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Effects of subconjunctival 5-fluorouracil injections on the corneal endothelium and ciliary epithelium

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Abstract. The effects of 14 successive daily subconjunctival 5-fluorouracil (5-FU) injections on corneal endothelial and ciliary epithelial cells were studied in pigmented rabbits. A total of 100 µl 5% or 1.25% 5-FU was injected subconjunctivally in one eye and vehicle solution was injected in the other eye. At 12 h or 14 days after the 14th and last injection, both eyes were processed for observation with scanning or transmission electron microscopy. In other groups of rabbits injected with 5% 5-FU, effects on the corneal thickness, corneal endothelial permeability, blood-aqueous barrier permeability, intraocular pressure and aqueous flow rate were also studied. In the fellow eyes and treated eyes examined 14 days after the last injection, no significant morphological changes were seen. In eyes examined 12 h after the last injection, the following findings were obtained. The corneal endothelial cells showed moderate cytoplasmic swelling and mitochondrial swelling and vacuolation. The changes were dose-dependent and most marked in the area near the injection site, whereas they were minimal in the area apposing it. In the ciliary body adjacent to the injection site, disarrangement of the basal infoldings of non-pigmented epithelial cells was seen in eyes injected with 5% 5-FU, whereas no significant change was observed in those injected with 1.25% 5-FU. However, the physiological parameters examined remained unaltered.

Introduction

Postoperative subconjunctival 5-fluorouracil (5-FU) injections have been reported to increase the success rate of filtering surgery in eyes with poor surgical prognosis [4, 6, 15, 17]. As 5-FU interferes with DNA and RNA synthesis [13], this drug is more toxic to proliferative than to non-proliferative cells, which corresponds with the clinical findings that corneal epithelial defects and conjunctival epithelial changes (wound leaks) are the most common complications observed with the clinical use of this drug [4, 6, 15, 17]. However, there is evidence that 5-FU also shows its toxic effect in cells with a lower population in the generation cycle, although these cells are much less sensitive [8, 14, 19]. Following intravitreal injection of 1 or 5 mg 5-FU in the rabbit eye, retinal damage was found on transmission electron microscopic examination [8]. When injected daily with 1.25 mg 5-FU for 7 days, rabbit eyes that had undergone lensectomy and vitrectomy showed marked corneal opacity and electroretinographic b-wave change [19], and perfusion of the rabbit eye cup with a solution containing 100 µg/ml 5-FU significantly decreased b-wave amplitude [14].

In the anterior segment of the eye, the corneal endothelium and ciliary epithelium are both metabolically very active and play essential roles in enabling the eye to function. It is likely that in glaucomatous eyes that are candidates for postoperative 5-FU subconjunctival injections, such as those that have previously undergone unsuccessful filtering surgeries or aphakic or neovascular glaucomatous eyes, the functionings of these tissues, especially that of the corneal endothelium, have sustained some prior damage. In eyes treated with gentamicin [9], betamethasone [18] or some ophthalmic preservatives [21], subconjunctival injection has been reported to cause various degrees of morphological change in the corneal endothelium.

However, to the best of our knowledge, it is not known as to which effects repeated subconjunctival 5-FU injections have on these tissues. In the present study, the effects of 14 successive daily subconjunctival injections of 5-FU on the morphology and function of the corneal endothelium and ciliary epithelium were investigated using pigmented rabbits.

Materials and methods

General experimental procedures

Since 5-FU shows an affinity for ocular melanin [20], adult pigmented rabbits of both sexes weighing 2.5–3.0 kg were used

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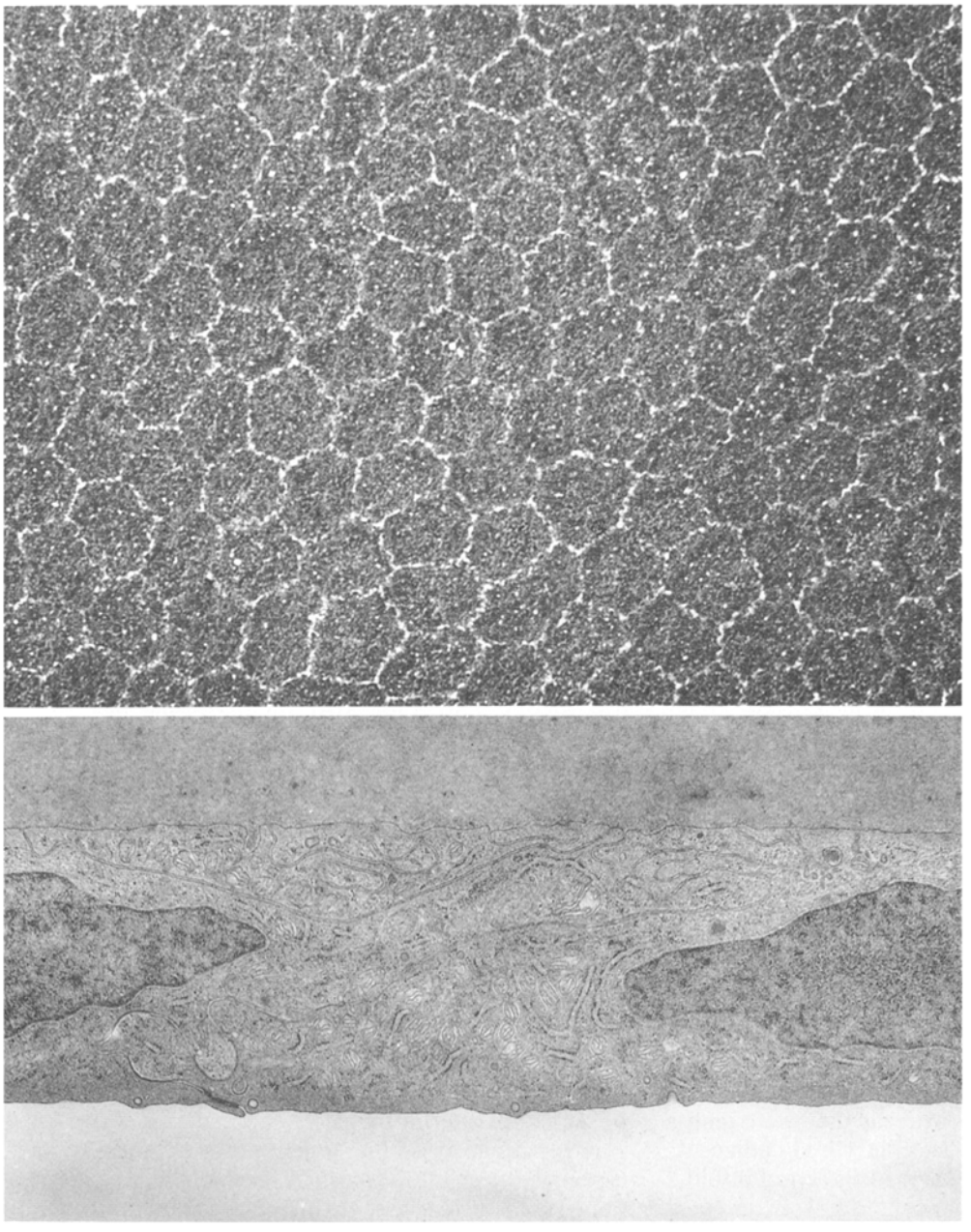

Fig. 1. Scanning and transmission electron micrographs (*top*, ×1200, and *bottom*, ×28000, respectively) of endothelial cells from the area near the site of vehicle solution injection. The eye was enucleated 12 h after the 14th and last subconjunctival injection. No apparent abnormality can be seen

throughout the present study. A 5-FU solution of 5% for intravenous injection (Kyowa Hakko Kogyo, Tokyo, Japan) was used for subconjunctival injection. The solution had a pH of 8.4 and osmolality of 865 mOsm and contained 5% (w/v) 5-FU and 8.47% (w/v) TRIS(hydroxymethyl)aminomethane, which was added to enhance the solubility of 5-FU, in distilled water. The 1.25% 5-FU solution was made by diluting the 5% solution with a 0.433 m*M* NaCl solution. For preparation of a control, the pH and osmolality of the 8.47% TRIS(hydroxymethyl)aminomethane solution were adjusted to pH 8.4 and 865 mOsm, respectively, by the addition of appropriate amounts of NaCl and 0.1 *N* NaOH.

In one randomly chosen eye of a animal, 100 µl 5% or 1.25% 5-FU (65 mg or 1.25 mg, respectively) was injected subconjunctivally with a 30-gauge needle in the superior temporal quadrant about 3 mm posterior to the corneal limbus at the 1:30 clock position using topical anesthesia. Penetration was made through the superior rectus muscle belly to prevent reflux of the injected material, and the conjunctival cul-de-sac was well rinsed with physiological saline. The fellow eye was injected with 100 µl control solution in the manner described above. The injections were carried out once a day at about 9 p.m. for 14 successive days. The examinations, described below, were carried out without knowledge of the laterality of the treatment. The anterior segment of the eye was checked using a portable slit-lamp microscope before each examination and all fluorometric examinations were carried out using a slit-lamp fluorometer (Tokyo Kogaku, Tokyo, Japan).

Morphological study

In 5 rabbits, 1.25% 5-FU was injected and 13 animals were injected with 5% 5-FU. At 12 h after the 14th and last injection of 5-FU, all rabbits injected with 1.25% 5-FU and seven animals injected with 5% 5-FU were killed by an intravenous overdose of sodium pentobarbital; the remaining six rabbits injected with 5% 5-FU were killed 14 days after completion of the 5-FU subconjunctival injections.

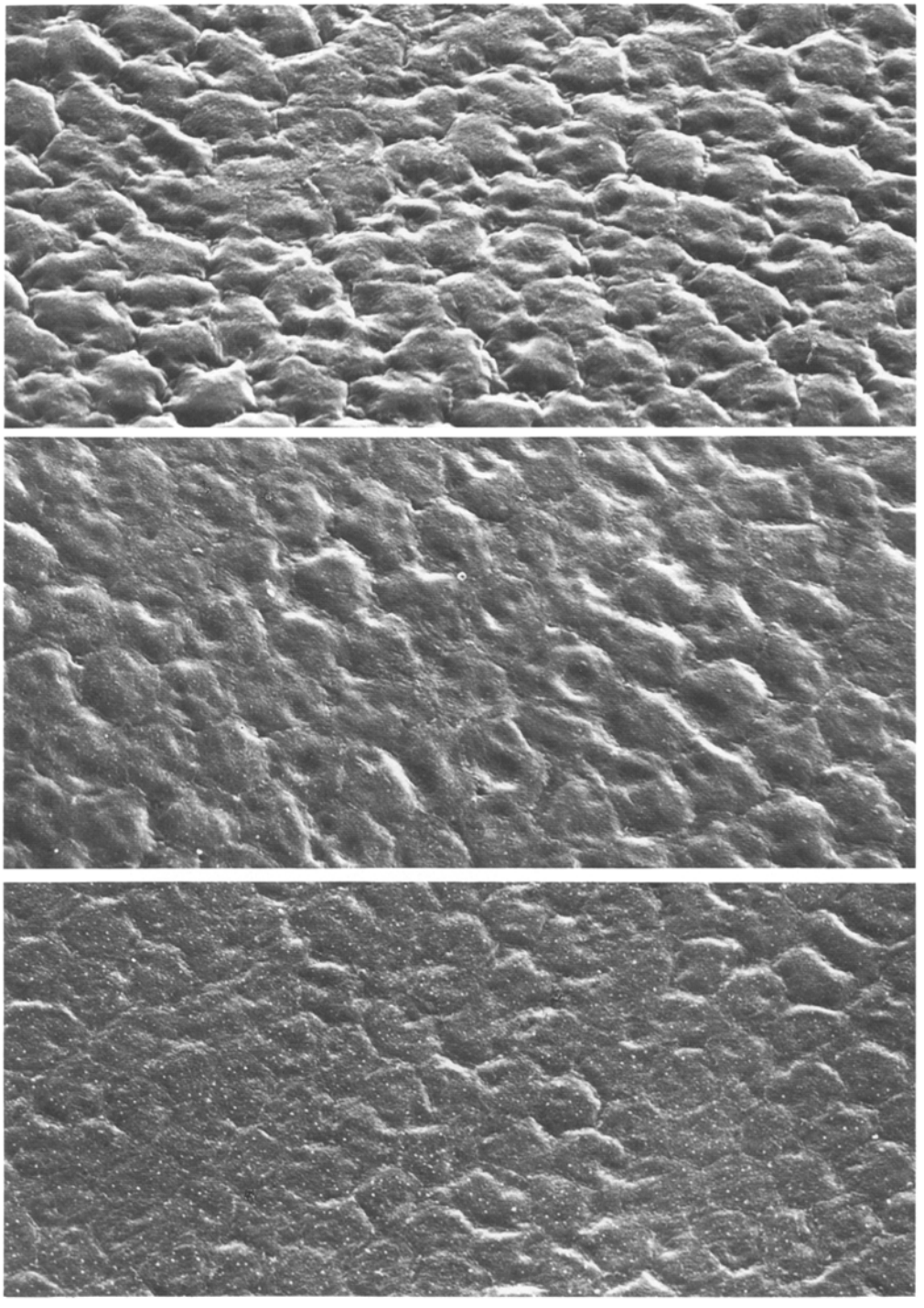

Fig. 2. Scanning electron micrographs of endothelial cells in eyes injected with 5% 5-FU (×1000). The eye was enucleated 12 h after the 14th and last subconjunctival injection. Endothelial cells from the area near the injection site show cytoplasmic swelling, but the mosaic-like pattern of endothelium is retained (*top*). Endothelial cells from the central area (*center*) and those from the area opposite the injection site (*bottom*) also show cytoplasmic swelling. Change is most marked in the area near the injection site, whereas it is moderate in the central area and minimal in the area opposite the injection site

Both eyes of each animal were enucleated and prefixed in ice-chilled fixative containing 2.5% glutaraldehyde and 2.0% paraformaldehyde. After 4 h, the whole cornea and the ciliary body adjacent to the injection site were cautiously dissected and the cornea was bisected by a meridian cut extending from the limbus at the 1:30 to the 7:30 clock position. The specimens were further fixed for 8 h at 4° C. One half of the cornea was further processed for transmission electron microscopy (TEM) and the other half, for scanning electron microscopy (SEM). For each corneal half, three parts of the corneal endothelium were examined: the peripheral part adjacent to the injection site, the central part and the peripheral part opposite the injection site. The ciliary body specimen was prepared for TEM only. Semithin sections for light microscopy (LM) were stained with toluidine blue and ultrathin sections for TEM, with uranyl acetate and lead citrate.

Physiological study

Effects of the 14 successive daily 5-FU subconjunctival injections on the corneal thickness, permeability of the corneal endothelium, permeability of the blood-aqueous barrier, and aqueous humor dynamics were studied. At 12 h after the 14th and last injection, the corneal thickness was measured in both eyes of 13 rabbits using a Mishima-Hedby pachometer.

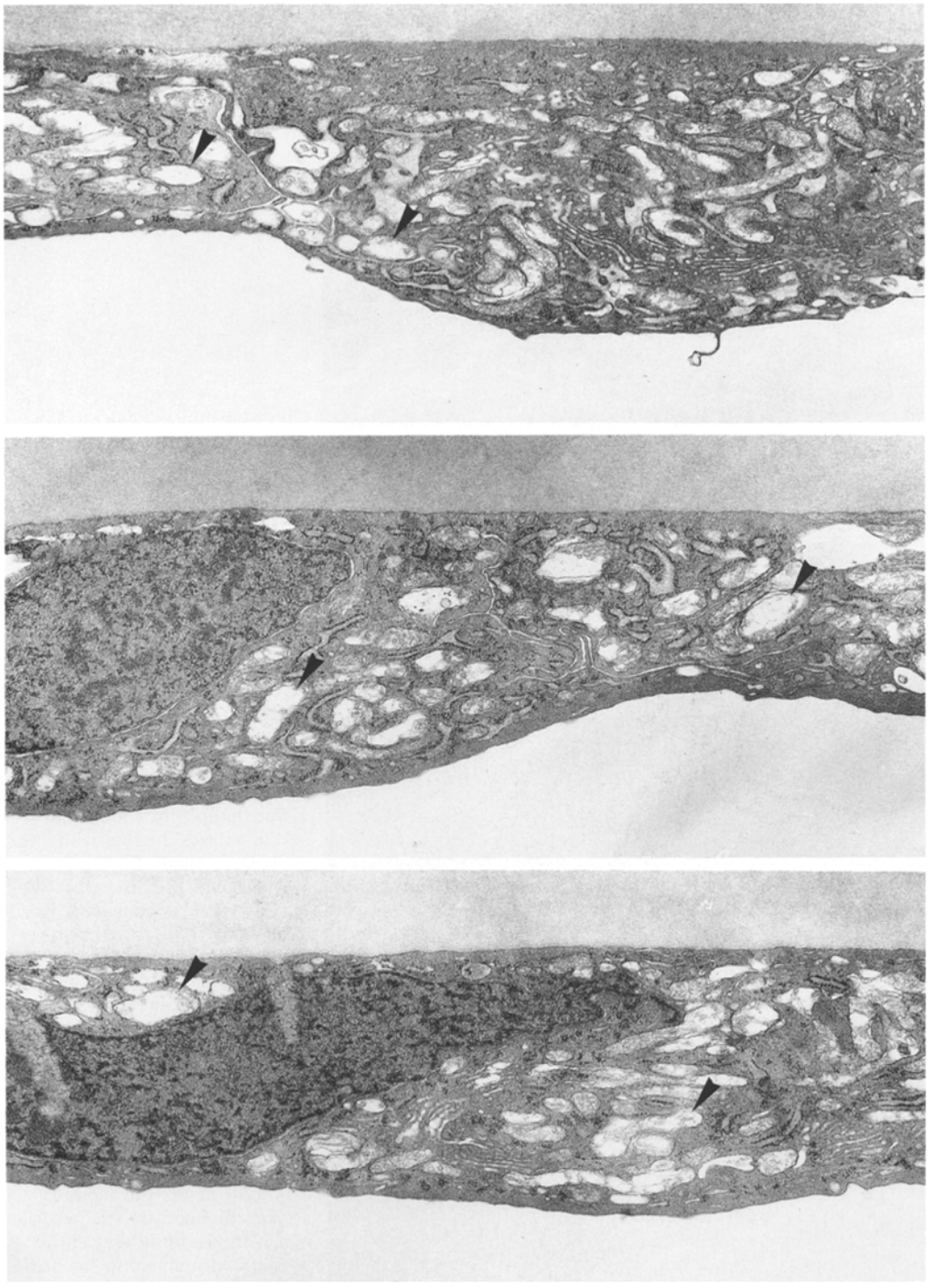

Fig. 3. Transmission electron micrographs from the areas shown in Fig. 2 (×30000). Cytoplasmic swelling and vacuolation and swelling of mitochondria (*arrowheads*) can be seen. Changes are most marked in endothelial cells from the area near the injection site (*top*). Changes are moderate and junctional complexes are unaltered in cells in the central area (*center*), and they are minimal in cells from the area opposite the injection site (*bottom*)

In six rabbits, the corneal endothelial permeability to carboxyfluorescein (P_{ac}) was measured according to the method of Araie [1]. At 12 h before the last 5-FU injection, 15 µl 0.5% carboxyfluorescein was injected into the vitreous cavity of each eye, and the apparent dye concentrations in the corneal stroma (F_s) and anterior chamber (F_a) were directly calculated from the fluorometric readings 20–25 h after the intravitreal dye injection at 1-h intervals. Although F_a is equal to the true dye concentration in the anterior chamber, C_a, F_s is not equal to that in the corneal stroma, C_s, since the corneal stroma affects the fluorescence of carboxyfluorescein [1]. Thus, based on the previous experiment, F_s was converted to C_s by multiplying by 1.6 [1]. The corneal thickness was also measured 24 h after the carboxyfluorescein injection as described above.

The P_{ac} was calculated as follows:

$$k_{c.ac}=A/(C_a/C_s-1/r_{ca}),\ P_{ac}=k_{c.ac}\times 0.9\times \mathrm{CT}, \quad (1)$$

where $k_{c.ac}$ is the aqueous-cornea transfer coefficient, A is the slope of the line fitted to the F_s data plotted on semi-logarithmic paper and r_{ca}, 1.62, is the cornea-aqueous distribution ratio of the dye. The details of the method were reported previously [1].

In eight rabbits, the permeability of the blood-aqueous barrier was estimated by intravenous injection of carboxyfluorescein solution at a dose of 10 mg/kg 12 h after the 14th and last 5-FU injection and measurement of the values for F_a in both eyes at 30 min after dye administration.

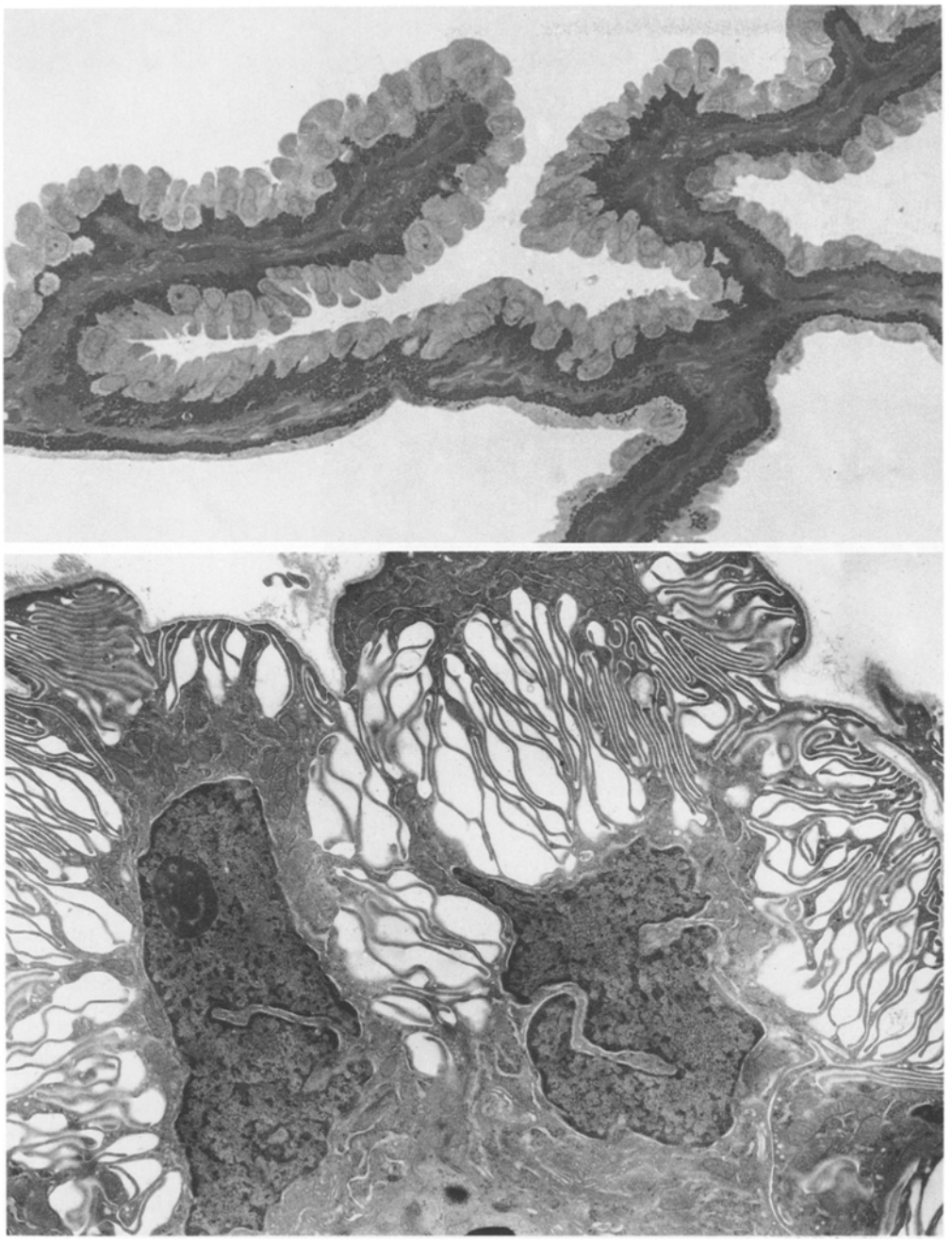

Fig. 4. Light and transmission electron micrographs of the ciliary process from the area adjacent to the injection site. The eye was enucleated 12 h after the 14th and last subconjunctival injection of 5% 5-FU. Little morphological change can be seen with light microscopy (× 1000, *top*), whereas disarrangement of the basal infolding of non-pigmented epithelial cells is visible with transmission electron microscopy (×18000; *bottom*)

Effects on the aqueous flow rate were studied using the method of Johnson and Maurice [5]. In seven animals under topical anesthesia, 15 µl 10% fluorescein isothiocyanate (FITC)-dextran solution with a nominal molecular weight of 65,600 daltons (FD-70S; Sigma Chemical Co., St. Louis, Mo.) was injected into the vitreous cavity of each eye through a 30-gauge needle. At 15 days after the FD-70S injection, the F_a in both eyes was measured at about 9 p.m. and the measurements were repeated at the same time for a period of 5 weeks at intervals of 2–5 days. In all, 14 daily subconjunctival 5-FU injections were carried out as described above from the 28th to the 41st day. On the 36th, 43rd and 50th days, the apparent dye concentration in the anterior vitreous just behind the lens (F_v) was also determined after the measurement of F_a after dilation of the pupil with one drop of 0.5% tropicamide. The intraocular pressure (IOP) in both eyes was measured using topical anesthesia and a calibrated Alcon pneumatonograph on the 25th, 36th, 43rd and 50th days.

Analysis

Numerical data are reported as the mean ± 1 SD. Student's paired or unpaired *t*-test was used when appropriate. The level of statistical significance was set at $P<0.05$.

Results

Morphological study

In eyes injected with the control solution, no morphological change was seen in any of the specimens obtained (Fig. 1). In the seven eyes injected with 5% 5-FU and enucleated 12 h after completion of the 14 daily 5-FU

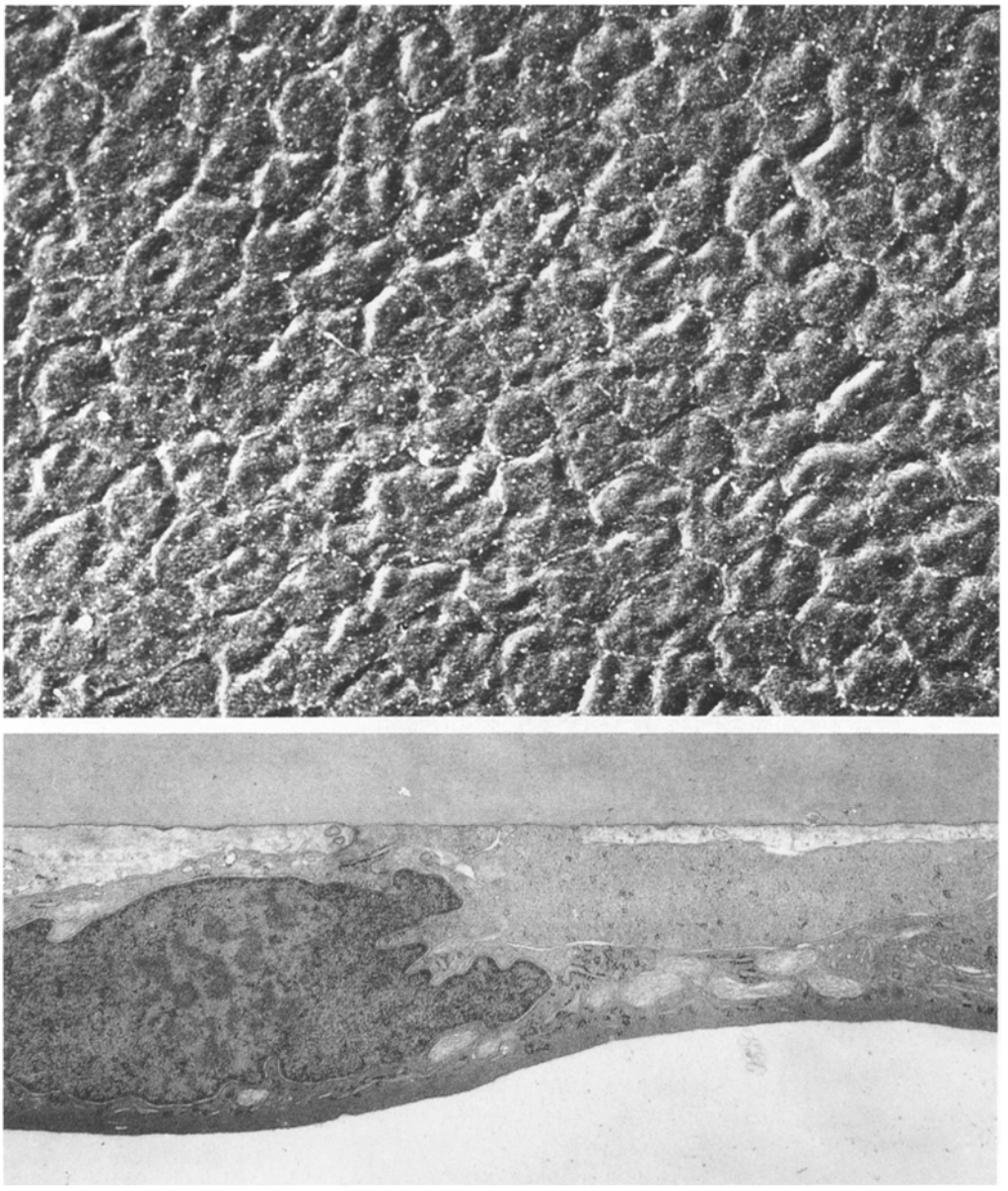

Fig. 5. Scanning and transmission electron micrographs (*top*, ×1000, and *bottom*, ×30000, respectively) of endothelial cells from the area near the site of injection of 1.25% 5-FU. The eye was enucleated 12 h after the 14th and last subconjunctival injection of 1.25% 5-FU. Slight swelling of endothelial cells can be seen, but cell organellae remain almost intact

injections, some morphological changes were observed in both the corneal endothelium and the ciliary epithelium. On SEM examination, the corneal endothelial cells showed cytoplasmic swelling; the change was most marked in the peripheral cornea adjacent to the injection site, whereas endothelial cells at the central part showed moderate changes and those at the peripheral part opposite the injection site were minimal. Intercellular junctions appeared to be intact and no focal detachment of the corneal endothelial cell was observed (Fig. 2). With TEM, moderate cytoplasmic swelling and vacuolation and swelling of mitochondria were seen in the endothelial cells in the peripheral part adjacent to the injection site (Fig. 3). These morphological changes were slighter in the central part and the junctional complexes remained unaltered. The endothelial cells in the peripheral part opposite the injection site showed only slight cytoplasmic swelling without apparent changes in the cell organellae (Fig. 3).

The semithin section of the ciliary process adjacent to the injection site showed little morphological change when observed with LM, but disarrangement of the basal infolding of non-pigmented epithelial cells was seen with TEM (Fig. 4).

In the five eyes injected with 1.25% 5-FU and enucleated 12 h after completion of the 5-FU injections, the morphological changes in the corneal endothelial cells were slighter than those observed in eyes injected with 5% 5-FU. Only the endothelial cells in the peripheral part adjacent to the injection site showed slight cytoplasmic swelling without apparent changes in the cell organellae (Fig. 5). No morphological change was seen in the ciliary epithelial cells on TEM examination.

In the six eyes injected with 5% 5-FU and enucleated 14 days after completion of the 14 daily subconjunctival injections of 5-FU, neither corneal endothelial cells nor ciliary epithelial cells showed morphological abnormalities (Fig. 6).

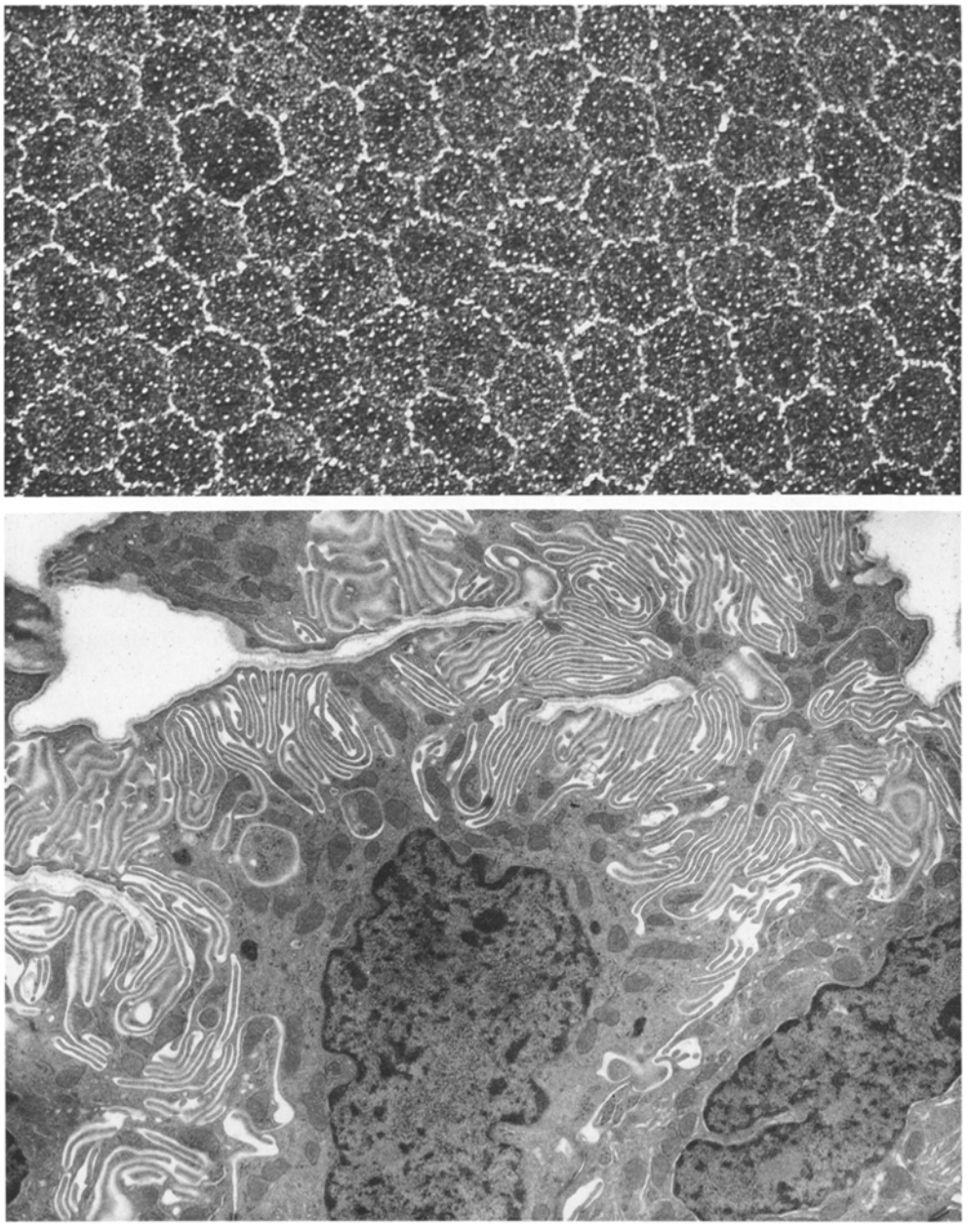

Fig. 6. Scanning electron micrograph of endothelial cells (×1200; *top*) and transmission electron micrograph of non-pigmented epithelial cells of the ciliary process (×18000; *bottom*) from the area adjacent to the site of injection of 5% 5-FU. The eye was enucleated 14 days after completion of the 14 successive daily subconjunctival injections of 5% 5-FU. No apparent morphological abnormalities can be seen

Physiological study

The corneal thickness was 0.38 ± 0.04 mm in the control eye and 0.39 ± 0.03 mm in the 5-FU-treated eye ($n = 13$). The value for P_{ac} was $3.2 \pm 0.9 \times 10^{-4}$ cm/min in the control eye and $3.2 \pm 0.8 \times 10^{-4}$ cm/min in the 5-FU-treated eye ($n = 6$). No significant difference in the corneal thickness or P_{ac} was seen between the control and 5-FU-treated eyes. The concentration of carboxyfluorescein in the anterior chamber averaged $6.11 \pm 1.86 \times 10^{-8}$ g/ml in the control eye and $5.73 \pm 1.62 \times 10^{-8}$ g/ml in the 5-FU-treated eye ($n = 8$); there was no significant difference between them.

After intravitreal injection of FITC-dextran, F_a declined at an almost constant rate and no significant difference in the time courses of F_a and F_v was seen between the control and 5-FU-treated eyes (Fig. 7). Between the 5-FU-treated and control eyes, no significant difference in the value for F_v/F_a was seen at any time measured, and the ratio of F_a in the 5-FU-treated eye to that in the control eye showed no significant difference at any time measured. There was no significant difference between the IOP of controls and that of 5-FU-treated eyes at any time during the study.

Discussion

In clinical use, 5-FU is usually given after glaucoma filtering surgery in eyes that had previously undergone unsuccessful filtering surgery. In the presence of postoperative inflammation and subconjunctival scar due to previous surgical treatment, the kinetics of subconjunctivally injected 5-FU should be considerably different

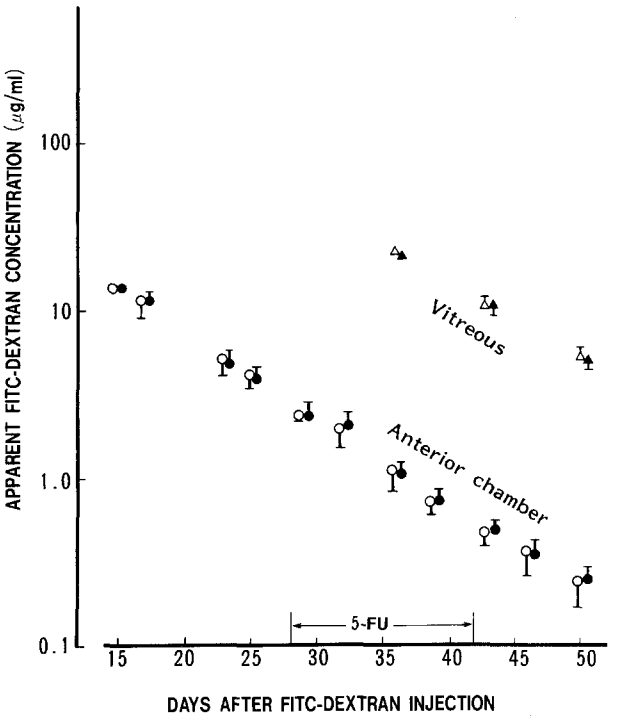

Fig. 7. Changes in fluorescent levels in the aqueous and anterior vitreous of 5-FU- and vehicle-injected eyes. Fluorescent levels in the anterior vitreous of 5-FU-injected eyes (▲), in the anterior vitreous of vehicle-injected eyes (△), in the aqueous of 5-FU-injected eyes (●) and in the aqueous of vehicle-injected eyes (○) are shown. Bar with brackets indicates SD in 7 eyes. In all, 14 successive daily subconjunctival injections of 5% 5-FU were carried out from the 28th to the 41st day

from that in normal rabbit eyes. Thus, in an attempt to better simulate clinical situations in the present study, it might have been better to use rabbit eyes that had undergone filtering surgery. However, filtering surgery itself would affect the corneal endothelium and ciliary epithelium, and the subconjunctival injection of antibiotics or steroids that should be carried out at the end of the surgery has been reported to damage the corneal endothelium [9, 18]. We therefore used normal rabbit eyes in the present study so as to observe isolated effects of 5-FU on these tissues.

In eyes that were enucleated 12 h after completion of the 14 daily subconjunctival injections of 5-FU, the corneal endothelial cells showed moderate morphological changes. The changes were most marked in the corneal endothelial cells near the injection site, whereas those in the central part and in the endothelial cells in the peripheral cornea opposite the injection site were only minimal. Fantes et al. [2] found that the 5-FU level in the cornea was related to the distance from the subconjunctival injection site, the highest level being achieved in the peripheral cornea adjacent to the injection site. Regional differences in the morphological change observed in endothelial cells in the present study are probably attributable to the intracorneal distribution of 5-FU after the subconjunctival injection.

On the other hand, the corneal thickness and the barrier function of the corneal endothelium measured at the central region showed no significant difference from those found in the control eye. Thus, the 14 successive daily subconjunctival injections of 5 mg 5-FU were thought to have little affected the functioning of the corneal endothelium, at least in the central part, although they caused some ultrastructual changes. Furthermore, the changes observed were thought to be reversible, as the specimens from eyes enucleated 14 days after completion of the 5-FU injections showed no significant morphological changes.

An in vitro experiment using animal and human corneas has demonstrated that corneal endothelial cells exposed to ≤ 1 mg/ml 5-FU for 2–4 h showed no significant morphological changes when examined with a vital staining technique and viewed at a magnification of 80 [10]. After a single subconjunctival injection of 5 mg 5-FU, the peak of the mean corneal 5-FU level was about 20 μg/g at 1 h [7, 16]. At 1 h after a single subconjunctival injection of 12.5 mg, the 5-FU level in the cornea near the injection site was reported to be about 3 times that in the central region and about 10 times that in the peripheral cornea opposite the injection site [2]. Based on these previous results [2, 7, 16], a rough estimation can be made of the intracorneal distribution of 5-FU after a single subconjunctival injection of 5 mg, and it would range from about 5 to 50 μg/g. As ocular melanin has an affinity for 5-FU [20], the corneal 5-FU level after 14 daily subconjunctival injections will be higher than the above figure, but it seems unlikely that it would reach the order of 1.0 mg/ml. The difference in the method of examination probably accounts for this apparent discrepancy.

The morphological changes observed in the ciliary epithelium were thought to be slighter than those observed in the corneal endothelium. The Johnson and Maurice method [5] was used in an attempt to detect a long-term effect of repeatedly injected 5-FU on the aqueous humor dynamics. This method entails the concentration measurements of FITC-dextran in the anterior chamber and vitreous many days after its intravitreal injection [5]. Originally, it was thought that a time change in the aqueous flow rate could be well estimated by measurement of the concentration changes of FITC-dextran in the anterior chamber. However, it was later found that the change in the fluid flow in the vitreous or the IOP per se could also influence the concentration change of FITC-dextran in the anterior chamber [3, 11]. In the present study, the IOP and the concentration changes of FITC-dextran in the anterior chamber and vitreous showed no significant difference between the 5-FU-treated and control eyes, at least at the times measured, throughout the experimental period. Furthermore, even at the time point at which ultrastructual changes in the ciliary epithelium were found in the 5-FU-treated eyes, the blood-aqueous barrier permeability of the treated eyes showed no significant change.

These results indicate that the 5-FU given in the present experiment had only a minor effect, if any, on the ciliary epithelium. However, it is possible that temporary effects might have been undetected because of the time

schedule for measurements used in the present experiment.

In summary, the subconjunctival injection of 5-FU at a dose comparable with that currently used in postoperative glaucoma patients caused some toxic effects on the corneal endothelial cells of the normal rabbit but was well tolerated. However, human corneal endothelial cells may be more sensitive to 5-FU than are rabbit cells. Unlike human corneal endothelial cells, rabbit cells show a remarkably proliferative activity [12]. In addition, it is likely that the corneal endothelium in glaucomatous eyes that are candidates for postoperative treatment with 5-FU has previously been somewhat damaged by accompanying ocular diseases or previous ocular surgery, and the intraocular penetration of 5-FU is more enhanced in postoperative glaucomatous eyes than in the normal rabbit eyes used in the present study. The postoperative use of 5-FU is not advised in eyes whose corneal endothelial cells have sustained previous damage. On the other hand, the present results suggest that the ciliary epithelium is less affected than the corneal endothelium by repeated subconjunctival injections of 5-FU.

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