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☐ Original Article ☐

The Effective Concentration and Exposure Time of Mitomycin-C for the Inhibition of Lens Epithelial Cell Proliferation in Rabbit Eyes

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Abstract

The proliferation of residual lens epithelial cells following cataract surgery is assumed to be a major cause of posterior capsular opacification. To assess the efficacy of mitomycin-C in preventing posterior capsular opacification, we determined the effective concentration and exposure time of mitomycin-C in inhibiting rabbit lens epithelial cell proliferation. The fourth-passaged rabbit lens epithelial cells were maintained for one day and then exposed to mitomycin-C for 1, 2, 3, and 5 minutes, respectively. There were 9 different plating concentrations of mitomycin-C with two-fold serial dilution. The maintenance of the phenotypic properties of lens epithelial cells was confirmed by continuous transcription of λ-crystalline mRNA determined by reverse transcription-polymerase chain reaction and the polymorphism of the restriction fragment. Cell proliferation was assayed with ^{3}H-thymidine incorporation into DNA. The fourth-passaged cells maintained the expression of λ-crystalline mRNA, suggesting that they are phenotypically authentic lens epithelial cells. The effective concentrations and exposure time of mitomycin-C were 0.1 mg/ml for 1 minute and 2 minutes, and 0.025 mg/ml for 2 minutes. By these results, we postulated that mitomycin-C at relatively short incubation times could be clinically used for prevention of posterior capsular opacification after cataract surgery.

Key Words: λ-crystalline mRNA, lens epithelial cell, mitomycin-C, posterior capsular opacity

INTRODUCTION

Many ophthalmic surgeons currently favor extracapsular cataract extraction for patients of all ages and not only for the young. The most frequent complications of extracapsular cataract extraction, mainly late opacification of the lens capsule, arise from two frequent complications of extracapsular cataract extraction, mainly late opacification of the lens capsule, arise from two distinct causes: fibrosis of the capsule and proliferation of lens epithelial cells on the capsule. The latter is more common and has been found in 32−50% of cases after 2−5 years follow-up.[1,2] Dense cell proliferation results in low visual acuity due to decreased transmissible light. Slight proliferation, on the other hand, causes glare due to light scattering.

Proliferation, migration and metaplasia to fibroblast of the residual lens epithelial cells primarily cause the pathogenesis of posterior lens capsule opacification when the extracapsular environment changes due to surgery.[3-7]

For prevention of posterior capsular opacification, several surgical methods such as mechanical scraping, vacuum cleaning, wet-field coagulation, cryocoagulation and ultrasonic cleaning at the time of surgery have been performed.[8,9] Intraocular lens design with a convex surface has been used for inhibiting the migration of lens epithelial cells and so preventing posterior capsular opacification.[10] But all attempts have shown limited efficacy. The only therapeutic option currently available for the opacified posterior capsule is to cut the capsule, either surgically or with the neodymium: YAG laser. Such a procedure, however, may sacrifice some of the advantages of preserving an intact capsule. Alternative ways of reducing posterior capsular opacification by using

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antimitotic drugs are being actively investigated.[11,12]

In an attempt to assess the possible efficacy of mitomycin-C in preventing posterior capsular opacification, we examined the effects of different application times and concentrations in inhibiting lens epithelial cell proliferation *in vitro*.

MATERIALS AND METHODS

Albino rabbit lenses were removed from freshly enucleated eyes under sterile conditions. The anterior capsules were isolated and the adherent lens epithelial cells were removed by incubation with 0.25% trypsin (Sigma, St. Louis, MO, USA) at 37°C for five minutes. Trypsinization was then curbed by the addition of Eagle's minimum essential medium (Gibco-BRL, Trand Island, NY, USA) with 100 μg/ml streptomycin, 100 mg/ml penicillin (MEME) and 20% fetal calf serum (Ginco-BRL, Grand Island, NY, USA) (MEME/20), and the cells were transferred to a 25 cm^2 tissue culture flask containing MEME/20. After reaching confluence, the albino rabbit lens epithelial cells (ALE) were trypsinized, resuspended in MEME/20, and transferred to a 75 cm^2 tissue culture flask containing MEME with 10% fetal calf serum. The cells were incubated at 37°C and 5% CO_2 and the medium was changed every 3 to 4 days. Albino rabbit lens epithelial cells were passed every 2 weeks. After 3 passages of albino rabbit lens epithelial cells, ALE was transferred to a 96-well cultured plate, with each well containing 1×10^4 cells.

Confirming the production of λ-crystalline m RNA in cultured cells

Reverse transcriptase-PCR (RT-PCR): The sequence of λ-crystalline m RNA was obtained by a genebank search of the National Institute of Health (NIH). Base sequences from the 78th to the 528th base among a total of 1,424 bases were amplified by RT-PCR. The segment that was used for RT-PCR proved not to be the same fragment in all DNA sequences of mammalians which were registered in the NIH genebank.

RNA was extracted from the cultured cells with acid guanidium thiocyanate. The synthesis of first-strand cDNA was taken by using reverse transcriptase. This was performed by the following methods: 1 μg total RNA was reverse transcribed in 50 mM Tris-HCl (pH 8.8), 6 mM $MgCl_2$, 10 mM DTT at room temperature. All four dNTPs were present at 10 mM concentration, and 5 μg random hexamer (Pharmacia, Upsala, Sweden)was used. Mo-MLV reverse transcriptase (Gibco-BRL, Grand Island, NY, USA) (400 units) was used in a total volume of 100 μl and incubated at 42°C for 1 hour.

Table 1. Primer for λ-crystalline RT/PCR

Primer	Sequence	Strand	Location
LC1	Cggcgatgtgctgatagttg	Sence	78−97
LC2	Gtatgatgtgggcgaccac	Antisence	509−528

LC, λ-crystalline.

PCR was then performed in a total volume of 100 μl containing template DNA [typically 3 μl of reverse transcription reaction <cDNA products derived form 2 μg RNA>, 500 ng λ-crystalline (LC) 1 and LC2 primer (Korea Biotech, Taejon, Korea) (Table 1), 100 mol/L each dNTP (Pharmacia, Upsala, Sweden), 2 U *Taq DNA polymerase* (Promega, Madison, WI, USA) and *Taq polymerase* buffer (1.5 mmol/L mgCl2, Promega)]. PCR reactions were performed using a DNA Thermal Cycler (Perkin Elmer-Cetus Corporation, Emerville, NY, USA). Forty cycles of PCR (denaturation at 94°C for 1 minute, annealing at the appropriate temperature for 1 minute, and extension at 72°C for 1 minute) were then followed by a final elongation step at 72°C for 4 minutes. Templates for amplification were initially denatured at 99°C for 15 minutes, followed by 2 minutes at 94°C. At this point, dNTPs and *Taq DNA polymerase* were added. PCR products were visualized by UV transillumination of ethidium bromide-stained 2% agarose gels.

Restriction mapping of RT-PCR products: Confirming c DNA to be the same as the original DNA, RT-PCR products were treated with a restriction enzyme such as *Pst I* (Boehringer Mannheim, Mannheim, Germany) and *Tag I* (Boehringer Mannheim, Mannheim, Germany) (Table 2). The enzyme-treated products were visualized by UV transillumination of ethidium bromide stained 3% agarose gel.

DNA synthesis analysis

The third-passaged lens epithelial cells were pre-

Table 2. Restriction Enzyme for λ-crystalline mRNA PCR Products

Restriction enzyme	Recognition sites	Position with DNA fragment	Size of expected fragment (bp)
Pst I	CA/CT	153	153,298
Tag I	T/CGA	279,221	279,42,130

pared in a 96-well culture plate as described. On day 2, the cells were treated with mitomycin-C (Sigma, ST. Louis, MO, USA) at 5 different concentrations with two-fold serial dilution (0.1, 0.05, 0.025, 0.0125, and 0.01 mg/ml) 24 hours prior to the addition of [methyl-^{3}H]-thymidine (specific activity: 6.7 Ci/mmol. NEN, Boston, MA, USA). For mitomycin-C treatment, the cells were treated with different concentrations of MMC for 1 and 2 minutes under light. And then mitomycin-C was then removed from the media with culture media 3 times. Control groups were treated with culture media the same as MMC treated groups. The cultures were pulsed with ^{3}H- thymidine and incubated at 37°C and 5% CO_2. After 18 hours, the cells were fixed with methanol and radioactive ^{3}H-thymidine incorporation was determined by scintillation count. Significant inhibition was defined as a decrease of more than 50% of the count per minute (CPM) value compared to the control. Repeated MMC treatment was taken for longer treated times (3 and 4 minutes) with different concentrations (0.125, 0.00625, 0.00315, 0.0015625, and 0.001 mg/ml), which might be ineffective for the inhibition of cell proliferation for 1 or 2 minutes.

RESULTS

Characteristics of cultured lens epithelial cells

λ-crystalline is a manor protein of the rabbit lens. λ-crystalline RT-PCR products were shown to be 451 bp, *Pst I* treated products were shown to be 153 and 298 bp and *Tag I* treated products were shown to be 130 and 299 bp (Fig. 1). This electrophoresis result was exactly the same as our expectation. It indirectly proved that the origins of cultured cells were rabbit lens epithelial cells by the continued transcription of λ-crystalline mRNA with a reverse-transcript polymerase chain reaction and restriction fragment length polymorphism.

Table 3. [^{3}H]-thymidine Uptake Proliferation Assay

Concentration (mg/ml)	CPM ratio			
	1 minute	2 minutes	3 minutes	5 minutes
0.1	0.206	0.285		
0.05	0.631	0.386		
0.025	0.606	0.459		
0.0125	0.876	0.799	0.574	0.604
0.01	0.892	0.776		
0.00625		1.028	0.642	0.705
0.003125			0.687	0.803
0.0015625			0.741	
0.001			0.856	

CPM (count/minute) ratio: CPM-MMC/CPM-Control.

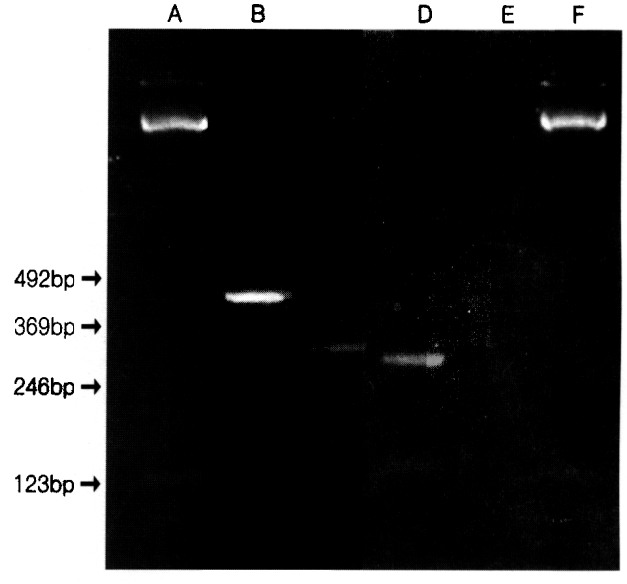

Fig. 1. Electrophoresis on agarose gel (3%) of 451 bp of λ -crystalline RT-PCR products and restriction fragment. Lane A: 123 bp ladder, Lane B: 451 bp of λ -crystalline RT-PCR products, Lane C: Pst I-treated products, Lane D: Tag I-treated products, Lane E: control, Lane F: 123 bp ladder.

Minimum effective concentration and application time of mitomycin-C

The effect of mitomycin-C on the lens epithelial cells was determined by measuring DNA synthesis. Significant inhibition of lens epithelial cell pro-

liferation was defined as DNA synthesis of less than 50% of control groups. A significant decrease of lens epithelial cell proliferation was achieved when mitomycin-C, 0.1 mg/ml was applied for 1 and 2 minutes, and 0.025 mg/ml for 2 minutes (Table 3). Although no additional significant inhibition of cell proliferation was shown for 3 and 5 minutes, more suppression of cell proliferation was revealed with increased times and drug concentrations (Fig. 2, 3). When mitomycin-C was included in the cell culture, DNA synthesis was inhibited in a dose and time dependent manner.

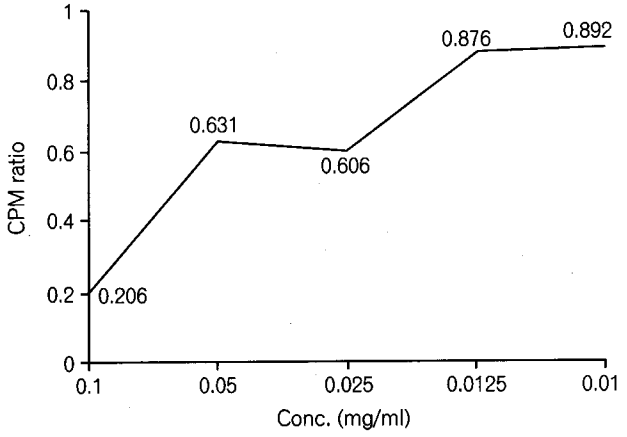

Fig. 2. Effect of mitomycin-C on DNA synthesis in rabbit lens epithelial cells: Confluent cultures of ALE were incubated in MEME containing 20% fetal calf serum, 5 Ci/ml ^{3}H-thymidine after treatment with mitomycin-C for 1 minute at each concentration. Inset shows significantly decreased DNA synthesis at 0.1 mg/ml (CPM (count/minute) ratio: CPM-MMC/ CPM-Control).

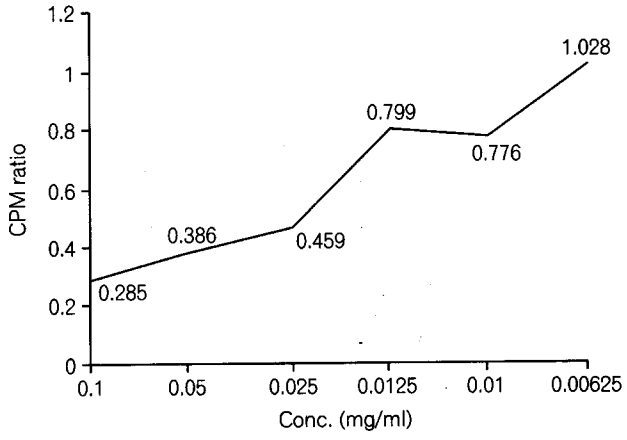

Fig. 3. Effect of mitomycin-C on DNA synthesis in rabbit lens epithelial cells: Confluent cultures of ALE were incubated in MEME containing 20% fetal calf serum, 5 Ci/ml ^{3}H-thymidine after treatment with mitomycin-C for 2 minutes at each concentration. Inset shows significantly decreased DNA synthesis at above 0.025 mg/ml (CPM (count/minute) ratio: CPM-MMC/CPM-Control).

DISCUSSION

Efforts to treat opacification of the posterior capsule surgically or with an argon laser while preserving the posterior capsule have been reported, but they have not been proven clinically applicable.[13,14] Lee and Shyn reported that distilled water irrigation is an effective method for the prevention of after-cataract, but distilled water may be harmful to corneal endothelium.[15] So clinical availability may be suspicious. Chan and Emery attempted to prevent capsule opacification in a primate model using pharmacological agents.[16] Most studies on the use of antiproliferative drugs to inhibit cellular proliferation in vitro, usually with regard to their possible use in preventing posterior capsular opacification after cataract surgery, have been performed by placing the experimental drugs in the media for 24 hours and then determining the amount of inhibition.[17] The technique used in this study, that of incubation for less than 5 minutes with mitomycin-C and replacement with a drug-free medium, may provide greater insight into the effects of a drug on cellular proliferation over an extended period of time. And these incubation times may be more useful in clinical states.

A significant decrease of lens epithelial cell proliferation appeared when mitomycin-C 0.1 mg/ml was applied for 1 minute and 0.025 mg/ml for 2 minutes. No further effect was observed for 3 and 5 minutes. Antiproliferative drugs have been limited for use in cataract surgery due to the damage to other ocular structures. The previous report showed the amount of leakage of Dispase into the anterior chamber as a percentage of the concentration of Dispase in the mixture with sodium hyaluronate injected into the capsular bag, 2.6% to 9.3% (mean: 4.8%).[18] When the minimal effective dosage of mitomycin-C in this study was injected into the capsular bag with sodium hyaluronate, the postulated amount of mitomycin-C into the anterior chamber ranged from 6.5×10^{-4} to 2.3×10^{-3} mg/ml. Seah reported the concentration of mitomycin-C in the anterior chamber after glaucoma filtering surgery to be $5\times10^{-6}-1.2\times10^{-4}$ mg/ml,

for which concentration of MMC may be safe for corneal endothelium and zonules.[19] The concentration of mitomycin-C leakage into the anterior chamber according to our postulation could be higher than that after filtering surgery. But if a small anterior capsulotomy is performed and a viscoelastic material is injected into the anterior chamber before injection of mitomycin-C and sodium hyaluronate mixture into the capsular bag, leakage could be further reduced and corneal endothelium and zonules could be protected.

To confirm that cultured cells sustained the characteristics of rabbit lens epithelial cells, RT-PCR and RFLP of λ-crystalline was performed. The RT-PCR and RFLP could provide a rapid, effective and simple methodology for proving the characteristics of cultured cells. This method can be performed without a specific anti-λ-crystalline antibody.

If further investigation is undertaken into the effectiveness and toxicity of mitomycin-C, as well as when mitomycin-C could be applied into the lens capsular bag at the time of surgery, then antimitotic agents such as mitomycin-C could prove their worth in preventing posterior capsular opacification after cataract surgery.

These findings suggest that mitomycin-C is able to inhibit cell proliferation of lens epithelial cells at relatively short incubation times and at low concentrations, and also that mitomycin-C could be used for the prevention of posterior capsular opacification after cataract surgery.

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