The Antiproliferative Effect of a Transferrin-Toxin on Human Retinal Pigment Epithelial Cells and Rabbit Fibroblasts

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Purpose. To determine the effect of a rabbit transferrin conjugated to recombinant ricin A chain (Tfr-rRA) and the carboxylic ionophore monensin on proliferating and density-arrested human retinal pigment epithelial cells and rabbit dermal fibroblasts.

Methods. Cells were seeded on 24-well plates at 20,000 cells/cm² and exposed to Tfr-rRA (0.1–10,000 ng/ml) with or without monensin (0.01 μM), and with or without human transferrin (65.7 μg/ml) for 5 minutes to 7 days. Cells were studied morphologically and counted at 1, 2, 4, and 7 days.

Results. Tfr-rRA (10–10,000 ng/ml) killed proliferating human retinal pigment epithelial cells and rabbit dermal fibroblasts in a dose-dependent manner (p < 0.01) up to a maximum of 86% and 93%, respectively. In contrast, Tfr-rRA had minimal effect on density-arrested human retinal pigment epithelial cells and rabbit dermal fibroblasts. The cytotoxicity of Tfr-rRA was inhibited by the addition of human transferrin (65.7 μg/ml), an effect that was partially overcome by longer treatment with Tfr-rRA. Monensin (0.01 μM) increased the cytotoxicity of Tfr-rRA by 4.8-fold over Tfr-rRA alone, shortened the onset of cell kill with Tfr-rRA from 48 to 24 hours (P = 0.04), and partially reversed the neutralizing effect of human transferrin.

Conclusions. The results indicate that Tfr-rRA effectively inhibited the proliferation of human retinal pigment epithelial cells and rabbit dermal fibroblasts in vitro. The inhibitory effect could be modified by the addition of human transferrin or monensin. Thus, this ricin A chain conjugate may interrupt the proliferation of cells necessary in the pathogenesis of proliferative vitreoretinopathy. Invest Ophthalmol Vis Sci. 1993;34:3419–3428.

Proliferative vitreoretinopathy (PVR) is the most common reason for failure of retinal reattachment surgery.1 In PVR, cells migrate and proliferate on the inner and outer surfaces of the retina, and in the vitreous cavity.1 Retinal glial cells, fibroblasts, macrophages, and especially retinal pigment epithelial (RPE) cells, have been implicated in the pathophysiology of this disease.1 Some of these cells secrete collagen and form a fibrocellular scar that can contract to cause traction retinal detachment or combined traction and rhegmatogenous retinal detachment.1 Surgical removal of these fibrocellular membranes is generally effective treatment of PVR. However, despite recent improvements in surgical technique and instrumentation, cellular reproliferation still may cause recurrent retinal detachment.

The Silicone Oil Study Group demonstrated that retinal reoperations, while increasing the anatomic success rate, had minimal effect on visual improvement.2 They suggested that new drugs that improve the primary anatomic success rate in surgery for PVR may ultimately improve visual results.2 In PVR, the ideal drug would kill pathological, proliferating cells without damaging nonproliferating, normal tissue. Transferrin, a glycoprotein involved in the transport of iron from the blood stream to cells, binds to transferrin receptors on the cell surface before it is internalized.3 More transferrin receptors are expressed on proliferating cells than nonproliferating cells.3 The
number of cell surface transferrin receptors is thus a marker for cell proliferation. One new antiproliferative drug, the antitransferrin receptor immunotoxin 454A12 MAb-rRA, composed of a monoclonal antibody directed against human cell surface transferrin receptors linked to recombinant ricin A chain toxin, exploits the differential expression of transferrin receptors by selectively killing actively proliferating cells without damaging nonproliferating cells. There is a rationale for the use of antitransferrin receptor immunotoxin in PVR; transferrin receptors have been identified on PVR membranes and cells in the subretinal space and vitreous fluid of patients with PVR. In addition, transferrin receptors are found in greater number on the surface of cultured, proliferating human RPE cells than density-arrested cells. We have shown that the antitransferrin receptor immunotoxin selectively kills proliferating, human retinal pigment epithelial (hRPE) cells while leaving density-arrested cells intact in vitro.

Immunotoxins have a potential shortcoming. They have been less potent as anticancer agents in vivo than in vitro, the slow onset of action limits their efficacy. Lysozymotropic agents have been used to potentiate the effect of immunotoxins. One such compound is the carboxylic ionophore monensin, which augments the cytotoxicity of immunotoxins against proliferating, cultured cells and increases the effectiveness of immunotoxins in localized malignancy. Recently, we showed that monensin potentiates the antiproliferative effect of antitransferrin receptor immunotoxin on cultured hRPE cells.

Ideally, we would like to test the efficacy of antitransferrin receptor immunotoxin (454A12 MAb-rRA) in an animal model of PVR before treating patients. A rabbit model of PVR, whereby fibroblasts injected into the vitreous humor induce a host cell proliferative response, is commonly used to study the efficacy of antiproliferative drugs. PVR still develops when irradiated fibroblasts are injected into the vitreous humor, demonstrating that the host cells, and not the injected cells, are responsible for the proliferative response. In animal models of PVR, the effectiveness of an antiproliferative drug is therefore dependent on its ability to inhibit host cell proliferation. Regardless, appropriate in vivo investigation in animal models of PVR is limited by the specificity of the monoclonal antibody component of 454A12 MAb-rRA for human tissue. Thus, it will not be possible to obtain detailed information about the safety and efficacy of this agent using an animal model of PVR.

A transferrin-ricin A chain conjugate has been shown to effectively kill cancer cells in culture. The binding of transferrin to transferrin receptors is much less species-specific than binding of 454A12 MAb (Handa JT, unpublished data, 1992). We hypothesized that a toxin composed of rabbit transferrin conjugated to recombinant ricin A chain toxin (Tfr-rRA) would kill both proliferating human and animal cells. We postulated that if Tfr-rRA is effective against rabbit cells, it could be tested in a rabbit model of experimental PVR, thereby providing useful in vivo information about the safety and efficacy of ricin A chain conjugates like 454A12 MAb-rRA and Tfr-rRA in the treatment of PVR. Furthermore, we hypothesized that if Tfr-rRA kills hRPE cells in vitro then its antiproliferative activity could be compared to that of antitransferrin receptor immunotoxin. Inhibition of proliferating human RPE cells would further support that Tfr-rRA itself would be useful in PVR. Finally, we theorized that because monensin potentiates the antiproliferative effect of antitransferrin receptor immunotoxin, it will also increase the cytotoxicity of Tfr-rRA, and would potentially improve the effectiveness of Tfr-rRA in vivo. Herein, we report the results of using Tfr-rRA and monensin to selectively kill cultured proliferating hRPE cells and rabbit dermal fibroblasts (rDFB).

MATERIALS AND METHODS

Cell Culture

Human RPE cells were explanted from eyes acquired from the Carolina Organ Procurement Agency, an affiliate of the North Carolina Eye and Human Tissue Bank using a previously described technique. Cells were maintained in Eagle’s minimal essential media (MEM; Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and incubated at 37°C in a humidified atmosphere of 5% CO2 and air. The purity of the RPE cultures was verified by immunohistochemical staining for cytokeratins as previously described. Rabbit dermal fibroblasts were harvested from the skin of Dutch belted rabbits and grown in MEM + 10% FBS. All cells were grown to confluence in 25 cm2 flasks (Costar Corp., Cambridge, MA). Medium was changed twice per week. Establishing the effect of Tfr-rRA on density-arrested RPE cells will yield information on its potential toxicity to the normal pigment epithelium in vivo. Basic fibroblast growth factor (bFGF) induces a polygonal morphology on density-arrested hRPE cells, and thus provides a better in vitro approximation to the in vivo RPE cell monolayer than cells not treated with 6FGF. In preliminary experiments, we found that 6FGF (1 ng/ml; Sigma Chemical Co., St. Louis, MO) did not alter the antiproliferative effect of antitransferrin receptor immunotoxin or Tfr-rRA in subconfluent cultures, compared to controls without 6FGF supplementation. Thus, to maintain identical experimental conditions, bFGF (1 ng/
ml) was added every other day to proliferating and density-arrested cells during proliferation assays. Only first through fifth passage cells were used.

**Tfr-rRA and Antitransferrin Receptor Immunotoxin Synthesis**

Recombinant ricin A chain was expressed in *Escherichia coli*, purified to 99% homogeneity, and linked to rabbit transferrin (Sigma Chemical Co., St. Louis, MO) via a disulfide bond using disulfide exchange. Antitransferrin receptor immunotoxin, 454A12 MAb-rRA, was synthesized by a previously described technique.

**Cell Proliferation**

Human RPE cells were seeded in quadruplicate wells of 24-well culture plates (Corning Glass Works, Corning, NY) at 20,000 cells/cm². Twenty-four hours later, approximately 18,000 cells/cm² were attached. To test the effect of Tfr-rRA, these proliferating cells were treated with 0.1 to 10,000 ng/ml (0.0009 to 90 nM) Tfr-rRA in MEM + 10% FBS for periods varying from 5 minutes to 7 days. To determine the potentiating effect of monensin, proliferating hRPE cells were treated with 0.1 to 10,000 ng/ml Tfr-rRA with and without 0.01 μM monensin (Sigma Chemical Company, St. Louis, MO) for 7 days. Before use, monensin was dissolved in absolute ethanol, added to MEM + 10% FBS, and diluted to the desired concentration. The volume of ethanol did not exceed 1% v/v and control studies showed no effect of ethanol on the number of cells during all assays. It is likely that monensin and Tfr-rRA would be administered concurrently in the treatment of PVR. Thus, to study the effect of various exposure periods to monensin that potentiatted the effect of Tfr-rRA, cells were treated simultaneously with these drugs. Proliferating hRPE cells were treated with 10,000 ng/ml Tfr-rRA for 1 hour, and 0.01 μM monensin for periods of 15 minutes to 7 days. For exposures to monensin that were shorter than 1 hour, cells were treated with Tfr-rRA and monensin for the time designated for monensin exposure, rinsed twice with phosphate-buffered saline, and treated with Tfr-rRA alone for the remainder of the 1-hour period. For exposures to monensin that were longer than 1 hour, proliferating hRPE cells were treated with both Tfr-rRA and monensin for 1 hour, rinsed, and treated with monensin for the duration of the designated exposure period. After the treatment period with both agents, cells were rinsed twice and incubated in MEM + 10% FBS alone for the duration of the 7-day experiment. To establish the effect of Tfr-rRA and monensin on density-arrested cultures, hRPE cells were grown until visually confluent. Cells were counted twice each week and used in experiments when cell number no longer increased (approximately 3 weeks in culture). These density-arrested hRPE cells were then treated with 0.1 to 10,000 ng/ml Tfr-rRA in MEM + 10% FBS with or without 0.01 μM monensin for 8 hours or 7 days.

On average, 65.7 mg/l transferrin is present in vitreous humor samples obtained from patients undergoing vitrectomy for PVR. To test the effect of Tfr-rRA in the presence of transferrin, proliferating hRPE cells were treated for 8 hours or 7 days with 0.1 to 10,000 ng/ml Tfr-rRA, with and without 0.01 μM monensin, in MEM + 10% FBS supplemented with 65.7 mg/l human transferrin (Sigma Chemical Company, St. Louis, MO). For comparison, hRPE cells were treated for 8 hours or 7 days with 10,000 ng/ml antitransferrin receptor immunotoxin (454A12MAb-rRA) in the presence or absence of 65.7 mg/l human transferrin.

To evaluate the effect of Tfr-rRA and monensin on rDFB, cells were plated at 20,000 cells/cm². After 24 hours, approximately 18,000 cells/cm² were attached; proliferating cells were treated with 0.1 to 10,000 ng/ml Tfr-rRA with or without 0.01 μM monensin for 7 days. The effect of Tfr-rRA and monensin was also tested on density-arrested rDFB. Density-arrested rDFB were produced by growing them for 4 weeks. After 4 weeks, cells were confluent and had slowed to 5 to 10% of their logarithmic growth rate. These "density-arrested" cells were then treated with 0.1 to 10,000 ng/ml Tfr-rRA with or without 0.01 μM monensin for 7 days.

After the exposure period, all cells were rinsed twice with phosphate-buffered saline to avoid carry-over effect from Tfr-rRA, monensin, or human transferrin, and incubated in fresh MEM + 10% FBS until the end of the experiment. At the end of the assay period, cells were rinsed with phosphate-buffered saline, trypsinized, and counted with a Coulter counter (Coulter Electronics, Hialeah, FL). Cell viability was determined by trypan-blue exclusion. Cells were examined morphologically with a phase-contrast microscope. Experiments were repeated at least once.

**Statistical Analysis**

A two-tailed nonpaired Student’s t-test was used to compare differences in cell count between experimental and control groups.

**RESULTS**

**Effect of Tfr-rRA on hRPE Cell Number**

When proliferating hRPE cells were treated for 7 days with Tfr-rRA, cell number was reduced in a dose-de-
pendent fashion ($P \leq 0.01$, for doses $\geq 100$ ng/ml) and half-maximal effect occurred at 300 ng/ml. In contrast, Tfr-rRA had minimal effect on density-arrested hRPE cells (Figure 1). Free ricin A chain (2000 ng/ml), a negative control, reduced the number of proliferating hRPE cells by only 15%. Similar treatment with the antitransferrin receptor immunotoxin (10,000 ng/ml), a positive control used to compare efficacy of a drug specific for transferrin receptors, reduced hRPE cell number by 87%. The viability of proliferating and density-arrested hRPE cells remaining after a 7-day treatment with Tfr-rRA was 85% to 95%, identical to untreated hRPE cells. In a second series of experiments, the onset of cytotoxic effect was determined 24, 48, 72, and 168 hours after addition of 10,000 ng/ml Tfr-rRA. Significant cytotoxic effect was first detected at 48 hours ($P = 0.0001$).

Human transferrin (65.7 mg/l) did not affect the number of hRPE cells compared to untreated controls, but it did reduce the cytotoxic effect of an 8-hour exposure to Tfr-rRA ($P \leq 0.0001$ for all doses of Tfr-rRA; Figure 2). The effect of human transferrin was partially overcome by longer incubation with higher doses of Tfr-rRA. For example, when 10,000 ng/ml Tfr-rRA was incubated with human transferrin, an 8-hour treatment decreased hRPE cell number by 57% and a 7-day treatment decreased cell number by 86% ($P = 0.0001$). In contrast, transferrin did not alter the cytotoxic effect of the antitransferrin receptor immunotoxin (10,000 ng/ml), which decreased hRPE cell number by 86% in the presence or absence of human transferrin.

**FIGURE 1.** Relative number of cells remaining after a single 7-day treatment with Tfr-rRA: proliferating hRPE cells (○), density-arrested hRPE cells (□). Proliferating cells started at 18,000 cells/cm². Density-arrested hRPE cells started at 418,000 cells/cm². The standard deviation was < 10% for each data point.

**FIGURE 2.** A representative experiment showing the number of proliferating hRPE cells remaining after an 8-hour treatment with Tfr-rRA in MEM + 10% FBS with (○), and without (□) 65.7 mg/l human transferrin. Results are presented as the mean (± SD) of four replicate wells.

**FIGURE 3.** A typical experiment showing the number of proliferating hRPE cells remaining after a 7-day treatment with Tfr-rRA alone (○), or with (●) 0.01 μM monensin. Results are presented as the mean (± SD) of four replicate wells.

**Effect of Tfr-rRA and Monensin on hRPE Cell Number**

A single 7-day treatment with 0.01 μM monensin did not decrease the number or viability of proliferating hRPE cells. Hence, this concentration of monensin was used in subsequent experiments. Monensin potentiated the cytotoxic effect of a 7-day treatment with Tfr-rRA ($P \leq 0.01$ for doses 100 ng/ml Tfr-rRA; Figure 3). With a 7-day treatment, the maximum increase
in potency was 2.6-fold and was attained with the combination of monensin and 100 ng/ml Tfr-rRA (P = 0.0001). In addition, monensin decreased the onset of cell kill by Tfr-rRA (10,000 ng/ml) from 48 to 24 hours (P = 0.04).

Treatment with the combination of monensin and Tfr-rRA, for the most part, had minimal effect on the number of density-arrested hRPE cells. An 8-hour treatment with all doses of Tfr-rRA and monensin did not decrease the number of density-arrested hRPE cells. Likewise, a 7-day treatment with doses below 10,000 ng/ml Tfr-rRA had no effect on the number of density-arrested cells. However, a 7-day treatment with the combination of Tfr-rRA (10,000 ng/ml) and monensin decreased density-arrested cell number by 45%. The viability of density-arrested hRPE cells remaining after treatment with monensin and Tfr-rRA (0.1–10,000 ng/ml), 85% to 90%, was identical that of controls.

The combination of monensin and Tfr-rRA partially neutralized the effect of human transferrin on proliferating hRPE cells. With human transferrin (65.7 ng/l) in the medium, a 7-day treatment with less than 1000 ng/ml Tfr-rRA alone or in combination with monensin did not reduce cell number, compared to controls. A 7-day exposure to 1000 ng/ml Tfr-rRA alone reduced cell number by 25% whereas the combination of Tfr-rRA and monensin decreased cell number 47% (P = 0.0008). With 10,000 ng/ml Tfr-rRA, cell number was decreased by 59% with Tfr-rRA alone and by 81% when combined with monensin (P = 0.0001).

Effect of Treatment Duration with Tfr-rRA and Monensin on hRPE Cell Number

The magnitude of reduction in the number of proliferating hRPE cells was dependent on the length of exposure to 10,000 ng/ml Tfr-rRA. Cell number was reduced by 32% (P = 0.0001) with a 5-minute exposure and by 83% by with a 24-hour treatment. There was no further statistically significant reduction in cell number when cells were treated with 10,000 ng/ml Tfr-rRA for longer than 24 hours (Figure 4A).

To determine the length of treatment by monensin that enhanced the cytotoxic effect of Tfr-rRA, proliferating hRPE cells were exposed to 10,000 ng/ml Tfr-rRA for 1 hour and to 0.01 μM monensin for varying times. A 1-hour exposure to monensin, the minimum time that enhanced the effect of Tfr-rRA, caused a 1.4-fold increase in potency over treatment with Tfr-rRA alone (P = 0.0001). Longer exposure to monensin further enhanced the effect of the 1-hour treatment with Tfr-rRA. Maximum decrease in cell number, 89%, and increased potency, 4.8-fold, was obtained by a 7-day treatment with monensin (Figure 4B).

Effect of Removing Tfr-rRA and Monensin on hRPE Cell Number

The cytotoxic effect of Tfr-rRA persisted in a dose-dependent manner after it was removed from the culture medium (P ≤ 0.0044 for all doses of Tfr-rRA tested; Figure 5). In contrast, the potentiating effect of mon-
FIGURE 5. A typical experiment showing the number of proliferating hRPE cells remaining after a 7-day treatment with Tfr-rRA and 0.01 \( \mu M \) monensin followed by a 7-day recovery period in medium devoid of both agents: control (○), 100 ng/ml Tfr-rRA alone (●), 100 ng/ml Tfr-rRA and monensin (▲), 1000 ng/ml Tfr-rRA alone (△), 1000 ng/ml Tfr-rRA and monensin (▲), 10,000 ng/ml Tfr-rRA alone (□), 10,000 ng/ml Tfr-rRA and monensin (▲). Arrow indicates when both drugs were removed from the medium. Results are presented as the mean (± SD) of four replicate wells.

FIGURE 6. A representative experiment showing the number of proliferating rDFB remaining after a 7-day treatment with Tfr-rRA with (●) and without (○) 0.01 \( \mu M \) monensin, and density-arrested cells treated with Tfr-rRA with (●) and without (□) monensin. Results are presented as the mean (± SD) of four replicate wells.

ensin was reversible; the number of cells remaining after recovery from a 7-day treatment with the combination of monensin and Tfr-rRA was equivalent to the number remaining after recovery from treatment with Tfr-rRA alone. The viability of hRPE cells remaining after the first and second weeks of these experiments was 85% to 90%, equivalent to that of untreated cells.

Effect of Tfr-rRA on Rabbit Dermal Fibroblast Cell Number

A 7-day exposure to Tfr-rRA caused a dose-dependent decrease in the number of proliferating rDFB up to a maximum of 93% inhibition (Figure 6). The number of density-arrested cells was unchanged after similar treatment with Tfr-rRA. A 7-day treatment with monensin alone did not decrease the number of proliferating rDFB, but enhanced the effect of Tfr-rRA (\( P \leq 0.008 \) for 0.1 to 1000 ng/ml Tfr-rRA). Monensin did not enhance the effect of 10,000 ng/ml Tfr-rRA because near maximal effect was achieved with this dose of Tfr-rRA alone. When density-arrested rDFB were treated with monensin and the highest dose of Tfr-rRA, 10,000 ng/ml, there was a 30% decrease in cell number. The number of density-arrested rDFB was not altered when cells were exposed for 7 days to the combination of monensin and all other concentrations of Tfr-rRA. The viability of proliferating and density-arrested cells remaining after treatment with Tfr-rRA alone, monensin alone, or both agents was 85% to 90%, similar to that of control cells.

Effect of Tfr-rRA and Monensin on Cell Morphology

Dose-dependent morphologic changes developed in human RPE cells after treatment with Tfr-rRA and monensin. In the central zone, untreated proliferating hRPE cells maintained a cuboidal morphology with extensive cell–cell contacts (Figure 7A) whereas in the peripheral zone, they were less cuboidal and had fewer cell–cell contacts. Untreated density-arrested hRPE cells were smaller and densely packed over the entire well. Proliferating hRPE cells treated with Tfr-rRA (100 to 10,000 ng/ml) lost their cuboidal shape and developed long spindle-shaped processes (Figure 7B,C) whereas density-arrested cells developed similar, less pronounced changes with 10,000 ng/ml Tfr-rRA. Proliferating and density-arrested hRPE cells treated with 0.01 \( \mu M \) monensin maintained normal morphology. When exposed to the combination of Tfr-rRA (100 to 10,000 ng/ml) and monensin (0.01 \( \mu M \)), proliferating hRPE cells developed mildly ballooned cell bodies and long spindle-shaped processes. Only with 1000 to 10,000 ng/ml Tfr-rRA combined with 0.01 \( \mu M \) monensin did density-arrested hRPE cells develop morphologic changes. These alterations were similar to, but less severe than those observed for proliferating cells.

Rabbit dermal fibroblasts developed mild changes in morphology. Untreated proliferating cells were spindle-shaped (Figure 8A). Proliferating cells treated
FIGURE 7. Phase-contrast microscopy of proliferating hRPE cells after 7-day treatment with Tfr-rRA: 0 ng/ml (A), 100 ng/ml (B), 10,000 ng/ml (C). Cells started at 18,000 cells/cm². Photographs were obtained from cells in the center of the culture well (original magnification × 40).

with 100 to 10,000 ng/ml Tfr-rRA developed exaggerated spindle morphology (Figure 8, B and C). When proliferating fibroblasts were treated with monensin alone, their appearance was not affected, but cells developed mildly ballooned cell bodies with long spindle-shaped processes when treated with monensin and 100 to 10,000 ng/ml Tfr-rRA. Untreated density-arrested fibroblasts resembled proliferating cells, but were densely arranged over the entire well. Density-arrested fibroblasts treated with Tfr-rRA appeared similar to untreated cells, but developed ballooned cell bodies with long spindle-shaped processes with a 7-day treatment with monensin and 10,000 ng/ml Tfr-rRA.

FIGURE 8. Phase-contrast microscopy of proliferating rDFB after a 7-day treatment with Tfr-rRA: 0 ng/ml (A), 100 ng/ml (B), 10,000 ng/ml (C). Cells started at 18,000 cells/cm². Photographs were obtained from cells in the center of the culture well (original magnification × 40).
DISCUSSION

In this study, Tfr-rRA selectively inhibited the growth of cultured proliferating hRPE cells. Tfr-rRA has been found to exert similar effects on cancer cells. The dose necessary to cause a 50% decrease in cell number was 0.03 nM on leukemia T-cells and 0.56 nM on Burkitt’s lymphoma cells. On proliferating hRPE cells, the potency of Tfr-rRA was at the low end of the range observed in cancer cells because a higher dose, 2.7 nM (300 ng/ml), was needed to produce a 50% reduction in cell number. This difference may be partly due to the inherently slower proliferation rate of nonmalignant hRPE cells compared to cancer cells. The density of transferrin receptors is a marker for cell proliferation. Cultured hRPE cells express a lower density of transferrin receptors than cancer cells. As a result, nonmalignant proliferating hRPE cells may bind fewer Tfr-rRA molecules than cancer cells, and hence will be less affected by Tfr-rRA. However, the potency of Tfr-rRA compares favorably with two antiproliferative agents investigated for the treatment of PVR; fluorouracil and daunorubicin have a 50% inhibitory dose of 3000 nM on hRPE cells and 40 nM on rDfB, respectively. These two drugs have effectively prevented the development of experimental PVR. By analogy, the data in this study suggest that Tfr-rRA may be effective in the treatment of experimental PVR.

The antiproliferative potency of Tfr-rRA is similar to that of antitransferrin receptor immunotoxin. For example, 10,000 ng/ml Tfr-rRA caused an 86% reduction in the number of proliferating hRPE cells, while similar treatment with 10,000 ng/ml antitransferrin receptor immunotoxin, in this study, inhibited cell number by 87%. Like antitransferrin receptor immunotoxin, the cytotoxic effect of Tfr-rRA on proliferating hRPE cells was greater than might be expected on the basis of transferrin receptor density. Although proliferating hRPE cells have approximately twice the number of transferrin receptors compared to nonproliferating cells, treatment with Tfr-rRA resulted in a 6.5-fold reduction in the number of proliferating cells and no reduction in the number of nonproliferating hRPE cells. We suspect that aspects of the proliferative response other than transferrin receptor density are important in the Tfr-rRA-mediated cytotoxicity. A recycling phenomenon occurs with the natural plant toxin ricin, composed of the toxic A subunit and carrier B subunit, whereby 50% of ricin molecules are released from cells within 20 hours after initial contact. This recycled ricin then kills remaining cells. Perhaps intact Tfr-rRA molecules are recycled and kill neighboring proliferating hRPE cells to amplify the cytotoxic effect of Tfr-rRA.

A goal in the treatment of PVR is to eliminate proliferating, pathological cells without injuring normal, nonproliferating ocular cells. In the nonpathologic state, unlike PVR, there is minimal intraocular cellular proliferative activity. The retinal pigment epithelium, in nonpathologic human eyes, has a low density of transferrin receptors. Similarly, in cell culture, density-arrested hRPE cells show minimal cellular proliferation and express a low density of transferrin receptors. In contrast, both cells in PVR membranes and proliferating hRPE cells in culture have a relatively high density of transferrin receptors. This study demonstrates that Tfr-rRA, which binds to cell surface transferrin receptors, selectively killed proliferating hRPE cells and minimally affected density-arrested cells. The relative safety of Tfr-rRA is supported by a preliminary toxicity study in rabbits. When Tfr-rRA (1000 ng) was injected into the anterior chamber of rabbits, there were no clinical signs of toxicity after 30 days of follow-up (Frank Murchison and associates, unpublished results, 1990). Taken together, these data indicate that Tfr-rRA will not affect nonproliferating ocular tissue thereby reducing the likelihood of toxicity in vivo, resulting in a wide therapeutic index. In contrast, fluorouracil and daunorubicin are not completely selective for proliferating cells and thus, have a narrow therapeutic index. However, further in vivo toxicity studies using ricin A chain conjugates are warranted.

Although RPE cells are the primary cell type, other cells, including glial and fibroblast-like cells, are involved in the pathophysiology of PVR. Some of these cells may be recruited into the proliferative process by RPE cells or macrophages. Antitransferrin receptor immunotoxin not only kills proliferating hRPE cells, but also has been found to selectively kill proliferating ocular cells, such as corneal epithelial cells, corneal endothelial cells, and Tenon’s capsule fibroblasts. We did not test the activity of Tfr-rRA on other cell types important in PVR. However, our data on Tfr-rRA and the results from studies of antitransferrin receptor immunotoxin in ocular cells suggest that ricin A chain conjugates will be active against other proliferating ocular cells involved in the development of PVR. Testing Tfr-rRA in an animal model of this disease, in which host cells are recruited to actively proliferate, will provide useful information about the effectiveness of this drug in PVR.

Tfr-rRA possesses other characteristics that are attractive for treatment of PVR. Tfr-rRA significantly inhibited the proliferation of hRPE cells after a short exposure. The degree of inhibition after 5 minutes of exposure to Tfr-rRA (32%) was similar to that obtained with a 5-minute exposure to antitransferrin receptor immunotoxin. Molecular weight, aqueous solubility, and molecular charge are factors involved in the clearance of a drug from the eye. In addition, cell membrane receptors that are specific for the drug
Tfr-rRA Inhibits RPE and Fibroblast Cell Proliferation

could influence the clearance rate. The nonpathologic eye has a low density of transferrin receptors. Thus, we would expect a low level of Tfr-rRA binding to ocular tissue, and a minimal contribution to the clearance of Tfr-rRA by a membrane receptor binding mechanism. Based on molecular weight only, Tfr-rRA would be cleared from the eye with a half-life of approximately 12 hours. Although the clearance rate of Tfr-rRA from the eye is unknown, the ability to inhibit proliferation after a short exposure suggests that Tfr-rRA could be effective before being cleared from the eye. Tfr-rRA also demonstrated a prolonged antiproliferative effect. Cell number declined for 7 days after removal of Tfr-rRA (1000 to 10,000 ng/ml) without a decrease in the viability of remaining cells. It appears that Tfr-rRA not only kills actively proliferating hRPE cells, but also prevents the proliferation of viable cells that survive treatment. It is possible that the recycling phenomenon, similar to that found with ricin, also could account for the sustained effect of Tfr-rRA. Regardless, a prolonged cytotoxic effect could increase the efficacy of Tfr-rRA in the treatment of PVR.

Tfr-rRA attaches to membrane-bound transferrin receptors on cancer cells. Transferrin blocks uptake of Tfr-rRA into cancer cells when incubated with Tfr-rRA, but is not inhibitory when added to medium after Tfr-rRA has been internalized into cells. In hRPE cells, we found that human transferrin partially reversed the cytotoxic effect of Tfr-rRA. This observation suggests that transferrin prevents the binding of Tfr-rRA to transferrin receptors, and that Tfr-rRA kills hRPE cells by a receptor-mediated uptake process. In contrast, the effect of antitransferrin receptor immunotoxin, 454A12MAb-rRA on hRPE cells was not blocked by coincubation with human transferrin. These results imply that the binding sites on the transferrin receptor for the 454A12 monoclonal antibody and transferrin are different.

Relatively high levels of transferrin are present in the vitreous humor of patients with PVR. Because transferrin partially blocked the effect of Tfr-rRA, our results suggest that endogenous vitreous transferrin could diminish the cytotoxic effect of Tfr-rRA in the treatment of PVR. Our data also indicate that the presence of transferrin in the vitreous humor will not interfere with the antiproliferative activity of antitransferrin receptor immunotoxin. The effect of transferrin on the cytotoxic effect of Tfr-rRA in the eye has not been tested. It is not known if the high levels of transferrin obtained from vitrectomy samples translate into increased levels of transferrin surrounding cells involved in PVR membrane formation. It should be possible to adjust the dose of Tfr-rRA to compensate for competitive inhibition by endogenous transferrin. Cotreatment with the carboxylic ionophore monensin had several effects that are potentially useful in the treatment of PVR. First, while treatment with monensin alone did not affect cell number, the combination of monensin and Tfr-rRA killed proliferating cells up to 4.8-fold higher than treatment with Tfr-rRA alone. For the most part, the selectivity of Tfr-rRA for proliferating cells was not altered by monensin; short exposures (8 hours) to both monensin and Tfr-rRA, and long exposures to monensin (7 days) combined with short (1 hour) treatment with Tfr-rRA did not alter the specific effect of Tfr-rRA on proliferating cells. However, a long exposure (7 days) to monensin and Tfr-rRA (10,000 ng/ml) caused a decrease in cell number of density-arrested cells. Extrapolated to use in vivo, these results indicate limiting the dose of Tfr-rRA below 10,000 ng/ml if combined with monensin. Second, the potentiating effect of monensin also partially reversed the neutralizing effect of human transferrin on Tfr-rRA. Finally, monensin reduced the onset of cell kill by Tfr-rRA from 48 to 24 hours. The ability of monensin to speed the onset of cell kill by ligand-drug conjugates is thought to be the principal reason for treatment success in vivo when ligand-drug conjugates were ineffective in the absence of monensin. In vivo, monensin could reduce the need for a long exposure to Tfr-rRA and could decrease the dose of Tfr-rRA needed in the treatment of PVR.

Our study demonstrates that Tfr-rRA selectively killed proliferating rabbit cells (dermal fibroblasts). A dose of 10,000 ng/ml Tfr-rRA reduced the number of proliferating rDFB by 93%, compared to controls, and had no effect on the number of density-arrested cells. These results support testing the antiproliferative effect of Tfr-rRA in a rabbit model of PVR. The data also show that the antiproliferative effects of Tfr-rRA are similar to the antitransferrin receptor immunotoxin 454A12MAb-rRA in vitro. Thus, study of Tfr-rRA in an experimental model of PVR may yield insight not only into the potential usefulness of this agent, but also the efficacy of the related immunotoxins.

Key Words
Transferrin-ricin, monensin, retinal pigment epithelial cells, cytotoxicity, proliferative vitreoretinopathy

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