

# Mitomycin-C Induces the Apoptosis of Human Tenon's Capsule Fibroblast by Activation of *c-Jun* N-Terminal Kinase 1 and Caspase-3 Protease

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**PURPOSE.** To investigate whether mitochondrial dysfunction and mitogen-activated protein kinase family proteins are implicated in apoptotic signaling of human Tenon's capsule fibroblasts (HTCFs) by mitomycin-C.

**METHODS.** Apoptosis was determined by Hoechst nuclei staining, agarose gel electrophoresis, and flow cytometry in HTCFs treated with 0.4 mg/mL mitomycin-C for 5 minutes. Enzymatic digestion of fluorogenic biosubstrate assessed the catalytic activity of caspase proteases, including caspase-3, caspase-8, and caspase-9. Phosphotransferase activity of *c-Jun* N-terminal kinase (JNK) 1 was measured by in vitro immune complex kinase assay using cJun<sub>1-79</sub> protein as a substrate. Mitochondrial membrane potential transition (MPT) was measured by flow cytometric analysis of JC-1 staining.

**RESULTS.** Mitomycin-C (0.4 mg/mL) induced the apoptosis of HTCFs, which was characterized as nucleic acid and genomic DNA fragmentation, chromatin condensation, and sub-G<sub>0</sub>/G<sub>1</sub> fraction of cell cycle increase. The catalytic activity of caspase-3 and caspase-9 was significantly increased and was accompanied by cytosolic release of cytochrome *c* and MPT in response to mitomycin-C. Treatment with mitomycin-C resulted in the increased expression of Fas, FasL, Bad, and phosphorylated p53 and a decreased level of phosphorylated AKT. Treatment with mitomycin-C also increased the phosphotransferase activity and tyrosine phosphorylation of JNK1, whose inhibitor significantly suppressed the cytotoxicity of mitomycin-C.

**CONCLUSIONS.** Mitomycin-C induced the apoptosis of HTCFs through the activation of intrinsic and extrinsic caspase cascades with mitochondrial dysfunction. It also activated Fas-mediated apoptotic signaling of fibroblasts. Furthermore, the activation of JNK1 played a major role in the cytotoxicity of mitomycin-C. (*Invest Ophthalmol Vis Sci*. 2005;46:3545-3552)

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The most common reason for failure in glaucoma-filtering surgery is scarring and fibrosis of the filtering bleb at the level of the subconjunctival fibroblasts.<sup>1,2</sup> Thus, the single intraoperative application of anticancer chemotherapeutic agents, including mitomycin-C and 5-fluorouracil (5-FU), is widely used to increase the success of glaucoma-filtering surgery by preventing fibroblast proliferation and excessive scar formation.<sup>3-5</sup> It has been suggested that antiproliferating effects of these agents are mediated by activation of the apoptosis-signaling pathway in conjunctival fibroblasts.<sup>6</sup> These drugs also induce the activation of apoptotic effector molecules, including *c-Jun* N-terminal kinase (JNK) and caspase-3, in human promyelocytic U937 cells. However, the pivotal mechanism of antiproliferating and apoptotic activity of mitomycin-C largely remains.

Recently, the effectiveness of mitomycin-C on the conjunctiva has focused on aspects of antiproliferating and apoptotic signaling for fibroblast functions, such as attachment and proliferation. Specifically, mitomycin-C is known to have a profound cytotoxic effect on cells in the mitosis and proliferation phases of capillary and corneal endothelial cells, corneal epithelial cells, and fibroblasts of conjunctiva and Tenon capsule.<sup>5</sup> Previous evidence suggests that mitomycin-C damages DNA by cross-linking bases in the same or adjacent strands of DNA and that it eventually triggers a powerful stimulus for apoptosis, including p53 induction. In addition, mitomycin-C induces the activation of NF-κB in human T-cell lines.<sup>7-9</sup>

Apoptosis is an active process that plays pivotal roles in the normal physiologic turnover of cells and in various pathologic processes. It is characterized by dramatic cellular alteration, particularly membrane blebbing, cell shrinkage, chromosome condensation, and DNA fragmentation.<sup>10</sup> Apoptosis can be triggered by a wide variety of cellular stresses, including UV radiation, ionizing radiation, heat shock, oxidative stress, and DNA-damaging agents.<sup>11</sup> JNK1 is also activated during apoptosis induced by UV light, heat shock, and ligation of the Fas antigen.<sup>12</sup> Activation of JNK is involved by dual phosphorylation of a Thr-Pro-Tyr motif, and it seems to contribute to the initiation of apoptosis. Furthermore, recent evidence suggests that activation of the cascade of cysteine proteases of the caspase family may play a pivotal role in the execution of apoptosis.<sup>13</sup> Caspases cleave a variety of substrates, including components of cellular DNA repair mechanisms such as poly ADP (adenosine diphosphate)-ribose polymerase (PARP), DNA-dependent protein kinase (DNA-PK), actin, fordins, lamin, and IκB, respectively. To clarify the mechanism by which mitomycin-C induces apoptosis, we examined the implications of the apoptosis-signaling pathway, including intrinsic and extrinsic caspase family proteases, mitochondrial dysfunction, p53, mitogen-activated protein (MAP) kinase family proteins, and AKT, in human Tenon's capsule fibroblasts (HTCFs).

## METHODS

### Cell Culture

A primary cell line of HTCFs was established from subconjunctival Tenon capsule isolated from patients during pterygium surgery. All patients gave their informed consent before surgery. This study was approved by the Yonsei University College Medicine ethics committee in accordance with the provisions of the Declaration of Helsinki. HTCFs were cultured at 37°C with 5% CO<sub>2</sub> in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 IU/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM). HTCFs in exponential growth phase were used between passages 2 and 6 for all experiments.

### Treatment of HTCFs with Mitomycin-C

An HTCF monolayer seeded in 24-well plates or 10-cm dishes overnight was washed with phosphate-buffered saline (PBS; pH 7.4) and then treated with a single application of 0.4 mg/mL mitomycin-C for 5 minutes. Control fibroblasts were treated with PBS for the same period. After treatment, cells were washed three times with PBS and were maintained in DMEM for subsequent experiments.

### Cell Viability

Cell viability was measured by using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT). HTCFs were cultured in triplicate in 96-well plates and were treated with 0.4 mg/mL mitomycin-C. HTCFs were maintained in DMEM for 72 hours and were further incubated with 500 µg/mL MTT for 4 hours. Cells that stained positively with MTT were considered viable cells and were expressed as a percentage compared with control cells.

### Hoechst Staining and DNA Laddering

HTCFs were plated in slide chambers and were treated with 0.4 mg/mL mitomycin-C, as described. Cells were then fixed in 3.7% formaldehyde in PBS at 37°C for 10 minutes and were washed with PBS. Cells were stained with 10 µg/mL bisbenzimidazole (Hoechst 33258) at 37°C for 30 minutes, washed three times in PBS, and examined by fluorescence microscopy. The ladder pattern of DNA strand break was analyzed by agarose gel electrophoresis. Genomic DNA was isolated with the Wizard Genomic DNA purification kit (Promega, Madison, WI). After ethanol precipitation, 50 µg DNA in each lane was subjected to electrophoresis on 1.5% agarose at 50 V for 3 hours. DNA was then visualized under UV transillumination by staining with ethidium bromide.

### Flow Cytometric Analysis of Apoptosis and Mitochondrial Membrane Potential Transition

Flow cytometric analysis of apoptotic cell death was performed as described.<sup>14</sup> HTCFs (10<sup>7</sup> cells/group) treated with 0.4 mg/mL mitomycin-C for 12 hours were harvested, washed with Hanks balanced salt solution (HBSS), and stained with 50 µg/mL propidium iodide in 0.1% Nonidet P-40. After the unbound dye was washed out, cells were subjected to flow cytometric analysis to estimate the sub-G<sub>0</sub>/G<sub>1</sub> fraction in cell cycle analysis. Data were collected and analyzed (Consort 3.0 program; Becton-Dickinson, Mountain View, CA). Mitochondria of HTCFs were incubated with 5 µg /mL JC-1 at 37°C for 30 minutes. After incubation, cells were washed three times with PBS and subjected to flow cytometric analysis.

### Western Blotting

HTCFs were lysed in 50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein was normalized to 100 µg/lane, resolved on 7.5% to 12.5% polyacrylamide gels, and blotted onto nitrocellulose membrane. The nitrocellulose membrane was incubated with blocking buffer (5% skim milk in TBS-T containing 0.05% Tween-20, 0.14 N NaCl, and 25 mM Tris-HCl, pH 7.4) for 1 hour and then was incubated with the primary

antibodies against PARP, cytochrome c, VDAC, Bcl-2, Bad, Fas, FasL, tBid, phospho-p53, p53, phospho-JNK1, JNK1, phospho-AKT, and AKT proteins (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G antibody (1:5000 diluted) was added to the membrane, which was further incubated for 1 hour. Immunoreactive bands were visualized with enhanced chemiluminescence reagents (ECL; Amersham Life Science, Amersham, UK). For reprobing, the same membrane was stripped with 0.1 M glycine (pH 2.5) at room temperature for 30 minutes and then incubated with the other primary antibody.

### In Vitro Kinase Assay of JNK1

HTCFs were lysed in ice-cold lysis buffer containing 10 mM Tris, pH 7.6, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% 2-mercaptoethanol, 1% aprotinin, 50 mM NaF, 2 mM PMSF, 5 µM phenylarsine oxide, and 100 mM sodium orthovanadate. The supernatant was incubated with 1 µg polyclonal anti-JNK1 antibody (Santa Cruz Biotechnology) for 1 hour and then was washed twice with PAN buffer containing 10 mM piperazine-N-N'-bis[ethanesulfonic acid (PIPES) pH 7.0, 1% aprotinin, and 100 mM NaCl. After incubation, a 10% solution of formalin-fixed *Staphylococcus aureus* was added to anti-JNK1 immune complex. Enzymatic activity of JNK1 was assayed by incorporation of [ $\gamma$ -<sup>32</sup>P]adenosine triphosphate (ATP) (2 µCi/sample) with 1 µg glutathione-S transferase (GST)-c-Jun N terminus<sub>1-79</sub> protein in a kinase reaction buffer containing 20 mM Tris, pH 7.5, 20 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, and 20 µM cold ATP at 30°C for 15 minutes. Phosphorylation reaction was terminated by an equal amount of 2× Laemmli sample buffer and was boiled for 5 minutes. The protein was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. <sup>32</sup>P-labeled GST-c-Jun N terminus<sub>1-79</sub> protein was detected by autoradiography and quantified on an analyzer (PhosphorImager; Fuji Co., Tokyo, Japan). After autoradiographic exposure to x-ray film, the nitrocellulose membrane was incubated with blocking buffer for 1 hour and then probed for phospho-JNK1 and JNK1 proteins by Western blot analysis.

### Caspase Activity Assay

To measure the catalytic activation of caspase family cysteine proteases, including caspase-3, caspase-8, and caspase-9, whole lysate of HTCFs was prepared in a lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM PMSF, 1 µg/mL leupeptin, 2 mM dithiothreitol [DTT], 10 mM Tris/HCl, pH 8.0) on ice for 30 minutes and centrifuged at 20,000g for 15 minutes. Equal amounts of total protein were quantified with a bicinchoninic acid kit (Sigma, St Louis, MO) in each lysate. Catalytic activity of caspase-3 from the cell lysate was measured by proteolytic cleavage of 100 µM 7-amino-4-methylcoumarin (AMC)-DEVD motif-specific peptide (Calbiochem Co., San Diego, CA) for 1 hour as a fluorogenic substrate and AMC as standard in an assay buffer (100 mM HEPES, 10% sucrose, 0.1% Chaps, pH 7.5, 1 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 2 mM DTT) at 380-nm excitation wavelength and 460-nm emission wavelength. Cleavage of fluorogenic substrates (Calbiochem Co.), including 100 µM Z-IETD-7-amino-4-trifluoromethylcoumarin (AFC) for caspase-8 and 100 µM Ac-LEHD-7-amino-4-trifluoromethylcoumarin (AFC) for caspase-9, was measured by spectrofluorometer (Jasco FR-777, Midland, Canada) at 405 nm and 505 nm in HTCF lysate.

### Preparation of Cytosolic and Mitochondrial Fractions

Preparation of cytosolic and mitochondrial fractions was performed as described in a previous report,<sup>15</sup> with some modifications. In brief, HTCFs were harvested, washed with ice-cold PBS, and incubated with 500 µL buffer A (250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10 µg/mL each of leupeptin, aprotinin, and pepstatin A) on ice for 30 minutes. Then cells were disrupted by 20 passages through a 26-gauge needle. Disrupted cells were centrifuged at 750g for 10 minutes. The supernatant was centrifuged at 10,000g for 25 minutes. After centrifugation, the cytosolic fraction was frozen at 70°C. The pellet contain-

ing mitochondria was washed with ice-cold buffer A and resuspended with cell lysis buffer. This resuspended pellet was incubated on ice for 30 minutes and then centrifuged at 10,000g for 25 minutes, and the supernatant was collected as a cytosolic fraction of HTCFs.

### Statistical Analysis

All values are represented as means  $\pm$  SD of triplicate experiments.  $P < 0.05$  was considered statistically significant.

## RESULTS

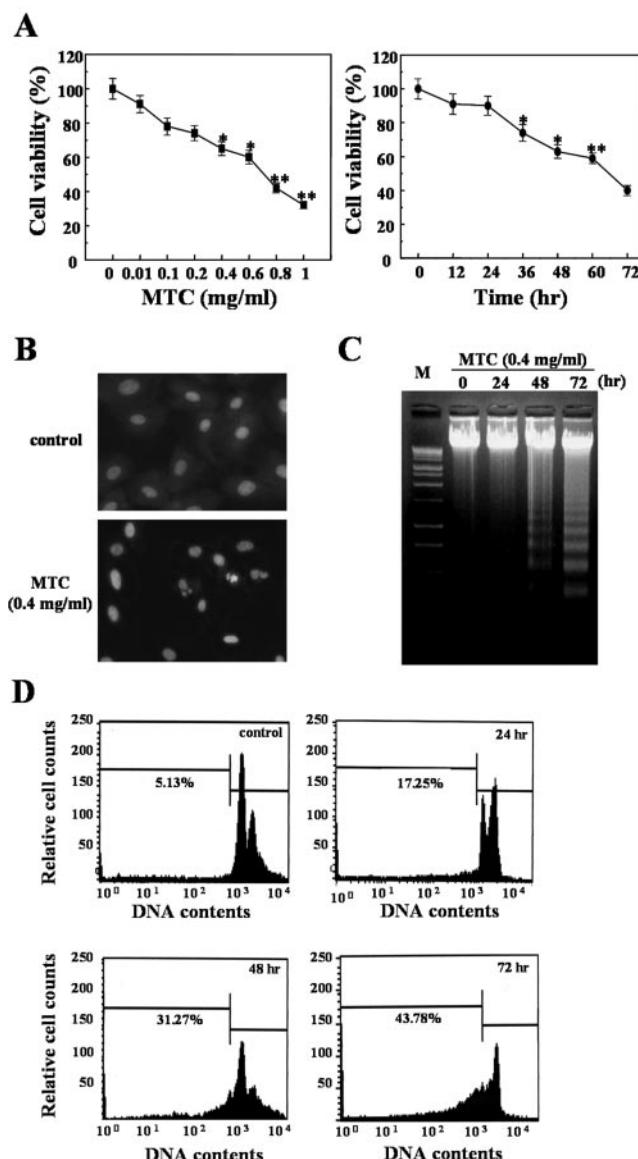
### Apoptotic Effect of Mitomycin-C on HTCFs

Exposing HTCFs to mitomycin-C led to time- and dose-dependent increases in the number of dead cells, as assessed by MTT assay (Fig. 1A). A single exposure to mitomycin-C for 5 minutes at the clinical concentration (0.4 mg/mL) in 62% of cells died after 48 hours. Time-dependent kinetics of cell viability on mitomycin-C demonstrated that short periods (<24 hours) of exposure did not exert significant cytotoxicity, but exposure became significant after 36 hours in HTCFs. Next, to determine the nature of the cytotoxicity of mitomycin-C in HTCFs, the phenotypic characteristics of apoptosis were examined. Cells were treated with 0.4 mg/mL mitomycin-C for 5 minutes, refreshed with new medium, and maintained for 48 hours. Then cells were stained with Hoechst dye and visualized under a fluorescence microscope (Fig. 1B). Nuclei of the control culture were oval round shape with homogeneous intensity, whereas those of cells treated with mitomycin-C were condensed, had fragmented shapes, and were irregular in staining homogeneity. To further verify the apoptotic characteristics, cells were cultured up to 72 hours after treatment with 0.4 mg/mL mitomycin-C for 5 minutes, and genomic DNA from cultures was extracted for 1.5% agarose gel electrophoresis (Fig. 1C). Consistent with the findings of the MTT assay, ladder-pattern fragmentation of genomic DNA appeared in a time-dependent fashion at 48 and 72 hours after treatment of mitomycin-C. DNA fragmentation by mitomycin-C was further analyzed as the sub-G<sub>0</sub>/G<sub>1</sub> fraction of cell cycle analysis by flow cytometry in HTCFs stained with propidium iodide (Fig. 1D). In contrast to the control culture, a single exposure of 0.4 mg/mL mitomycin-C for 5 minutes dose dependently increased the sub-G<sub>0</sub>/G<sub>1</sub> fraction of HTCFs. Collectively, these data indicated that mitomycin-C treatment decreased HTCF viability by apoptosis.

### Catalytic Activation of Caspase-3 in Mitomycin-C-Treated HTCFs

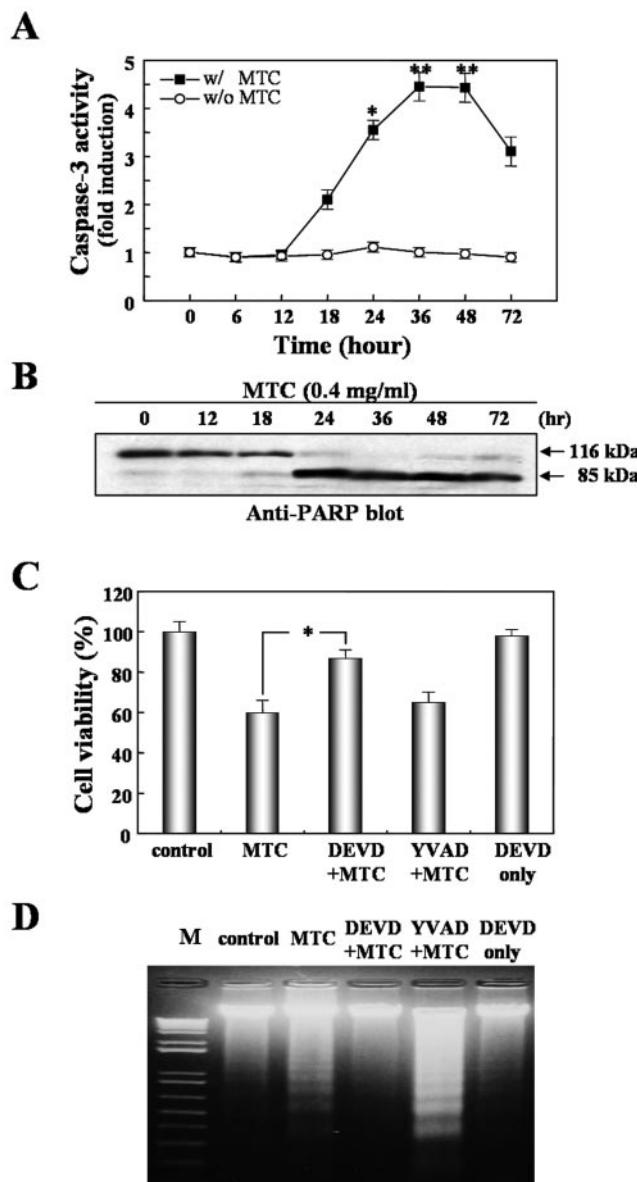
To ascertain the signaling pathway in apoptosis of HTCFs, cells were maintained in the presence of 0.4 mg/mL mitomycin-C for 5 minutes, and lysate was used to measure the catalytic activation of caspase-3 at various time periods (Fig. 2A). The enzymatic activation of caspase-3 started after 12 hours and attained its peak at 36 hours. It sustained that level for 48 hours and then declined. To further confirm the activation of caspase-3, the cleavage of PARP, an intracellular biosubstrate of the enzyme, was measured by Western blot analysis (Fig. 2B). Cleavage of PARP from 116 kDa to 85 kDa was clearly demonstrated at 24 hours after a single application of 0.4 mg/mL mitomycin-C for 5 minutes.

We next applied the pharmacologic inhibitors of caspase family proteases to test whether inhibition of these proteases might have affected the cytotoxic effect of mitomycin-C on HTCFs (Fig. 2C). The effect of pharmacologic inhibitors of caspase-3 and caspase-1, including DEVD-AMC and YVAD-AMC tetrapeptide, on the cytotoxicity of mitomycin-C was measured by MTT assay. Cells were pretreated



**FIGURE 1.** Apoptosis of HTCFs occurred by mitomycin-C (MTC) application for 5 minutes. (A) Dose-dependent (left) and time-dependent (right) effects of MTC on viability were determined by MTT assay at 48 hours with various doses or up to 72 hours at the 0.4-mg/mL concentration. Data represent mean  $\pm$  SD of three independent experiments (\* $P < 0.05$ ; \*\* $P < 0.01$ ). (B) Cell nuclei with Hoechst staining were observed under a fluorescence microscope 48 hours after treatment with 0.4 mg/mL mitomycin-C for 5 minutes. (C) Genomic DNA of fibroblasts was separated on 1.5% agarose gel. (D) Cell cycle analysis was performed by flow cytometry after staining of propidium iodide in cells treated with mitomycin-C.

with inhibitors of caspase proteases, followed by the addition of 0.4 mg/mL mitomycin-C for 5 minutes, and were further maintained for 48 hours. Pretreatment with the caspase-3 inhibitor DEVD-AMC (30  $\mu$ M) kept 60% to 87% ( $P < 0.05$ ) of the cells viable, whereas the caspase-1 inhibitor YVAD-AMC did not have any effect on the cytotoxicity of mitomycin-C. We further confirmed the inhibitory effect of the caspase-3 inhibitor on the apoptosis of HTCFs by mitomycin-C in 1.5% agarose gel electrophoresis (Fig. 2D). Consistent with the results of the MTT assay, caspase-3 inhibition clearly abolished the ladder-pattern fragmentation of genomic DNA in mitomycin-C-treated HTCFs.



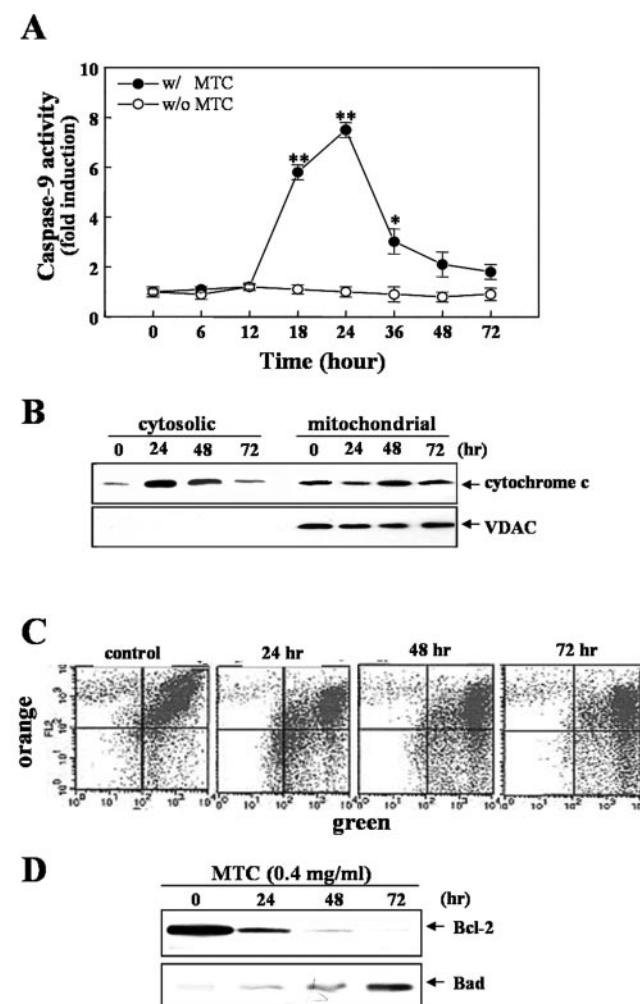
**FIGURE 2.** Application of mitomycin-C caused the activation of caspase-3 in HTCFs. Cells were treated with 0.4 mg/mL mitomycin-C for 5 minutes and were maintained in fresh medium for the indicated periods. (A) Catalytic activation of caspase-3 was measured by cleavage of the fluorogenic biosubstrate DEVD tetrapeptide (\* $P < 0.05$ ; \*\* $P < 0.01$ ). (B) Cytosolic expression of PARP was determined by Western blot analysis with polyclonal anti-PARP antibody in cells treated with mitomycin-C. (C) Pharmacologic inhibitors of the caspase-3 inhibitor DEVD-AMC (30  $\mu$ M) and the caspase-1 inhibitor YVAD-AMC (30  $\mu$ M) were pretreated, and cell viability was measured by MTT assay (\* $P < 0.05$ ). (D) Cells were pretreated with DEVD-AMC or YVAD-AMC and added to 0.4 mg/mL mitomycin-C for 5 minutes, and then genomic DNA was separated on 1.5% agarose gel.

### Catalytic Activation of Caspase-9 with Mitochondrial Dysfunction in Mitomycin-C-Treated HTCFs

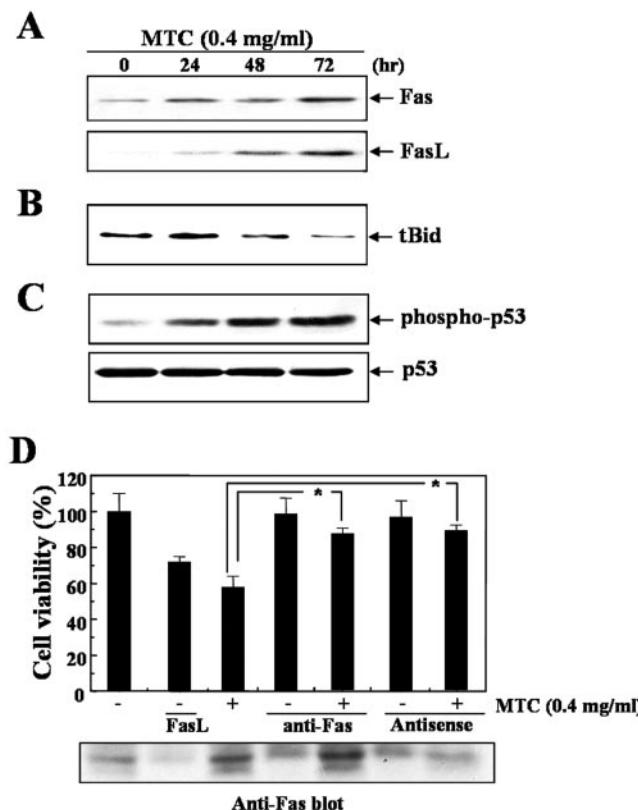
The enzymatic activation of caspase-3 could be initiated by upstream protease, either caspase-9 with mitochondrial dysfunction or caspase-8 with stimulation of the death receptor Fas/FasL system. To test whether the activation of caspase-3 resulted from activation of the intrinsic caspase cascade and from mitochondrial dysfunction, HTCFs were treated with 0.4 mg/mL mitomycin-C for 5 minutes and maintained up to 72

hours, and then lysate was used to measure the catalytic activity of caspase-9, the cytosolic release of cytochrome *c*, and mitochondrial membrane potential transition (MPT). The catalytic activity of caspase-9 started to increase at 12 hours and reached a maximum at 24 hours (6.7-fold) after the application of mitomycin-C (Fig. 3A). After fractionation of the cell lysate into mitochondrial and cytosolic parts, two fractions separately were used to measure the expression of cytochrome *c* by Western blot (Fig. 3B). Cytochrome *c* in cytosolic fraction was markedly increased at 24 hours after mitomycin-C treatment and was time dependently diminished in expression. Consistently, the immunoreactive band of mitochondrial cytochrome *c* at 24 hours was the weakest in intensity among other lanes. The purity of the mitochondrial fraction was verified by Western blot analysis with anti-VDAC antibody.

We next determined the MPT of HTCFs after 0.4 mg/mL the application of mitomycin-C for 5 minutes. Extranuclear mitochondria in the control culture primarily emitted as orange, whereas mitochondria with MPT diffusely located through whole cells were detected as green particles on JC-1 staining.



**FIGURE 3.** Mitomycin-C induced the activation of caspase-9 with mitochondrial dysfunction in HTCFs. (A) Catalytic activity of caspase-9 was measured by cleavage of the fluorogenic biosubstrate Ac-LEHD-7-AFC tetrapeptide (100  $\mu$ M) (\* $P < 0.05$ ; \*\* $P < 0.01$ ). (B) Cytosolic release of cytochrome *c* was measured by Western blot analysis in cytosolic and mitochondrial fractions. Mitochondrial fraction was verified with anti-VDAC antibody. (C) Mitochondrial MPT was determined by flow cytometric analysis with JC-1 staining. (D) Expression of Bcl-2 and Bad was determined by Western blot in cells treated with 0.4 mg/mL mitomycin-C for 5 minutes.



**FIGURE 4.** Mitomycin-C induced the activation of the Fas/FasL system and p53 in HTCFs. Cells were treated with 0.4 mg/mL mitomycin-C for 5 minutes and then were maintained in fresh medium for the indicated periods. (A–C) Cellular expression of Fas, FasL, tBid, phosphorylated p53, and p53 was determined by Western blot analysis. (D) Cells were pretreated with neutralizing anti-Fas antibody or *Fas* antisense oligonucleotide, followed by the addition of 0.4 mg/mL mitomycin-C for 5 minutes. Then cell viability was measured by MTT assay.

Flow cytometric analysis of MPT demonstrated that the green signal (Fig. 3C, lower right quadrant) of control culture was 15% and changed to 28% at 24 hours, 35% at 48 hours, and 41% at 72 hours, respectively, after treatment with mitomycin-C. Furthermore, mitomycin-C treatment markedly decreased anti-apoptogenic Bcl-2 expression, whereas it increased apoptotic Bad expression in a time-dependent fashion (Fig. 3D). These data indicated that mitomycin-C induced mitochondrial dysfunction, which eventually activated the intrinsic caspase cascade, including caspase-9 and caspase-3, in the apoptosis signaling of HTCFs.

#### Increase in Expression of Fas/FasL, Bid Truncation, and p53 Phosphorylation by Mitomycin-C

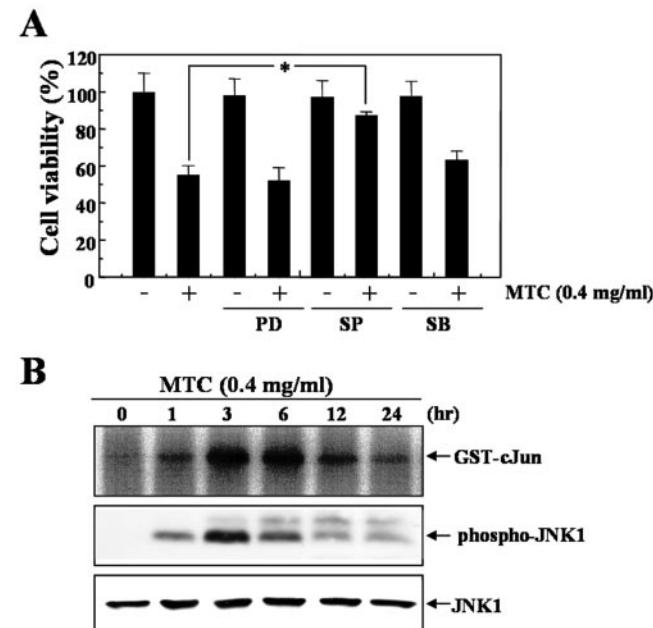
We further questioned whether the death receptor Fas/FasL system was implicated in the apoptosis of HTCFs by mitomycin-C. Cells were treated with 0.4 mg/mL mitomycin-C for 5 minutes and were lysed for Western blot analysis at the indicated time periods. Treating cells with mitomycin-C markedly induced the increase in the expression of Fas and FasL proteins in a time-dependent fashion with different kinetics (Fig. 4A). We also measured the catalytic activity of caspase-8, a downstream target of Fas. However, the enzymatic activation of caspase-8 was not detectable in cells treated with mitomycin-C for 72 hours (data not shown). We were further interested in the translocation of truncated Bid (tBid), from cytosol to mitochondria, which eventually resulted in mitochondrial dysfunc-

tion. Our results showed that treating mitomycin-C time dependently decreased the expression of cytosolic tBid, indicating that tBid translocated into the mitochondria (Fig. 4B). In addition, treating mitomycin-C resulted in an increase of p53 phosphorylation at the serine 15 residue in a time-dependent fashion, even though mitomycin-C did not affect the accumulation or degradation of the protein (Fig. 4C).

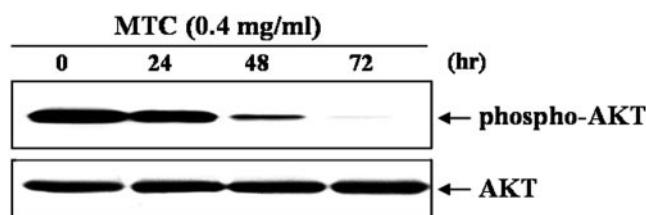
Furthermore, we tested the functional role of Fas receptor in mitomycin-C-mediated HTCF apoptosis at 48 hours (Fig. 4D). Cells were pretreated with the neutralizing anti-Fas antibody or with the antisense oligonucleotide of *Fas*. This was followed by the addition of 0.04 mg/mL mitomycin-C for 5 minutes, and viability was measured by MTT assay. Results showed that blocking Fas signaling with the neutralizing anti-Fas antibody or with the antisense oligonucleotide of the *Fas* gene significantly antagonized the cytotoxicity of mitomycin-C ( $P < 0.05$ ).

#### Suppression of Mitomycin-C Cytotoxicity by Pharmacologic Inhibition of JNK1

MAP kinase family proteins have been implicated in the proliferation, differentiation, and death of cells.<sup>16</sup> Therefore, we pretreated HTCFs with specific inhibitors of MAP kinase family proteins—including MEK, JNK, and p38—in the presence of mitomycin-C for 5 minutes and maintained this for 48 hours. Only the pharmacologic inhibition of JNK1 with SP600125 antagonized the cytotoxicity of mitomycin-C, by 20% ( $P < 0.05$ ), whereas other inhibitors, including PD98059 for MEK and SB203580 for p38, did not (Fig. 5A). To verify these results, in vitro kinase assay and Western blot for JNK1 were performed in HTCFs treated with 0.4 mg/mL mitomycin-C for 5 minutes. Mitomycin-C increased the phosphotransferase activity of JNK1 toward the c-Jun N-terminal protein and the tyrosine phosphorylation of JNK1 at 3 to 12 hours after treat-



**FIGURE 5.** Inhibition of JNK1 activation antagonized the cytotoxicity of mitomycin-C in HTCFs. (A) Cell viability was measured by MTT assay in fibroblasts pretreated with 100  $\mu$ M MEK inhibitor PD98059, 5  $\mu$ M JNK inhibitor SP600125, and 25  $\mu$ M p38 inhibitor SB203580, followed by the addition of mitomycin-C (MTC). (B) Phosphotransferase activity of JNK1 was measured by in vitro immune complex kinase assay (upper panel). Tyrosine phosphorylation of JNK1 was tested by Western blot with anti-phospho-JNK1 antibody.



**FIGURE 6.** Application of mitomycin-C (MTC) caused the phosphorylation of AKT in HTCFs. Phosphorylation of AKT in cells maintained in DMEM supplemented with 10% FBS and 100 nM EGF for 72 hours, treated with 0.4 mg/mL MTC for 5 minutes, was measured by Western blot with anti-phospho-AKT antibody. Anti-AKT immunoblot revealed that the same amount of protein was loaded.

ment. Equal amounts JNK1 protein were confirmed by Western blot with polyclonal anti-JNK1 antibody (Fig. 5B).

### Decrease in AKT Phosphorylation of HTCFs by Mitomycin-C

We tested the phosphorylation of AKT, of which activation is well documented in the process of cell proliferation.<sup>12,17</sup> After 5-minute exposure of HTCFs to 0.4 mg/mL mitomycin-C, cells were maintained in DMEM supplemented with 10% FBS and 100 nM EGF (Genzyme, Cambridge, MA) for 72 hours, and the lysate was used for Western blot analysis of AKT and phospho-AKT (Fig. 6). Phosphorylation of AKT was observed in resting cells but was time dependently decreased by the addition of mitomycin-C at 48 and 72 hours. Western blot analysis for AKT protein verified that equal amounts of the protein were separated on 10% SDS-PAGE.

## DISCUSSION

In this study, we provide mechanistic evidence that mitomycin-C induces the apoptosis of HTCFs at clinically a relevant dose of 0.4 mg/mL. Traditionally, mitomycin-C was widely used as a chemotherapeutic agent for the treatment of many types of cancer. Recently, the mechanism of mitomycin-C has been intensely investigated on fibroblast functions, including proliferation and migration, in relation to conjunctival wound healing after trabeculectomy. Mitomycin-C suppresses the mitosis and proliferation of ocular compartments, including capillary, corneal endothelium and epithelium, conjunctival fibroblasts, and Tenon's capsule fibroblasts, respectively.<sup>5,17,18</sup>

Occleston<sup>19</sup> first suggested that mitomycin-C was cytotoxic to Tenon's capsular fibroblasts. Shields et al.<sup>20</sup> further demonstrated, through histologic examination of mitomycin-C-treated draining blebs, that rather than inhibiting their proliferation, mitomycin-C induced the death of fibroblasts. Later, the nature of mitomycin-C cytotoxicity was revealed as apoptosis by Crowstone et al.<sup>21</sup> and Kim et al.<sup>22</sup> Recently, Kim et al.<sup>22</sup> showed a cotreatment effect of mitomycin-C with alcohol on the apoptosis of cultured corneal fibroblasts.<sup>23</sup> Consistent with previous results, we here present the apoptogenic effects of mitomycin-C on HTCFs. In our study, apoptotic features in mitomycin-C-treated cells were apparent, demonstrating chromatin condensation with nuclear fragmentation, ladder-pattern fragmentation of genomic DNA, and increased sub-G<sub>0</sub>/G<sub>1</sub> fraction of cell cycle analysis.

In apoptosis-signaling cascades, two different initiation machineries, including extrinsic death receptor-mediated signaling and intrinsic caspase family cysteine protease-mediated signaling, play major roles in a variety of cell types.<sup>24,25</sup> We reconfirmed that the effector caspase of mitochondrial downstream caspase-3 plays a critical role in death of HTCFs by mitomycin-C. These results are consistent with previous findings showing that preincubation of CPP32-like protease inhib-

itor (Z-VAD-FMK) caused a decrease in LDH activity in mitomycin-C-treated keratinocytes.<sup>6,22</sup> Mitochondrial dysfunction in apoptotic signaling of mitomycin-C has not been specifically addressed, even though several studies have already proposed that Tenon capsular fibroblast undergoes apoptosis by topical application of anti-metabolite drugs, including 5-FU and mitomycin-C.<sup>6,26</sup> We present the first evidence that mitomycin-C treatment induces mitochondrial dysfunction with the activation of caspase-9 in HTCFs. Caspase-9 is one of the upstream activators of caspase-3; thus, we measured the activity of the enzyme in this experimental system. Mitomycin-C increases the catalytic activity of caspase-9 in a time-dependent fashion. However, we could not detect the time difference of activation between caspase-3 and caspase-9. Furthermore, characteristic markers of mitochondrial dysfunction, including cytosolic translocation of cytochrome c, MPT, and changes in Bcl-2 and Bad expression, were clearly observed in mitomycin-C-treated HTCFs. On the contrary, our data showed a decrease in expression of Bcl-2 after mitomycin-C treatment, high dose (1 μg/mL) mitomycin-C could transiently increase the expression of Bcl-2, which rapidly fade out at 2 hours in human gastric cancer.<sup>27</sup>

Cytosolic release of cytochrome c is essentially required in forming apoptosome complex, containing pro-caspase-9, Apaf-1 and ATP, which triggers the autolytic activation of caspase-9.<sup>28</sup> Also, MPT, revealed by JC-1 staining, could cause the cytosolic release of cytochrome c from mitochondria in mitomycin-C treated HTCFs. Furthermore, the changes in expression of mitochondrial associated proteins, including Bcl-2 and Bad, may contribute to mitochondrial dysfunction, including MPT and cytosolic release of mitochondrial cytochrome c, by mitomycin-C. Among the death receptors on the cellular surface, investigation into the apoptotic mechanism of Fas (CD95) has been interested in fibroblast.<sup>29,30</sup> Earlier studies demonstrated that CD95L was expressed in HTCFs as well as in lens epithelial cells and it mediated the activation of caspase-3 and caspase-9 in apoptosis.<sup>13,29</sup> Consistent with the idea of Crowstone et al.,<sup>21</sup> we found an increase in expression of Fas and FasL proteins in relation to the concentrations of mitomycin-C in HTCFs. However, the expression of FasL in resting control cells was less obvious compared to the work of Hueber et al.<sup>29</sup> In our experimental model, Fas/FasL expression of cells was inducible by the addition of mitomycin-C in a time- and dose-dependent manner. Whereas both expression and apoptosis activity of CD95L in NIH 3T3 did not require *de novo* synthesis of RNA or proteins.<sup>29</sup> Interestingly, treatment of mitomycin-C could not result in induction of FasL and Fas expression in human gastric adenocarcinoma cells.<sup>31</sup> Consistent with TUNEL-sensitive DNA breaks in the works of Hueber et al, pretreatment of both neutralizing anti-Fas antibody and antisense oligonucleotide of Fas revealed that mitomycin-C could activate the Fas/FasL system to transduce signals for apoptosis in HTCFs. The implication of Fas/FasL system in mitomycin-C mediated apoptosis was further evidenced by the decrease in expression of tBid after treatment of mitomycin-C in this study.

The decrease in cytosolic expression of tBid might affect both cytosolic release of mitochondrial cytochrome c and MPT in Fig. 3. However, we could not present the apparent activation of caspase-8, a bridge between Fas and Bid, in cell free assay system (data not shown). However, caspase-8 was activated on mitomycin-C in SNU-16 human gastric adenocarcinoma cells.<sup>31</sup> We also found that p53 was phosphorylated at serine 15 residue, which may also contribute the digestion of Bid protein, unless changes in the accumulation of p53 protein are present. The phosphorylation of p53 is generally accepted as a hall marker of apoptogenic responder in various cell types against genotoxic agents.<sup>32</sup>

MAP kinases play a critical role in proliferation, differentiation and degeneration of cells.<sup>16</sup> In studies of pharmacological inhibition of MAP kinases, including MEK inhibitor PD98059, JNK inhibitor SP600125, and p38 inhibitor SB203580, we demonstrated that activation of JNK1 was required in apoptosis of HTCFs by mitomycin-C. Interestingly, JNK1 activation occurred at an early point in time around 1 to 12 hours after treatment of mitomycin-C in HTCFs before cellular morphologic changes of death, apparently showing after 24 hours. Moreover, phosphorylation of AKT was markedly disappeared by mitomycin-C in HTCFs maintained in DMEM supplemented with 10% FBS and 100 nM EGF for 72 hours. However, PKC- $\alpha$  was transiently increased within 2 hours after a high dose (1  $\mu$ g/mL) mitomycin-C and subsequently disappeared in human gastric cancer MKN-74 cells.<sup>31</sup>

The effectiveness of mitomycin-C on other conjunctival scarring diseases, including ocular cicatricial pemphigoid and Stevens-Johnson syndrome, mostly depends on its apoptotic ability to induce apoptosis of fibroblasts. Earlier studies demonstrated that effectiveness of mitomycin-C in intraoperative application to improve the postoperative outcome in the surgical treatment of cicatricial shrinkage of conjunctival fornices and in subconjunctival inoculation to prevent progression of subconjunctival cicatrization in patients with ocular cicatricial pemphigoid.<sup>33,34</sup> Conversely, Foster et al.<sup>35</sup> showed that subconjunctival mitomycin was not efficacious in controlling conjunctival inflammation and scarring. They suggested that combined immunoglobulin therapy with methotrexate was more effective at treating ocular cicatricial pemphigoid. In addition, we could not reach a definitive conclusion to suggest that mitomycin resistance of fibroblasts usually occurred because of the ability of cells to escape apoptosis. At least two major compartments, including fibroblasts and inflammatory cells, are part of the process of ocular scarring (e.g., autoimmune scarring diseases, wound healing). Thus, the termination process of tissue damage to form scar tissue is, in part, dependent on the role of immune cells, including inflammatory response, cytokines, and tissue growth factors, at the local site. Taken together with those of previous reports, our results demonstrate that the apoptosis of fibroblasts tends to be beneficial only in limited conditions of ocular tissue scarring, including intraoperative and postoperative topical application, subconjunctival application of ocular cicatricial pemphigoid, and subconjunctival treatment before transplantation of corneal epithelial stem cells, respectively.

In summary, our data represent that mitomycin-C simultaneously induces activation of the extrinsic apoptotic pathway, the Fas/FasL system, and the intrinsic caspase cascades with mitochondrial dysfunction in HTCF culture. Our data indicated that the predominant apoptotic role of mitomycin-C in HTCFs may be attributed to the activation of Fas/FasL-mediated signaling. Two pieces of evidence support this. First, along with increased Fas/FasL expression, blocking of Fas protein and mRNA by neutralizing anti-Fas antibody or antisense of *Fas* significantly inhibited the cytotoxicity of mitomycin-C, even though we could not observe the catalytic activation of caspase-8. Second, decreased cytosolic expression of truncated Bid by mitomycin-C might have contributed to the disruption of mitochondrial membrane potential, indicating that mitochondrial dysfunction occurred. Findings from Kim et al.<sup>22</sup> showing that pharmacologic inhibition of caspase-8 significantly decreased LDH activity in mitomycin-treated-HTCFs support this hypothesis.

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