Increased Neovascularization in Mice Lacking Tissue Inhibitor of Metalloproteinases-3

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PURPOSE. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is a matrix-bound inhibitor of matrix metalloproteinases (MMPs). The authors have previously determined a novel function of TIMP-3 to inhibit vascular endothelial growth factor (VEGF)-mediated angiogenesis. Here, the authors examined the in vivo angiogenic phenotype of ocular vessels in mice deficient in TIMP-3.

METHODS. VEGF-mediated corneal neovascularization and laser-induced choroidal neovascularization (CNV) were examined in TIMP-3-null mice. The effects of the absence of TIMP-3 on the phosphorylation status of the VEGF-receptor-2 (VEGFR-2) and the downstream signaling pathways were evaluated biochemically. In addition, the activation state of MMPs in the retina of TIMP-3-deficient mice was examined by in situ zymography.

RESULTS. The results of these studies determine an accentuation of pathologic VEGF-mediated angiogenesis in the cornea and laser-induced CNV in mice lacking TIMP-3. In the absence of the MMP inhibitor, pathophysiological changes were observed in the choroidal vasculature concomitantly with an increase in gelatinolytic activity. These results suggest that an imbalance of extracellular matrix homeostasis, together with a loss of an angiogenesis inhibitor, can prime vascular beds to be more responsive to an angiogenic stimulus.

CONCLUSIONS. In light of the recent studies suggesting that genetic variants near TIMP-3 influence susceptibility to age-related macular degeneration, these results imply that TIMP-3 may regulate the development of the choroidal vasculature and is a likely contributor to increased susceptibility to choroidal neovascularization. (*Invest Ophthalmol Vis Sci.* 2011;52:6117–6123) DOI:10.1167/iovs.10-5899

Angiogenesis is a multistep physiological process involving the degradation of the capillary basement membrane, endothelial cell migration, capillary tube formation, and endothe-

lial cell proliferation. Sprouting of new blood vessels from preexisting capillaries requires precise spatial and temporal proteolysis of the extracellular matrix (ECM), which is critical for initiating the process of endothelial cell invasion into the ECM¹ and for recruiting endothelial progenitor cells to the angiogenic site. The matrix metalloproteinases (MMPs) are zinc-dependent enzymes that can degrade most components of the ECM² and that play a critical role in the process of neovascularization.^{3–7}

Tissue inhibitor of metalloproteinases (TIMPs) constitute a family of distinct MMP inhibitors whose members are TIMP-1, TIMP-2, TIMP-3, and TIMP-4. The TIMPs show 35% to 40% identity at the amino acid level and are capable of inhibiting the MMP family of enzymes with equal efficacy in in vitro assays.⁸ TIMP-1, TIMP-2, and TIMP-4 are “soluble” proteins and are present in numerous body fluids.⁹ TIMP-3 is distinct from these proteins in that it is sequestered in the ECM. Our previous work has shown that TIMP-3 is a potent angiogenesis inhibitor¹⁰ and suppresses vascular endothelial growth factor (VEGF)-mediated angiogenesis independently of its MMP inhibitory properties.¹¹ Its angiostatic function is mediated by inhibition of the binding of VEGF₁₆₅ to its receptor, VEGFR-2 (KDR).¹¹

VEGF is an essential growth factor that plays a critical role in the development of the retinal^{12–14} and choroidal vasculature.^{15,16} Previous studies have demonstrated choroidal vascular abnormalities in mice deficient in TIMP-3 along with enhanced capillary sprouting of aortic explants from these mice.¹⁷ VEGF has also been shown to contribute significantly to the development of choroidal neovascularization (CNV) in age-related macular degeneration (ARMD). Although no mutations were found in the coding region of TIMP-3 in a small study,¹⁸ a more recent large-scale genome-wide association scan for ARMD¹⁹ identified genetic variants near TIMP-3 that were associated with increased susceptibility to the disease. In this study, we explored the role of TIMP-3 in VEGF-mediated angiogenesis in vivo by comparing pathologic neovascularization in TIMP-3-deficient mice and corresponding wild-type (WT) controls.

MATERIALS AND METHODS

Animals

All animal studies were approved by the Animal Care and Use Committee guidelines of the Cleveland Clinic and conformed to the National Institutes of Health Guide for the Care and Use of Animals in Research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. *Timp-3*^{−/−} mice on a C57BL/129SV background have been previously described.^{20,21} The *Timp-3*^{−/−} mice were backcrossed seven times or more and then bred on a pure C57BL/6 background and on an FVB-BALB/c background separately.

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Labeling of Choroidal Vasculature

Albino *Timp-3*^{-/-} mice ($n = 6$) and WT littermates at 3 months of age were perfused by intracardiac injection of India ink under deep anesthesia. The eyes were enucleated and fixed with 4% paraformaldehyde for 16 hours. The extraocular tissue was removed, and the posterior view of whole eye was examined and photographed. After this the anterior segment (cornea, lens) and retina were carefully dissected and removed. Flat mounts of India ink-labeled choroid with the underlying sclera were made by advancing radial incisions centrally toward the optic nerve and were placed on glass slides for microscopy and imaging. Flat mount images of India ink-labeled choroids were quantitatively analyzed for avascular regions and vascular density retinal images using customized (fully automated) batch processing macros and algorithms generated in image processing software (Image-Pro Plus 6.1; Media Cybernetics, Silver Spring, MD). To determine the total choroid tissue area, each image was converted to grayscale, a spectral filter was applied to enhance or equalize the appearance of the vasculature, and a morphologic "closing" filter was used to fill in regions between vessels. For segmentation of the vascular area labeled with India ink, a high-pass spectral filter was applied to enhance the vasculature, followed by fixed-size and intensity thresholds. Vascular density was calculated as the sum of these thresholded vascular pixels divided by the total choroid area, and the avascular area was calculated by subtracting the total segmented vascular area from the total tissue area.

Scanning Laser Ophthalmoscopy

Scanning laser ophthalmoscopy (SLO) was performed as described previously.²² After anesthesia and papillary dilation, fluorescein and ICG angiograms were conducted using subcutaneous injections of Na-fluorescein (Fluorescein 10%; Alcon Laboratories, Fort Worth, TX; 75 mg/kg body weight) and ICG (IC-Green; Akron, Buffalo Grove, IL; 50 mg/kg body weight). The fundus was examined with a scanning laser ophthalmoscope (HRA II SLO; Heidelberg Engineering, Dossenheim, Germany) modified for use in mice.

In Situ Zymography

Fluorescein-quenched gelatin (DQ; Molecular Probes/Invitrogen, Carlsbad, CA) was added to unfixed 10- μ m mouse posterior pole sections at a concentration of 100 μ g/mL and incubated in the dark at 37°C for 1 to 4 hours. Parallel sections were overlaid with unconjugated gelatin as a negative control. The sections were mounted in medium (Vectashield; Vector Laboratories, Burlingame, CA) with DAPI and photographed using a fluorescence microscope (Olympus, Tokyo, Japan).

Gelatin Zymography

Protein extracts were prepared from choroid/sclera preparations of mouse eyes using homogenization in lysis buffer (125 mM Tris-HCl, pH 7.0, 100 mM NaCl, 0.1% Genapol [Sigma-Aldrich, St. Louis, MO]). Five micrograms of protein extract was subjected to gelatin zymography on a 7.5% SDS-PAGE gel containing 0.1% gelatin, without any reducing agent. After electrophoresis, gels were processed as described previously.¹¹ Briefly, gels were incubated in a solution of 25 mg/mL Triton X-100 to remove SDS and to promote the renaturation of proteases and inhibitors. After the removal of triton with water, the gels were incubated for 16 hours in 50 mM Tris-HCl (pH 7.5) containing 5 mM CaCl₂ and 0.2 mg/mL sodium azide at 37°C. The gels were stained with 5 mg/mL dye (Coomassie Blue R-250; Invitrogen) in acetic acid/methanol/water (1:3:6) for 2 hours and destained with acetic acid/methanol/water (1:3:6).

Mouse Corneal Micropocket Assay

Hydron pellets containing 25 ng recombinant human VEGF165 (kindly provided by Genentech, South San Francisco, CA), were inserted into corneal micropockets (1 mm from the limbus) of *Timp-3*^{-/-} and control WT littermates ($n = 5$). Corneas were examined with the aid

of a surgical microscope to monitor angiogenic responses to VEGF. To photograph the neovascular response, animals were perfused with India ink to label the vessels. After enucleation and fixation, the corneas were excised, flattened, and photographed. A positive neovascularization response was recorded only if sustained directional ingrowth of capillary sprouts and hairpin loops toward the pellet was observed. A negative response was recorded when either no growth was observed or only an occasional sprout or hairpin loop was detected. All responses were compared to a negative control (pellet containing buffer). Angiogenic response was analyzed for mean vascular extension and total skeletal (vascular) length using image processing software (Image-Pro Plus 6.1; Media Cybernetics). Before performing vessel measurements, images were processed using best-fit equalization filters, spectral filters, and large pixel-width background removal filters to enhance vasculature and eliminate image artifacts. For total skeletal length measurements, processed images were skeletonized, summing the pixel lengths of resultant skeletal segments. To determine mean vessel extension, processed images were thresholded for vasculature, filling in holes between adjacent vessels using morphologic filters. The resultant image, a single segmented object representing the overall dimensions of the vascular bed, was analyzed for maximum box height (i.e., extent of vessel penetration).

Laser-Induced CNV Assay

Laser photocoagulation-induced rupture of Bruch's membrane was used to generate CNV, as previously described.²³ Briefly, 4- to 5-week-old C57BL/6J mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight) and xylazine (10 mg/kg body weight) followed by 1% tropicamide (Alcon Laboratories, Inc., Fort Worth, TX) for pupillary dilation. Three burns of 532-nm diode laser photocoagulation (Oculight; Iridex, Mountain View, CA; 50- μ m spot size, 0.1-second duration, 200 mW) were delivered to each retina using a slit lamp delivery system and a hand-held coverslip as a contact lens. Burns were performed in the 9, 12, and 3 o'clock positions of the posterior pole of the retina. The production of a bubble at the time of lasering indicated a successful burn. Two weeks later, mice were anesthetized and perfused with fluorescein-labeled dextran (2×10^6 average molecular weight; Sigma-Aldrich), choroidal flatmounts were prepared, and the CNV area was measured. Five mice were used for each group with three burns in each eye ($n = 15$ –18 successful burns in each group). For quantitative analysis of lesion intensity and size, CNV images were batch processed using a custom macro generated in image processing software (Image-Pro Plus 5.1; Media Cybernetics). For each image, a region of interest (ROI) was traced around the lesion using a wand tool (a manual trace was performed if the lesion was not significantly brighter than the background). Mean intensity (range, 10–255 gray levels), perimeter, area, and mean diameter (pixels) were calculated for each ROI and exported to data analysis (Excel; Microsoft, Redmond, WA). Analyses were performed in a blinded fashion to eliminate user bias.

Immunoprecipitation and Immunoblotting

Cell fractions or immunoprecipitates of the lysates with the indicated antibodies were subjected to SDS-PAGE. Proteins were probed with antibody and detected with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Amersham Pharmacia Biotechnology, Piscataway, NJ) followed by enhanced chemiluminescence. The blots were restripped with solution (Western ReProbe; GBiosciences, Maryland Heights, MO) for 30 minutes and reprobed as indicated.

Staining of Choroid Mast Cells

Whole mount preparations of choroid and the attached underlying transparent sclera, prepared from albino *TIMP-3*^{-/-} and WT controls, were fixed in 10% formalin overnight. Choroid preparations were stained with 0.1% toluidine blue staining for 1 minute, followed by gentle washing with ddH₂O for 5 minutes and mounted with a glass coverslip. Choroid mast cells were identified based on the presence of

red- and purple-stained cytoplasmic granules. Mast cell density was evaluated by counting the cell profile centrally in the posterior choroid surrounding the optic nerve and in the peripheral choroid using 40 $\times$ magnification and a grid overlay.

Statistical Analysis

Data are presented as mean $\pm$ SEM. The statistical significance of differential findings observed between experimental and control groups was determined using one-way analysis of variance and considered significant if $P < 0.05$.

RESULTS

Increased VEGF-Mediated Neovascularization in the Corneas of TIMP-3-Deficient Mice

To determine whether TIMP-3-deficient mice had an accentuated angiogenesis response to VEGF, micropellets of the slow release polymer-hydrone containing recombinant VEGF (25 ng) were implanted into the corneas of *Timp-3*^{-/-} mice (Fig. 1d) and their WT littermates (Fig. 1b). The implanted corneas were analyzed for the induction of angiogenesis after India ink perfusion at day 7. Increased neovascularization was observed in TIMP-3-deficient mice that peaked at day 7 with a mean extension length of 43.1 ± 6.9 pixels compared with 19.5 ± 3.13 pixels in WT littermates (Fig. 1e). All responses were compared with pellets containing buffer as a negative control (Figs. 1a, 1c).

Absence of TIMP-3 Contributes to Increased CNV

To determine whether the absence of TIMP-3 contributes to the development of CNV, we examined the laser-induced CNV

FIGURE 1. Increased VEGF-induced corneal neovascularization in TIMP-3^{-/-} mice. Representative images of mouse corneas 7 days after implantation of micropellets containing (a) PBS (control) in WT mice, (b) VEGF (25 ng) in WT mice, (c) PBS (control) in TIMP3^{-/-} mice, and (d) VEGF (25 ng) in TIMP3^{-/-} mice. (e) Mean vessel extension was calculated. * $P = 0.01$.

FIGURE 2. Exacerbation of CNV in TIMP-3^{-/-} mice. Representative images of mouse choroids at 14 days after laser photocoagulation in WT mice (a-c) and TIMP-3^{-/-} mice (d-f). CNV area (g) and diameter (h) were calculated. ** $P \leq 0.01$.

lesions in TIMP-3-deficient mice and their corresponding WT littermate controls. Quantitative image analysis determined that the CNV lesions of TIMP-3-deficient mice were significantly larger in area and diameter (Figs. 2d-h) than those of WT controls (Figs. 2a-c, 2g, 2h). To determine whether the increase in CNV lesions in TIMP-3-deficient mice was a consequence of increased signaling with VEGF and VEGFR-2, we examined the posterior eyecups of mice subjected to laser-induced CNV and their corresponding nonlasered controls for expression of VEGFR-2 phosphorylation and for downstream ERK signals. In nonlasered mice, the RPE-choroid showed no evidence of a basal increase in VEGFR-2 autophosphorylation (Fig. 3a), VEGFR-2 levels (Fig. 3c), or ERK phosphorylation (Fig. 3e) in TIMP-3^{-/-} mice. Five days after the laser induction of CNV, significantly increased VEGFR-2 phosphorylation (Fig. 3b) and ERK phosphorylation (Fig. 3f) was observed in the TIMP-3-null mice compared with their WT littermates. No differences in VEGF levels were observed in the RPE/choroids of WT and TIMP-3^{-/-} mice 5 days after laser injury (data not shown). These results suggest that the absence of TIMP-3, a potent inhibitor of VEGF-mediated neovascularization, results in increased CNV.

TIMP-3-Deficient Mice Show Abnormalities in the Choroidal Vasculature

Recent studies have established an essential role for RPE-derived soluble VEGF in the development and maintenance of the

FIGURE 3. Increased VEGF-mediated signaling in *TIMP-3*^{-/-} with CNV. Phosphorylation of VEGFR-2 in the choroid/RPE was evaluated in WT and *TIMP-3*^{-/-} mice in (a) the absence of laser induction (no laser control) and (b) 5 days after laser induction of CNV. Lysates from choroid/RPE were separated by SDS-PAGE (10%) and analyzed by immunoprecipitation with an anti-VEGFR-2 antibody followed by immunoblotting with an anti-phosphotyrosine antibody, 4G.10. (c, d) VEGFR-2 protein was analyzed in the same blot after stripping and reprobing with anti-VEGFR-2 antibody. (e, f) Phosphorylation of ERK1 and ERK2 was detected by immunoblotting with phospho-specific ERK antibodies. (g, h) Total protein levels of ERK was determined by stripping the blot and immunoblotting with anti-ERK1/2 antibodies.

choriocapillaris.^{15,16} Overexpression of VEGF in photoreceptors or RPE of mice did not result in CNV but showed abnormalities in the choroidal vasculature.^{24–26} A previous study examining the choroidal vasculature in *TIMP-3*-deficient mice has reported the development of abnormal choroidal vessels in these animals.¹⁷ In our study, using fluorescein and ICG angiography in *Timp-3*^{-/-} mice and their WT littermates, we also observed changes in the choroidal vasculature in mice lacking *TIMP-3*. SLO with fluorescein angiography revealed fairly normal retinal vasculature in these mice (data not shown). ICG angiography, however, demonstrated the presence of hyperfluorescent spots and dilated choroidal vessels in all *TIMP-3*-null mice examined (Figs. 4a, 4b). To further evaluate these abnormalities, we injected India ink into the vasculature of albino mice and visualized the scleral (outer) surface (Figs. 4c, 4d) before preparing flat mounts to visualize the vitreal (inner) surface (Figs. 4e, 4f). Although no significant abnormalities were observed in the major vessels, posterior ciliary artery, long posterior ciliary artery, or long posterior ciliary vein, a generalized increase in the diameter and tortuosity of choroid vessels was seen in mice deficient in *TIMP-3*. The presence of irregularly dilated capillaries, some of which coalesced to form sinusoid-like structures (Figs. 4g, 4h), were probably responsible for the hyperfluorescent areas seen in *Timp-3*^{-/-} mice on SLO. Interestingly, most of the abnormalities in the large vessels and in the capillaries were limited to the posterior choroid, with sparing of the peripheral areas. Quantitative analysis measuring vascular density and avascular area confirms a significant increase in vascular density in the choroidal vasculature of *Timp-3*^{-/-} mice (Figs. 4i, 4j).

Increased Gelatinase Activity in the RPE/Choroid of *TIMP-3*-Deficient Mice

MMP2 and MMP9 have been shown to synergize in promoting CNV.²⁷ We performed gelatin zymography to determine whether gelatinases A and B were endogenously activated in the absence of *TIMP-3*. The RPE/choroid tissue of *Timp-3*^{-/-} mice showed increased levels of latent MMP-9 and

active MMP-2 (Fig. 5a). In situ zymographic analysis using DQ gelatin spatially localized the gelatinase activity to the choroid and RPE, with increased activity observed in the choroidal vasculature of *TIMP-3*-deficient mice (Figs. 5c, 5e). Incubation of sections with gelatin revealed no autofluorescence (Figs. 5b, 5d).

Accumulation and Altered Distribution of Mast Cells in the Choroid of *TIMP-3*-Deficient Mice

Mast cells have recently been shown to play an important role in vascular wall remodeling and have been implicated in the pathogenesis of abdominal aortic aneurysms.^{28–31} Given the abnormalities observed in the choroidal vasculature of *TIMP-3*-deficient mice, we analyzed the distribution and density of mast cells in choroid using 0.1% toluidine blue. Mast cells were identified by the presence of red and purple cytoplasmic granules. In WT mice, the mast cells were predominantly localized centrally around the optic nerve and radiated outward parallel to the blood vessels (Fig. 6a). We observed a significant increase in the total number of mast cells in the periphery of the choroid and a decrease in the central choroid in *Timp-3*^{-/-} mice compared with WT controls (Figs. 6b–d).

DISCUSSION

CNV is a significant complication in patients with ARMD and contributes to most of the blindness associated with this disease. The risk factors for the disease are both genetic and environmental,^{32–38} but the association of these genetic variants with disease progression is still unclear.³⁹ Although mutations in the *TIMP-3* gene were found to not be associated with ARMD,^{18,40} its association with progression to CNV has not been examined. Recent genomewide association scan analysis suggests *TIMP-3* loci to be associated more strongly with neovascular ARMD.¹⁹ Our results suggest that in the absence of *TIMP-3*, the vascular structure of the choroid is altered in a manner that renders it more susceptible to neovascularization

FIGURE 4. Abnormal choroidal vasculature in TIMP-3^{-/-} mice. Choroidal vasculature of 12-week-old adult (a) WT and (b) TIMP-3^{-/-} mice was visualized by ICG angiograms and SLO. Scleral view (c, d), vitreal aspect (e, f), and higher magnification (g, h) of India ink-labeled vasculature in 6- to 8-week-old albino WT (c, e, g) and TIMP-3^{-/-} (d, f, h) mice. Quantitation of vascular density (i) and avascular area (j). * $P < 0.01$; ** $P < 0.001$.

under the appropriate stimulus. This, then, leads to the question of whether the choroidal vasculatures of patients with CNV are primed in some manner for increased neovascularization by virtue of a genetic predisposition. Although VEGF inhibitors, ranibizumab, and bevacizumab have been successful in reducing vision loss from CNV, polymorphisms in the *VEGF* gene are not associated with progression to CNV.⁴¹ It would be interesting to speculate that alterations in angiogenesis inhibitors or matrix-degrading enzymes would be able to prime the choriocapillaris to be more sensitive to angiogenic insults that result in neovascularization. It could be hypothesized that increased matrix-degrading enzymes could lead to thinning of the vascular wall or the capillary basement membrane that could result in an increased sensitivity to angiogenic stimulators. Alternatively, sequence variations in the *TIMP-3* gene in association with other ARMD susceptibility loci or environmental insults could contribute to progression to CNV. In the case of the TIMP-3 knockout mouse, the absence of TIMP-3 (an angiogenesis inhibitor) and an increase in gelatinases in the choroid (a consequence of loss of TIMP-3, an inhibitor of matrix metalloproteinases) could likely contribute to the altered morphology of the choroidal vasculature and the increased susceptibility to CNV.

Mutations in TIMP-3 cause Sorsby fundus dystrophy (SFD),⁴² an autosomal dominant retinal dystrophy that closely resembles ARMD and is characterized by loss of central vision as a consequence of CNV. Whether the primary pathology in SFD is a consequence of gain or loss of function has not yet been determined. Langton et al.^{43,44} proposed the hypothesis that an accumulation of functional mutant TIMP-3 in the RPE/Bruch's membrane as a result of reduced turnover was responsible for the disease phenotype. If this hypothesis holds true, then the results obtained in this study with TIMP-3 deficiency may not have any direct implications for SFD. Interestingly, mice with the *Timp3*^{S156C} mutation knocked in do not show a classic SFD phenotype.⁴⁵

Hemovasculogenesis—the differentiation of hematopoietic, erythropoietic, and endothelial cells from a precursor hemangioblast—contributes to the development of early embryonic and fetal choriocapillaris.^{46,47} Fenestrations and pericyte investment occurs late during maturation, which occurs concomitantly with the connection to the intermediate and deep vessels of the choroid. VEGF signaling plays a critical role in both the development and the maintenance of the choriocapillaris.^{15,48,49} Although remodeling of the primary choroid vascular plexus during development appears to be regulated by

inductive signals from the RPE, the roles of ECM integrity and matrix-degrading enzymes in this process have not been identified. Whether the developmental changes in the choriocapillaris of TIMP-3-deficient mice are a consequence of increased MMP activity or loss of VEGF inhibition has yet to be determined. Aged *Timp-3*^{-/-} mice show increased alveolar enlargement associated with decreased collagen²⁰ and altered myocardial remodeling,⁵⁰ which suggests that TIMP-3 may be critical for branching morphogenesis.

The observation of increased mast cells in the choroids of TIMP-3-deficient mice is an interesting phenomenon given the previous suggestions of a possible role for mast cells in angiogenesis.⁵¹ More recently, there has been increasing evidence that mast cells may be critical in the “angiogenic switch” during tumor growth.⁵² TNF- α has been shown to play an important role in regulating mast cell function and tumor growth. TIMP-3 is the only TIMP that can inhibit the catalytic activity of tumor necrosis factor converting enzyme (TACE)⁵³ and prevent the release of ectodomains of transmembrane proteins. TIMP-3-deficient mice have attenuated tumor growth and metastasis.⁵⁴ In addition to secreting proangiogenic compounds, mast cells also release proteases that are likely to be involved in tissue remodeling. Recently, mast cell chymase was shown to play a critical role in the formation of the mouse abdominal aortic aneurysm^{29,30} through the activation of cysteine protease cathepsins, elastin degradation, and vascular remodeling. Similar pathways may be involved during the development of the choriocapillaris and the choroid in TIMP-3-null mice. Further studies to determine the exact role of mast cells in this process will involve studies with *Kit*^{W^{sh}/W^{sh}} mice, which are deficient in mast cells. Both the choriocapillaris and

FIGURE 5. Accentuated gelatinase activity in TIMP-3^{-/-} choroid/RPE. (a) Lysates prepared from the RPE/choroid of WT and TIMP-3^{-/-} eyes ($n = 3$) were analyzed by zymography and compared with recombinant active MMP-2 (lane 1) and MMP-9 (lane 2). (b–e) In situ zymography was performed using fresh frozen cryosections from WT (b, c) and TIMP-3^{-/-} (d, e) eyes of mice that had been injected with Evans blue dye. Gelatinolytic activity was visualized as green fluorescence that was present on the cleavage of DQ gelatin (c, e) and compared with background fluorescence obtained with gelatin alone (b, d).

FIGURE 6. Accumulation and altered distribution of mast cells in the choroid of TIMP-3^{-/-} mice. Representative photographs of whole-mount choroids, central area (a, c), or peripheral area (b, d) stained with 0.1% toluidine blue from WT mice (a, b) or TIMP-3^{-/-} mice. (e) The total number of mast cells was counted in the central and peripheral areas of the choroid.

the capillaries of the kidney glomerulus are similar in that they are large, fenestrated, and have a lobular pattern. Mast cell-mediated glomerular remodeling has been demonstrated in mice lacking mast cells. It will be interesting to determine whether there is an abnormal localization of mast cells and altered vascular remodeling in the glomerular vascular bed in TIMP-3-deficient mice.

In summary, though the loss of angiogenesis inhibitory function in this mouse model does not induce CNV, it is more susceptible to CNV after laser injury.

References

1. Pepper MS, Montesano R, Mandriota SJ, Orci L, Vassalli JD. Angiogenesis: a paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. *Enzyme Protein*. 1996; 49:138–162.
2. Matrisian LM. The matrix degrading metalloproteinases. *Bioessays*. 1992;14:455–463.
3. Lafleur MA, Handsley MM, Edwards DR. Metalloproteinases and their inhibitors in angiogenesis. *Expert Rev Mol Med*. 2003;5:1–39.
4. Werb Z, Vu TH, Rinkenberger JL, Coussens LM. Matrix-degrading proteases and angiogenesis during development and tumor formation. *APMIS*. 1999;107:11–18.
5. Hiraoka N, Allen E, Apel IJ, Gyetko MR, Weiss SJ. Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. *Cell*. 1998;95:365–377.
6. Stetler-Stevenson W, Corcoran M. Tumor angiogenesis: functional similarities with tumor invasion. *EXS*. 1997;79:413–418.

7. Stetler-Stevenson WG. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest.* 1999; 103:1237-1241.
8. Apte SS, Olsen B, Murphy G. The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. *J Biol Chem.* 1995;270:14313-14318.
9. Murphy G. The regulation of connective tissue metalloproteinases by natural inhibitors. In: *AAS 35: Progress in Inflammation Research and Therapy*. Basel: Birkhauser; 1991:69-76.
10. Anand-Apte B, Pepper MS, Voest E, et al. Inhibition of angiogenesis by tissue inhibitor of metalloproteinase-3. *Invest Ophthalmol Vis Sci.* 1997;38:817-823.
11. Qi JH, Ebrahim Q, Moore N, et al. A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. *Nat Med.* 2003;9:407-415.
12. Haigh JJ. Role of VEGF in organogenesis. *Organogenesis.* 2008;4: 247-256.
13. Haigh JJ, Morelli PI, Gerhardt H, et al. Cortical and retinal defects caused by dosage-dependent reductions in VEGF-A paracrine signaling. *Dev Biol.* 2003;262:225-241.
14. Stalmans I, Ng YS, Rohan R, et al. Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. *J Clin Invest.* 2002;109:327-336.
15. Saint-Geniez M, Kurihara T, Sekiyama E, Maldonado AE, D'Amore PA. An essential role for RPE-derived soluble VEGF in the maintenance of the choriocapillaris. *Proc Natl Acad Sci U S A.* 2009;106: 18751-18756.
16. Marneros AG, Fan J, Yokoyama Y, et al. Vascular endothelial growth factor expression in the retinal pigment epithelium is essential for choriocapillaris development and visual function. *Am J Pathol.* 2005;167:1451-1459.
17. Janssen A, Hoellenriegel J, Fogarasi M, et al. Abnormal vessel formation in the choroid of mice lacking tissue inhibitor of metalloproteinase-3. *Invest Ophthalmol Vis Sci.* 2008;49:2812-2822.
18. Stone EM, Sheffield VC, Hageman GS. Molecular genetics of age-related macular degeneration. *Hum Mol Genet.* 2001;10:2285-2292.
19. Chen W, Stambolian D, Edwards AO, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2010;107:7401-7406.
20. Leco KJ, Waterhouse P, Sanchez OH, et al. Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). *J Clin Invest.* 2001;108:817-829.
21. Mohammed FF, Smookler DS, Taylor SE, et al. Abnormal TNF activity in *Timp3*^{-/-} mice leads to chronic hepatic inflammation and failure of liver regeneration. *Nat Genet.* 2004;36:969-977.
22. Seeliger MW, Beck SC, Pereyra-Munoz N, et al. In vivo confocal imaging of the retina in animal models using scanning laser ophthalmoscopy. *Vision Res.* 2005;45:3512-3519.
23. Tobe T, Ortega S, Luna JD, et al. Targeted disruption of the *FGF2* gene does not prevent choroidal neovascularization in a murine model. *Am J Pathol.* 1998;153:1641-1646.
24. Oshima Y, Oshima S, Nambu H, et al. Increased expression of VEGF in retinal pigmented epithelial cells is not sufficient to cause choroidal neovascularization. *J Cell Physiol.* 2004;201:393-400.
25. Schwesinger C, Yee C, Rohan RM, et al. Intrachoroidal neovascularization in transgenic mice overexpressing vascular endothelial growth factor in the retinal pigment epithelium. *Am J Pathol.* 2001;158:1161-1172.
26. Tee LB, Penrose MA, O'Shea JE, Lai CM, Rakoczy EP, Dunlop SA. VEGF-induced choroidal damage in a murine model of retinal neovascularisation. *Br J Ophthalmol.* 2008;92:832-838.
27. Lambert V, Wielockx B, Munaut C, et al. MMP-2 and MMP-9 synergize in promoting choroidal neovascularization. *FASEB J.* 2003;17:2290-2292.
28. Mayranpaa MI, Trosien JA, Fontaine V, et al. Mast cells associate with neovessels in the media and adventitia of abdominal aortic aneurysms. *J Vasc Surg.* 2009;50:388-395, discussion 395-386.
29. Sun J, Sukhova GK, Yang M, et al. Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice. *J Clin Invest.* 2007;117:3359-3368.
30. Sun J, Zhang J, Lindholt JS, et al. Critical role of mast cell chymase in mouse abdominal aortic aneurysm formation. *Circulation.* 2009;120:973-982.
31. Tsuruda T, Kato J, Hatakeyama K, et al. Adventitial mast cells contribute to pathogenesis in the progression of abdominal aortic aneurysm. *Circ Res.* 2008;102:1368-1377.
32. de Jong PT. Age-related macular degeneration. *N Engl J Med.* 2006;355:1474-1485.
33. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science.* 2005;308:421-424.
34. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2005;102:7227-7232.
35. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science.* 2005;308:419-421.
36. Jager RD, Mieler WF, Miller JW. Age-related macular degeneration. *N Engl J Med.* 2008;358:2606-2617.
37. Klein R, Peto T, Bird A, Vannewkirk MR. The epidemiology of age-related macular degeneration. *Am J Ophthalmol.* 2004;137: 486-495.
38. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science.* 2005;308: 385-389.
39. Scholl HP, Fleckenstein M, Fritsche LG, et al. CFH, C3 and ARMS2 are significant risk loci for susceptibility but not for disease progression of geographic atrophy due to AMD. *PLoS ONE.* 2009;4:e7418.
40. De La Paz MA, Pericak-Vance MA, Lennon F, Haines JL, Seddon JM. Exclusion of TIMP3 as a candidate locus in age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 1997;38:1060-1065.
41. Boekhoorn SS, Isaacs A, Uitterlinden AG, et al. Polymorphisms in the vascular endothelial growth factor gene and risk of age-related macular degeneration: the Rotterdam Study. *Ophthalmology.* 2008;115:1899-1903.
42. Weber BH, Vogt G, Pruett RC, Stohr H, Felbor U. Mutations in the tissue inhibitor of metalloproteinase-3 (TIMP-3) in patients with Sorsby's fundus dystrophy. *Nat Genet.* 1994;8:352-356.
43. Langton KP, McKie N, Curtis A, et al. A novel tissue inhibitor of metalloproteinases-3 mutation reveals a common molecular phenotype in Sorsby's fundus dystrophy. *J Biol Chem.* 2000;275: 27027-27031.
44. Langton KP, McKie N, Smith BM, Brown NJ, Barker MD. Sorsby's fundus dystrophy mutations impair turnover of TIMP-3 by retinal pigment epithelial cells. *Hum Mol Genet.* 2005;14:3579-3586.
45. Weber BH, Lin B, White K, et al. A mouse model for Sorsby fundus dystrophy. *Invest Ophthalmol Vis Sci.* 2002;43:2732-2740.
46. Baba T, Grebe R, Hasegawa T, et al. Maturation of the fetal human choriocapillaris. *Invest Ophthalmol Vis Sci.* 2009;50:3503-3511.
47. Hasegawa T, McLeod DS, Bhutto IA, et al. The embryonic human choriocapillaris develops by hemo-vasculogenesis. *Dev Dyn.* 2007; 236:2089-2100.
48. Saint-Geniez M, D'Amore PA. Development and pathology of the hyaloid, choroidal and retinal vasculature. *Int J Dev Biol.* 2004;48: 1045-1058.
49. Saint-Geniez M, Maldonado AE, D'Amore PA. VEGF expression and receptor activation in the choroid during development and in the adult. *Invest Ophthalmol Vis Sci.* 2006;47:3135-3142.
50. Kassiri Z, Defamie V, Hariri M, et al. Simultaneous transforming growth factor beta-tumor necrosis factor activation and cross-talk cause aberrant remodeling response and myocardial fibrosis in *Timp3*-deficient heart. *J Biol Chem.* 2009;284:29893-29904.
51. Norrby K. Mast cells and angiogenesis. *APMIS.* 2002;110:355-371.
52. Coussens LM, Raymond WW, Bergers G, et al. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev.* 1999;13:1382-1397.
53. Amour A, Slocumbe P, Webster A, et al. TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett.* 1998;435:39-44.
54. Cruz-Munoz W, Kim I, Khokha R. TIMP-3 deficiency in the host, but not in the tumor, enhances tumor growth and angiogenesis. *Oncogene.* 2006;25:650-655.