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TIMP-1 promotes VEGF-induced neovascularization in the retina

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Summary. Proteolysis of vascular basement membranes and surrounding extracellular matrix is a critical early step in neovascularization. It requires alteration of the balance between matrix metalloproteinases (MMPs) and proteins that bind to and inactivate MMPs, tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 has been demonstrated to inhibit neovascularization in chick chorioallantoic membranes. However, TIMP-1 has also been shown to either promote or inhibit cell proliferation and migration in different settings. To determine whether genetic alteration of the MMP/TIMP-1 ratio would alter retinal neovascularization, we crossed mice that express vascular endothelial growth factor (VEGF) in photoreceptors with TIMP-1-deficient mice or mice that overexpress TIMP-1. Compared to *VEGF* transgene-positive/TIMP-1-sufficient mice, *VEGF* transgene-positive/TIMP-1-deficient mice showed smaller neovascular lesions. There was also no difference between the two groups of mice in the appearance of the neovascularization by light or electron microscopy. Compound *VEGF*/TIMP-1 transgenic mice had increased expression of both *VEGF* and TIMP-1 in the retina, and had more neovascularization than mice that had increased expression of VEGF alone. These gain- and loss-of-function data suggest that alteration of the TIMP-1/MMP ratio modulates retinal neovascularization in a complex manner and not simply by altering the proteolytic activity and thereby invasiveness of endothelial cells.

Key words: TIMP-1, *VEGF*, retinal neovascularization, proteinases, proliferative retinopathies

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Introduction

Neovascularization (NV) occurs by the formation of endothelial cell sprouts from pre-existent blood vessels. This process involves degradation of vascular basement membranes and surrounding tissue (Ausprunk and Folkman, 1977; Kalebic et al., 1983). Angiogenic factors cause endothelial cells to increase their expression of one or more matrix metalloproteinases (MMPs), a family of homologous enzymes that digest a wide variety of extracellular matrix (ECM) components including collagens, fibronectin, laminin, and various proteoglycans (Moscatelli et al., 1980, 1985; Gross et al., 1983; Mignatti et al., 1989). Each of the MMPs is secreted in zymogen form and requires proteolytic cleavage to become active (Woessner, 1991). Their activity is also modulated by a family of inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Four members of this family have been cloned, TIMPs-1, -2, -3, and -4 (Docherty et al., 1985; Carmichael et al., 1986; Stetler-Stevenson et al., 1989; Pavloff et al., 1992; Greene et al., 1996; Leco et al., 1997). α_2-Macroglobulin, which has broad inhibitory activity, also inhibits MMPs (Kan et al., 1985).

There is mounting evidence suggesting that invasive cell behavior, including metastasis and NV, is controlled in part by the local balance between MMPs and their inhibitors. Tumor necrosis factor-α, an angiogenesis factor, increases expression of collagenase and decreases expression of TIMP-1 in human dermal microvascular endothelial cells (Cornelius et al., 1995). Cartilage, an avascular tissue that resists vascular invasion, has high levels of TIMPs which inhibit NV *in vivo* (Moses et al., 1990; Johnson et al., 1994). TIMP-1 is also present in substantial levels in the vitreous, an avascular tissue in the eye (Bunning et al., 1984). Therefore, TIMP-1 may help to protect tissues from invasion by endothelial cells and the level of protection may be altered by changes in TIMP-1 expression. This possibility is supported by recent studies demonstrating that expression of a transfected TIMP-1 gene in melanoma cells lowers their

tumorigenic and metastatic potentials (Khokha, 1994) and targeted disruption of the *Timp-1* gene causes enhanced invasion of normal differentiated cells and increased metastatic potential of transformed cells (Alexander and Werb, 1992). But, it is not the case that an increase in the TIMP/MMP ratio always increases invasive cell behavior such as angiogenesis, or that a decrease in the ratio always decreases it. An excess of proteolysis or inhibitors may have opposite or similar effects, depending upon whether critical amounts of ECM components required for attachment, survival, and proliferation are present (Yoshiji et al., 1998; Zucker et al., 1998). Some tumor cells grow and metastasize less well in the absence of *TIMP-1* (Soloway et al., 1996), suggesting that *TIMP-1* may act as a growth and survival factor.

The vascular supply of the retina is highly specialized due to requirements related to visual function. Retinal blood vessels supply the inner retina via a superficial capillary bed in the nerve fiber layer and deep capillary beds in the inner nuclear and outer plexiform layers. Normally there are no blood vessels in the outer retina; photoreceptors are maintained by diffusion from choroidal vessels. We have previously demonstrated that expression of *VEGF* in photoreceptors of transgenic mice results in vascular invasion of the outer retina (Okamoto et al., 1997; Tobe et al., 1998). In these mice, endothelial cells migrate from the deep capillary bed of the retina, through the outer nuclear layer into the subretinal space where they form new blood vessels that gradually enlarge. No NV originating from the choroidal vessels has been identified. It is possible that like other avascular tissues, TIMP-1 may regulate the extent of NV originating from retinal vessels and vascular invasion from the choroid. In this study, we used mice with targeted disruption of the Timp-1 gene and transgenic mice that overexpress *Timp-1* to investigate whether *Timp-1* contributes to a critical balance that limits the progression and extent of NV in the outer retina and subretinal space.

Materials and methods

Mice with targeted deletion of Timp-1

Timp-1 null mice were generated by homologous recombination and identified by Southern blotting as previously described (Soloway et al., 1996). Since *Timp-1* is located on the X chromosome, all male offspring of female *Timp-1*-null mice and wild type males are null.

Model of intraretinal and subretinal NV

The production and screening of transgenic mice with overexpression of *VEGF* in photoreceptors (*rho-VEGF* transgenics) has been previously described (Okamoto et al., 1997). Mice from the V-6 line heterozygous for the rhodopsin/*VEGF* transgene develop NV that originates from the deep capillary bed of the retina and grows into the subretinal space where it gradually spreads and enlarges. Heterozygous transgenic males were mated with female *Timp-1* -/- or *Timp-1*+/+ mice of the same genetic background. At six weeks of age, male rho-*VEGF*/*Timp-1* -/0 and rho-*VEGF*/*Timp-1*+/0 (controls) were perfused with fluorescein-labeled dextran and their eyes were used to make retinal flat-mounts or the mice were sacrificed without perfusion and their eyes were used for light and electron microscopy.

Transgenic mice that overexpress Timp-1

Heterozygous transgenic mice overexpressing the human TIMP-1 gene under the control of the ubiquitous ß-actin promoter (ß-actin-TIMP-1 mice) (Alexander et al., 1996) were mated with *rho-VEGF* transgenic mice. At 3 weeks of age, the offspring were perfused with fluorescein-labeled dextran and retinal whole mounts were prepared. The mice were genotyped by PCR and Southern blotting of tail DNA.

Northern Blot Analysis

RNA blot hybridization analysis was done as previously described (Campochiaro et al., 1994) using 10 μg of total retinal RNA. The cDNA probe was the 598 bp *Bam*HI fragment of h*VEGF* labeled with ^{32}P by hexanucleotide random priming (Okamoto et al., 1997). Hybridization temperature was 42 °C. The membrane was washed twice for 30 minutes at 60 °C in 1 x SSC, 0.2% SDS, followed by two washes of 30 minutes each at 65 °C in 0.2 x SSC, 0.2% SDS. Washed blots were exposed to XRP film (Kodak, Rochester, NY) and multiple exposure times were used to obtain exposures within the linear range for autoradiographic signals. Blots were stripped and rehybridized with a 0.8 kb cDNA fragment coding for most of exon 2 and all of exon 3 of the human FGF2 gene (Abraham et al., 1986). After exposure, the blots were stripped and hybridized with a probe for 18S ribosomal RNA to control for potential differences in RNA loading.

Retinal flat mounts

Retinal flat mounts were prepared by modification of a previously described technique (Smith et al., 1994; Tobe et al., 1998). Mice were anesthetized, the descending aorta was clamped, the right atrium was cut, and they were perfused through the left ventricle with 1 ml of phosphate-buffered saline containing 50 mg/ml of fluorescein-labeled dextran ($2x10^6$ average mw, Sigma, St. Louis, MO). The eyes were removed and fixed for 1 hour in 10% buffered formalin phosphate. The cornea and lens were removed and then the entire retina was carefully dissected from the eyecup, radially cut from the edge of the retina to the equator in all 4 quadrants, and flat-mounted in Aquamount with photoreceptors facing

upward. Flat mounts were examined by fluorescence microscopy, photographed with T-64 film (Kodak, Rochester, NY), and slides were scanned with a QuickScan 35 scanner (Minolta, Osaka, Japan). Image files were imported into Adobe Photoshop 4.0, labeled, and printed with a Fujix pictography 3000 printer (Fuji Photo Film Co., Tokyo, Japan).

Quantitation of retinal NV

Retinal flat mounts were examined by fluorescence microscopy at 400x magnification, which provides a narrow depth of field so that when focusing on NV on the outer edge of the retina the remainder of the retinal vessels are out-of-focus allowing easy delineation of the NV. The outer edge of the retina, which corresponds to the subretinal space *in vivo*, is easily identified and therefore there is standardization of focal plane from slide to slide. Images were digitized using a 3 CCD color video camera (IK-TU40A, Toshiba, Tokyo, Japan) and a frame grabber. Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) was used to delineate each of the lesions and calculate the number in each retina, the area of each lesion, and the total area of neovascularization per retina. Measurements were repeated three times for each retina and the mean was used for one experimental value; there was little variability among triplicate measurements.

Light and electron microscopy

Eyes were fixed in 4% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 24 hours at 4 °C. The anterior segments were removed and the posterior eyecups were divided into 4 parts. Segments of tissue were postfixed with 1% osmium tetroxide/cacodylate buffer (pH 7.4), dehydrated through a series of graded alcohols, and embedded in Poly/Bed 812 resin (Polysciences, Warrington, PA). One micron serial sections were cut, stained with Toluidine Blue, and examined with an Axioscope microscope (Zeiss, Thornwood, NY). Sections were photographed, scanned, labeled, and printed as described above. For electron microscopy, ultrathin sections were cut, counterstained with 2.0% uranyl acetate and 0.3% lead citrate, and examined with a transmission electron microscope (JEOL 100CX).

Table 1. Quantitation of neovascularization in the photoreceptor layer in *VEGF+/Timp-1*-wild type and *VEGF+/Timp-1*-null mice 6 weeks after birth.

Timp-1	N	LESIONS/ RETINA	AREA/LESIONS (mm^2x10^{-3})	NV AREA/RETINA (mm^2x10^{-3})
Wild type	20	25.15±1.96	6.05±0.52	147.47±16.29
Null	14	28.07±2.94$^+$	4.20±0.23*	116.19±11.41$^+$

*: p=0.0034 for difference from wild type by a t-test for populations with unequal variances; $^+$: not significant for difference from wild type by a t-test for populations with equal variances.

Results

TIMP-1-deficient mice have normal-appearing retinas

Evaluation of sections from the eyes of TIMP-1-deficient mice through 6 weeks of age by light microscopy showed normal-appearing retinas with no identifiable retinal phenotype (not shown).

Rho-VEGF/TIMP-1-deficient mice have no difference in total amount of neovascularization, but have smaller neovascular lesions than those in rho-VEGF/TIMP-1-sufficient mice

As noted previously in mice with a C57BL/6J genetic background (Okamoto et al., 1997; Tobe et al., 1998) F1(129Sv x C57BL/6J) mice, that were TIMP-1-sufficient and inherited a *rhodopsin/VEGF* transgene, developed NV originating from the deep capillary bed of the retina that extended through the photoreceptors into the subretinal space (Fig. 1A,B). TIMP-1-deficient mice that inherited a rhodopsin/*VEGF* transgene, also showed NV extending from retinal vessels into the subretinal space (Fig. 1C,D). There were no identifiable qualitative morphological differences by light microscopy in the NV in TIMP-1-deficient compared to TIMP-1-sufficient mice and neither showed evidence of NV originating from the choroid.

The amount of NV in *rho-VEGF*/TIMP-1-deficient and *rho-VEGF*/TIMP-1-sufficient mice was measured on retinal flat mounts after perfusion with fluorescein-labeled dextran, a technique that has previously been demonstrated to provide good quantitation of NV (Tobe et al., 1998). Figure 2A shows the entire retina from a *rho-VEGF*/TIMP-1-sufficient mouse. At this low magnification it is difficult to resolve the NV, but the pigmented cells (arrows) associated with NV can be seen. At higher magnification, the new vessels that are surrounded by pigmented cells are easily seen (Fig. 2B, arrows). The amount of pigment seen at low magnification (Fig. 2C, arrows), and the new vessels seen at higher magnification (Fig. 2D, arrows) are comparable in *rho-VEGF*/TIMP-1-deficient mice. Quantitation of the NV by image analysis showed that rho-*VEGF*/TIMP-1-deficient mice had smaller neovascular lesions than *rho-VEGF*/TIMP-1-sufficient mice and there was no significant difference in the number of NV lesions or the total area of NV per retina (Table 1).

There is no identifiable difference in the ultrastructure of NV in rho-VEGF/TIMP-1-deficient mice compared to rho-VEGF/TIMP-1-sufficient mice

Electron microscopy of the subretinal NV in *rho-VEGF*/TIMP-1-sufficient mice showed that many of the

new vessels were completely surrounded by retinal pigmented epithelial (RPE) cells in the plane of the section, and as previously noted (Tobe et al., 1998), most of these vessels had fairly thick endothelial cells with no fenestrations (Fig. 3A). A few of the vessels surrounded by RPE cells in the plane of the section, and all vessels not surrounded by RPE cells, had thin endothelial cells with fenestrations (Fig. 3B). New vessels with each type of morphology were also seen in *rho-VEGF*/TIMP-1-deficient mice (Fig. 4).

Rho-VEGF/ß-actin-TIMP-1 mice have more NV than rho-VEGF mice with the same genetic background

While the investigations comparing the amount of NV in 6 week old *rho-VEGF*/TIMP-1-deficient and *rho-VEGF*/TIMP-1-sufficient mice (Table 1) were in progress, we completed time course experiments and found that 3 weeks of age provides an even better time point for assessment of modulation of NV in *rho-VEGF* mice (Tobe et al., 1998). Therefore, we investigated the effect of increased expression of TIMP-1 on *VEGF*-induced NV at the 3 week time point. Retinal whole mounts of 3 week old *rho-VEGF*/ß-actin-TIMP-1 mice showed extensive intraretinal and subretinal NV (Fig. 5A,B) that was greater than that seen in littermates that inherited the *VEGF* transgene, but not the TIMP-1 transgene (Fig. 5C,D). Quantitation of the NV by image analysis demonstrated significantly more neovascular lesions per retina and greater total area of NV per retina in *rho-VEGF*/ß-actin-TIMP-1 mice (Fig. 6). Mice that carried only the TIMP-1 transgene did not have any NV. Northern blots showed only small, inconsistent differences in *VEGF* or FGF2 mRNA levels in the retinas of *rho-VEGF* mice that over-expressed TIMP-1 compared to the levels in mice with normal TIMP-1 levels (Fig. 7). These small differences in mRNA levels do not provide an explanation for the difference in NV.

Discussion

VEGF is a stimulator of NV in the retina. Transgenic

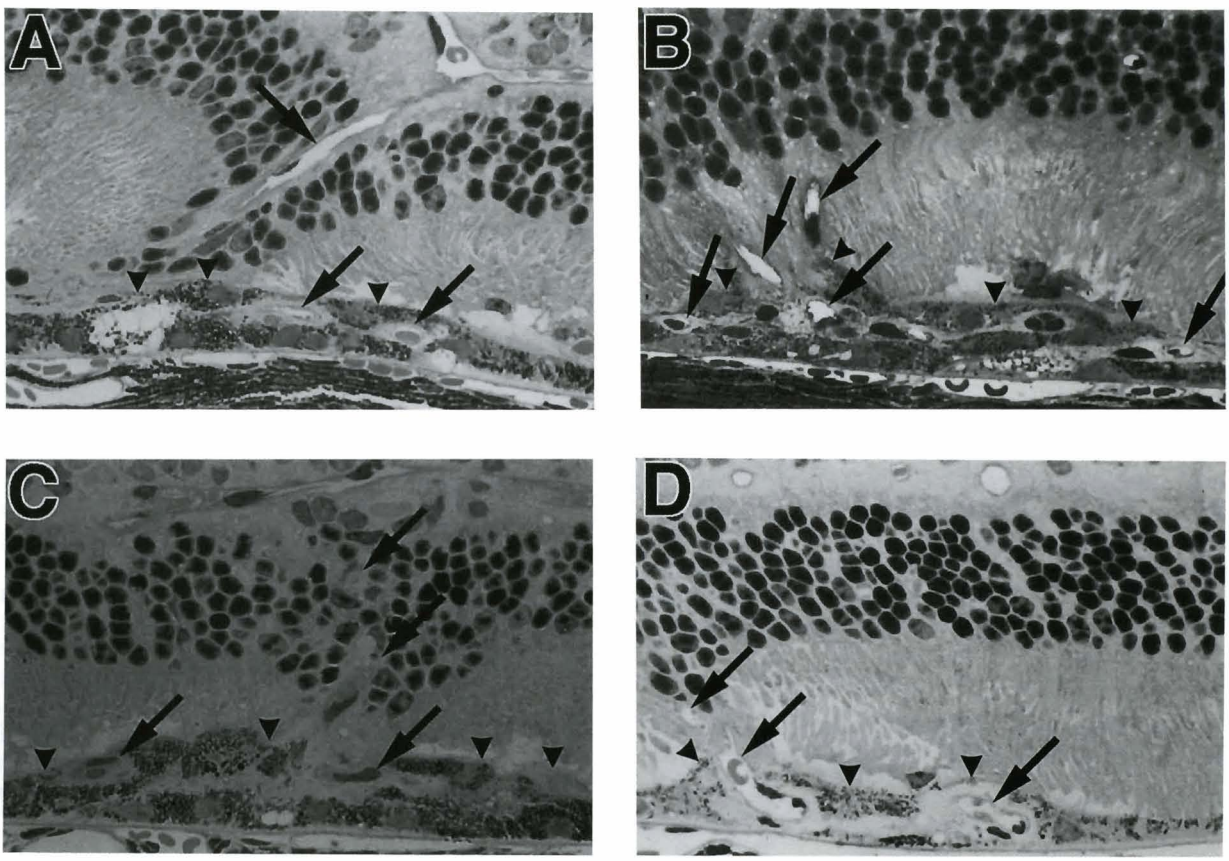

Fig. 1. Intraretinal and subretinal neovascularization in *Timp-1*-wild type (A and B) and *Timp-1*-deficient (C and D) mice that overexpress *VEGF* in photoreceptors. **A.** 6 week-old *VEGF+/Timp-1*-wild type mouse has a large blood vessel extending from the inner nuclear layer, through the outer nuclear layer (uppermost arrow) to a plexus of blood vessels in the subretinal space (lower arrows). The blood vessels are partially surrounded by RPE cells (arrowheads). **B.** Another 6 week-old *VEGF+/Timp-1*-wild type mouse shows similar findings to those seen in (A). **C.** A 6 week-old *VEGF+/Timp-1*-deficient mouse shows a network of blood vessels (arrows) in the subretinal space surrounded by RPE cells (arrowheads) with a feeder vessel from the inner retina seen in the outer nuclear layer (upper arrows). Another 6 week-old *VEGF+/Timp-1*-deficient mouse shows findings similar to those seen in A-C. x 400

mice with ectopic expression of *VEGF* in photoreceptors develop sprouts of NV from retinal vessels in the deep capillary bed of the retina extending through the outer nuclear layer into the subretinal space (Okamoto et al., 1997; Tobe et al., 1998). The NV becomes surrounded by RPE cells and gradually spreads and coalesces. Although choroidal vessels are located closer to photoreceptors, where *VEGF* is produced, no choroidal NV occurs. These observations raise the question, does something in the outer retina and/or something produced by the RPE, protect against and limit vascular invasion of the outer retina? TIMP-1, along with other MMP inhibitors, has been postulated to play such a role (Bunning et al., 1984) and it is produced by cultured and in situ RPE cells (Alexander et al., 1990; Padgett et al., 1997). To investigate the hypothesis that TIMP-1 acts as an inhibitor of NV in the outer retina, we crossed *rho-VEGF* transgenic mice with TIMP-1-deficient or TIMP-1 overexpressing mice.

VEGF transgene-positive mice deficient in TIMP-1 showed significantly smaller neovascular lesions compared to *VEGF* transgene-positive, TIMP-1-sufficient mice with the same genetic background. This difference suggests that TIMP-1 may not limit NV in the outer retina, and raises the question of whether loss of TIMP-1 antagonizes the development of NV in the retina. If this is the case, it might be expected that increased expression of TIMP-1 would promote the development of NV. Evaluation of *rho-VEGF/ß-actin-TIMP-1* mice suggests that this is the case, because they showed significantly greater total area of NV per retina than littermates that inherited the *VEGF* transgene, but not the TIMP-1 transgene. Taken together with the data in TIMP-1-deficient mice, these data suggest that in the presence of excess *VEGF*, TIMP-1 functions as a positive regulator of NV in the retina.

While proteolytic activity is needed for breakdown of the basement membrane to allow endothelial cell

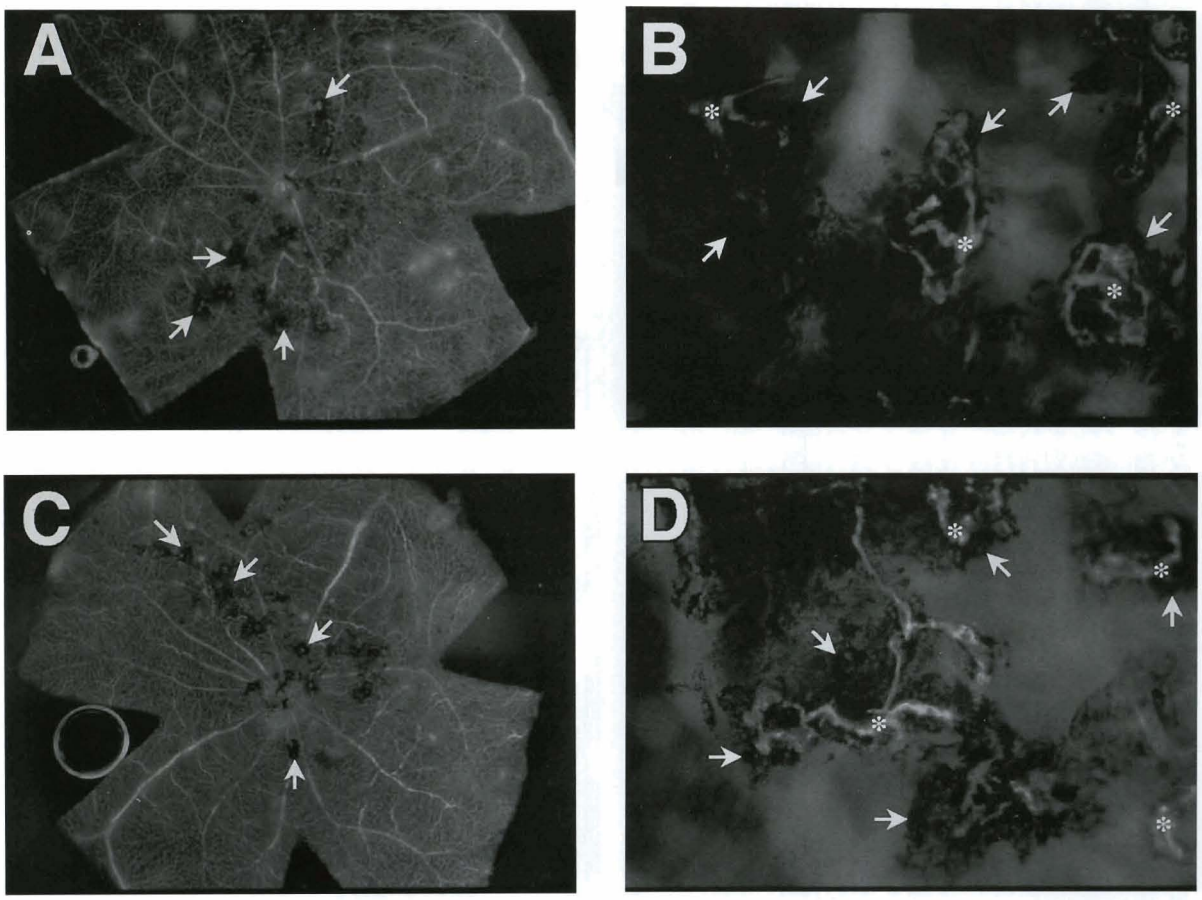

Fig. 2. Retinal whole-mounts in mice perfused with fluorescein-labeled dextran. **A.** A low magnification (25x) view of the entire retina in a *VEGF* transgene-positive mouse with a wild type *Timp-1* allele. It is difficult to resolve the neovascularization, but the pigmented cells (arrows) associated with neovascularization give a rough idea of the total amount of neovascularization. **B.** Higher magnification (1000x) of the retina seen in (A), the neovascularization (some, but not all of which is marked by asterisks) is easily distinguished. Note the adjacent retinal pigmented epithelial cells (arrows). **C.** At low magnification (25x) of a retina from a *VEGF* transgene-positive/*Timp-1*-deficient mouse, there are pigmented cells (arrows) roughly comparable to those seen in (A). **D.** Higher magnification (1000x) of the retina seen in (C), shows the neovascularization (asterisks) and adjacent retinal pigmented epithelial cells (arrows).

sprouting and growth of new vessels through avascular tissue, alteration of the MMP/TIMP-1 ratio could affect the process of NV in other ways. The ECM provides signals to endothelial cells that can either potentiate the effects of angiogenic stimuli or promote differentiation and decrease responsiveness to angiogenic stimuli (Schwartz and Ingber, 1994; Ingber et al., 1995; Dike and Ingber, 1996). It is likely that a decrease in the MMP/TIMP-1 ratio results in decreased ECM turnover and alteration of its content, which could perturb the differentiation-promoting effects of the ECM and make endothelial cells more prone to form sprouts in the presence of *VEGF*. Also, TIMP-1 can regulate *VEGF* production and stability (Yoshiji et al., 1998). Another possibility is that NV is kept in check by an endogenous inhibitor(s), such as transforming growth factor-ß (TGF-ß, that requires activation by MMPs. Pericytes that contact endothelial cells in cocultures inhibit endothelial cell growth by secretion of TGF-ß that is activated by proteinases at the cell surface (Antonelli-Orlidge et al., 1989; Sato et al., 1990). Rats with experimental autoimmune uveoretinitis (EAU) express high levels of *VEGF* and TGF-ß in the retina and do not develop retinal NV, while rats with ischemic retinopathy express high levels of *VEGF* and low levels of TGF-ß and develop retinal NV (Vinores et al., 1998). Therefore, TGF-ß may be an endogenous negative regulator of retinal angiogenesis and since MMPs participate in its activation, a decrease in the MMP/TIMP-1 ratio might result in a decrease in active TGF-ß and thereby greater

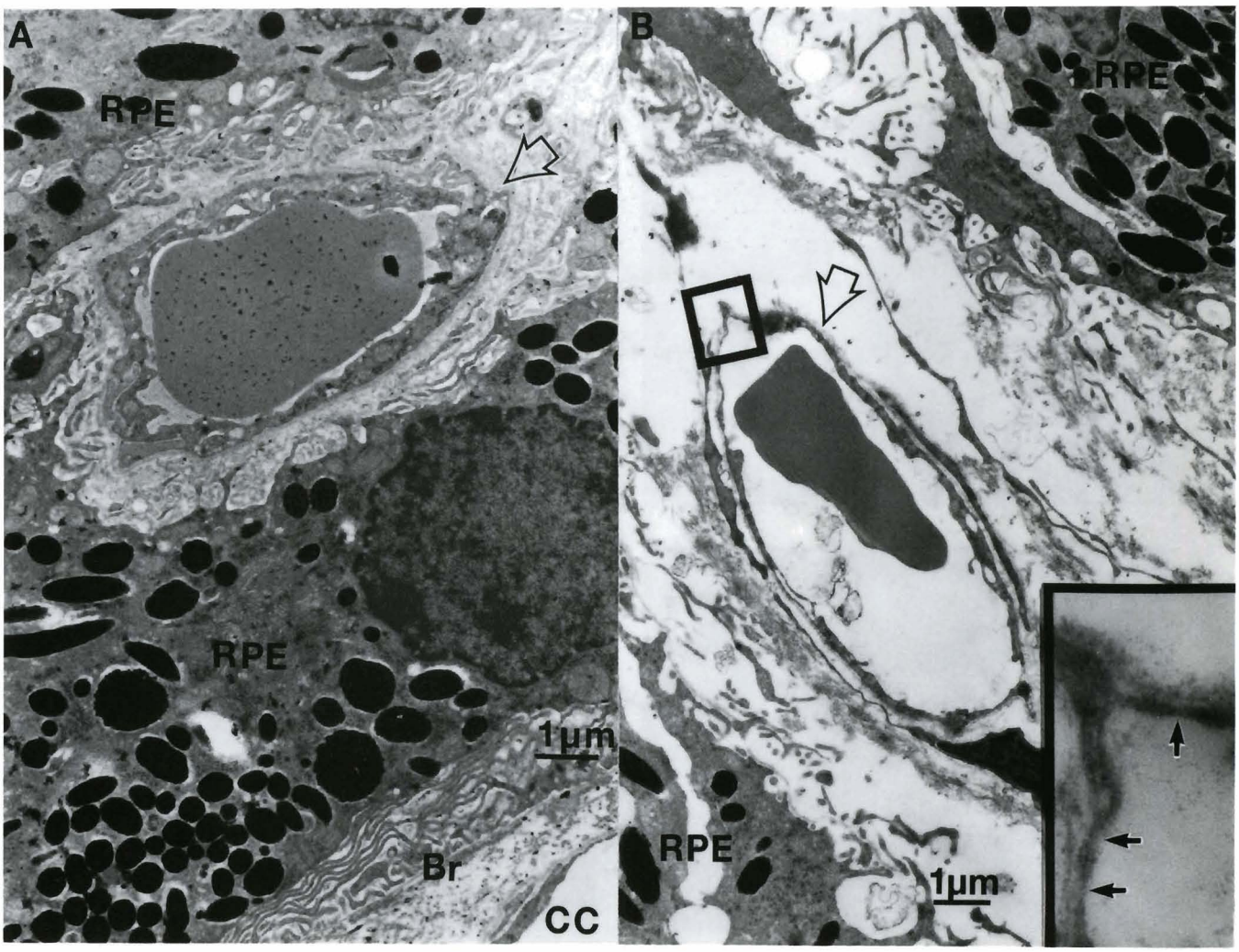

Fig. 3. Electron microscopy of subretinal neovascularization in *VEGF* transgenic mice with a wild type *Timp-1* allele. **A.** A new vessel (arrow) in the subretinal space of a *VEGF* transgene-positive mouse with a wild type *Timp-1* allele has thick endothelial cells with no fenestrations. Beneath the vessel there is an intact layer of retinal pigmented epithelial cells (RPE) adjacent to Bruch's membrane (Br) and the choriocapillaris (CC). There are also RPE cells above the vessel and although they were excluded from this micrograph, there were RPE cells completely surrounding the vessel in the plane of the section. **B.** Another *VEGF* transgene-positive mouse with a wild type *Timp-1* allele has a new blood vessel in the subretinal space that is partially surrounded by RPE cells and has thin endothelial cells with fenestrations (inset, arrows).

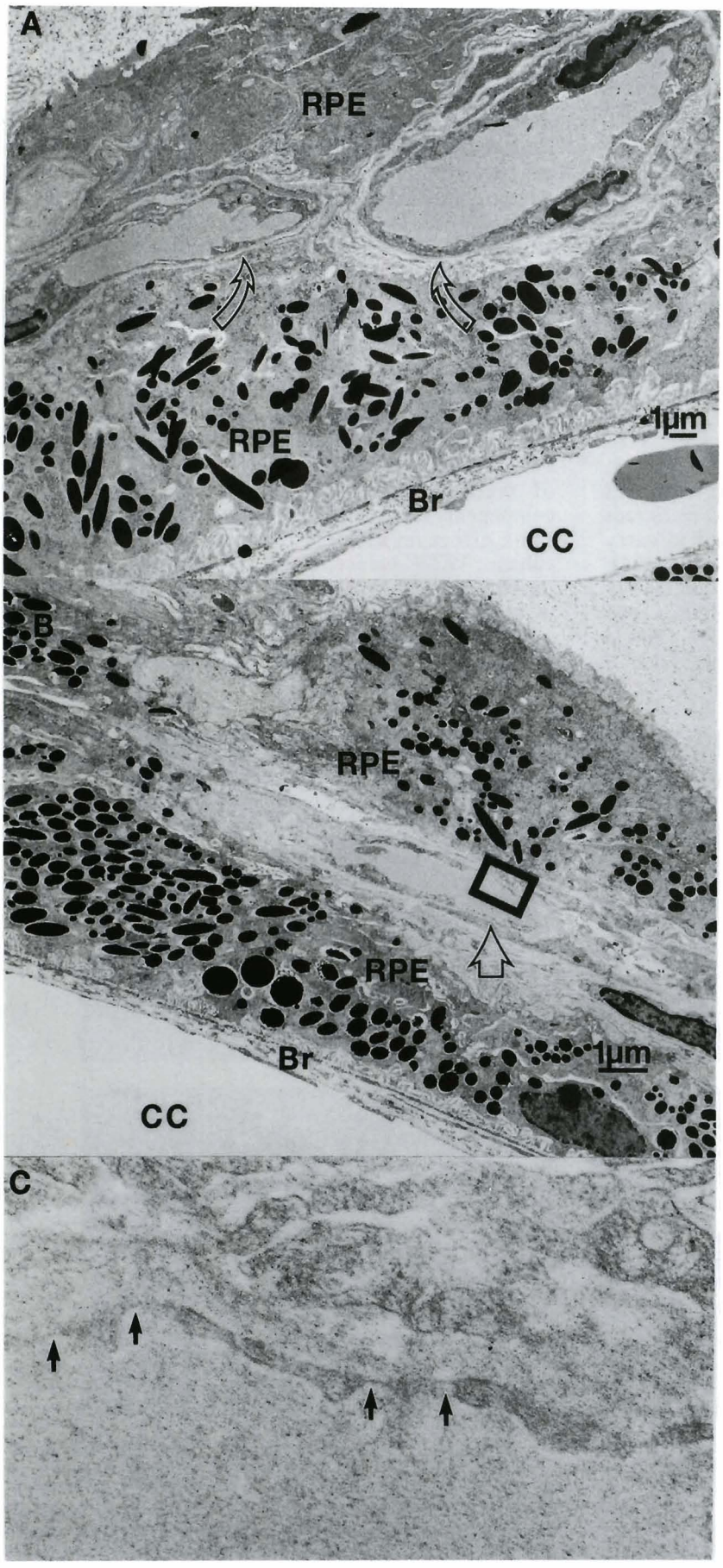

Fig. 4. Electron microscopy of subretinal neovascularization in *VEGF* transgenic mice deficient in *Timp-1*. **A.** New blood vessels in the subretinal space of a *VEGF* transgene-positive/*Timp-1*-deficient mouse are surrounded by RPE cells and have relatively thick endothelial cells with no fenestrations. The RPE cells above the vessels have few pigment granules, while those adjacent to Bruch's membrane (Br) and the choriocapillaris (CC) have numerous pigment granules. **B.** Another *VEGF* transgene-positive/*Timp-1*-deficient mouse shows a new vessel in the subretinal space that is completely surrounded by RPE cells, but has thin endothelial cells with many fenestrations. **C.** The region within the box in (B) is shown at higher magnification to show fenestrations (arrows).

VEGF-induced retinal NV. Similarly, angiostatin (O'Reilly et al., 1994) and endostatin (O'Reilly et al., 1997) are angiogenesis inhibitors that are cleavage products of large proteins generated by MMPs and a decrease in the MMP/TIMP-1 ratio may promote retinal NV in part by decreasing angiostatin and/or endostatin levels.

Our results should not be interpreted to suggest that inhibition of proteolysis does not provide a protective influence against the development of NV in the outer retina and subretinal space, because protection may be mediated by inhibitors other than TIMP-1. In some systems, TIMP-2 inhibits endothelial cell processes associated with angiogenesis to a greater extent than TIMP-1 (Murphy et al., 1993), and if this is the case in the retina, TIMP-2, but not TIMP-1, may help to prevent retinal neovascularization. Individuals in two pedigrees with Sorsby's fundus dystrophy were found to carry mutations in the TIMP-3 gene (Weber et al., 1994). Sorsby's fundus dystrophy is an autosomal dominantly inherited disease in which patients have a high incidence of early-onset choroidal NV (Sorsby et al., 1949; Hoskin et al., 1981; Capon et al., 1988). Within the eye, TIMP-3 is differentially expressed in the RPE (Della et al., 1996; Ruiz et al., 1996) and therefore perturbation of TIMP-3 activity in RPE cells may predispose to choroidal NV. Although TIMP-1 and TIMP-3 have a similar antiproteolytic spectrum of activity, TIMP-3 has greater affinity for ECM (Pavloff et al., 1992) and association with the ECM may be critical for limitation of proteolysis in the subretinal space.

The mechanism by which increased expression of TIMP-1 increases *VEGF*-induced retinal NV is unclear. It may be due to an activity of TIMP-1 other than its inhibition of proteolysis. For instance, TIMP-1 has been demonstrated to stimulate the proliferation of several cell types *in vitro* (Bertaux et al., 1991; Hayakawa et al., 1992). This may occur by TIMP-1-mediated protection of matrix-bound growth factors or other ECM components critical for cell survival. If TIMP-1 has the same effect on endothelial cells *in vivo*, this could enhance *VEGF*-induced retinal NV. On the other hand, if the stimulatory effect of TIMP-1 on retinal NV is related to its inhibition of metalloproteinase activity, then it is unlikely that synthetic proteinase inhibitors will provide therapeutic benefit in patients with retinal NV.

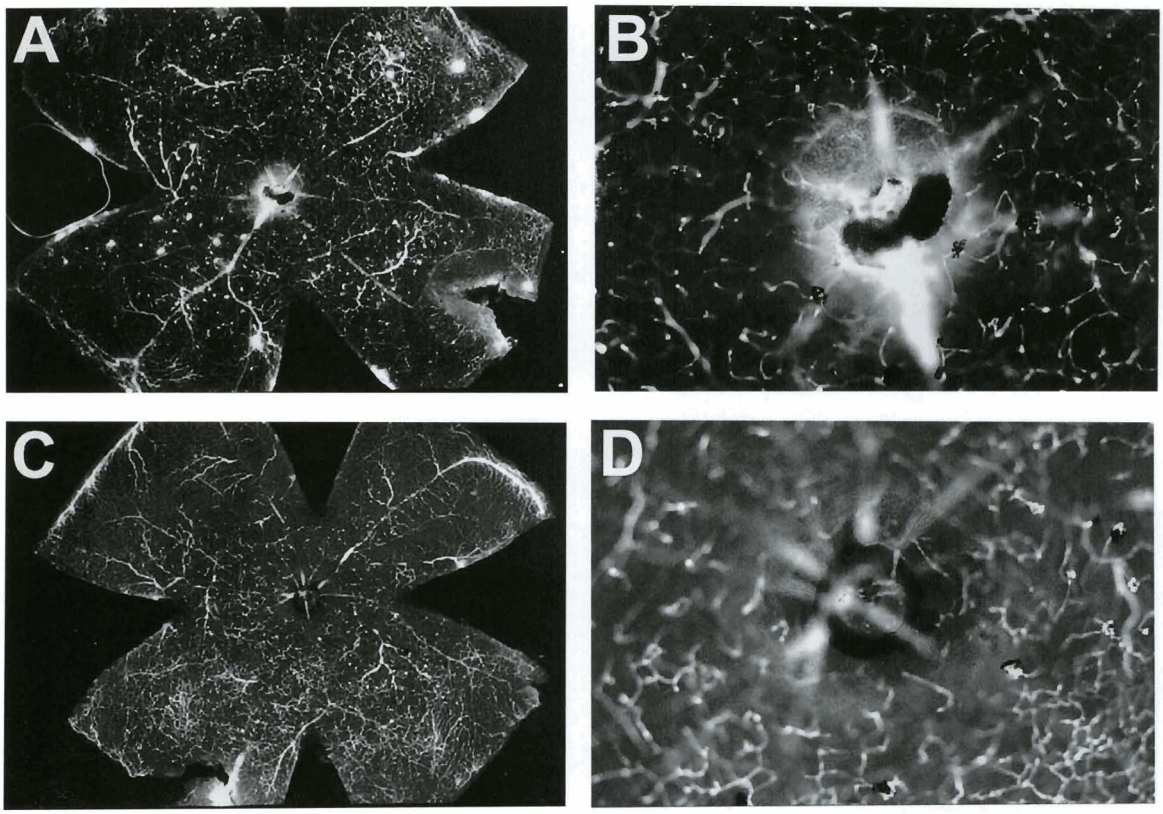

Fig. 5. Mice that overexpress TIMP-1 have increased *VEGF*-induced retinal neovascularization at 3 weeks of age compared to littermate controls **A.** Double transgenic mice that overexpress TIMP-1 and *VEGF* in the retina show many small hyperfluorescent spots corresponding to areas of neovascularization. x 25. **B.** At higher magnification, focusing on the outer edge of the retina, numerous neovascular lesions partially surrounded by retinal pigmented epithelial cells are seen. The dark area marked is the region where the optic nerve formerly entered the retina. x 100. **C.** A littermate that overexpresses *VEGF*, but not TIMP-1, shows fewer hyperfluorescent spots corresponding to neovascularization than seen in (A). x 25. **D.** At higher magnification, focusing on the outer edge of the same retina shown in (C), a few neovascular lesions are seen, but many fewer than in (B). x 100

Neovascularization is a complex process that is likely to result from disturbance of a critical balance between stimulatory and inhibitory factors, and the factors involved may vary from tissue to tissue. Identification of the factors involved in any particular tissue is likely to be a difficult task, but is worthwhile because it can help direct development of pharmacologic and gene therapies. Our strategy for the retina, is to use leads obtained from *in vitro* studies or models in other tissues and methodically test them using genetically engineered mice. Our studies thus far suggest that *VEGF* is an important stimulatory factor for NV in the retina, and while TIMP-1 plays an important role in limiting cellular invasion in several tissues, and NV in some tissues, it facilitates NV in the retina and subretinal space.

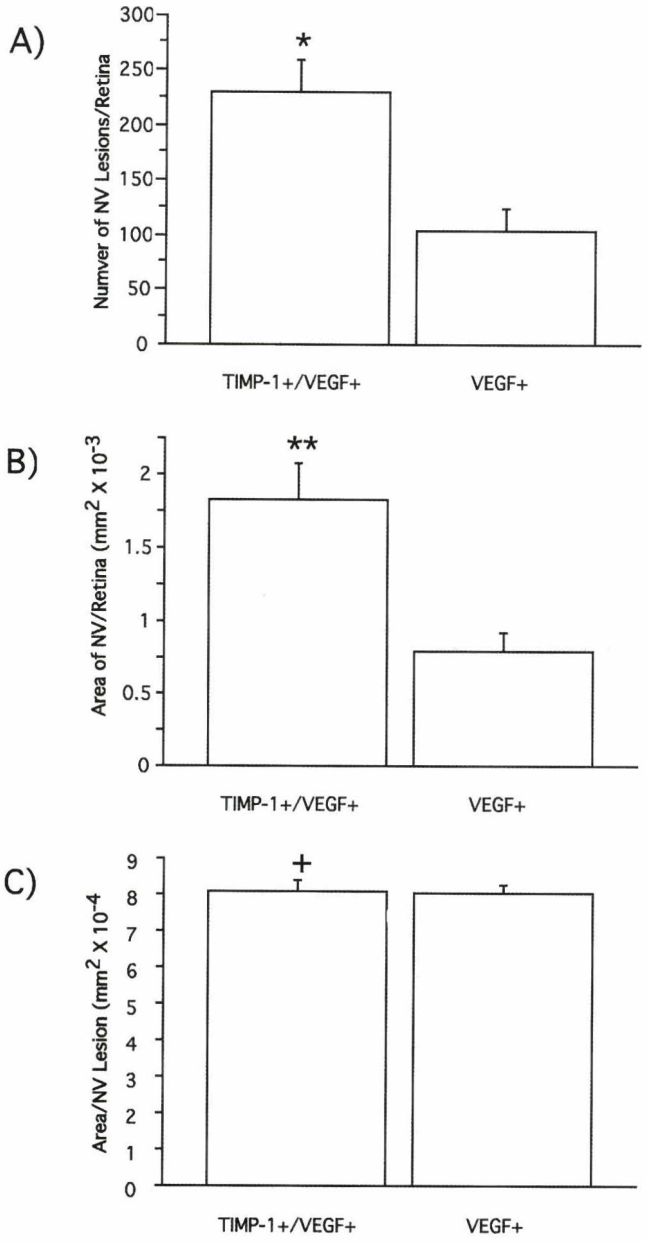

Fig. 6. Mice that overexpress TIMP-1 and *VEGF* in the retina have more neovascular lesions and a greater total area of neovascularization than mice that overexpress *VEGF*, but not TIMP-1. Retinal whole mounts of fluorescein-labeled dextran perfused 3 week old mice that overexpress TIMP-1 and *VEGF* (TIMP-1+/*VEGF*+; n=19) and littermates that overexpress *VEGF*, but not TIMP-1 (*VEGF*+; n=11) were examined by fluorescence microscopy and the number of neovascular lesions per retina, the area per lesion, and the total area of neovascularization per retina were quantitated by image analysis. The bars represent the mean ± SEM. *: p=0.0053; **: p=0.0039 for difference from *VEGF*+ littermates by a t-test for populations with unequal variances +p=0.91 for difference from *VEGF*+ littermates by a t-test for populations with equal variances.

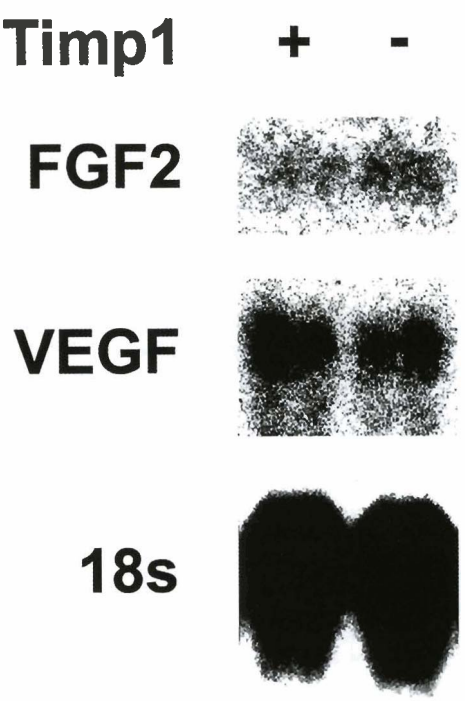

Fig. 7. There are no differences in *VEGF* or FGF2 mRNA levels to explain the difference in neovascularization in *rho/VEGF* mice that overexpress TIMP-1 compared to those that have normal TIMP-1 levels. Northern blots demonstrate only small differences in *VEGF* and FGF2 mRNA in the retinas of 3 week old *rho-VEGF* mice that express increased levels of TIMP-1 compared to those with normal TIMP-1 expression. Blots were also hybridized with a probe for 18S which shows no difference in loading.

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