Transferrin Receptor Expression by Retinal Pigment Epithelial Cells in Proliferative Vitreoretinopathy

Ch. Daudouin,* F. Brignole,† D. Fredj-Reygrobeller,‡ F. Nègre,* J. Boyle,† and P. Gastaud*

Immunotoxins directed against a membrane marker of cell proliferation, transferrin receptor, were investigated to inhibit the growth of retinal pigment epithelial (RPE) cells in proliferative vitreoretinopathy (PVR). We undertook an immunocytological study in specimens of vitreous, subretinal fluid, and epiretinal membranes from patients with PVR to address the expression of transferrin receptor by proliferating pigment epithelial cells during the course of PVR and in normal human ocular structures. Thirty four specimens of vitreous and subretinal fluid, as well as seven epiretinal membranes, were immunocytologically examined using monoclonal antibodies to transferrin receptor. They showed a strong expression of this marker by a large majority of the cells in these two periretinal fluids (mean percentages 80 and 91% in vitreous and subretinal fluid, respectively). In contrast, only a few cells within epiretinal membranes were found to express transferrin receptor. In normal human eye sections conjunctival and corneal epithelial cells, subcapsular epithelium of the lens strongly expressed transferrin receptor, whereas RPE cells remained negative to antitransferrin receptor antibodies. A few iris or ciliary pigment epithelial cells reacted weakly. Thus, this study shows that most intravitreal and subretinal fluid proliferating cells strongly express transferrin receptor on their surface. Also confirmed is that immunotoxins to this membrane antigen could constitute potentially useful therapeutic agents in PVR. Invest Ophthalmol Vis Sci 33:2822-2829, 1992

Proliferative vitreoretinopathy (PVR) remains the main complication that occurs after rhegmatogenous retinal detachment and accounts for most of the failures in retinal detachment surgery. PVR is characterized by extensive formation of proliferative tissues on the surfaces of retina and within the vitreous cavity. Various types of cells have been documented in newly formed periretinal membranes, such as retinal pigment epithelial (RPE) cells, glial cells, fibroblast-like cells, and macrophage-resembling cells. During the course of PVR, those cells actively divide and spread throughout the vitreous body and the subretinal space.

Despite recent advances in vitreoretinal surgery, visual outcome in PVR remains impaired by recurrent periretinal proliferation. Therefore, various adjuvant therapeutic agents have been experimented on for a long time. It has not been possible, however, to develop drugs that would be efficient on proliferating cells without being toxic to normal ocular structures. To specifically inhibit the growing cells, recent studies have shown the potential use of immunotoxins directed against a membrane marker related to cell proliferation—transferrin receptor. Exposure of subconfluent cultures of RPE cells to immunotoxins composed of a monoclonal antibody directed against transferrin receptor and conjugated to ricin A chain was shown to inhibit protein synthesis and to significantly decrease the number of proliferating RPE cells.

In previous studies, we had been working on epiretinal membranes and intravitreal and subretinal cells, surgically obtained in patients with PVR. We found that during the course of PVR, RPE cells undergo striking morphologic changes, and that they acquire membrane markers of immunocompetent cells—class II histocompatibility antigens. Moreover, in a preliminary report, we noted that intravitreal and subretinal cells also may express transferrin receptor. Therefore, the aim of the present study was to investigate the presence of transferrin receptor on proliferating pigment epithelial cells in patients suffering from retinal detachment with PVR, and to evaluate the expression of this marker in normal human ocular structures.

Material and Methods

This study was performed in 16 specimens of vitreous and 18 specimens of subretinal fluid obtained surgically in 28 patients with retinal detachment, as previ-
ously described. Patients' ages ranged from 32–84 yr, and all patients had retinal detachment with various degrees of periretinal retraction (Table 1), graded according to the updated classification of the Retina Society. The whole vitreous gel was removed by posterior vitrectomy and cytologically examined. Subretinal fluids were sampled using a cut down through the sclera after heat coagulation of sclera and choroid to avoid bleeding. Only in six patients could both vitreous and subretinal fluid be obtained. All of these specimens were first centrifuged at 1600 rpm for 10 min. Cytocentrifuge smears were prepared (1200 rpm for 10 min) and fixed in methanol at 4°C.

In addition, seven epiretinal membranes were mechanically peeled off the retina with microscissors. Specimens were immediately embedded in Tissue Tek (OCT compound; Miles, Elkhart, IN) and frozen in liquid nitrogen. Ten normal eyes also were obtained at autopsies performed within 6 hr after the deaths of five 55 to 70-year-old subjects who had no known ocular disease. Eyeballs were embedded in Tissue Tek and frozen in liquid nitrogen-cooled isopentane. Epiretinal membrane and whole eye frozen sections 4 μm thick were prepared using a cryostat and were reacted as follows.

Standard cytologic examination was conducted using the May-Grünwald giemsa staining procedure. Immunocytologic examination was performed on intravitreal and subretinal cells, as well as on cryosections of epiretinal membranes and normal human eyes, using two different monoclonal antibodies to transferrin receptor CD71 (OKT9, Ortho Diagnostic Systems, Raritan, NJ; and CD71, Aster Biotechnologies, La Gaude, France) and a monoclonal antibody against 56 kD cytokeratin (clone KL1; Immunotech,) to identify pigment epithelial cells. Indirect immunofluorescence procedures were performed using previously described methods. Primary monoclonal antibodies, in a 1:50 dilution, were layered onto slides for 1 hr. After washing in phosphate-buffered saline (PBS), specimens were incubated for 30 min with the secondary antibodies—fluorescein isothiocyanate-labeled antimouse immunoglobulin antiserum (Dakopatts, Copenhagen, Denmark). The slides were washed again in PBS, counterstained with propidium iodide to identify cell nuclei, and mounted in mounting medium (AF1; Citifluor Ltd.) before examination. Regarding intravitreal and subretinal cells, the percentages of reactive cells were determined by counting at least 100 cells for each antibody. Because two different monoclonal antibodies to transferrin receptor were used, percentages of transferrin receptor expressing cells were calculated by measuring the proportion of reactive cells in at least 200 cells by sample. Specimens with unexplained discrepancies between the different anti-CD71 antibodies were systematically controlled on two more slides. Damaged cells were not considered. In all specimens, negative controls were prepared by substituting the primary antibody with nonimmune mouse serum in the same dilution.

Results

Cytologic examination of vitreous and subretinal specimens showed the five major cellular patterns, as previously described: large typical pigment epithelial cells, containing melanin pigment or autofluorescent lipofuscin granules; large partially pigmented cells; large macrophage-resembling totally nonpigmented cells; smaller nonpigmented cells, occasionally taking spindle-shaped fibroblast-like features; and rare lymphocytes. Immunocytologic procedures with anticytokeratin monoclonal antibodies confirmed that, although they were morphologically different, the first four cellular patterns widely expressed epithelial marker cytokeratin (50–100% of positive cells) and were of pigment epithelium origin. Moreover, the majority of these cells (Tables 2 and 3) expressed transferrin receptor on their membrane (Figs. 1 and 2). Cells with different morphologies similarly expressed transferrin receptor, even though strongly pigmented cells remained more nonconstantly positive than poorly pigmented and nonpigmented ones. Percentages of positive cells were similar in vitreous and subretinal fluid (mean positivity 80% and 91%, respec-

### Table 1. Grading of periretinal retraction in 35 patients suffering from retinal detachment with PVR, following the updated classification of the Retina Society

<table>
<thead>
<tr>
<th>Grade</th>
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<tr>
<td>Vitreous</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>9</td>
<td>4</td>
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<tr>
<td>SRF</td>
<td>2</td>
<td>6</td>
<td>5</td>
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<td>3</td>
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<tr>
<td>ERM</td>
<td>0</td>
<td>0</td>
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<td>5</td>
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SRF, subretinal fluid; ERM, epiretinal membrane.

* Some patients may have associated types of periretinal proliferation, such as percreetal and subretinal, anterior and posterior retraction.
Table 2. Mean immunoreactivity of cellular components from 16 specimens of vitreous gel (V) and 18 of subretinal fluid (SRF) with anticytokeratin and anti-transferrin (TRF) receptor antibodies in proliferative vitreoretinopathy

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Mean percentages of positive cells (SD)</th>
<th>Range (%)</th>
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<tbody>
<tr>
<td>Cytokeratin</td>
<td>V: 76 (22.3) SRF: 71 (17.6)</td>
<td>50-90</td>
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<tr>
<td>TRF receptor</td>
<td>V: 80 (21.6) SRF: 91 (7.7)</td>
<td>50-100</td>
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Percentages were calculated by counting at least 100 cells in each specimen and for each monoclonal antibody. Standard deviations are given in parentheses.

No correlation could be found between the immunostaining and the importance of periretinal retraction or duration of retinal detachment (Table 3). No difference in patterns of reactivity nor in percentages of positive cells could be found with the different antibodies to transferrin receptor. As expected, negative controls did not show reactivity.

Sections of epiretinal membranes (Table 4) showed numerous isolated nonpigmented cells and rounded lipofuscin-containing cells embedded in large amounts of extracellular matrix. Some of these cells, pigmented or nonpigmented ones, positively reacted with monoclonal antibody to cytokeratin. Some cells of both types also expressed transferrin receptor, and serial sections showed cells positive for both antibodies. However, a majority of cells within epiretinal membranes did not express any reactivity (Fig. 3).

Regarding normal human eye sections, three ocular structures showed a strong reactivity to antitransferrin receptor antibodies, conjunctival epithelium, corneal epithelium, and subcapsular epithelium of the lens (Fig. 4). A weak and inconstant reactivity also was found on some pigmented and nonpigmented epithelial cells of pars plana and ciliary processes. Rare cells of the iris pigment epithelium similarly expressed transferrin receptor (Fig. 5). Retina did not show reactivity in neuroretinal layers nor in retinal vessels. The other ocular structures, including retinal pigment epithelium and choroid, remained negative to antitransferrin receptor antibodies.

**Discussion**

Iron is needed for many metabolic processes, including DNA and protein synthesis. It is carried in the bloodstream in association with an 80 kD glycoprotein, transferrin, which is synthesized by various types of cells, such as hepatocytes, lymphocytes, muscle cells, or brain cells. Transferrin also has been detected in vitreous and in newly formed membranes from patients with proliferative vitreoretinal disorders. Transferrin binds to transferrin receptors on the surface of target cells, and transferrin receptor-iron complex is internalized into vesicles that release iron into the cytoplasm.

Transferrin is an essential requirement for cell growth and mitosis, and transferrin receptor density at the cell surface has been shown to be increased in proliferating cells compared to quiescent cells. Recent reports have addressed the eventual use of immunotoxins directed against transferrin receptors as adjuvant therapeutic agents in the treatment of PVR. Theoretically, immunotoxins to transferrin receptors would constitute an ideal antiproliferative agent by selectively inhibiting growing cells, avoiding toxic effects to normal nonproliferating intraocular cells. Because RPE cells have been shown to be major components of epiretinal membranes, cultured human RPE cells were exposed to an immunotoxin composed of a monoclonal antibody to transferrin receptors conjugated to a toxin—recombinant ricin A chain. A significant decrease in the number of RPE cells and protein synthesis in actively dividing RPE cells was found in a dose-dependent manner. In contrast, nondividing confluent RPE cells, when treated with maximal doses of immunotoxins, did not disclose morphologic change, nor decrease in number. Thus, such results indicated that immunotoxins to transferrin receptor might constitute potentially use-

Table 3. Comparative percentages of immunostaining reactivity of intravitreal and subretinal fluid cells with anti-cytokeratin and anti-transferrin receptor antibodies according to grading of retinal retraction

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ful antiproliferative agents in patients suffering from PVR.

These two studies, however, have been performed on cultured RPE cells, and the eventual use of immunotoxins in patients requires additional works to determine whether transferrin receptors would be expressed in high density at the surface of proliferating cells in pathologic conditions and to evaluate transferrin receptor expression by quiescent cells from normal ocular structures. A previous immunohistologic work performed in 14 epiretinal membranes obtained from patients with PVR or proliferative diabetic retinopathy...
nopathy had shown transferrin receptor expression in 11 membranes at the surface of some isolated cells.

Epiretinal membrane formation, however, appears to be the result of complex proliferative phenomena that occurs very early in the course of PVR and involves the vitreous gel that usually contains numerous brown pigmented dots and clumps even when epiretinal membrane formation has not begun. As we indicated in a previous study, although they show different morphologic patterns, RPE cells were found to be major components of vitreous body and subretinal fluid in patients with PVR. This morphologic heterogeneity could be the result of strong ultrastructural and functional changes that occur in proliferating pigment epithelial cells, from the typical heavily pigmented RPE cells to the totally nonpigmented ones, occasionally taking macrophage or spindle-shaped fibroblast-like patterns.

The present study shows that a large majority of these cells, within vitreous gel and subretinal fluid, strongly express transferrin receptors on their membranes. We also confirmed the presence of transferrin receptors at the surface of scattered cells within epiretinal membranes. Some of them were keratin-positive epithelial-derived cells. However, it was noteworthy that, although a large majority of vitreal and subretinal fluid cells expressed transferrin receptors, only some isolated cells within the membranes reacted with antibodies to transferrin receptors. The absence of immunostaining to transferrin receptor may indicate that negative cells would not be in proliferating stages of expressed small amounts of transferrin receptor, not detected by standard immunohistologic procedures.

Moreover, we showed that some human ocular structures—conjunctival and corneal epithelia and subcapsular epithelium of the lens—strongly express transferrin receptors in the normal human eye. These results suggest a potential role for immunotoxins in specific epithelial proliferations within the anterior segment, such as epithelial downgrowth or capsular opacification after cataract surgery, as Goins et al described in cultures of lens epithelial cells. Retina did not react with antitransferrin receptor antibodies, but a few cells from iris, ciliary processes, or pars plana were positively stained, whereas the large majority of epithelial cells from these structures remained negative. Such differences in pattern reactivity suggest various levels of transferrin receptor expression, possi-

Table 4. Immunostaining reactivity of cellular components in seven epiretinal membranes from patients with PVR

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<thead>
<tr>
<th>Antibodies</th>
<th>Mean percentages of positive cells (SD)</th>
<th>Range (%)</th>
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<tbody>
<tr>
<td>Cytokeratin</td>
<td>15 (15.3)</td>
<td>5–30</td>
</tr>
<tr>
<td>TRF receptor</td>
<td>21 (11.6)</td>
<td>5–35</td>
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Percentages of positive cells were calculated by counting at least 100 cells on each slide. Standard deviations are given in parentheses.

Fig. 3. Preretinal membrane from a patient with PVR, showing some transferrin receptor-expressing cells (arrows); most of the cells within this membrane, identified by propidium iodide counterstain of their nuclei, remain negative, however (×550).
Fig. 4. Immunofluorescence staining of subcapsular epithelial cells of the lens with anti-transferrin receptor antibodies (arrows), in autopsy specimen of normal human eye. The bright white band (arrowheads) is the autofluorescent lens capsule (×350).

We did not find transferrin receptor expression by RPE cells in normal human eyes. In vitro, as shown by Hunt et al,17 RPE cells possess high and low affinity transferrin receptors on their surface, associated with the cytoskeleton. In vivo, in normal quiescent tissues, even if RPE cells (which are involved in high

Fig. 5. Normal eye section showing cells from the posterior epithelium of iris reacting positively (arrows) with anti-transferrin receptor antibodies (×550).

bly related to heterogeneity in cellular metabolism and activity. Nevertheless, although only very few cells expressed high levels of transferrin receptors, their presence may be responsible for the eventual side effects of immunotoxins and must be considered before any therapeutic use.
metabolic processes) possess low rates of transferrin receptors, these antigens could not be detected by standard cytologic procedures. This is in contrast to those expressed by intravitreal or subretinal cells and by conjunctival, corneal, and lens epithelia. In experimental procedures, immunotoxins to transferrin receptors were found to be nontoxic on quiescent RPE cells, but this was not directly correlated to low rates of transferrin receptor, as confluent cells had approximately half the number of receptors found in proliferating RPE cells. This indicates that additional mechanisms could be responsible for the antiproliferative effect of immunotoxins to transferrin receptor.

The present work also stresses the striking functional and metabolic changes that occur in pigment epithelial cells during the course of PVR. We previously found that proliferating cells in PVR express class II histocompatibility antigens HLA DR and DQ, in epiretinal membranes and in intraocular fluids, suggesting inflammatory and even immunologic disturbances in PVR. Now we show expression of transferrin receptor, which is known to be related to proliferative phenomena. Mechanisms of such expression are not well known, but in human fibroblasts, various growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor, or insulin-like growth factor type-I (IGF-I), have been shown to cause a transient increase in expression of transferrin receptors on the cell surface. These growth factors have been found in great amounts within surgically removed epiretinal membranes. They also have been shown to enhance the production of gamma interferon, a lymphokine known to induce expression of class II antigens at the surface of various types of cells, including RPE cells, and recently identified in the vitreous of patients with PVR. Thus, one or some of these growth factors could be liberated when neuroretinal layers detach from the pigment epithelium, inducing activation and multiplication of the target cells, as well as morphologic and functional changes at their level, as they migrate and proliferate in the periretinal spaces.

Mechanisms of intraocular proliferation are not well known, but they appear to result from strong interactions between a large number of biologic agents, involving growth factors and mediators of the immune system. These works must be extended in vivo, on experimental models, and in vitro, to gain a better understanding of pathophysiologic events that occur in proliferative intraocular disorders and to evaluate the potential use of therapeutic agents that would specifically inhibit aberrant intraocular proliferation.

Key words: proliferative vitreoretinopathy, retinal detachment, retinal pigment epithelium, transferrin receptor, vitreous

References

17. Hunt RC, Dewey A, and Davis AA: Transferrin receptors on...