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# Identification and Partial Characterization of TGF-β1 Receptors on Trabecular Cells

Navaneet S. C. Borisuth, Brenda J. Tripathi, and Ramesh C. Tripathi

**By using two specific receptor assays, we identified and partially characterized receptors for transforming growth factor-β1 (TGF-β1) on porcine trabecular cells in vitro. Cultured trabecular cells were incubated with labeled TGF-β1 and analyzed by flow cytometry. Pretreatment with trypsin or preincubation with cold TGF-β1 or a neutralizing antibody to TGF-β1 inhibited the binding of labeled TGF-β1. $^{125}$I-TGF-β1 was cross-linked covalently to cell surface receptors on the trabecular cells. By SDS-PAGE and autoradiography, we identified three labeled macromolecular species of receptors, two of which had apparent molecular weights greater than 212 kDa and one of which had an apparent molecular weight of approximately 80–90 kDa under reducing conditions. The low and high molecular weight species probably represent type II and type III TGF-β1 receptors, respectively. At concentrations of 0.5 and 1 ng/ml, activated TGF-β1 caused retraction and a marked decrease in the rate of proliferation and in the motility of trabecular cells in vitro. Our findings implicate TGF-β1 in the modulation of the functional homeostasis of trabecular cells and suggest that the aqueous humor contains a level of TGF-β1 which, once activated, is sufficient to exert a biologic effect on the trabecular meshwork. Invest Ophthalmol Vis Sci 33:596–603, 1992**

The transforming growth factor-βs (TGF-βs) are a family of homodimeric polypeptides with a molecular weight of approximately 25 kD[1,2] that exhibit a broad range of activities.[3] Recently, significant amounts of TGF-β1 and -β2 have been reported in aqueous humor from human, murine, porcine, bovine, rabbit, and canine eyes.[4–7] Although the action of TGF-β in the aqueous humor is presumed to be mediated through its binding to specific surface receptors, no information exists on the presence of TGF-β binding protein(s) in the avascular tissues that border the anterior and posterior chambers of the eye.

In the absence of information on the existence of TGF-β receptors or of studies in vitro on the effect of TGF-βs on cells of the anterior segment of the eye, the role of this family of growth factors in the modulation of aqueous outflow and in ocular wound healing remains speculative.[5,6] In this study, we identified and biochemically characterized TGF-β1 receptors on the surface of cultured porcine trabecular cells and determined that TGF-β1, at the concentrations in the aqueous humor, can modulate the growth and function of trabecular cells in vitro.

From the Department of Ophthalmology and Visual Science, The University of Chicago, Chicago, Illinois.

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Reprint requests: Professor Ramesh C. Tripathi, Visual Sciences Center, The University of Chicago, 939 East 57th Street, Chicago, IL 60637.

## Material and Methods

We used purified human platelet TGF-β1 and chicken anti-human platelet TGF-β1 neutralizing antibody from Oncomembrane Inc. (Seattle, WA); porcine platelet TGF-β1, human platelet TGF-β2, and the fluorokine TGF-β1 flow cytometry system from R & D Systems (Minneapolis, MN); avidin-phycoerythrin (avidin-PE) from Calbiochem Immunochemicals (La Jolla, CA); reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) from Hoefer (San Francisco, CA); Eagle's minimal essential medium from Gibco Laboratories (Grand Island, NY); and (ethylenedinitrilo)tetraacetic acid, disodium salt (EDTA) from J. T. Baker, Inc. (Phillipsburg, NJ). $^{125}$I-TGF-β1 was a gift of Dupont Co. (Boston, MA).

### Trabecular Cell Culture

We used porcine trabecular meshwork explants to initiate primary cell cultures as described previously.[8] Briefly, porcine eyes were obtained from a local abattoir within 20 min after decapitation and were opened by a circumferential incision at the ora serrata. After placing the anterior segment in a petri dish with its

external surface down and removing the lens, we cut the anterior segment into halves. The iris was lifted carefully from the inner corneal surface, and the pectinate ligaments were cut at their insertion into the periphery of the cornea and the uveal meshwork. The samples of trabecular meshwork were excised and placed in tissue culture medium (Eagle's minimal essential medium supplemented with 15% fetal calf serum). After the meshwork was cut into 3–4 mm wide pieces, the explants were placed in tissue culture dishes and incubated at 37°C in culture medium. Outgrowth of porcine trabecular cells occurred within 1–2 weeks after explantation. All receptor studies were performed on confluent primary cultures, whereas the motility and proliferation assays were conducted on preconfluent primary cultures after the original explants were removed.

### Receptor Binding Assay

We detected TGF-β1 receptors on trabecular cells by using biotinylated TGF-β1 and a fluorokine flow cytometry kit according to the manufacturer's specifications (R & D Systems). After aspiration of the culture medium, cultures were washed twice with phosphate-buffered saline (PBS; 0.15 M, pH 7.2) to remove of any residual serum components. The cells were treated with 0.5 mM EDTA for 10 min, which facilitated their removal from the substrate. A single cell suspension of viable trabecular cells was obtained by vigorous pipetting. The trabecular cells were then centrifuged at 500 × *g* for 5 min and washed twice in binding buffer (PBS, bovine serum albumin [BSA], 0.02% sodium azide, pH 7.4). The cells were counted with a hemocytometer and suspended in binding buffer at a final concentration of $4 \times 10^6$ cells/ml.

To test the sensitivity of the receptors to trypsin, cultured cells were disaggregated by mild trypsinization (0.2% trypsin, 5–10 min) and analyzed immediately for TGF-β1 binding. Alternatively, after trypsinization, the cells were incubated for an additional 7 hr at 37°C in culture medium on a rocker platform and then analyzed by flow cytometry. This incubation period allows for regeneration of the receptors.[9,10] The cells were then washed and suspended in binding buffer as described above.

For flow cytometry, 10 μl of biotinylated TGF-β1 (10 μg/ml) was added to 25 μl of washed cell suspension in a siliconized glass tube. After incubation for 1 hr at 4°C, the cells were washed twice with 2 ml of binding buffer. The samples were then resuspended in 150 μl of binding buffer containing 10 μl of avidin conjugated to fluorescein isothiocyanate (FITC) (10 μg/ml). After an additional incubation in the dark for 30 min at 4°C, the cells were washed three times with 2 ml of binding buffer and analyzed by flow cytometry. The background level of fluorescence was determined by incubation of the trabecular cells in avidin-FITC alone.

Specific competition experiments were performed by preincubation of 25 μl of the trabecular cell suspension with 200 ng of unlabeled TGF-β1 or unlabeled TGF-β2 for 1 hr at 4°C. This suspension was incubated with biotinylated TGF-β1 as already described. An additional control included preincubation of biotinylated TGF-β1 with 1 mg/ml of neutralizing antibody to TGF-β1 (37°C, 30 min) before the mixture was added to trabecular cells for analysis. We calculated the percent inhibition of competition by setting the binding of the biotinylated TGF-β1 by the trabecular cells in the absence of unlabeled TGF-β or of neutralizing antibody at 100%. The percent inhibition is the proportion of binding that is suppressed by the unlabeled TGF-β1 or by the neutralizing antibody.[10]

### Flow Cytometry

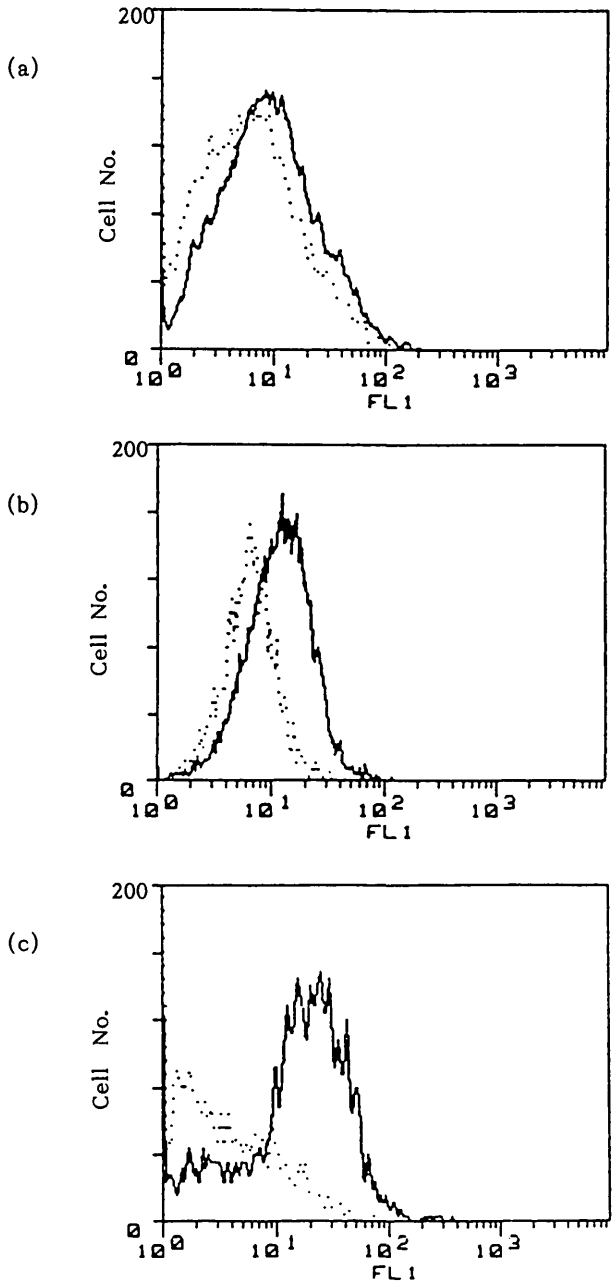
We used a Becton Dickinson (Mountain View, CA) FACScan equipped with an argon laser. We used a forward-angle versus a 90°-angle light scatter gating and a forward-angle threshold to exclude clumped cells and doublets from analysis. All data analysis was performed on a Hewlett-Packard (Cupertino, CA) Consort 30 computer work station. A minimum of 5000 gated events were collected for each analysis, and frequency distribution plots of the fluorescence signals were generated.

### Affinity Cross-Linking of $^{125}$I-TGF-β1 to Trabecular Cells

Affinity labeling of receptors on trabecular cells was accomplished by using a previously described method.[11] Confluent cultures of trabecular cells were washed once with binding buffer (128 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.2 mM $CaCl_2$, 50 mM HEPES, pH 7.5, 2 mg/ml BSA) at 4°C and were allowed to equilibrate in a second wash of binding buffer for 30 min at 4°C. After the wash was aspirated, the trabecular cells were incubated with labeling buffer (50 pM of 2.18 μCi/pmol $^{125}$I-TGF-β1 in 4 ml binding buffer per dish) for 4 hr at 4°C on an oscillating platform. The unbound material was aspirated, and the monolayer was washed five times with ice-cold binding buffer.

Cross-linking of the radiolabel to the trabecular cells was accomplished by adding 0.14 mM dissuccinimidyl suberate (DSS) in binding buffer without BSA. A 27 mM stock solution of DSS in dimethyl sulfoxide was prepared prior to the incubation. After 15 min at

4°C, the monolayers were rinsed briefly with detachment buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4, 0.3 mM phenylmethylsulfonyl fluoride [PMSF]) and removed with a disposable Teflon scraper in the presence of 1 ml detachment buffer. Each dish was rinsed with a second aliquot of 0.5 ml detachment buffer. The cells were sedimented by centrifugation at 12,000 *g* for 2 min, and the supernatant was discarded. The pellets were then incubated, with frequent shaking, for 40 min at 4°C in a solubilization buffer (125 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.0, 1% volume/volume Triton X-100 supplemented with 10 μl/ml protease inhibitor cocktail that consisted of 1 mg/ml each of leupeptin, antipain, pepstatin, and bestatin; 5 mg/ml aprotinin, 10 mg/ml each of soybean trypsin inhibitor and benzamidine hydrochloride; and 30 mM PMSF). The insoluble cell debris was removed by centrifugation of the mixture for 15 min at 12,000 *g*. The supernatant was processed for SDS-PAGE and autoradiography as described below.




### SDS-PAGE and Autoradiography

Aliquots of $^{125}$I-TGF-β1, TX-100 cell membrane extract, or $^{125}$I-TGF-β1-labeled receptors were mixed in electrophoresis buffer (70 mM Tris, pH 6.8, 2% SDS, 3% 2-mercaptoethanol, 10% glycerol, and 0.01% pyronin-Y) and heated at 100°C for 5 min. The polypeptide fractions were separated by discontinuous SDS-PAGE on 0.75 mm-thick, 6% total acrylamide gels using a Laemmli buffer system.[12] The polypeptides separated on the gels then were either stained by a sensitive silver method[12,13] or dried and subjected to autoradiography at −80°C with X-Omat Kodak AR film (Eastman Kodak, Rochester, NY) and DuPont Lightning Plus intensifying screens (Dupont Co., Boston, MA). We used high- and low-molecular-weight protein markers to determine the molecular weight of the samples.

### Motility and Proliferation Assay

Preconfluent primary cultures of trabecular cells were exposed to TGF-β1 in fresh medium at a concentration of 0.5 or 1 ng/ml for 48 hr. These concentrations were chosen because they correspond to the levels of TGF-β1 in the aqueous humor of man and animals.[6,7] The medium and growth factor were replaced every 24 hr. The cultured cells were monitored continuously and analyzed for alterations in morphology and motility, as well as rate of mitosis with a

**Fig. 1.** Results of flow-cytometric analysis of the binding of fluorescinated TGF-β1 to trabecular cells. FL1: intensity of green fluorescence ($\log_{10}$) plotted on X axis; number of cells plotted on Y axis. The continuous line denotes trabecular cells treated with biotinylated TGF-β1 followed by avidin-FITC. The dotted line denotes trabecular cells treated with avidin-FITC only and represents the level of autofluorescence. (**a**) Analysis of trabecular cells obtained after disaggregation with 0.25% trypsin for 5 min and treated immediately with biotinylated TGF-β1. Trypsin treatment cleaves receptors for TGF-β1. There is no significant difference in the intensity of fluorescence of labeled cells and that of autofluorescence. (**b**) Analysis of trabecular cells that were treated with trypsin as above, but were incubated for an additional 7 hr at 37°C in culture medium, and then analyzed by flow cytometry; this incubation period allows for partial regeneration of the receptors. The intensity of fluorescence of labeled cells is increased two-fold compared with the peak level of autofluorescence. (**c**) Analysis of trabecular cells that were treated with 0.5 mM EDTA for 10 min. The peak of the intensity of fluorescence of the labeled cells is increased ten-fold compared with the peak level of autofluorescence.

phase contrast microscope equipped with an ultra-low-light time-lapse video recorder. The rate of mitosis is expressed as the number of mitotic events/10 × field/2 hr.

## Results

### TGF-β1 Binding Proteins on Trabecular Cells

Through flow cytometry, we demonstrated the presence of trypsin-sensitive binding proteins for TGF-β1 on trabecular cells in vitro. Analysis of trabecular cells immediately after treatment with trypsin revealed only a small shift in the fluorescence intensity (Fig. 1a). The intensity of the fluorescence peak increased two-fold compared to the level of autofluorescence when trypsinized trabecular cells that had been incubated in culture medium for 7 hr on a rocking platform were analyzed (Fig. 1b). However, for labeled trabecular cells that had been detached and disaggregated by EDTA treatment and vigorous pipetting, respectively, a ten-fold increase in the fluorescence intensity compared to the mean level of autofluorescence was obtained (Fig. 1c). We observed only a modest inhibition (19%) of the binding of biotinylated TGF-β1 upon preincubation of trabecular cells in the presence of a two-fold excess of unlabeled TGF-β1. No inhibition of the binding was observed when the cells were preincubated with unlabeled TGF-β2. For the experiments in which TGF-β1 was preincubated with the neutralizing antibody, a 55% decrease in the binding between biotinylated TGF-β1 and the trabecular cells was observed (data not shown).

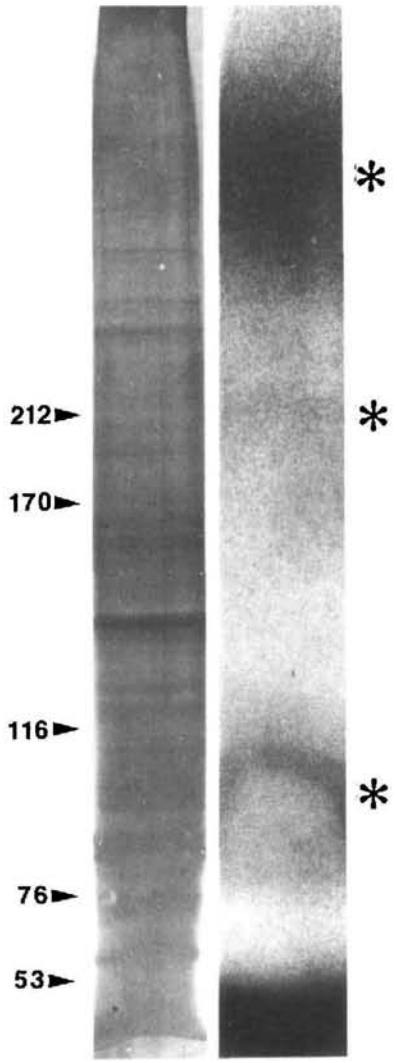



Fig. 2. Affinity labeling of intact porcine trabecular cells with $^{125}$I-TGF-β1. Confluent cultures of porcine trabecular cells were incubated with 50 pM of 2.18 μCi/pmol $^{125}$I-TGF-β1 for 4 hr at 4°C. Cross-linking was accomplished by addition of 0.14 mM DSS. The cells were then scraped off the dishes, sedimented, and extracted with 1% Triton X-100 buffer. Aliquots were subjected to SDS-PAGE under reducing conditions on 0.75 mm thick 6% gels and either stained with silver (left) or subjected to autoradiography (right). Note the three affinity-labeled fractions (asterisk) that correspond to the type II and III receptors for TGF-β1. The molecular weight markers were 212 kDa myosin, 170 kDa $\alpha_2$ macroglobulin, 116 kDa β-galactosidase, 76 kDa transferrin, and 53 kDa glutamic dehydrogenase.

### Affinity Cross-Linking of $^{125}$I-TGF-β1 to Trabecular Cells

The membrane binding sites for TGF-β1 on trabecular cells were further characterized by affinity cross-linking. Analysis by SDS-PAGE under reducing conditions revealed three macromolecular species representing the products of the cross-linking reaction (Fig. 2, right lane). Two of these species migrated as a diffuse fraction with approximate molecular weight of 280–350 kDa, and as a single fraction of approximately 220 kDa. These findings are consistent with type III receptors reported by others. We also detected an ~80–90 kDa affinity-labeled fraction, which is consistent with the reported molecular weight of type II receptors.[3,10,14,15] These affinity-labeled species are compared to the silver-stained gel of the $^{125}$I-TGF-β1-labeled TX-100 cell membrane extract in Figure 2 (left lane).

### Motility and Proliferation

In medium supplemented with serum, the cells at confluence averaged three to five mitotic figures/10 × field/2 hrs. A single dose of activated TGF-β1 at concentrations of 0.5 ng/ml and 1 ng/ml caused a marked inhibition of proliferative (<1 mitotic figure/10 × field/2 hrs; Figs. 3A and B) and migratory activity of trabecular cells. This effect lasted for approximately 24 hr. After 24 hr, the activity of the trabecular cells recovered to its pretreatment level. In addition,

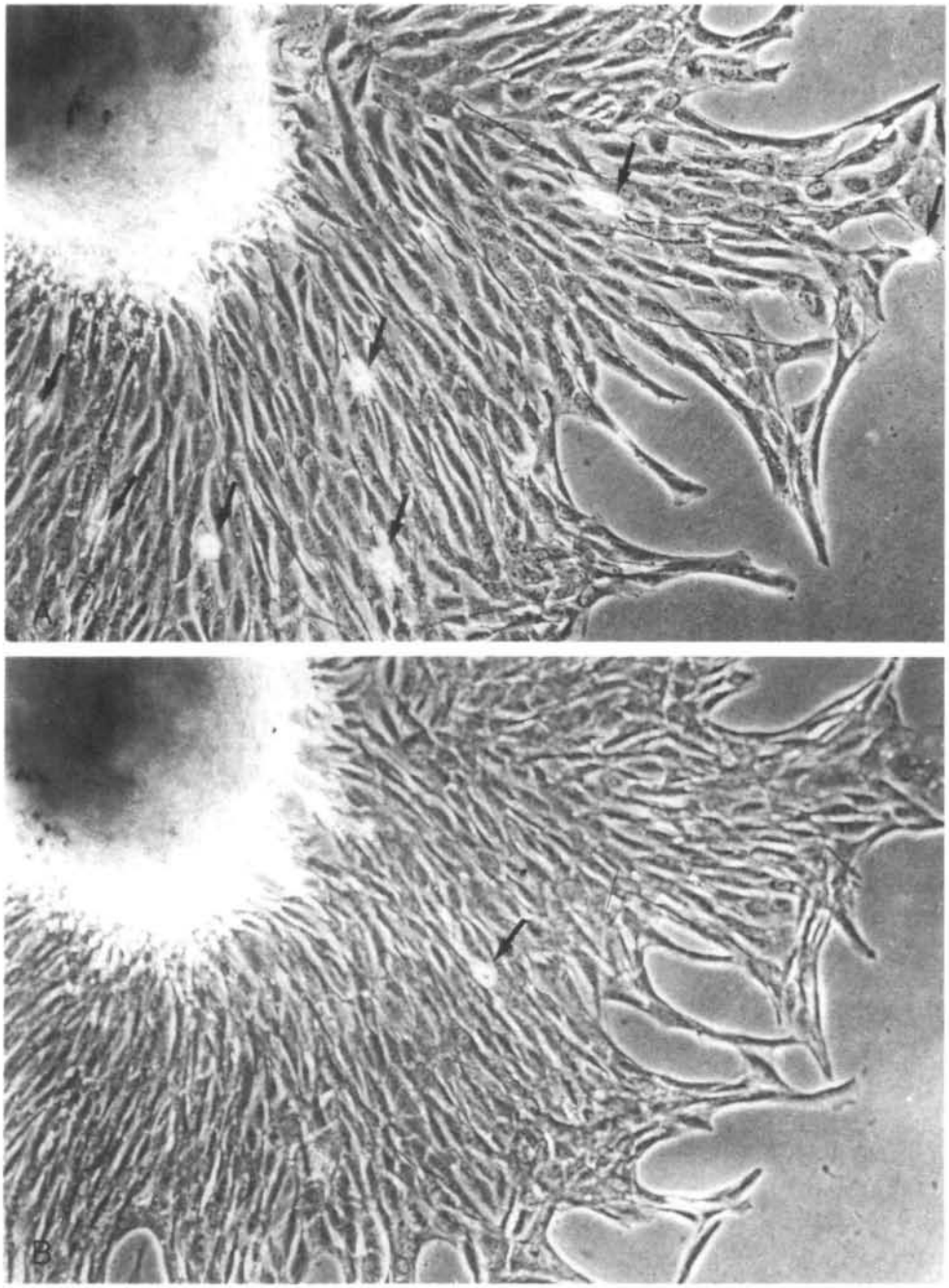


**Fig. 3.** Porcine trabecular cells in vitro. **(A)** Two-week-old culture of trabecular cells in MEM supplemented with 15% fetal calf serum. Note the abundance of mitotic figures (arrows) in this field. The proliferative response was approximately three to five mitotic figures/×10 objective field/2 hr. **(B)** Same field after exposure to a single dose of activated TGF-β1 at a concentration of 0.5 ng/ml for 20 hr. The cells demonstrated a marked inhibition of proliferative activity. Cell division (arrows) was reduced to <1 mitotic figure/×10 objective field/2 hrs. The migratory activity of trabecular cells was also markedly inhibited. Phase-contrast micrographs, ×95.

we observed that cells often retracted within one hour of treatment (Figs. 4A and B).

## Discussion

Our data are consistent with the presence of one or more trypsin-sensitive cell-surface binding proteins for TGF-β1 on trabecular cells. The binding proteins or receptors are regenerated to a limited extent after 7 hr of incubation.[10,16] Preincubation of the biotinylated TGF-β1 with neutralizing antibody resulted in a 55% decrease in labeling of the receptors, which is consistent with the capacity of TGF-β1-neutralizing antibodies to prevent the binding of TGF-β1 to its receptor and thereby block its biological activity.[3] The specific competition experiments demonstrate that a two-fold excess of unlabeled TGF-β1, but not TGF-β2, partially inhibits the binding of biotinylated TGF-β1 with its receptor. Other investigators[10,14] used a 100-fold excess of TGF-βs to demonstrate inhibition of binding of TGF-β1. However, the limited availability of unlabeled TGF-β1 and TGF-β2 precludes us from elaborating on these experiments. Nevertheless, the above experiments are consistent with the exis-

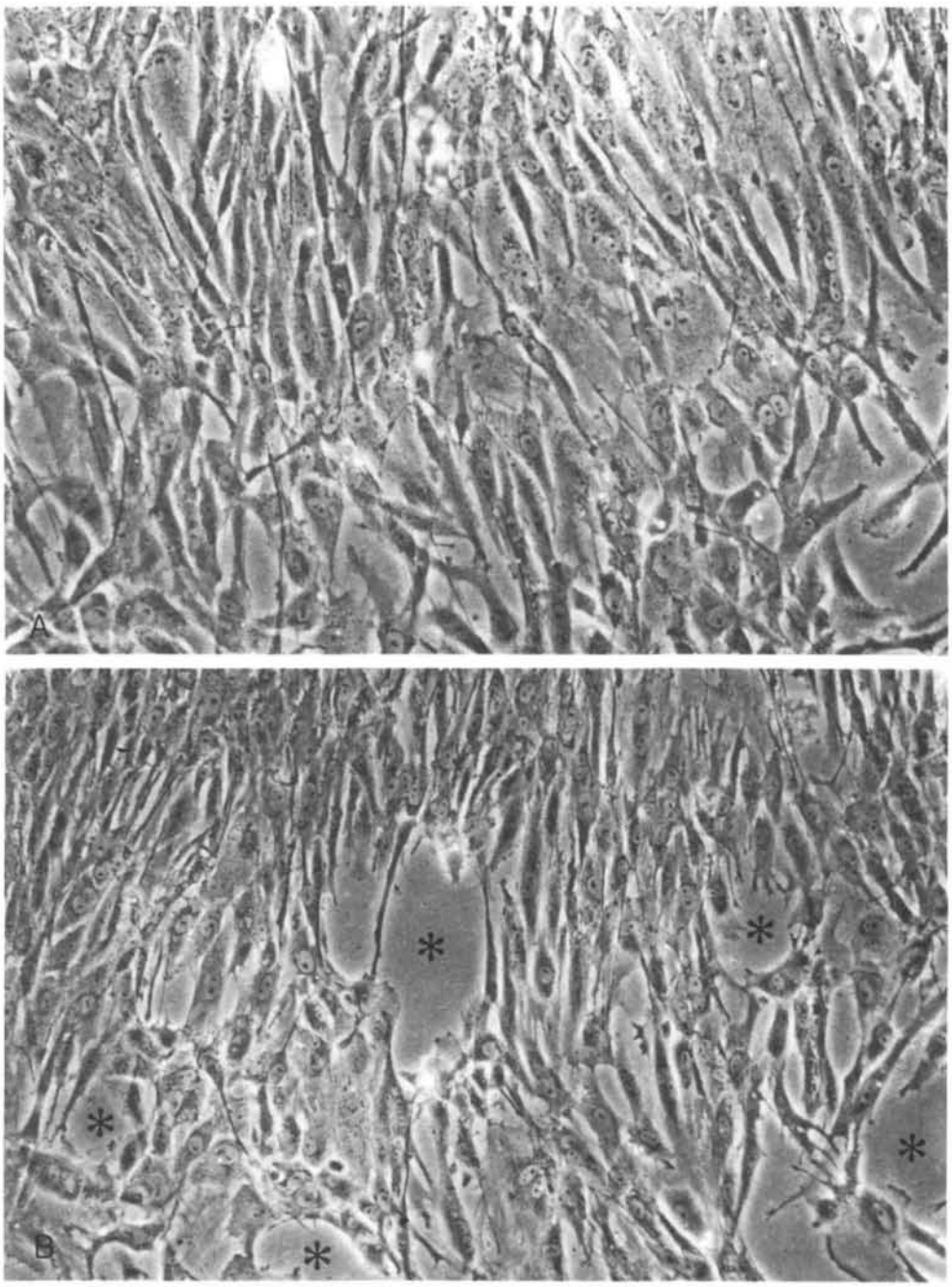



**Fig. 4.** (A) Three-week-old culture of trabecular cells in MEM supplemented with 15% FCS prior to exposure to activated TGF-β1. (B) One hour after the addition of 0.5 ng/ml TGF-β1 to the medium, the cells demonstrated marked retraction as evidenced by the loss of confluence between adjacent cells (asterisk). Same field as (A). Phase-contrast micrographs, original magnification ×145.

tence of a subset of trypsin-sensitive TGF-β1 cell-surface-binding proteins that do not bind TGF-β2 or have different affinities for the two isoforms of TGF-β.

To further characterize the binding protein(s) for TGF-β1, we used the homobifunctional linking agent DSS. This affinity-labeling compound reacts with primary amino groups and results in the formation of stable covalent complexes between the labeled ligand and its receptors after their initial binding. Affinity cross-linking with DSS has been applied successfully to the characterization of a variety of growth factor receptor proteins, including those for insulin-like growth factors,[17] platelet-derived growth factors,[18] and basic fibroblast growth factor.[19]

Based on the ability to extract the ligand-receptor complex with Triton X-100, our experiments reveal two classes of integral cell membrane components in trabecular cells that bind TGF-β1. The type III affinity-labeled receptor, which migrates as a broad fraction between 250 and 350 kDa, has been described previously in other tissues[3,10,11,15] and is believed to be the reduced species of a high-affinity, disulfide-linked receptor complex of ~600 kDa.[11] The single high-

molecular weight fraction of ~220 kDa is probably also a type III receptor that has been demonstrated in aortic smooth muscle cells grown in culture.[10] These reduced type III species generally are considered to be the ligand binding site(s) for a larger 600 kDa complex and are composed of proteoglycan subunits.[10,14] Type III receptors are the major receptor species in mammalian and avian fibroblasts and epithelial cells and mediate the actions of TGF-β1, TGF-β2, and TGF-β1.2 equivalently.[3,15] The 80–90 kDa affinity-labeled band is similar in molecular weight to that of class II receptors, which are believed to mediate the action of TGF-β1 selectively.[14] Further investigations are required to elucidate the role of each class of receptors in maintaining the function of trabecular cells.

Although evidence for the physiologic and pathophysiologic roles of TGF-β1 remains circumstantial, our data implicate this growth factor in the modulation of the functional homeostasis of trabecular cells and perhaps in the maintenance of the viability of the aqueous outflow pathway. The presence of TGF-β1 in aqueous humor of different species[4–7] and, more importantly, the demonstration in this study of specific receptors for TGF-β1 on trabecular cells, suggest that this growth factor, once activated, can bind to receptors and affect the angle tissue. Our studies in vitro indicate that TGF-β1 exerts an antiproliferative effect on trabecular cells. However, it is not known whether activated TGF-βs are responsible for the lack of significant mitotic activity in vivo of trabecular cells or other cells, such as the corneal endothelium, in the anterior segment of normal eyes.

The relevance of receptors for the TGF-βs in the trabecular meshwork attains special significance in view of the amounts of these growth factors in aqueous humor. In vitro, the action of the different TGF-β isoforms, particularly that of TGF-β1, depends upon many parameters, including the type of cell and its state of differentiation, the growth conditions, and the presence of other growth factors.[3,6] In vivo, the bioavailability of TGF-βs in the anterior chamber will depend on their activation in the aqueous humor or at the site of the target tissues. The precise mechanism for activation of TGF-βs in the normal eye is not known. However, in conditions of breakdown of the blood-aqueous barrier, TGF-β1 is present in an activated form in the plasmoid aqueous humor (unpublished personal observations). Therefore, it is conceivable that certain substances, such as plasminogen activators circulating in the aqueous humor or present in the trabecular meshwork/Schlemm's canal system,[20,21] are associated with activation in vivo of TGF-βs.

In vivo and in vitro, the TGF-βs specifically enhance the interactions between fibroblastic cells and components of the extracellular matrix in several ways. TGF-β1 increases the levels of mRNA and upregulates the synthesis of many matrix proteins, such as collagen types I, III, IV, and V, fibronectin, thrombospondin, elastin, and glycosaminoglycans.[3,22,23] It also controls the expression of cell membrane receptors (integrins) for these cell adhesion proteins.[3] TGF-βs enhance the accumulation of extracellular matrix components by decreasing the synthesis of proteases such as plasminogen activator and matrix metalloproteinases[24] and by increasing the synthesis of protease inhibitors such as plasminogen activator inhibitor.[25] The presence of these macro-molecules in the trabecular meshwork/Schlemm's canal system[20,26,27] creates a microenvironment that may be closely regulated by TGF-βs and other growth factors, whether synthesized locally or supplied by the aqueous humor.

**Key words:** aqueous outflow pathway, autoradiography, flow cytometry, receptor binding assay, SDS-PAGE, tissue culture, transforming growth factor-β, wound healing.

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