

Mitomycin C-Induced Reduction of Keratocytes and Fibroblasts after Photorefractive Keratectomy

Tae-im Kim, Jhang Ho Pak, Sun Young Lee, and Hungwon Tchah

PURPOSE. To investigate the effects of mitomycin C (MMC) on the number of keratocytes and the proliferation of fibroblasts after photorefractive keratectomy (PRK) and exposure to ultraviolet B (UV-B) irradiation.

METHODS. The right eyes of New Zealand White rabbits in Groups 1, 2, and 3 ($n = 18$ each) underwent PRK to correct -10 diopters with 5 mm optical zone. Sponges soaked with 0.02% MMC were applied to the right eyes of Group 1 rabbits for 2 minutes. Antibiotic ointment was applied daily to all rabbits until the epithelium healed completely, after which 0.02% MMC eye drops were applied twice daily to the right eyes in Group 2 until 4 weeks after PRK. Three weeks after PRK, the right eyes of all the remaining rabbits were exposed to 100 mJ/cm² C UV-B radiation. Corneal haziness was assessed biomicroscopically using the Fantes scale every 3 weeks. Six eyes of each group were each enucleated 3, 6, and 12 weeks after PRK, and tissue specimens were stained with hematoxylin and eosin and with TUNEL stain. The tissues were evaluated immunohistochemically with antibody to α -smooth muscle actin (SMA). Cellular changes in the anterior stroma and epithelial basement membrane were evaluated by electron microscopy.

RESULTS. Corneal haze was observed after PRK and was aggravated by UV-B irradiation. A single intraoperative application of MMC immediately after PRK induced opacity and apoptosis of keratocytes. Twelve weeks after PRK, MMC significantly reduced corneal haze, the number of keratocytes, apoptotic cells, and fibroblasts, even after UV-B irradiation. Relatively large numbers of apoptotic and SMA-positive cells were found only in PRK-treated, non-MMC treated rabbits (Group 3), even after 12 weeks. Three weeks after PRK, dying stromal cells showed cell shrinkage, and chromatin condensation was observed in all treated groups by electron microscopy. Twelve weeks after PRK, fewer keratocytes and inflammatory cells were observed just beneath the epithelial layer in Group 1 than in any of the other groups.

CONCLUSIONS. MMC is a potent inhibitor of corneal haze induced by PRK. MMC reduced the number of keratocytes and fibroblasts after PRK and UV-B irradiation. Although MMC would improve the clinical results of PRK, it has significant toxicity on corneal keratocytes, which did not disappear until

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The early disappearance of keratocytes after epithelial injury is mediated by apoptosis.¹⁻³ The remaining keratocytes begin to proliferate, which may lead to the generation of myofibroblasts.^{4,5} Inflammatory cells have also been observed to enter the stroma. This complex cellular response contributes to stromal remodeling and modulates healing of the overlying epithelial cells.⁴ The advent of refractive surgery has greatly stimulated interest in understanding this apoptotic response and the subsequent repopulation of the area of cell death. Similarly, keratocyte activation after surgery and trauma has become an area of interest, since corneal wound healing shows individual variability after photorefractive keratectomy (PRK) or LASIK.⁶⁻⁹ Modulation of this process at the cellular and molecular level is thought to be critical for ideal refractive surgery, with early keratocyte apoptosis regarded as a promising target for controlling later events in the wound healing cascade. To date, pharmacological efforts to control early keratocyte apoptosis have not been successful, but research is ongoing to identify agents that can regulate this phenomenon and its sequelae.

Mitomycin C (MMC), which suppresses the proliferation of rapidly growing cells by inhibiting DNA synthesis secondary to alkylation,¹⁰ is used in glaucoma filtering surgery¹¹ and to prevent recurrence of pterygium.¹² In addition, MMC has been suggested for treating conjunctival and corneal intraepithelial neoplasia.^{13,14} In experimental models, MMC acts as a potential modulator of wound healing after PRK.¹⁵⁻¹⁸ Although topical application of MMC prevents haze and recurrence of subepithelial fibrosis after refractive corneal surgery,¹⁹⁻²² adverse effects of this agent have also been reported,^{16,18,23} making its safety of critical importance.

Exposure of the eye to UV radiation results in the development of photokeratitis after a few hours.²⁴ The degree of damage depends on several factors, including the wavelength of incident light and the length of time of exposure. UV-induced keratopathy results in reactive production of hyaluronan in keratocytes.²⁵ Similarly, hyaluronan formation has been found to occur after excimer laser surgery.²⁶ UV-B exposure during post-PRK stromal healing exacerbates the damage and prolongs the healing response, as manifested by subepithelial haze augmentation.^{27,28} UV-B exposure also modulates tissue response to excimer laser treatment.

In this study, the modulating effect of MMC on the wound healing process after PRK, as well as the safety of this agent, were evaluated. The effect of MMC on keratocyte and myofibroblast proliferation in the special environment resulting from UV-B irradiation after PRK was also studied.

MATERIALS AND METHODS

Surgical Technique and Clinical Protocol

Animals used in this study were treated in accordance with the ARVO Resolution on the Use of Animals in Research. Seventy-two New Zealand White rabbits, weighing between 2 and 2.5 kg, were divided into four groups. Rabbits in Groups 1 to 3 ($n = 18$ each) were

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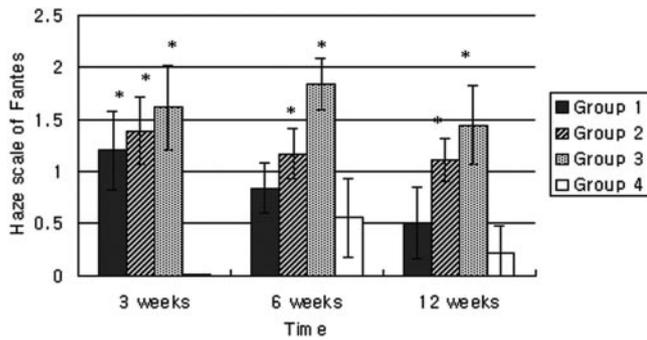


FIGURE 1. Quantitation of corneal haze after PRK, in Group 1 (■), Group 2 (▨), Group 3 (▩), and Group 4 (□). Error bars: SD of the mean; *statistically different from Group 4 ($P < 0.01$).

anesthetized with intramuscular injections of a mixture of ketamine (25 mg/kg) and xylazine (2 mg/kg). Proparacaine hydrochloride eye drops were instilled into each right eye, and a speculum was used to open the eyelids. PRK (5 mm optical zone, -10 diopters) was performed on the right eye of each rabbit with a flying spot excimer laser (Technolas 217z; Bausch & Lomb Surgical, Munchen, Germany). The left eyes of Groups 1-3 and both eyes of Group 4 were not treated.

Immediately after PRK, a sponge soaked with 0.02% MMC was placed for 2 minutes on each exposed corneal bed of the right eyes in Group 1, and each eye was subsequently irrigated vigorously with a 30 mL-balanced salt solution. An antibiotic (0.3% ofloxacin) ointment was applied for 3 days onto each right eye in Groups 1 to 3. After complete epithelial healing and until 4 weeks after PRK, 0.02% MMC eye drops were applied twice daily to the right eyes of the rabbits in Group 2.

Three weeks after PRK, 12 rabbits in each of the four groups were anesthetized as above and placed in a standard dermatologic UV light chamber. The right eyes were kept open using a speculum and exposed for 1 to 2 minutes to UV light at a wavelength in the UV-B range (290 to 315 nm) and with total equivalent energy of 100 mJ/cm². Stromal haziness was assessed biomicroscopically²⁹ every 3 weeks thereafter.

Tissue Processing and Sections

Six rabbits in each group were killed at weeks 3, 6, and 12, by intravenous injection of 10 mL air, and their eyes were enucleated. The corneas were fixed in 4% paraformaldehyde, embedded in paraffin wax, and sectioned into 5 μ m slices. Sections were double-stained with hematoxylin and eosin.

TUNEL Stain and Immunohistochemical Assay

The number of apoptotic cells in each cornea was determined using the In Situ Cell Death Detection Kit (Roche Diagnostics Korea Co., Seoul, Korea) and the fluorescein simplified TUNEL assay. Fluorescein quantitates cell death (apoptosis) by labeling DNA strand breaks in individual cells, allowing their detection by fluorescence microscopy. The assay uses an optimized terminal transferase (TdT) to label free 3'OH ends in genomic DNA with fluorescein-dUTP. Photographs were obtained with a fluorescence microscope (LEITZ DM LB2; Leica, Wetzlar, Germany).

For immunohistochemical assays, the sections were incubated for 1 hour at room temperature with a monoclonal anti-SMA antibody (DAKO, Glostrup, Denmark), a myofibroblast marker, and assayed using a Ventana: i View DAB detection kit (Ventana, Tucson, AZ). All sections were counterstained with hematoxylin to detect nuclei. The sections were viewed and photographed with a microscope equipped with a digital camera (Coolpix 950; Nikon, Tokyo, Japan).

Transmission Electron Microscopy

Central corneal sections obtained with a flat cutter were fixed overnight at 4°C in 2.5% glutaraldehyde and washed twice with PBS for 5

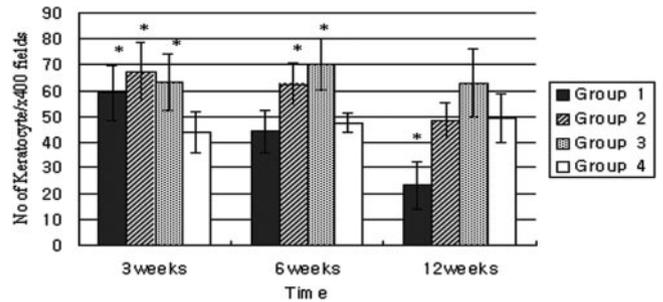


FIGURE 2. Quantitation of number of keratocytes after PRK, in Group 1 (■), Group 2 (▨), Group 3 (▩), and Group 4 (□). Error bars: SD of the mean; *statistically different from Group 4 ($P < 0.01$) ($\times 10^3/\mu\text{m}^2 \pm \text{SD}$).

minutes each. Each cornea was bisected, and a 1-mm strip was obtained from its center, fixed in 1% OsO₄ in phosphate buffer for approximately 90 minutes at room temperature, washed twice in phosphate-buffered fixative vehicle, and dehydrated using a graded ethanol series. The transition from 100% ethanol to epoxy was mediated by two changes of propylene oxide, and a pure epoxy medium was used for infiltration and embedding. The fragments were mounted in flat molds and hardened at 80°C overnight before sectioning. Both 600-1000 nm and 60-80 nm TEM sections were cut onto polyvinyl butyral-coated grids (Pioloform; Sigma, St Louis, MO) and stained with saturated aqueous uranyl acetate and lead citrate. Evaluation was performed by TEM (JEM1200 EX2; Jeol LTD, Tokyo, Japan).

Cell Counting and Statistical Analysis

Six specimens from each group were used for counting at each time point, with the cells in five non-overlapping, stromal fields counted by one observer (TK) in a X400 power field. To reduce possible investigator bias, this procedure was performed in a blinded fashion on serially numbered slides. Data were analyzed using SPSS software (SPSS Inc., Chicago, IL). Statistical comparisons between the groups were performed using ANOVA test with Bonferonni correction.

RESULTS

Clinical Course

Subepithelial haze was observed in the ablation area of all treated rabbits 3 weeks after PRK. During the early postoperative period, rabbits in Groups 1, 2, and 3 displayed a similar degree of corneal haze (Fig. 1). Six weeks after PRK, corneal haze increased in all rabbits in Groups 2 and 3. Twelve weeks after PRK, Group 1 rabbits exhibited a lesser degree of haze than did the other groups ($P < 0.01$). Although the Group 2

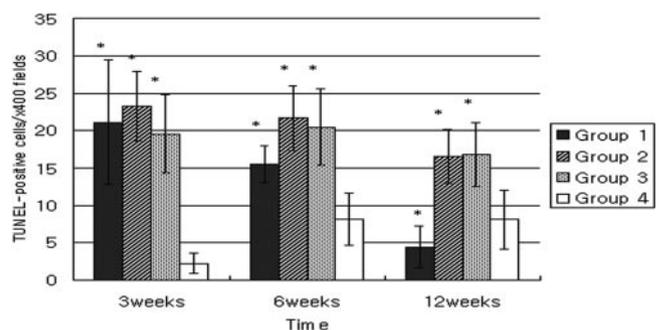


FIGURE 3. Quantitation of TUNEL-positive cells in the stroma after PRK, in Group 1 (■), Group 2 (▨), Group 3 (▩), and Group 4 (□). Error bars: SD of the mean; *statistically different from Group 4 ($P < 0.01$).

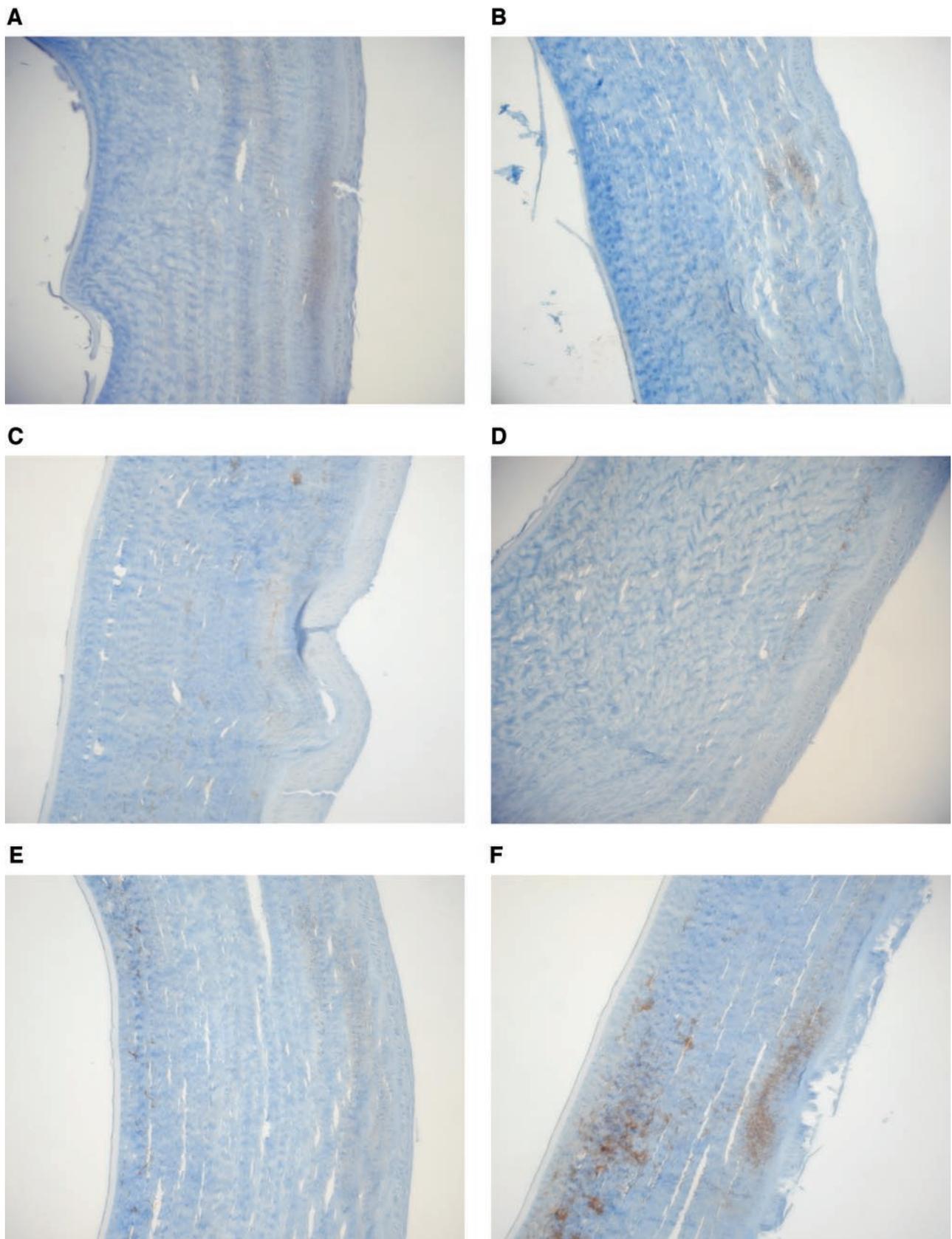


FIGURE 4. Immunolocalization of SMA at 3 weeks (A, B, C) and 12 weeks (D, E, F) after PRK. SMA appears as *brown or black* and the hematoxylin counterstain is *blue*. Three weeks after PRK, all treated groups showed some anti-SMA binding in subepithelial and anterior stroma in the corneal wound area. However, 12 weeks (D, E, F) after PRK, SMA-positive cells were observed in anterior and posterior stroma. Group 1 (D) showed some SMA-positive stromal cells at anterior stroma and Group 2 (E) showed SMA-positive stromal cells at anterior and posterior stroma. Especially in Group 3 (F), the number of positive cells continued to be significantly greater than other groups at 3 months after surgery. Magnification, $\times 200$.

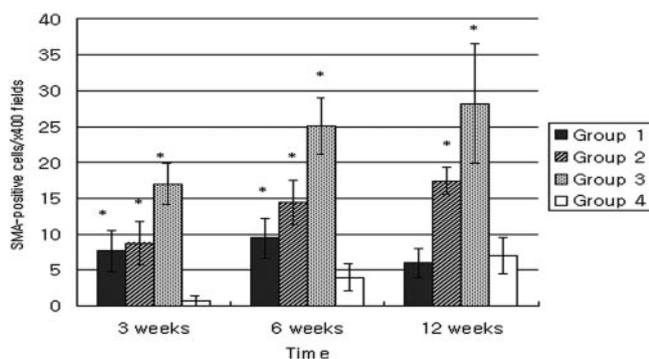


FIGURE 5. Quantitation of SMA-positive cells in the stroma after PRK, in group 1 (■), group 2 (▨), group 3 (▩), and group 4 (□). Error bars: SD of the mean; *statistically different from Group 4 ($P < 0.01$).

rabbits showed slightly lower grade haze than the Group 3 rabbits, this difference was not statistically significant. After UV irradiation, rabbits in Group 4 also showed mild haze formation.

Histologic Findings

Three weeks after PRK, light microscopy was unable to detect any statistically significant between-group differences in the number of keratocytes, except for differences between Group 4 and the other groups (Fig. 2). In all PRK-treated rabbits, morphologic changes were evident, including epithelial irregularity, stromal edema, irregular scar tissue, and the presence of collagen.

Six weeks after PRK, the number of keratocytes was slightly decreased in Groups 1 and 2 and slightly increased in Groups 3 and 4. Twelve weeks after PRK, the number of keratocytes decreased in Groups 1, 2, and 3. Compared with the rabbits in Group 3, the rabbits in Groups 1 and 2 had significantly lower numbers of keratocytes, with the most significant decrease observed in Group 1.

Evaluation of Apoptosis

Chromatin condensation is a marker of apoptosis, which can be detected using TUNEL stain. Three weeks after PRK, apoptotic keratocytes were detected in the corneas of all PRK-treated rabbits, mostly in the anterior portion of the corneas. Compared with Group 3, the rabbits in Groups 1 and 2 had relatively severe apoptotic processes (Fig. 3).

Six weeks after PRK, apoptotic keratocytes were observed in the entire stromal layer, but Group 1 had a significantly lower number of these cells ($P < 0.01$). Twelve weeks after PRK, the number of apoptotic cells had decreased in all groups, most significantly in Group 1 ($P < 0.01$).

Immunohistochemical Assay

Three weeks after PRK, Group 3 eyes had a greater number of SMA-positive cells than the other groups ($P = 0.001$; Fig. 4), but no SMA-stained cells were observed in unwounded stroma. All PRK-treated rabbit eyes had small numbers of anti-SMA binding cells in the subepithelial and anterior stroma of the corneal wound area (Fig. 5). Twelve weeks after PRK, however, SMA-positive cells were observed in both the anterior and posterior stroma, with significant differences among Groups 1, 2, and 3 ($P < 0.01$), but no difference between Groups 1 and 4. Three months after surgery, the number of positive cells was significantly greater in Group 3 than in any of the other groups.

Transmission Electron Microscopy

The central corneas were examined by electron microscopy 3, 6, and 12 weeks after PRK. At 3 weeks after PRK, dying stromal cells had shrunk, and chromatin condensation was observed (Fig. 6), with some of the dead cells having undergone apoptosis. Irregular epithelial basement membrane and infiltration of many polymorphonuclear leukocytes and monocytes were observed in the corneas of Groups 1, 2, and 3 rabbits. In addition, their collagen bundles had become irregular, and there were deposits of disorganized matrix material and large amounts of rough endoplasmic reticulum (RER). There were no significant differences in morphology, however, among these three groups.

Twelve weeks after PRK, there were very few keratocytes or inflammatory cells in the Group 1 corneas. Although the collagen bundles were less irregular, the epithelial basement membranes did not recover their consistency, having been replaced by amorphous linear structures. Consistency of the epithelial basement membrane was observed in Group 2 corneas, with few inflammatory cells and restoration of some myofibroblasts or keratocytes. Relatively patent epithelial basement membrane was found in Group 3 corneas, but there was some irregularity, and the collagen fibers were more irregular than in Group 1. In addition, relatively large amounts of RER were observed in Group 3 myofibroblasts.

DISCUSSION

Epithelial injury or surgical trauma, such as that associated with PRK, induces keratocyte apoptosis,^{7,8} leading the surrounding keratocytes to proliferate and replenish the anterior stroma.^{6,8} During this process, some activated keratocytes differentiate into fibroblasts or myofibroblasts.⁶ This wound healing process may be related to regression and haze formation after PRK.

MMC, which prevents corneal haze, is the most commonly used pharmacologic agent for modulating the wound healing process after PRK.^{19,20} MMC has been reported to decrease the number of keratocytes after PRK,¹⁶ however, suggesting that there are long-term safety concerns in the use of this agent.

The present study showed that MMC reduced corneal haze after PRK and after UV-B irradiation subsequent to PRK. A single intraoperative application of MMC was more effective than its topical application in preventing corneal haze. At 3 weeks after PRK, there was no statistically significant difference in the number of keratocytes among Groups 1, 2, and 3. There was significant decrease in cell density in the Group 1 rabbits at week 12. Moreover, cell density of the rabbits in Group 1 was lower than that of the control, untreated rabbits (Group 4) after 3 weeks. These findings can be compared with those of an earlier report,¹⁸ showing that MMC decreased the number of keratocytes 1 week after PRK; the number of cells slowly increased thereafter, becoming normalized after approximately 12 weeks. This study differs from the earlier report in that corneas were irradiated with UV-B 3 weeks after PRK. Moreover, while treatment with PRK and MMC caused an initial increase in cell number, the number of keratocytes was significantly lower than normal and slowly decreased over time, but was not influenced by UV-B irradiation. Although the clinical importance of this reduction in cell density is not known, it may influence long-term corneal survival.

In our previous study,¹⁷ MMC induced apoptosis of activated keratocytes. The results shown here confirm the apoptotic effects of MMC, as detected by TUNEL staining. While epithelial injury and surgical damage induced apoptotic changes,⁷⁻⁹ MMC was expected to have additional apoptotic effects.¹⁷ Six

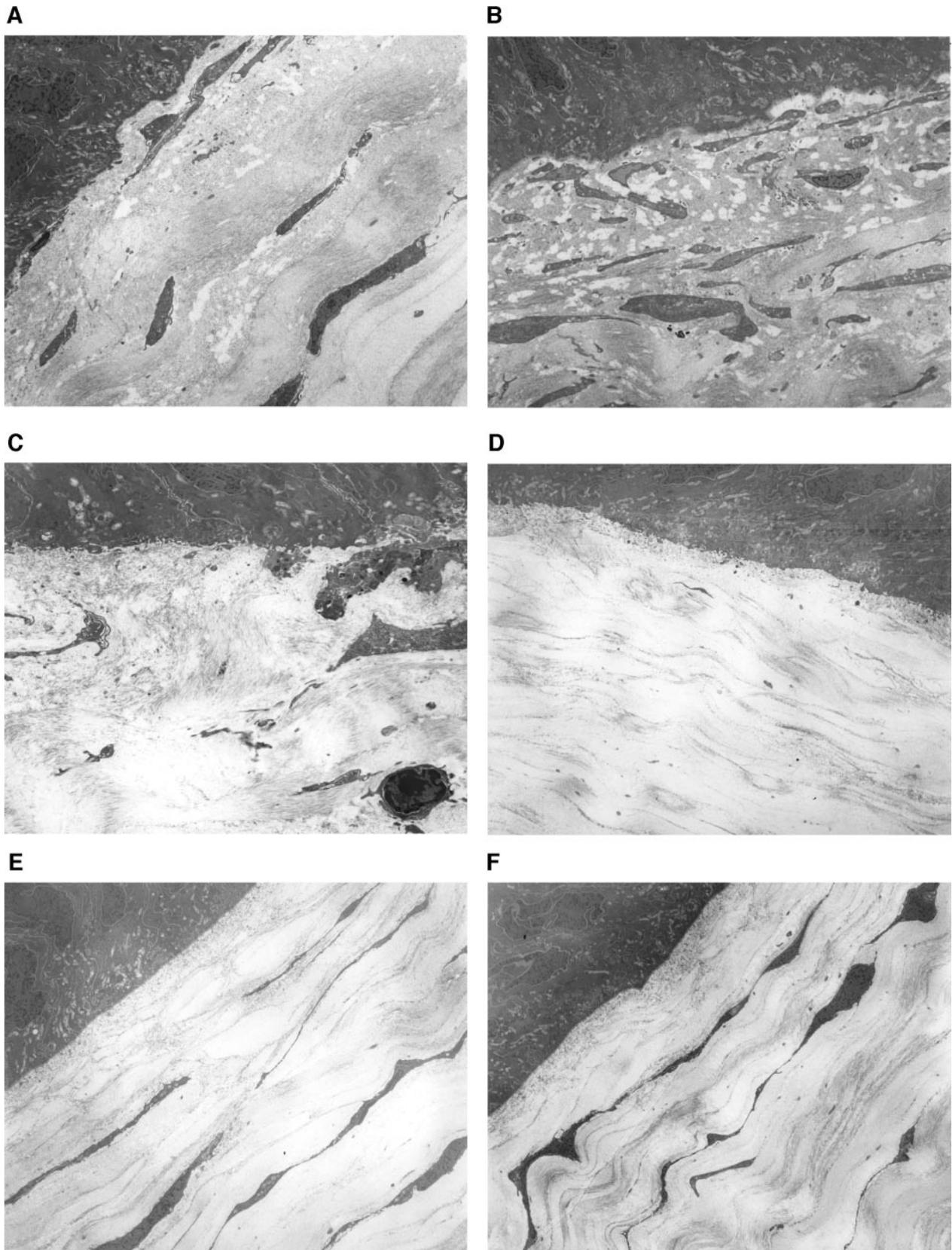


FIGURE 6. Transmission electron microscopy of the central treatment area of rabbit cornea at 3 weeks (A, B, C) and 12 weeks (D, E, F) after PRK. Dying stromal cells with cell shrinkage, chromatin condensation, irregular epithelial basement membrane, and infiltration of many polymorphonuclear leukocytes and monocytes were observed in Groups 1 (A), 2 (B), and 3 (C) at 3 weeks after PRK. At 12 weeks after PRK, Group 1 (D) showed very few keratocytes and inflammatory cells and inconsistent epithelial basement membrane. Group 2 (E) showed the linear epithelial basement membrane and rare inflammatory cells. Group 3 (F) showed a relatively patent epithelial basement membrane and irregular collagen. Relatively high amounts of rough endoplasmic reticulum (RER) were observed in myofibroblasts. Magnification, $\times 3000$

weeks after PRK, rabbits treated intraoperatively with MMC had fewer TUNEL-positive cells than did rabbits treated with MMC drops or those that were not treated with MMC. In addition, UV-B treatment alone increased the number of TUNEL-positive cells, which confirms a previous report that UV light of wavelength 310 nm can induce keratocyte apoptosis through the full thickness of the cornea.³⁰

Four hours after PRK for high myopia (−9.0 D) in rabbits, there are large numbers of TUNEL-positive cells, which become fewer over time and disappear after 4 weeks.⁹ In our study, however, TUNEL-positive cells were observed 6 weeks after PRK, which may be due to the induction of apoptosis in the remaining keratocytes by UV irradiation at week 3. In addition, significantly fewer TUNEL-positive cells were observed in rabbits intraoperatively treated with MMC, perhaps due to the decreased number of remaining keratocytes.

When PRK-, MMC-treated rabbits were compared with control rabbits after UV-B irradiation, 6 weeks after PRK, the number of keratocytes was similar, but the number of TUNEL-positive cells was higher in the former group. While the apoptotic response in the control group was caused by UV irradiation only, the other group had experienced surgical damage and MMC treatment as well as UV irradiation. These findings suggest that the different degrees of apoptosis may have been due to the residual effects of MMC.

After the initial apoptotic response, the remaining keratocytes begin to proliferate. Any myofibroblasts generated^{5,6} can be identified by immunohistochemical staining for SMA. Statistically significant differences were observed in SMA-stained cells between MMC-treated and -untreated rabbits at every time point. MMC had the most potent effect on rapid proliferating cells, suppressing the number of SMA-positive cells, which may explain the clinical differences in outcome, such as corneal haze.

TEM revealed that the rabbits in Groups 1, 2, and 3 had similar percentages of apoptotic or necrotic cells and inflammatory change. Twelve weeks after PRK, rabbits treated intraoperatively with MMC did not have re-established epithelial basement membrane, perhaps due to the increased susceptibility to epithelial damage caused by minimal insults and delayed healing. The corneas in this group showed scant cellular components and a relatively regular pattern of collagen fibers. Shown by confocal microscopy, after PRK, keratocyte density in the anterior stroma is not restored to the high-density level observed preoperatively.³¹ In the present study, MMC induced a more profound reduction of keratocytes, suggesting that, when combined with PRK, MMC application may have long-term deleterious effects on the cornea stroma.

Application of MMC may prevent corneal haze after PRK by inducing apoptosis of activated keratocytes. A single intraoperative application of MMC was more effective than topical application. Since intraoperative MMC was applied directly onto the surgical bed, it was easily able to penetrate into the stroma. In contrast, application of eye drops onto the intact epithelium did not result in effective penetration of MMC into the stroma.

In conclusion, MMC induces apoptosis of activated keratocytes and reduces corneal haze after PRK, even when combined with UV irradiation. Cell loss resulting from the application of MMC is a serious problem, however, lasting at least 3 months after PRK. Our results indicate that the long-term safety of MMC should be considered before its clinical adoption.

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