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Antiproliferative effect of mycophenolate mofetil on cultured human Tenon fibroblasts

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Abstract *Background:* Wound healing after glaucoma filtering surgery is often complicated by exaggerated scarring of the subconjunctival Tenon's layer. Therefore, antiproliferatives are commonly employed. The immunosuppressive drug mycophenolate mofetil (MMF) is used to prevent graft rejection after kidney or liver transplantation. The effect is mediated by inhibition of lymphocyte proliferation. In this study we investigated the effect of MMF on human Tenon fibroblast proliferation in cell culture. *Methods:* Human Tenon fibroblasts (HTF) were cultivated with 10% fetal calf serum. Cells were incubated with MMF concentrations of 0.1 µM to 3000 µM for up to 20 days. In a second set of experiments HTF were incubated for 10 min only in MMF solutions. Cell counts were performed to evaluate the proliferation rate. The proliferation was also assessed by Ki67 staining. Morphological changes were documented by vimentin staining. *Results:* Growth inhibition of HTF by MMF was concentration dependent. IC_{50} was 0.85±0.05 µM for 6 days of incubation. Brief exposure to MMF leads to a reversible growth arrest for up to 14 days with concentrations of 1000 µM or higher. Ki67 staining confirmed the concentration dependent proliferation rate. *Conclusion:* MMF has a concentration-dependent antiproliferative effect on HTF without any detected cytotoxicity in the applied concentration range. Brief incubation also leads to a growth arrest; therefore, intraoperative MMF application might prevent exaggerated scarring after glaucoma filtering surgery.

Introduction

The success of glaucoma filtering surgery depends on the response of human Tenon fibroblasts (HTF) to the mechanical trauma performed by surgery. It is now understood that the conjunctival wound healing is a complex process in which HTF play a major role. HTF respond by increased proliferation or by secretion of growth factors [25, 37, 44]. Currently antiproliferatives such as mitomycin C (MMC) or 5-fluorouracil (5-FU) are used during or after glaucoma surgery to control exaggerated scarring. New approaches try to influence the production of transforming growth factor beta 2 (TGFβ2) by using monoclonal antibodies against TGFβ2 [9]. TGFβ2 is one of the major factors to promote activation and proliferation of HTF [10].

The immunosuppressive agent mycophenolate mofetil (MMF) is successfully used to prevent graft rejection in kidney, liver and pancreas transplantation [4, 39, 50, 51]. First clinical trials in cornea transplantation have been performed [40, 41], and a positive influence on controlling inflammatory eye diseases is reported [27]. MMF has a known effect on the proliferation of lymphocytes in cell culture in vitro and in vivo [16, 30, 31]. Only few in vitro studies show that there is also an influence on other cells, e.g. mesangial and endothelial cells [15, 19, 21]. MMF and the active metabolite mycophenolic acid lead to a reversible inhibition of the key enzyme inosine monophos-

phatase dehydrogenase and of the de novo synthesis of guanosine monophosphate (GMP) [18, 47]. Shortage of GMP leads to shortage of guanosine triphosphate (GTP) which is necessary for the production of deoxyguanosine, an essential part for DNA and RNA synthesis.

In the this study we set out to investigate the effect of MMF on HTF in vitro. For that reason we performed proliferation assays with cell counts and immunohistochemical staining with Ki67, which is present in proliferating cells of all active phases from late G1 through the M phase [17, 26]. Experiments were carried out with long-term or brief exposure to MMF, and reversibility of the effects was observed.

Methods

Cell culture

Human Tenon samples were obtained from tissue explants from two male white patients (65 and 70 years old) without any topical eye treatment who underwent routine cataract surgery with their informed written consent. After removal, tissue was immediately transferred into cold phosphate-buffered saline (PBS) supplemented with 15% heat-inactivated fetal calf serum (FCS; Seromed, Germany). Tenon samples were attached in drops of FCS in 60-mm^2 tissue growth dishes (Corning, N.Y., USA) overnight at 37°C. After fixation to the surface, cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 µg/ml), D-glucose (4.5 mg/ml), insulin (10 µg/ml; all Sigma, Deisenhofen, Germany) and mercapto-ethanol (5×10^{-5} M). Primary cultures were passaged at confluence by trypsinization into 75-cm^2 tissue flasks (Falcon, Becton Dickinson, Heidelberg, Germany). For subsequent passages cells were split 1:2 or 1:3 in DMEM with 10% FCS. Cells from passages 4 to 11 were used for all experiments. During cultivation cells exhibited the same morphological phenotype. Cells were grown at 37°C in a humidified incubator with 10% CO_2.

Cell proliferation studies with long-term MMF exposure

After trypsinization 2×10^5 cells were grown with medium and 10% FCS in tissue flasks (175 mm^2; Corning). MMF (Cell Cept solution for intravenous application; Roche, Grenzach-Wyhlen, Germany) was originally dissolved in sterile water and diluted to different concentrations ranging from 0.1 µM to 3000 µM. The medium was not changed during the proliferation tests. Cells were trypsinized and centrifuged at 800 *g* for 10 min in preparation for counting. After resuspension in a small volume (0.5–1.0 ml) of PBS, 20 µl of trypan blue (Sigma) was added to stain necrotic cells. The cell counts were performed in a Rosenthal counting chamber (three times) by using a phase-contrast microscope (Zeiss Oberkochen, Germany) at days 6, 13 and 20. All long-term tests were performed in quadruplicate.

Cell proliferation studies with brief exposure to MMF

Cells were prepared and counted in the same way as described above. MMF was added 3 h after cells were seeded in the flasks in concentrations from 100 µM to 3000 µM. Incubation times with MMF were limited to 10 min, followed by two washes with PBS. All brief exposure tests were performed in duplicates.

Proliferation staining with Ki67antibody

For the Ki67 staining 1×10^4 cells were seeded on cover slips (10 mm in diameter) in 48-well tissue culture plates. Cells were incubated for 7, 13 and 20 days with MMF concentrations ranging from 1 µM to 10 µM in triplicate. They were fixed with ethanol acetic acid (95:5) at –20°C for 7 min and then incubated with rabbit anti-human monoclonal Ki67 antibody (Dako, Garching, Germany, no. A0047, dilution 1:100) for 30 min at room temperature. Cover slides were washed with PBS three times before fluorescein-conjugated anti-rabbit IgG (Dianova, Hamburg, Germany, 711-226-152, 1:100) antibody was added, diluted in blocking buffer, for 30 min at room temperature. Glasses were washed with PBS again and evaluated by fluorescence microscopy (Zeiss).

Staining with vimentin

Cover slips with HTF cells were prepared in the same way as for Ki67 staining. Cells of the 10th passage were used. The vimentin mouse anti-human antibody was diluted 1:50 (Dako). Cells were then incubated with FITC-conjugated donkey anti-mouse IgG (h+l chain, Fab2 fragment, Dianova) antibody diluted 1:100.

MMF influence on human endothelium in corneal organ culture

Two pairs of human corneas which were unsuitable for organ transplantation because of positive or inconclusive donor serology for hepatitis B, C or HIV, or low endothelial cell count (below 2000 cells/mm^2) were cut into quarters and then incubated with 300, 1000 or 3000 µM of MMF for 15 days. The organ culture was performed after the standard operating procedures of the Cornea Bank of Essen without changing the culture medium. Cell counts of each specimen were performed at days 0, 3, 6 and 15 with induced swelling of endothelial cells by hypoosmotic balanced salt solution (hBSS), which allows visualization of the intercellular spaces. On day 15 corneas were also stained with trypan blue.

Statistics

Representative data or means ± standard deviation (SD) of independent experiments are shown. Concentrations of MMF for 50% inhibition of HTF proliferation (IC_{50}) were obtained by fitting of the data to the general dose–response equation for a single binding component [13].

Results

Concentration dependence of the antiproliferative effect of long-term exposure of MMF on HTF

Incubation with different concentrations of MMF showed an antiproliferative effect on Tenon fibroblasts in cell culture. In all series growth inhibition was above 90% with concentrations of 5 µM or higher of MMF (Fig. 1). The concentration necessary for 50% inhibition (IC_{50}) was calculated as 0.85±0.05 µM for 6 days of incubation. For other incubation times the IC_{50} was 0.45±0.05 µM for 13 days and 0.39±0.04 µM for 20 days. Using concentrations higher than 10 µM for up to 11 days leads to total growth arrest (data not shown in

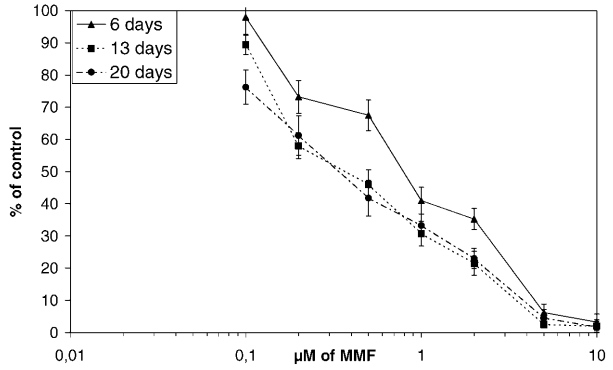

Fig. 1 Proliferation rate of human Tenon fibroblasts after 6, 13 and 20 days of incubation with mycophenolate mofetil (*MMF*) at concentrations from 0.1 µM to 10 µM. For each duration, inhibition of proliferation was concentration dependent. IC_{50} was 0.85±0.05 µM for 6 days of incubation. Data are means ± SD of four independent experiments

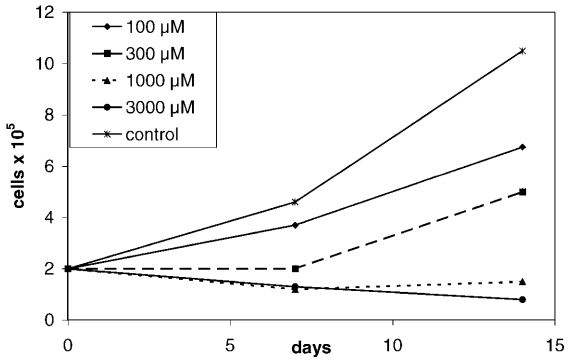

Fig. 2 Cell number of human Tenon fibroblasts at 7 and 14 days after incubation with mycophenolate mofetil for 10 min at concentrations from 100 µM to 3000 µM. Concentrations of 300 µM and higher led to growth inhibition for 7 days. Growth arrest for 14 days could be achieved with concentrations of 1000 µM or higher. Data represent the mean of two independent experiments

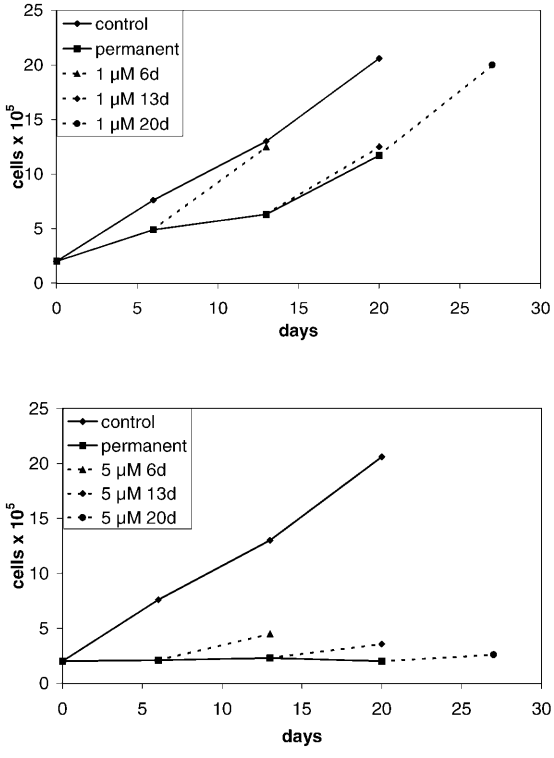

Fig. 3 Proliferation after cessation of mycophenolate mofetil (MMF) incubation with 1, 2, 5 or 10 µM of MMF for 6, 13 or 20 days; *dotted lines* show incubation without MMF. Proliferation resumed with every concentration used, depending on incubation time and concentration

Fig. 1) without any observed cytotoxic effect. Only with permanent concentrations of 300 µM and higher could some cytotoxic effects could be seen. In all other counts no significant number of trypan blue-positive dead cells were observed.

Concentration dependence of the antiproliferative effect of brief incubation with MMF on HTF

Brief incubation with MMF for 10 min also showed growth inhibition. Concentrations higher than 300 µM did not lead to any proliferation during the 7 days after brief exposure to MMF. After 14 days, total growth inhibition was observed only with concentrations of 1000 µM or higher (Fig. 2). Even at the highest used concentration of 3000 µM no significant number of necrotic cells was detected. No significant difference between the two experiments was seen.

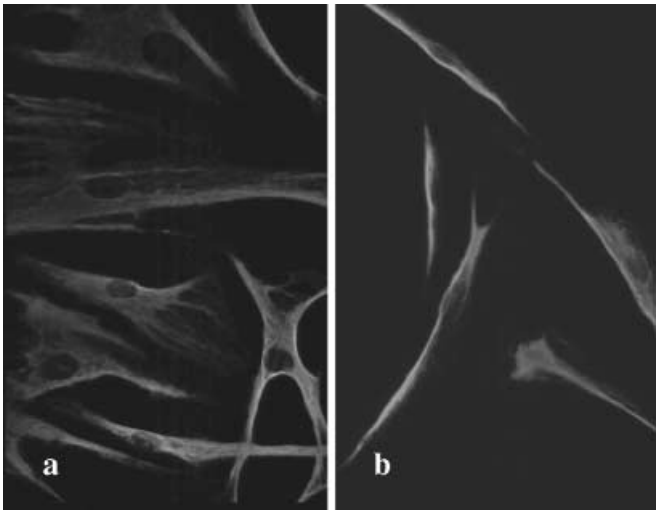

Fig. 4a, b Morphological effect on human Tenon fibroblasts stained with vimentin after incubation: **a** without mycophenolate mofetil (MMF); **b** with 10 µM MMF for 13 days. Incubation with MMF shows long cell branches with small nuclei. The typical unaffected appearance is spindle-shaped with a wider cell body and shorter branches

Reversibility of the antiproliferative effect of MMF

After long-term incubation with MMF the inhibition of proliferation was temporary. Proliferation rates without MMF in the 6 days following incubation with MMF at different concentrations for 6, 13 or 20 days were measured. HTF resumed proliferation after cessation of MMF incubation. Proliferation recovered with every used concentration of MMF. With increased incubation time and higher concentration of MMF the proliferation rate was lower (Fig. 3).

Staining with proliferation marker Ki67

When incubating HTF without MMF for 7 days a rate of 25% of Ki67-positive cells was seen in the control. A concentration-dependent effect was found with different MMF concentrations. The highest concentration of 10 µM showed only one to three Ki67-positive cells on each cover slip.

Morphological effects after incubation with MMF

Vimentin, a cytoscleral filament, was used to visualize the differences in the morphologic appearance of HTF before and after incubation with MMF. After incubation the cells had long branches with small nuclei shown by vimentin antibody staining. Without MMF the cells had the typical spindle-shaped appearance with a wider cell body and shorter branches (Fig. 4).

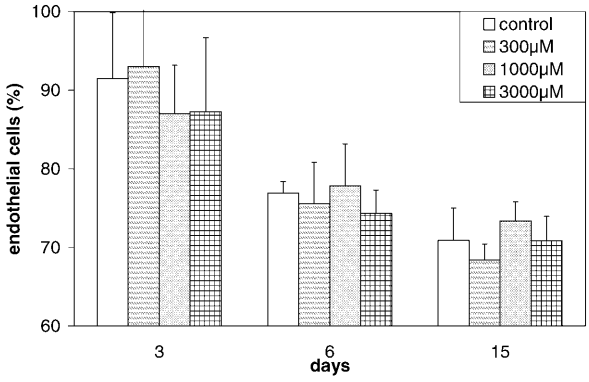

Fig. 5 Endothelial cell number after incubation with mycophenolate mofetil (MMF) for 15 days show no significant influence of different concentrations of MMF compared with the control. Data are means ± SD of four independent experiments related to the control

MMF influence on human endothelium in corneal organ culture

There was no difference in swelling patterns compared to the control specimen with the specimen exposed to MMF. At all time points intercellular spaces could be immediately visualized, which is indicative of vitality of corneal endothelial cells. No change in color or clarity of medium was observed. Compared to cell number per square millimeter in the control specimens there was no significant cell loss at any concentration (Fig. 5) or any difference in the number of trypan blue-positive cells at the end of the tests.

Discussion

Human Tenon fibroblasts play a central role in wound healing after glaucoma filtering surgery by proliferating and also by secreting growth factors due to surgical trauma. The use of antiproliferatives has improved success rates [20, 48] by modulating the response of the HTF, mainly leading to long-term growth arrest [22, 23, 24]. Growth-arrested HTF still produce growth factors which lead to a scarring response of the surrounding unaffected HTF [12, 37]. This may explain why, in some patients, glaucoma filtration surgery fails despite the usage of antiproliferatives. Patients treated with MMC often present with cystic blebs surrounded by cicatricial conjunctiva. Histologic examination of MMC-treated drainage blebs showed that the overlying treated area was virtually devoid of fibroblasts [45]. The clinical effect of the antimetabolites MMC and 5-FU has been well documented in many studies [3, 7, 42]. Many other antiproliferatives, such as mithramycin, daunorubicin and bleomycin [29], have been mainly investigated for in vitro use. Other approaches reduced HTF proliferation by using growth fac-

tor inhibitors such as decorin and suramin [35, 36]. Other groups combined antiproliferatives with electric pulses [38] or single application of β-radiation [8] to modulate activation of fibroblasts. The actions of TGFβ appear to be an important component of the conjunctival scarring response [9, 37]. TGFβ stimulates HTF proliferation, migration and collagen contraction [9]. As a conclusion, targeting of TGFβ, especially TGFβ2, by means of monoclonal anti-TGFβ2 antibodies is an important way of modulating the scarring response [10].

Since there are many known complications, such as late bleb leakage, endophthalmitis, ciliary toxicity, retinal bleeding or epitheliopathy, when using MMC or 5-FU in glaucoma filtering surgery [1, 5, 11, 32, 43, 49], and because of the lack of predictability of their antiproliferative effect, there is a need for new approaches to modulate wound healing.

The immunosuppressive drug mycophenolate mofetil, with its active agent mycophenolic acid, is an uncompetitive reversible inhibitor of the de novo synthesis of guanosine nucleotides [18, 47]. In many other cells a salvage pathway exists to produce guanosine nucleotides by the adenine or guanine nucleotide pathway. In lymphocytes MMF blocks inosine monophosphate dehydrogenase (IMPDH), leading to a depletion of GTP. Consequently a decrease in lymphocyte proliferation occurs because lymphocytes rely on the de novo synthesis of GTP. Other authors focus mainly on effects of MMF on lymphocytes to suppress immunoreactions, e.g., after kidney or other solid organ transplantation. An in vitro effect of MMF on other cells has only rarely been documented [15, 19, 21]. Systemic application of MMF appears to improve clinical symptoms of patients with ocular pemphigoid or other chronic cicatricial conjunctivitis [34, 53]. These effects may be mediated by inhibition of fibroblast proliferation as well as the anti-inflammatory effect.

Our data show that there is a concentration-dependent inhibition of HTF proliferation in vitro. These results could be achieved by cell counts and with Ki67 staining. The IC_{50} obtained for HTF was marginally higher than the IC_{50} seen in other nonlymphoid tissue [21], but in the same concentration range. We conclude that HTF predominantly rely on the de novo synthesis of GTP, because proliferation can be suppressed by blocking this pathway with MMF.

The resumption of proliferation after cessation of MMF incubation can also be found using other antiproliferatives [52]. Our data suggest that there is no exaggerated proliferation following incubation of HTF with MMF for up to 14 days. Higher doses of MMF are necessary to reach an effect on fibroblast proliferation when HTF are incubated for only 10 min with MMF. Brief exposure to concentrations higher than 1000 µM leads to growth arrest for at least 14 days without any significant cytotoxic effect. The absence of cytotoxicity at effective doses of MMF appears to represent its most significant advantage over commonly used antiproliferatives. Toxic effects of MMC or 5-FU used in glaucoma surgery on corneal endothelium have been described [14, 33, 46]. No toxic effect of high concentrations of MMF on human corneal endothelium was detected in this study.

We conclude that MMF might have a positive effect on scarring after glaucoma surgery. The efficacy of brief application suggests that intraoperative use may be beneficial. Our investigations focused on the cell proliferation rates of HTF. On the other hand, the production of, for example, growth factor and cell adhesion molecules by lymphocytes is also very important in ocular wound healing [6]. By depletion of GTP the glycosylation of adhesion molecules of lymphocytes is suppressed [2, 28]. Thus beside growth inhibition MMF may also influence wound healing by modulating other components of the complex system. Mycophenolate mofetil provides a novel approach to the influencing of fibroblasts in different clinical situations such as wound healing or cicatricial conjunctivitis.

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