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Identification and Quantification of Transferrin Receptors on Trabecular Cells

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The authors identified and quantified the receptor for transferrin on trabecular meshwork (TM) cells cultured from porcine eyes by using two receptor assays. Flow-cytometric analysis of TM cells that were incubated with a monoclonal antibody to the transferrin receptor revealed such receptors, which decreased in number as the cells passed from the pre- to the post-confluent phase. Quantitative characterization by radioligand binding of ^{125}I-transferrin to trabecular cells followed by Scatchard analysis showed that pre-confluent cultures expressed 23,839 ± 6746 high-affinity receptors/cell and post-confluent cultures expressed 5104 ± 3639 receptors/cell. The expression of the receptor for transferrin reflects the index of mitotic activity and can be correlated with the proliferative state of the trabecular cells. Further characterization of the receptors for transferrin in the TM may open up the possibility of a pharmacologic approach that enhances the TM cell population, which is known to decrease with age and in glaucomatous states of the eye. Invest Ophthalmol Vis Sci 33:3449–3454, 1992

Transferrin is a polymorphic glycoprotein (molecular weight 79.5 kD) that has two binding sites for iron and circulates in serum and in other body fluids.[1] Several isoforms of transferrin, including the so-called cerebrogenic marker, "tau fraction," have been described in the aqueous humor of humans and other species.[2–4] In addition to serving as an essential factor for delivering ferric iron to target tissues, transferrin acts as a growth-promoting substance for many tissues,[5,6] including the avascular structures that border the anterior chamber of the eye, such as the corneal endothelium, trabecular meshwork (TM), and lens epithelium.[7–9]

Although the use of transferrin is believed to be mediated by specific receptors, no direct evidence exists for the presence of these receptors on the trabecular cells. Our previous studies indicated that transferrin in the aqueous humor has an affinity for the TM because it is not removed by extensive washing of the tissue.[10] In the present study, we identified and quantified the receptors for transferrin on cultured trabecular cells. We report that the expression of these receptors is related to the state of proliferation of the trabecular cells in vitro.

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Materials and Methods

We used a biotinylated mouse monoclonal anti-human transferrin receptor antibody from Oncogene Science Inc. (Uniondale, NY). The specificity of the monoclonal antibody to transferrin receptor (clone T56/14) has been characterized extensively by immunoprecipitation studies.[11–13] Phycoerythrin (PE)-conjugated avidin was obtained from Calbiochem (San Diego, CA); ^{125}I(tyrosyl)-transferrin from Amersham Corporation (Arlington Heights, IL); holotransferrin from ICN Biomedicals (Irvine, CA); Eagle's minimal essential medium (MEM), newborn calf serum (NCS), Hanks' balanced salt solution, and trypsin from Gibco Laboratories (Grand Island, NY); phenylmethylsulfonyl fluoride from Eastman Kodak Company (Rochester, NY); and (ethylenedinitrilo)tetraacetic acid disodium salt (EDTA) from J. T. Baker Inc. (Phillipsburg, NJ).

Trabecular Cell Cultures

We obtained porcine eyes from a local abattoir within 20 min of decapitation and initiated the cultures of trabecular cells as described previously.[14] Briefly, a circumferential incision was made at the ora serrata, and the anterior segment of the eye was placed in a petri dish with the corneal epithelial side down. The iris was lifted from the inner corneal surface, and the pectinate ligaments were cut at their insertion into the periphery of the cornea so the entire iris could be reflected posteriorly. After we made two parallel incisions, one posterior to Schwalbe's line and the other anterior to the ciliary musculature, we excised the tra-

becular meshwork and placed the tissue in culture medium (MEM, 15% NCS, 0.1% penicillin, and 0.1% streptomycin, pH 7.4). Three to four millimeter-wide segments of the meshwork were placed in tissue culture dishes that contained culture medium. The culture plates were incubated at 37°C, and the original explants were removed from the culture dishes 2–4 wk later. We initiated secondary cultures from primary cultures that were pre-confluent, confluent, or post-confluent. For flow cytometry, pre-confluent cells were incubated for 2 wk, and post-confluent cells were incubated for 6 wk.

Flow Cytometry

We aspirated the culture medium from secondary cultures and washed the cells with 0.15 mol/l phosphate-buffered saline (PBS, pH 7.2) three times. The cells then were treated with 5 mmol/l EDTA for 15 min to remove them from the underlying substrate, and a single-cell suspension was obtained by vigorous pipetting.[15] The cells were centrifuged at 500 × *g* for 5 min; washed twice in binding buffer that consisted of PBS, 0.2% bovine serum albumin (BSA), and 0.02% sodium azide (pH 7.4); counted with a hemocytometer; and resuspended in binding buffer at a final concentration of 2–3 × 10^6 cells/ml. After the optimal antibody concentration was determined, 15 μl of biotinylated transferrin receptor antibody (100 μg/ml) was added to 1 × 10^5 trabecular cells in 85 μl of binding buffer in a polystyrene tube. The cells were incubated in the antibody for 40 min at 4°C, washed three times in PBS-0.01% sodium azide (NaN_3), and incubated with 2 μl of PE-conjugated avidin (1 mg/ml) in 150 μl of binding buffer for 20 min at 4°C in the dark. The cells were again washed in PBS-NaN_3 (three times, 2 ml each) and analyzed by flow cytometry. We determined the background level of fluorescence by incubating the TM cells in avidin-PE alone (2 μl of 1 mg/ml stock solution). To determine whether the binding site for transferrin on its receptor is the same as the antibody recognition site, we pre-incubated the TM cells with 10 μl of transferrin (10 mg/ml) for 1 hr before adding transferrin receptor antibody.

The labeled cells were analyzed with a Becton Dickinson (San Jose, 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 (Palo Alto, CA) Consort 30 computer work station. A minimum of 10,000 gated events were collected for each analysis, and frequency distribution plots of the fluorescence signals were generated.

Quantitation of Transferrin Receptors

We quantitated receptors for transferrin by using a radiolabeled ligand binding technique. Primary cultures of trabecular cells were treated with 0.25% trypsin with 5 mmol/l EDTA that facilitated their removal from the dish. The cells were seeded at an average density of 100,000 cells/well in 16 mm plates at 37°C for 24 hr to permit regeneration of the receptors for transferrin as well as for other growth factors.[16–18] Pre-confluent cells were analyzed 24 hr after they attached to the plates, whereas for post-confluent studies, the cells were incubated for an additional 4 wk.

Before the assay, the monolayers were incubated in fresh culture medium with or without serum for an additional 24 hr. The cells were rinsed twice in ice

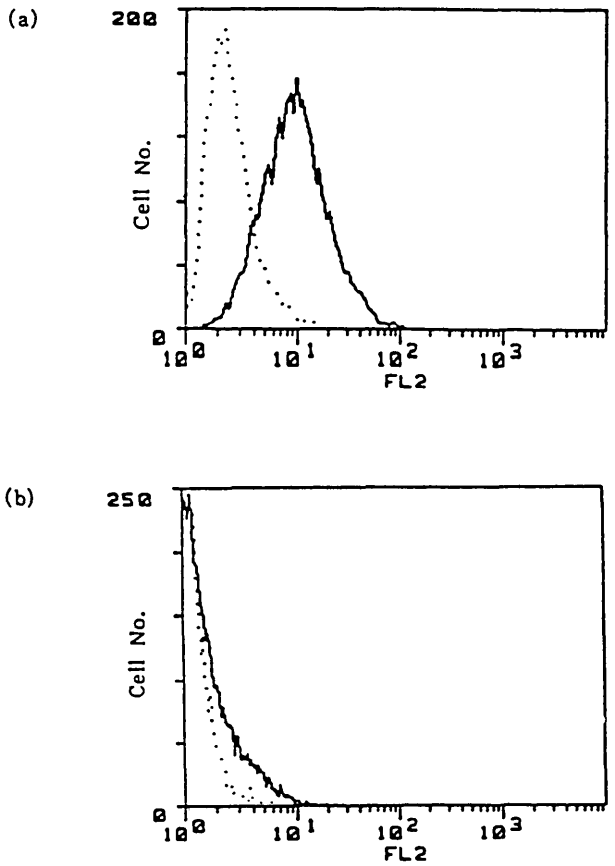

Fig. 1. Flow-cytometric analysis of the binding of monoclonal antibody (T56/14) to transferrin receptors on trabecular cells. The intensity of fluorescence (log_{10}) is plotted on the X axis, and the number of cells is plotted on the Y axis. The dotted curve denotes cells treated with PE-avidin only and represents the mean level of autofluorescence. The solid curve represents cells treated with biotinylated antibody and PE-avidin. (**a**) Preconfluent trabecular cells. (**b**) Post-confluent cells. The intensity of fluorescence of labeled preconfluent cells was increased sixfold compared to the mean level of autofluorescence. For the post-confluent cells, the peak fluorescence was increased threefold compared to autofluorescence.

cold serum-free culture medium and incubated in binding buffer (120 mmol/l NaCl, 6 mmol/l KCl, 1.2 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 35 mmol/l HEPES, pH 7.4, and 0.2% BSA) for 30 min at 4°C for dissociation of 85–95% of bound endogenous transferrin.[19,20] We added ^{125}I-labeled transferrin to each well at eight different concentrations, ranging from 0.19–40 nM, and incubated for 2.5 hr at 4°C to yield a standard curve. A nonspecific binding curve was generated by the simultaneous addition of ^{125}I-transferrin and a 1000-fold excess of unlabeled transferrin to trabecular cells. After incubation, the medium was aspirated and the trabecular cells were washed with ice-cold binding buffer (three times, 1 ml each) and solubilized in 900 μl of 1 N NaOH for 30 min. The solubilized cell extracts were aspirated and counted in a gamma counter. The cells from the unlabeled wells were trypsinized and counted after the assay was completed. We determined the amount of transferrin specifically bound by subtracting the nonspecific binding from the total transferrin bound. The receptor affinity and the number of receptors per cell were calculated by Scatchard analysis.[21]

Results

Identification of Transferrin Receptors on Trabecular Cells

Analysis of pre-confluent trabecular cells that were incubated with monoclonal antibody to transferrin receptor for flow cytometry revealed a sixfold increase in the mean fluorescence peak compared to the mean autofluorescence peak (Fig. 1a). In contrast, post-confluent trabecular cells showed only a threefold shift in the peak fluorescence (Fig. 1b). Pre-incubation of the trabecular cells for 1 hr with 10 μl of transferrin (10 mg/ml) did not inhibit the binding of the monoclonal anti-transferrin receptor antibody. As quantitated by the FACScan, the mean number of cells that demonstrated transferrin receptors was approximately 15% higher than in cultures not treated with transferrin.

Transferrin Receptor Quantitation Assay

Saturation of the transferrin receptors on the trabecular cells was achieved at a concentration greater than 10 nM of ^{125}I-labeled transferrin (Fig. 2). Addition of a 1000-fold excess of unlabeled transferrin decreased the binding of ^{125}I-transferrin to 30% of the total binding. Scatchard analysis revealed a single component binding curve that consisted of high-affinity receptors with a dissociation constant (K_d) of 5.9 ± 1.5 nM (Fig. 3). Pre-confluent cells that were proliferating actively in secondary cultures expressed 23,839 ± 6746 receptors/cell, and post-confluent cells had 5104 ± 3639 receptors/cell. There was no significant difference in the number of transferrin receptors per cell regardless of the age of the primary cultures.

Discussion

The receptor for transferrin is a dimeric cell-surface glycoprotein (molecular weight 180 kD) composed of two identical subunits linked by a disulfide bond.

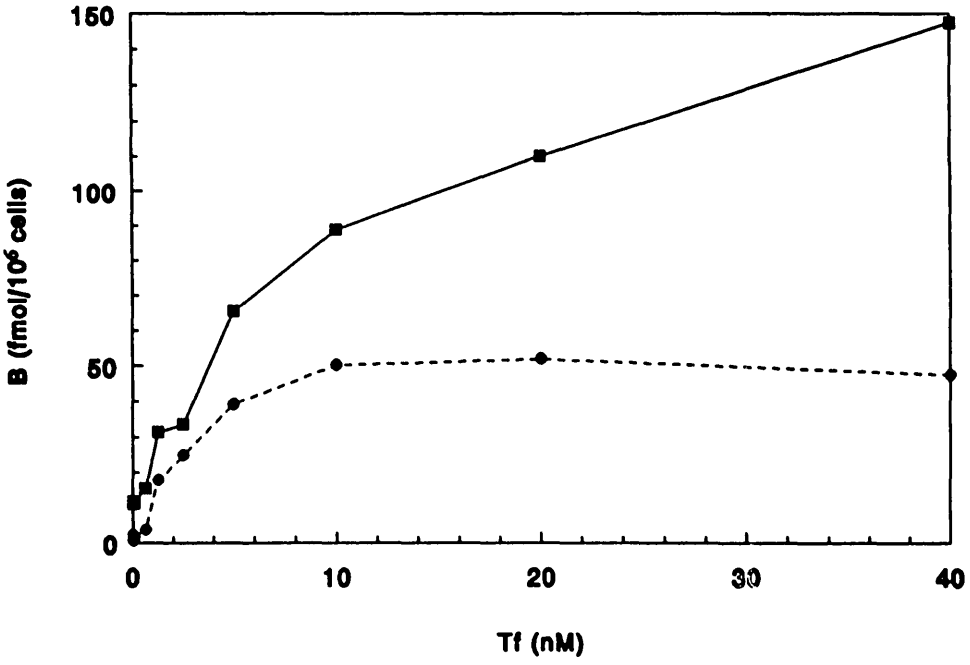

Fig. 2. Saturation curves for the binding of ^{125}I-transferrin to trabecular cells. The total binding (filled squares) of ^{125}I-transferrin was assessed after solubilization of the cells in 1 N NaOH. Nonspecific binding was obtained in the presence of a 1000-fold excess of unlabeled transferrin. Specific binding (filled circles) was calculated by subtracting the nonspecific binding from total binding. The experiments were performed in triplicate.

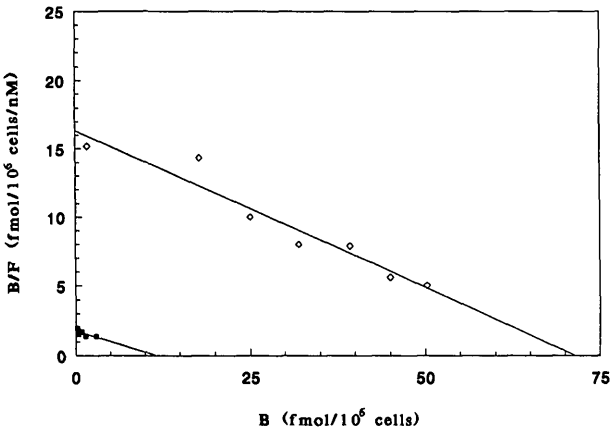

Fig. 3. Scatchard analysis of ^{125}I-transferrin binding to preconfluent (open diamonds) and post-confluent (filled squares) trabecular cells. The X axis represents ^{125}I-transferrin bound (fmols/10^6 cells), and the Y axis represents the ratio of specific bound to free ^{125}I-transferrin. The negative slopes of the linear Scatchard plots are equivalent (K_d = 5.9 ± 1.5 nM).

Each subunit binds one molecule of transferrin.[22] Transferrin receptors have been identified in and isolated from many normal cells, such as reticulocytes, placental trophoblasts, and kidney cells, and from transformed cells, as well as from vitreous and preretinal membranes in proliferative vitreoretinopathy.[20,23–29] In the present study, we used two receptor assays to identify and quantitate transferrin receptors on trabecular cells.

The binding of transferrin to its receptor on TM cells is concentration dependent at low levels and saturable at high levels. The dissociation constant (K_d) of 5.9 ± 1.5 nM for transferrin receptors on trabecular cells is consistent with that for high-affinity receptors that have a K_d in the range of 0.3–26 nM.[27,30–32] The expression of high-affinity receptors in our cell system reflects the capacity of trabecular cells in vitro, and the capacity of the parent cell during ontogeny, to proliferate. Our findings are consistent with previous reports on the expression of transferrin receptors in rapidly proliferating cells such as hepatocytes in regenerating liver and transformed lymphoid and melanoma cells.[6,31–33] Quiescent cells, such as normal resting B cells, demonstrate a significantly reduced expression of transferrin receptors.[33]

Scatchard analysis revealed that the number of receptors was fivefold greater on pre-confluent trabecular cells than on post-confluent cells and confirms the data we obtained by flow cytometry. The reduced proliferative activity of post-confluent cells in vitro, as seen in our experiments, may be analogous to senescence of cells in vivo. Because the density of transferrin receptors on the cell surface reflects the mitotic potential of the tissue,[12,34] our findings implicate transferrin in the functional homeostasis of the TM in health and disease. Trabecular cells normally have a limited capacity for regeneration,[35] which could be related to a downregulation of receptors for transferrin and other factors. The loss of TM cells associated with aging may lead to a further decrease in the expression of the transferrin receptors.

The reduction in the number of cells in the trabecular meshwork in eyes with POAG[36,37] is particularly relevant to the role of transferrin receptors in regulating the proliferative activity of trabecular cells. Studies in vitro have shown that only limited growth occurs in trabecular cell cultures initiated from explants obtained from patients with POAG.[36,37] Iron and transferrin are known to regulate the expression and recycling of surface transferrin receptors.[38,39] Our experiments demonstrated an upregulation in the number of transferrin receptors when TM cells were preincubated with transferrin. Further investigation is needed to determine whether the proliferation and rejuvenation of trabecular cells after laser trabeculoplasty[40] is related to the upregulation of transferrin receptors on the trabecular cells secondary to an influx of transferrin and other growth factors in the secondary aqueous humor.[41,42]

The receptors for transferrin interact in a complex manner with several growth factors in vitro. Epidermal growth factor induces a transient mobilization of internalized transferrin receptors to the cell surface.[16,19] Platelet-derived growth factor, insulin-like growth factor-I, and interleukin-2 cause a prolonged up-regulation of transferrin receptors that lasts for several hours, but does not alter their affinity for transferrin.[19,43] However, treatment of fibroblasts with interferon-α decreases the expression of transferrin receptors.[44] A disruption in the balance between these growth factors might play a vital role in the abnormal hyperplastic response of the lens epithelium that often results after the breakdown of the blood-aqueous barrier. Because the receptor for transferrin is expressed at high density on rapidly proliferating cells, therapeutic modalities based on the targeting of cytotoxic agents to specific cells have been envisioned. Anti-transferrin receptor antibodies linked to an immunotoxin inhibited the proliferation of human and baboon lens epithelial cells in vitro.[45] Protein synthesis was inhibited specifically in actively dividing retinal pigment epithelial cells in vitro, but not in confluent, nondividing cells.[46] Mitotoxins linked to transferrin act as a suicide antagonist at the transferrin receptor and may be used to selectively control

cell proliferation, for example, of Tenon's capsule fibroblasts after glaucoma filtration procedures.

Key words: aqueous outflow pathway, flow cytometry, glaucoma, high-affinity receptors, mitotic activity, receptor binding assay, Scatchard analysis, tissue culture

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