Localization of TIMP-3 mRNA Expression to the Retinal Pigment Epithelium

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Purpose. To evaluate ocular sites of expression of the tissue inhibitor of metalloproteinases-3 gene (TIMP-3).

Methods. In situ hybridization was performed on frozen sections of albino mouse eyes using riboprobes generated to the 3' untranslated region of TIMP-3.

Results. TIMP-3 mRNA expression was detected strongly in the retinal pigment epithelium (RPE) and to a minor extent in the ciliary epithelium, but not at any other site within the eye.

Conclusions. Expression of TIMP-3 in the RPE is consistent with the recent demonstration of TIMP-3 mutations in patients with Sorsby's fundus dystrophy, a condition marked by the early onset of choroidal neovascularization in the macula. Unlike many of the recently described genes that cause human retinal disease, TIMP-3 is preferentially expressed in the RPE of the normal eye, as opposed to the photoreceptors. Invest Ophthalmol Vis Sci. 1996;37:1921-1924.

Choroidal neovascularization (CNV) is responsible for severe loss of vision in many retinal diseases, particularly age-related macular degeneration (AMD). Despite the magnitude of its clinical impact, there is only limited understanding of the pathophysiology of CNV. Sorsby's fundus dystrophy is a rare, autosomal, dominantly inherited condition characterized by a high incidence of early-onset CNV. Even with initially successful laser treatment, recurrent CNV usually leads to macular scarring and significant loss of central vision. In this respect, Sorsby's dystrophy is similar to AMD, which ranks as the most common cause of blindness in developed countries. Recently, persons in two pedigrees with Sorsby's dystrophy were found to carry mutations in the tissue inhibitor of metalloproteinases-3 gene (TIMP-3). This finding implies that perturbation of the inhibitory action of TIMP-3 on proteinases in or around the subretinal space may play an important role in the development of CNV. At present, little is known about the distribution of TIMP-3 in the normal eye, and the current study was undertaken to investigate the pattern of expression of TIMP-3 in the albino mouse eye.

METHODS. Generation of Riboprobes. Total RNA was extracted from cultured human RPE cells, freshly isolated bovine RPE, murine brain, and murine retina using the acid guanidinium extraction method. Because the sample of murine retina contained some underlying RPE, this sample was referred to as mouse retina-RPE. The human RPE was isolated from eyes obtained from the Old Dominion Eye Bank of Virginia. In particular, use of this collection of human tissues complied with the tenets of the Declaration of Helsinki, including appropriate informed consent and approval. The nonhuman tissues used in this study were obtained from animals handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Oligo dT-primed reverse transcription of 1 to 3 μg of each RNA sample was carried out with avian myeloblastosis virus reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a 20 μl reaction volume by standard methods. Aliquots of 2 μl of each cDNA, together with controls incubated without reverse transcriptase, were amplified by the polymerase chain reaction (PCR) in a Perkin Elmer (Foster City, CA) 9600 machine for 30 cycles using parameters of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. The oligonucleotide primers used in this reaction were designed to amplify a 580-base pair (bp) fragment located approximately 450 bp downstream of the stop codon in the 3' untranslated region (UTR) of the murine TIMP-3 gene. The 5' primer (5'-ATGCTAAAGGCAAGCAAGCA-3') corresponded to bp 1415-1435, and the 3' primer (5'-ACCATTGAATGAATCT GTGTCTC-3') corresponded to bp 1986-1962 of the murine TIMP-3 sequence (EMBL accession number Z30970). The PCR product from murine brain was subcloned. It was filled in with klenow fragment of DNA polymerase (Boehringer Mannheim, Indianapolis, IN), purified by agarose gel electrophoresis, and then ligated into the EcoRV site of pCR-Script SK (Stratagene, La Jolla, CA). Subclones were checked by dieoxy DNA sequencing (Sequenase; US Biochemical, Cleveland, OH). Clones with both orientations were selected to allow use of the same RNA polymerase (T3) to generate sense and antisense riboprobes.
The TIMP-3-containing vectors were linearized with SalI, phenol–chloroform extracted, and ethanol precipitated. In vitro transcription was performed using digoxigenin-labeled uridine triphosphate (Boehringer Mannheim), according to the manufacturer’s protocol. A 1 μl aliquot of each probe was assessed for size and approximate yield by formaldehyde agarose gel electrophoresis. In addition, the yield of riboprobe was estimated by spotting a dilution series onto a nylon membrane (Genescreen; New England Nuclear, Boston, MA) and comparing with a known digoxigenin-labeled RNA standard, as described in the manufacturer’s protocol.

Processing of Tissues and In Situ Hybridization. BALB/cj mice were killed by cervical dislocation, and the eyes were enucleated and rapidly frozen in embedding medium (OCT; Miles Diagnostics, Elkhart, IN) over dry ice-cooled isopentane. Albino mice were chosen to enable careful analysis of TIMP-3 expression in areas of the eye normally obscured by melanin. Sections (12 μm) were thaw-mounted onto glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA), freeze-dried on dry ice, and thawed and fixed in 4% paraformaldehyde for 10 minutes in phosphate-buffered saline containing 0.1% Triton X-100. Hybridization of tissue sections and associated prehybridization and posthybridization processing of these sections was carried out by a modification of published methods.3’4 In particular, Pronase E (70 μg/ml for 10 minutes at room temperature; Sigma, St. Louis, MO) was used to pretreat the sections to improve probe access to the target mRNA. Hybridization was performed overnight at 57°C using a riboprobe concentration of 1 μg/ml, and a posthybridization ribonuclease digestion (20 μg/ml of RNase A for 30 minutes at 37°C; Sigma) was included to reduce background. Sections were blocked with 40% heat-inactivated lamb serum (Sigma) in Tris-buffered saline before the addition of the anti-digoxigenin antibody (1:2000; Boehringer Mannheim). The alkaline phosphatase-mediated color reaction was performed as described.4 Sections were mounted in an aqueous glycerol-based mounting medium. Photographs were taken with a Zeiss (Oberkochen, Germany) Axioskop photomicroscope using bright-field or Nomarski optics and Kodak T-64 35 mm film (Eastman Kodak, Rochester, NY).

RESULTS. Reverse transcription–PCR (RT–PCR) using primers designed to amplify a 580 bp region of the 3’ UTR of the murine TIMP-3 gene yielded appropriate size products using total RNA from murine retina–RPE, murine brain, bovine RPE, and cultured human RPE cells (data not shown). To identify more specifically the ocular sites of TIMP-3 expression, in situ hybridization was performed. A 3’ UTR probe was chosen because the 3’ UTRs of the TIMP-1, -2, and -3 genes are divergent, but the coding regions are highly conserved. The 580 bp RT–PCR product amplified from murine brain was subcloned and verified by sequencing to be made identical to the known murine TIMP-3 sequence. This subcloned DNA fragment was used as a template to generate sense and antisense TIMP-3 riboprobes.

The results of hybridization of the TIMP-3 riboprobes to tissue sections of the albino mouse eye are presented in Figure 1. Positive signal localized to the RPE is observed with the antisense probe (Fig. 1B). There are no apparent differences between the central and peripheral regions of the RPE monolayer. The sense controls do not show detectable signal (Figs. 1C, 1E). High-power views of the RPE (Fig. 1D) reveal TIMP-3 signal in RPE cytoplasm, with cell nuclei readily apparent as discontinuities in the reaction product. There is no detectable TIMP-3 expression in the neural retina. The only other ocular sites of apparent TIMP-3 expression are the ciliary epithelium and, to a barely detectable extent, the iris (Fig. 1G). The level of expression in the ciliary epithelium appeared considerably lower than in the RPE, and it was not possible to determine whether this expression was confined to the pigmented or nonpigmented epithelium, or to both. Some signal was seen in the corneal epithelium with the antisense probe, but, because a similar appearance was noted with the sense probe, it probably represented nonspecific background (data not shown).

DISCUSSION. The turnover of extracellular matrix (ECM) in many tissues reflects opposing actions of the matrix metalloproteinases (MMPs) and their inhibitors, the TIMP family of proteins.5 The MMPs are a diverse group of zinc-containing proteinases that degrade collagen and proteoglycans. There are three known proteinase inhibitors in the TIMP family, TIMP-1, TIMP-2, and TIMP-3. They share a conserved N-terminal domain as well as other individual conserved cysteine residues, and they show broad-spectrum inhibition of the MMPs.6 The most recently cloned member of this family is TIMP-3. By Northern blot analysis, expression of TIMP-3 was observed in various tissues in humans and mice, particularly in the placenta, kidneys, lungs, ovaries, and uterus.7’8 Expression appears to be lower in the brain and the liver. This tissue distribution of TIMP-3 transcripts differs from that of TIMP-1 or TIMP-2, and the TIMP-3 protein displays prominent ECM association in the mouse.9 However, the eye was not specifically examined in these published studies by TIMP-3 expression.

The importance of TIMP-3 in the human eye was underscored by the finding that, in some patients, Sorsby’s fundus dystrophy is caused by mutations in the TIMP-3 gene.2 In another report published recently, TIMP-3 was observed to be expressed in the degenerating retinas of patients with simplex retinitis...
FIGURE 1. In situ hybridization analysis of tissue inhibitor of metalloproteinases-3 (TIMP-3) mRNA expression in the eye of the BALB/cJ albino mouse. Low-power views (bright field) of the retina, choroid, and sclera (A, counterstained with 0.1% thionin) hybridized with antisense (B) or sense TIMP-3 riboprobes (C) reveal expression specifically in the retinal pigment epithelium (RPE). High-power views (Nomarski optics) show signal in the cytoplasm of the RPE cells with antisense TIMP-3 probe (D), but absence of signal with sense probe (E). View of the peripheral anterior segment (F, bright field, counterstained with 0.1% thionin) hybridized with antisense TIMP-3 probe (G, Nomarski optics) shows a signal in the ciliary epithelium that is not seen with the sense probe (H, Nomarski optics). Magnification scale bars = 50 μm (A,B,C,F,G); = 20 μm (D,E). INL = inner nuclear layer; IPL = inner plexiform layer; IS = inner segments; ONL = outer nuclear layer; OS = outer segments; ac = anterior chamber; ce = ciliary epithelium; i = iris.

pigmentosa but not in normal neural retina. The possibility of TIMP-3 expression in the RPE of these eyes could not be assessed because of the masking effect of melanin and lipofuscin pigments.

This study implicates the RPE as the major site of TIMP-3 expression in the albino mouse eye. If the same pattern of expression exists in the human, which is probably based on the high degree of functional conservation between TIMP-3 in mice and humans, TIMP-3 would be one of the few genes responsible for human retinal disease normally expressed in the RPE and not in the photoreceptors. Ornithine aminotransferase, mutated in patients with gyrate atrophy, is similar to TIMP-3 because its broad pattern of expression includes the RPE and not the photoreceptors.

The role of TIMP-3 in the RPE is unknown but is likely to involve ECM homeostasis, particularly in Bruch's membrane. The RPE is known to participate in local ECM production. Certain MMPs and TIMP-1 have been reported to be secreted by cultured human RPE cells. In addition, the presence of MMP activity, including a 72 kDa MMP, and MMP inhibitory activity, specifically for TIMP-1, have been detected in the bovine interphotoreceptor matrix. The relationship between perturbation of TIMP-3 function in the RPE and the development of choroidal neovascularization warrants detailed investigation. Previous work has shown that MMP inhibition produced by...
vascular endothelial cells can inhibit neovascularization. From a clinical viewpoint, AMD is the most common cause of blindness in developed countries, but it is a genetically heterogeneous condition. The similarities between Sorsby's fundus dystrophy and AMD highlight the importance of understanding TIMP-3 function in the RPE and evaluating its possible involvement in AMD.

**Key Words**
extracellular matrix, neovascularization, proteinases, retinal pigment epithelium, tissue inhibitor of metalloproteinases-3

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**References**

**Effect of Tumor Necrosis Factor α on Rabbit Corneal Endothelial Permeability**

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**Purpose.** Tumor necrosis factor α (TNFα) is present in the iris and the lacrimal gland, and its concentration is increased during inflammation and after corneal wounding. Although TNFα has been shown to increase keratoctye and corneal epithelial interleukin production, no definitive effects of TNFα on corneal endothelial cells have been reported. TNFα has been shown to disrupt barrier function in vascular endothelial monolayers through f-actin depolymerization. A reduction in intracellular cyclic adenosine monophosphate (cAMP) concentration may play a role in this response. This study was designed to examine the role and signal transduction mechanisms of TNFα modulation of endothelial permeability in the cornea. In addition, it is the first examination of the effects of TNFα on the barrier function of a noncultured cell monolayer.

**Methods.** Rabbit corneal endothelial superfusions were performed under an in vitro specular microscope. Corneas were processed for permeability measurements or f-actin staining.

**Results.** TNFα superfused corneas had significantly higher permeabilities than controls. F-actin staining re-