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Synthesis of a Thrombospondin-Like Cytoadhesion Molecule by Cells of the Trabecular Meshwork

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Thrombospondin is a large extracellular-matrix protein that is released by smooth muscle cells and platelets and is distributed widely in mammalian brain. By using western blotting with a monoclonal antibody against the calcium-binding domain of thrombospondin, the authors detected a 180-kD glycated polypeptide in the trabecular meshwork tissue of normal human and porcine eyes. The epitope-bearing polypeptide was soluble in sodium dodecylsulfate/urea (SDS/urea), and apparently it was stabilized in part by disulfide bonding to the Triton X-100 and SDS/urea-insoluble pellet of this tissue. Treatment of the insoluble matrix with beta-mercaptoethanol led to an enriched extraction of the approximately 160-kD form of thrombospondin. On immunohistochemical study, the thrombospondin antibody also reacted positively with the extracellular matrix and intracellular structures of trabecular cells in primary monolayer culture. This suggested that the presence of thrombospondin in the trabecular meshwork was probably due to local synthesis. These findings are relevant to the establishment of a model in vitro for assessment of adhesion of trabecular cells. Because thrombospondin is a cell–substrate adhesion molecule, its role in the loss of cellularity that occurs in the trabecular meshwork of the aging eye and in eyes with primary open-angle glaucoma is worthy of further investigation. Invest Ophthalmol Vis Sci 32:181–188, 1991

The trabecular meshwork/Schlemm's canal system undergoes age-related alterations in both general protein composition[1] and cellularity.[2,3] These changes indicate an abnormal conformation of the extracellular matrix, a decrease in cytoadhesion, and an overall hyalinization of this specialized connective tissue.[4,5] Currently, the normal and abnormal composition of both the protein and glycosaminoglycan components of the extracellular matrix in the trabecular meshwork is being elucidated.[1,6,7] Direct information obtained by a biochemical approach could be related to the morphologic alterations that have been documented in the trabecular meshwork with advanced age and in primary open-angle glaucoma.[8]

We applied microanalytic methods to individual samples of the trabecular meshwork for the identification and characterization of the molecules that can mediate cell–cell and cell–substrate interactions. Such molecules are almost invariably large glycoproteins.[9,10] We report on the detection of a macromolecular glycated polypeptide that shares immunologic reactivity with thrombospondin and is expressed in the extracellular matrix of trabecular tissue, both in vivo and in vitro, obtained from human and porcine eyes.

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Materials and Methods

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were prepared from six normal human globes (patient age, 42–65 yr) that were obtained at autopsy within 12 hr after death and from four normal adult porcine eyes that were obtained within 2 hr after death. The sections were deparaffinized with xylene and by sequential immersion in descending grades of ethanol, followed by rehydration in distilled water. The primary monoclonal antibody (A6.1) used for immunohistochemical study was raised against the calcium-dependent domain of thrombospondin; this antibody has been characterized previously.[11] Sections were incubated in 1:10 to 1:200 dilutions of A6.1 in phosphate-buffered saline (PBS), pH 7.4, for 2–4 hr at 21°C. For the negative controls, adjacent sections were incubated in a nonimmune mouse serum in place of the antithrombospondin antibody. A streptavidin-biotin-amplified peroxidase staining method was used to detect antibody binding (Biogenex Laboratories, Dublin, CA). We used 3-amino-9-ethylcarbazole as the chromogenic substrate.[12,13]

Finally, the slides were rinsed in distilled water and analyzed by light microscopy.

Trabecular Cell Culture

We used porcine trabecular meshwork explants to initiate primary cell cultures as described previously.[14] The cells were grown on glass cover slips in Eagle's medium supplemented with 15% newborn calf serum and 1% penicillin and streptomycin antibiotics, pH 7.4, for 10–14 days. Confluent primary monolayer cultures were stained by means of a minor modification of the technique of Raugi et al.[15] Briefly, the cells were washed in PBS and fixed in 3% paraformaldehyde for 1 hr at 4°C. After three rinses with PBS, the cells were permeabilized with 0.01% Triton X-100 (TX-100) for 90 sec at 4°C and washed three more times in PBS. The cells were then immunostained with the antibody A6.1 by the streptavidin-biotin-peroxidase method as described for the tissue sections.

Tissue Solubilization

Ten samples of normal human trabecular meshwork were excised within 12 hr after death from seven individuals (age range, 40–57 yr) who had no history of ocular disease or trauma.[1] Individual tissue samples were extracted three times in 250 μl of 0.2% (v/v) TX-100, 2 mM phenylmethylsulfonic fluoride, pH 7.4, for 30 min at 4°C. The TX-100 extract was obtained as the supernatant after centrifugation at 13,000 g for 15 min. To extract the insoluble pellet, we used several 100-μl volumes of 1% (w/v) sodium dodecylsulfate (SDS)/40% (w/v) urea, pH 7.4, at 4°C. The samples were homogenized until no protein was detected in 4-μl aliquots of this SDS/urea extract. The 13,000 g pellet was then placed in 500 μl of 1% (w/v) SDS/40% (w/v) urea/3% (v/v) beta-mercaptoethanol (BME) at 100°C for 5 min, and the homogenate was clarified by centrifugation at 13,000 g, yielding the SDS/urea/BME extract.

Ten eyes from adult male pigs were obtained within 30 min after the animals were killed by decapitation at a local abattoir, and the trabecular meshwork was excised.[14] The samples were solubilized as three extracts (TX-100, SDS/urea, and SDS/urea/BME) as described for the human samples. All porcine and human extracts were stored at −90°C until analyzed further.

Gel Electrophoresis and Immunoblotting

Before electrophoresis, all samples were brought to final concentrations in 1% SDS, 3% BME, and 0.01% (w/v) Pyronin-Y (Sigma Chemical Co., St. Louis, MO), and heated to 100°C for 3 min. They were analyzed by discontinuous SDS-polyacrylamide gel electrophoresis on minigels (43 × 50 × 0.45 mm) of 7.5% total acrylamide with 5% total acrylamide stacking gels. Each lane was loaded with 4 μl of the trabecular meshwork/Schlemm's canal tissue extract, and they underwent electrophoresis with Phastgel buffer strips (Pharmacia, Picataway, NJ) composed of 0.20 M Tricine, 0.20 M Tris, and 0.55% (w/v) SDS for 60 V-hr at 10.0 mA/3.0 W/15°C. The TX-100, SDS/urea, and SDS/urea/BME extracts from each sample were analyzed on the same gel, and the gels were stained with silver. The amount of total protein applied to each lane was normalized by dilution with sample buffer (1% SDS/3% BME, pH 7.4), as determined with an Ultroscan XL scanning laser densitometer (LKB, Piscataway, NJ) and accompanying Gelscan XL software.

Equal amounts of total protein (2–5 μg) were analyzed for the presence of thrombospondin by immunoblotting with the monoclonal antibody A6.1 by using a modification of the method of Towbin et al.[16] Briefly, unstained gels were transferred to nitrocellulose (0.2 μm; Schleicher and Schuell, Keene, NH) in 25 mM Tris, 192 mM glycine, 20% methanol for 5 V-hr at 25.0 mA/1.0 W/15°C. The nitrocellulose sheets were blocked overnight with 5% (w/v) bovine serum albumin in buffer consisting of 150 mM Tris, 5 mM ethylendiaminetetraacetic acid, 150 mM NaCl, and 0.05% Nonidet-P 40 (NET, Sigma), pH 7.4. All subsequent washes and antibody dilutions were performed in NET buffer. We incubated the nitrocellulose sheets with monoclonal A6.1 at a 1:200 dilution[11] for 4 hr at 21°C. After three 10-min washes, a peroxidase-conjugated secondary antibody consisting of rabbit anti-mouse immunoglobulins was applied at a concentration of 0.06 g/l for 1 hr at 21°C. The nitrocellulose was washed again three times, and we detected the binding of the secondary antibody by means of 0.04% (w/v) diaminobenzidine-tetrahydrochloride and 0.03% hydrogen peroxide.[17]

Purified thrombospondin isolated from platelets[11,18] was used as a positive control for immunoblotting. In some experiments, as a further control, nonimmune mouse serum was substituted for the primary antibody. The relative molecular weights of immunoreactive polypeptides were calculated by linear regression based on the migration of calibration proteins (myosin, 212 kD; alpha-macroglobulin, 170 kD; beta-galactosidase, 116 kD; transferrin, 76 kD; and glutamic dehydrogenase, 56 kD) that had been stained with Ponceau-S.[19]

Results

Immunohistochemistry

In the tissue sections of the human and porcine globes, a positive immunoreaction with the anti-

thrombospondin antibody was observed in the trabecular meshwork (Figs. 1A,2A). Positive staining was also observed in the corneal endothelium, episclera, meninges of the optic nerve, and the retinal and ciliary blood vessels. Staining of the sclera and ciliary epithelium was irregular. No immunoreactivity was observed in control sections exposed to goat serum in place of the A6.1 antibody (Figs. 1B,2B).

Cell Cultures

Immunohistochemical staining of primary confluent cultures of porcine trabecular cells with A6.1 revealed both an intracellular and an extracellular location of thrombospondin. Patchy staining was observed on the plasma membrane of the trabecular cells, and a granular immunoreaction was present in the cytoplasm around the nucleus (Fig. 3). Controls, in which nonimmune goat serum was used in place of the A6.1 primary antibody, showed no detectable reaction product deposited either intracellularly or on the cell surface (Fig. 4).

Immunoblotting

Thrombospondin was detected as a single immunoreactive fraction at approximately 180 kD in the SDS/urea-soluble fractions of all samples of human and porcine trabecular meshwork/Schlemm's canal tissue analyzed (Fig. 5). Purified thrombospondin comigrated with this polypeptide and bound A6.1 on the same immunoblots (Fig. 5). The A6.1 antibody also recognized a fraction at approximately 160 kD that was present exclusively in the SDS/urea/BME extracts of these samples from both human and porcine eyes. No binding of the antibody was seen to the TX-100-soluble fractions from any samples or on control blots when a nonimmune serum was substituted for the primary antibody.

Discussion

Thrombospondin is a high molecular-weight (420 kD), sialated glycoprotein which is composed of three apparently identical subunits joined by disulfide bonds.[20] Originally described as the major component of platelet alpha granules,[21] it is now known to mediate platelet aggregation[11,18] and to be distributed widely in mammalian brain. This polypeptide is present in basement membranes, vessel walls, and glandular connective tissue.[22] It is apparently synthesized for secretion by several distinct cell types, such as smooth muscle cells,[23] endothelial cells,[24,25] fibroblasts,[26] glial cells,[27] megakaryocytes,[28] osteoblasts,[29] macrophages,[30] and keratinocytes.[31,32]

In a recent preliminary study, thrombospondin was detected in the cornea.[33] The distribution of thrombospondin in the extracellular matrices of other ocular structures has not been investigated previously. Our immunohistochemical results for tissue sections of human and porcine globes suggested that thrombospondin was a major component of many structures of the eye, including the aqueous outflow pathway. However, the goal of the current study was to investigate thrombospondin in the trabecular meshwork/Schlemm's canal system and not to elaborate on its general distribution in the eye.

To investigate further the presence of thrombospondin in the aqueous outflow pathway, we analyzed pure samples of trabecular meshwork by using immunoblotting. Our results support at least three conclusions: (1) the thrombospondin that is present in the TX-100-insoluble extract of the trabecular meshwork is composed of the approximately 180 kD monomers (under reducing conditions); (2) some of the thrombospondin in the meshwork tissue can be solubilized only by reduction of intermolecular disulfide bonding; and (3) this latter population of thrombospondin migrates on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) exclusively as the 160-kD form of the monomer. Although the molecular weight of thrombospondin is 420 kD as determined by sedimentation experiments, it is well recognized that its monomers will migrate at apparent molecular weights ranging from 140–185 kD on SDS-PAGE depending on the conditions of solubilization and electrophoresis.[20] Thus, our findings are consistent with those reported previously. Because the smaller monomeric form probably results from proteolytic cleavage near the amino terminus of thrombospondin,[20] it is possible that the prolonged solubilization time that we used for obtaining the SDS/urea/BME extract accounts for complete conversion to its apparent 160-kD form. Alternatively, the process of heat denaturation and reduction of intermolecular disulfides by BME may result in an increased susceptibility of the thrombospondin to proteolytic cleavage.

The monoclonal antibody A6.1 recognizes the calcium-dependent domains in the secondary structure of thrombospondin which are located near the carboxy terminus.[11] The binding of the antibody is reduced considerably when calcium is bound to thrombospondin; therefore, our immunoblotting experiments were done in the presence of a chelating agent. The apparent absence of these calcium-binding structures from other substrate adhesion molecules, such as fibronectin,[34] and the binding of A6.1 to pure thrombospondin in control experiments, support the specificity of this monoclonal antibody.

Because smooth muscle cells are known to secrete thrombospondin,[23] the results of our immunoblot-

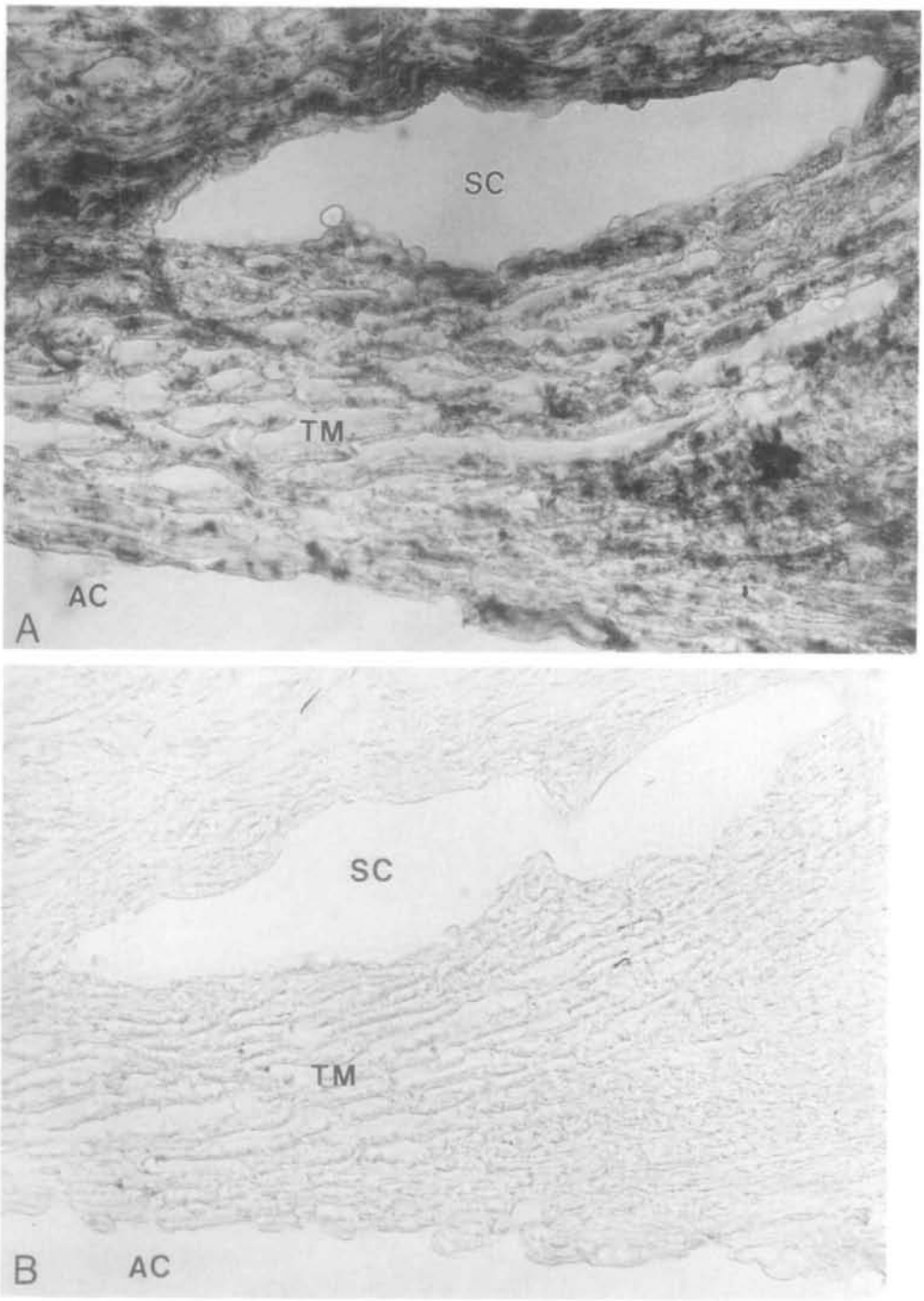

Fig. 1. (**A**) Section of human eye stained immunohistochemically with antithrombospondin antibody. The cells and the beams of the trabecular meshwork (TM), as well as the inner wall of Schlemm's canal (SC) show a positive immunoreaction. (**B**) Negative control. The section was incubated in nonimmune serum in place of the antithrombospondin antibody. No staining of any structure is seen. AC, anterior chamber (original magnification ×540).

ting experiments do not exclude the possibility that this molecule is synthesized by neighboring structures, such as the ciliary muscle, and deposited in the trabecular meshwork. Therefore, we investigated whether thrombospondin is present in trabecular cells that have been propogated as primary cell cultures. The results clearly showed that the thrombospondin which is incorporated in the extracellular matrix of the trabecular meshwork is produced by the trabecular cells. The binding of A6.1 to the plasma membrane and that in perinuclear cytoplasmic granules correspond exactly to the distribution pattern of thrombospondin that has been found in glomerular mesangial cells[15,35] and the pattern observed in cultured osteoblasts.[36] The perinuclear distribution of thrombospondin in the trabecular cells is probably related to its presence in the endoplasmic reticulum, Golgi bodies, and secretory granules,[15,35] and it corroborates the notion of local synthesis of the molecule in the trabecular meshwork.

Thrombospondin possesses discrete domains that have the capacity to interact with various molecules

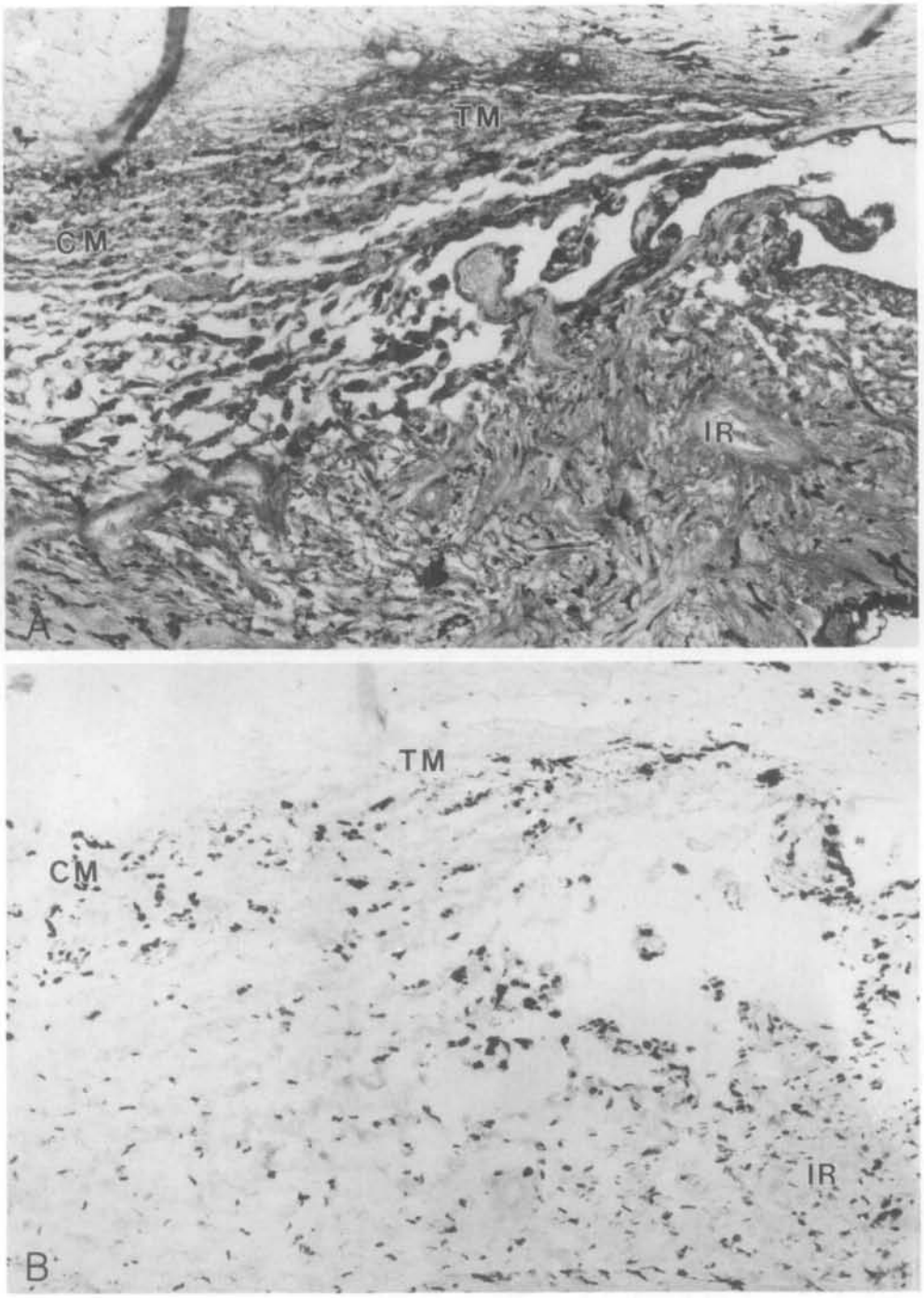

Fig. 2. (A) Section of porcine eye stained immunohistochemically with antithrombospondin antibody. Positive immunoreaction is present in the trabecular meshwork (TM), iris (IR), and ciliary muscle (CM). (B) Control section incubated in nonimmune serum in place of the anti-thrombospondin antibody. No immunostaining of any structure is present. The presence of pigmented cells in the angular region is a normal finding. TM, trabecular meshwork; IR, iris; CM, ciliary muscle (original magnification ×150).

such as type IV collagen, fibrinogen, fibronectin, laminin, plasminogen, plasminogen activator, and heparan sulfate.[37] Several of these polypeptides are present in the trabecular meshwork.[38–43] Because of its capacity to bind and colocalize with many components of the extracellular matrix, thrombospondin is well suited to serve a structural role in the aqueous outflow pathway. However, further investigations are needed for determining whether an increase in the amount of this sialoglycoprotein accounts for accumulation of extracellular matrix material in the trabecular meshwork of eyes with primary open-angle glaucoma[8,44] or whether alterations in its sialation contribute to the increase in sialic acid moieties that has been implicated in abnormal aqueous outflow.[45]

Like fibronectin and several of the other substrate adhesion molecules,[46] thrombospondin contains an arginine-glycine-aspartic acid (RGD) sequence in its polypeptide structure[47] that modulates cell adhesion. By detecting thrombospondin in the trabecular meshwork, we identified another candidate for mediation of the reduced cellularity that occurs with age

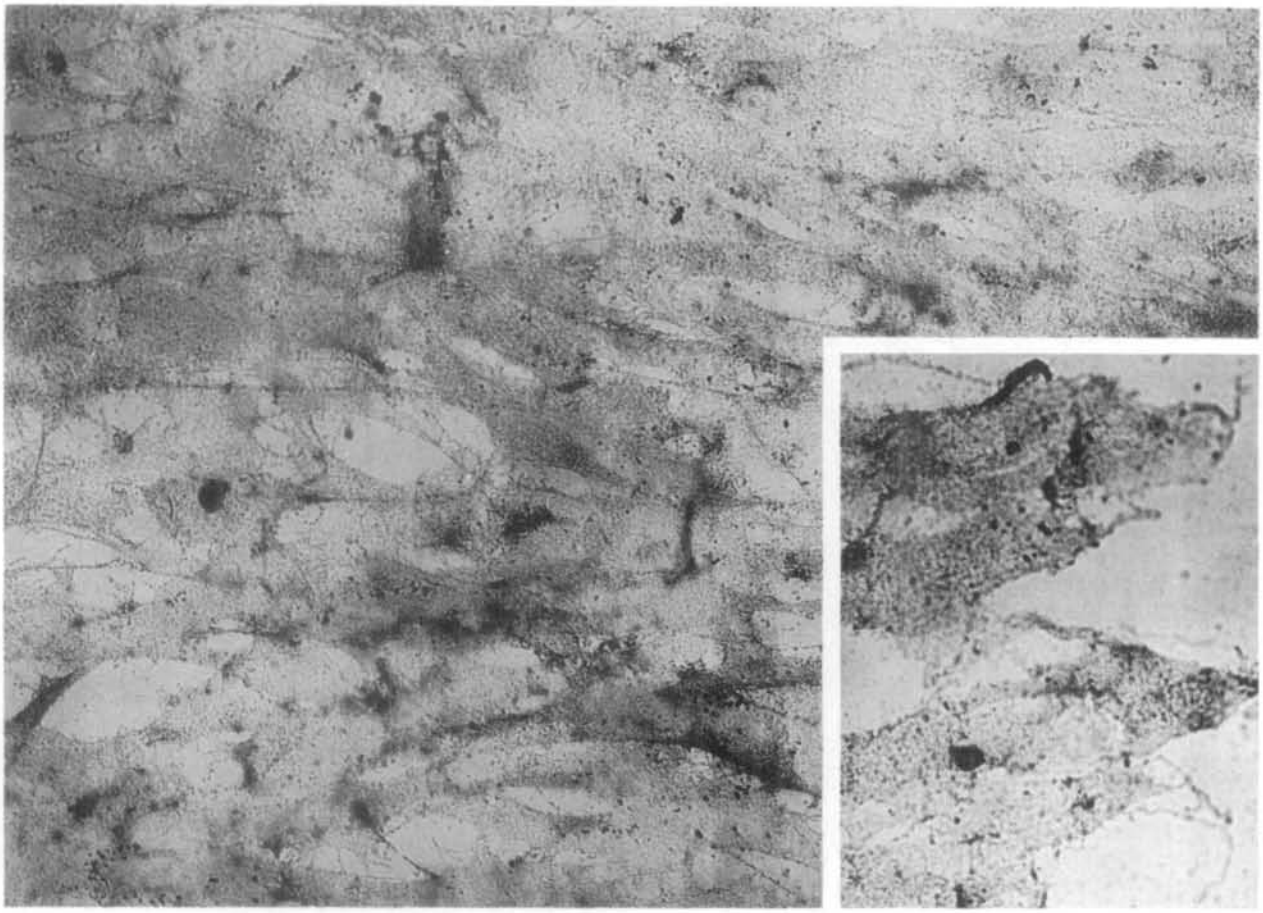

Fig. 3. Primary confluent culture of porcine trabecular cells stained immunohistochemically with anti-thrombospondin antibody. A granular positive immunoreaction is present within the cytoplasm, on the plasma membrane, as well as in the extracellular matrix (×275). Inset shows higher magnification of individual cells at the periphery of the culture (×550).

Fig. 4. Negative control. Primary culture of porcine trabecular cells incubated in nonimmune goat serum in place of the A6.1 thrombospondin primary antibody. No reaction product is seen. N, nucleus. Nomarski optics (original magnification ×550).

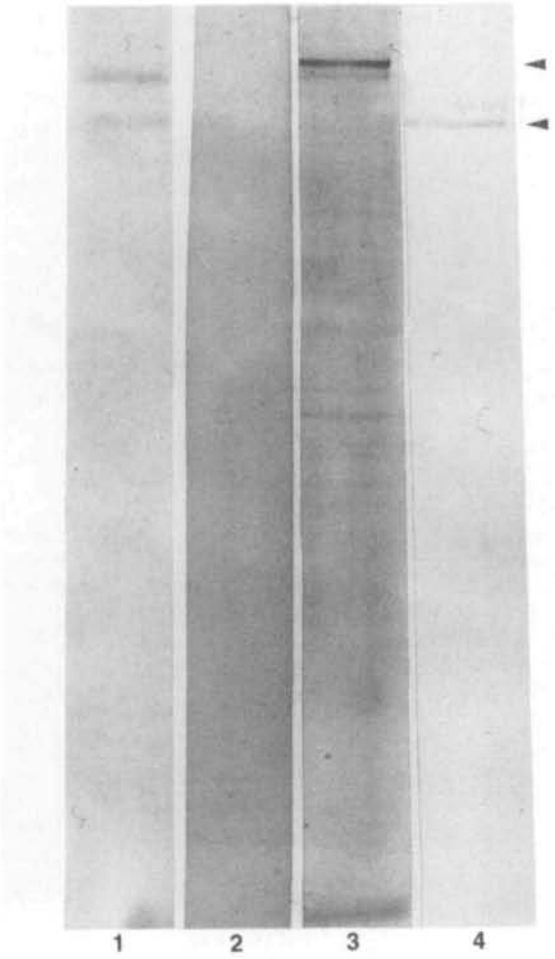

Fig. 5. Detection of thrombospondin in human trabecular meshwork on immunoblots with antibody A6.1. Lane 1: Pure thrombospondin (positive control). Lane 2: TX-100 extract. Lane 3: SDS/urea extract. Lane 4: SDS/urea/BME extract. An immunoreactive fraction is present at approximately 180 kD in lanes 1 and 3, and at approximately 160 kD in lane 4 (upper and lower arrows, respectively). No reaction is present in lane 2.

and with the progression of primary open-angle glaucoma.[2,3] Although thrombospondin is also known to influence cellularity by its interaction with trophic factors such as platelet-derived growth factor, transforming growth factor-β, and epidermal growth factor,[48,49] at present we can only speculate as to its role in exerting such an influence in the trabecular meshwork. However, our documentation that porcine trabecular cells in primary culture synthesize and secrete thrombospondin opens up the possibility of using this model to evaluate the effects of structural perturbation or of the addition of exogenous mediators of adhesion (ie, growth factors or tissue plasminogen activator) to delineate more precisely the role of thrombospondin in the maintenance of adhesion of trabecular cells to the beams and, consequently, in the modulation of outflow of aqueous humor in normal and glaucomatous eyes.

Key words: aqueous outflow pathway, cell culture, cell–substrate adhesion, extracellular matrix, gel electrophoresis, glycoprotein, immunohistochemistry, primary open-angle glaucoma, western blotting

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