Electroporation and Bleomycin in Glaucoma-Filtering Surgery

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Purpose. To develop a new treatment in glaucoma-filtering surgery using combined treatment with electroporation and the antiproliferative drug bleomycin.

Methods. Pigmented rabbits were treated with both bleomycin (10 μg/ml) and localized electric pulses (EP) using a special probe (5 V/cm, 100 msec, 8 pulses) (n = 10; group A). After EP treatment, bleomycin was washed out with 50 ml balanced salt solution, and then a posterior lip sclerectomy was performed on the same area. We also studied rabbits undergoing a posterior lip sclerectomy with bleomycin treatment alone (n = 10; group B), a posterior lip sclerectomy with EP treatment alone (n = 5; group C) and a posterior lip sclerectomy alone using the same operation (n = 5; group D, negative control). The intraocular pressure (IOP) was measured before and 1, 3, 5, 7, 10, 15, and 20 days after surgery. The formation of blebs, the conjunctiva, and the cornea were periodically examined by slit-lamp biomicrography.

Results. In every group, the IOP decreased until day 7, and no significant difference was observed among the four groups. In groups B, C, and D (control), the IOP increased gradually from day 10 and thereafter returned to the preoperative level after 15 days. However, in group A, the IOP remained lower than the preoperative level for 20 days; it was also significantly lower than each of the other three groups (P < 0.01). The survival rate of a filtering bleb was significantly higher in group A than in groups B, C, or D, but the survival rate in group B was not higher than groups C or D. No adverse effects were clinically observed in the ocular tissue, such as the cornea and conjunctiva.

Conclusions. The combined treatment with EP and bleomycin was found to decrease IOP more prominently than EP or bleomycin treatment alone in filtering surgery. This treatment thus makes filtering surgery effective by decreasing the dose of the antiproliferative drug and by possibly localizing the drug delivery. Invest Ophthalmol Vis Sci. 1997;38:2864–2868.
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All operations were performed by the same surgeon (Y. Oshima). Japan pigmented rabbits (2.5 to 3 kg) were used. After an intramuscular injection of ketamine hydrochloride (50 mg/kg), a lid speculum was inserted and the superotemporal conjunctiva was incised near the fornix with spring scissors. The conjunctiva was carefully dissected anteriorly to the limbus. Next, Tenon's tissue overlying the sclera was excised.

In group A (10 eyes), the conjunctiva of the upper limbal area was draped over the cut cellulose sponge (5 × 5 × 5 mm) soaked with bleomycin (Nihon Kayaku, Tokyo, Japan; 10 μg/ml) for 5 minutes, and then the sponge was removed. The optimal dose of bleomycin was determined based on our previous in vitro and in vivo studies (data not shown). The cup-shaped electric probe (Fig. 1A) was placed in the anterior position and the bleomycin and EP (5 V/cm, 50 msec, 8 pulses) were delivered (Fig. 1B). The EP was produced by a generator (BTX, San Diego, CA) and the electroporation was performed transconjunctivally. The power of the EPs was also determined in our preliminary study. Using this procedure, no thermal changes or damages directly related to EP were observed. Immediately after that, the bleomycin was washed away with 50 ml balanced salt solution (BSS), and thereafter a posterior lip sclerectomy was performed. For a posterior lip sclerectomy, a groove incision was

FIGURE 1. Photographs of the surgical procedure of electroporation. (A) A cup-shaped electric probe specially designed for glaucoma-filtering surgery. (B) The surgical procedure. (C) A filtering bleb of the rabbit eye on day 20 (group A). (D) The scar of a filtering bleb of the rabbit eye on day 20 (group D). The electric currency was transferred between the metal bars (arrows). The cup-shaped electric probe is placed on the rabbit eye and the electric pulse is delivered (B). A filtering bleb is well formed in a eye treated with electric pulses and bleomycin (arrows) (C), but only a scar was present in an eye of the control group (arrowhead) (D).
made with a razor blade and then was extended anteriorly into the corneal stroma. The area was cauterized over the sclera to prevent bleeding. The anterior chamber was entered through the filtering side and a 3 × 3 mm block of scleral tissue and trabecular meshwork was excised using Vannas scissors. A small peripheral iridectomy was also made with small scissors. The conjunctiva was closed with a continuous 8-0 virgin silk suture to produce a watertight closure.

In group B (10 eyes), a cut cellulose sponge (5 × 5 × 5 mm) soaked with bleomycin (10 μg/ml) was also put on the limbal area of the upper sclera for 5 minutes, and then the sponge was removed. The electric probe was placed in the anterior position where bleomycin was delivered, but no EP was given. The limbal area was washed with 50 ml BSS to remove bleomycin, the same as in group A, and a posterior lip sclerectomy was performed, as in group A.

In group C (five eyes), the effect of EP alone on the IOP and filtering bleb was examined: a cut cellulose sponge containing BSS was put on the limbal area of the upper sclera for 5 minutes. The cup-shaped electric probe was placed on the anterior position where the sponge was placed, and the limbal area was washed with 50 ml BSS. Electric pulses were delivered to the limbal area (5 V/cm, 50 msec, 8 pulses).

In group D (control, five eyes), a cut cellulose sponge (5 × 5 × 5 mm) containing BSS was also put on the limbal area of the upper sclera for 5 minutes. The electric probe was placed on the anterior position where the sponge was placed, but no EP was given. The limbal area was washed with 50 ml BSS, the same as in group A. In addition, a posterior lip sclerectomy was also performed, the same as in group A. Ofloxacin eye drops were given 5 times a day for 7 days after surgery.

**Intraocular Pressure Measurement.** Intraocular pressure was measured and a slit-lamp biomicroscopic examination of the anterior segment was performed before surgery and 1, 3, 5, 7, 10, 15, and 20 days after surgery. The IOPs of all animals before surgery were 15 to 17 mm Hg, and there was no significant difference in the average IOP among the experimental groups (data not shown). IOP was measured at the same time of the day (8 PM) using a pneumotonograph (Alcon Applanation Pneumotonograph; Digilab, Cambridge, MA), which had been calibrated for use in the rabbit by relating pneumotonometerically the IOP readings to the actual IOPs obtained manometrically. Before measuring the IOP, 1 drop of 0.4% oxybuprocaine hydrochloride was applied topically to each eye. No general anesthesia was used while measuring the IOP.

**Filtering Bleb.** The survival of the filtering bleb was evaluated by a slit-lamp biomicroscopic examination 1, 3, 4, 7, 10, 15, and 20 days after surgery. The date that the bleb appearance was first determined to be flat was considered to be the bleb failure date. The treated area was photographed and projected on a screen, and the presence of a bleb was determined by three masked observers (Figs. 1C and 1D). When two of the observers agreed to a diagnosis and the other did not, the majority opinion was chosen. The filtering bleb and the surrounding tissue (e.g., conjunctiva, cornea, anterior chamber, iris, lens, and sclera) were also clinically examined at the same time.

**Statistical Analysis.** The IOP change after surgery was used as an index of the filtering bleb surgery. Differences in the IOP change among the experimental groups were analyzed using the Kruskal-Wallis test and the Wilcoxon rank-sum test. The survival rate of the filtering bleb was estimated using the Kaplan-Meier method. Differences in the survival curves among the four groups were analyzed using the log rank test. A probability value < 0.05 was considered significant; however, the probability value was adjusted by Bonferroni's procedure to correct for multiple comparisons.

**RESULTS. Surgical Procedure.** The surgical procedure caused no systemic changes in the animal. In addition, no abnormal reaction related to the EP treatment (e.g., cardiac arrest, unusual muscular movement) occurred either during or after the procedure.

**Intraocular Pressure Measurement.** In every group, IOP decreased on the day after surgery until day 7. There was no statistically significant difference among the four groups. In groups B, C, and D, IOP increased gradually after day 10 and returned to the preoperative level after 15 days. In group A, IOP remained lower than the preoperative level for 20 days; the IOP change in group A was also significantly greater than each of the other three groups (P < 0.01) (Fig. 2). The comparisons of IOP of groups...
B and C, groups B and D, and groups C and D showed no significant difference.

**Filtering Bleb.** In all eyes from each group, the filtering blebs were well formed and survived at least 5 days. In group A, the survival rate was 80% on day 20; in groups B, C, and D, the survival rates on day 20 were 0%. The survival rate of the filtering bleb in group A was significantly higher than in group B ($P < 0.005$), group C ($P < 0.005$), or group D ($P < 0.001$). The survival rates of group B, however, were not higher than in group C or D. There was also no significant difference in survival rate between groups C and D (Fig. 3). Conjunctival injection was noted for 3 days after surgery in every group but disappeared after 5 days. No significant adverse changes (e.g., corneal erosion, corneal opacification, neovascularization, cataract, presumed bleb leak, endophthalmitis, or moderate to severe inflammation on the sclera) were noticed in any of the eyes in each group.

**DISCUSSION.** In this study, the combined treatment of electroportation and the antiproliferative agent bleomycin in filtering surgery significantly decreased IOP in rabbit eyes from 10 days to 20 days. This treatment also improved the survival rate of filtering blebs. Bleomycin treatment alone did not increase the survival rate of the filtering bleb nor lower the IOP level for 20 days. In addition, EP treatment alone (5 V/cm, 50 msec, 8 pulses) did not lower the IOP or improve the survival rate of filtering surgery compared with filtering surgery alone (group D). Therefore, the combined treatment is considered to be important for obtaining a sufficiently low IOP and filtering bleb in this experimental system.

In our previous study, administering EPs alone or with low-dose bleomycin had no effect on the proliferation of the retinal pigment epithelial cells in vitro. However, the combined treatment with both of them could greatly augment the inhibitory effect of bleomycin by inducing apoptosis (100 times greater than bleomycin alone). Therefore, a similar biologic mechanism might also play a role in this combined therapy. Excessive fibrosis and fibroblast proliferation are the major causes of failure for glaucoma-filtering surgery. The use of antiproliferative drugs such as mitomycin C or 5-fluorouracil has helped to improve glaucoma-filtering surgery greatly by inhibiting both excessive fibroblast proliferation and excessive fibrosis.6–10 The excessive fibrosis and proliferation of conjunctival fibroblasts might be inhibited by this combined treatment.

Clinically, bleomycin is widely used to inhibit cancer cell proliferation. It is a water-soluble antibiotic, and its inhibitory effect on cell proliferation is believed to be related to its ability to induce single- and double-strand DNA breaks. The exact mechanisms by which EP enhances the effect of bleomycin are unclear but can be speculated from previous studies. The plasma membrane is known to limit the uptake of bleomycin into cytosol, which thus prevents bleomycin from working effectively.12 Electric pulses make reversible pores in the cell membrane and thus allow for the direct internalization of molecules into the cytoplasm. This direct membrane-internalizing effect of EP may therefore allow bleomycin to enter the cytosol easily, thus increasing bleomycin's inhibitory effect on cell proliferation.

The possible advantages of this treatment are as follows. First, the use of a low-dose drug can decrease the unwanted effects on the surrounding tissue. In this study, 10 μg/ml (6.7 μM) bleomycin and EP treatment was effective enough to decrease IOP, but a much higher dose was necessary to obtain a low IOP in filtering surgery in rabbits treated with mitomycin C (200 μg/ml [425 μM]) or 5-fluorouracil (50,000 μg/ml [384 mM]). It is inappropriate to compare the biologic effect of different drugs based on their doses, but the dose of bleomycin in this combined therapy was 10 times lower than that of bleomycin alone to obtain the same effect in our pilot study (data not shown). Indeed, EP treatment could possibly reduce the amount of bleomycin required to keep IOP low after filtering surgery. We are now investigating the effect of EP on other drug effects.

Second, the present treatment could also possibly help deliver the drug to highly selective areas. The antiproliferative effect of bleomycin is beneficial to the survival of the bleb, but it can also be harmful to the cornea or adjacent conjunctival tissue. Because the augmentative effect by EP on the drug is caused only by the transient permealization of bleomycin through the cellular membrane,12 the area without EP treatment can be free from (or only minimally influenced by) bleomycin. Although the local concentration of bleomycin could not be obtained be-
cause of the sensitivity of the assay system (data not shown), the effect of bleomycin was supposed to be localized where EP was delivered. If the surrounding conjunctiva had remained intact without the influence of any antiproliferative drugs, additional treatment might have been easy to administer, even in the case of complications.

So far, pharmacologic therapy has been a major treatment for ocular disease. Although a variety of drugs have been proven to modulate cellular functions in vitro, not all of these have yet been proven appropriate for clinical treatment. One of the major reasons for that is the lack of an appropriate drug-delivery system to the tissue in vivo, and the effectiveness of drugs is often limited by their insufficient selectivity. Even by local administration of a drug into the eye (e.g., direct injection to the vitreous), the intracellular concentration sufficient to modulate the pathologic cells is often so strong that it impairs the surrounding ocular tissue, including the retina and cornea. This is a large obstacle in the clinical application of drugs. Therefore, a highly selective drug-delivery system is preferred for the pharmacologic treatment of ocular tissue. Until now, drug-delivery systems have attempted to use the drug conjugation either with tissue-specific antibody or an alteration of protein structures coding for the functional domains. How- ever, none of these methods have been found to be appropriate for drug delivery to very small areas in the same tissue.

The present target drug-delivery system by EP has no such limitations. In addition, we found no damage caused by EP either locally or systemically. Transdermal drug delivery by EP has already been attempted in clinical trials with cutaneous melanoma, and the power of the EP range used in this study is thus considered safe enough for use in humans. However, further analysis is necessary to prove the safety of this procedure in humans: the eye contains very sensitive tissue, and unpredictable effects might be induced locally or systemically.

Our study demonstrated that combined drug treatment with EP offers a potentially beneficial way to minimize the amount of antiproliferative agent used and possibly to localize the effect of anticellular proliferation, resulting in potentially safer and more effective filtering surgery. We are now investigating the combined effect of EP and mitomycin C or other antiproliferative drugs, and our results will be reported soon. The eye is an easily accessible organ during surgery, and future modifications in surgical devices or an electric probe are thus expected to make not only glaucoma surgery but also the treatment of various other diseases safer and more effective.

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Key Words

antiproliferative drug, electric pulse, 5-fluorouracil, mitomycin C, target drug delivery

References