Adenovirus-Mediated Gene Therapy Using Human p21WAF-1/Cip-1 to Prevent Wound Healing in a Rabbit Model of Glaucoma Filtration Surgery

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Objective: To determine if adenovirus-mediated p21WAF-1/Cip-1 (p21) gene therapy can prevent fibroproliferation and wound healing in a rabbit model of glaucoma filtration surgery.

Methods: In vitro studies were performed using rabbit Tenon fibroblasts harvested from fresh tissue. In vivo studies were conducted in New Zealand white rabbits. A full-thickness sclerotomy was performed under a limbal-based conjunctival flap. Reagents tested included a replication-deficient recombinant adenovirus containing the human p21 gene (rAd.p21); the nonspecific marker gene for green fluorescent protein or β-galactosidase; mitomycin, 0.5 mg/mL; and balanced saline solution. Each treatment was applied episclerally for 5 minutes before the sclerotomy using a soaked cellulose sponge placed under the surgically created conjunctival flap. Independent experiments were conducted to (1) monitor changes in intraocular pressure during a 30-day period after treatment and examine surgical site histological features, (2) examine changes in bleb morphologic features over 30 days, (3) determine outflow facility 14 days after treatment, and (4) examine the localization and persistence of rAd.p21 expression between 3 and 60 days after treatment.

Results: Treatment of Tenon fibroblasts with rAd.p21 resulted in a dose-dependent inhibition of DNA synthesis and cell growth in vitro. In vivo, rAd.p21 inhibited wound healing and fibroproliferation after filtration surgery, comparably to mitomycin. Mitomycin caused notable thinning of the bleb wall. In addition, 2 of the 5 mitomycin-treated eyes exhibited an abscess with hypopyon and hyalitis 30 days after surgery, which was not observed in any of the rAd.p21-treated eyes. None of the treatments resulted in a significantly sustained decrease in intraocular pressure during the 30-day period, although mitomycin treatment resulted in a significant (P = .02) increase in outflow facility 2 weeks after surgery in separate animals. Mitomycin- and rAd.p21-treated eyes had functioning blebs at the end of the experiment based on slitlamp examination.

Conclusions: Mitomycin and rAd.p21 were effective in preventing fibroproliferation and wound healing in a rabbit model of glaucoma surgery. Mitomycin treatment increased outflow facility in normal-pressure eyes.

Clinical Relevance: Gene therapy with rAd.p21 may provide an effective antiproliferative for glaucoma filtration surgery, without the complications associated with mitomycin.

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Treatment of cancer has been a primary focus of gene therapy protocols and clinical trials using cell cycle regulatory genes such as p53. However, other medical conditions that result from nonmalignant cell proliferation are candidates for gene therapy with cell cycle regulators. One example is the fibroproliferation that oc-
MATERIALS AND METHODS

ANIMALS

New Zealand white rabbits (Pasteurella free) were used for this study. Experimental procedures were conducted according to the guidelines established by the Association for Research in Vision and Ophthalmology for the use of animals. All animal procedures were approved by the Animal Use Committee at the University of Wisconsin, Madison. These animals were used to evaluate the effect of rAd.p21 on glaucoma filtration surgery in 18 independent experiments. A total of 20 rabbits were used to evaluate changes in intraocular pressure (IOP) and surgical site histological features. 13 were used to measure the localization and persistence of rAd.p21 expression. All experimental analyses using rabbits were performed in a masked fashion, including surgery, IOP measurements, bleb scoring, outflow facility measurements, and the determination of histological features. An additional 12 rabbits were used for alternate reagents that provide the desired antipro- liferative effect without the associated complications. 12 13 In this study, we examined the potential of gene therapy using human p21 to prevent wound healing in a rabbit model of filtration surgery. The results show that an adenovirus containing the human p21 gene (rAd.p21) can effectively block the proliferation of Tenon fibroblasts and inhibit scarring during a 30-day period in the rabbit eye, without some of the extreme effects found with mitomycin.

RECOMBINANT ADENOVIRUS

An E1/partial E3–deleted recombinant adenovirus was used for gene transfer in these experiments. Essentially, the p21 coding region, driven by the human cytomegalovirus immediate early promoter, was cloned into the E1 region of the adenovirus (rAd.p21). Control viruses were created in a similar manner; they encode bacterial β-galactosidase, jellyfish green fluorescent protein, or no added transgene (rAd..control) in place of the p21 coding sequence. Deletion of the E1 region of adenovirus renders the virus replication deficient. Details of the construction of the recombinant adenovirus are published elsewhere. 15 Recombinant viruses were grown and propagated in the human embryonic kidney cell line 293 (American Type Culture Collection, Rockville, Md) and purified using standard protocols. 16

IN VITRO STUDIES

Tenon fibroblasts were harvested from explants of the Tenon capsule dissected from freshly enucleated rabbit eyes and grown in Dulbecco Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS); penicillin G sodium, 100 U/mL; streptomycin sulfate, 100 µg/mL; and amphotericin B, 0.25 µg/mL. Only cells before passage 10 were used for in vitro analyses. Before recombinant adenovirus treatment, cells were made quiescent by serum starvation (with 0.3% FBS) for 24 hours. For thymidine incorporation experiments, quiescent cells plated in 96-well plates were incubated overnight with rAd.p21 or rAd.control (in triplicate) with increasing virus particle concentrations (4 × 10^3–3 × 10^4 virus particles per milliliter). Cells were then washed and refed with complete media containing tritiated thymidine. After 24 hours, plates were harvested and incorporated tritiated thymidine was counted (TopCount Microplate Scintillation Counter; Packard BioScience Co, Meriden, Conn).

For cell growth assays, quiescent cells were incubated with rAd.p21 or rAd.control (6.7 × 10^7 virus particles per milliliter) for 24 hours, after which the recombinant adenovirus was removed by washing. The cells were stimulated to grow by the addition of complete media. Five days later, cell growth was evaluated by counting viable cells using trypan blue exclusion.

For cell cycle analyses, primary human ocular fibroblasts from the Tenon capsule obtained during surgery were isolated and cultured as previously described. Cells were arrested by culturing in DMEM plus 0.9% FBS for 3 days. Arrested cells were treated overnight with 1 × 10^6 virus particles per milliliter of rAd.p21 or rAd.control. Cells were washed in DMEM plus 10% FBS to remove the adenovirus, and released from G0 (the quiescent phase of the cell cycle) or G1 by culturing in DMEM plus 10% FBS for 24 hours, at which time they were treated with trypsin and fixed in 70% ethanol at 4°C overnight. Cells were washed with phosphate-buffered saline (PBS), centrifuged at 1500 rpm, and treated with RNase, 5 µg/mL, for 30 minutes at 37°C. Cells were stained with propidium iodide, 50 µg/mL (Molecular Probes, Inc, Eugene, Ore), for 30 minutes, followed by flow cytometric analysis. Computer software (Cell Quest; Becton, Dickinson and Company, Franklin Lakes, NJ) was used to quantify percentages of cells in each phase of the cell cycle.

SCLEROSTOMY PROTOCOL

Surgery was conducted on one eye of each rabbit for IOP and bleb evaluation studies. In some cases, both eyes of one rabbit were operated on in animals used for outflow facility measurements. The bilateral surgery protocol was approved by the Animal Use Committee at the University of Wisconsin, providing that one eye received balanced saline solution (BSS) only as the antiproliferative agent. Rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride, 40 mg/kg body weight, and xylazine hydrochloride, 5 mg/kg body weight. A wire eyelid speculum was placed, and the eye was fixed with a corneal suture (6-0 nylon). A limbal-based flap of the conjunctiva and the Tenon capsule was made in the superior nasal quadrant of either left or right eyes, and the sclera was cauterized. Each reagent was soaked into a 1-mm cellulose sponge, which was placed on the sclera under the flap for 5 minutes. After application, the area was irrigated extensively with BSS. The irrigation step...
RESULTS

IN VITRO STUDIES

Treatment of quiescent rabbit Tenon fibroblasts with rAd.p21 resulted in a dose-dependent inhibition of DNA synthesis within 24 hours after infection, as measured by tritiated thymidine incorporation (Figure 1A). No inhibition was observed with control treatments. Fluorescent-activated cell sorter analysis of similarly treated human Tenon fibroblasts showed that inhibition of DNA synthesis by rAd.p21, but not by rAd.control, resulted in a G1 arrest of the cell cycle (Table 1). The rAd.p21-treated cells were viable, as there was no sub-G1 population indicative of debris from dead cells (not shown). To demonstrate a prolonged inhibition of cell growth, quiescent cells were infected with 6 × 10⁵ virus particles per milliliter; this dose was chosen based on results from the dose-response thy-
G1 phase by serum withdrawal, during which they were infected with 1 of the 2 viruses. Cells were then allowed to continue to proliferate for 24 hours by the addition of media containing serum. The percentage of cells that have accumulated in different phases of the cell cycle was determined by measuring DNA content. Fibroblasts released from G0 or G1 arrest are typically spread between the G1 and G2 or M phases of the cell cycle.

Table 1. Fluorescent-Activated Cell Sorter Analysis of Human Tenon Fibroblasts Treated With rAd.control or rAd.p21*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phase</th>
<th>No. of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>G1</td>
<td>36.7 ± 10.2</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>8.3 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>G2 or M</td>
<td>46.0 ± 8.9</td>
</tr>
<tr>
<td>rAd.control</td>
<td>G1</td>
<td>40.7 ± 9.3</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>9.7 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>G2 or M</td>
<td>44.7 ± 7.6</td>
</tr>
<tr>
<td>rAd.p21</td>
<td>G1</td>
<td>75.7 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>2.0 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>G2 or M</td>
<td>18.3 ± 5.7</td>
</tr>
</tbody>
</table>

*Data are given as mean ± SD percentage of cells in each phase (3 independent experiments were conducted). Cells were first held in the G0 or G1 phase by serum withdrawal, during which they were infected with 1 of the 2 viruses. Cells were then allowed to continue to proliferate for 24 hours by the addition of media containing serum. The percentage of cells that have accumulated in different phases of the cell cycle was determined by measuring DNA content. Fibroblasts released from G0 or G1 arrest are typically spread between the G1 and G2 or M phases of the cell cycle. Fibroblasts treated with rAd.control exhibit a similar distribution, but most cells infected with rAd.p21 have accumulated in the G1 phase, indicating a G1 arrest. rAd.control indicates control recombinant adenovirus (no added transgene); rAd.p21, recombinant adenovirus containing the human p21 gene; G1, early interphase; S, period of DNA replication; G2, late interphase; M, nuclear division and cytokinesis; and G0, quiescent period of G1 phase.

Slitlamp Examination of Blebs

Rabbits were examined for bleb height at various points after surgery. Figure 4 shows the change in the bleb score during a 30-day period for eyes treated with rAd.p21 compared with PBS containing 3% sucrose and mitomycin. Statistical evaluation of the data during the experiment showed significantly larger blebs for mitomycin- and rAd.p21-treated eyes relative to the eyes treated with PBS containing 3% sucrose (P<.001, unpaired t test), while blebs in the mitomycin-treated rabbits were slightly larger than blebs in the rAd.p21-treated rabbits (P=.03).
Histological Evaluation of Wound Healing

The surgical sites were examined 30 days after surgery from sections cut through the surgical site and stained with Gomori trichrome. Figure 5 shows a panel of representative sections from eyes of each treatment group. Eyes treated with either BSS or control virus (recombinant adenoviruses containing either green fluorescent protein or β-galactosidase) exhibited nearly complete scarring over the sclerostomy site, including evidence of new collagen deposition in the scleral gap created by surgery (Figure 5A and B, respectively). In contrast, eyes treated with rAd.p21 had moderate bleb cavities and minimal evidence of new collagen deposition in the sclera (Figure 5C). Eyes treated with mitomycin exhibited large acellular bleb cavities with substantial damage to surrounding tissues, including thinning of the conjunctiva and Tenon layer in the region of the surgery (Figure 5D).

Sections (range, 5-15) through each surgical site were masked and scored by 2 pathologists for the 2 criteria of fibroproliferation and cellular infiltrate into the surgical site. The consensus score of each criterion, for each eye, is shown in Table 2. For fibroproliferation, most eyes treated with BSS or control virus received scores of 3, indicative of a completely healed wound, while 5 of 6 rAd.p21-treated eyes received scores of 4, indicative of a completely healed wound.
and 4 of 5 mitomycin-treated eyes received scores of less than 3. On average, the eyes treated with BSS, control adenovirus, or rAd.p21 displayed minimal cellular infiltrate. Eyes treated with mitomycin, however, showed mild to moderate signs of inflammatory cells, including 2 that exhibited an abscess with hypopyon and hyalitis extending from the bleb cavity into the anterior chamber (Figure 6).

Intraocular Pressure

Intraocular pressure measurements taken on awake rabbits with a handheld applanation tonometer exhibited a high degree of variability. In general, the IOP decreased below baseline immediately as a result of the sclerostomy regardless of treatment. Surgical procedures that failed did so within 10 days, as determined by a return to the baseline IOP (data not shown). The data for each treatment are as follows:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IOP Change Over 30 d, Mean (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS (n=4)</td>
<td>−0.4 (4.4)</td>
</tr>
<tr>
<td>A control recombinant adenovirus containing the gene for green fluorescent protein (n=3)</td>
<td>−0.3 (3.1)</td>
</tr>
<tr>
<td>rAd.p21 (n=6)</td>
<td>−2.7 (6.2)</td>
</tr>
<tr>
<td>Mitomycin (n=5)</td>
<td>−2.9 (4.4)</td>
</tr>
</tbody>
</table>

The data were taken directly from the handheld applanation tonometer and not calibrated to true millimeters of mercury for the rabbit eye. The rAd.p21- and the mitomycin-treated eyes showed a trend toward maintaining a reduced IOP during the experiment, although these data do not show a significant decrease over the BSS- or control vector–treated eyes.

Outflow Facility

Figure 7 shows the results of outflow facility measurements in rabbit eyes 14 days after surgery, after the point when BSS-treated eyes were observed to fail based on IOP measurements. The mean ± SD outflow facility of unoperated on rabbit eyes (n=11) was 0.26 ± 0.04 μL/min per millimeter of mercury. A sclerostomy using BSS or rAd.p21 had no significant (P=.10, Mann-Whitney test) influence on the outflow facility of a normal-tension eye after 14 days, but mitomycin treatment created a significant increase in facility vs unoperated on eyes (P=.02).

COMMENT

EFFECT OF rAd.p21 IN PREVENTING WOUND HEALING

Several measurements were taken to judge the effect of p21 gene therapy in controlling wound healing in the rab-
bit eye. Observations of the bleb characteristics and histological evaluation of the wound indicate that the p21 transgene attenuates fibroproliferation and scarring in the rabbit model at a level comparable to mitomycin-treated eyes. Intraocular pressure measurements were also taken as a measure of performance of the sclerostomies. Although there was a trend toward a decrease in IOP for eyes treated with rAd.p21 and mitomycin, these data were variable and not significantly different from those of eyes treated with BSS or the control virus. Several investigations with the rabbit model have used IOP as an indicator of efficacy for antiproliferative treatments. Jampel and Moon12 showed that paclitaxel (Taxol) and mitomycin were able to prevent wound healing as judged by the change in IOP relative to the unoperated on eye of each animal. Intraocular pressure was also used to evaluate antibodies against transforming growth factor β2 by Cordeiro and coworkers.13 Their study failed to find any effect on IOP in rabbits, although effects on bleb morphologic characteristics were detected. Other studies have questioned the usefulness of IOP as a measure of successful filtration surgery and bleb function in normal-tension rabbit eyes because the ciliary epithelium and residual iris have a tendency to extend forward into the anterior chamber and block the fistula.22 This problem is evident in histological sections taken through the eyes used in this study (Figure 5), which often show the iris either pushing into the fistula or extended toward the cornea.

Our study also included examination of outflow facility in treated compared with unoperated on eyes 14 days after surgery, when control blebs had typically failed (based on IOP measurements). No change in outflow facility was noted for the rAd.p21-treated eyes relative to the BSS-treated eyes or the unoperated on eyes. However, the sclerostomy provided only a small option for outflow in these eyes, which contained an undamaged outflow pathway that was presumably still functioning normally around the entire remaining circumference. It is not surprising that a functioning bleb may not be reflected in these measurements. Mitomycin-treated eyes did show a significant increase in outflow relative to normal eyes. This observation is consistent with studies23 showing an increased risk of hypotonia in human eyes that undergo trabeculectomies using mitomycin. Mitomycin also produced complications in our study that were not observed with rAd.p21. The most noticeable were the increased incidence of inflammatory cells in mitomycin-treated eyes at 30 days after surgery (2 of 5 eyes) and the thinning of the Tenon capsule through the sclerostomy and into the anterior and posterior chambers of the eye (original magnification ×100).

Table 2. Fibroproliferation and Cellular Infiltration Scores for Rabbit Eyes Prepared 30 Days After Sclerostomy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibroproliferation</th>
<th>Overall Cellular Infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>BSS</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>BSS</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>BSS</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>rAd.LacZ</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>rAd.GFP</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>rAd.GFP</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>rAd.GFP</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>rAd.p21</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>rAd.p21</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>rAd.p21</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>rAd.p21</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>rAd.p21</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>3</td>
<td>3†</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>1</td>
<td>3†</td>
</tr>
</tbody>
</table>

*Scores are the consensus between 2 masked pathologists who viewed successive sections through the entire surgical site of each eye (0 indicates no effect; 1, minimal effect; 2, mild effect; 3, moderate effect; and 4, severe effect). rAd.LacZ and rAd.GFP are control adenoviruses. BSS indicates balanced saline solution; rAd.LacZ, nonspecific marker gene for β-galactosidase; rAd.GFP, recombinant adenovirus containing green fluorescent protein; and rAd.p21, recombinant adenovirus containing the human p21 gene.
†Granulomatous/suppurative inflammation present.
cases it causes cell cycle arrest. Treatment of cultured Tenon fibroblasts treated with mitomycin induces permanent growth arrest and extensive tissue damage.

Tenon fibroblasts with rAd.p21 results in reduced DNA synthesis and cell division, which disrupts the subsequent wound-healing cascade. Mitomycin is an alkylating agent that has numerous toxic effects on cells, although its primary mode of action is to cross-link DNA.

Cultured Tenon fibroblasts treated with mitomycin undergo apoptotic cell death, and electron microscopic evaluations of mitomycin-treated rabbit conjunctiva and Tenon capsule show extensive tissue damage.

Previous studies of p21 function show that this protein can also cause apoptotic cell death in some circumstances, but in most cases it causes cell cycle arrest. Treatment of cultured Tenon fibroblasts with rAd.p21 results in reduced DNA synthesis and cell division, which disrupts the subsequent wound-healing cascade.

The present in vivo experiments do not conclusively demonstrate that p21 inhibits wound healing by preventing cell division and subsequent extracellular matrix deposition, but Gomori trichrome staining clearly shows reduced collagen deposition and fibroproliferation in the surgical area. To expand these results, experiments are under way to characterize the time course of extracellular matrix deposition and to demonstrate inhibition of cellular proliferation in situ.

In summary, our results demonstrate the p21-specific inhibition of fibroproliferation and wound healing in a rabbit model of glaucoma surgery, which resulted in patent fistulas. These results are comparable to those obtained with mitomycin, but the toxic adverse effects, such as thin filtration blebs and persistent inflammation, observed with mitomycin use are eliminated. Consequently, p21 gene therapy may make an attractive alternative to mitomycin in those undergoing trabeculectomy.

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THE MECHANISM OF ACTION OF THE p21 TRANSGENE

The sequence of events that lead to wound healing includes the proliferation and migration of cells to the site of damage, the deposition of new extracellular matrix, the contraction of the wound margins, and the subsequent programmed death of infiltrating fibroblasts at the end of the scarring process. The strategy of using antiproliferative agents to prevent wound healing is to block the proliferation step, which disrupts the subsequent wound-healing cascade. Mitomycin is an alkylating agent that has numerous toxic effects on cells, although its primary mode of action is to cross-link DNA.

Cultured Tenon fibroblasts treated with mitomycin undergo apoptotic cell death, and electron microscopic evaluations of mitomycin-treated rabbit conjunctiva and Tenon capsule show extensive tissue damage. Previous studies of p21 function show that this protein can also cause apoptotic cell death in some circumstances, but in most cases it causes cell cycle arrest. Treatment of cultured Tenon fibroblasts with rAd.p21 results in reduced DNA synthesis and cell division, which disrupts the subsequent wound-healing cascade.

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REFERENCES


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