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Effect of mitomycin on normal dermal fibroblast and HaCat cell: an in vitro study

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Abstract: Objective: To evaluate the effects of mitomycin on the growth of human dermal fibroblast and immortalized human keratinocyte line (HaCat cell), particularly the effect of mitomycin on intracellular messenger RNA (mRNA) synthesis of collagen and growth factors of fibroblast. Methods: The normal dermal fibroblast and HaCat cell were cultured in vitro. Cell cultures were exposed to 0.4 and 0.04 mg/ml of mitomycin solution, and serum-free culture medium was used as control. The cellular morphology change, growth characteristics, cell proliferation, and apoptosis were observed at different intervals. For the fibroblasts, the mRNA expression changes of transforming growth factor (*TGF*)-*β1*, basic fibroblast growth factor (*bFGF*), *procollagen I,* and *III* were detected by reverse transcription polymerase chain reaction (RT-PCR). Results: The cultured normal human skin fibroblast and HaCat cell grew exponentially. A 5-min exposure to mitomycin at either 0.4 or 0.04 mg/ml caused marked dose-dependent cell proliferation inhibition on both fibroblasts and HaCat cells. Cell morphology changed, cell density decreased, and the growth curves were without an exponential phase. The fibroblast proliferated on the 5th day after the 5-min exposure of mitomycin at 0.04 mg/ml. Meanwhile, 5-min application of mitomycin at either 0.04 or 0.4 mg/ml induced fibroblast apoptosis but not necrosis. The apoptosis rate of the fibroblast increased with a higher concentration of mytomycin ($p<0.05$). A 5-min exposure to mitomycin at 0.4 mg/ml resulted in a marked decrease in the mRNA production of *TGF-β1, procollagen I and III*, and a marked increase in the mRNA production of *bFGF*. Conclusions: Mitomycin can inhibit fibroblast proliferation, induce fibroblast apoptosis, and regulate intracellular protein expression on mRNA levels. In addition, mitomycin can inhibit HaCat cell proliferation, so epithelial cell needs more protecting to avoid mitomycin's side effect when it is applied clinically.

Key words: Mitomycin, Fibroblast, HaCat cell, Apoptosis, TGF-β1, bFGF, Procollagen
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1 Introduction

Extensive postsurgical fibrosis and scaring, such as laryngeal stenosis, adhesion and stricture of nasal cavity, restenosis or atresia of external ear canal, have long presented a challenge to otolaryngologist. To take external ear canal stenosis for example, stents and skin grafts are used at the time of primary canaloplasty; surgical debridement, silver nitrate cauterization, corticosteroid injection and topical and systemic medications are used in the postoperative period. Despite these efforts, recalcitrant granulation tissue formation and resultant canal and mastoid cavity occlusion may require revision surgery. A nonsurgical method for eliminating or reducing such unwanted granulation tissue growth and thereby allowing external ear canal epithelialization warrants further consideration (Banthia and Selesnick, 2003).

Mitomycin is a kind of antineoplastic drug that

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can cause cross-linking of DNA and inhibit RNA and protein synthesis. When used topically, it can inhibit fibroblast proliferation, but the exact mechanism is not quite clear. Since its success in maintaining trabecular patency in glaucoma surgery (Tahery and Lee, 1989), the topical application of mitomycin has surged in popularity among otolaryngologist in order to: (1) prevent aerodigestive tract and external ear restenosis; (2) maintain the patency of tympanic membrane, choana, and laryngotrachea, and (3) avoid the recurrence of head and neck Keloids after surgery (Prasad *et al.*, 2002; Rahbar *et al.*, 2002; Battelino *et al.*, 2005; Ragab, 2005; Simpson and James, 2006; Stewart and Kim, 2006).

Wound healing is a complicated process with overlapping mechanisms. Epithelium and fibroblasts are the most important cells in this process. Growth factors contribute to wound healing by modulating epithelielization, angiogenesis, and collagen metabolism. Basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-β1 are two major growth factors which affect wound healing directly. Fibrosis and scaring are characterized by the excessive deposit of extracellular matrix, particularly the deposit of collagen. Collagen types I and III play a major role in wound healing and scar formation. By manipulating growth factors and modulating extracellular matrix expression by modulators, such as mitomycin, it may be possible to modify the wound healing process in different clinical states to achieve the desired healing response.

To date, there have been no comparative studies on the effects of mitomycin as a modulator on dermal fibroblasts and epithelium cells cultured in vitro. Furthermore, the study of mitomycin's effect on the messenger RNA (mRNA) synthesis of growth factors and collagen of cultured dermal fibroblast is sparse. The purpose of this study is to understand the growth characteristics of dermal fibroblasts and human keratinocyte line (HaCat) cells in vitro, and analyze the mRNA expression of growth factor and collagen of fibroblasts exposed to mitomycin.

2 Materials and methods

2.1 Establishing fibroblast cell lines

Normal dermal fibroblast cell lines were acquired from facial skin which would be discarded in operation. The use of operative specimens was approved by the Human Subjects Committee of Central South University. The dermal specimens were cut into approximately 1 mm^3 fragments on a dish. Dulbecco's phosphate-buffered saline (PBS) solution with 5% (50 g/L) penicillin was then used to wash the fragments. The specimens were then placed in 75 cm^2 tissue culture flasks (Falcon) with 10 ml Dulbecco's modified Eagle's media (DMEM) in which there was 10% fetal bovine serum (Gibco) and 1% L-glutamine (Gibco). The flasks were incubated in humidified 5% CO_2 atmosphere at 37 °C. After 48 h, the culture media was changed. The culture media should be changed every 3 d before fibroblasts were growing out of the skin fragments. Then the skin fragments were removed, the cells were subcultured into 75 cm^2 culture flasks, changing culture media every few days.

HaCat cell lines came from the China Center for Type Culture Collection (Wuhan, China).

Fibroblasts cells in the second to seventh passage were used for experiments. PBS was used to washed cells, 0.05% (0.5 g/L) trypsin was used to release cells from flasks, and DMEM with 10% fetal bovine serum was used to inactivate the trypsin.

2.2 Cell counts and growth curve

Cells were seeded in a 24-well plate, with approximately 1 ml (~2×10^4) cells in each well. Twenty-four hours later, when the fibroblasts and HaCat cells attached to the walls of the wells, the experiment began. Mitomycin (Zhejiang Hisun Pharmaceutical Co., Ltd.) was dissolved in DMEM without fetal bovine serum, yielding different concentrations of mitomycin (0.4 and 0.04 mg/ml). In the treatment wells, these different concentrations of mitomycin were added to the wells and left in for 5 min. In contrast, the control wells were filled with DMEM for 5 min. The wells were then rinsed with PBS and filled with culture media. Cell counts were performed in triplicate on Days 1, 3, 5, 7, 9, and 11. Growth curves of the fibroblasts and HaCat cells were then made. Cell morphology was observed by inverted microscope.

2.3 MTT assay

About 2×10^4 cells were seeded in each well of a 96-well plate (Falcon) in a volume of 100 μl per well.

Twenty-four hours later, when the fibroblasts and HaCat cells had attached to the walls of the wells, the experiments began. In the treatment wells, mitomycin at 0.04 or 0.4 mg/ml was applied to cells for 5 min. Then the wells were rinsed with PBS and filled with DMEM which contained 10% fetal bovine serum. Cell inhibition rate was calculated in triplicate using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on Days 1, 3, 5, 7, and 10. And there were blank controls. Assays read using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad).

2.4 Acridine orange (AO)/propidium iodide (PI) staining

About 2×10^4 cells fibroblasts were seeded in each well of a 24-well plate (Falcon) in a volume of 11 ml per well. Twenty-four hours later, when the fibroblasts had attached to the walls of the wells, the experiments began. In the treatment wells, mitomycin at 0.04 or 0.4 mg/ml was applied to cells for 5 min. In contrast, the control wells were exposed to DMEM for 5 min. After 5 min, the wells were rinsed with PBS and filled with DMEM which contained 10% fetal bovine serum. On Days 1, 3, 5, and 7, the cultural medium was discarded. Then 20 ng/ml acridine orange (AO, Sigma) and 40 ng/ml propidium iodide (PI, Sigma) were filled in each well in a volume of 100 μl separately and stained for 10 min. Fluorescence microscope was used to observe fibroblasts' and HaCat cells' morphology change and count fibroblasts apoptosis rate.

2.5 Isolation of total RNA

Fibroblasts were seeded at a density of approximately 2×10^4 cells in 6 cm^2 cultural discs in a volume of 5 ml. Experiments began after fibroblasts were nearly overgrown. Mitomycin at 0.4 mg/ml was applied to cells for 5 min. After 5 min, the wells were rinsed with PBS and filled with DMEM which contained 10% fetal bovine serum. Total cellular RNA was isolated by extraction in Trizol (Sigma, St. Louis, MO, USA), and after low speed centrifugation to remove cellular debris, the RNA was pelleted through a cesium chloride gradient. After a series of ethanol precipitation, the concentrations of total RNA were measured spectophotometrically at 260 nm and the purity was detected with a 260/280 optical density (OD) spectrophotometer readings. Total RNA was size-fractionated on 1.0% (10 g/L) agarose gels, and stained with ethidium bromide for verification of RNA integrity and loading equivalency.

2.6 Complementary DNA (cDNA) synthesis

Samples containing 2 μg of total RNA were denatured in distilled water for 10 min at 70 °C and then placed on ice. Two μl 10 mmol/L deoxyribonucleoside triphosphate (dNTP) (Promega, Fitchburg, WI), 4 μl 5× reaction buffer (Promega), 1 μl Ribolock™ ribonuclease transcriptase (Promega), 1 μl ribonuclease inhibitor (Promega), 1 μl oligo(dT_{18}) primer (Promega) and RNase-free ddH_2O (Promega) were added up to a final volume of 20 μl. The mixture was incubated at 42 °C for 1 h, then 70 °C for 10 min. Before amplification by polymerase chain reaction (PCR), the samples were stored at −20 °C.

2.7 Primer selection

For amplification of *TGF-β1*, *bFGF*, *procollagen I*, *III*, and *β-actin*, the following primers were employed separately:

sense: 5′-agcacagagcctcgccttt-3′; anti-sense: 5′-agggtgaggatgcctctctt-3′.

sense: 5′-agacacccatccgtgaacc-3′; anti-senses: 5′-cttgatgtgagggtcgctct-3′.

sense: 5′-aagaggaaggccaagtcgag-3′; anti-senses: 5′-ataagacagctggggagcaa-3′.

sense: 5′-gcagggaacaacttgatggt-3′; anti-senses: 5′-taggagcagttggaggctgt-3′.

sense: 5′-gtacctgaacccgtgttgct-3′; anti-senses: 5′-gtccttgcggaagtcaatgt-3′.

All oligonucleotide primers were designed by the software of Primer 3 (ABI, USA) and synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. The expected sizes for the *TGF-β1*, *bFGF*, *procollagen I*, *III*, and *β-actin* resulting products were 258, 438, 428, 370, and 486 bp, respectively.

2.8 PCR

PCR mixtures were prepared containing the appropriate set of primers 0.4 μl sense and 0.4 μl antisense, 3.0 μl sample cDNA, 1.0 μl 10× long and accurate (LA) PCR buffer II (TaKaRa Bio. Inc., Tokyo, Japan), 0.2 μl dNTP mixture (TaKaRa), 0.2 μl LA Taq (TaKaRa), and ddH_2O was added up to a final

volume of 10 μl. The tubes were placed in a thermocycler (GeneAmp 9600 PCR system) to start the PCR reaction.

Reaction conditions for *β-actin*, *TGF-β1*, and *procollagen III*: denaturation: 95 °C for 5 min on initial cycle, 30 s on the rest; annealing: 58 °C for 30 s; extension: 72 °C for 1 min, 10 min on last cycle; number of cycles: 25.

Reaction conditions for *bFGF* and *procollagen I*: denaturation: 95 °C for 5 min on initial cycle, 30 s on the rest; annealing: 56 °C for 30 s; extension: 72 °C for 1 min, 10 min on last cycle; number of cycles: 30.

2.9 Analysis and quantification of the PCR products

The PCR products were run on a 1.5% agarose gel containing ethidium bromide. Gels were photographed using an electronic camera connected to a computerized Image Quant program (Bio-Rad, Marnes la Coquette, France) and the intensities of the PCR products were scanned and quantified. The densities of *TGF-β1*, *bFGF*, *procollagen I*, and *III* mRNA bands were divided by that of *β-actin*.

2.10 Statistical analysis

Results are expressed as means, using SPSS. Statistical significance was determined using *t*-test and analysis of variance (ANOVA) test. Differences were considered statistically significant at a value of $p<0.05$.

3 Results

The microscopic analysis of control cells showed a denser and more organized cellular pattern compared with the treated cells, which gradually lost their regular morphology, organization pattern, and density. The morphology change was especially obvious when the cells were exposed to 0.4 mg/ml of mitomycin (Figs. 1 and 2).

In the control group, fibroblasts and HaCat cells survived and replicated during the 10-d period of study. They roughly demonstrated exponential proliferation. Comparisons were made between the control group and the treatment group, which was treated with mitomycin for 5 min. The population of fibroblasts exposed to 0.4 mg/ml of mitomycin for 5 min declined over the 10-d period (Fig. 3). Fibroblasts exposed to 0.04 mg/ml of mitomycin for 5 min showed a decline in population over the initial 5 d period, followed by slight proliferation over Days 5–10 after the exposure (Fig. 3). HaCat cell exposed to both 0.4 and 0.04 mg/ml of mitomycin for 5 min showed a dramatic decline in population during the 10-d period (Fig. 4).

MTT assay of fibroblasts and HaCat cells showed that for fibroblast, after the exposure to 0.4 mg/ml of mitomycin, the inhibition rate rose gradually day after day, but after the exposure to 0.04 mg/ml of mitomycin, the inhibition rate rose in the first 5 d then declined (Fig. 5). The inhibition rate

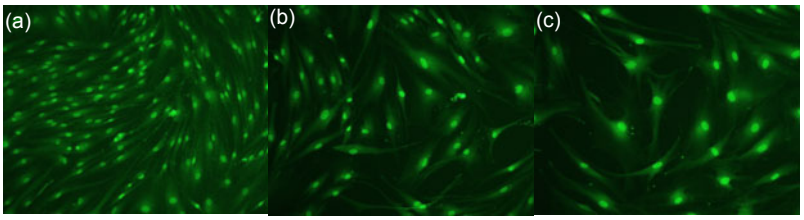

Fig. 1 Microscopic analyses of control and treated fibroblasts stained by AO
(a) Control on Day 3; (b) Mitomycin 0.04 mg/ml treated on Day 3; (c) Mitomycin 0.4 mg/ml treated on Day 3

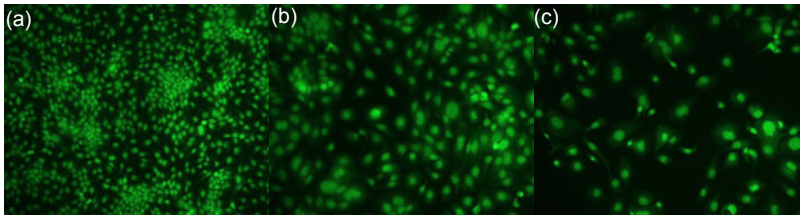

Fig. 2 Microscopic analyses of control and treated HaCat cell stained by AO
(a) Control on Day 3; (b) Mitomycin 0.04 mg/ml treated on Day 3; (c) Mitomycin 0.4 mg/ml treated on Day 3

in the 0.4 mg/ml group was significantly higher than that in 0.04 mg/ml (p<0.05). For HaCat cells, the inhibition rate in the 0.4 mg/ml group was higher than that in 0.04 mg/ml group, but there was no significant difference between different concentrations of mitomycin (p>0.05) (Fig. 6).

AO/PI staining showed that exposure to mitomycin for 5 min could induce fibroblast apoptosis (Fig. 7). The change trend of fibrosis apoptosis rate is the same as that of the fibrosis inhibition rate (Fig. 8).

As shown in Fig. 9, the mRNA expression of *TGF-β1* decreased gradually after fibroblasts were exposed to 0.4 mg/ml of mitomycin and the mRNA expressions of *procollagen I* and *III* showed a similar trend (Figs. 10 and 11). In contrast, the mRNA expression of *bFGF* increased day by day (Fig. 12).

4 Discussion

The most common concentrations of mitomycin typically used in clinical practice are 0.4 and 0.04 mg/ml, so we chose these concentrations to apply on fibroblasts and HaCat cells. One study showed that mitomycin at 0.1 mg/ml for 5 min suppressed the proliferation of certain Keloid fibroblast cell lines in

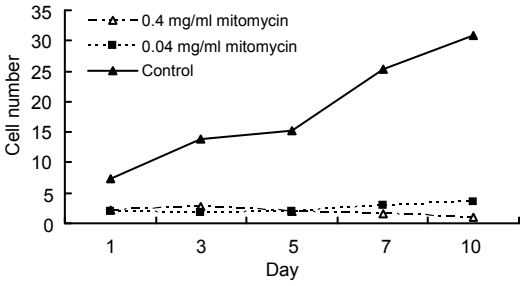

Fig. 3 Growth curve of fibroblasts after mitomycin exposure

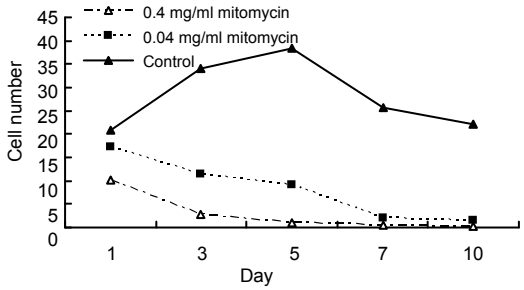

Fig. 4 Growth curve of HaCat cells after mitomycin exposure

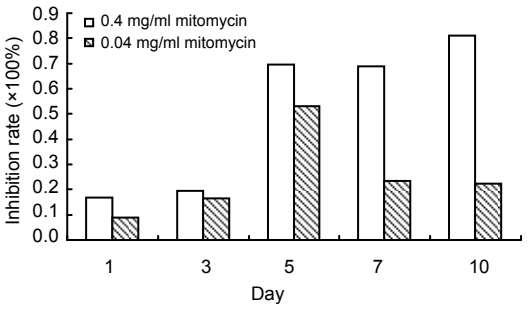

Fig. 5 Inhibition rate of fibroblast after the exposure to mitomycin

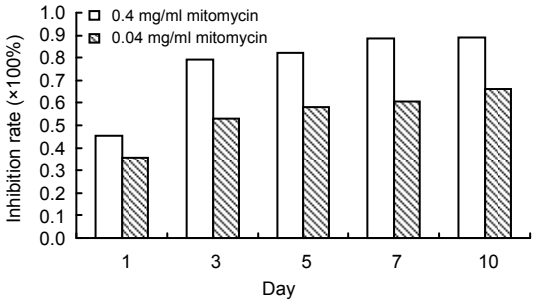

Fig. 6 Inhibition rate of HaCat cell after the exposure to mitomycin

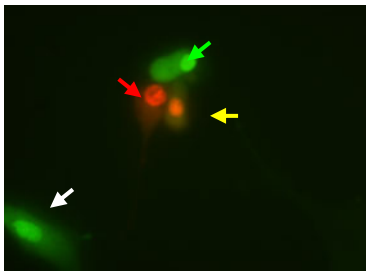

Fig. 7 Different apoptosis stage of fibrosis after the exposure to mitomycin

The cell white arrow pointed was normal; the cell green arrow pointed was in the early stage of apoptosis; the cell yellow arrow pointed was in the middle stage; the cell red arrow pointed was in the late stage

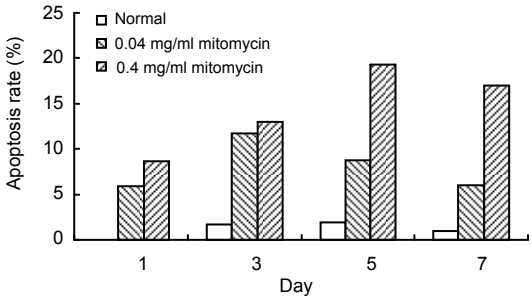

Fig. 8 Apoptosis rate of fibroblast after mitomycin application

Apoptosis rates of fibroblasts exposed to mitomycin were much higher than the control (p<0.05). However, there was no statistically significant difference of apoptosis rate between 0.4 and 0.04 mg/ml groups

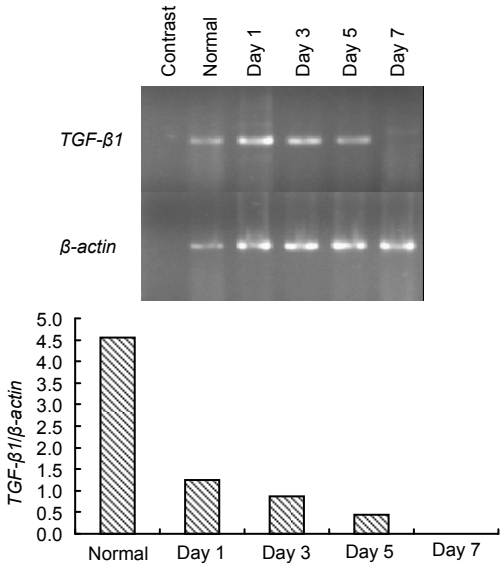

Fig. 9 Analyses of *TGF-β1* mRNA expression and *β-actin* using RT-PCR
Each mRNA value is corrected for *β-actin* mRNA value (*TGF-β1*/*β-actin* mRNA ratio)

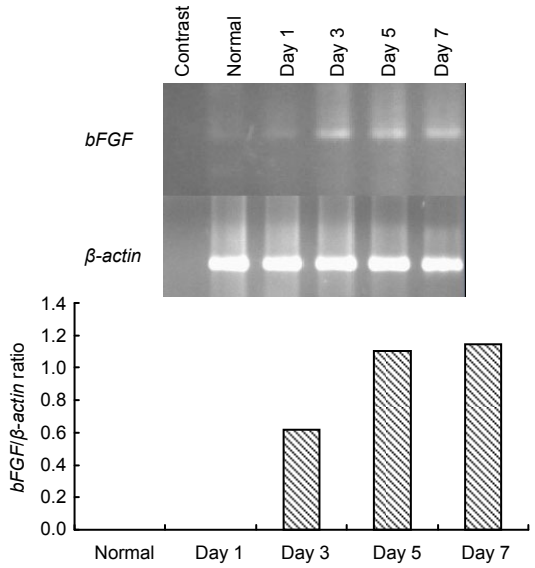

Fig. 10 Analyses of *bFGF* mRNA expression and *β-actin* using RT-PCR
Each mRNA value is corrected for *β-actin* mRNA value (*bFGF*/*β-actin* mRNA ratio)

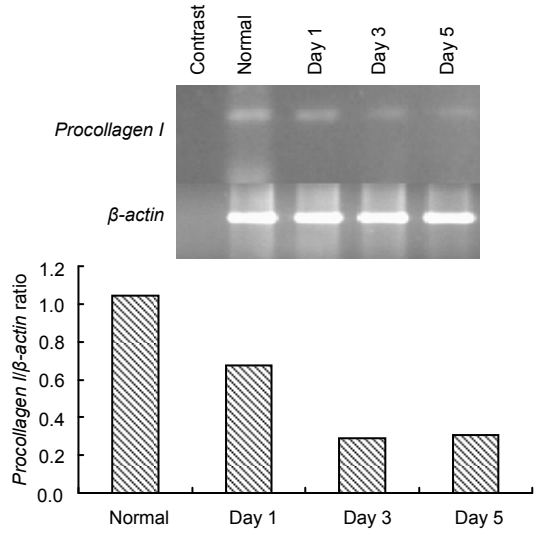

Fig. 11 Analyses of *procollagen I* mRNA expression and *β-actin* using RT-PCR
Each mRNA value is corrected for *β-actin* mRNA value (*procollagen I*/*β-actin* mRNA ratio)

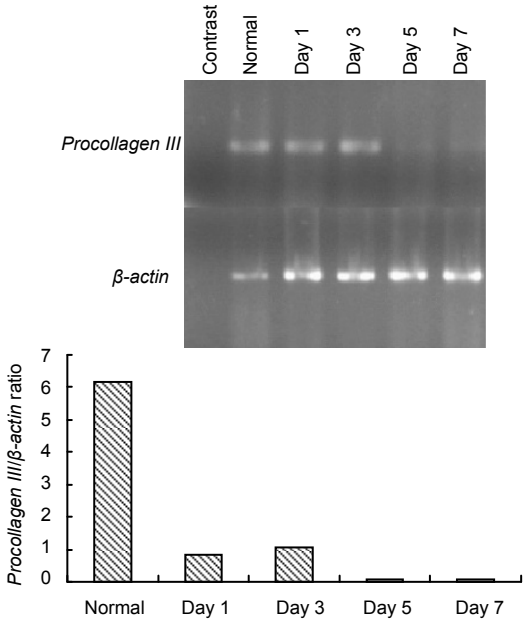

Fig. 12 Analyses of *procollagen III* mRNA expression and *β-actin* using RT-PCR
Each mRNA value is corrected for *β-actin* mRNA value (*procollagen III*/*β-actin* mRNA ratio)

vitro for a period of three weeks (Simman *et al.*, 2003). Recently, Chen *et al*. (2006) showed that after human normal dermal fibroblasts were exposed to either 0.4 or 0.04 mg/ml mitomycin, the proliferation of fibroblasts decreased in a period of 7 d. The results of our study are consistent with these findings. Compared with the control group, after a single 5-min exposure to either 0.4 or 0.04 mg/ml of mitomycin, fibroblast proliferation was significantly depressed, apoptosis rate increased, and cells gradually lost their

regular shape, organization pattern and density. In our study, the antiproliferative effect of 0.4 mg/ml mitomycin on fibroblasts was stronger and longer than that of 0.04 mg/ml.

Apoptosis is an active process that plays a pivotal role in the normal physiological turnover of cells and in various pathological processes. It is characterized by dramatic cellular alteration, particularly membrane blebbing, cell shrinkage, chromosome condensation, and DNA fragmentation (Schwartzman and Cidlowski, 1993). AO and PI served as fluorescent markers for the simultaneous visualization of both live and dead cells. Because AO is a weak base that readily enters living cells, at the low dye concentrations used here, AO caused live fibroblasts to fluoresce green. PI is an analog of ethidium bromide, which selectively binds to nucleic acids. It functions as an exclusion dye entails that it cannot penetrate living cells but rather readily enters dead or dying cells. Once PI penetrates through the cell membrane, it binds to nucleic acids and causes them to fluoresce bright red (Bank, 1988). Therefore, AO/PI double staining can differentiate vital cells (VC), apoptotic cells (AC) and necrotic cells (NC). VC's nuclear acids is normal and fluoresces green (Fig. 7); AC's nuclear acid is condensed or fractured, and fluoresces green or orange in the early or middle stage and red in the late stage (Fig. 7); NC's nuclear acid is normal and fluoresces red.

Mitomycin can induce cell apoptosis which has been reported in other studies. Kim *et al*. (2003; 2004) described that mitomycin together with ethanol could cause human cornea fibroblast apoptosis. In another study, after exposure to 0.4 mg/ml mitomycin, the human Tenon's fibroblasts showed typical apoptotic morphology change (Seong *et al.*, 2005), such as chromosome condensation and DNA fracture. Similar results were reported in the study of Crowston *et al.* (2002). The results of our study are consistent with these findings. We observed that 5 min exposure of normal human skin fibroblast to mitomycin in concentrations of 0.4 and 0.04 mg/ml could induce cell death via apoptosis but not necrosis, in a dose- and time-dependent manner.

HaCat cells are immortalized human keratinocytes. They have similar growth characteristics with normal human skin keratinocytes. The results from our experiments showed that mitomycin has a significantly stronger antiproliferative effect on HaCat cells than on fibroblasts, but there was no difference between 0.4 and 0.04 mg/ml.

Given this, we believed that 0.4 mg/ml of mitomycin would be more appropriate and effective than 0.04 mg/ml to inhibit fibroblasts proliferation. However, skin keratinocytes needed more protection.

Multiple studies have examined mitomycin effect on growth factor production, often with conflicting results.

TGF-β1 is a 2.5 kDa polypeptide, which has influence on cell cycle progression, extracellular matrix production, and granulation tissue formation (Moses *et al.*, 1990). TGF-β1 can increase collagen production in Keloid dermal fibroblasts in vitro (Peltonen *et al.*, 1991; Kikuchi *et al.*, 1995). Alternatively, anti-TGF-β1 antibodies can decrease collagen production (Younai *et al.*, 1994).

bFGF is a kind of single chain peptide, which is known for its mitogenic, chemoattractant, regulatory, and angiogenic abilities (Kikuchi *et al.*, 1995). In specific fibroblast tissue cultures, bFGF can promote mitosis, increase cell survival rate, inhibit collagen synthesis, and stabilize cell phenotype (Hong *et al.*, 1999). The application of bFGF to Keloid fibroblasts cultured in vitro can inhibit the excessive expression of collagen I (Gospodarowicz, 1991).

A study showed that after the application of mitomycin to human Tenon's capsule fibroblasts, the synthesis of TGF-β1 and bFGF initially increased as compared to the control group, but decreased 48 h later (Occleston *et al.*, 1997). Another study showed that the level of secreted TGF-β1 and bFGF of human normal fibroblasts rose in 5 d after mitomycin's application in a serum-free culture medium (Chen *et al.*, 2006). Our study's emphasis was on the effects of mitomycin on mRNA synthesis of *TGF-β1* and *bFGF*. After a 5-min exposure to 0.4 mg/ml mitomycin, the mRNA expression of *TGF-β1* decreased gradually and the mRNA expression of *bFGF* increased day after day. These results are inconsistent with the studies mentioned above. The changes of *TGF-β1* and *bFGF* mRNA levels were favorable to the inhibition effect of mitomycin to fibroblasts' collagen sysnthesis.

In our RT-PCR tests, similar to *TGF-β1*, the expressions of *procollagen I* and *III* were down-regulated at the mRNA level after a 5-min application of 0.4 mg/ml mitomycin. This was consistent with a

six-week animal study, in which the level of tissue procollagen mRNA was down-regulated by mitomycin which was applied locally or injected subcutaneously (Gray *et al.*, 2003). Mitomycin inhibited collagen I and fibronectin's synthesis of human Tenon's capsule fibroblasts (Occleston *et al.*, 1997). Ferguson *et al*. (2005) showed that mitomycin decreased the mouse skin collagen and fibronectin's expression eight weeks after its application.

5 Conclusions

In summary, a 5-min exposure to 0.4 or 0.04 mg/ml of mitomycin resulted in inhibition of fibroblast proliferation, and 0.4 mg/ml might be better than 0.04 mg/ml for mitomin's clinical application. Due to the same inhibition effect of mitomycin on HaCat cells, kerotinocyte requires more protection when mitomycin used in clinic. A 5-min exposure to 0.4 or 0.04 mg/ml of mitomycin induced fibroblast apoptosis rather than necrosis. A concentration of 0.4 mg/ml of mitomycin which was applied to human skin fibroblast could down-regulate *TGF-β1*, *procollagen I,* and *III* expressions and up-regulate *bFGF* expression on mRNA levels. This observation may lead to the use of mitomycin to prevent fibrosis and scar formation. Further studies assessing the side effects of mitomycin are warranted before it can be considered in clinical practice.

References

Bank, H.L., 1988. Rapid assessment of islet viability with acridine orange and propidium iodide. *In Vitro Cel. Dev. Biol.*, **24**(4):266-269. [doi:10.1007/BF02628826]

Banthia, V., Selesnick, S.H., 2003. Mitomycin-C in the Post Surgical Ear Canal. American Academy of Otolaryngology/Head and Neck Surgery. Annual Meeting, Orlando, Florida, USA, p.882-886.

Battelino, S., Hocevar-Boltezar, I., Zargi, M., 2005. Intraoperative use of mitomycin C in fibrous atresia of the external auditory canal. *Ear Nose Throat J.*, **84**(12): 776-779.

Chen, T., Kunnavatana, S.S., Koch, R.J., 2006. Effects of mitomycin-C on normal dermal fibroblasts. *Laryngoscope*, **116**(4):514-517. [doi:10.1097/01.MLG.0000205590.62824.0A]

Crowston, J.G., Chang, L.H., Constable, P.H., Daniels, J.T., Akhar, A.N., Khaw, P.T., 2002. Apoptosis gene expression and death receptor signaling in mitomycin C-treated human Tenon capsule fibroblasts. *Invest. Ophthalmol. Vis. Sci.*, **43**(3):692-699.

Ferguson, B., Gray, S.D., Thibeault, S., 2005. Time and dose effects of mitomycin C on extracellular matrix fibroblasts and proteins. *Laryngoscope*, **115**(1):110-115. [doi:10.1097/01.mlg.0000150694.08259.80]

Gospodarowicz, D., 1991. Biological activities of fibroblast growth factors. *Ann. N. Y. Acad. Sci.*, **638**:1-8. [doi:10.1111/j.1749-6632.1991.tb49012.x]

Gray, S.D., Tritle, N., Li, W., 2003. The effect of mitomycin on extracellular matrix proteins in a rat wound model. *Laryngoscope*, **113**(2):237-242. [doi:10.1097/00005537-200302000-00008]

Hong, R., Lum, J., Koch, R.J., 1999. Growth of keloid-producing fibroblasts in commercially available serum-free media: a comparative study. *Otolaryngol. Head Neck Surg.*, **121**(4):469-473. [doi:10.1016/S0194-5998(99)70239-1]

Kikuchi, K., Kakano, T., Takehara, K., 1995. Effects of various growth factors and histamine on cultured keloid fibroblasts. *Dermatology*, **190**(1):4-8. [doi:10.1159/000246625]

Kim, T.I., Tchah, H., Lee, S., Cho, B.J., Kook, M.S., 2003. Apoptosis in keratocytes caused by mitomycin C. *Invest. Ophthalmol. Vis. Sci.*, **44**(5):1912-1917. [doi:10.1167/iovs.02-0977]

Kim, T.I., Tchah, H., Cho, E.H., Kook, M.S., 2004. Evaluation of safety of cultured corneal fibroblasts with cotreatment of alcohol and mitomycin C. *Invest. Ophthalmol. Vis. Sci.*, **45**(1):86-92. [doi:10.1167/iovs.03-0520]

Moses, H.L., Yang, E.Y., Peitenpol, J.A., 1990. TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell*, **63**(2):245-257. [doi:10.1016/0092-8674(90)90155-8]

Occleston, N.L., Daniels, J.T., Tarnuzzer, R.W., Sethi, K.K., Alexander, R.A., Bhattacharya, S.S., Schultz, G.S., Khaw, P.T., 1997. Single exposures to antiproliferatives: long-term effects on ocular fibroblasts wound-healing behavior. *Invest. Ophthalmol. Vis. Sci.*, **38**(10):1998-2007.

Peltonen, J., Hsiao, L.L., Jaakkola, S., Sollberg, S., Aumailley, M., Timpl, R., Chu, M.L., Uitto, J., 1991. Activation of collagen gene expression in keloids: co-localization of type I and VI collagen and transforming growth factor-β1 mRNA. *J. Invest. Dermatol.*, **97**(2):240-248. [doi:10.1111/1523-1747.ep12480289]

Prasad, M., Ward, R.F., April, M.M., Bent, J.P., Froehlich, P., 2002. Topical mitomycin as an adjunct to choanal atresia repair. *Arch. Otolaryngol. Head Neck Surg.*, **128**(4): 398-400.

Ragab, S., 2005. The effect of radiofrequency and mitomycin C on the closure rate of human. tympanostomy. *Otol. Neurotol.*, **26**(3):355-360. [doi:10.1097/01.mao.0000169760.70302.f1]

Rahbar, R., Jones, D.T., Nuss, R.C., Roberson, D.W., Kenna, M.A., McGill, T.J., Healy, G.B., 2002. The role of mitomycin in the prevention and treatment of scar formation in the pediatric aerodigestive tract. *Arch. Otolaryngol. Head Neck Surg.*, **128**(4):401-406.

Schwartzman, R.A., Cidlowski, J.A., 1993. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr. Rev.*, **14**(2):133-151. [doi:10.1210/edrv-14-2-133]

Seong, G.J., Park, C., Kim, C.Y., Hong, Y.J., So, H.S., Kim, S.D., Park, R., 2005. Mitomycin-C induced the apoptosis of human Tenon's capsule fibroblast by activation of c-Jun N-terminal kinase 1 and caspase-3 protease. *Invest. Ophthalmol. Vis. Sci.*, **46**(10):3545-3552. [doi:10.1167/iovs.04-1358]

Simman, R., Hashim, A., Frances, W., 2003. Effect of mitomycin-C on keloid fibroblasts: an in vitro study. *Ann. Plast. Surg.*, **50**(1):71-76. [doi:10.1097/00000637-200301000-00012]

Simpson, C.B., James, J.C., 2006. The efficacy of mitomycin-C in the treatment of laryngotracheal-stenosis. *Laryngoscope*, **116**(10):1923-1925. [doi:10.1097/01.mlg.0000235934.27964.88]

Stewart, C.E.IV, Kim, J.Y., 2006. Application of mitomycin-C for head and neck keloids. *Otolaryngol. Head Neck Surg.*, **135**(6):946-950. [doi:10.1016/j.otohns.2005.07.026]

Tahery, M.M., Lee, D.A., 1989. Review: pharmacologic control of wound healing in glaucoma filtration surgery. *J. Ocul. Pharmacol.*, **5**(2):155-179. [doi:10.1089/jop.1989.5.155]

Younai, S., Nichter, L.S., Wellisz, T., Reinisch, J., Nimni, M.E., Tuan, T.L., 1994. Modulation of collagen synthesis by transforming growth factor-beta in keloid and hypertrophic scar fibroblasts. *Ann. Plast. Surg.*, **33**(2):148-151. [doi:10.1097/00000637-199408000-00005]