Inhibition of Human Subconjunctival Fibroblast Proliferation By Immunotoxin

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The ability to target proliferating cells is important for agents used to modulate wound healing by decreasing the growth of fibroblasts. Proliferating cells are known to express increased numbers of transferrin receptors and have increased receptor turnover. 454A12 Mab-rRA, an immunotoxin containing anti-human transferrin receptor monoclonal antibody conjugated to recombinant ricin A chain, was shown to inhibit the proliferation of human subconjunctival fibroblasts in vitro. A dose-related reduction of cell counts was observed in proliferating cells. More than 90% inhibition was achieved with an immunotoxin concentration of 10 ng/ml per 20,000 cells plated. In contrast, confluent fibroblasts were markedly less sensitive to the immunotoxin at equivalent concentrations. Comparative experiments demonstrated that 5-fluorouracil has less specificity for proliferating cells, with significant death of confluent fibroblasts at high drug concentrations. Invest Ophthalmol Vis Sci 33:2293–2298, 1992

Subconjunctival cicatrization is a common cause of failure in glaucoma filtering surgery.1,2 The capability to modulate wound healing holds great promise for improving the outcome of these procedures.3 The response to surgical injury provides several stages in which pharmacologic agents might act to favorably influence the postoperative course. Fibroblast proliferation is one such step that has received recent attention. Blumenkranz and coworkers have suggested using fibroblast tissue culture as a method to evaluate agents for the potential treatment of ocular proliferative disease.4 Two studies demonstrated that the chemotherapeutic drug 5-fluorouracil (5-FU) inhibits the proliferation of nonconfluent subconjunctival fibroblasts in vitro,5,6 and a recent clinical trial demonstrated its efficacy in reducing the failure rate of filtering surgery in certain high risk cases.3 However, potential side effects and a difficult mode of delivery involving multiple subconjunctival injections have led researchers to investigate alternative agents.

Immunotoxins may provide an alternative class of compounds to decrease fibroblast proliferation. They have been investigated for the treatment of malignant disease and, more recently, for the potential therapy of nonmalignant ocular proliferative disease.6,7,8,9 Immunotoxins possibly can minimize toxicity to cells with low proliferative activity,9 and this may help limit adverse effects. To assess the response of such tissue in vitro, confluent cell cultures can be used that, due to contact inhibition, have a slowed rate of cell division. The purpose of this study was to compare the effects of the immunotoxin 454A12 Mab-rRA and 5-FU on confluent and nonconfluent cultures of human subconjunctival fibroblasts.

Materials and Methods

Immunotoxin

The immunotoxin 454A12 Mab-rRA (Cetus Corporation, Emeryville, CA) consists of a murine monoclonal antibody (454A12 Mab) conjugated to recombinant ricin A chain (rRA). The 454A12 antibody (IgG1) is specific for the human transferrin receptor. Production of each component and their subsequent conjugation have been previously described.6 The stock concentration of 2 mg/ml was serially diluted with Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY) under sterile conditions to create varying immunotoxin concentrations for immediate use in cell culture experiments.

5-Fluorouracil

Fifty milligrams per milliliter of 5-FU for intravenous injection (Quad Pharmaceuticals, Indianapolis, IN), was serially diluted with DMEM under sterile conditions. The various concentrations were immediately used for cell culture experiments.
Subconjunctival Fibroblasts

Tenonectomy specimens routinely excised during glaucoma filtering surgery were used to establish primary tissue cultures. Specimens were grown as explants on the bottom of 25 cm² polystyrene tissue culture flasks (Corning, Corning, NY) in DMEM with 10% fetal bovine serum (FBS; Gibco) at 37°C in a 5% CO₂ atmosphere. Each liter of medium included 10 ml of antibiotic-antimycotic agent (Gibco). Bovine fibroblast growth factor (FGF; Sigma, St. Louis, MO) was added to flasks every other day at a concentration of 10 ng/ml of media. No iron or transferrin was added during the experiments.

When cultures reached confluence, they were passaged as follows. Medium was aspirated, followed by placement of 0.05% trypsin-0.52 mmol/l edetic acid mixture (Gibco). When cell beading was noted, the mixture was aspirated. Multiple washings with culture medium were performed to dislodge cells from the flask bottom. The cell suspension was centrifuged at 1500 rpm for approximately 8 min. Medium was aspirated and the cells were resuspended in fresh DMEM. After counting with a Coulter counter (Coulter, Hialeah, FL), cells were plated for subculture.

Immunotoxin and 5-FU Experiments

Twenty-thousand second passaged fibroblasts were plated on 35 mm polystyrene culture dishes (Becton Dickinson, Lincoln Park, NJ) with 2 ml of DMEM with 10% FBS. Twenty-four hours later, the proliferating cultures were treated with (1) rRA at 2000 ng/ml; (2) 454A12 Mab at 2000 ng/ml; (3) F9 mAb-rRA, an irrelevant immunotoxin, at 2000 ng/ml; (4) 454A12 Mab-rRA at varying concentrations (Fig. 1); or (5) 5-FU at varying concentrations (Fig. 2); or (6) were left untreated to serve as negative controls or to determine cell counts at the time of drug exposure. Other plates were allowed to grow to confluence as determined by microscopic evaluation. After cultures became confluent, they were left untreated or were exposed to 454A12 Mab-rRA or 5-FU at concentrations shown in Figures 1 and 2, respectively. All plates were incubated for 7 d from the time of treatment. The medium was not changed during this interval. FGF (10 ng/ml) was added every other day for a total of three doses to each plate. Toxicity could be observed in the cultures as affected cells became dysmorphic and then detached from the plates. At the end of 7 d, attached cells were harvested from each dish and counted. Phase contrast photomicrographs of these cultures were taken at days four and seven.

Additional confluent cultures were treated with 454A12 Mab-rRA at 1 X 10³ ng/ml or 5-FU at 1 X 10⁵ ng/ml. These concentrations were selected because they produced at least 90% inhibition of cell proliferation in the present study. The medium was changed 24 hr after these confluent cells were exposed to the toxic agents. The cultures then were allowed a "recovery" period of 1, 4, or 7 d. Proliferation of these treated fibroblasts was induced after the allotted recovery time by harvesting the cells and plating them for nonconfluent cultures (20,000 cells per dish). Each proliferating culture was counted after 7 d of growth.

Harvesting of cultures and cell counting was carried out as follows. After the medium and floating cell debris were aspirated, each dish was treated with 1 ml of the trypsin mixture and incubated at 37°C for 10 min. The culture dishes were carefully washed with trypsin to dislodge all cells. Cell counts then were determined with a Coulter counter.

All experiments were performed in duplicate. The mean cell count of each duplicate culture was calcu-
lated. Dose-response curves for 454A12 Mab-rRA and S-FU were generated by plotting the percent of control cell count (treated plate cell count/negative control plate cell count × 100) versus drug concentration for nonconfluent and confluent cultures. A graph of cell growth versus recovery time was also generated. This used data from confluent fibroblasts exposed to each drug and then allowed a recovery period before cell division was stimulated.

**Results**

Untreated control cultures counted 24 hr after plating contained slightly more than the 20,000 cells plated. Figures 1 and 2 demonstrate the respective effects of 454A12 Mab-rRA and 5-FU on confluent and nonconfluent fibroblast populations. Nonconfluent cells were highly sensitive to the immunotoxin, with over 90% reduction of cell growth induced by concentrations of 10 ng/ml and greater. Unconjugated 454A12 Mab and rRA at 2000 ng/ml had little effect on nonconfluent cells, as did F9 Mab-rRA (data not shown). Nearly equivalent inhibition of cellular proliferation was achieved with 5-FU but at notably higher concentrations of $1 \times 10^5$ ng/ml or more. Confluent populations, in contrast, were decreased only 20% or less by all levels of immunotoxin and all but the highest concentration of 5-FU, $1 \times 10^6$ ng/ml, which did result in 90% cell loss.

Microscopic evaluation of treated cells supported the cell count data. Affected cells were observed to become dysmorphic, bead up, and then detach from the plates with both agents. Figure 3 depicts the marked cell loss and dysmorphism observed with nonconfluent cells exposed to 100 ng/ml of 454A12 Mab-rRA. The effect of $1 \times 10^6$ ng/ml 5-FU, seen in Figure 3, was comparable but less dramatic, with less dysmorphism of the more numerous remaining cells. Confluent cells exhibited severe cell loss and dysmorphism when treated with $1 \times 10^5$ ng/ml 5-FU in contrast to the appearance of confluent tissue exposed to $1 \times 10^5$ ng/ml 454A12 Mab-rRA (Fig. 4). 5-FU and, to a greater extent, the immunotoxin exerted profoundly toxic effects at these doses on proliferating, nonconfluent fibroblasts.

Confluent cells that were exposed to one of the drugs and then induced to proliferate had greater cell growth with the immunotoxin than with 5-FU (Fig. 5). Fibroblasts stimulated to divide after a 1 d "recovery" from 5-FU exposure achieved only 16% of con-

![Fig. 3. Phase-contrast photomicrographs of proliferating nonconfluent human subconjunctival fibroblasts (original magnifications ×100). (A) untreated control culture after 5 days of growth. (B) Cells after 4-day continuous exposure to 100 ng/ml of immunotoxin. (C) Cells after 4-day continuous exposure to 1,000,000 ng/ml of 5-FU.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933392/ on 05/29/2017)
Fig. 4. Phase-contrast micrographs of confluent human subconjunctival fibroblasts (original magnification ×100). (A) Untreated control culture. (B) Cells after 7-day continuous exposure to immunotoxin at a concentration of 100,000 ng/ml. (C) Cells after 7-day continuous exposure to 1,000,000 ng/ml of 5-FU.

trol growth. A linear increase in cell counts occurred at 4-day and 7-day recoveries. Confluent cells treated with 454A12 Mab-rRA retained an average of about 80% control growth when stimulated to proliferate after the recovery periods.

Discussion

Immunotoxins are hybrid molecules that consist of two components: an antibody directed against a specific cell surface antigen and a cellular toxin. The antibody imparts a high degree of specificity for cells expressing the antigen while also providing an avenue for the toxin to bind to its target. The immunotoxin used in the present study, 454A12 Mab-rRA, selectively acts on human cells that have the transferrin receptor on their surfaces. Its cytotoxic effect against malignant and nonmalignant cells in vitro has been demonstrated previously. The antibody binds to a locus on the receptor distant from the transferrin binding site so that it does not interfere with transferrin binding. Once bound, the immunotoxin-receptor complex undergoes internalization and it is assumed that cleavage of the disulfide bond linkage releases the antibody from recombinant ricin A chain. The free ricin A chain enzymatically attacks ribosomal RNA, inhibiting protein synthesis and causing cell death. Exposure of human cells to 454A12 Mab-rRA in vitro for as little as 5 min can cause significant toxicity. Unconjugated ricin A chain penetrates cell membranes ineffectively, as demonstrated by the poor inhibitory effect on proliferation it exerted in vitro.
Proliferating cells have increased levels of protein and DNA synthesis and, therefore, are more susceptible to agents that interfere with these processes. Thus, it is expected that 5-FU, an inhibitor of thymidylate synthetase and RNA function, and 454A12 Mab-rRA will be more toxic to proliferating cells. The immunotoxin, however, has an additional feature that may contribute to such selectivity. Dividing cells have an increased requirement for the iron transport protein transferrin. Various cell types in a proliferative state, including Tenon’s fibroblasts, express an increased density of transferrin receptors on the cell membrane. This result in a significantly higher rate of 454A12 Mab-rRA entry into dividing compared to nondividing cells. In contrast, 5-FU may not exert such a degree of specificity at the level of internalization. The absence of this added mechanism to limit toxicity in nonproliferating tissue could explain why the present study demonstrates significant confluent cell loss at high doses of 5-FU.

Fulcher and Jaffe showed a greater toxicity of 454A12 Mab-rRA on nonconfluent than on confluent human corneal endothelial and retinal pigment epithelial cells, respectively. The present study demonstrates this same specificity for rapidly proliferating human subconjunctival fibroblasts. The selectivity of the immunotoxin was greater than that observed with 5-FU. 454A12 Mab-rRA showed greater potency than 5-FU in limiting the proliferation of nonconfluent fibroblasts. Confluent fibroblast populations, however, were markedly less affected by the immunotoxin compared to 5-FU at its highest dose. Although this confluent cell toxicity occurred at doses three log units greater than those needed to inhibit nonconfluent cells greater than 80%, this window of safety may be smaller than is apparent from the dose-response curves. Because of the nearly 50 times greater cell number in confluent cultures, these cells would have been exposed to less drug relative to those in nonconfluent cultures. The behavior of confluent cells that were stimulated to proliferate after treatment with 5-FU or the immunotoxin underscores the more selective effect of 454A12 Mab-rRA. The presumed greater ability of 5-FU to enter confluent cells may have allowed it to exert a greater toxic effect on these cells when they were stimulated to proliferate.

One limitation of this study concerns the inability to obtain truly confluent, nonproliferating cell cultures. Conclusions regarding data from confluent cultures must be made with caution because confluence was not found to adequately inhibit proliferation of the fibroblasts. Instead, we observed stacking of cells in multiple layers in confluent cultures. This continued proliferative activity undoubtedly contributed to decreased cell counts in confluent experiments for both agents. However, it is unlikely that the residual proliferation would account for the widespread cell loss in “confluent” cultures caused by 5-FU at its highest concentration. Although the effects of both drugs on cell counts with truly confluent populations probably is less than these data illustrate, we cannot conclude that 454A12 Mab-rRA has no effect on confluent tissue. Fulcher and coworkers found a decrease in protein synthesis of confluent corneal endothelial cells exposed to the immunotoxin. Jaffe and coworkers demonstrated dysmorphism of confluent human retinal pigment epithelial cells at higher immunotoxin concentrations. We also noted morphologic changes of confluent cells treated with higher doses of 454A12 Mab-rRA, although stacking of the fibroblasts made this more difficult to assess. These findings are consistent with the observation that nonproliferating cells express some level of transferrin receptors.

Caution also must be exercised in attempting to translate these in vitro results to a potential clinical setting. The greater selectivity for proliferating cells observed in vitro makes the immunotoxin theoretically more attractive because toxicity might be minimized in ocular tissues with a low level of cell division. However, 5-FU at lower doses also demonstrated specificity for proliferating cells, and its in vivo use may act within this window of selectivity. The common adverse effects of 5-FU—conjunctival wound leaks and corneal epithelial defects—remain potential problems with 454A12 Mab-rRA. The proliferating conjunctival epithelium and basal cells of the corneal epithelium should be sensitive to the immunotoxin and 5-FU. It is possible that the less active, nonbasal corneal epithelium expresses fewer transferrin receptors, so these cells may be less sensitive to 454A12 Mab-rRA than to 5-FU.

Interestingly, human corneal epithelial cells appear to be less sensitive to this immunotoxin in vitro than were our fibroblasts (unpublished data). The presumed ability of 5-FU to more readily enter nondividing cells than 454A12 Mab-rRA could potentiate the effects of 5-FU when these cells undergo proliferation, as the data illustrated in Figure 5 may demonstrate. This could offer a theoretical advantage of 454A12 Mab-rRA regarding toxicity. The potential for an immune reaction to the murine antibody would be of some concern. The relatively small doses needed compared to systemic administration and the local
nature of ocular delivery could help to limit this possibility. Finding the optimal delivery system for each agent should play a significant role in determining the ultimate risk-benefit ratio for these drugs. How these multiple considerations may finally interact clinically awaits in vivo studies. An important obstacle to such research in an animal model is the immunotoxin's specificity for human cells. Immunotoxins with antibody against transferrin receptors of other species might be useful in this regard.

While the specificity of 454A12 Mab-rRA presents problems for in vivo studies, it also offers the potential for advantages over 5-FU. The observations from the present study are believed to be sufficiently encouraging to stimulate continued research with 454A12 Mab-rRA, and possibly other immunotoxins, for the modulation of wound healing after glaucoma filtration surgery.

Key words: fibroblast proliferation, wound healing, immunotoxin, tissue culture, filtering surgery

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