Abnormal Vessel Formation in the Choroid of Mice Lacking Tissue Inhibitor of Metalloprotease-3

Andreas Janssen, Julia Hoellenriegel, Martin Fogarasi, Heinrich Schreve, Mathias Seeliger, Ernst Tamm, Andreas Ohlmann, Christian Albrecht May, Bernhard H. F. Weber, and Heidi Stöhr

PURPOSE. Tissue inhibitor of metalloprotease (TIMP)-3 is an inhibitor of matrix metalloprotease (MMP) and regulates angiogenesis. In the eye, TIMP3 is tightly associated with Bruch's membrane. In this study, the authors analyzed mice lacking TIMP3 for retinal abnormalities.

METHODS. Mice with targeted disruption of the Timp3 gene were generated (Timp3<sup>−/−</sup>) and bred into C57/Bl6 and CD1 backgrounds. Eyes were analyzed by light and electron microscopy. Vasculature was examined by scanning laser ophthalmoscopy, corrosion casts, and whole mount preparations. MMP activity was assessed by in situ zymography, angiogenic potential was evaluated by tube formation, and aortic ring assays and signaling pathways were studied by immunoblotting.

RESULTS. TIMP3-deficient mice develop abnormal vessels with dilated capillaries throughout the choroid. Enhanced MMP activity in the choroid region of Timp3<sup>−/−</sup> eyes was detected when compared with controls. Timp3<sup>−/−</sup>-derived tissue showed an increased angiogenic activity over wild-type, an effect that could specifically be inhibited by recombinant TIMP3. Moreover, the antiangiogenic property of TIMP3 was demonstrated to reside within the C-terminal domain. When VEGFR2 inhibitor was added to Timp3<sup>−/−</sup> aortic explants, endothelial sprout formation was markedly reduced, which provided evidence for an unbalanced VEGF-mediated angiogenesis in Timp3<sup>−/−</sup> animals. Finally, angiogenic signaling pathways are activated in Timp3<sup>−/−</sup>-derived cells.

CONCLUSIONS. These findings suggest that the distinct choroidal phenotype in mice lacking TIMP3 may be the result of a local disruption of extracellular matrix and angiogenic homeostasis, and they support an important role of TIMP3 in the regulation of choroidal vascularization. (Invest Ophthalmol Vis Sci. 2008; 49:2812–2822) DOI:10.1167/iovs.07-1444

Tissue inhibitor of metalloprotease (TIMP)-3 was initially identified as a 21-kDa protein that strongly binds to the cell substratum of cultured chicken embryo fibroblasts infected with Rous sarcoma virus. Further studies revealed complex and multifunctional properties of this molecule. TIMP3 is the only member of the TIMP protein family that exclusively localizes to the extracellular matrix (ECM), a dynamic fibrillar structure that surrounds cells and exhibits scaffolding and signaling functions. Within the ECM, TIMP3 forms noncovalent complexes with numerous secreted and membrane-type matrix metalloproteinases (MMPs; e.g., MMP-1, -2, -3, -9, MT1-MMP), a large group of zinc-dependent endopeptidases. The N-terminal domain of TIMP3 binds the active site of MMPs to inhibit their proteolytic activity. In addition, the C-terminal domain of TIMP3 interacts with MMP2 and MMP9 proenzymes to facilitate their activation. MMPs are responsible for the enzymatic digestion of ECM components and the liberation of signaling molecules from the ECM. Coordinating local MMP and TIMP levels is crucial for normal ECM remodeling, which is involved in various physiological processes including development, wound healing, angiogenesis, apoptosis, migration, and ovulation. As a consequence, disturbances in the delicate MMP/TIMP balance underlie a number of pathologic conditions such as arthritis, cardiovascular disease, and tumor invasion. Besides MMPs, TIMP3 inhibits selective members of the ADAM family (a disintegrin and a metalloproteinase domain) and aggrecanases (ADAM with thrombospondin-like repeats). Notably, TIMP3 binding of ADAM17 (alias TNF-α–cleaving enzyme [TACE]) prevents the shedding of TNF-α from its receptor and, thus, suggests a role for TIMP3 in inflammation.

Recently, mice carrying a targeted deletion of the Timp3 gene have been generated to examine the role of this protein in vivo. TIMP3-deficient animals develop spontaneous pulmonary air space enlargement accompanied by an enhanced degradation of collagen in the peribronchiolar space. In addition, a reduced number of bronchioles and dilated tubes indicates an impaired bronchiole branching morphogenesis in the null mouse. TIMP3 ablation in the mouse also causes spontaneous left ventricular dilation, cardiomyocyte hypertrophy, and contractile dysfunction reminiscent of human dilated cardiomyopathy. The pathogenic changes observed in lung and heart are believed to be caused by enhanced matrix degradation by MMPs and uncontrolled cleavage of the cytokine TNF-α by TACE in the absence of TIMP3. Elevated TNF-α levels in TIMP3-null mice may also be responsible for chronic hepatic inflammation and impaired liver regeneration after partial hepatectomy and enhanced inflammatory response in antigen-induced arthritis. TIMP3-deficient mice have also been used to investigate the role of TIMP3 in tumorigenesis. The absence of TIMP3 in host stroma was demonstrated to promote tumor growth with increased angiogenesis and extensive vessel dila-
tion within the tumor. Furthermore, TIMP3-null mice exhibit enhanced metastatic colonization by tumor cells. The promotion of different stages of tumor development has been attributed to the loss of TIMP3 protease inhibitory activity. Earlier studies have shown that overexpression of recombinant TIMP3 in cancer cells inhibits tumor growth in vivo and that TIMP3 gene expression is reduced in several human tumors because of promoter hypermethylation. Taken together, these findings indicate an important but complex role of TIMP3 in cancer development and progression.

One of the few recognized cellular activities of TIMP3 that seems to be independent of its inhibitory activity on proteolytic enzymes is its ability to specifically bind VEGFR2 in vitro, thereby blocking the interaction between VEGFR2 and its ligand VEGF. This feature of TIMP3 is considered to be responsible for the suppression of VEGF-mediated angiogenesis. A direct link between the antiangiogenic effect of TIMP3 and the inhibition of VEGF/VEGFR2 interaction in vivo, however, still requires confirmation.

The RPE in the outer eye wall is one of the major sites of TIMP3 expression. After secretion from RPE cells, TIMP3 accumulates in Bruch’s membrane, a five-layered sheet of connective ECM material located between the RPE and the choroid. Here, TIMP3 is assumed to be one of the crucial regulators of Bruch’s membrane turnover. An excessive amount of TIMP3 protein in abnormal Bruch’s membrane deposits was found in a human donor eye with Sorsby fundus dystrophy (SFD), a late-onset autosomal dominant macular disease caused by mutations in TIMP3. In addition, increased thickening of Bruch’s membrane and breakdown of the RPE microvilli labyrinth has been observed in mice carrying an SFD-related mutation (S156C) in the murine Timp3 gene. In SFD patients but not in Timp3S156C-knock-in mice, new thin-walled blood vessels frequently grow into the subretinal space, causing severe macular damage and blindness.

To further understand the physiological role of TIMP3 at the level of the RPE/Bruch’s membrane/choroid interface, we have generated mice with a targeted disruption of the Timp3 gene independently of a previous model and the eye phenotype analyzed for pathologic abnormalities.

**MATERIALS AND METHODS**

**Generation of Timp3−/− Mice**

The Timp3 gene locus was isolated and cloned from a 129/SvJ mouse genomic DNA library, as described earlier. The neo expression cassette of plasmid PGKneoA was inserted into the HincII site of exon 3 (Fig. 1A). To permit negative selection against random integration, the HSV-TK expression cassette was introduced at the 3′ end for negative selection. Positions of the 5′ and 3′ primer pairs used to PCR screen for correctly targeted alleles in ES cells and offspring of chimeric animals, as well as the sizes of the PCR products, are indicated. (B) Exemplary PCR amplification of genomic DNA from tails of Timp3+/+ and Timp3−/− mice confirm the correct targeting of Timp3 in the knock-out animals. (C) Western blot analysis of ECM extracts obtained from primary fibroblast cultures of wild-type and Timp3−/− mice. TIMP3 protein is absent in Timp3−/− ECM. Immunoblot with laminin B2 antibodies served as a control.
construct (Figs. 1A, 1B). The injection of mutant ES cells into C57BL/6 blastocysts, the generation of chimeras, and the breeding to C57BL/6 mice was performed as described elsewhere. Genotyping of animals was performed by PCR amplification of DNA extracted from mouse tails, as described. Expression of TIMP3 and laminin B2 in ECM extracts from murine fibroblasts was monitored by Western blotting using rabbit anti–mouse TIMP3 and mouse anti–laminin B2 (Upstate Biotechnology, Lake Placid, NY) antibodies, as described. Before analysis, mice carrying the mutant allele were backcrossed more than six times into the C57BL/6 and the C57BL strains. Mice were housed under specific-pathogen-free conditions at the Central Animal Facility of Regensburg University and maintained under the guidelines established by the institution for their use. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Light and Electron Microscopy and Scanning Laser Ophthalmoscopy

Enucleated mouse eyes were pierced with a fine needle and placed in iced fixative for 24 hours. Subsequently, the eyes were washed overnight in cacodylate buffer, postfixed with Oso4, dehydrated, and embedded in Epon (Roht, Karlsruhe, Germany). Semithin sections (1 μm) were stained with methylene blue for histologic analysis. Ultrathin sections were stained with uranyl acetate and lead citrate and were viewed with an electron microscope (EM 902; Zeiss, Göttingen, Germany) using an infrared wavelength of 795 nm. The retinas were then fixed on a holder and sputtered with gold. Vascular corrosion casts were examined with a confocal scanning laser ophthalmoscope (Heidelberg Engineering GmbH, Heidelberg, Germany) using an infrared wavelength of 795 nm.

Vascular Corrosion Casts

Mice (n = 4) were perfused through the heart with liquid plastic containing araldite CY 223 (45%), hardener HY (25%), and acetone (30%). After 24 hours, the eyes were enucleated and macerated in several changes of concentrated KOH. The eyes were then fixed on a holder and sputtered with gold. Vascular corrosion casts were examined with a scanning electron microscope (Stereoscan 90; Cambridge Instruments, Cambridge, UK).

Fluorescein-Dextran Angiography and Whole Mount Lectin Staining

For fluorescein-dextran angiography, mice were deeply anesthetized intramuscularly with ketamine (120 mg/kg) and xylazine (8 mg/kg) and perfused through the left ventricle with 1 mL PBS containing 50 mg 2 million molecular weight fluorescein-conjugated dextran (Sigma Chemical Co., St. Louis, MO). Eyes were enucleated and fixed in 4% paraformaldehyde for 4 hours. Retinas were dissected and flat mounted using fluorescence mounting medium (Dako, Hamburg, Germany). For whole mount staining, fixed retinal tissue was passed through a graded series of ethanol to xylene and returned through alcohol to PBS. Before blocking in 1% milk and 0.05% Tween 20 in PBS for 30 minutes at room temperature, retinal tissues were incubated in 25% Triton-X-100, 25% Tween 20, and 50% 1X PBS overnight at 4°C. FITC-conjugated isoelectin B4 (1:200; Vector Laboratories, Burlingame, CA) was applied for 4 hours at 37°C. The retinas were then washed with PBS and labeled with polyclonal rabbit antibodies against pericyte marker NG2 (1:200; Chemicon, Temecula, CA; a gift from Ludwig Aigner, University of Regensburg, Germany), and secondary goat anti–rabbit IgG conjugated to Alexa 594 (1:1000; Molecular Probes, Eugene, OR).

In Situ Zymography and Immunohistochemistry

DQ gelatin from pig skin, fluorescein conjugated (Molecular Probes), was reconstituted in ISZ buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM CaCl2, 0.2 mM sodium azide) at a concentration of 80 μg/mL. Before addition to unfixed 10-μm retinal cryosections, the gelatin was diluted 1:1 in 2% agarose in ISZ buffer. The sections were incubated at 37°C for 12 hours. As a control, 15 mM EDTA was added to ISZ buffer. Immunohistochemistry was performed on 4% paraformaldehyde-fixed retinal cryosections, as described, using rabbit collagen XVIII antibodies diluted 1:100 (a generous gift from Ritva Heljasvaara, University of Oulu, Finland), and secondary goat anti–rabbit IgG conjugated to Alexa 488 (Molecular Probes). Sections and whole mount preparations were analyzed under a fluorescence microscope (Axioskop-2; Zeiss).

Aortic Ring Assay

Mice were humanely killed by CO2 asphyxiation. A midline incision that included splitting the sternum was made into the abdominal and thoracic cavities. Thoracic aortas were excised and placed in ice-cold PBS. After thorough removal of blood and fibroadipose tissue, the aortas were cross-sectioned into 1 mm-long rings (approximately 12–16 rings per mouse aorta). The rings were placed in a 48-well tissue culture dish containing 400 μL endothelial basal medium-2 (Lonza GmbH, Wuppertal, Germany) supplemented with 0.25% fibrinogen and 0.5% 5-amino-n-caproic acid (Sigma Chemical Co.). Per well, 0.05 U thrombin (Calbiochem, La Jolla, CA) was added, and the gel was allowed to clot at 37°C, 5% CO2, for 50 minutes. An equal volume of EBM medium (Lonza GmbH, Wuppertal, Germany) was added. In some assays, EBM was supplemented with purchasable recombinant VEGF (rVEGF; R&D Systems GmbH, Wiesbaden, Germany) or rTIMP3 (EMD Chemicals, San Diego, CA). In addition, rTIMP1, rTIMP2, rTIMP3, N-terminal rTIMP3 (aa 1–121), C-terminal rTIMP3 (aa 122–188), and rTIMP4 were expressed in Escherichia coli with a C-terminal hexahistidine tag and purified from inclusion bodies by a Ni-NTA agarose column. Protein refolding was achieved by dropwise addition of 1 mL denatured protein to 100 mL refolding buffer (100 mM Tris-HCl, 1 M arginine, 100 mM NaCl, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, pH 8.0) at 4°C. Recombinant proteins were used in angiogenesis assays at a concentration of 2 μg/mL. VEGFR2 inhibitor ZM528881 was obtained from Merck Biosciences (Darmstadt, Germany). Aortic rings were kept at 37°C and 5% CO2 for 6 days with a medium change once at day 3. Quantification of angiogenesis was performed on day 6. Aortic rings were photographed, and the number of microvessels, including branches, was counted as described. Each set of experiments was repeated three times.

Isolation of Aortic Endothelial Cells

Isolated thoracic aortas were cut into pieces of approximately 3 mm, placed in a 6-cm gelatin-coated cell culture well, and maintained in EBM. Explants were cultured at 37°C and 5% CO2; medium was routinely changed every other day. Aortic explants were removed when cells covered 80% of the cell culture dish. Confluent cells were transferred to 10-cm dishes without gelatin coating (passage 1) and were propagated. To purify aortic endothelial cells (AECs), cells from passage 4 were labeled with 5 μg/mL di-I-acetylated-LDL (Molecular Probes, Leiden, Netherlands) in EBM at 37°C, 5% CO2, overnight. Cells that underwent sorting (FACSAracell; Becton Dickinson, Franklin Lakes, NJ) were subsequently cultured in EBM containing 20% FCS and termed passage 1. The MHEC5-T transformed murine endothelial cell line served as a positive control. FAC-sorted AECs expressed endothelial cell surface markers CD31 and VE-cadherin and formed tube-like structures on basement membrane matrix (Matrigel; BD Biosciences, San Jose, CA).

Fibrin Bead Assay

FACS-sorted AECs were mixed with microcarriers (CytoMed 3; GE Healthcare Europe GmbH, Munich, Germany) at a concentration of 400 AECs per bead in 10 mL EBM-2. Beads were incubated for 4 hours at 37°C and 5% CO2 with occasional shaking. Cell-coated beads were transferred to a 25-cm2 tissue culture flask and left overnight in 5 mL EBM-2. After washing with EBM-2, the cell-coated beads were resuspended in fibrinogen solution at a concentration of 100 beads/mL. Then 500 μL fibrinogen/bead solution was added to 0.05 U thrombin.
in one well of a 24-well tissue culture plate. The fibrinogen/bead solution was allowed to clot for 20 minutes at 37°C, 5% CO₂. Lung fibroblasts were layered on top of the clot at a concentration of 20,000 cells/well. After attachment of the cells, 1 mL fresh EBM supplemented with various recombinant TIMPs was added as described. Plates were kept in a humidified incubator at 37°C for 6 days. To quantify vessel outgrowth, in vitro high-resolution images of cell-coated beads were taken, and the number of sprouts per bead was counted. Sprouts larger than half a length of particle diameter were included. Each set of experiments was repeated at least three times.

**Western Blotting**

FACS-sorted AECs (passage 3) were resuspended and sonicated in buffer (Phosphosafe Buffer; Novagen, Madison, WI) and were centrifuged for 20 minutes at 14,000 g. Loading buffer (125 mM Tris-HCl [pH 6.8], 10% sucrose, 2% SDS, 5% β-mercaptoethanol, 0.05% bromphenol blue) was added, and the extracts were electrophoretically separated on polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Immunoblotting was performed with primary antibodies toward ERK1/2, phospho ERK1/2, p38, phospho p38 (all from Sigma Chemical Co.), VEGFR2 (Abcam, Cambridge, MA), and phospho VEGFR2 (Upstate Biotechnology, Lake Placid, NY) and secondary goat anti–mouse antibodies conjugated to horseradish peroxidase (EMD Chemicals, San Diego, CA). Blots were developed using enhanced chemiluminescence substrate (GE Healthcare Europe GmbH).

**RESULTS**

**Generation of TIMP3-Deficient Mice**

Disruption of the mouse Timp3 gene was achieved by inserting a neomycin-resistance gene expression cassette (PGKneobPA) into exon 5 of Timp3 (Fig. 1A). This leads to a frameshift and the generation of a premature stop codon. Thus, the sequence of the targeted allele after homologous recombination should direct expression of a truncated and nonfunctional TIMP3 mRNA. Successful germ line transmission of the correctly targeted allele was confirmed by two locus-specific genomic PCR reactions. With tail DNA from mice carrying the recombinant allele, 2.8- and 2.9-kb fragments were amplified, representing sequences 5’ and 3’ from the PGKneobPA cassette, respectively (Figs. 1A, 1B). Absence of Timp3 protein in fibroblast ECM extracts derived from mice homozygous for the disrupted Timp3 gene (Fig. 1C) indicated that the targeted allele indeed represented a true null allele.

**Regular Appearance of the Retina, RPE, and Bruch’s Membrane in TIMP3-Deficient Mice**

Histologic examination of semithin retinal sections from Timp3−/− eyes of different ages (2–18 months) revealed no morphologic abnormalities in the structure of the different retinal layers, Bruch’s membrane, or the RPE compared with wild-type littermates at comparable ages (Figs. 2A, 2B). Similarly, transmission electron microscopy of ultrathin sections of eyes from TIMP3-null mice (ages 2–18 months) showed a regular appearance of RPE cells and Bruch’s membrane, with its characteristic five-layered structure composed of a central elastic layer flanked by an inner and an outer collagenous layer and two basal membranes derived from the RPE and the choriocapillaris, respectively (Figs. 2C, 2D). There was no significant difference in the thickness of Bruch’s membrane between Timp3−/− and control mice.

**Choroidal Vascular Abnormalities in TIMP3-Null Mice**

To detect retinal defects that might not be obvious by analyzing retinal cross-sections, we performed ICGA using in vivo scanning laser ophthalmoscopy. This technique provides detailed information on the structural integrity of the fundus by sequential viewing of the different planes of the posterior pole from the surface of the retina down to the choroid. Importantly, ICGA uses the near-infrared light and highly protein-
bound dye ICG to effectively visualize the choroidal vasculature. In ICG angiograms, normal development of the retinal vasculature was observed in mice lacking TIMP3. In contrast, irregularities at the level of the choroid were seen in TIMP3-deficient C57BL/6 mice that were absent in control animals. This includes hyperfluorescent larger caliber vessels and prominent balloon-like spots associated with the vascular layer of the choroid (Figs. 5A, 5B). Because ICG fluorescence is partially obscured by the RPE in pigmented mice, the Timp3−/− genotype was backcrossed onto the CD1 albino outbred background. Subsequently, scanning laser ophthalmoscopic images of CD1-TIMP3-null mice showed more prominent changes of the deep choroid with numerous dilated vessels clearly not present in control littermates (Figs. 3C–F). The choroidal phenotype was observed in all Timp3−/− mice tested (n = 9) without an apparent progression of the vascular lesions. To further examine the nature of the vessel abnormalities in Timp3−/− mice, vascular corrosion casts of Timp3−/− (C57BL/6) and wild-type eyes were analyzed by scanning laser electron microscopy. Although wild-type animals exhibited a regular network of choroidal blood vessels (Fig. 3G), the choroid of Timp3−/− mice revealed strongly dilated or fused vessel formations (Fig. 3H) that likely corresponded to the abnormalities seen by scanning laser ophthalmoscopy (Fig. 3B).

Close examination of whole mounted Timp3−/− and control retinas after fluorescein-dextran perfusion (Figs. 4A–D, F–I) or isolectin B4-staining (data not shown) revealed regular morphologies of the retinal vascular plexi in Timp3−/− mice without any signs of neovascular pathology (Figs. 4F–I). These observations confirm the ICGA findings. In addition, the localization and morphology of pericytes accompanying endothelial sprouts in deep retinal capillary layers were similar in Timp3−/− and wild-type retinas (Figs. 4E, 4I). Taken together, these data suggest that normal retinal vascularization in mice is apparently not influenced by the lack of TIMP3 in mice.

**Enhanced MMP Activity in TIMP3-Deficient Eyes**

To dissect the molecular pathway leading to choroidal abnormalities in TIMP3-null mice, we first performed in situ zymographic analyses of cryosections derived from wild-type and Timp3−/− eyes. Fluorescence signals in this assay indicate gelatin degradation by active MMPs (MMP2 and MMP9). For both genotypes, we observed fluorescence of similar intensity in the inner and outer nuclear layers and the ganglion cells (Figs. 5A, 5E). In contrast, a significant enhancement of fluorescence signal along the basel aspect of the RPE monolayer was repeatedly observed in Timp3−/− sections (Figs. 5E, 5F) compared with wild-type (Figs. 5A, 5B), indicating locally elevated gelatinase (MMP2 and MMP9) activity in the knock-out animals. The fluorescence pattern resulting from gelatinolytic activity at this site differed from the immunolabeling with collagen XVIII antibodies in consecutive cryosections (Figs. 5D, 5H) in that the latter explicitly stained Bruch’s membrane in Timp3−/− control eyes whereas MMP activity was found more externally toward the choroid and in ECM domains that interdigitate between individual choriocapillaris lobules. In addition, the intensity of collagen XVIII staining of Bruch’s membrane was similar for both genotypes, demonstrating that the enhanced fluorescence produced by increased MMP2 and MMP9 activity in Timp3−/− was not caused by artifacts in section preparations but instead reflected functional differences in TIPM3 activities. Together these findings suggest that the lack of TIMP3 causes a marked disruption of the TIMP/MMP balance in the choroid region. In situ zymography in the presence of metalloprotease inhibitor (EDTA) abolished the fluorescence signals (Figs. 5C, 5G), confirming that gelatin cleavage in the assays is mediated by MMPs.
Enhanced Capillary Sprouting of Aortic Explants in TIMP3-Deficient Mice

VEGF overstimulation is known to produce abnormally enlarged blood vessels and vascular hyperfusion. Dysregulation of VEGF-induced angiogenesis may therefore be involved in the development of the choroidal vascular abnormalities observed in TIMP3-null mice. We examined the consequences of TIMP3 deficiency on VEGF-mediated angiogenesis using the ex vivo aortic ring angiogenesis assay. Aortic explants from Timp3/+/H11002 mice reproducibly showed a significantly enhanced spontaneous microvessel outgrowth when compared with explants from wild-type littermates (Figs. 6A, 6Aa). The addition of recombinant TIMP3 (Fig. 6Bb) reduced capillary sprouting of Timp3/+/H11002 aortic rings to the level seen in wild-type (Fig. 6B). Treatment with recombinant VEGF greatly stimulated vessel formation in wild-type aortas to a level comparable to spontaneous vessel outgrowth from Timp3/+/H11002 mice (Fig. 6C). Furthermore, addition of VEGFR2 inhibitor to Timp3/+/H11002 aortic rings dramatically reduced capillary sprouting by more than 80% (Figs. 6D, 6Dd). Supplementation of control and Timp3/+/H11002 explants with recombinant TIMP3, but not with recombinant TIMP2, decreased vessel outgrowth, indicating that the angio-inhibitory effect is specific to TIMP3 (Figs. 6E, 6F). Taken together, these data suggest that a lack of competitive interaction between TIMP3 and VEGF for binding to VEGFR2 leads to the strong sprouting activity in TIMP3-deficient aortic explants.

Antiangiogenic Activity Resides within the C-Terminal Domain of TIMP3

To define the region of the TIMP3 protein responsible for the angiogenic activity, we compared the capillary vessel outgrowth of Timp3/−/− aortic rings supplemented with recombinant proteins representing full-length TIMP3 (aa 1–188), the N-terminal part (aa 1–121) containing the MMP inhibitory domain, or the C terminus (aa 122–188; Figs. 7B–E). In contrast to N-terminal TIMP3, the addition of recombinant full-length TIMP3 or C-terminal TIMP3 drastically diminished capillary sprouting in aortic rings from Timp3/−/− mice to a level lower than the spontaneous microvessel outgrowth from wild-type explants (Fig. 7A). The assessment of angiogenesis by fibrin bead assays (Figs. 7F–J) showed that isolated Timp3/−/− AECs significantly promote the formation of capillary-like vessels when compared with AECs from wild-type animals (Figs. 7F, 7G). Similar to the capillary sprouting, enhanced tube formation of Timp3/−/− AECs was specifically blocked by the addition of recombinant full-length TIMP3 or C-terminal TIMP3 (Figs. 7H–J). These independent data demonstrate that the C-terminal domain of TIMP3 is sufficient to exert the antiangiogenic properties of TIMP3.

Activation of Angiogenic Signaling Pathways in the Absence of TIMP3

VEGF induces several endothelial cell functions through activation of its cell surface receptor, VEGFR2, and downstream...
signaling molecules. To further understand how the lack of TIMP3 promotes VEGF-mediated angiogenesis, we evaluated the effect of TIMP3 deficiency in AECs of Timp3/H11002/H11002 mice on the activation of VEGF/VEGFR2 signaling pathways. Immuno- blot analyses show that the autophosphorylation of VEGFR2 and the phosphorylation of ERK1/2 and p38 mitogen-activated protein kinases (MAPKs), which are downstream signaling molecules of VEGFR2, were enhanced in AECs of Timp3/H11002/H11002 animals when compared with wild-type (Fig. 8). This suggests that the absence of TIMP3 leads to a constitutive activation of VEGF/VEGFR2 signaling pathways. Lack of TIMP3 had no influence on the levels of dephosphorylated VEGFR2, ERK1/2, and p38 proteins.

DISCUSSION

We demonstrated that TIMP3 deficiency in the mouse causes a distinct choroidal, but not retinal, phenotype characterized by prominently enlarged choroidal blood vessels. Thus, this study provides the first in vivo evidence in support of the hypothesized role of TIMP3 in the regulation of choroidal vascularization of the retina.

The choroidal vascular system starts to form early during mouse eye development, namely at the time of optic vesicle invagination. Primitive periocular blood vessels are generated from a perineural vascular plexus at embryonic day 11.5 and extend around the outer layer of the optic cup. This initial network develops into a complex vasculature by a process that involves sprouting and nonsprouting angiogenesis accompanied by extensive remodeling and maturation of blood vessels. The persistent interaction between blood vessels and the adjacent RPE is thereby crucial for the formation and maintenance of an intact choroidal vasculature. It has been shown that the suppression of RPE differentiation inhibits choroidal development, whereas biochemical or mechanical destruction of the RPE monolayer causes loss of endothelial fenestration, atrophy of the choriocapillaris and underlying choroidal vessels, and abnormal collagen deposition in the choroid. Growth factors released by the RPE play an important role in conducting the modulation and survival effect of the RPE on the choroidal vascular system. This includes VEGF, a crucial vascular permeability and angiogenic factor involved in all stages of blood vessel formation in the course of vasculogenesis and angiogenesis. RPE cells express...
FIGURE 6. Enhanced vessel sprouting in aortic explants from TIMP3-deficient mice is mediated by unbalanced VEGF-induced angiogenesis. Aortic ring assays showing spontaneous vessel outgrowth in Timp3+/− (A) versus Timp3−/− (Aa) aortic rings, spontaneous vessel outgrowth in Timp3+/− (B) versus vessel sprouting in Timp3−/− aortic rings supplemented with recombinant TIMP3 (Bb), vessel sprouting in Timp3+/− supplemented with recombinant VEGF (C) versus spontaneous vessel outgrowth in Timp3−/− (Cc) aortic rings, and spontaneous vessel outgrowth in Timp3−/− (D) versus vessel sprouting in Timp3−/− aortic rings supplemented with VEGFR2 inhibitor (Dd). Quantification of sprouting at days 2, 4, and 6 is depicted by light- and dark-shaded columns in charts beside the respective photographs (taken at day 6 of the assay); d, day. Capillary sprouting of aortic explants from (E) Timp3+/− or (F) Timp3−/− aortic rings supplemented with recombinant TIMP2 and TIMP3. Light-shaded columns represent vessel outgrowth at day 2, dark-shaded columns at day 4, and black columns at day 6. All data are presented as mean (n = 5; bars) ± SD (y-axis; sprouts/aorta).
VEGF during choroidal development and continue to secrete VEGF toward the basal choroidal side throughout adulthood. In support of a VEGF-mediated paracrine relation between the RPE and the choroid, VEGFR2 was shown to localize at the inner choriocapillaris. In coculture experiments, RPE-derived VEGF induces tube formation of underlying choroidal endothelial cells, a process that can be inhibited by VEGF antibodies. The observation that mice with a conditional inactivation of endogenous Vegf gene expression in the RPE are characterized by an absence of choroidal tissue and abnormalities in RPE and Bruch’s membrane morphology eventually confirms the fundamental role of VEGF in choroidal formation.

TIMP3 was shown to inhibit VEGF-induced angiogenesis in a chick embryo chorioallantoic membrane assay, with recombinant TIMP3 interfering with VEGF-induced endothelial cell migration and proliferation in vitro. The antiangiogenic properties of TIMP3 were attributed to its ability to bind VEGFR2, thereby blocking the activation of intracellular downstream signaling molecules required to stimulate various endothelial cell functions. In accordance with this hypothesis, we observed an increased angiogenic response in ex vivo angiogenesis assays using organ explants from TIMP3-deficient mice, an effect that could be reversed by the addition of VEGFR2 inhibitor. It is therefore reasonable to assume that under physiological conditions, the absence of TIMP3 protein in Bruch’s membrane perturbs VEGF-mediated paracrine signaling between the RPE and the choroid. As a consequence, enhanced stimulation of VEGFR2 signaling pathways by unopposed VEGF binding to endothelial cells may be responsible for increased cell proliferation and migration, leading to the formation of enlarged choroidal vessels. Consistent with this model are data demonstrating that transgenic mice with RPE cells overexpressing VEGF under the RPE65 promoter exhibit a thickened choroid with dilated vessels and wide lumina. In addition, exogenous VEGF stimulation is known to induce malformation, fused vessels with enlarged lumens, and elevated VEGF concentrations enhance vessel diameter through VEGF2 pathways in vitro.

Our immunoblot experiments with whole AEC cell extracts show differences in the activation of VEGFR2 and downstream MAPK signaling between Timp3<sup>−/−</sup> and wild-type mice. Signal transmission of several growth factors occurs through activated MAPK pathways, and it is likely that factors other than VEGF added to the enhanced phosphorylation of ERK1/2 and p38. One such candidate would be FGF-2, which has recently been shown in extracellular matrix plug and gel foam assays to be a stronger angiogenic stimulus than VEGF in Timp3<sup>−/−</sup> mice. Assuming that more than one angiogenic pathway is affected by TIMP3 deficiency, the analysis of the role of specific angiogenic factors in the development of choroidal abnormalities represents an important next step to further understand TIMP3 function in choroidal vascularization.

Higher levels of active and inactive forms of MMP-2 and MMP-9 were previously found in primary fibroblasts isolated from the lungs of our Timp3 knock-out mice, correlating with heightened metalloprotease levels detected in lung and heart of an independently generated TIMP3-null mouse line. Although a notable local increase of gelatinase activity in the choroid region of Timp3<sup>−/−</sup> eyes was easily detected by our in situ zymography experiments, MMP activity within Bruch’s membrane is difficult to assess. The observation that TIMP3-deficient mice do not show alterations in thickness and integrity of the individual layers of Bruch’s membrane, moreover, argues against a profound imbalance between MMPs and TIMPs within this specific matrix. MMPs are known to regulate vascular morphogenesis in multiple ways. Besides their prominent role in ECM degradation to facilitate the migration and proliferation of endothelial cells, MMPs are required to activate, liberate, and modify proangiogenic, but also antiangiogenic, factors sequestered in the pericellular ECM. For example, MMPs have been implicated in the coordination of VEGF release from extracellular stores by matrix breakdown and directly through intramolecular processing. Enhanced MMP activity in the absence of TIMP3 might therefore trigger a cascade of events contributing to the vascular abnormalities seen in TIMP3-deficient eyes.

Vessel abnormalities have not been reported in other organs of mice with a targeted TIMP3 deficiency. Injection of melanoma cells into TIMP3-null mice, however, was shown to result in increased tumor growth and angiogenesis characterized by an enhanced formation of blood vessels with larger diameters than those in wild-type littermates. This may indicate that the impact of TIMP3 ablation on vascular morphogenesis is subtle and dependent on the cellular microenvironment. In addition, phenotypic variability in TIMP3-null mice with different genetic backgrounds was observed in this and a previous study. Such an effect is generally attributable to modifier genes that act in concert with the causative gene. Further studies are needed to evaluate the influence of TIMP3 on vessel growth and remodeling.
deficiency on vasculogenesis and angiogenesis in different mouse strains. Modulation of VEGF-induced angiogenesis has been demonstrated to be independent of the MMP-inhibitory properties of TIMP3. Consistent with this, we mapped the region of the TIMP3 protein responsible for its antiangiogenic activity to its C-terminal domain. Interestingly, all 10 TIMP3 mutations underlying SFD are located in or affect the C-terminal part of TIMP3. A next step will be to determine whether the antiangiogenic properties are retained in mutant TIMP3 proteins. This should contribute to unraveling the still unknown molecular mechanism of TIMP3-related pathology in SFD.

Acknowledgments

The authors thank Barbara Bathke for help with breeding and genotyping of the mouse lines.

References


