Apoptosis in Keratocytes Caused by Mitomycin C

Tae-im Kim, Hungwon Tchah, Seung-ah Lee, Kyungrim Sung, Beom Jin Cho, and Michael S. Kook

Purpose. The purpose of this study was to quantify the effect of mitomycin C on rabbit keratocytes, with a view to determining its potential in modulating corneal stromal wound healing. In addition, the pathway by which this regulation occurs was investigated.

Methods. Keratocytes were isolated from New Zealand White rabbits and cultured. Hoechst staining and flow cytometric analyses with annexin V were used to identify the nature of the keratocyte response to mitomycin C. The response of cultured keratocytes to 0.005%, 0.01%, 0.02%, 0.04%, and 0.06% mitomycin C was evaluated with the lactate dehydrogenase (LDH) assay. In addition, after exposure of keratocytes to 0.01% mitomycin C, the LDH assay was performed at different times of 6, 12, and 24 hours. Keratocytes were preincubated with various concentrations of CPP32-like protease inhibitor (Z-VAD-FMK), caspase-8 inhibitor (Z-IETD-FMK), and caspase-9 inhibitor (Z-LEHD-FMK) and treated with 0.01% mitomycin C. The LDH assay was performed after 12 hours. Cytochrome *c* immunostain was performed after exposure to 0.01% mitomycin C.

Results. Hoechst staining revealed shrinkage of the cytoplasm, formation of apoptotic bodies, and nuclear fragmentation. Apoptotic changes in cells were detected by flow cytometry. LDH activities increased significantly at concentrations of 0.005% mitomycin C or greater and were time dependent until 24 hours. Treatment with a CPP32-like protease inhibitor caused a decrease in LDH activity, although the results were not statistically significant. Specific inhibitors of caspase-8 and -9 significantly reduced the LDH activity induced by mitomycin C. Cytochrome *c* immunostaining of keratocytes pretreated with mitomycin C showed strongly positive findings.

Conclusions. Mitomycin C induced apoptosis, not necrosis, in cultured corneal keratocytes through the caspase pathway—specifically, caspase-8 and -9—related to the mitochondrial pathway. (*Invest Ophthalmol Vis Sci.* 2003;44:1912–1917) DOI:10.1167/iovs.02-0977

Apoptosis is a process of natural cell death. Unlike necrosis, apoptosis constitutes an active, physiologic, noninflammatory, and irreversible pathway in a single cell. The apoptosis system removes individual cells without damaging the surrounding environment. Cells undergoing apoptosis display membrane blebbing, shrinkage, protein fragmentation, chromatin condensation, DNA degradation, and rapid engulfment of apoptotic cell bodies by neighboring cells, among other characteristics. There are two major apoptotic pathways in mammalian cells: One is the death receptor pathway triggered by members of the death receptor superfamily including CD95. The other is the mitochondrial pathway, which is activated in response to extracellular cues and internal insults, such as DNA damage.[1–14]

The signals and pathways that trigger apoptosis remain to be elucidated. Various factors, including viral infection, DNA damage, cell membrane damage, mitochondrial damage, Fas ligand, and TNF, induce apoptosis. These pathways are related to p53, ceramide, CED4, Fas receptor, and cytokine receptor.[1–10,14] Mechanical injury and viral infection of corneal epithelial cells are major factors that trigger the apoptosis of underlying keratocytes.[11–13] Several studies suggest that keratocyte death is mediated by cytokines released by the injured epithelium.[11,14,15] Apoptosis of keratocytes is reportedly an essential initiator of the wound-healing response that occurs in the cornea after surgical procedures.[12,13] Therefore, control of this apoptotic pathway may provide a method for regulating the corneal wound-healing response.

Several studies have demonstrated that mitomycin C is a powerful modulator of corneal wound healing (Talamo JH, Lee K, Puliafito CA, Steinert RF, ARVO Abstract 1247, 1991).[16] Mitomycin C reduces keratocytes in the anterior stroma after corneal refractive surgery, leading to a decrease in activated fibroblasts, production of extracellular matrix, and formation corneal haze.[17–20]

Mitomycin C, an alkylating antibiotic agent derived from *Streptomyces caespitosus*, blocks DNA and RNA replication and protein synthesis. The compound is metabolized by liver enzymes to form an alkylating agent that nonspecifically crosslinks with DNA in a cell-cycle–dependent manner.[21] Mitomycin C inhibits mitosis and the proliferation of capillary and corneal endothelial cells, corneal epithelial cells, conjunctival cells, Tenon's capsule fibroblasts, and other fibroblasts.[22–24]

In this study, we investigated the effects of mitomycin C on keratocyte apoptosis in vitro and the specific pathway through which this cell death occurs.

Methods

Keratocyte Culture

This study was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We obtained keratocyte primary cultures from New Zealand White rabbit corneas. Stromal explants were prepared by removing the epithelium and endothelium and culturing in 10% FBS DMEM at 37°C in 5% CO_2, in a six-well tissue culture plate.

Keratocytes were allowed to migrate from the explant to the surface of the wells. Cells reached confluence within 15 to 21 days. These were enzymatically detached with 0.05% trypsin at 37°C for 3 minutes. Suspended keratocytes were centrifuged at 1400 rpm for 5 minutes, and the supernatant was removed. Cells were resuspended in 20 mL medium and cultured in 75-mL flasks at 37°C in 5% CO_2 until confluence was reached. They were then serially trypsinized and passaged three times for use in experiments. Cells were plated at concen-

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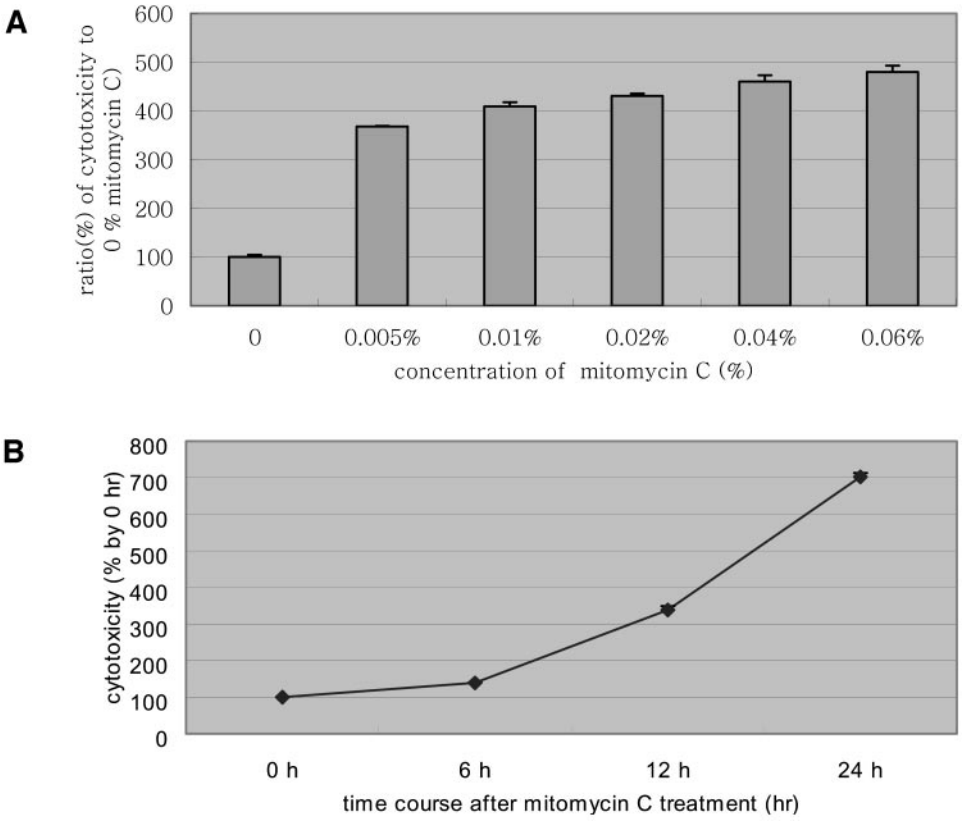

FIGURE 1. Cell death in rabbit keratocytes caused by mitomycin C application was determined by the LDH assay. Data show mean ± SD of results in three samples at each concentration. (**A**) Dose–dependent cytotoxicity of mitomycin C was evaluated at 24 hours after application. LDH activities were significantly increased at the concentration of 0.005% and higher, compared with the LDH activity without mitomycin C ($P < 0.01$). (**B**) Time course of cell death after application of 0.01% mitomycin C. Until 24 hours, LDH activity was increased. LDH activity showed a time-dependent course.

trations of 3×10^3 to 5×10^3 per well, in 96-well tissue-culture plates. These were incubated in 1 mL 10% FBS DMEM at 37°C in 5% CO_2 for 24 to 48 hours.

Evaluation of Responses Induced by Mitomycin C

Cell Death by Mitomycin C. We evaluated dose-dependent cell death at 24 hours after application of various concentrations of mitomycin C (0.005%, 0.01%, 0.02%, 0.04%, and 0.06%). The time course of cell death was determined after treatment with 0.01% mitomycin C at 0, 6, 12, and 24 hours. The lactate dehydrogenase (LDH) assay (CytoTox 96R Non-Radioactive Cytotoxicity Assay; Promega, Madison, WI) was used for evaluating cellular damage.[2]

Identification of the Responses Induced by Mitomycin C. *Hoechst-EthD Staining.* Cells were treated with 0.01% mitomycin C for 6 hours, stained with 1 μg/mL Hoechst 33342 (Molecular Probes, Leiden, The Netherlands), and photographed with fluorescence and phase-contrast microscopes.

Flow Cytometric Analysis. Keratocytes were either left untreated or were treated with 0.01% mitomycin C for 3, 6, and 24 hours. Cells were incubated (with annexin V FITC, Caltag Laboratories, Burlingame, CA), in buffer containing PI, and analyzed by flow cytometry.[3]

Analysis of the Apoptotic Pathway. After preincubation of keratocytes with 0, 10, 100, 1,000 or 10,000 nM CPP32-like protease inhibitor (Z-VAD-FMK; Calbiochem, Bad Soden, Germany) for 1 hour, 0.01% mitomycin C was added. We evaluated LDH activity after 6 hours. In addition, after preincubation with 0, 50, or 100 μM of the specific caspase-8 inhibitor (LIETD-FMK; Calbiochem) and the caspase-9 inhibitor (Z-LEHD-FMK; Calbiochem) for 1 hour, cells were treated with 0.01% mitomycin C, and LDH activity was evaluated after 6 hours.

Cells were immunostained with cytochrome *c* rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and apoptotic changes were observed by phase-contrast microscope.

Statistical Analyses

The ANOVA test was used to evaluate keratocyte apoptosis. Statistical significance was determined at a $P < 0.05$. Data were analyzed on computer (SPSS, ver. 10.0 for Windows; SPSS, Inc., Chicago, IL).

RESULTS

Experiments with different concentrations of mitomycin C revealed that LDH activity was considerably increased at concentrations of 0.005% and higher ($P < 0.01$). No significant differences in activity were noted at concentrations of 0.005%, 0.01%, 0.02%, 0.04%, and 0.06% (Fig. 1A). LDH activity after treatment with 0.01% mitomycin C was time dependent and continued to increase until 24 hours (Fig. 1B).

In the group treated with mitomycin C, Hoechst 33342 staining clearly revealed apoptotic characteristics, such as nuclear condensation and shrinkage of cytoplasm (Fig. 2). In flow cytometric analyses of apoptotic cells using annexin V-FITC, untreated cells (Fig. 3A) were primarily annexin V-FITC and PI negative, indicating viability and no apoptosis. After treatment with 0.01% mitomycin C, a significant number of cells were annexin V-FITC positive and PI negative (Figs. 3B, 3C), signifying that cells were in the early stages of apoptosis, but still viable. After 24 hours, the cells progressed to a later stage of apoptosis and stained positively for both PI and annexin V, indicating nonviability (Fig. 3D).

The CPP32-like inhibitor Z-VAD-FMK did not affect LDH activity triggered by 0.01% mitomycin C ($P > 0.05$; Fig. 4). However, LDH activity was significantly decreased by the caspase-8 inhibitor, IETD-CHO, and the caspase-9 inhibitor, Z-LEHD-FMK ($P = 0.03, 0.05$, respectively; Figs. 5 and 6).

On immunostaining with cytochrome *c*, positive findings, such as a punctate appearance around the cytosol, were clearly noted in keratocytes (Figs. 7B, 7C) photographed

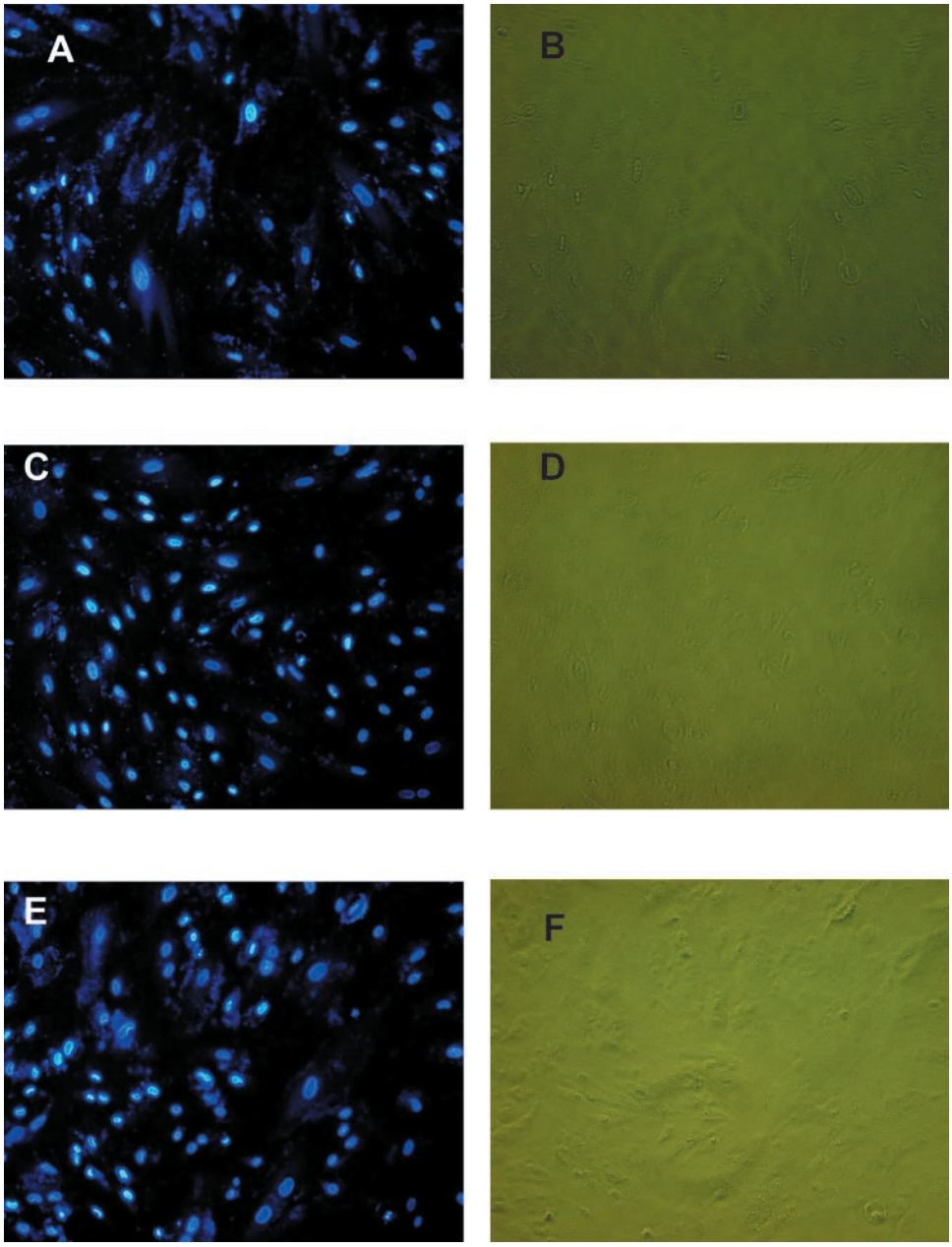

FIGURE 2. Micrographs of rabbit keratocytes exposed to 0.01% mitomycin C for 4 (**C**, **D**) and 6 (**E**, **F**) hours and control cells (**A**, **B**). (**A**, **C**, **E**) Stained with 1 μg/mL Hoechst 33342 and photographed by fluorescence microscopy. More fluorescence was detected in (**C**) and (**E**) than in (**A**), (**B**), (**D**), and (**F**). Photographed by phase-contrast microscopy. Apoptotic appearances, such as nuclear condensation and perimembranous vesicles, were clearly visible (**D**, **F**). Magnification, ×200.

after exposure to 0.01% mitomycin C for 4 (Fig. 7B) or 6 (Fig. 7C) hours.

DISCUSSION

The disappearance of superficial keratocytes after corneal epithelial injury has been demonstrated in several studies.[25–27] This cell loss is mediated by programmed cell death (apoptosis)[11] related to the release of IL-1[11] or Fas-ligand[14] from the epithelium and reactive oxygen radicals.[29] Keratocytes replenish the anterior stroma within a few days of epithelial injury, but repopulating keratocytes are in an activated state with dark nuclei and prominent intracellular organelles. Activated keratocytes are associated with increased collagen deposition and disorganization, manifesting as haze and regression after refractive surgical procedures. Transepithelial PRK may cause less opacity and regression.[11,29,30]

Recent reports have shown that mitomycin C decreases corneal opacity after refractive surgery. In clinical trials, mitomycin C was applied before PRK. The 9-month follow-up revealed that the surgically treated corneas remained clear.[18] Majmudar et al.[31] demonstrated that topical application of mitomycin C (0.02%) successfully prevented the recurrence of epithelial fibrosis after debridement. Eight corneas undergoing mitomycin C treatment after debridement remained clear, with no recurrence or adverse reaction throughout the follow-up periods (6–25 months). After the application of mitomycin C during PRK in rabbit corneas, keratocyte repopulation was not detected at the anterior stroma.[17] Moreover, mitomycin C inhibited the activation of humane keratocytes and displayed cytotoxicity to these cells.[19]

Our Hoechst-EthD staining and annexin V binding assays demonstrate that cell loss by mitomycin C is due to apoptosis. Apoptosis in keratocytes induced by mitomycin C occurs in relation to the mitochondrial pathway. This was confirmed by

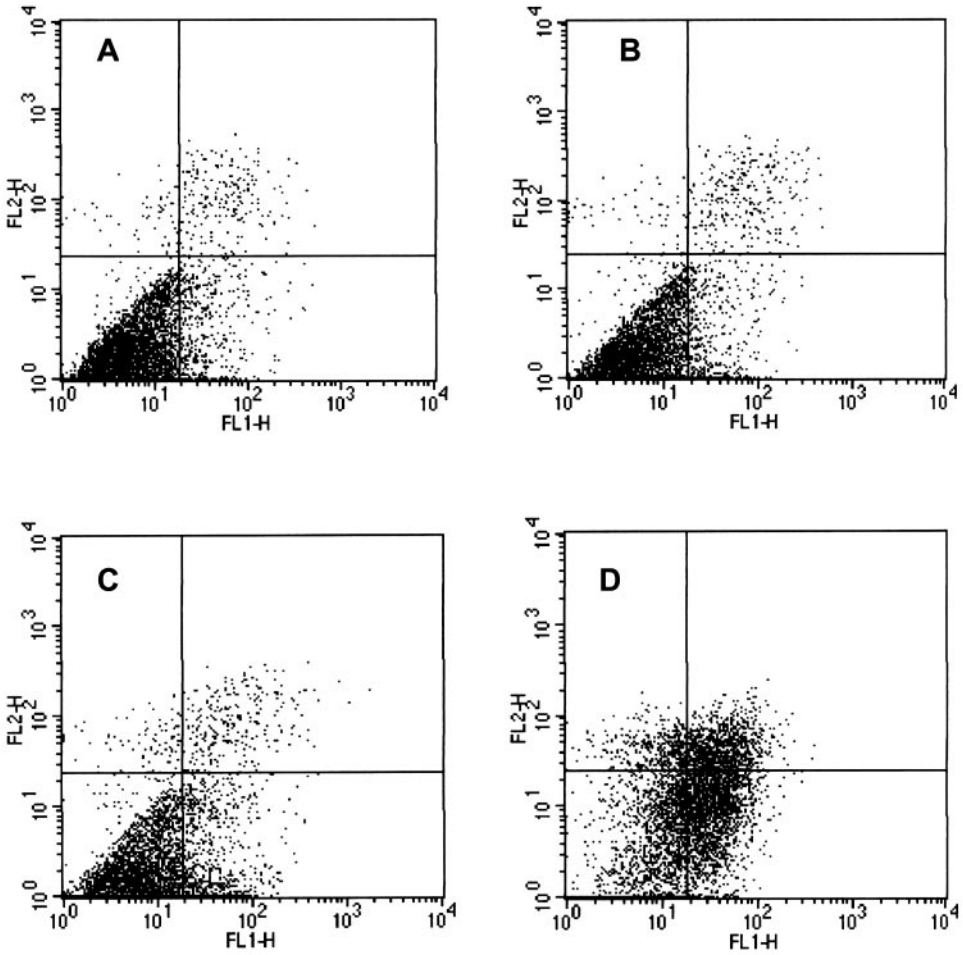

FIGURE 3. Flow cytometric analysis of apoptotic cells using annexin V-FITC. Rabbit cornea keratocytes cells were left untreated (**A**) or were treated for 3 (**B**), 6 (**C**), and 24 (**D**) hours with 0.01% mitomycin C. Cells were incubated with annexin V-FITC in a buffer containing PI and analyzed by flow cytometry. Untreated cells were primarily annexin V-FITC and PI negative (**A**, *bottom left quadrant*) indicating that the cells were viable and not undergoing apoptosis. After treatment with 0.01% mitomycin C, a significant number of cells are annexin V-FITC positive and PI negative (**B**, **C**, *bottom right quadrant*), indicating that the cells were in an early stage of apoptosis and still viable. A population of cells progressed to a later stage of apoptosis and stained positively for both PI and annexin V, indicating that the cells were no longer viable (**D**).

the finding that keratocytes treated with mitomycin C were stained positively for cytochrome *c*.

Caspase-8 and -9 inhibitors significantly decreased the apoptotic effect of mitomycin C. Although data were not statistically significant, the diffuse caspase inhibitor, Z-VAD, suppressed apoptosis, consistent with previous studies. Z-VAD prevented the apoptosis of keratocytes after corneal scraping, but caused necrosis.[32] The compound is therefore not useful for modulating corneal wound healing. The apoptosis pathway induced by mitomycin C is related to the caspase pathway, in particular, caspase-8 and -9. To date, the mechanism of keratocyte apoptosis is unknown. Wilson et al.[14] demonstrated that mRNA coding for several mediators (Bax, BCL-2, BCL-X1, and ICE) of the common final pathway of apoptosis were expressed in corneal epithelial, stromal fibroblast, and endothelial cells in primary culture. Elucidation of the pathways and mediators related to the apoptosis of keratocytes should facilitate the control of their cellular behavior.

Our experiments showed that apoptotic responses were time dependent. Until 6 hours, this response was identified as apoptosis with Hoechst-EthD staining and annexin V–binding

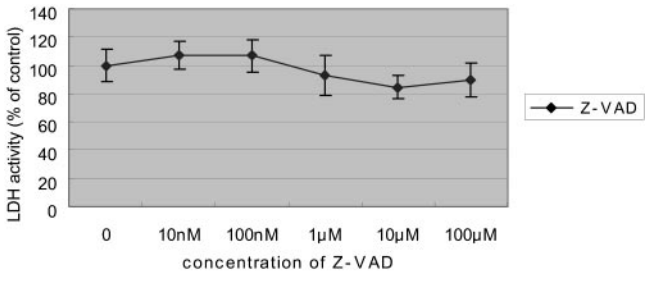

FIGURE 4. The effect of a CPP32-like protease inhibitor on keratocytes. Keratocytes were preincubated for 1 hour in various concentrations of CPP32-like inhibitor Z-VAD-FMK. At 12 hours after 0.01% mitomycin C application, cell toxicity was assayed using the LDH assay. Data are the mean ± SD of results in three samples at each concentration. LDH activity was somewhat but not significantly reduced ($P > 0.05$).

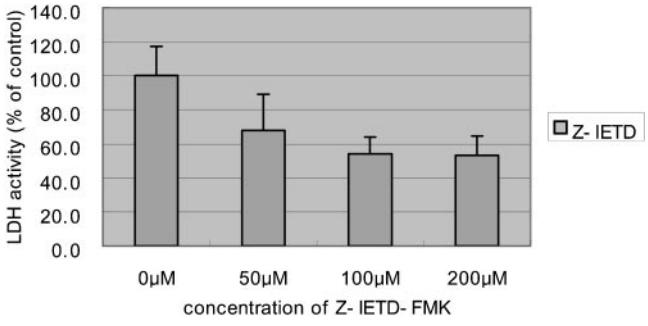

FIGURE 5. The effect of the caspase-8 inhibitor, Z-IETD-FMK. Keratocytes were preincubated for 1 hour in various concentrations of IETD-CHO. At 12 hours after 0.01% mitomycin C application, cell toxicity was assayed with the LDH assay. Data are the mean ± SD of results in three samples at each concentration. LDH activity in each group treated with Z-IETD-FMK was significantly reduced compared with the control ($P = 0.03$).

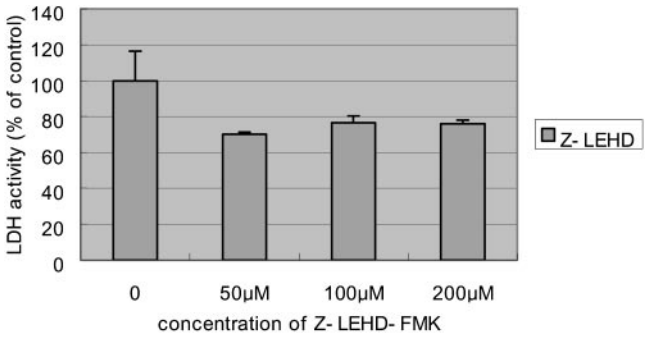

FIGURE 6. The effect of the caspase-9 inhibitor, Z-LEHD-FMK. Keratocytes were preincubated for 1 hour in various concentrations of Z-LEHD-FMK. At 12 hours after 0.01% mitomycin C application, cell toxicity was determined with the LDH assay. Data are the mean ± SD of results in three samples at each concentration. LDH activity in each group treated with Z-LEHD-FMK was significantly reduced compared with the control ($P = 0.05$).

assays. After 24 hours, LDH activity increased significantly. These responses may have been related to other kinds of cytotoxicity such as necrosis.[33] Therefore, it is important to determine the optimal duration for application of mitomycin C. No significant differences in LDH activity were observed at concentrations greater than 0.005% mitomycin C, signifying that this concentration was adequate to induce apoptosis in rabbit keratocytes. This result is evidently not applicable to human keratocytes, and therefore establishing a safe and effective concentration and duration of treatment is needed in humans.

After refractive surgery, apoptotic changes in keratocytes beneath the surgical wound are an inevitable result of epithelial injury, mechanical ablation by excimer laser, or reactive oxygen radicals. Although numerous investigations have focused on inhibiting keratocyte apoptosis to prevent subsequent corneal opacity, no promising results have been obtained to date.[22,23] Mitomycin C prevented this recruitment and activation of remnant keratocytes through the induction of apoptosis in activated keratocytes. We believe that keratocyte death has different consequences, depending on whether it is induced in by surgical procedures or occurs in the course of wound healing.

There are two problems to be solved. One is whether mitomycin C induces apoptosis only in rapidly proliferative keratocytes or in inactivated cells as well. In vivo, keratocytes are surrounded by very compact collagen and are thus inactivated without proliferation or production of excessive collagen. However, in vitro, keratocytes have no inhibitory compact surroundings and may therefore be activated. If mitomycin C causes apoptosis in both activated and inactivated cells, the loss of keratocytes may cause corneal thinning years after a single application. Therefore, the extent and duration of mitomycin C activity are still to be determined. Although Smith et al.[24] reported that mitomycin C disappears rapidly from the ocular tissue and that concentration of the chemical is significantly reduced by irrigating the tissue afterward, long-term effects of mitomycin C should be investigated in detail.

The other problem is the evaluation of effective and safe concentrations of mitomycin C in clinical trials. Sadeghi et al.[19] reported inhibitory and cytotoxic concentrations of mitomycin C in cultured human keratocytes. However, the report was based on in vitro data and does not reflect the in vivo state.

Corneal haze and refractive regression are two problematic areas after refractive surgery. Current attention is focused on modulating the postoperative wound-healing process. Corticosteroid and noninflammatory drugs have been investigated for their potential in regulating stromal haze and refractive regression. Mitomycin C, which modulates the corneal wound-heal-

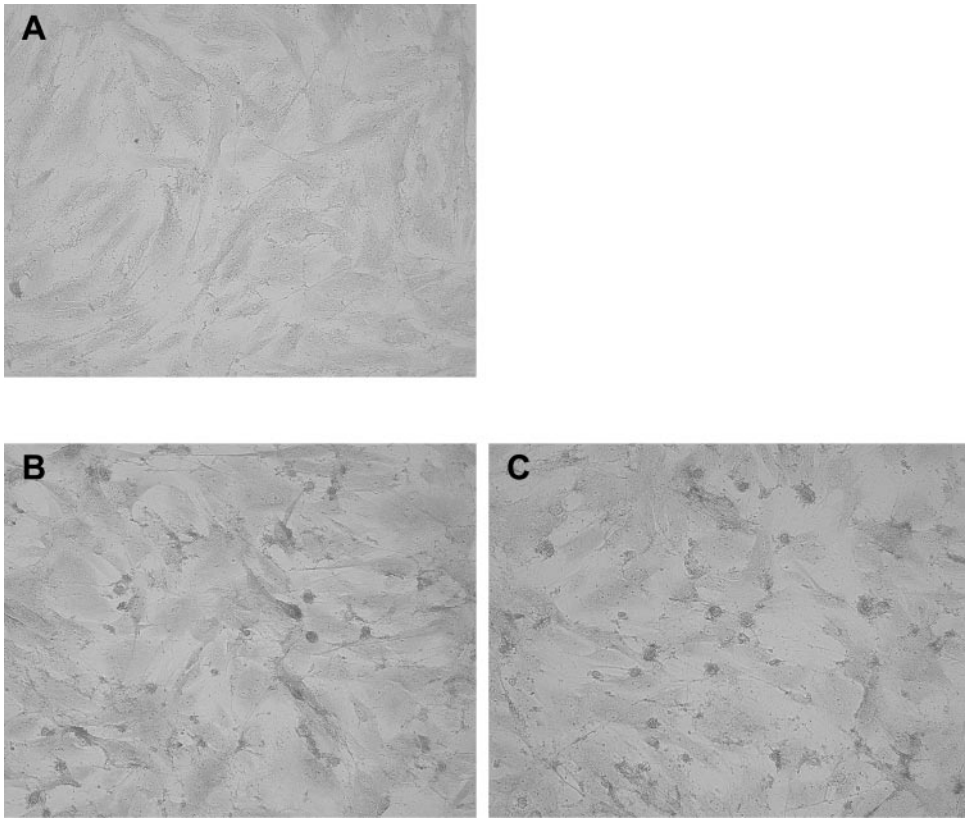

FIGURE 7. Effect of cytochrome *c* on mitomycin C–induced apoptosis. Micrographs of rabbit keratocytes exposed to 0.01% mitomycin C for 4 (**B**) or 6 (**C**) hours and control cells (**A**). Cells were stained with cytochrome *c* and photographed by phase-contrast microscopy. Apoptotic changes such as punctate appearance around the cytosol were clearly visible (**B**, **C**). Magnification, ×200.

ing process, has been investigated for the same purpose. In our study, mitomycin C induced apoptotic changes through the caspase cascade related to the mitochondrial pathway in rabbit keratocytes. This finding is useful for the control of keratocyte apoptosis and provides an experimental background for application of mitomycin C to prevent corneal opacity in refractive surgery.

References

1. Arends MJ, Wyllie AH. Apoptosis: mechanisms and roles in pathology. *Int Rev Exp Pathol.* 1991;32:223–254.
2. Adachi S, Cross AR, Babior BM, Gottlieb RA. Bcl-2 and the outer mitochondrial membrane in the inactivation of cytochrome c during Fas-mediated apoptosis. *J Biol Chem*. 1997;272:21878–21882.
3. Gudz TI, Tserng KY, Hoppel CL. Direct inhibition of mitochondrial respiratory chain complex III by cell-permeable ceramide. *J Biol Chem*. 1997;272:24154–24158.
4. Mignotte B, Vayssiere JL. Mitochondria and apoptosis. *Eur J Biochem*. 1998;15;252:1–15.
5. Bellomo G, Perotti M, Taddei F, et al. Tumor necrosis factor alpha induces apoptosis in mammary adenocarcinoma cells by an increase in intranuclear free Ca2+ concentration and DNA fragmentation. *Cancer Res*. 1992;52:1342–1346.
6. Obeid LM, Linardic CM, Karolak LA, Hannun YA. Programmed cell death induced by ceramide. *Science*. 1993;259:1769–1771.
7. Varfolomeev EE, Boldin MP, Goncharov TM, Wallach D. A potential mechanism of "cross-talk" between the p55 tumor necrosis factor receptor and Fas/APO1: proteins binding to the death domains of the two receptors also bind to each other. *J Exp Med*. 1996;183:1271–1275.
8. Wilson SE, Pedroza L, Beuerman R, Hill JM. Herpes simplex virus type-1 infection of corneal epithelial cells induces apoptosis of the underlying keratocytes. *Exp Eye Res*. 1997;64:775–779.
9. Jaroszewski L, Rychlewski L, Reed JC, Godzik A. ATP-activated oligomerization as a mechanism for apoptosis regulation: fold and mechanism prediction for CED-4. *Proteins*. 2000;15;39:197–203.
10. Mohan RR, Liang Q, Kim WJ, Helena MC, Baerveldt F, Wilson SE. Apoptosis in the cornea: further characterization of Fas/Fas ligand system. *Exp Eye Res*. 1997;65:575–589.
11. Wilson SE, He YG, Weng J, et al. Epithelial injury induces keratocyte apoptosis: hypothesized role for the interleukin-1 system in the modulation of corneal tissue organization and wound healing. *Exp Eye Res.* 1996;62:325–338.
12. Wilson SE, Kim WJ. Keratocyte apoptosis: implications on corneal wound healing, tissue organization, and disease. *Invest Ophthalmol Vis Sci.* 1998;39:220–226.
13. Helena MC, Baerveldt F, Kim WJ, Wilson SE. Keratocyte apoptosis after corneal surgery. *Invest Ophthalmol Vis Sci.* 1998;39:276–283.
14. Wilson SE, Li Q, Weng J, et al. The Fas-Fas ligand system and other modulators of apoptosis in the cornea. *Invest Ophthalmol Vis Sci.* 1996;37:1582–1592.
15. Mohan RR, Kim WJ, Mohan RR, Chen L, Wilson SE. Bone morphogenic proteins 2 and 4 and their receptors in the adult human cornea. *Invest Ophthalmol Vis Sci.* 1998;39:2626–2636.
16. Talamo JH, Gollamudi S, Green WR, De La Cruz Z, Filatov V, Stark WJ. Modulation of corneal wound healing after excimer laser keratomileusis using topical mitomycin C and steroids. *Arch Ophthalmol.* 1991;109:1141–1146.
17. Cheng Z, Li J, Cai K, et al. Effects of mitomycin C on haze after photorefractive keratectomy for myopia in rabbits [in Chinese]. *Chung Hua Yen Ko Tsa Chih.* 1998;34:454–456.
18. Winkler von Mohrenfels C, Hermann W, Gabler B, Muller M, Marshall J, Lohmann CP. Topical Mitomycin C for the prophylaxis of recurrent haze after excimer laser photorefractive keratectomy (PRK): a pilot study of 5 patients [in German]. *Klin Monatsbl Augenheilkd.* 2001;218:763–767.
19. Sadeghi HM, Seitz B, Hayashi S, LaBree L, McDonnell PJ. In vitro effects of mitomycin-C on human keratocytes. *J Refract Surg.* 1998;14:534–540.
20. Chew SJ, Beuerman RW, Kaufman HE. In vivo assessment of corneal stromal toxicity by tandem scanning confocal microscopy. *Lens Eye Toxic Res.* 1992;9:275–292.
21. Crooke ST, Bradner WT. Mitomycin C: a review. *Cancer Treat Rev.* 1976;3:121–139.
22. Jampel HD. Effect of brief exposure to mitomycin C on viability and proliferation of cultured human Tenon's capsule fibroblasts. *Ophthalmology.* 1992;99:1471–1476.
23. Khaw PT, Sherwood MB, MacKay SL, Rossi MJ, Schultz G. Five-minute treatments with fluorouracil, floxuridine, and mitomycin have long-term effects on human Tenon's capsule fibroblasts. *Arch Ophthalmol.* 1992;110:1150–1154.
24. Smith S, D'Amore PA, Dreyer EB. Comparative toxicity of mitomycin C and 5-fluorouracil in vitro. *Am J Ophthalmol.* 1994;118:332–337.
25. Nakayasu K. Stromal changes following removal of epithelium in rat cornea. *Jpn J Ophthalmol.* 1988;32:113–125.
26. Crosson CE. Cellular changes following epithelial abrasion. In: Beuerman RW, Crosson CE, Kaufman HE, eds. *Healing Processes in the Cornea.* Houston, TX: Gulf Publishing; 1989:3–14.
27. Campos M, Szerenyi K, Lee M, McDonnell JM, Lopez PF, MacDonnell PJ. Keratocyte loss after corneal deepithelialization in primate and rabbits. *Arch Ophthalmol.* 1994;112:254–260.
28. Bilgihan K, Bilgihan A, Adiguzel U, et al. Keratocyte apoptosis and corneal antioxidant enzyme activities after refractive corneal surgery. *Eye*. 2002;16:63–68.
29 Hanna KD, Pouliquen YM, Waring GO III, Savoldelli M, Fantes F, Thompson KP. Corneal wound healing in monkeys after repeated excimer laser photorefractive keratectomy. *Arch Ophthalmol.* 1992;110:1286–1291.
30. Del Pero RA, Gigstad JE, Roberts AD, et al. A refractive and histopathologic study of excimer laser keratectomy in primates. *Am J Ophthalmol.* 1990;109:419–429.
31. Majmudar PA, Forstot SL, Dennis RF, et al. Topical mitomycin-C for subepithelial fibrosis after refractive corneal surgery. *Ophthalmology.* 2000;107:89–94.
32. Kim WJ, Mohan RR, Mohan RR, Wilson SE. Caspase inhibitor z-VAD-FMK inhibits keratocyte apoptosis, but promotes keratocyte necrosis, after corneal epithelial scrape. *Exp Eye Res*. 2000;71:225–232.
33. Kim YH, Kim EY, Gwag BJ, Sohn S, Koh JY. Zinc-induced cortical neuronal death with features of apoptosis and necrosis: mediation by free radicals. *Neuroscience.* 1999;89:175–182.