Antiproliferative Effect of Mitomycin C on Experimental Proliferative Vitreoretinopathy in Rabbits

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To investigate the therapeutic potential of mitomycin C (MMC) in the management of proliferative vitreoretinopathy (PVR), antiproliferative effect of MMC on rabbit retinal pigment epithelial (RPE) cells, its intraocular toxicity, and its preventive effect on experimental PVR were investigated. Cultured rabbit RPE cells were exposed to various concentrations of MMC ranging from $1.0 \times 10^{-3}$ to 1.0 $\mu$g/ml for 72 hours. The RPE cells were then cultured in a medium without MMC for another 7 days, and the cells were harvested and counted. Toxicity of MMC to rabbit retina was evaluated after intravitreal injection of MMC by means of clinical observation, electrophysiologic test, and histopathologic examination. To test antiproliferative effect of MMC on experimental PVR, 200,000 cultured RPE cells were injected into the vitreous cavity of pigmented rabbits, and either 0.2 $\mu$g or 1.0 $\mu$g MMC was injected intravitreally 24 hours after RPE cell injection. Two to four weeks later, the vitreoretinal status was compared between MMC-treated eyes and control eyes. The antiproliferative effect of MMC on RPE cells was evident at the concentration of $1.0 \times 10^{-2}$ $\mu$g/ml. The drug concentration required for 50% inhibition of growth was $3 \times 10^{-2}$ $\mu$g/ml. Nontoxic intraocular doses of MMC were 2.0 $\mu$g in rabbit eyes with normal vitreous and 1.0 $\mu$g in rabbit eyes with gas-compressed vitreous. The rates of traction retinal detachment after intravitreal RPE cell injection were reduced in the eyes treated with MMC compared with control eyes. These results indicate that MMC may have clinical application to the treatment of PVR.

Key words: antiproliferative effect, mitomycin C, proliferative vitreoretinopathy, retinal detachment, retinal pigment epithelial cell

INTRODUCTION

Proliferative vitreoretinopathy (PVR) is still the most common cause of failure in both primary and secondary retinal reattachment surgery. Intravitreal injection of various antiproliferative agents for the management of PVR is promising but not practical yet because their effects last only for a short period. Mitomycin C (MMC), one of the antineoplastic agents, is now clinically used as an adjunct for trabeculectomy and pterygium surgery. It is well-known for its prolonged antiproliferative effect, but its pharmacological effect on PVR is not well investigated yet. In this experiment, effective antiproliferative concentration of MMC for retinal pigment epithelial (RPE) cell, which is the main cellular component of proliferative membrane, was estimated, and then nontoxic dose of intravitreal MMC was estimated. Finally the preventive effect of MMC on the experimental PVR was investigated.
ANTIPROLIFERATIVE EFFECT OF MITOMYCIN C

MATERIALS AND METHODS

RPE cell culture

RPE cells were harvested from pigmented rabbits and were cultured as a monolayer in Dulbecco’s modified Eagles’ medium (GIBCO) with 20% fetal bovine serum (DMEM/20) in a 37°C, 5% carbon dioxide incubator. Cells at the third or fourth passages were used in this experiment.

Proliferation assay

RPE cells were placed in 24-well, 16-mm tissue culture plates (4.0 \times 10^3 cells/well) (Falcon, Lincoln Park, NJ) containing DMEM/20. The cells were incubated for 24 hours at 37°C in 5% CO₂, being allowed to attach to the bottom of the plates. The cells were then washed with phosphate-buffered saline (PBS) (pH 7.0). Fresh DMEM/20 containing the following concentrations of MMC (Kyowa, Tokyo) was added: 1.0 \times 10^{-3}, 1.0 \times 10^{-2}, 2.0 \times 10^{-2}, 5.0 \times 10^{-2}, 1.0 \times 10^{-1}, 2.0 \times 10^{-1}, 5.0 \times 10^{-1}, and 1.0 \mu g/ml. MMC-free wells were used as controls. The cells were incubated for 72 hours at 37°C. The drug-containing medium was then removed and the cells were washed three times in PBS without magnesium or calcium, and the cells were incubated in DMEM/20 without MMC for another 7 days. The culture medium was discarded and the wells were washed with PBS and incubated at 37°C for 10 minutes in 0.3 ml of 0.05% trypsin-EDTA (GIBCO). The trypsin enzymatic reaction was stopped with 0.7 ml of DMEM/20 after incubation. Cell suspensions were immediately pipetted into vials containing 10 ml of Isotone II (Fisher Scientific, Springfield, NJ). Each vial was counted four times consecutively with the Coulter counter (Coulter electronics, Luton, Beds., England) and the counts for each well were averaged for the cell number value of that well. Experiments were repeated four to six times for each drug concentration.

Percent growth of the RPE cells relative to the control (percent control growth) was defined in each concentration of MMC as (B/A) \times 100, where A is average cell number in MMC-free controls and B is average cell number in MMC-treated wells. Results were plotted in a semilogarithmic fashion with percent control growth on the y-axis and log concentration of MMC on the x-axis. The inhibitory concentration required to produce a 50% reduction in proliferation (ID₅₀) was plotted on this diagram.

Toxicity evaluation of intravitreal MMC

Pigmented rabbits weighing 2-3 kg were used in this study. Animals were anesthetized with intramuscular injection of ketamine (40 mg/kg) mixed with xylazine (4 mg/kg), while the pupils were dilated with topical applications of 10% phenylephrine HCl and 1% tropicamide. Under the direct visualization, 0.1 ml of distilled water containing various amounts of MMC was injected into the center of the vitreous cavity from a site 5 to 6 mm posterior to the corneoscleral limbus inferotemporally.

The rabbits were divided into two groups and the right eyes were used. In group 1, MMC was injected into the normal vitreous. In group 2, the eyes underwent gas-mediated vitreous compression prior to intravitreal injection of MMC. Gas-mediated vitreous compression has been developed by Thresher et al. We used the method with some modifications. A 30-gauge needle attached to a perfluoropropane-filled tuberculin syringe was inserted into the center of the vitreous cavity from a site 5 to 6 mm posterior to the corneoscleral limbus inferonasally. Using indirect ophthalmoscopy, 0.4 ml of gas was slowly injected. To reduce the possibility of ocular damage to acute pressure rise, a portion of aqueous was removed by anterior chamber paracentesis after initial 0.2 ml injection of gas. A gas-fluid exchange with balanced salt solution was performed four days after gas injection when the gas bubble expanded completely to fill the entire vitreous cavity. We used the eyes at least 5 days after gas-fluid exchange for these studies. MMC was administered in doses of 0.5, 1, 2, 4, 8, and 16 μg with 0.1 ml of distilled water in each group. Control eyes were injected with 0.1 ml of distilled water without MMC in each group. More than four eyes were analyzed for each dose of MMC in each group.

Toxicity of MMC to rabbit retina was evaluated after intravitreal injection of MMC by means of clinical observation, electrophysiologic test, and
histopathologic examination, and then nontoxic dose of intravitreal MMC was determined. The eyes were examined for three weeks with a slit lamp and binocular indirect ophthalmoscopy. Electroretinography was performed 1, 2, 3 weeks before and after injection, using Neuropack-II plus (Nihon Kohden, Japan). Maximal b-wave amplitudes were used to evaluate the results. The eyes were enucleated 3 weeks after MMC injection. Anterior segments of the eyes were removed, and the small sections of tissue including retina, choroid, and sclera were fixed in 2% glutaraldehyde for 24 hours. The specimens were rinsed in several changes of phosphate buffer for one hour. The tissues were postfixed in 2% osmium tetroxide, washed in distilled water, dehydrated in a graded ethanol series, placed in propylene oxide, and embedded in epoxy resin. The tissues were examined by light and electron microscopy.

Antiproliferative effect of MMC on experimental PVR

Fourty eyes of forty pigmented rabbits were divided into three groups; 12 eyes treated with 0.2 μg of MMC, 12 eyes treated with 1.0 μg of MMC, and 16 eyes as a control. To make experimental PVR, we injected RPE cells into the vitreous cavity which had been compressed by perfluoropropane gas as described earlier. Under the operating microscope, 0.1ml of RPE cell suspension (2.0 \times 10^{5} cells) was injected with a 26-gauge needle into the middle of the vitreous cavity from a site 5 to 6 mm posterior to the corneoscleral limbus inferotemporally. Twenty-four hours later, either 0.2 μg or 1.0 μg MMC in 0.1ml of distilled water was injected intravitreally in MMC-treated group and 0.1ml of distilled water without MMC was injected in control group. These injections were done with a 30-gauge needle from a separate site superotemporally.

The eyes were examined with indirect ophthalmoscopy and slit-lamp daily for the first week and then by weekly periods. The rabbits were killed with an overdose of intravenous pentobarbital four weeks after RPE cell injection. Clinical severity of PVR was graded from stages 0 to 5 according to a classification published by Fastenberg et al. The eyes were enucleated and immersed in a fixative solution containing 10% formaldehyde. After dehydration in graded alcohol, the specimens were embedded in paraffin, and sections were stained with hematoxylin and eosin. The tractional retinal changes were examined in MMC-treated eyes and control eyes.

RESULTS

Inhibitory dose of MMC for the cultured cells

Most of the cells in MMC-free control wells became confluent at the end of this experiment. The average number of cells in the control wells was 9.2 \times 10^{4}. Morphologic changes in the cells were apparent at high concentrations of MMC (Fig. 1). The cells treated with 2.0 \times 10^{-1} μg/ml of MMC were shrunk and elongated, resulting in scanty cellular density. MMC inhibited proliferation of RPE cells in a dose-dependent manner (Table 1) and had a significant antiproliferative effect from the concentration of 1.0 \times 10^{-2} μg/ml. The drug concentration required for 50% inhibition of growth (ID_{50}), as interpolated from the concentration - percent control growth curve, was 3.0 \times 10^{-2} μg/ml (Fig. 2).

Retinal toxicity of MMC in the vitreous

As the intravitreal dosage of MMC increased, inflammatory reaction of the anterior chamber was developed, but the reaction regressed spontaneously in two or three weeks after injection. Electrophysiologically, in group 1, b-wave amplitude did not show significant change in the eyes injected with up to 2.0 μg MMC. In the eyes injected with 4.0 μg MMC, b-wave amplitude decreased down to 34.5% and no b-wave was recorded in the eyes injected with 8.0 μg. In group 2, the eyes injected with equal to or more than 2.0 μg MMC showed considerable decreases in the b-wave amplitude (Table 2).

Histologically, all the eyes treated with up to 2 g MMC in group 1 and up to 1 g MMC in group 2 had normal appearances so much as control eyes. Of four eyes treated with 2.0 g MMC in group 2, two eyes showed the decrease of basal infoldings and the increased intracytoplasmic vacuoles of RPE cells in mild degree, although the retina in these
eyes seemed to be normal in light microscopic examination. The eyes treated with more than these levels showed toxic changes in the retina from slight loss of photoreceptor outer segments to destruction of whole layer of the retina, complete atrophy of the retina, and chorioretinal scar with increasing severity according to dose of MMC (Fig. 3). From the results of the above experiments, nontoxic doses of MMC in the vitreous were determined as 2 μg in

eyes with normal vitreous and 1 μg in eyes with compressed vitreous.

Antiproliferative effect of MMC on experimental PVR

Among 40 eyes which were used in this experiment, five eyes were excluded due to severe
Table 2. Average % of baseline value of b-wave amplitude after intravitreal injection of mitomycin C in rabbit eyes.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>Eyes with normal vitreous</th>
<th>Eyes with compressed vitreous</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1 wk</td>
<td>2 wks</td>
</tr>
<tr>
<td>0</td>
<td>111.5</td>
<td>95.5</td>
</tr>
<tr>
<td>0.5</td>
<td>99.3</td>
<td>97.5</td>
</tr>
<tr>
<td>1</td>
<td>136.3</td>
<td>106.0</td>
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<td>2</td>
<td>96.8</td>
<td>90.3</td>
</tr>
<tr>
<td>4</td>
<td>75.8</td>
<td>69.3</td>
</tr>
<tr>
<td>8</td>
<td>10.8</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>8.0</td>
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</tr>
</tbody>
</table>

Fig. 3. Transmission electron microscopy of the outer retina 3 weeks after intravitreal injection of mitomycin C (MMC) in rabbit eyes. A, 1 µg of MMC. The photoreceptor outer segments (OS) and the retinal pigment epithelial cells (RPE) have normal appearance. B, 4 µg of MMC. The photoreceptor outer segments (OS) are scanty, and numerous intracytoplasmic vacuoles, swollen smooth endoplasmic reticulum, and swollen mitochondria are shown in the retinal pigment epithelial cells (RPE). Bar = 3 µm.
Table 3. Stages of proliferative vitreoretinopathy (PVR) four weeks after intravitreal injection of retinal pigment epithelial cells in control eyes and eyes treated with mitomycin C (MMC)

<table>
<thead>
<tr>
<th>Stage of PVR</th>
<th>Control eyes (n = 13)</th>
<th>0.2 μg MMC (n = 11)</th>
<th>1.0 μg MMC (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (No pathologic change)</td>
<td>0</td>
<td>1 (9.1%)</td>
<td>2 (18.2%)</td>
</tr>
<tr>
<td>1 (Intravitreal membrane)</td>
<td>0</td>
<td>3 (27.3%)</td>
<td>4 (36.4%)</td>
</tr>
<tr>
<td>2 (Focal traction)</td>
<td>0</td>
<td>3 (27.3%)</td>
<td>3 (27.3%)</td>
</tr>
<tr>
<td>3 (Localized detachment of medullary ray)</td>
<td>2 (15.4%)</td>
<td>3 (27.3%)</td>
<td>2 (18.2%)</td>
</tr>
<tr>
<td>4 (Extensive retinal detachment)</td>
<td>5 (38.5%)</td>
<td>1 (9.1%)</td>
<td>0</td>
</tr>
<tr>
<td>5 (Total retinal detachment)</td>
<td>6 (46.1%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

vitreous hemorrhage or intraocular infection during the follow-up period. By two weeks, focal tractional changes (stage 2, 3) occurred in 5 (45.5%) of 11 eyes treated with 0.2 μg MMC, 4 (36.4%) of 11 eyes treated with 1.0 μg MMC, and in 6 (46.2%) of 13 control eyes, while advanced retinal detachment (stage 4, 5) occurred in none of the eyes treated with MMC and in 7 (53.8%) of 13 control eyes. By four weeks, tractional change progressed slightly but preventive effect of MMC was maintained during this period and the rates of developing advanced retinal detachment were remarkably reduced in MMC-treated eyes (9.1%, 0% vs. 84.6%) (Table 3).

**DISCUSSION**

PVR is an abnormality complicated by proliferation of membranes, and is still the most important cause of failure in retinal reattachment surgery. Two major events in PVR are the formation and contraction of epiretinal membrane and the vitreous gel contraction. Many types of cells, such as RPE cell, fibrocyte, glial cell, and macrophage, have been known as cellular components of epiretinal membrane. Especially, RPE cell is known as the major cellular component of the membrane, and it produces extracellular matrix and humoral factors such as TGF-β. For this reason, RPE cells were used both in vitro and in vivo in our study.

For the last 10 years, the use of chemotherapeutic agents in the treatment of PVR has evoked increased interest. Antimetabolites, such as 5-fluorouracil (5-FU), have been used to inhibit RPE cell and fibroblast growth both in experimental models of retinal detachment and in cultured cell models. However, the use of 5-FU is not successful clinically because intravitreal concentration of 5-FU decreased rapidly and its antiproliferative effect lasted only for a short period. Repetitive injection of 5-FU was advocated but this method is not applicable to human. MMC is an antitumor antibiotic isolated from Streptomyces caesipitosus. In ophthalmology, the drug is used after pterygium surgery to suppress its recurrence and during filtering surgery of glaucoma. MMC has been reported to have an antiproliferative effect similar to other antineoplastic agents such as 5-FU, but it has much more prolonged effect after a short-term exposure. Therefore, MMC may have a significant antiproliferative effect in the management of PVR with a single intraocular application, where multiple intraocular injection of drug is not so applicable. However, little work has been done to evaluate inhibitory potentials of MMC in experimental models of PVR.

Previous in vitro studies suggest that MMC produce a significant antiproliferative effect on cultured fibroblasts at various concentrations ranging from $2.0 \times 10^{-3}$ μg/ml to 3.94 μg/ml depending on the exposure time and total culture period. Recently in vitro study on the antiproliferative concentration of MMC on cultured RPE cells reported a similar result to that on fibroblasts. The ID$_{50}$ for MMC in this study was approximately $3.0 \times 10^{-2}$ μg/ml. This result is comparable to that by Muldoon et al. To determine the therapeutic range of MMC, toxicity and safe dose of MMC in the vitreous cavity were evaluated in our study. The vitreous was
compressed by perfluoropropane gas in order to permit even diffusion of MMC. The nontoxic dose was estimated as 2.0 μg in rabbit eyes with normal vitreous and 1.0 μg in eyes with compressed vitreous. Under these levels of MMC, the eyes treated with MMC did not show any toxic effect clinically, electrophysiologically, and histologically. Histologically the outer retina appeared to be damaged initially in the higher dose. As the dose increased, inner portion of the retina was also damaged. In a very high concentration of MMC, an atrophic chorioretinal scar completely replaced normal retinal structures. These results were similar to the previous reports on the toxicity of various antineoplastic agents. Nontoxic dose in eyes with normal vitreous was smaller than that in eyes with compressed vitreous in spite of the faster clearance in vitrectomized eyes. This result implies that the mechanism of MMC toxicity may be related to peak drug concentration rather than duration of tissue exposure.

Either 0.2 μg or 1.0 μg of MMC was selected as the intravitreal dose in experimental PVR, because these amounts were much larger than the effective in vitro antiproliferative dose and in the therapeutic range in our study. Before intravitreal injection of RPE cells, vitreous was compressed by perfluoropropane gas to simulate the clinical setting in the management of PVR. The intravitreal injection of RPE cells or fibroblasts in rabbits has been shown to be effective in eliciting tractional retinal changes that are characteristic of PVR, and has been used in evaluating the inhibitory potential of various antineoplastic drugs. A dosage of 200,000 cells, which was used in our study, has been shown to be sufficient to elicit extensive traction retinal detachment.

Even in the same concentration of MMC, the degrees and extents of tractional change were variable, which was similar to the findings by others using fluorouracil. Tractional changes were reduced in MMC-treated eyes when compared with control eyes. Especially, severe tractional changes such as total retinal detachment were remarkably decreased in MMC-treated eyes. The preventative effect of MMC on experimental PVR persisted for four weeks after the RPE cell injection. When compared with control eyes, the rates of developing tractional retinal detachment were much more reduced in eyes treated with 1.0 μg of MMC than in those treated with 0.2 μg of MMC, and the preventive effect was dependent on the dosage of MMC.

In our study, MMC effectively prevented the proliferation of RPE cells in vitro and in experimental PVR model, and the preventive effect on experimental PVR persisted during the follow-up period of four weeks after single injection. These results indicate that MMC may have clinical application to the treatment of PVR, although clinical trials are needed to confirm the efficacy of MMC in the prevention of PVR.

REFERENCES

ANTIPROLIFERATIVE EFFECT OF MITOMYCIN C


