Use of Immunotoxin to Inhibit Proliferating Human Corneal Endothelium

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Transferrin plays a central role in cellular proliferation and proliferating cells have been shown to express transferrin receptors with increased density. We examined the effect of an immunotoxin consisting of anti-transferrin receptor monoclonal antibody (454A12) conjugated to recombinant ricin A chain (rRTA) on the proliferation of human corneal endothelium (HCE) in vitro. In proliferating cultures an immunotoxin (454A12-rRTA) concentration of 50 ng/mL reduced cell counts at day 7 by at least 89%, with no effect observed at 0.01 ng/mL. In contrast, cell counts were only minimally reduced in confluent cultures, even after 7 days' exposure to high concentrations of immunotoxin. These data suggest that 454A12-rRTA may be used to prevent growth of human corneal endothelium in pathological conditions such as the iridocorneal endothelial (ICE) syndrome. Invest Ophthalmol Vis Sci 29:755-759, 1988

The hyperproliferation of nonmalignant tissue causes a wide spectrum of clinically significant ocular disease such as proliferative vitreoretinopathy, the iridocorneal endothelial syndromes, fibrous or epithelial downgrowth and posterior capsular fibrosis. Methods currently used to treat these conditions include surgery, laser surgery, cryotherapy and chemotherapy using 5-fluorouracil. These methods often fail completely or are unsatisfactory because they do not successfully differentiate between unwanted, proliferating cells from normal tissue.

The use of immunotoxins offers a way to selectively deliver drugs to proliferating cells for treatment of some ocular diseases. Central to the use of immunotoxins is the development of monoclonal antibodies that specifically react with antigens on the surfaces of cells and that can be used to carry potent cellular toxins to target cells. Several toxins or fragments of toxins such as diphtheria toxin, ricin and ricin A chain have been coupled chemically to antibodies to form immunotoxins.12 Ricin A chain is an enzyme that inhibits protein synthesis by chemically modifying 28 S rRNA on the 60 S ribosomal subunit to prevent protein synthesis.3 Because of the presence of the antibody in the conjugate, immunotoxins recognize the surface of specific cells. If internalization occurs, the target cell may be killed selectively. We tested the ability of such an immunotoxin directed against transferrin receptor to selectively inhibit the growth of proliferating human corneal endothelial cells in vitro.

Transferrin is a growth factor that is required for the growth of all cells, and through endocytosis using the transferrin receptor contributes to the maximal proliferation of cells in vitro.45 Other growth factors such as erythropoietin and epidermal growth factor influence cell proliferation and the expression of transferrin receptors.57 Because transferrin receptor is located at the cell surface, is present in increased amounts on the surface of proliferating cells,5-10 and is rapidly internalized after it binds to transferrin,8,10 it has been an effective target for the inhibition of malignant cells by immunotoxins.1,2,11-13 Because actively proliferating cells express a higher number of transferrin receptors, we think that proliferating cells may be killed preferentially over resting cells by an immunotoxin directed toward transferrin receptors.

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In this study, we show that an immunotoxin that contains recombinant ricin A chain and an antibody directed against transferrin receptor selectively inhibits proliferation of dividing human corneal endothelial cells compared to confluent cell cultures.

**Materials and Methods**

**Production and Characterization of Monoclonal Antibodies**

Murine monoclonal antibody 454A12 (IgG2a isotype) is specific for human transferrin receptor and was produced by standard fusion techniques described previously. This antibody binds to cell surface transferrin receptors, immunoprecipitates the 95K MW subunit transferrin receptor, recognizes an antigenic site distant to the actual transferrin binding site and does not interfere with transferrin binding to its receptor or to the internalization of transferrin. Ammonium sulfate precipitation, DEAE-Sepharose chromatography, and Sephacryl S-300 gel filtration were used to purify the antibody contained in ascites fluid from Balb/c mice.

**Immunotoxin Synthesis**

Recombinant ricin A chain was obtained from *E. coli* and purified to over 99% homogeneity. Iminothiolane was used to introduce a thiol group onto the surface of 454A12 and a mixed disulfide bond was made using Ellman's reagent \( [5,5'-dithiobis(2-nitrobenzoic acid), DTNB] \). Disulfide exchange between the mixed disulfide bond and the free thiol group of rRTA formed a disulfide bond between 454A12 and rRTA. The immunotoxin was purified by a combination of chromatography and gel filtration; the final product contained no detectable unconjugated rRTA or 454A12.

**Cell Cultures**

Under sterile conditions, human corneal endothelial cells were scraped from donor corneas within 2 hr of harvesting. Stock cultures were seeded on gelatinized 35 mm dishes (Becton/Dickenson, Oxnard, CA) in medium 199 (GIBCO, Grand Island, NY) containing 15% fetal calf serum (Sterile Systems, Logan, UT), Earle's balanced salts (GIBCO), and 1% of 200 mM glutamine (Sigma, St. Louis, MO). The cells were maintained in 5% CO₂ at 37°C with fibroblast growth factor (University of California, San Francisco) added every other day. Once confluent, cells were trypsinized and seeded in 2 ml of medium on gelatinized 35 mm dishes at 2 X 10⁴ cells/ml for individual experiments.

**Assay of Immunotoxin Activity**

The effect of 454A12-rRTA on cellular proliferation was assayed using two sets of duplicate plates containing serial dilutions of immunotoxin. Four sets of controls were used, including plates with unconjugated rRTA, with purified unconjugated 454A12 antibody, with rRTA conjugated to MOPC21 (an irrelevant immunotoxin) or without any additives. Proliferating cells were exposed continuously to a single dose of 454A12-rRTA or control protein for all 7 days of culture, whereas confluent cultures were exposed for 7 days after achieving confluence. Dead cells were observed to detach from the dishes and were aspirated prior to counting; unexposed plates did not contain detached nonviable cells. No detached cells were viable as assessed by trypan blue exclusion. The number of live cells was determined with a Coulter counter after 7 days of incubation with immunotoxin or control protein.

The effect of immunotoxin on amino acid incorporation was assayed in duplicate with 3 X 10⁴ cells in 0.5 mL of medium seeded in borosilicate glass vials (ICN Radiochemicals, Irvine, CA) coated with 0.2% gelatin. After 48 hr of proliferation, cells were incubated in duplicate for 18 hr in the presence or absence of 454A12-rRTA. The cells were then rinsed three times with phosphate-buffered saline (PBS) and incubated with 0.2 mL of leucine-free 1640 RPMI (GIBCO) containing 2.0 μCi of [H³H]leucine (Du Pont NEN Research Products, Boston, MA). Fetal calf serum (10%) was added to maintain cellular adherence during the incubation with [H³H]leucine. After 3 hr of incubation, the cells were washed three times with PBS and the protein was precipitated with 5% trichloroacetic acid (GIBCO). Counts were measured in a Tricarb® liquid spectrometer scintillation counter with 5 mL of scintillation fluid (Research Products International, Elk Grove, IL).

**Data Analysis**

The effects of 454A12-rRTA on cellular proliferation and [H³H]leucine incorporation were determined by comparing the growth or incorporation in plates or vials containing immunotoxin with control plates or vials from the same stock to which no 454A12-rRTA was added. Results from plates or vials without additives were defined as representing 100% of control numbers for that experiment and were compared to results from plates or vials with 454A12-rRTA, rRTA or 454A12 alone. Percent cell survival and [H³H]leucine incorporation were calculated for plates or vials containing additives as compared to negative controls. These data were plotted against the log dose (ng/ml) of 454A12-rRTA.
Results

Inhibition of Proliferation With Immunotoxin

Proliferating HCE cells were significantly inhibited by 454A12-rRTA in a dose-dependent fashion (Fig. 1). Addition of 454A12-rRTA at 50 ng/ml resulted in at least an 89% decrease in the number of viable cells compared with controls without 454A12-rRTA, and the few remaining live cells were dysmorphic on light microscopy. Little or no effect was observed at 0.01 ng/ml. Cell survival at an immunotoxin concentration of 50 ng/ml ranged from 50% to 75% of the original number plated.

Unconjugated rRTA or 454A12 alone failed to diminish cell numbers (Fig. 1). The irrelevant immunotoxin, MOPC21-rRTA, diminished cell counts compared with controls by 25% at a concentration of 250 ng/ml, presumably through nonspecific binding and internalization (data not shown). In a separate experiment, the effect of the immunotoxin was shown to be specific since its action could be readily blocked by the presence of unconjugated 454A12 at a concentration of 2500 ng/ml (Fig. 2).

In contrast to the relative sensitivity of proliferating HCE cells, confluent cultures were much less affected by 454A12-rRTA, as shown by measuring the number of live cells after treatment with various concentrations of immunotoxin (Fig. 1). There was no significant drug effect up to a concentration of 2500 ng/ml. At 50 ng/ml, a concentration that completely inhibited the growth of proliferating cells, the ratio of immunotoxin to cell number was 6.95 pmol per one million cells. A concentration of immunotoxin 50 times higher (2.5 μg/ml, equivalent to 28.7 pmol per million cells) had no effect on confluent cells. HCE cells in confluent cultures did not become dysmorphic after exposure to 454A12-rRTA; however, proliferating HCE cells quickly became dysmorphic.

The stability of the immunotoxin during the course of the study was demonstrated by removing samples of the culture medium and assaying for the presence of unbound immunotoxin on a sensitive cell line (MCF-7). There was no change in specific activity of unbound immunotoxin over the course of the experiment.

Inhibition of Protein Synthesis

Protein synthesis, as measured by the incorporation of [H3H]leucine into protein, was significantly inhibited in a dose-dependent fashion by 454A12-rRTA in both proliferating and confluent HCE cells compared to unexposed controls (Fig. 3). The average cell density for confluent cells was four times that of...
proliferating cells during [H^3H]leucine incorporation studies. Although cells grown at either density were inhibited, there was a differential sensitivity to the immunotoxin. After correcting for the number of cells by using the ratio of immunotoxin to one million cells, proliferating cells (2.32 pmol per one million cells) were estimated to be at least 2.5-fold more sensitive than confluent cells (5.8 pmol per one million cells). This ratio was calculated from the concentrations of immunotoxin that inhibited 50% of the protein synthesis, 100 ng/ml and 1000 ng/ml for proliferating and confluent cells, respectively.

**Discussion**

An increase in the expression of transferrin receptors occurs with cell division in both hemoglobin- and non-hemoglobin-producing cells, as described by many investigators. Immunotoxins directed against the transferrin receptor or transferrin conjugated to rRTA are efficient inhibitors of cellular proliferation. Most attention has been directed to the use of such conjugates to inhibit malignant cells, and little work has been done to investigate the possibility of using these conjugates to inhibit the hyperproliferation of normal tissue.

Hyperproliferation of ocular cell types occurs in a wide spectrum of ocular diseases, including proliferative vitreoretinopathy, epithelial downgrowth, the ICE syndromes, recurrent pterygium, filtration surgery failure and posterior capsular fibrosis after extracapsular cataract extraction. Methods currently used to treat these conditions include surgery, 5-fluorouracil, cryotherapy and laser applications. By the selective inhibition of proliferating cells with specific immunotoxins, treatment of some of these diseases may be possible.

Alvarado et al have recently discussed the establishment of long-term culture techniques for HCE cells. We chose HCE cells as the target tissue because of the potential application of immunotoxin in the ICE syndrome and because of our experience in working with these cells. Our data clearly demonstrate that proliferating HCE cells may be selectively killed by 454A12-rRTA as compared to confluent HCE cells. This suggests that it may be possible to treat hyperproliferative eye disorders in vivo without significantly damaging surrounding eye tissue. In this regard it is important to show that whether surrounding eye tissues do not express active transferrin receptors in significant amounts or are otherwise resistant to the detrimental effects of the immunotoxin.

Normal human tissues express transferrin receptors in varying density at rest or during cell division. Tissues that express higher densities of transferrin receptors include basal epidermis, hepatocytes, Kupffer cells, pituitary, tests and endocrine pancreas. Tissues that would also be theoretically at risk to 454A12-rRTA include those with rapid rates of turnover, such as hematopoietic cells or cells lining the gastrointestinal tract. However, for many regional applications such as in the eye, the amount of immunotoxin administered would be small and should not reach significant levels systemically. The ocular condition that could benefit from the immunotoxin must be defined precisely, with consideration given to the dose, method of administration and results of toxicity studies. In vivo studies are needed to better address these issues prior to the clinical use of immunotoxins.

The best measure of cell death in vitro is obtained from counting the number of living cells, and this assay clearly shows differential sensitivity between confluent and proliferating cells. These data derived from cell counts are encouraging, but it appears from the [H^3H]leucine data that functional transferrin receptor activity in confluent HCE cells is not completely eliminated. In this case the inhibition of protein synthesis observed in the confluent HCE cells may represent a nonlethal insult to the resting cell. Although one can postulate that a small proliferating subpopulation of HCE cells in the confluent cultures accounts for the decrement in [H^3H]leucine incorporation, this appears unlikely since baseline protein synthesis per cell was not diminished in confluent HCE cell controls compared with proliferating HCE controls.

Although these data suggest that selectivity for proliferating HCE cells can be achieved in vitro, it is not clear that similar results will be obtained in vivo. It is clear that immunotoxins directed against transferrin receptors are capable of preventing tumor cell growth in vivo, and that recombinant ricin A chain can be substituted for ricin A chain obtained from plants. It would be helpful to study the effects of 454A12-rRTA or similar immunotoxins in suitable in vivo models of ocular tissue hyperproliferation, or in in vitro models of wound healing to better ascertain the potential application of such agents in the treatment of clinical ocular disease. Further investigation into the role of transferrin and transferrin receptors in ocular cell division may provide new avenues to promote or reduce cell proliferation in the eye. Our findings with proliferating and confluent HCE cells suggest the possible eventual therapeutic use of immunotoxins in hyperproliferative conditions of the eye.

**Key words:** immunotoxin, cell proliferation, corneal endothelium, transferrin, transferrin receptor
Acknowledgments

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