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Mycophenolic acid suppresses human pterygium and normal tenon fibroblast proliferation in vitro

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ABSTRACT

Aims To investigate whether mycophenolic acid (MPA) exerts antifibrotic effects on pterygium fibroblasts (PFB) with and without stimulation with fibrogenic cytokines, and to compare the efficacy of MPA with mitomycin (MMC) and dexamethasone (DXM) on PFB and tenon fibroblasts (TFB).

Methods TFB and PFB were obtained from tissue explants during strabismus or pterygium surgery. Proliferation of subconfluent fibroblasts±basic fibroblast growth factor (bFGF) (10 ng/ml) was assessed by using the (3H) thymidine-incorporation assay. Cell cultures were incubated with MPA, MMC or DXM. Apoptosis was evaluated by quantifying Annexin V and propidium iodide positive cells with flow cytometry.

Results MPA showed a concentration-dependent inhibition of proliferation of PFB±bFGF as well as TFB±bFGF. The antiproliferative effect of MPA was comparable with that of MMC and DXM. Short exposure of PFB to MPA under profibrogenic conditions was significantly inhibitory. No apoptotic effect was found on TFB.

Conclusions MPA suppressed tenon and pterygium fibroblast proliferation in vitro under basal and profibrogenic conditions. It was comparable with MMC under long-term exposure, but MMC was more suppressive under short-term exposure. MPA may be safer than MMC due to a more specific mechanism of action and lack of cytotoxicity. Further investigation is warranted regarding MPA concentrations that will lead to a potent antiproliferative effect in vivo.

INTRODUCTION

Pterygium is a pathological growth of fibrovascular tissue continuous with the conjunctiva and extending onto the cornea, leading to sight impairment. It is characterised by progressive stromal fibroblast proliferation, angiogenesis and inflammatory cell infiltrate.¹ Several growth factors play an important role in the pathogenesis of pterygium, such as connective tissue growth-factor (CTGF), transforming growth factor- β (TGF β) and basic fibroblast growth factor (bFGF).^{2,3}

Intraoperative application of mitomycin C (MMC) significantly reduced the recurrence rate in pterygium excision to less than 10%.¹⁻⁴ MMC is an alkylating agent, which inhibits the synthesis of DNA, RNA and protein. Reported complications included delayed corneal epithelialisation, prolonged postoperative epithelial and stromal oedema and corneal perforation.^{5,6}

The immunosuppressive drug mycophenolate mofetil (MMF; Cellcept, Roche Pharma, Basel,

Switzerland) is used nowadays in the prevention of allograft rejection⁷ and was found to have a positive effect on refractory inflammatory eye diseases.^{8,9} It acts by selectively inhibiting the production of guanosine nucleotides needed for DNA and RNA synthesis, thus inhibiting lymphocyte proliferation.¹⁰ Recent evidence suggests that MMF is also capable of inhibiting the proliferation of non-immune cells. In various cell lines, for example smooth muscle cells and fibroblasts, mycophenolic acid (MPA) reduced proliferation in response to proliferative stimuli.^{11,12} Due to its intrinsic antifibrotic properties, it is currently in clinical use in a spectrum of fibrotic conditions such as systemic sclerosis and Graft-versus-Host Disease.^{13,14}

The aim of this work was to investigate whether MPA exerts antifibrotic effects on cultured pterygium fibroblasts (PFB) and tenon fibroblasts (TFB). Proliferation index was measured using [³H]thymidine-incorporation assay under basal and profibrogenic conditions, simultaneously comparing MPA with the conventional therapies including MMC and dexamethasone (DXM). The effects on TFB necrosis and apoptosis were also assessed using annexin V/propidium iodide (PI) double staining in flow cytometric analysis. We demonstrate that MPA inhibits PFB proliferation under basal and profibrogenic conditions in the same manner as it inhibits TFB proliferation and that it is comparable with conventional accepted therapies such as MMC and dexamethasone (DXM). Moreover, MPA did not exert any cytotoxic effects on TFB.

MATERIALS AND METHODS

Materials

MPA (from *Penicillium brevicompactum* ($\geq 98\%$)), MMC (from *Streptomyces caespitosus*) and Hepes were obtained from Sigma Aldrich (St Louis, Missouri). Dulbecco modified Eagle medium (DMEM), L-glutamine solution, fetal calf serum (FCS), trypsin EDTA solution A (trypsin 0.25%, EDTA 0.02%) and penicillin–streptomycin–amphotericin B were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Nunclon flat 96-well plates and Flasks Nunclon Δ Surface were obtained from Nunc Brand Products (Roskilde, Denmark). [Methyl-³H] Thymidine was obtained from Amersham radiochemical batch analysis (TRK120, Batch 499A). Dexamethasone was obtained from Merck (Rahway, New Jersey), whereas bFGF (recombinant (*Escherichia coli*) human sequence) was from R&D systems (Minneapolis, Minnesota).

Methods

Tenon and pterygium fibroblast cell culture

Tenon samples were obtained from tissue explants from paediatric patients undergoing strabismus surgery. Pterygium samples were obtained from tissue explants from adults (age 33 to 66 years) undergoing pterygium excision. Tissue was immediately transferred into normal saline tube. Tissue samples were attached in drops of FCS and DMEM in 60 mm² tissue growth dishes overnight at 37°C. After fixation to the surface, cells were grown as monolayers in DMEM supplemented with 10% FCS, penicillin (100 units), streptomycin (100 µg), amphotericin B (0.25 µg) and 2 mM glutamine and Hepes (0.01 M). Primary cultures were passaged at confluence by trypsinisation into 75 cm² tissue flasks. For subsequent passages, cells were split 1:2 or 1:3 in DMEM with 10% FCS. Cells were grown at 37°C in a humidified incubator with 5% CO₂. Cells from passages 2 to 8 were used in all experiments.

Tenon and pterygium fibroblast proliferation assay

Proliferation of subconfluent fibroblasts was assessed by using the [³H]thymidine-incorporation assay. Fibroblasts were seeded in 96-well plates (5×10³/well) in 200 µl of supplemented DMEM/10% FCS. After overnight for TFB and after 48 h for PFB (due to relatively slow growth), medium was aspirated, and MPA/MMC/DXM were added in DMEM 2% into separate wells in increasing concentrations±bFGF (10 ng/ml). [³H]Thymidine was added for 24 h (1 µCi/well).

When PFB and TFB were both seeded in the same 96-well plates, medium was aspirated after 48 h for both types of fibroblasts.

After a total of 48 h with MPA/MMC/DXM, medium was aspirated, and trypsin was added, and the resuspended cells were collected by an Automash (Dynatech, Cambridge, Massachusetts), transferred to scintillation phials, and counted in a beta-counter. Data from [³H]thymidine-incorporation assay were expressed as counts per minute per well (cpm/well).

Pterygium proliferation assay—short exposure

PFB were seeded in 96-well plates (5×10³/well) in 200 µl of supplemented DMEM/10% FCS. After 48 h, medium was aspirated, and the fibroblasts were washed carefully with DMEM. MMC at 10⁻³ M and MPA at 10⁻³ M were added into separate wells for 5 min. Then, the supernatants were aspirated again, and the cells were carefully washed three times with DMEM and replaced with DMEM 2% with or without bFGF (10 ng/ml). [³H]Thymidine was added for 24 h (1 µCi/well). After a total of 48 h, the procedure was carried out as described above.

Detection of apoptotic cells

Cells undergoing apoptosis were identified by annexin V/PI double staining. Apoptosis was detected by the analysis of phosphatidylserine (PS) translocation to the outer leaflet of plasma membrane by flow cytometry, using annexin V conjugated with fluorescein isothiocyanate (FITC) and PI conjugated with phycoerythrin (PE). TFB were seeded in Petri dishes at the concentration of 10⁵. After gentle trypsinisation of subconfluent TFB (cultured with and without the addition of MPA for 24 h), TFB were dissociated and collected. Then, cells were washed in PBS and incubated with FITC-conjugated annexin V (5 µl) in binding buffer (0.01 M Hepes, 0.14 M NaCl, 2.5 mM CaCl₂, pH 7.4) for 15 min at 37°C in the dark. After incubation, cells were washed and resuspended in 200 µl PBS with 1% FCS, and then additionally incubated with 2 µl of a PI solution. The annexin V positive (+)/PI negative (-) cells, indicating early apoptotic

cells, and the annexin V positive (+)/PI positive (+), indicating late apoptotic cells, were detected using a FACS Calibur (BD Biosciences, San Jose, California). The results were analysed using the CellQuest software (BD Biosciences). Annexin V—FITC conjugates were detected with the FL1 channel of the FACS Calibur machine. PI—PE conjugates were read on the FL2 channel.

Statistical analysis

Results are expressed as mean±SEM. Statistical analyses on quantitative data were performed by analysis of variance (ANOVA), using the Kruskal–Wallis non-parametric test.

The p value for each variable was calculated using the Mann–Whitney test, and a Bonferroni correction applied. For all experiments, p values <0.01 were considered significant.

RESULTS

Effect of MPA, MMC and DXM on TFB proliferation with and without bFGF

MPA caused a concentration-dependent inhibition of TFB proliferation, which was significant at MPA concentrations of ≥10⁻⁷ M (p<0.01). At the maximal MPA concentration of 10⁻³ M, fibroblast proliferation was inhibited by 96.85% (reduction from a mean of 804.56 cpm/well in wells incubated only with DMEM 2% to 25.33 cpm/well).

The effect of MMC on TFB proliferation was also found to be concentration-dependent and significant at concentrations ≥10⁻⁶ M (p<0.001). For MMC, the maximal inhibition of proliferation was 98.52% at a concentration of 10⁻³ M (reduction from a mean of 804.56 cpm/well in wells incubated only with DMEM 2% to 11.88 cpm/well).

DXM was found to cause significant inhibition of proliferation at concentrations of 200 µg/ml and 400 µg/ml (p<0.001), with a reduction of 77.1% in TFB proliferation at maximal concentration (from mean of 804.56 cpm/well in wells incubated only with DMEM 2% to 184.25 cpm/well).

Figure 1 summarises the added effect of bFGF on TFB proliferation. Addition of bFGF at concentration of 10 ng/ml demonstrated an increase of 628.07% in TFB proliferation compared with cells incubated with only DMEM 2%. MPA showed a concentration-dependent inhibition of bFGF-induced TFB proliferation. The effect was significant at MPA concentrations ranging from 10⁻⁷ to 10⁻³ M. At an MPA concentration of 10⁻³ M, bFGF-induced TFB proliferation was inhibited by 99.54% (from a mean of 5053.22 cpm/well in wells with

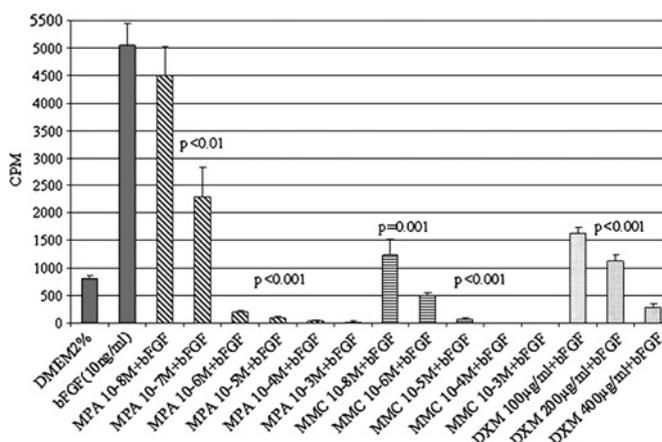


Figure 1 Effect of mycophenolic acid, mitomycin-C and dexamethasone on bFGF-induced tenon fibroblast proliferation.

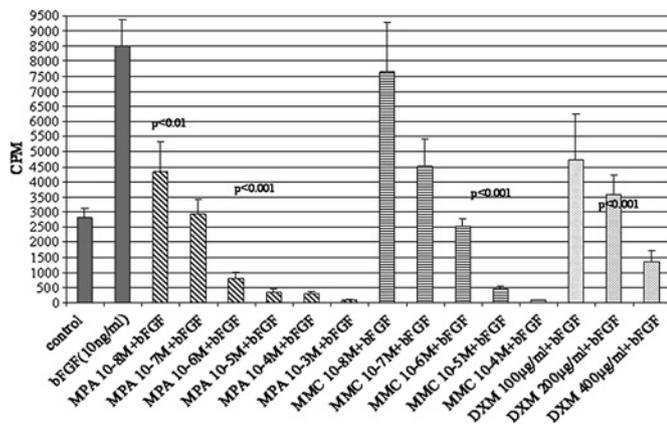


Figure 2 Effect of mycophenolic acid, mitomycin-C and dexamethasone on bFGF-induced pterygium fibroblast proliferation.

bFGF only to 23 cpm/well), with no significant change compared with MPA 10^{-3} M alone. The proliferation-inducing effect of bFGF was significant only when added to MPA concentrations of $\leq 10^{-6}$ M (mean of 109.78 cpm/well at MPA 10^{-6} M vs 203.33 cpm/well at MPA 10^{-6} M + bFGF, $p < 0.01$). Concentration-dependent inhibition of bFGF-induced TFB proliferation was also demonstrated with MMC and was significant at all concentrations. The maximal proliferation inhibition was 99.86% at concentration of 10^{-3} M (from a mean of 5053.22 cpm/well in wells with bFGF only to 7.13 cpm/well). The proliferation-inducing effect of bFGF was significant only when added to MMC concentration of 10^{-6} M (mean of 169 cpm/well at MMC 10^{-6} M vs 504.8 cpm/well at MMC 10^{-6} M + bFGF, $p = 0.001$). The addition of DXM demonstrated a significant reduction of bFGF-induced TFB

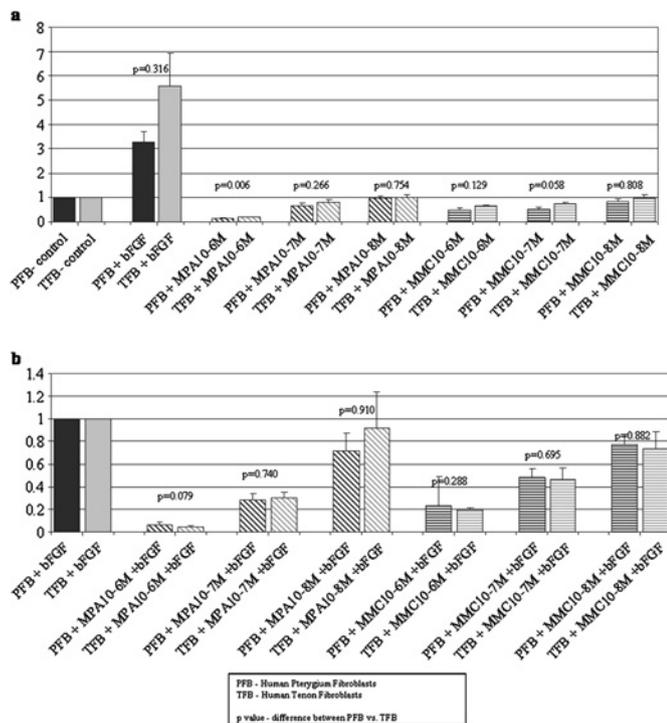
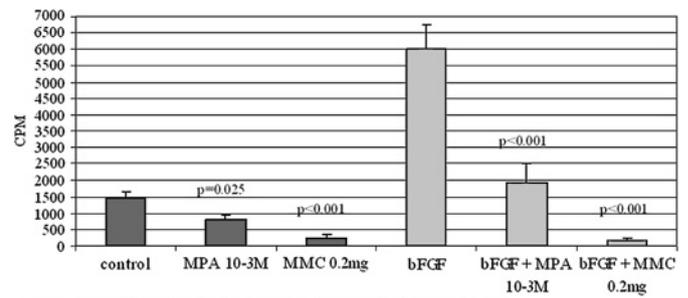


Figure 3 (A) Effect of mycophenolic acid and mitomycin-C on tenon fibroblast versus pterygium fibroblast proliferation. (B) Effect of mycophenolic acid and mitomycin-C on bFGF-induced tenon fibroblast versus pterygium fibroblast proliferation.



p value - calculated for control groups (control and bFGF) respectively.

Figure 4 Effect of short-exposure of tenon fibroblasts and pterygium fibroblasts to mycophenolic acid with and without the addition of bFGF.

proliferation at all concentrations ($p < 0.001$), with a maximal inhibition of 94.24% at DXM concentration of 400 μ g/ml (from a mean of 5053.22 cpm/well in wells with bFGF only to 291.17 cpm/well).

Effect of MPA, MMC and DXM on PFB proliferation with and without bFGF

MPA led to a concentration-dependent inhibition of PFB proliferation, which was significant at MPA concentrations of $\geq 10^{-6}$ M. At the maximal MPA concentration of 10^{-3} M, PFB proliferation was inhibited by 98.52% (from a mean of 2817.79 cpm/well in wells incubated only with DMEM 2% to 92.18 cpm/well).

The effect of MMC on PFB proliferation was also found to be concentration-dependent and significant at concentrations of $\geq 10^{-6}$ M ($p < 0.01$).

DXM was found to cause significant inhibition of proliferation only at concentrations of 200 μ g/ml and 400 μ g/ml ($p < 0.001$).

Figure 2 summarises the added effect of bFGF on PFB proliferation. The addition of bFGF demonstrated an increase of 301.97% in PFB proliferation compared with cells incubated with DMEM 2% only. MPA showed a concentration-dependent inhibition of bFGF-induced PFB proliferation. The effect was significant at all MPA concentrations. At an MPA concentration of 10^{-3} M, bFGF-induced PFB proliferation was inhibited by 98.88%. Concentration-dependent inhibition of bFGF-induced PFB proliferation was also demonstrated with MMC, and was significant at concentrations of 10^{-4} to 10^{-6} M ($p < 0.001$). DXM also demonstrated a significant reduction of bFGF-induced PFB proliferation at concentrations of 200 and 400 μ g/ml ($p < 0.001$), with maximal inhibition of 83.89% at DXM concentration of 400 μ g/ml.

Effect of MPA and MMC on pterygium versus tenon fibroblasts

To evaluate if PFB were more sensitive to the effects of MMC and MPA than TFB, both were incubated under the same conditions (figure 3). Subconfluent monolayers were incubated

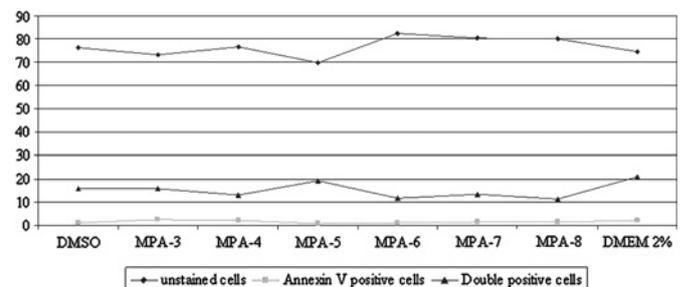


Figure 5 Effect of mycophenolic acid on the apoptosis of TFB.

with increasing concentrations of MPA and MMC with and without the addition of bFGF. Only the lower concentrations were used, as both drugs were very effective in suppressing fibroblast proliferation at higher concentrations. When comparing the two groups, there appears to be almost no statistically significant difference between the results of the TFB group and the PFB group, with the exception of the group incubated with MPA 10^{-6} M without b-FGF (87% reduction in PFB proliferation vs 80% reduction in TFB proliferation, $p=0.006$).

Pterygium proliferation assay—after short exposure to MMC and MPA

To assess the effects of short exposure (5 min) of PFB to MMC and MPA, subconfluent monolayers were washed (as described above) and exposed for 5 min to either MMC (10^{-3} M) or MPA (10^{-3} M). After a 5 min exposure, cells were washed and then incubated \pm bFGF. Results are displayed in figure 4.

In the first group (with DMEM 2% only), inhibition of PFB proliferation was 82% with MMC and 44% with MPA, and when comparing the proliferation rate with the control group, it was statistically significant for the MMC group ($p<0.001$).

In the second group (with b-FGF), MMC reduced proliferation by 96% and was statistically significant, as was MPA with reduction of 68% ($p<0.001$).

In both groups, the results strongly indicate that at the concentrations tested, MMC was significantly more effective than MPA ($p<0.001$).

Tenon fibroblast apoptosis assay

To evaluate whether MPA exerted any cytotoxic effects on TFB, cells undergoing apoptosis were identified by annexin V/PI double staining. In cells undergoing apoptosis, PS is exposed at the outer leaflet of plasma membrane. Annexin V binds specifically to PS, allowing viable cells to be distinguished from apoptotic cells. TFB were stained with annexin V, and with PI, thus allowing detection of cells with a disrupted membrane. The annexin V positive (+)/PI negative (-) cells, indicating early apoptotic cells, and the annexin V (+)/PI (+) cells, indicating late apoptotic cells, were detected by flow cytometry and quantified. As can be seen in figure 5, no increase in the percentages of early and late apoptotic cells was detected using the different MPA concentrations.

DISCUSSION

This study demonstrates a direct suppressive effect of MPA on cultured pterygium and tenon fibroblasts. This was evident by suppression of proliferation of normal and pathological fibroblasts that were cultured in the presence of MPA. An increased proliferation of the cells, triggered by activation with bFGF, was reversed and inhibited when these cells were cultured with MPA. This suppressive effect was comparable with the conventional therapies used clinically, namely MMC and DXM. No marked difference was noted between the influences of MPA on pterygium versus normal tenon fibroblasts (except for a slightly more inhibition of PFB at MPA concentration of 10^{-6} M). Short exposure of PFB to MPA under profibrogenic conditions, that is after stimulation with bFGF, was also significantly inhibitory, though this suppressive effect was not marked under basal culture conditions. MPA did not exert cytotoxic effects on TFB.

Growth factors such as bFGF and CTGF are involved in pterygium pathogenesis.^{2,3} First and foremost, this study established that MPA is an effective inhibitor of the proliferative

stimulation of bFGF on PFB and TFB. This is especially important since bFGF was described as important in recurrent pterygium.² MPA was also described to inhibit TGF β -induced cultured human mesangial cell proliferation¹⁵ and CTGF expression.¹⁶ In addition, MPA has been shown to inhibit PDGF-induced proliferation of vascular smooth muscle cells¹⁷ and to exert an inhibitory effect on IL-6 production and secretion by fibroblasts, as well as by other cell types such as endothelial cells.^{18,19} We therefore speculate that MPA could be particularly effective in inhibiting PFB proliferation, as it would downregulate most of the growth factors reported so far in its pathogenesis.

Angiogenesis plays an important role in the pathological process in pterygium.²⁰ Recent reports have demonstrated that anti-VEGF injections subconjunctivally resulted in prompt regression of conjunctival microvessels in the pterygial bed.²¹ MPA was also demonstrated to have an antiangiogenic effect.²² Domham *et al*²² reported that MPA potently inhibited in vitro endothelial cell and fibroblast proliferation, invasion/migration and endothelial cell tube formation. These works suggest that MPA may influence the inter-related factors involved in pterygium pathogenesis and thus may halt pterygium growth and facilitate vascular regression. Moreover, immunological mechanisms were described in pterygium where stromal infiltrates of plasma cells, T cells and abnormal deposits of IgE and IgG were shown.²³ MPA is a potent immunosuppressive agent that acts by selectively inhibiting inosine monophosphate dehydrogenase (IMPDH) expressed in activated lymphocytes.¹⁰ MPA has a more potent cytostatic effect on lymphocytes than on other cell types. Thus, by exerting its anti-inflammatory effects in pterygium, it may adversely affect the recruitment of inflammatory cells and the subsequent production of growth factors and angiogenic factors.

In conclusion, MPA may be a promising therapeutic agent in the management of ocular fibrotic conditions, as it was shown here to potently suppress PFB proliferation also in the presence of profibrogenic factors. It was also shown to have effects comparable with the currently available therapies like DXM and MMC. It exerted potent antiproliferative effects on PFB proliferation when applied for 48 h as well as in a profibrogenic environment when applied for only 5 min. Many reports have already described its anti-inflammatory and antiangiogenic effects. Moreover, it may be superior to MMC because of a potentially better side-effect profile due to a more specific mechanism of action and lack of cytotoxicity. Further investigations are warranted, regarding MPA concentrations that will lead to a potent antiproliferative effect in vivo.

Competing interests None.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

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