Suppressed Expression of Tubedown-1 in Retinal Neovascularization of Proliferative Diabetic Retinopathy

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Purpose. Retinal neovascularization occurring as a complication of diabetes mellitus can cause vision loss and blindness. The identification and study of novel genes involved in retinal angiogenesis may define new targets to suppress retinal neovascularization in diabetes and other ocular diseases. A novel acetyltransferase subunit, tubedown-1 (tbdn-1), has been isolated, the expression of which is regulated during blood vessel development. Tbdn-1 is not detected in most adult vascular beds but persists at high levels in the adult ocular vasculature. The purpose of this study was to gain insight into the possible role of tbdn-1 in retinal blood vessels by characterizing its expression patterns in adult homeostasis and in retinal neovascularization associated with diabetes.

Methods. Western blot analysis and immunohistochemistry were performed to study the expression patterns of tbdn-1 during adult homeostasis in normal human retinas, in a model of choroid–retina endothelial capillary outgrowth in vitro, and in retinas showing neovascularization in patients with proliferative diabetic retinopathy (PDR).

Results. In adults during homeostasis, tbdn-1 was expressed highly in normal endothelium of retinal and limbic blood vessels. Tbdn-1 was also expressed in RF/6A, a rhesus macaque choroid-retina–derived endothelial cell line. In an in vitro model system using the RF/6A cell line, tbdn-1 expression was downregulated during the outgrowth of these cells into capillary-like structures on a reconstituted basement membrane matrix. Similar to this in vitro model, tbdn-1 expression is specifically suppressed in the endothelial cells of blood vessels and capillary fronds in vivo in both the neural retinal tissue and in preretinal membranes in eyes of patients with PDR.

Conclusions. High levels of expression of tbdn-1 are associated with ocular endothelial homeostasis in adults. Conversely, low levels of tbdn-1 expression are associated with endothelial capillary outgrowth in vitro and retinal neovascularization in vivo. Because the tbdn-1 acetyltransferase subunit is a member of a family of regulatory enzymes that are known to control a range of processes, including cell growth and differentiation, through posttranslational modification, the current results support a hypothesis that tbdn-1 may be involved in maintaining homeostasis and preventing retinal neovascularization. (*Invest Ophthalmol Vis Sci.* 2001;42:3000–3007)

Diseases involving ocular neovascularization can cause visual loss and blindness. Ocular disease associated with diabetes mellitus is initially characterized by retinal ischemia, which progresses to a proliferative stage involving both neovascularization of the retina, optic disc, or iris and fibrosis.[1,2] In proliferative diabetic retinopathy (PDR), high levels of proangiogenic factors are thought to lead to increased neovascularization, which contributes to a positive feedback cycle of fibrovascular growth, retinal dysplasia, scarring, and eventual retinal detachment.[3] A range of angiogenic growth factors (VEGF, bFGF, and insulin-like growth factor [IGF])-1), integrins and extracellular matrix components probably contribute to and have been associated with pathologic neovascularization in PDR.[3–5] However, increased production of VEGF in the retina may be a determining factor in the later proliferative neovascularization that leads to pathologic sequelae in later stages of PDR.[5–7] The current effective treatment for PDR involves ablative therapy that can cause complications (retinal vein occlusion, loss of visual acuity, vitreous hemorrhage) or even sometimes fails altogether.[1,2]

Treatments specifically targeting either VEGF and its receptors or specific integrins have been found effective in reducing but not abolishing retinal neovascularization in animal models.[8–12] Because a range of angiogenic factors is probably involved in the microenvironment promoting retinal blood vessel proliferation, the targeting of a single factor for antiangiogenic therapy may not completely counter the neovascularization in PDR. The characterization of common regulators that act downstream of these angiogenic signals mediating retinal neovascularization are key to identifying targets that could have a more global effect on controlling retinal neovascularization.

At present, there is little known about the intracellular regulatory pathways controlling retinal neovascularization and the presumed disturbances in such pathways during PDR. Elucidation of these regulatory pathways and the identification of the associated molecular effectors could reveal potential targets for blocking neovascularization and restoring normal function to the diseased retina. We have isolated a novel gene that we named tubedown-1 (*tbdn-1*), because it is downregulated during the formation of capillary structures in IEM vascular endothelial cells in vitro and during the development of most vascular beds in vivo.[13] *Tbdn-1* encodes a novel 69-kDa polypeptide associated with an acetyltransferase activity.[13] *Tbdn-1* displays homology with the previously characterized yeast N-terminal acetyltransferase subunit NAT1 and contains other motifs suggesting a regulatory function.[13] In contrast to most vascular structures in adults, *tbdn-1* expression persists in ocular vascular endothelium in adulthood. In the present study, we investigated the expression regulation of *tbdn-1* in normal and diseased eyes to better understand the potential role of this novel regulatory protein in retinal vessel homeostasis.

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Materials and Methods

Cell Culture

RF/6A rhesus macaque choroid-retina endothelial cells[14,15] were obtained from the American Type Culture Collection (Manassas, VA) and were grown in DMEM supplemented with 10% fetal bovine serum (FBS) plus 2 mM glutamine and nonessential amino acids. The spontaneously immortalized RF/6A choroid-retina endothelial cell line, derived from the choroid-retina of a rhesus macaque, retains the expression of endothelial markers[14,15] including the VEGF receptor type-2 tyrosine kinase (VEGFR-2; our unpublished observation, 2000). Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA) and grown in DMEM plus 10% FBS, 2 mM glutamine, 1 ng/ml bFGF, and a mixture of insulin, transferrin, and selenium (Gibco, Rockville, MD). The IEM cell line, from which *tbdn-1* was originally isolated, was grown as previously described[16] and was initially derived by immortalizing differentiation products of embryonic stem cell cultures using simian virus (SV)40 large T antigen. The IEM line expresses endothelial markers and can be induced to form capillary structures in a synthetic basement matrix (Matrigel; Collaborative Research, Inc., Bedford, MA) after induction with bFGF and leukemia inhibitory factor.[16] IEM cells can also contribute to vascular structures in embryonic chimeras in vivo after blastocyst injections.[16] *Tbdn-1* RNA and protein become downregulated as IEM cells differentiate into capillaries on synthesized basement membrane.[13]

Capillary Formation

For capillary induction, cultures of RF/6A cells were treated with 10 ng/ml bFGF plus 10 ng/ml VEGF for 48 hours before being transferred to the synthetic basement membrane for a further 96 hours for capillary formation, as previously described.[13,16,17] RF/6A capillary colonies were collected by gently lifting the colonies, together with the membrane on which they were growing, from the culture dishes with a fine spatula. Control cultured RF/6A cells were harvested from tissue culture dishes by scraping the cells from the dishes and were collected by gentle centrifugation. The pellets of cultured RF/6A cells and the RF/6A capillary colonies were then fixed in 4% buffered paraformaldehyde and immobilized by embedding in small blocks of low-melting-temperature agarose. The agarose blocks containing the pellets of cultured RF/6A cells and the RF/6A capillary colonies were next fixed in 4% paraformaldehyde and embedded in paraffin blocks for histologic processing and analysis.

Anti-tbdn-1 Antibody

As described in our initial report of the cloning and characterization of *tbdn-1*,[13] an anti-tbdn-1 IgY antibody (Ab1272) was generated by immunizing chickens with a keyhole limpet hemocyanin (KLH)-conjugated 10mer peptide sequence in the *tbdn-1* open reading frame. The peptide sequence used was MDEAQALDTA (tbdn-1 amino acids 160-170). IgY was isolated to 90% purity from preimmune and immune egg yolks using an extraction agent (Eggstract; Promega, Madison, WI). We have previously demonstrated the specificity of Ab1272 for detecting tbdn-1 protein in IEM cell lysates by Western blot analysis and in tissue sections by immunohistochemistry.[13]

Tissue Specimens and Immunohistochemistry

Immunocytochemistry was performed on paraformaldehyde-fixed, paraffin-embedded sections of cultures of untreated RF/6A cells, RF/6A capillary colonies, and human eye tissues to detect tbdn-1 and endothelial and pericyte marker expression. Four normal human adult eye specimens and five specimens from patients with PDR were studied. All human eye tissue specimens were obtained under the approval of the Institutional Review Boards of the Smith Kettlewell Eye Research Institute, San Francisco, and Childrens Hospital Medical Center, Cincinnati. All research on human specimens followed the tenets of the Declaration of Helsinki at all times. Specimens of human eyes were obtained either from the University of San Francisco Department of Ophthalmologic Pathology or procured for us from certified eye banks through services of the National Disease Research Interchange (Philadelphia, PA). Normal control eye specimens were obtained as either whole globes or posterior poles from donors with no history of eye disease. Procurement criteria for PDR specimens received through the National Disease Research Interchange were that donors must have diabetic retinopathy, must be 21 to 100 years of age, but could be of either sex and any race.

Eyes of patients with diabetes (either whole globes or posterior poles) were procured at autopsy within 8 hours of death and formalin fixed within 12 hours of death. Formalin fixed eyes were processed and embedded in paraffin blocks. The health history and details of the diabetic retinopathy eye specimens analyzed were as follows: The first specimen was from an enucleation in a 74-year-old man with long-standing insulin-dependent diabetes mellitus (IDDM) with a history of unresolved PDR. The second specimen was from a 78-year-old man with long-standing IDDM and a history of unresolved diabetic retinopathy who had died of myocardial infarction. The third specimen was from a 70-year-old woman with long-standing IDDM with a history of unresolved diabetic retinopathy who had died of acute myocardial infarction. The fourth specimen was from a 60-year-old man with long-standing IDDM and a history of unresolved diabetic retinopathy who had been found unresponsive, had undergone attempted cardiopulmonary resuscitation, and was declared dead on arrival at the hospital. The fifth specimen was from a 62-year-old man with long-standing IDDM and a history of unresolved diabetic retinopathy who died of acute renal failure.

Specimens were embedded in paraffin blocks and were sectioned. They were then deparaffinized, rehydrated, and subjected to immunohistochemistry. All conditions and procedures for processing RF/6A cells and RF/6A capillary colonies were identical. After a 1-hour blocking step in 2% normal goat serum, sections were incubated with either a 1:100 dilution of chicken anti-tbdn-1 IgY (Ab1272[13]) or an equal concentration of preimmune IgY. For an endothelial cell marker, rabbit anti-von Willebrand factor (Dako, Glostrup, Denmark) was used for labeling endothelial cells in blood vessels in adjacent sections. An anti-α-smooth muscle actin (ASMA) monoclonal antibody directly conjugated to alkaline phosphatase (Sigma, St. Louis, MO) was used in conjunction with the anti-tbdn-1 IgY antibody to double label sections of normal human eyes for simultaneous localization of tbdn-1 and pericytes. Anti-tubulin mouse monoclonal antibody (Sigma) was used as a positive ubiquitous staining control for RF/6A cells and capillary colonies. After a rinse in phosphate-buffered saline (PBS), reactions were developed using the appropriate alkaline phosphatase–conjugated, species-specific secondary reagents (anti-rabbit IgG, anti-mouse IgG, or anti-chicken IgY; Promega). Red color reactions were generated using naphthol-AS-MX phosphate in the presence of fast red and levamisole (to block endogenous tissue alkaline phosphatase activity). In double-labeling experiments, the anti-tbdn-1 reaction was developed first using anti-IgY horseradish peroxidase and a diaminobenzidine (DAB) substrate kit (Sigma) to yield a dark brown color reaction for tbdn-1 expression, whereas the alkaline phosphatase anti-ASMA reaction was developed immediately after using fast red and levamisole, as stated above. Anti-tbdn-1/anti-ASMA double-labeled reactions were slightly overdeveloped to enable clearly revealing the locations of both epitopes. Slides were counterstained lightly using a 0.5% aqueous solution of methyl green. Sections were then rinsed, dried, and mounted (Permount, Fisher, Pittsburgh, PA) before viewing and photography using a microscope-mounted digital camera (DC120; Eastman Kodak, Rochester, NY). Differences in immunohistochemical staining of tbdn-1 were quantitatively analyzed by measuring the total area of red chromogen in high-power fields of identical dimensions sampled from the retinal areas in normal and PDR specimens. Measurements were made using the magic wand and histogram command tools of an image-management program (Photoshop; Adobe, Mountain View, CA) run on a computer (Macintosh G3; Apple Computer, Cupertino, CA),

as described in a previously published method.[18] Results are expressed as mean red chromogen pixels per high-power field ± SEM.

Western Blot Analysis

Cell lysates were prepared using Triton X-100 lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Triton X-100) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 0.3 U/ml aprotinin, and 10 μg/ml leupeptin) and phosphatase inhibitors (1 mM sodium orthovanadate, 25 mM sodium fluoride, and 10 mM β-glycerophosphate). Lysates were clarified by centrifugation, the protein concentration was quantified and samples analyzed by SDS-PAGE. Western blot analysis was performed by standard procedures using chemiluminescence detection (ECL Plus reagent; Amersham Pharmacia Biotech, Piscataway, NJ), except for low-salt buffer (25 mM NaCl) conditions for Ab1272 incubations and washes. For experiments demonstrating the specificity of the Ab1272 antibody in Western blot analysis of RF/6A cells, RF/6A cell clones overexpressing *tbdn-1* cDNA sequences 1-1413 in an antisense orientation were generated using zeocin selection from the pcDNA3.1/Zeo vector (Invitrogen, San Diego, CA). Lysates were prepared from several of these antisense tbdn-1 RF/6A transfectants and from parental RF/6A cells, as described earlier, and then used in Western blot analysis experiments for testing the specificity of Ab1272 in detecting tbdn-1 in RF/6A cells.

Results

Tbdn-1 Expression in Endothelial Cells

We first made a comparison of tbdn-1 expression in endothelial cell lines from different species. To establish that tbdn-1 is equally detectable by Ab1272 anti-tbdn-1 antibody in primate and human retinal endothelial cells as it is in mouse vascular endothelial cells,[13] we first performed Western blot analysis using the Ab1272 antibody on whole-cell lysates prepared from the rhesus macaque RF/6A choroid-retina endothelial cell line,[14,15] the mouse IEM embryonic endothelial cell line,[13,16] and HUVECs. Western blot analysis indicated the presence of a 6-kDa tbdn-1 protein band in all these endothelial cell lines (Fig. 1, left). As we have described previously, IEM cells display a 69-kDa doublet that could correspond to acetylated and nonacetylated forms of tbdn-1.[13] Furthermore, Ab1272 Western blot analysis of several RF/6A cell clones stably overexpressing an antisense *tbdn-1* cDNA fragment, which was designed to block endogenous tbdn-1 expression, showed a significant decrease or complete absence of the 69-kDa band representing tbdn-1 (Fig. 1, right). These results indicate that the Ab1272 antibody is specific for detecting tbdn-1 protein in RF/6A cells. The marked decrease of the tbdn-1 band in RF/6A cells harboring *tbdn-1* antisense cDNA is similar to what we had previously shown using IEM cells harboring antisense *tbdn-1* cDNA.[13] These results indicate that tbdn-1 can be specifically detected by Ab1272 in primate choroid-retina endothelial cells as well as in mouse and human endothelial cells.

Tbdn-1 Expression in Ocular Endothelial Homeostasis in Adults

Tbdn-1 immunolocalization was performed in normal adult human eye specimens to determine the levels of tbdn-1 expression in normal adult ocular blood vessels. In four of four normal human adult eye specimens, both limbic (Fig. 2A) and retinal (Fig. 2C, 2E) blood vessels showed high levels of tbdn-1 expression in the endothelial cells lining these vessels. We also detected a very similar pattern of tbdn-1 expression in normal choroidal blood vessel endothelium (see choroidal vessels stained in Fig. 2G). The limbic and retinal blood vessels in normal adult human specimens showed the same staining pattern using an anti-von Willebrand factor antibody (retinal vessels are shown in Figs. 2B, 2D), whereas adjacent sections incubated with either normal rabbit serum or preimmune IgY control antibodies showed no staining (an IgY reacted section is shown in Fig. 2F). These results indicate that, in contrast to most vascular beds, tbdn-1 is expressed at high levels in endothelial linings of normal adult ocular blood vessels during homeostasis.

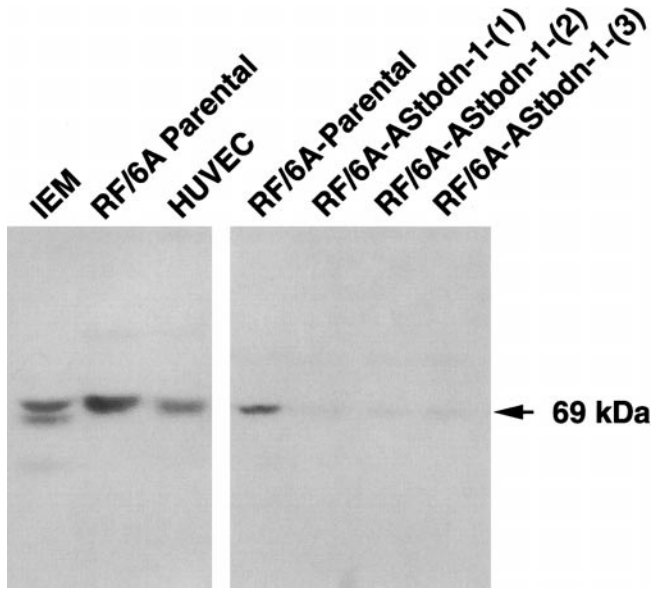

Figure 1. Tbdn-1 was specifically detected by anti-tbdn-1 Ab1272 antibody in mouse and human vascular endothelial cells and in rhesus macaque choroid-retina endothelial cells. Expression analysis of tbdn-1 protein in 50 μg of whole-cell lysate of the mouse IEM embryonic vascular endothelial cell line (IEM), the untrasfected rhesus RF/6A choroid-retina endothelial cell line (RF/6A Parental), human umbilical vein endothelial cells (HUVECs), and three separate clones of RF/6A cells stably expressing a *tbdn-1* antisense cDNA fragment (RF/6A-AStbdn-1-3), as indicated. *Arrow*: 69-kDa tbdn-1 band, which resolves as a doublet in the IEM cells.[12]

To assess whether tbdn-1 is expressed by retinal pericytes in vivo, we also analyzed normal human eye sections double stained for tbdn-1 and ASMA, a marker expressed by pericytes and perivascular contractile cells and not by endothelial cells. Figure 3 shows a representative view of a normal human retinal blood vessel double stained for tbdn-1 (Fig. 3, dark brown stain) and ASMA (bright red stain). The tbdn-1 and ASMA stains did not colocalize in retinal blood vessels in normal human eye sections. These results indicate that tbdn-1 does not appear to be expressed in retinal pericytes at the same high levels at which it is expressed in retinal endothelial cells in vivo.

Suppression of Tbdn-1 Expression during Capillary Formation of a Choroid-Retina Endothelial Cell Line

Our previous work has shown that tbdn-1 protein expression is downregulated during capillary formation of the IEM embryonic vascular endothelial cell line in vitro.[13] Because tbdn-1 expression is maintained at high levels in adult ocular blood vessels, contrary to most other vascular beds, we tested in the current study whether tbdn-1 was regulated in a manner different from IEM cells using a model of choroid-retina endothelial cell capillary outgrowth in vitro. We have previously developed an in vitro capillary formation assay using the IEM cell line[13,16,17] and used the RF/6A endothelial cell line derived from rhesus choroid-retina tissue for a similar application in the current study. By treating either IEM or RF/6A cells with an-

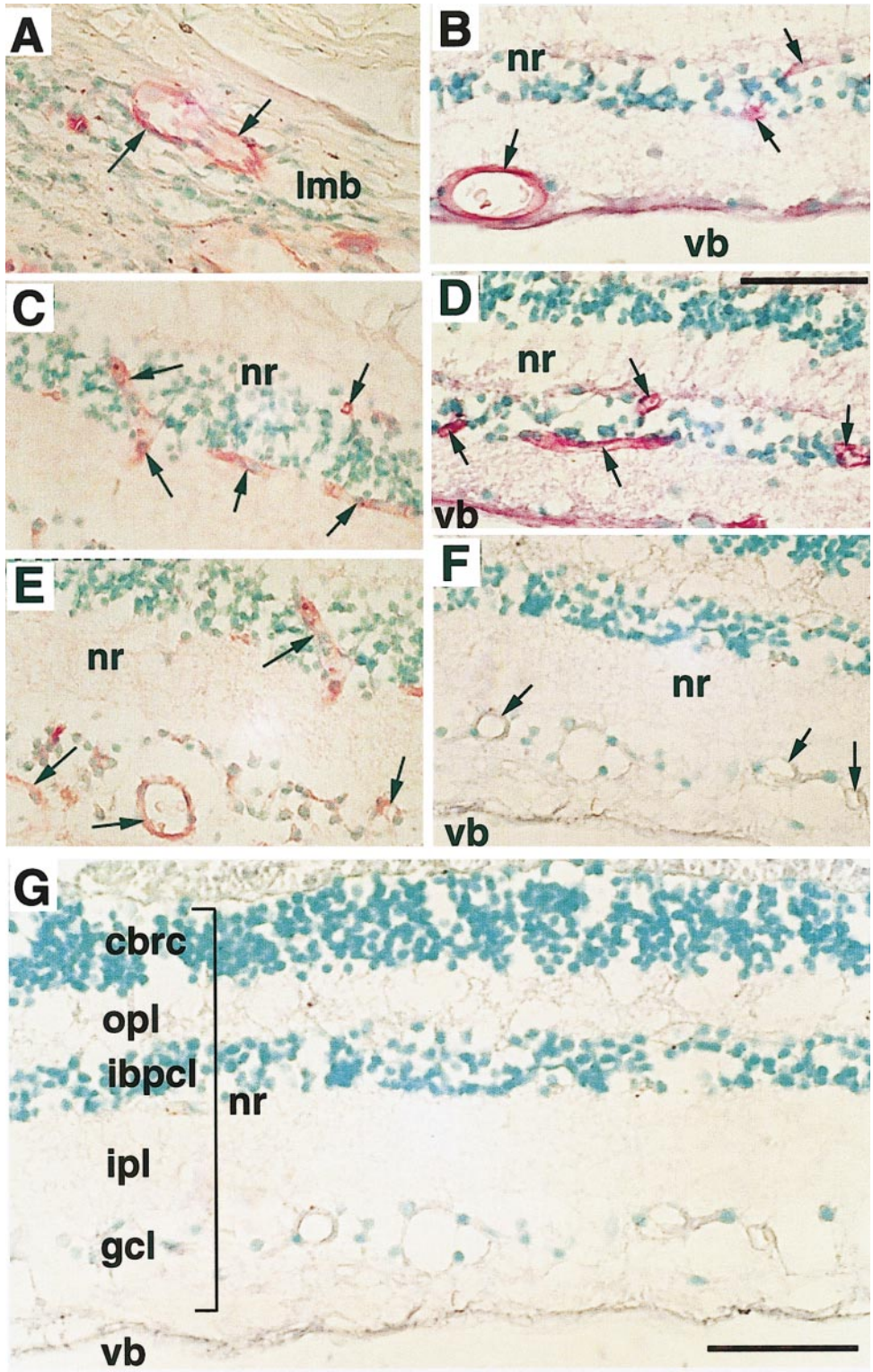

FIGURE 2. Tbdn-1 protein and endothelial marker expression in sections of normal adult human eye. (**A**) Limbic vessel tbdn-1 expression (*red* stain; *arrows*: tbdn-1–positive endothelial cells in a limbic blood vessel). (**C**, **E**) Retinal endothelial tbdn-1 expression in longitudinal- and transverse-sectioned blood vessels in normal adult eye (*red* stain; *arrows:* tbdn-1–positive endothelial cells in retinal blood vessels. (**B**, **D**) Retinal endothelial von Willebrand factor expression in longitudinal- and transverse-sectioned blood vessels in normal adult eye (*red* stain, *arrows*: von Willebrand factor–positive endothelial cells in retinal blood vessels). Adjacent sections stained with equal concentrations of preimmune IgY control antibody showed no staining (**F**). (**G**) A low-power and labeled view of a methyl green–stained section of the retinal areas shown in (**A**–**F**) is provided for orientation purposes. Sections were developed using alkaline phosphatase and fast red substrate; methyl green counterstain. lmb, limbic region of cornea; nr, neural retina; vb, vitreous body; cbrc, cell bodies of rods and cones; opl, outer plexiform layer; ibpcl, integrating bipolar cell layer; ipl, inner plexiform layer; gcl, ganglion cell layer. Scale bar, 50 μm.

giogenic growth factors and then plating the stimulated cells onto a layer of synthetic basement membrane (Matrigel; Collaborative Research, Inc.), we can reproducibly generate colonies of cells sprouting capillary structures (Fig. 4A). These capillary colonies can then be fixed, embedded, and histologically sectioned for immunocytochemical studies as we have previously described for IEM capillary colonies.[13] Of note, little to no staining for tbdn-1 protein was detected in histologic sections of fixed, paraffin-embedded RF/6A cultures induced to form capillary outgrowths in the membrane (Fig. 4 B). However, high levels of tbdn-1 expression were present in histologic sections of fixed, paraffin-embedded RF/6A cells maintained in tissue culture in the absence of any treatment for 48 hours (Fig. 4C). Detection of α-tubulin immunostaining in sections of the preparations of RF/6A capillary cultures (Fig. 4B, inset) confirmed retention of antigenicity in these fixed, paraffin-embedded, and sectioned capillary colony preparations. These results show that a suppression of tbdn-1 expres-

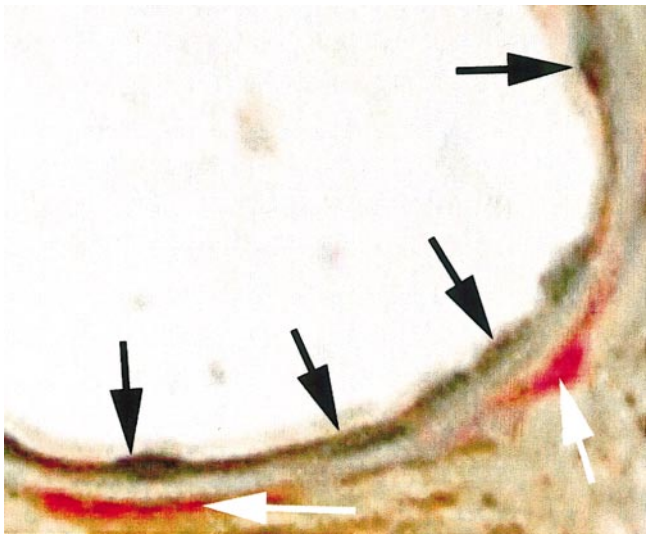

FIGURE 3. Double staining for tbdn-1 and (ASMA) in a retinal vessel of a normal human eye section. Shown is a representative view of a normal human retinal blood vessel double stained for tbdn-1 (*dark brown* peroxidase stain) and ASMA (*bright red* alkaline phosphatase stain). The tbdn-1 and ASMA stains did not colocalize in these retinal blood vessels in normal human eye sections. *Black arrows*: locations of tbdn-1 expression (*brown* staining) in endothelial cells; *white arrows*: locations of ASMA expression (*bright red* staining) in pericyte and perivascular contractile cells. Note that anti-tbdn-1/anti-ASMA double-labeled reactions were overdeveloped to enable clearly revealing the locations of both epitopes.

sion accompanies the induction of capillary formation of RF/6A choroid-retina endothelial cells, in a manner similar to that observed during capillary outgrowth of the IEM cells.

Suppression of Tbdn-1 Expression during Retinal Neovascularization in PDR

Tbdn-1 immunolocalization was performed in diabetic adult human eye specimens in parallel with the normal samples to determine whether the expression characteristics of tbdn-1 in retinal blood vessels change during PDR. Sections of five of five eyes from patients with PDR that were processed and stained simultaneously with the normal human eye samples showed a dramatically lower level of expression of endothelial tbdn-1 protein in the diseased areas of the retinas showing neovascularization. Tbdn-1 was suppressed or completely absent from abnormal proliferating blood vessels and fronds in both preretinal membranes and neural retinal areas in the PDR specimens (Fig. 5C–F for several representative specimens). Quantitative analysis of the red chromogen representing tbdn-1 staining in normal versus PDR specimens by computer (Photoshop; Adobe)[18] revealed a significant decrease of tbdn-1 staining in PDR (10,663 ± 4,740 mean pixels per high-power field; $n = 5$ separate fields from five different eyes) versus normal eyes (173,325 ± 31,042 mean pixels per high-power field; $n = 5$ separate fields from four different eyes). The difference in tbdn-1 staining between normal and PDR specimens was significant ($P < 0.01$ by Student's *t*-test). PDR specimens showed no change in tbdn-1 levels in the limbic vessels in the anterior portion of the eye in the same sections (compare Fig. 5C–F and 4B). Thus, the suppression of tbdn-1 expression occurred in blood vessels within the neural retina and preretinal membranes but did not occur in limbic vessels in the anterior portions of the same PDR specimens. The limbic vessel expression of tbdn-1 in PDR also served as an internal positive control for tbdn-1 expression in these specimens. We also observed that tbdn-1 was downregulated in the choroidal vessels in the PDR specimens in comparison to choroidal vessels in normal specimens (Fig. 5, low-power view). Expression of the endothelial marker von Willebrand factor was detected at high levels, similar to normal retinal blood vessels, in blood vessels showing decreased tbdn-1 expression from the same PDR specimens (Fig. 5C, 5F, insets). These results indicate that tbdn-1 expression is suppressed in abnormal proliferating blood vessels of the neural retina and preretinal membranes in PDR.

DISCUSSION

Tbdn-1 expression peaks during early to middle stages of development of most blood vessels and is downregulated at later stages of maturation, suggesting it may be involved with regulating specific stages of blood vessel maturation during embryogenesis.[13] This is exemplified by tbdn-1 expression in yolk sac vasculature development, in which tbdn-1 is expressed most highly during early stages of yolk sac vasculature formation and is downregulated at the later stages of development during which time angiogenesis of the vitelline vasculature occurs.[13]

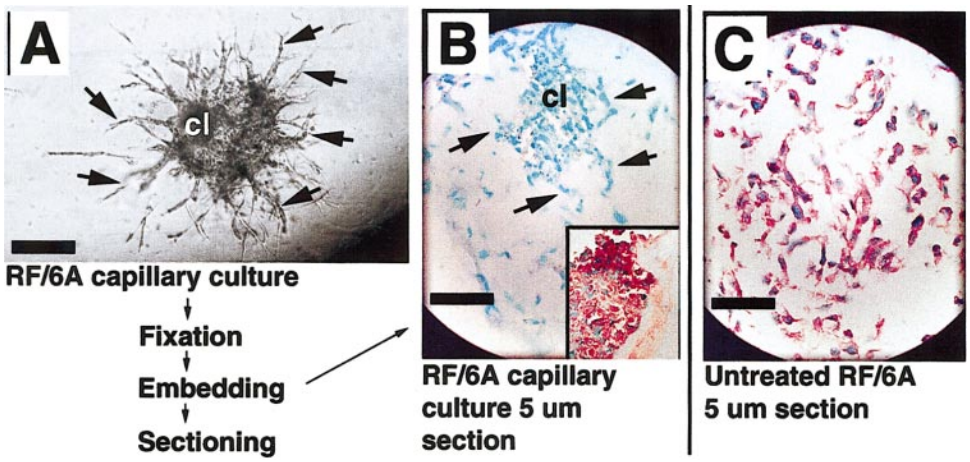

FIGURE 4. Suppression of tbdn-1 protein expression in RF/6A cells in vitro during induction of capillary formation on synthetic basement membrane. (**A**) RF/6A capillary colonies shown in culture before processing. (**B**) After fixation, embedding, sectioning, and staining with anti-tbdn-1 Ab1272, RF/6A capillary colonies showed low staining levels for tbdn-1. (**B**, *arrows*) Similar capillary sprouts as indicated by *arrows* in (**A**); cl indicates main body of the colony. Sections of RF/6A capillary colonies from the same preparation stained very strongly with anti-tubulin positive control antibody (**B**, *inset*, *dark red* stain). (**C**) Sections of RF/6A cells harvested from untreated cultures that were fixed and paraffin embedded were highly positive when stained using anti-tbdn-1 Ab1272 (*dark red* stain). Sections of RF/6A cells and capillary colonies stained with equal concentrations of preimmune IgY were negative (not shown). Staining of sections was developed using alkaline phosphatase and fast red substrate. Methyl green counterstain in (**B**) reveals the capillary sprouts (shown by *arrows* in the capillary colony before processing, in **A**, and after processing, in **B**). cl, sprouting from the RF/6A colonies. Scale bar, 50 μm.

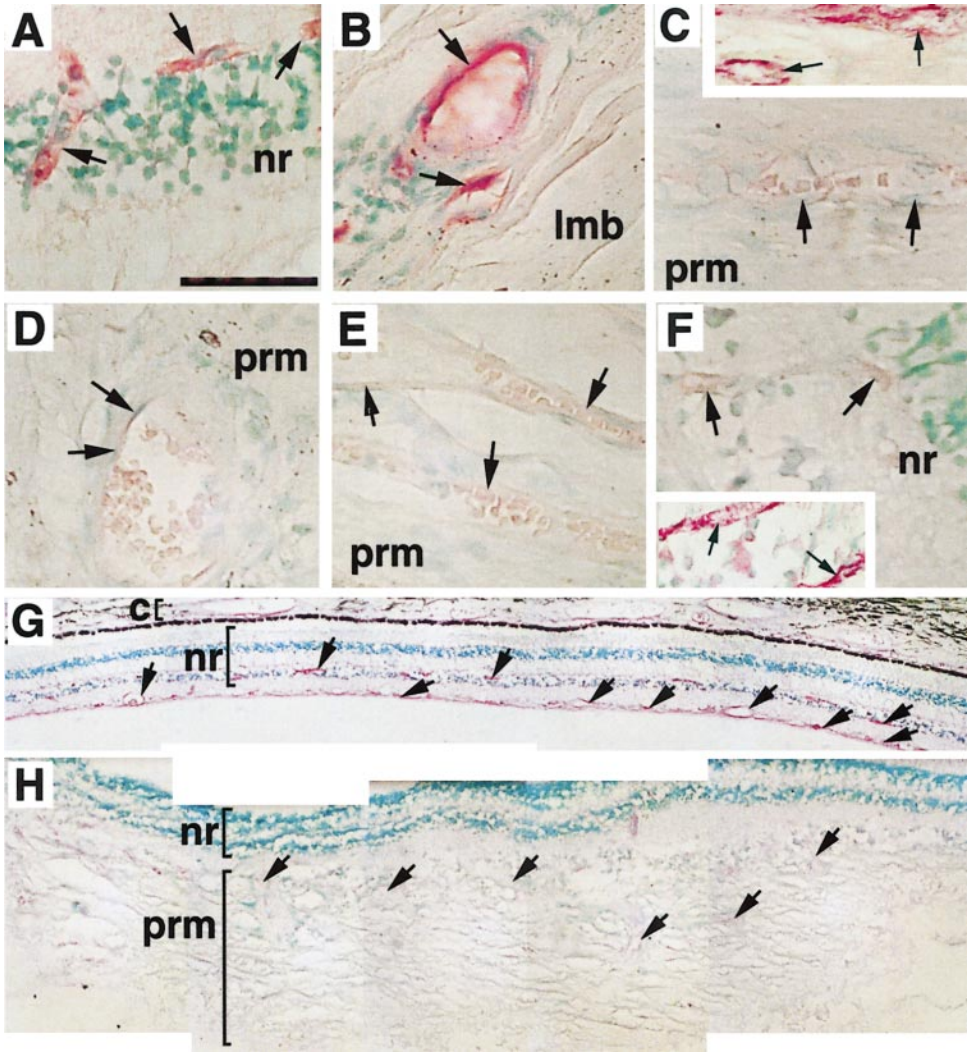

FIGURE 5. Tbdn-1 protein expression was suppressed in specimens of eyes from patients with PDR. (**A**) Retinal endothelial tbdn-1 expression (*arrows*: retinal blood vessels stained *red*) in normal adult eye. (**C–E**) Tbdn-1 staining in blood vessels in preretinal membranes in sections of eyes from three separate representative patients with PDR. (**F**) Tbdn-1 staining in blood vessel fronds cut longitudinally in a neural retinal area in a section of eye from a fourth and separate representative patient. (**C**, **F**, *insets*) von Willebrand factor staining of abnormal blood vessels (*arrows*) in sections from the same PDR specimens and adjacent to those stained for tbdn-1. Blood vessels in the diseased retinal tissue showed either very low levels of tbdn-1 expression or no detectable tbdn-1 expression, compared with normal specimens, whereas the same abnormal blood vessels expressed von Willebrand factor (see also the Results section for quantitative analysis of tbdn-1 expression levels in these sections). (**B**) Tbdn-1 staining (*arrow*, *red*) of limbic blood vessels in the anterior part of the same section as that shown in (**D**) to exemplify normal tbdn-1 expression in unaffected areas of PDR-affected eyes. All sections were also incubated with equal concentrations of preimmune IgY and showed no staining (see example in Fig. 2). Low-power views of a normal retina (**G**) and a diabetic retina with a preretinal membrane (**H**), both stained for tbdn-1 are provided for orientation purposes. Sections were developed using alkaline phosphatase and fast red substrate with methyl green counterstain. lmb, limbic region of cornea; nr, neural retina; vb, vitreous body; c, choriocapillaris; preretinal membrane. Scale bar, 50 μm.

In the adult, tbdn-1 is not ubiquitously expressed in all blood vessels but is restricted to the endothelium of highly specialized vascular beds (e.g., atretic ovarian vasculature and atrial endocardium.[13]).

These studies suggest that tbdn-1 may play a role in some specialized vascular beds during adulthood as well. The results of the present study provides two lines of evidence to suggest that tbdn-1 expression may be involved in maintaining ocular blood vessel homeostasis. First, tbdn-1 expression persisted at high levels in normal adult ocular blood vessels. Second, retinal endothelial tbdn-1 expression was suppressed in neovascularization of PDR. In interpreting these data, it could be argued that the observed loss of tbdn-1 was due simply to loss of endothelial cells or, alternatively, to loss of pericytes, if it were the case that tbdn-1 was also expressed by retinal pericytes. Our present results and the results of others[19] have shown that diseased blood vessels in PDR specimens retain expression of the endothelial marker von Willebrand factor, indicating that the decrease in tbdn-1 expression is not merely a consequence of loss of all vascular endothelial cells in these blood vessels, as has been reported for some vascular beds in certain stages of retinopathy in other studies.[20,21]

Furthermore, to assess whether tbdn-1 could be expressed by pericytes in vivo, we analyzed human eye sections double stained for tbdn-1 and ASMA, a cytoskeletal isoform of vascular actin expressed by pericytes and nonendothelial perivascular contractile cells.[22] Because these markers did not show an obvious colocalization pattern in normal human retina sections, retinal pericytes do not appear to express the same high levels of tbdn-1 as found in retinal endothelial cells in vivo. Our results do not exclude the possibility that tbdn-1 may be expressed in pericytes at very low levels below the limit of detection by these methods.

Nevertheless, all these results taken together indicate that suppression of retinal blood vessel tbdn-1 expression in neovascularization of PDR is a reflection of a decrease in tbdn-1 levels in retinal endothelial cells rather than a reflection of cell loss. Because tbdn-1 is expressed in normal retinal endothelium but is suppressed in retinal endothelium of PDR, our results prompt speculation that a possible functional role for tbdn-1 in normal retinal capillaries may be to participate in a mechanism that may dampen capillary outgrowth. Conversely, because tbdn-1 suppression is associated with the abnormal retinal capillary outgrowth occurring during neovascularization in PDR, removal of such a potential dampening influence of tbdn-1 may permit outgrowth of retinal capillaries in the diabetic environment.

To study tbdn-1 in ocular endothelium in vitro, we used the rhesus RF/6A choroid-retina endothelial cell line[14,15] as a model system. The fact that the primate RF/6A cell line is evolutionarily closer to human than the mouse or bovine renders the RF/6A model more attractive than nonprimate models. Furthermore, RF/6A cells were derived by spontaneous immortalization rather than with the use of exogenous transforming oncogenes. However, it is not known whether RF/6A cells were derived entirely from the choroid, the retina, or a mixture of both structures.[14,15] Therefore, although RF/6A cells may not be a genuine representation of either choroid or retina endothelial cells as they occur in vivo, this may not be a critical factor for the purpose of our study, because tbdn-1 is expressed in both retinal and choroidal vessels in vivo. The RF/6A cell line possesses a number of properties consistent with and characteristic of vascular endothelium,[14,15] and our unpublished observation, 2000, and thus retains some value as an in vitro model system for studies of tbdn-1. Tbdn-1 expression during in vitro capillary outgrowth of RF/6A cells was significantly reduced or absent compared with control cells growing under normal, unstimulated culture conditions in vitro. Our results obtained with the choroid-retina RF/6A in vitro model correlate with downregulation of tbdn-1 during IEM cell in vitro capillary outgrowth.[13]

Most important, downregulation of tbdn-1 during capillary outgrowth of RF/6A correlates with the suppression of tbdn-1 expression we observed in abnormal neural retinal blood vessels, blood vessels and fronds in preretinal membranes, and choroidal blood vessels in PDR specimens. Although retinal and choroidal capillaries are anatomically and physiologically different, choroidal pathologic neovascularization occurs in PDR.[20] The suppression of tbdn-1 in diseased retinal and choroidal vessels is consistent with the pathologic course of PDR. Because the regulation pattern of tbdn-1 in RF/6A cell capillary outgrowth correlates with the regulation pattern of tbdn-1 in retinal neovascularization of PDR, our results indicate that the RF/6A system may serve as a useful model for studies of retinal capillary outgrowth.

Our results suggest that the microenvironment in the disease-affected regions in PDR retinas may harbor a local milieu that supports the downregulation of tbdn-1, in that limbic vascular tbdn-1 levels were not different from normal in the PDR specimens we analyzed (see the Results section and Fig. 5, comparing 5C–F with 5A and 5B). This hypothesis is also supported by our observation that both blood vessels and capillary fronds showed a suppression of tbdn-1 expression in the tissues of PDR-affected retinas. The PDR microenvironment may include factors present in PDR retinal tissue that may lead to downregulation of tbdn-1 levels. Furthermore, the abnormal death of cells such as pericytes in the retinal vascular wall may cause derangements in the diabetic retinal microenvironment to which the remaining and viable retinal endothelial cells become exposed.[21]

A range of angiogenic growth factors (VEGF, bFGF, and IGF-1), integrins, and derangements of extracellular matrix (ECM) components (such as collagen type IV) are associated with pathologic neovascularization in PDR, any or all of which could potentially affect tbdn-1 expression.[3–5,23–27] However, our recent data suggest that tbdn-1 expression levels may be altered by ECM components rather than by direct actions of angiogenic growth factors, such as VEGF and bFGF (Paradis H, Gendron RL, unpublished observations, 2001). Our observation of a similar suppression of tbdn-1 expression during RF/6A choroid-retina capillary outgrowth in vitro is consistent with this evidence, because the synthetic membrane used is a reconstitution of basement membrane components (Matrigel; Collaborative Research, Inc.) and is known to contain a range of ECM components, such as collagen type IV, heparan sulfate proteoglycans, laminin, and entactin.[28,29]

Despite the likely caveats associated with interpreting the regulation of endothelial behavior in reconstitution experiments in vitro and during PDR in vivo, our results indicate a correlation between suppression of tbdn-1 expression and retinal capillary formation occurring in choroid-retina capillary outgrowth in vitro and during neovascularization of PDR in vivo. We are currently in the process of identifying the ECM components that may regulate tbdn-1 expression.

Of particular interest, the expression of tbdn-1 in normal adult retinal blood vessels parallels the expression of pigment epithelium derived factor (PEDF) in adult retina, a recently described novel antiangiogenic serpin family member produced by the normal retinal pigment epithelium. Decreases in the expression levels of PEDF have been observed during oxygen-induced retinal neovascularization in mice and rats,[30,31] and systemic administration of PEDF to mice with ischemia-induced retinopathy prevents retinal neovascularization in this model.[32] It has not yet been determined whether PEDF expression levels are decreased in retinal tissues in human PDR specimens, but it can be predicted that this would be the case. We also do not know at this time whether tbdn-1 can be regulated either directly or indirectly by PEDF.

Although animal models of retinal neovascularization have been studied, little information is available about the intracellular mechanisms in retinal vascular cells that are associated with neovascularization during PDR in human specimens. Polymorphisms of the aldose reductase gene, which may alter aldose reductase mRNA levels within cells, are thought to predispose patients with diabetes to retinopathy through possible disturbances in the polyol pathway and subsequent vascular damage.[33] In diabetes, increases in retinal pericyte expression levels of the proapoptotic protein Bax have been associated in a recent study with increased apoptotic death of retinal pericyte cells.[34] Although they detected no changes in endothelial Bax expression in the specimens analyzed, the investigators indicate that Bax may be regulated with a different time course in retinal endothelial cells in diabetes.[34]

Our finding of high levels of tbdn-1 expression in adult ocular blood vessel endothelial cells during homeostasis and the loss of this expression of tbdn-1 during retinal capillary outgrowth occurring in PDR sheds light on the intracellular processes that are disregulated during neovascularization associated with PDR. The re-expression of tbdn-1 in diseased vessels in PDR may be necessary to restore homeostasis and stop neovascularization. Tbdn-1 is associated with an acetyltransferase activity and contains protein–protein interaction and DNA binding-like motifs.[13] Therefore, if tbdn-1 is indeed mechanistically involved in regulating neovascularization in the eye, it can be speculated that it may act through acetyltransferase activity and/or protein–protein interactions similar to its yeast homologue, the N-terminal acetyltransferase subunit NAT1.[35–38]

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