Objective: To determine if adenovirus-mediated p21<sup>WAF-1/Cip-1</sup> (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.

Arch Ophthalmol. 2002;120:941-949
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 statement for the use of animals in research and approved by the Animal Use Committee at the University of Wisconsin, Madison. These animals were used to evaluate the effect of rAd.p21 in glaucoma filtration surgery in 3 independent series of experiments. A total of 20 rabbits were used to evaluate changes in intraocular pressure (IOP) and surgical site histological features, 18 were used to determine changes in bleb characteristics, and 15 were used to measure outflow facility. An additional 12 rabbits were used for separate experiments to determine the localization and persistence of rAd.p21 expression. All experimental analyses using rabbits were conducted in a masked fashion, including surgery, IOP measurements, bleb scoring, outflow facility measurements, and the determination of histological features.

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. 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.

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 0.5% 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 × 10^5-3 × 10^7 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–basal 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 4 × 4 × 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 cured as part of the normal wound healing response after trabeculectomy for glaucoma, resulting in failure of the surgery. The use of antiproliferative agents, such as fluorouracil and mitomycin, has greatly increased the success of filtration surgery, but these agents are associated with complications ranging from corneal epithelial defects to late-onset bleb leaks that can lead to endophthalmitis. These complications have prompted the search for alternative reagents that provide the desired antiproliferative effect without the associated complications. 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.
was omitted in some cases, with no effect on any of the variables examined in this study (such as the level of transduction of fibroblasts). For the sclerostomy, a limbal groove angled toward the anterior chamber was first made with a No. 57 blade, followed by the production of a fistula first with a No. 75 blade and then with a Kelly punch. About 2 to 3 punches were generally taken in each eye. An iridectomy was then performed through the fistula, and the conjunctiva was closed with a 9-0 polyglactin suture. After surgery, 1 cm each of 1% atropine sulfate ophthalmic ointment and neomycin sulfate, 3.5 mg/g; polymixin B sulfate, 10 000 U; and dexamethasone, 1 mg/g, ointment was applied to the eye.

TRANSGENE EXPRESSION AND DISTRIBUTION OF VIRAL INFECTION

Immediately following euthanasia, eyes were enucleated and rinsed in PBS. The surgical site was dissected, placed in a cryovial, and snap frozen in liquid nitrogen. Total RNA was extracted from approximately 100 mg of tissue harvested from the surgical sites of 3 eyes, using a reagent (Tri-Reagent; Molecular Research Center, Inc, Cincinnati, Ohio), per the manufacturer’s protocol. Total RNA was treated with DNase I (Roche/Boehringer-Mannheim, Berkeley, Calif) to remove residual DNA. Quantification of human p21 messenger RNA was performed using reverse transcription–polymerase chain reaction with real-time detection (PE Applied Biosystems, Foster City, Calif), as described previously.17 The primers used were as follows: forward primer, 5’-AACGGAATCCGACCGGATCGG-TAMRA-3’; reverse primer, 5’-TTCTGACATGGCGCCTACT-3’; and probe, 5’-FAM-TCCGATCAGCGGATCGG-TAMRA-3’. The protein from the remaining tissue homogenate was then extracted for the detection of p21 by enzyme-linked immunosorbent assay per the manufacturer’s protocol (Oncogene Research Products, San Diego, Calif).

Expression of the p21 transgene in vivo was detected by immunohistochemistry using a monoclonal antibody against human p21 that exhibited no cross-reactivity to the rabbit homolog (Oncogene Sciences, Inc, Uniondale, NY). Briefly, globes from rabbits treated with each reagent were harvested at different times after surgery, fixed in 4% paraformaldehyde in phosphate buffer (pH, 7.2) for 48 hours at 4°C and embedded in paraffin. Paraffin sections (5 µm) were cut and stained for p21 expression, as previously described.

EVALUATION OF EFFICACY

IOP Measurements

Intraocular pressure was measured in awake rabbits before and after surgery using an applanation tonometer (Tono-Pen XL; Mentor Corp, Norwell, Mass). All IOPs were obtained at the same time each day by one of us (J.A.K.). Baseline IOPs were determined from average measurements taken one to several days before surgery after the rabbits became familiar with their handler. Intraocular pressure was monitored every 2 to 3 days after surgery for the first 10 days and every 3 to 4 days thereafter for the remaining 20 days.

Bleb Evaluation

Blebs were evaluated during slitlamp examinations on a qualitative scale of 1+ to 4+, reflecting increasing bleb height and size as follows: 1+, minimal height, conjunctival thickening, and no microcysts; 2+, microcysts are present; 3+, elevated bleb covering 3 to 4 clock hours of the eye; and 4+, greatly elevated bleb covering longer than 5 clock hours of the eye. A score of 0 indicates no observable bleb. Controls for these experiments were conducted using sponges soaked in PBS containing 3% sucrose, which is a solution used to maintain viral stability during cryostorage.

Histological Evaluation

For this study, the rabbits were killed humanely 30 days after surgery. A small burn was made with an ophthalmic cautery in the cornea of each eye to mark the surgical area before enucleation. An incision was made 90° away from the surgical site, and the whole globe was fixed in 4% paraformaldehyde in phosphate buffer (pH, 7.2) for 48 hours at 4°C and embedded in paraffin. Serial sections were then cut through the sclerostomy site. Approximately every fifth section was stained using Gomori trichrome.20 Sections were masked and evaluated by 2 pathologists who scored the extent of fibroproliferation and cellular infiltrate using the following scale: 0, no change; 1, minimal change; 2, mild change; 3, moderate change; and 4, severe change. A final score for each variable was made by consensus between the 2 observers.

Outflow Facility

Total outflow facility was determined using 2-level constant-pressure anterior chamber perfusion with Bárány21 solution. Animals were evaluated 14 days after surgery, after the time when sclerostomies performed with no antifibrotic agent typically fail. Generally, each eye was perfused for 60 minutes, during which 9 outflow facility measurements were taken. The calculated outflow facility for each eye was then taken as the average of these measurements.

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 millilitre; this dose was chosen based on results from the dose-response th-

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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 incorporated was demonstrated at rAd.p21 concentrations of 1 x 10^7 P/mL or greater. B, Trypan blue counts of viable cells 5 days after treatment with 6.7 × 10^7 P/mL of rAd.p21 or rAd.control. Treatment with rAd.p21 significantly reduced the proliferation of Tenon fibroblasts in culture (P<.001, unpaired t test), whereas the rAd.control did not (P>.05). Data are given as mean ± SD, rAd.p21 indicates recombinant adenovirus containing the human p21 gene; rAd.control, control recombinant adenovirus; and P/mL, virus particles per milliliter.

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>% of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>G1</td>
<td>36.7 ± 10.2</td>
</tr>
<tr>
<td>rAd.control</td>
<td>S</td>
<td>8.3 ± 1.5</td>
</tr>
<tr>
<td>rAd.p21</td>
<td>G2 or M</td>
<td>46.0 ± 8.9</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.

Figure 1. Treatment of rabbit Tenon fibroblasts with rAd.p21 inhibits DNA synthesis and proliferation in vitro. A, Tritiated thymidine incorporation, plotted as a percentage of untreated cells, is inversely correlated with rAd.p21 concentration. No effect was observed with rAd.control treatment. Complete inhibition of incorporation was demonstrated at rAd.p21 concentrations of 1 × 10^7 P/mL or greater. B, Trypan blue counts of viable cells 5 days after treatment with 6.7 × 10^7 P/mL of rAd.p21 or rAd.control. Treatment with rAd.p21 significantly reduced the proliferation of Tenon fibroblasts in culture (P<.001, unpaired t test), whereas the rAd.control did not (P>.05). Data are given as mean ± SD, rAd.p21 indicates recombinant adenovirus containing the human p21 gene; rAd.control, control recombinant adenovirus; and P/mL, virus particles per milliliter.

### IN VIVO STUDIES

#### Characteristics of Transgene Expression

Experiments were conducted to determine the pattern and persistence of transgene expression. Immunohistochemical staining for human p21 protein in rabbit eyes 14 days after rAd.p21 treatment and surgery showed numerous fibroblasts expressing the transgene throughout the flap region (Figure 2B). No p21 staining was evident in eyes treated with BSS (Figure 2A), rAd.control, or mitomycin (data not shown). Other experiments, conducted with the β-galactosidase–expressing vector to identify transduced cells, showed that vascular endothelial and conjunctival epithelial cells were transduced in addition to Tenon fibroblasts. No evidence of p21 expression in scleral fibroblasts, ciliary body epithelial cells, retina, iris, or any cells of the cornea was found using this mode of recombinant adenovirus application (data not shown).

The persistence of p21 transgene expression was investigated at the messenger RNA and protein levels. Quantitative reverse transcription–polymerase chain reaction analysis identified transgene-specific transcripts as late as 60 days after treatment, with levels decreasing throughout the experiment (Figure 3A). Enzyme-linked immunosorbent assay analysis of protein fractions from the same eyes showed detectable recombinant protein up to 28 days after infection (day 60 was not evaluated) (Figure 3B), which also decreased with time.

#### 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 scar formation 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

Figure 2. Immunohistochemical localization of human p21 protein in cells of treated rabbit eyes 14 days after sclerostomy. A, Nomarski interference photomicrograph of the wound area in the conjunctiva and Tenon capsule of an eye treated with balanced saline solution. No staining is evident. The asterisk indicates a small open area remaining from the surgical bleb that is predominantly filled with fibroblasts. B, Micrograph of the corresponding region in an eye treated with a recombinant adenovirus containing the human p21 gene. Cells lining the open areas of the bleb (asterisks) are positive for human p21 protein. The overlying conjunctival flap (con) is positioned at the top of each image (original magnification ×400).

Figure 3. In vivo p21 transgene expression persists beyond 30 days. A, Reverse transcription–polymerase chain reaction analysis to detect recombinant adenovirus containing the human p21 gene messenger RNA shows detectable transcripts in homogenates of surgical sites up to at least 60 days after treatment by sponge application. The mean copy number of messenger RNA is calibrated against a standard of known copies of template. B, Enzyme-linked immunosorbent assay analysis indicates that p21 protein persists up to 28 days after treatment (day 60 not done). Protein concentration is indicated as human p21 units, based on an internal standard of purified human p21 protein. Data are given as mean ± SD.

Figure 4. Bleb scores for rabbit eyes treated with mitomycin, 0.05 mg/mL; rAd.p21; or V-PBS. The mean ± SEM for 6 eyes at each treatment is shown over 30 days from the day of surgery and treatment. Mitomycin-treated eyes exhibited significantly larger blebs relative to rAd.p21 (P = .03, unpaired t test) and V-PBS (P < .001) during all stages of the experiment. Eyes treated with rAd.p21 exhibited significantly larger blebs than those treated with V-PBS (P < .001). rAd.p21 indicates recombinant adenovirus containing the human p21 gene; V-PBS, phosphate-buffered saline containing 3% sucrose.

Figure 5. 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. In contrast, eyes treated with rAd.p21 had moderate bleb cavities and minimal evidence of new collagen deposition in the sclera. 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.
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</td>
<td>−0.3 (3.1)</td>
</tr>
<tr>
<td>gene for green fluorescent protein (n=3)</td>
<td></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 Moon 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.

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 studies 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 and conjunctiva in the surgical area (5 of 5 eyes). Although the numbers of cases reported in this study were too few to assign a definitive correlation with mitomycin use, these complications were not observed in any of the other eyes. These data are also consistent with numerous reports that mitomycin treatment can lead to excessively leaky and thin-walled blebs that are prone to late-onset infections.

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.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>3</td>
<td>0</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.

Figure 6. Gomori trichrome–stained section of the surgical site of an eye treated with mitomycin. The cornea is to the right of the micrograph and the conjunctiva (con) is at the top. The region of the sclerostomy is indicated with arrows. At the time of humane killing, 30 days after surgery, this eye exhibited severe infiltration of inflammatory cells extending from the Tenon capsule through the sclerostomy and into the anterior and posterior chambers of the eye (original magnification ×100).
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, while fluorescent-activated cell sorter analysis shows that these cells accumulate in the G1 phase of the cell cycle.

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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Corresponding author and reprints: Robert W. Nickells, PhD, Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison, 1300 University Ave, Room 6640 MSC, Madison, WI 53706-1532 (e-mail: nickells@facstaff.wisc.edu).

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, while fluorescent-activated cell sorter analysis shows that these cells accumulate in the G1 phase of the cell cycle.

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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Figure 7. Outflow facility of normal-tension rabbit eyes 14 days after sclerostomy with mitomycin, rAd.p21, or BSS, compared with unoperated on eyes. The mean ± SEM of a minimum of 4 eyes per group is shown. The BSS- and rAd.p21-treated eyes have no significant increase in outflow facility (P > .2, Mann-Whitney test), while mitomycin causes a significant increase in facility relative to untreated eyes (P < .02). rAd.p21 indicates recombinant adeno-virus containing the human p21 gene; BSS, balanced saline solution.
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