Mycophenolate mofetil inhibits rat and human mesangial cell proliferation by guanosine depletion

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Abstract

**Background.** Mycophenolate mofetil (MMF) is used for immunosuppression after renal transplantation because it reduces lymphocyte proliferation by inhibiting inosine monophosphate dehydrogenase (IMPDH) in lymphocytes and GTP biosynthesis. In the present study we asked if therapeutic concentrations of MMF might interfere with mesangial cell (MC) proliferation which is involved in inflammatory proliferative glomerular diseases.

**Methods.** Rat and human MCs were growth-arrested by withdrawal of fetal calf serum (FCS) and stimulated by addition of FCS, platelet-derived growth factor (PDGF) or lysophosphatidic acid (LPA). Different concentrations of MMF (0.019–10 µM) were added concomitantly in the presence or absence of guanosine. MC proliferation was determined by [³H]thymidine incorporation. Cell viability was assessed by trypan blue exclusion. Apoptotic nuclei were stained using the Hoechst dye H33258. Cytosolic free Ca²⁺ concentrations were determined with the fluorescent calcium chelator fura-2-AM.

**Results.** MMF inhibited mitogen-induced rat MC proliferation with an IC₅₀ of 0.45 ± 0.13 µM. Human MCs proved to be even more sensitive (IC₅₀ 0.19 ± 0.06 µM). Inhibition of MC proliferation was reversible and not accompanied by cellular necrosis or apoptosis. Addition of guanosine prevented the antiproliferative effect of MMF, indicating that inhibition of IMPDH is responsible for decreased MC proliferation. Early signalling events of GTP-binding-protein-coupled receptors, such as changes in intracellular Ca²⁺ levels were not affected by MMF.

**Conclusions.** The results show that MMF has a concentration-dependent antiproliferative effect on cultured MCs in the therapeutic range, which might be a rationale for the use of this drug in the treatment of mesangial proliferative glomerulonephritis.

Key words: glomerulonephritis; immunosuppressive therapy; inosine monophosphate dehydrogenase; mesangial cell proliferation; mycophenolate mofetil; purine synthesis

Introduction

Mycophenolate mofetil (MMF) is a new immunosuppressive agent which is effective in preventing acute allograft rejection by inhibiting lymphocyte proliferation [1–4]. In vitro studies showed that the antiproliferative effect of MMF and of its active metabolite mycophenolic acid is mediated through reversible inhibition of inosine monophosphate dehydrogenase (IMPDH), which is the key enzyme of purine synthesis in lymphocytes [5,6]. Application of MMF in a restenosis injury model of the carotid artery suggested an additional antiproliferative effect of MMF in vascular smooth-muscle cells [7].

Renal mesangial cells (MCs) are located in the pericapillary mesangial space of the glomerular capillary tuft and can be considered as specialized smooth-muscle cells [8]. During inflammatory glomerular diseases MCs take part in the disease process by enhanced secretion of growth factors, increased cell proliferation and matrix deposition leading to glomerular scarring [9,10]. In kidney biopsies of patients with mesangial proliferative glomerulonephritis, proliferation of MCs and deposition of extracellular matrix represent prominent histopathological changes [11,12]. Specific inhibition of MC proliferation combined with an anti-inflammatory mode of action could be a therapeutic approach to limit disease progression of mesangial proliferative glomerulonephritis. In the present study, we investigated the effect of MMF on DNA synthesis and proliferation of rat and human MCs in vitro. MMF was used in non-toxic, therapeutic concentrations. Our data demonstrate that MMF has a strong concentration-dependent antiproliferative effect on MCs mediated by guanosine depletion.
Methods

Reagents

Stock solutions of mycophenolate mofetil (MMF, 5 mg/ml, kindly provided by Roche, Palo Alto, USA), mycophenolic acid and guanosine (Sigma, Deisenhofen, Germany) were made by solubilization in dimethyl sulphoxide (DMSO). Potential effects of DMSO on MC growth were assessed by appropriate controls. Hoechst 33258, lysophosphatidic acid (LPA) and MCDB 302 medium were obtained from Sigma, Deisenhofen, Germany. All other cell culture materials were from Biochrom (Berlin, Germany). Human recombinant platelet-derived growth factor BB (PDGF BB) was kindly provided by Dr J. Hoppe (Biozentrum University of Würzburg, Germany). All other substances were from commercial sources and of highest analytical grade available.

Cell culture

Rat MCs were isolated, characterized, and cultured as described by Lovett et al. [13]. Cells were grown in DMEM supplemented with 2 mM L-glutamine, 5 µg/ml insulin, 4.5 g/l glucose, 100 U/ml of penicillin, and 100 U/ml streptomycin containing 10% FCS and were growth-arrested by serum deprivation in DMEM without FCS. For the experiments, MCs were used between passages 10 and 25. Human MCs were isolated and cultured as described previously [14]. Human MCs were maintained in RPMI 1640 supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 1 x non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin containing 10% FCS. Cells between passages 3 and 12 were used for experiments. Cells were growth-arrested in MCDB 302, supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 100 U/ml of penicillin and 100 U/ml streptomycin without FCS.

Human vascular smooth-muscle cells (SMCs) from the iliac artery of an organ donor were obtained from the Correll Institute for Medical research (Camden NJ). The smooth-muscle cell nature of the cultures was confirmed by immunofluorescence staining for α-actin and negative staining for factor VIII related antigen. SMCs were cultured in medium M199, enriched with 20% (FCS), 0.1 mg/ml bovine hypotalamic growth factor (ECGF), 1 mM sodium pyruvate, 0.05 mg/ml heparin, 100 U/ml penicillin and 100 U/ml streptomycin. The SMCs were used for experiments between passages 8 and 15 [15].

Cell proliferation studies

Rat MCs were seeded into 96 well plates (5000 cells/well) and grown to confluence for 24 h in DMEM with 10% FCS. Then the cells were washed once with PBS and incubated with serum-free DMEM for 24 h for growth-arrest. Human MCs (5000 cells/well) were growth-arrested in MCDB 302 medium and human vascular SMCs (5000 cells/well) were growth-arrested in serum-free medium M199 without ECGF and subsequently stimulated with different mitogens for 20 h. If not indicated otherwise MMF or mycophenolic acid (0.02–10 µM) were added together with 10% FCS. DNA synthesis was assessed by adding 37 kBq/ml [3H]thymidine (925 Gbq/mmol, Amersham, Braunschweig, Germany) for further 24 h. Thereafter, the cells were washed twice with PBS, trypsinized with 0.05% trypsin/0.02% EDTA for 10 min and harvested with a cell harvester. Incorporation of [3H]thymidine was quantified by liquid-scintillation counting.

Detection of apoptosis and necrosis of mesangial cells

Cytosolic free 

= 0.05 were considered significant.

Results

Effect of mycophenolate mofetil on PDGF-, LPA-, and FCS-stimulated mitogenesis of rat mesangial cells

The mitogenic properties of growth factors that use different signal transduction pathways were examined in growth-arrested rat MCs. Platelet-derived growth factor (PDGF, 20 ng/ml), lysophosphatidic acid (LPA, 10 µM) and fetal calf serum (FCS, 10%) increased [3H]thymidine incorporation about tenfold over basal values (Figure 1). Treatment of rat MCs with different concentrations of MMF (1.25 and 2.5 µM) produced
Fig. 1. Interference of mycophenolate mofetil with mesangial cell proliferation. Growth-arrested rat mesangial cells (MCs) were stimulated with platelet-derived growth factor (PDGF; 20 ng/ml), lysophosphatidic acid (LPA; 10 μM) or fetal calf serum (FCS; 10%) for 24 h in the presence or absence of different concentrations of mycophenolate mofetil (MMF) as indicated. (Co): untreated MCs. Proliferation was determined by [3H] thymidine incorporation during the last 4 h of incubation. Data are means ± SD of a typical experiment with six replicate measurements.

A concentration-dependent decrease of PDGF-, LPA-, and FCS-stimulated proliferation: 2.5 μM MMF, a concentration which is in the therapeutic range of this immunosuppressant caused >90% inhibition of PDGF-, LPA-, or FCS-stimulated rat MC proliferation. Similar effects were also observed with mycophenolic acid, the active metabolite of MMF (data not shown). Therefore in all subsequent experiments, 10% FCS and MMF were used for cell proliferation studies.

Concentration-dependence of the antiproliferative effect of mycophenolate mofetil on rat and human mesangial cells

A concentration-dependent inhibition of FCS-induced cell proliferation was observed in rat (Figure 2A) and human MCs (Figure 2B). In rat MCs more than 90% of cell proliferation was inhibited by therapeutically relevant concentrations between 2.5 and 10 μM MMF. The concentration of MMF for 50% inhibition of [3H] thymidine incorporation (IC50) was calculated to be 0.45 ± 0.12 μM (means ± SD, n = 3). Cell numbers were accordingly reduced by MMF (data not shown). When the IC50 of human MCs (0.19 ± 0.06 μM; n = 3) was compared with that of rat MCs, it turned out to be significantly lower (P < 0.05). Maximal inhibition of [3H] thymidine incorporation into human MCs of >90% was already present at a therapeutic concentration of 0.6 μM.

Reversibility of the antiproliferative effect of mycophenolate mofetil

The inhibitory effect of MMF on FCS-stimulated proliferation was completely reversible, when cells were first treated with MMF for 24 h and then incubated with fresh culture medium containing 10% FCS without MMF for additional 24 h (Figure 3). The ability of MCs to recover from the growth-inhibitory effect of MMF indicated that MMF was not cytotoxic. Viability of cells was also confirmed by their ability to extrude trypan blue. More than 95% of control and MMF-treated cells (10 μM) extruded trypan blue and were viable (data not shown). Possible effects of MMF on MC apoptosis were examined by staining of cell nuclei with the Hoechst dye H33258. MMF did not induce DNA fragmentation even if the highest concentration of MMF (10 μM) used for proliferation assays was administered for 24 h. The number of apoptotic cells was less than 10% under all conditions tested (data not shown).

Effect of mycophenolate mofetil on human vascular smooth-muscle cell proliferation

As MCs are closely related to SMCs, the action of MMF on FCS-stimulated growth of human SMCs was investigated. The proliferation rate of SMCs was reduced to 43 ± 8% (n = 3) at the maximal concentration of 10 μM MMF. These data indicate that the antiproliferative effect of MMF on human MCs was much more pronounced than its effect on human SMCs. The proliferation rate of human umbilical vein endothelial cells was also investigated but did not change significantly at concentrations up to 10 μM MMF (data not shown).

Dependence of the antiproliferative effect of mycophenolate mofetil on GTP-depletion

As IMPDH inhibition is the primary mechanism of action of MMF in human lymphocytes [6,11], we tested whether the antiproliferative effect of MMF in MCs was dependent on guanosine depletion. Guanosine alone did not significantly affect cell proliferation. When FCS-stimulated cell proliferation was set to 100% (7259 ± 593 c.p.m. for rat MCs and 2319 ± 118 c.p.m. for human MCs; n = 3; Figure 4A,B),
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**Fig. 2A,B.** Concentration-dependent inhibition of rat and human mesangial cell proliferation by mycophenolate mofetil. Growth-arrested rat (A) or human (B) mesangial cells (MCs) were incubated with fetal calf serum (FCS; 10%) and increasing concentrations of mycophenolate mofetil (MMF) as indicated for 24 h. Proliferation of rat MCs was determined by \[^{3}H\]thymidine incorporation in the absence of MMF was set to 100%. 100% equals 7042 ± 929 c.p.m. for rat MCs and 2555 ± 328 c.p.m. for human MCs. Data are means ± SD of three independent experiments.

**Fig. 3.** Reversibility of mycophenolate mofetil-induced inhibition of mesangial cell proliferation. Growth-arrested rat mesangial cells (MCs) were stimulated with fetal calf serum (FCS; 10%) and treated without (-) or with 0.6 μM mycophenolate mofetil (MMF) for the first (0–24 h) and/or second (24–48 h) half of the incubation period as indicated. Proliferation was determined by \[^{3}H\]thymidine incorporation during the last 4 h of a total incubation period of 48 h. Data are means ± SD of a typical experiment with six replicate measurements.

**Discussion**

Mycophenolic acid, the active metabolite of MMF, is a selective, uncompetitive reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), the key enzyme of guanine nucleotide synthesis [6, 15]. Lymphocytes are thought to be especially sensitive to proliferation with guanosine alone was 97 ± 27% for rat MCs and 100 ± 7% for human MCs. FCS-induced MC proliferation that had been inhibited by 0.6 μM MMF to 36 ± 4% in rat MCs and to 10 ± 2% in human MCs could be completely restored when guanosine was added together with MMF (92 ± 10% in rat and 93 ± 12% in human MCs). These data indicate that the growth-inhibitory effect of MMF on MCs is mediated by IMPDH inhibition.

**Effect of mycophenolate mofetil on early GTP-dependent signalling in mesangial cells**

Incubation of MCs with ATP or serotonin leads to a rapid activation of phosphatidylinositol-specific phospholipase C and subsequent release of Ca\(^{2+}\) from internal stores which is dependent on trimeric GTP-binding proteins [17]. To determine possible effects of MMF on early signalling steps in MCs, ATP- and serotonin-stimulated increase of cytosolic free calcium concentrations was determined. As an example, the ATP-mediated biphasic increase in intracellular Ca\(^{2+}\) is shown in Figure 5 (ATP 100 μM). There was no alteration when the MCs had been preincubated with MMF (10 μM) overnight. Analogous results were obtained with 1 μM serotonin (data not shown).
inflammatory processes in vivo. The antiproliferative effect of MMF is not solely dependent on the inhibition of IMPDH, but also on the capacity of cells to synthesize purine nucleotides by the salvage pathway rather than the de novo pathway of guanosine synthesis [2]. The metabolic activity of these different pathways has not yet been investigated in MCs. However, our experiments clearly show that the effect of MMF is due to GTP depletion, because addition of exogenous guanosine as source for IMPDH-independent GTP synthesis completely reversed the MMF-mediated inhibition of MC proliferation. In contrast MMF had no antiproliferative effect on human umbilical venous endothelial cells. This observation is in agreement with a previous study in which we showed that the effect of MMF on cell adhesion molecules in human endothelial cells was not reversed by addition of exogenous guanosine [21]. Both studies suggest that in endothelial cells the salvage pathway for guanosine synthesis might be more active than in vascular smooth-muscle and mesangial cells.

Although GTP depletion led to an almost complete cessation of DNA synthesis in mesangial cells, as evidenced by [3H]thymidine uptake, there was no effect on GTP-dependent signalling: preincubation of MCs with 10 μM MMF did not interfere with ATP-stimulated release of Ca2+ from internal stores, which is mediated by purinergic receptors that are coupled to trimeric GTP-binding proteins [17]. The antiproliferative effect of MMF on MCs did not depend on the mitogenic stimulus used. At about 1 μM MMF, inhibition was the same for PDGF binding to tyrosine kinase receptors as for lysophosphatidic acid, which induces proliferation by binding to receptors that couple to trimeric GTP-binding proteins [22]. These data support the notion that MMF does not critically interfere with basic cell metabolism but that its effects become evident in situations of increased IMPDH activity and enhanced demand of guanosine nucleotides.

MMF was not cytotoxic for MCs, inducing neither necrosis nor apoptosis. Instead, the inhibition of MC proliferation was fully reversible when MMF-treated MCs were subsequently restimulated with FCS. These in vitro data are in accordance with observations in vivo which showed that MMF treatment is not associated with cytotoxic effects on glomerular cells [23]. On the contrary, in this rat model of chronic allograft rejection, glomerular sclerosis and proteinuria were reduced in MMF-treated animals in comparison with the control group [23].

Compared to other non-lymphoid cells tested, human MCs proved to be especially sensitive to the inhibitory effect of MMF (IC50 about 200 nM). The therapeutically relevant blood trough levels of MMF are in the low micromolar range corresponding to 1–5 μg/ml (4.3 μg/ml = 10 μM) and are reached with a daily dosage of 2–3 g MMF in adult transplant recipients [24]. Therefore the concentrations used in our in vitro studies with MCs are within the range of clinically appropriate concentrations reached in transplant

Fig. 4A,B. Exogenous guanosine overcomes mycophenolate mofetil-mediated inhibition of mesangial cell proliferation. Growth-arrested rat (A) or human (B) mesangial cells (MCs) were stimulated with fetal calf serum (FCS; 10%) in the presence or absence of guanosine (100 μM) and/or mycophenolate mofetil (MMF; 0.6 μM) as indicated. Proliferation was determined by [3H]thymidine incorporation. Data are means ± SD of a typical experiment with six replicate measurements.

Fig. 5. Mycophenolate mofetil does not inhibit early GTP-dependent signalling steps. Rat mesangial cells (MCs) were preincubated with or without 10 μM mycophenolate mofetil (MMF) for 12 h and then stimulated with ATP (100 μM). Changes in cytosolic free Ca2+ concentrations were determined using the fluorescent calcium chelator fura-2-AM.
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recipients treated with MMF for the prophylaxis of allograft rejection.

MC hyperplasia is a key feature of many glomerular diseases, caused by immunological or other mechanisms of injury, including IgA nephropathy, membranoproliferative glomerulonephritis or lupus nephritis. Specific treatment for proliferative glomerulopathies is presently lacking. In an experimental model of lupus nephritis, Corra et al. [25] demonstrated improved survival, less proteinuria and decreased renal damage in MMF-treated animals compared with controls. Based on clinical experience with eight patients, it has also been suggested that MMF-treatment might be an alternative in the therapy of glomerular diseases of different origin [26]. These preliminary indications for the therapeutic efficacy of MMF in glomerular disease are in keeping with our in vitro results identifying human and rat MCs as sensitive targets of the antiproliferative action of MMF. Other compounds with a different mode of action such as the HMG-CoA reductase inhibitor lovastatin have been shown to inhibit mesangial cell proliferation as well [27]. It is tempting to speculate that a combination therapy using low induced Ca$^{2+}$ would minimize renal damage in proliferative glomerular diseases without causing the undesirable side effects of a high-dose monotherapy.

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