(34)

Antiproliferative properties of mycophenolic acid on human retinal pigment epithelial cells in vitro

Antyproliferacyjny wpływ kwasu mykofenolowego na komórki nabłonka barwnikowego siatkówki in vitro

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Summary:

Purpose: Mycophenolate mofetil (MMF) is an immunosuppressive agent used in the prophylaxis of graft rejection in transplantology. Its antyproliferative effects on lymphocyte, monocytes, vascular smooth muscle cells and fibroblasts are well known, but to our knowledge there are no reports on its action on the retinal epithelial (RPE) cells in vitro.

Material and methods: In all experiments we used mycophenolic acid (MPC), which is the biologically active form of MMF. Its activity was assessed on the cultures of immortalized non-transformed cells line from a human donor (ARPE19). Cells were seeded and incubated in vitro with deferent concentrations of MPC: 0.0025 μg/ml, 0.025 μg/ml, 0.25 μg/ml, 2.5 μg/ml, 25 μg/ml and 250 μg/ml. After 24 and 72 hours of incubation, proliferative activity was assessed by 5'-bromo-2'-deoxyuridine (BrdU) incorporation into cellular DNA and the amount of cell proliferation was determined using the 3-(4.5-dimethylthiazol-2yl)-2.5--diphenyltetrazolium bromide (MTT) assay. Additionally, to determine cytotoxicity of MPC, ARPE19 cells were grown to confluence and subsequently cultured in a serum-deficient medium and then, after 24 hours of incubation with diferent concentrations of MPC, the MTT test was performed.

Results: The BrdU assay showed the decrease of DNA synthesis activity for increasing concentrations of MPC starting with 0.025 μg/ml to 250 μg/ml. The number of RPE cells assessed with MTT test decreased after the exposition to the drug concentrations of 25 μg/ml and 250 μg/ml after 24 and 72 hours of incubation, and additionally for the concncentrations of 0.25 μg/ml and 2.5 μg/ml after 72 hours of incubation.

Conclusions: MMF influences the proliferation of immortalized ARPE19 cells without evident cytotoxic effect.

Słowa kluczowe: witreoretinopatia proliferacyjna, mykofenolan mofetilu, kwas mykofenolowy, komórki nabłonka barwnikowego siatkówki.
Key words: proliferative vitreoretinopathy, mycophenolate mofetil, mycophenolic acid, retinal pigment epithelial cells.

Introduction

Proliferative vitreoretinopathy represents an exuberant wound healing process in retinal surgery and refers to the migration and proliferation of cells into the subretinal space and vitreous cavity and onto the retinal surface. Subsequent collagen production and cell-mediated contraction of the collagen scaffolding leads to the retinal detachment and loss of vision with eventual blindness. Although refinements in surgical techniques and equipment have improved the success rate for the treatment of PVR in recent years, recurrent PVR secondary to reproliferation is not uncommon and is the leading cause of failure of surgery after retinal detachment or trauma (1).

The current strategy in the management of PVR comprises the operative removal of the vitreous including all proliferative mediators like blood and growth factors. But even meticulous surgery with removal of all tractions cannot prevent reproliferation with subsequent deleterious development. Especially severely traumatized eyes show strong susceptibility to proliferative activity within the first months necessitation for multiple revisions.

Various pharmacologic adjuncts have been evaluated for the reduction of the formation of fibrocellular membranes in PVR. Chemotherapeutic drugs like 5-fluorouracyl (2) or daunorubicin (3) are successfully tested in RPE-cell cultures. However, until now these agents are limited in their clinical usefulness, because of their small therapeutic windows or short half-time duration in the vitreous body.

Mycophenolate mofetil (MMF), and its bioactive compound – mycophenolic acid (MPA), is an immunosuppressive agent used in the prophylaxis of graft rejection in transplantology. MMF inhibits lymphocyte proliferation both in vitro and in vivo; and in vitro affects monocytes, vascular smooth muscle cells (4) and fibroblasts (5). Its mechanism of action relies on reversible inhibition of inosine monophosphate dehydrogenase (IMPDH) – a key enzyme in the de novo synthesis of guanosine monophosphate (GMP). The deficiency in GMP leads to

deficiency in guanosine triphosphorane (GTP), a necessary compound for desoxyguanosine production. Absence of desoxyguanosine disrupts the syntheses of DNA and RNA. MMF blocks the proliferation of human pulmonary fibroblasts and the fibroblasts of the Tenon's capsule (6), but its effect on the RPE cells is unknown.

Our study was undertaken to determine the effect of MMF on RPE cells in vitro with view to assess the possibility to use this drug in the management of PVR.

Materials and methods

Reagents

Cell culture reagents and chemicals were purchased from Invitrogen-Gibco (Rockville, MD, USA). In all experiment we used mycophenolic acid (MPA-Sigma, Germany), the bioactive form of MMF.

Cell Culture

Human retinal pigment epithelial cells (ARPE19; ATCC, Manassas, USA; passage 17-20) were cultured in Dulbecco's modified Eagle's medium containing 3 mM L-glutamine, 10% fetal bovine serum (FBS), 100 μg/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37° C in an environment containing 95% O_2 and 5% CO_2. All cells were observed by phase contrast microscopy in vivo and checked for viability by means of vital staining with fluorescein diacetate (FDA) and ethidium bromide testing.

For the proliferation assays, cell suspensions of 3 x 10^3 cells/ml were seeded onto five 96-well tissue culture plates. After overnight incubation, the cells were washed with phosphate-buffered saline (PBS) before fresh medium containing MPC in the appropriate concentrations was reintroduced. Adequate negative (cells without MPC) and positive controls for toxic effect (cells treated with 0.3% Triton X in PBS) were performed. All experiments were performed in triplicate.

BrdU ELISA

ARPE19 cell proliferation was monitored by incorporation of 5'-bromo-2'-deoxyuridine (BrdU) into cellular DNA using the Cell Proliferation ELISA, BrdU (colorimetric) (Boehringer, Mannheim, Germany) according to the manufacturers instructions. Briefly, 100 μl/well of cell suspension in medium containing 10% FBS were placed on 96-well plates (Becton Dickinson, Franklin Lakes, USA) at a concentration of 3 x 10^3 cells/ml. After appropriate drug exposure, the cellular DNA was labeled for 24 hours using BrdU labeling reagent containing 10 μg/ml BrdU. After fixation, the cells were incubated with 1 μg/ml monoclonal anti-BrdU antibody for 90 minutes at room temperature. The cells were washed, then incubated for 30 minutes with peroxidase-conjugated goat anti-mouse IgG. After the addition of substrate solution the reaction was stopped with H_2SO_4 and analyzed in an ELISA reader (SLT Spectra, Salzburg, Austria) at dual wavelengths of 450 and 540 nm within 30 minutes.

MTT ELISA

The amount of cell proliferation was measured by an assay of mitochondrial function. The tetrazolium salt 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT; Sigma, Germany) was dissolved in PBS at 5 mg/ml stock concentration and sterilized by passage through a Millipore filter (0.22 μm). After appropriate drug exposure, the cells were washed with phosphate-buffered saline (PBS) and diluted stock solution of MTT at 0.5 mg/ml was added. After 2 hours of incubation, formazan extraction was performed with isopropanol and the quantity of viable cells was measured colorimetrically with an ELISA reader (SLT Spectra, Salzburg, Austria) at dual wavelengths of 570 and 690 nm.

Proliferation assessment

Cell seeding and preparation were performed as described above. After overnight incubation the cells were washed with PBS and fresh medium containing 0.0025 μg/ml, 0.025 μg/ml, 0.25 μg/ml, 2.5 μg/ml, 25 μg/ml and 250 μg/ml of MPA was added. After incubation for 24 and 72 hours, MTT and BrdU tests were performed.

Cytotoxicity tests

The cytotoxicity assay was designed to assess the level of direct toxicity of MPA in a confluent growth-arrested cell layer. A quantity of 5 x 10^4 cells was grown to confluence in 96-well tissue culture plates. Then medium containing 0.002516 μg/ml, 0.025 μg/ml, 0.25 μg/ml, 2.5 μg/ml, 25 μg/ml and 250 μg/ml of MPA was added. After another 24 hours of incubation, MTT quantification of viable cells was performed as described above.

For statistical analysis the results were expressed as units of mean absorbance of formazon at 570 nm ± SD and of BrdU at 450 nm ± SD. Mean values were compared using the Mann-Whitney U test, and a p value of less than 0.05 was considered significant and marked with an asterisk. Five individual samples per group were measured in triplicate.

Results

Proliferation experiments

RPE cells incubated with MPA in a concentration range from 0.0025 μg/ml to 250 μg/ml displayed a dose-dependent response towards MPA in increasing concentrations (Fig. 1, 2). MPA caused a significant reductions in cell proliferation at two highest concentration (250 μg/ml, and 25 μg/ml) and this effect was observed after both 24 and 72 hours of incubation.

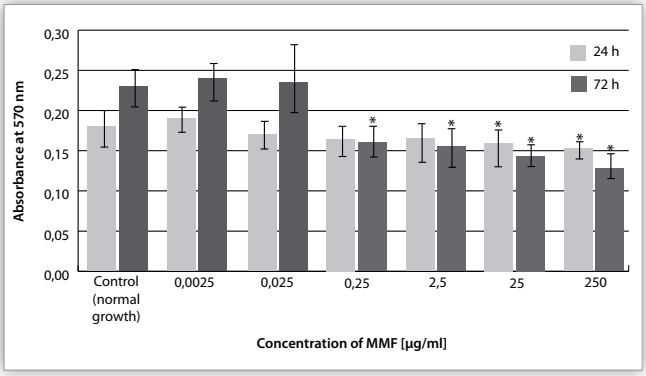

Fig. 1. Proliferation of ARPE19 cells in the presence of various concentrations of MPC for 24 and 72 hours of incubation. MTT assay at 570 nm. Mean values were compared using the Mann-Whitney U test. An asterisk indicates a statistically significant difference ($p<0.05$).

Ryc. 1. Proliferacja ludzkich komórek nabłonka barwikowego siatkówki (ARPE 19) w obecności różnych koncentracji kwasu mykofenolowego (MPC) inkubowanych przez 24 i 72 godz. Próba w roztworze MTT w 570nm. Średnie wartości były porównane za pomocą testu U Manna-Whitneya. Gwiazdki wskazują różnice statystycznie znamienne ($p<0,05$).

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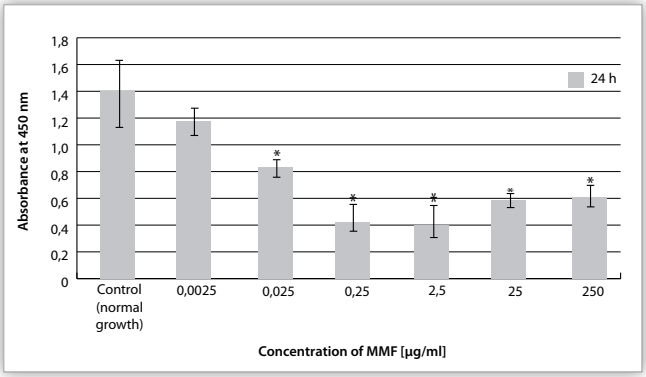

Fig. 2. Proliferation experiments. Analysis of BrdU incorporation into ARPE19 cells during incubation for 24-hours with various concentrations of MPC. Significant reductions in nuclear proliferative activity were observed at all the examined concentrations. BrdU assay at 450 nm. Mean values were compared using the Mann-Whitney U test. An asterisk indicates a statistically significant difference ($p<0.05$).

Ryc. 2. Eksperyment proliferacyjny. Analiza wbudowywania BrdU w komórki ARPE 19 podczas inkubacji przez 24 godz. w różnych koncentracjach kwasu mykofenolowego. Próba BrdU w 450 nm. Średnie wartości porównano za pomocą testu U Manna-Whitneya. Gwiazdki wskazują różnice statystycznie znamienne ($p<0,05$).

At the concentration of 2.5 μg/ml and 0.25 μg/ml a significant reduction of proliferation was noted only after a longer period of incubation (72 h). At the lowest concentrations (0.0025 μg/ml, 0.025 μg/ml) the drug did not cause significant differences in cell proliferation.

Quantitative analysis of BrdU-positive cell nuclei showed a more pronounced inhibition of proliferative activity for concentrations from 250 μg/ml to 0.025 μg/ml (Fig. 2).

MTT stationary toxicity assay

A stationary confluent cell culture is better suited to discriminate a toxic drug effect than a proliferating culture and more closely resembles the natural situation within the eye. There was no cytotoxicity detected for the dissolved MPA at any of the evaluated concentrations (Fig. 3). Within a healthy confluent cellular layer, no significant decrease in the total amount of viable cells was observed after 24 hours.

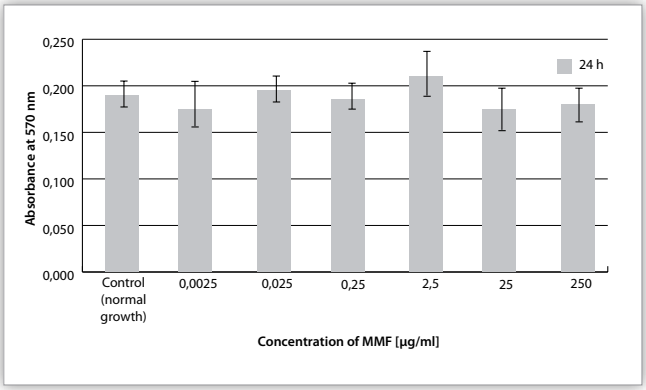

Fig. 3. MTT stationary toxicity assay. 24-hour exposure to all the examined concentrations of MPC demonstrated no significant cytotoxic effect to confluent ARPE19 cells.

Ryc. 3. Próba miejscowej toksyczności MTT. 24-godzinna ekspozycja dla wszystkich stężeń MPC nie wykazała znaczącej cytotoksyczności dla zrastających się komórek ARPE 19.

Discussion

The clinical impotence effectiveness of antyproliferative drugs is well known from glaucoma surgery, where the use of antiproliferative medications such as mitomycine and 5-fluorouracyl improves the rate of successes treatment (7). Unfortunatelly, in the field of retinal surgery no such an adjunctive agent, which would be useful in clinical practice, has been identified so far. Many antiproliferative agents were tested in view of their use in the prevention of PVR, such as glycocorticosteroids (8), colchicine (9), retinoid acid (10) and daunorubicin (11), but none of these are used in the clinical settings. The most advanced research was done with 5-fluorouracil and heparin. However randomized placebo controlled trials with thsese agents gave ambiguous results (12,13).

In this study we demonstrated that MPA inhibits the proliferation of ARPE cells in vitro in a dose-dependent manner without toxic effect for the concentrations evaluated. These features of this agent are crucial in its potential use for the prevention of PVR development after trauma and retinal surgery.

MMF can also enhance the expression of matrix metalloproteinases and alpha-smooth muscle actin (14), which are involved in the development and contraction of extracellular matrix. In these two ways MPA can inhibit the development of proliferative membranes by both inhibiting the cell proliferation and by direct effect on the fibrotic process. The results of our study seem promissing but Reed further confirmation.

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