantitation of angiogenesis and anti-angiogenesis in the chick embryo chorioallantoic membrane. *Microvasc. Res.* 47, 31–40 (1994).
22. Spurbeck, W.W., Ng, C.Y., Strom, T.S., Vanin, E.F. & Davidoff, A.M. Enforced expression of tissue inhibitor of matrix metalloproteinase-3 affects functional capillary morphogenesis and inhibits tumor growth in a murine tumor model. *Blood* 100, 3361–3368 (2002).
23. Bond, M. *et al.* Localization of the death domain of tissue inhibitor of metalloproteinase-3 to the N terminus. Metalloproteinase inhibition is associated with proapoptotic activity. *J. Biol. Chem.* 275, 41358–41363 (2000).
24. Ahonen, M., Baker, A.H. & Kahari, V.M. Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinase-3 inhibits invasion and induces apoptosis in melanoma cells. *Cancer Res.* 58, 2310–2315 (1998).
25. Baker, A.H., Zaltsman, A.B., George, S.J. & Newby, A.C. Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death *in vitro*. TIMP-3 promotes apoptosis. *J. Clin. Invest.* 101, 1478–1487 (1998).
26. Baker, A.H., George, S.J., Zaltsman, A.B., Murphy, G. & Newby, A.C. Inhibition of invasion and induction of apoptotic cell death of cancer cell lines by overexpression of TIMP-3. *Br. J. Cancer* 79, 1347–1355 (1999).
27. Langton, K.P., Barker, M.D. & McKie, N. Localization of the functional domains of human tissue inhibitor of metalloproteinases-3 and the effects of a Sorsby's fundus dystrophy mutation. *J. Biol. Chem.* 273, 16778–16781 (1998).
28. Bergers, G. *et al.* Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat. Cell Biol.* 2, 737–744 (2000).
29. Coussens, L.M., Tinkle, C.L., Hanahan, D. & Werb, Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 103, 481–490 (2000).
30. Hoegy, S.E., Oh, H.R., Corcoran, M.L. & Stetler-Stevenson, W.G. Tissue inhibitor of metalloproteinases-2 (TIMP-2) suppresses TKR-growth factor signaling independent of metalloproteinase inhibition. *J. Biol. Chem.* 276, 3203–3214 (2001).
31. Fariss, R.N., Apte, S.S., Luthert, P.J., Bird, A.C. & Milam, A.H. Accumulation of tissue inhibitor of metalloproteinase-3 in human eyes with Sorsby's fundus dystrophy or retinitis pigmentosa. *Br. J. Ophthalmol.* 82, 1329–1334 (1998).
32. Kamei, M. & Hollyfield, J. TIMP-3 in Bruch's membrane: changes during aging and in age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* 40, 2367–2375 (1999).
33. Della, N.G., Campochiaro, P.A. & Zack, D.J. Localization of TIMP-3 mRNA expression to the retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 37, 1921–1924 (1996).
34. Ruiz, A., Peterson, B. & Bok, D. Localization and quantification of TIMP-3 mRNA in the human retina. *Invest. Ophthalmol. Vis. Sci.* 37, S1143 (1996).
35. Blaauwgeers, H.G. *et al.* Polarized vascular endothelial growth factor secretion by

human retinal pigment epithelium and localization of vascular endothelial growth factor receptors on the inner choriocapillaris. Evidence for a trophic paracrine relation. *Am. J. Pathol.* 155, 421–428 (1999).
36. Bian, J. *et al.* Suppression of *in vivo* tumor growth and induction of suspension cell death by tissue inhibitor of metalloproteinases (TIMP)-3. *Carcinogenesis* 17, 1805–1811 (1996).
37. Ahonen, M. *et al.* Antitumor activity and bystander effect of adenovirally delivered tissue inhibitor of metalloproteinases-3. *Mol. Ther.* 5, 705–715 (2002).
38. Sun, Y. *et al.* Molecular cloning of mouse tissue inhibitor of metalloproteinases-3 and its promoter. Specific lack of expression in neoplastic JB6 cells may reflect altered gene methylation. *J. Biol. Chem.* 270, 19312–19319 (1995).
39. McElligott, A.M., Baker, A.H. & McGlynn, H. Matrix metalloproteinase and tissue inhibitor of metalloproteinase regulation of the invasive potential of a metastatic renal cell line. *Biochem. Soc. Trans.* 25, 147S (1997).
40. Airola, K. *et al.* Human TIMP-3 is expressed during fetal development, hair growth cycle, and cancer progression. *J. Histochem. Cytochem.* 46, 437–447 (1998).
41. Byrne, J.A. *et al.* The tissue inhibitor of metalloproteinases-3 gene in breast carcinoma: identification of multiple polyadenylation sites and a stromal pattern of expression. *Mol. Med.* 1, 418–427 (1995).
42. Powe, D.G. *et al.* TIMP-3 mRNA expression is regionally increased in moderately and poorly differentiated colorectal adenocarcinoma. *Br. J. Cancer* 75, 1678–1683 (1997).
43. Zeng, Z., Sun, Y., Shu, W. & Guillem, J.G. Tissue inhibitor of metalloproteinase-3 is a basement membrane-associated protein that is significantly decreased in human colorectal cancer. *Dis. Colon Rectum* 44, 1290–1296 (2001).
44. Uria, J.A., Ferrando, A.A., Velasco, G., Freije, J.M. & Lopez-Otin, C. Structure and expression in breast tumors of human TIMP-3, a new member of the metalloproteinase inhibitor family. *Cancer Res.* 54, 2091–2094 (1994).
45. Murphy, G. & Willenbrock, F. Tissue inhibitors of matrix metalloendopeptidases. *Methods Enzymol.* 248, 496–510 (1995).
46. Pavloff, N., Staskus, P.W., Kishnani, N.S. & Hawkes, S.P. A new inhibitor of metalloproteinases from chicken: ChIMP-3. A third member of the TIMP family. *J. Biol. Chem.* 267, 17321–17326 (1992).
47. Nguyen, M., Shing, Y. & Folkman, J. Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane. *Microvasc. Res.* 47, 31–40 (1994).