Mycophenolic acid inhibits mesangial cell activation through p38 MAPK inhibition

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Abstract

Mesangial cell (MC) proliferation and extracellular matrix (ECM) accumulation are major pathologic features of chronic renal disease including chronic allograft nephropathy (CAN). Mycophenolic acid (MPA), a potent immunosuppressant, has emerged as a treatment to prevent CAN because it inhibits MC proliferation and ECM synthesis, but the mechanism involved has not been clarified. The present study examined relative role of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (p38 MAPK) activation in inhibitory effect of MPA on MC activation. Growth arrested and synchronized primary rat MC (passages 7–11) were stimulated by PDGF 10 ng/ml in the presence and absence of clinically attainable dose of MPA (0–10 μM). Cell proliferation was assessed by [3H]thymidine incorporation, fibronectin and the activation of ERK and p38 MAPK by Western blot analysis, and total collagen by [3H]proline incorporation. PDGF increased cell proliferation by 4.6-fold, fibronectin secretion by 3.2-fold, total collagen synthesis by 1.8-fold, and the activation of ERK and p38 MAPK by 5.6-fold and 3.1-fold, respectively, compared to control. MPA, at doses inhibiting PDGF-induced MC proliferation and ECM synthesis, effectively blocked p38 MAPK activation but reduced ERK activation by 23% at maximal concentration tested (10 μM). Exogenous guanosine partially reversed the inhibition of MPA on p38 MAPK activation. Inhibitor of ERK or p38 MAPK suppressed PDGF-induced MC proliferation and ECM synthesis. In conclusion, MPA inhibits p38 MAPK activation leading to inhibiting proliferation and ECM synthesis in MC. Guanosine reduction is partially responsible for inhibitory effect of MPA on p38 MAPK activation in MC.

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Introduction

Chronic allograft nephropathy (CAN), despite the recent improvements for the short-term graft survival, remains the leading cause of graft failure after the first year following transplantation (Häyry et al., 1993; Colvin, 2003). Vascular remodeling and glomerulosclerosis are two major features of CAN (Waller and Nicholson, 2001; Shimizu et al., 2002) and glomerulosclerosis is characterized by mesangial cell (MC) proliferation and extracellular matrix (ECM) accumulation (Kashgarian and Sterzel, 1992; Johnson et al., 1992a). It is, therefore, important to effectively block MC proliferation and ECM accumulation in order to prevent the development and progression of CAN.

Mycophenolic acid (MPA), a potent immunosuppressive agent, has the potential for preventing and treating CAN (Keunecke et al., 2000; Ojo et al., 2000) because it effectively inhibits proliferation and ECM synthesis in vascular smooth muscle cell (VSMC) (Mohacsi et al., 1997; Park et al., 2004) and MC (Ziswiler et al., 1998; Hauser et al., 1999; Dubus et al., 2002, 2003) in addition to lymphocyte proliferation (Allison and Eugui, 2000). Understanding the mechanisms involved in inhibitory effect of MPA on the activation of VSMC and MC would provide a rational treatment for CAN. MPA is a selective, noncompetitive, and reversible inhibitor
of inosine monophosphate dehydrogenase (IMPDH) which is the rate-limiting enzyme in the de novo biosynthesis of guanosine nucleotide. Guanosine depletion has been, in fact, reported to be a possible mechanism that MPA inhibits MC proliferation (Ziswiler et al., 1998; Hauser et al., 1999). However, mechanisms involved in inhibiting ECM synthesis have not been reported. Unlike immune cells, the role of IMPDH in guanosine nucleoside biosynthesis of mesenchymal cells including MC is limited (Allison and Eugui, 1993). The inhibitory effect of MPA on MC activation would be connected to other mechanisms in addition to IMPDH inhibition. In this connection, we (Park et al., 2004) recently reported that MPA inhibits VSMC proliferation, in part, through inhibiting reactive oxygen species (ROS)-extracellular signal-regulated kinase 1/2 (ERK1/2) and -p38 mitogen-activated protein kinase (p38 MAPK) activation.

ERK1/2 and p38 MAPK are serine/threonine protein kinases transmit signals from the membrane to the nucleus (Seger and Krebs, 1995; Cobb, 1999). The function of MAPK is often cell type and stimuli specific. In endothelial cells, major function of ERK1/2 is to maintain cell viability while p38 MAPK plays multiple roles in controlling cell proliferation, viability, and morphogenesis during tube formation cultured in 3-dimensional collagen matrices (Yang et al., 2004). In VSMC, urokinase-induced migration depends on ERK1/2 but proliferation on both ERK1/2 and p38 MAPK (Nicholl et al., 2005), and platelet-derived growth factor (PDGF)-induced migration are regulated by both ERK1/2 and p38 MAPK but proliferation by ERK1/2 (Zhan et al., 2003). PDGF-induced MC proliferation is mainly regulated by ERK1/2 (Kawano et al., 2003). In cardiac fibroblasts, ERK1/2 stimulates but p38 MAPK inhibits mechanical load-induced procollagen α1(I) gene expression (Kimoto et al., 2004). In human retinal pigment epithelial cells ARPE-19, biochemical blockade of p38 MAPK, but not ERK1/2 activation, inhibited transforming growth factor-β2 (TGF-β2)-induced collagen I mRNA and protein synthesis (Papakri-vopoulou et al., 2004).

The present study, therefore, examined the relative role of ERK1/2 and p38 MAPK in inhibitory effect of MPA on proliferation and ECM synthesis in MC and the results were compared with the data from VSMC (Park et al., 2004). PDGF was employed to stimulate MC as an in vitro model for CAN because that PDGF, especially the PDGF-β chain, is a potent stimulator of cell proliferation and ECM accumulation (Heldin and Westmark, 1999; Floege and Johnson, 1995), that either acute rejection episode or cyclosporine treatment upregulates PDGF and its receptor expression and induces glomerulosclerosis in rat and human kidney transplantation (Savikko et al., 2002; Floege et al., 1992, 1993; Iida et al., 1991; Johnson et al., 1992b), and that PDGF antagonism inhibits glomerular disease including CAN (Ludewig et al., 2000; Johnson et al., 1992c; Ostendorf et al., 2002).

Materials and Methods

All the chemicals, unless otherwise stated, were obtained from Sigma Chemical Company (St. Louis, MO, USA). All tissue culture plastics were purchased from Becton Dickinson Labware (Lincoln Park, NJ, USA).

MC culture

Primary rat MC were obtained by culturing glomeruli isolated from kidneys of 100 to 150 g male Sprague–Dawley rats by conventional sieving methods as described previously and characterized (Oh et al., 1998; Ha et al., 2002). Cells between the 7th and 11th passages were cultured in Dulbecco’s modified Eagle’s medium (DMEM: Gibco BRL, Grand Island, NY, USA) containing 5.6 mM glucose, 20% fetal bovine serum (FBS: Gibco BRL), 100 μg/ml penicillin, and 100 μg/ml streptomycin, 44 mM NaHCO3, and 14 mM N-hydroxy-ethylpiperazine-N’-2-ethane sulfonic acid (HEPES).

Cells were cultured in 96-well plates for [3H]thymidine incorporation, 24-well plates for [3H]proline incorporation, and 6-well plates for Western blot analysis and RT-PCR. Near confluent cells were incubated with serum-free DMEM for 24 h to arrest and synchronize cell growth. Media were then replaced with fresh serum-free DMEM containing 10 ng/ml PDGF-BB, and cells were incubated for up to 48 h. MPA up to 10 μM was administered 1 h before the addition of PDGF. Guanosine 100 μM with or without MPA was administered to investigate the role of de novo synthesis of guanosine in connection with the inhibitory effect of MPA on PDGF-induced MC activation. PD98059 (Calbiochem, San Diego, CA, USA), a MEK inhibitor, and p38 MAPK inhibitor [2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one: Calbiochem] were also treated 1 h before PDGF to determine the role of ERK and p38 MAPK on PDGF-induced MC activation, respectively. Each experiment was compared with the non-PDGF treated group.

[3H]thymidine incorporation

DNA synthesis was assessed by [3H]thymidine incorporation as described (Park et al., 2004). One μCi/ml of [3H] thymidine (Du Pont Co., Wilmington, DE, USA) was added to each well for the last 12 h of the end of the experimental periods. The cells were then washed twice with PBS and trypsinized before harvesting with a cell harvester (Titertek Cell Harvester 550, Flow Laboratories, Irvine, Scotland, UK) onto glass-fiber filters (Flow Laboratories). They were placed in a 3 ml scintillation cocktail, and their radioactivities were measured by using a β-counter (TL 5000s, Beckman Instruments Inc., Fullerton, CA, USA).

[3H]proline incorporation

Total collagen synthesis was assessed by [3H]proline incorporation. One μCi/ml [3H]proline (Du Pont Co.) was added to each well for the last 12 h of the end of the experimental periods. The cells were fixed with methanol and washed twice with PBS, and then they were incubated for 10 min with 10% trichloroacetic acid (TCA) at room temperature and subsequently reacted with 0.2 N NaOH and
0.5% SDS for 30 min. Finally, all the reactions were stopped with 1 N HCl, and then harvested materials were placed in a 3 ml scintillation cocktail. Their radioactivity was measured using a β-counter (Beckman Instruments Inc.).

Western blot analysis

Fibronectin secretion and the activation of ERK1/2 and p38 MAPK were assessed by Western blot analysis as described (Park et al., 2004; Oh et al., 1998). After the experiments, the medium was collected, centrifuged to remove cell debris and used for fibronectin protein assays. For the measurement of ERK1/2 and p38 MAPK activity, the cells were washed twice with PBS and lysed in a buffer containing 1.0% Triton X-100, 10% glycerol, 20 mM Tris–HCl (pH 7.0), 137 mM sodium chloride, 5 mM EDTA, 20 μM leupeptin, 1 μg/ml aprotinin, 1 mM PMSE, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM EGTA (pH 8.0), 1 mM pyrophosphate, and 1 mM β-glycerophosphate (Park et al., 2004). Insoluble materials were removed by centrifugation.

Cellular protein was measured using a Bio-Rad protein analysis kit (Bio-Rad, Hercules, CA, USA). Protein loadings (aliquots of media or cell lysate) were normalized based on the cellular protein. Each sample was mixed with a sample buffer (12 mM Tris–HCl, pH 8.0, 0.5% glycerol, 0.4% SDS, 2.88 mM 2-mercaptoethanol, 0.02% bromophenol blue). The mixture was heated at 95 °C for 5 min. The proteins were resolved in SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with specific antibodies against ERK1/2 (1:1000: cell signaling, Beverly, MA, USA) and p38 MAPK (1:1000: cell signaling) followed by a reaction with a peroxidase conjugated secondary antibody (HRP-conjugated anti-rabbit IgG; 1:2000: Santa Cruz, CA, USA) for 1 h. The peroxidase-conjugated polyclonal rabbit anti-human fibronectin antibody (1:5000: DAKO Japan Co.) bound with primary and secondary antibody was used for the detection of fibronectin secretion into the media. Secondary antibodies were detected by using an ECL kit (Enhanced Chemiluminescence, Amersham, Buckinghamshire, UK). The activation of ERK1/2 and p38 MAPK were normalized with the ratio of phosphorylation versus total protein. Each density was quantified using the Tina 2.1 program and compared to a control (density =1).

Statistical analysis

All experimental results and measurements are expressed as means±standard deviation (SD), and statistical comparisons between two groups were evaluated by Student’s t-test, and comparisons between multiple groups were done using ANOVA followed by the Tukey method for the multiple comparison.

Results

Effects of MPA on PDGF-induced MC proliferation

MPA up to 10 μM did not affect lactate dehydrogenase release (data not shown) and basal [3H]thymidine incorporation (Fig. 1A). [3H]thymidine incorporation in rat MC cultured 48 h under serum-free condition was 57.61 ±13.25 cpm (Fig. 1A). PDGF at 10 ng/ml stimulated [3H]thymidine incorporation up to 270.72±90.10 cpm

![Figure 1](https://example.com/figure1.png)
1A), which was significantly increased than control. MPA inhibited PDGF-induced \[^{3}H\]thymidine incorporation in a dose-dependent manner (Fig. 1A). The IC\textsubscript{50} of MPA on cell proliferation was 0.1 nM ∼ 1 μM.

**Effects of MPA on PDGF-induced ECM production**

PDGF increased MC fibronectin secretion by 3.2-fold (Fig. 1B) and total collagen protein synthesis by 1.8-fold (Fig. 1C) compared to the control at 48 h. MPA at above 100 nM significantly inhibited PDGF-induced fibronectin secretion (Fig. 1B) and total collagen synthesis (Fig. 1C). MPA did not affect either basal fibronectin secretion or total collagen synthesis.

**Effects of MPA on PDGF-induced ERK1/2 and p38 MAPK activation**

PDGF increased ERK1/2 and p38 MAPK phosphorylation in MC (Fig. 2). ERK1/2 phosphorylation reached a maximal increment of 5.6-fold at 10 min (Fig. 2A) and p38 MAPK phosphorylation peaked at 3.0-fold at 5 min (Fig. 2B).

MPA up to 1 μM did not affect PDGF-induced ERK1/2 phosphorylation, and MPA at 10 μM reduced PDGF-induced ERK1/2 phosphorylation by 23% (Fig. 3), whereas PDGF-induced p38 MAPK phosphorylation was effectively inhibited by MPA in a dose-dependent manner (Fig. 4A). MPA up to 10 μM did not affect basal ERK1/2 (Fig. 3) and p38 MAPK phosphorylation (Fig. 4A). Exogenous guanosine 100 μM partially overcame the inhibition of MPA on PDGF-induced p38 MAPK activation (Fig. 4B). p38 MAPK activation in MC cultured under PDGF, guanosine, and

Fig. 2. PDGF-induced ERK1/2 (A) and p38 MAPK (B) activation in MC. After incubation of quiescent MC with 10 ng/ml PDGF for 5 min up to 48 h, ERK1/2 and p38 MAPK phosphorylation were measured as described in the text. Each upper panel shows a representative Western blot and the lower panel is relative changes presented as mean±SD of 5 experiments. *p<0.05 compared to control, †p<0.05 compared to PDGF-stimulated control.

Fig. 3. Effect of MPA on PDGF-induced ERK1/2 activation in MC. After incubation of quiescent MC with 10 ng/ml PDGF in the presence of different concentrations of MPA (0–10 μM) for 10 min, ERK1/2 phosphorylation was measured as described in the text. Upper panel shows a representative Western blot and lower panel is relative change presented as mean±SD of 5 experiments. *p<0.05 compared to control, †p<0.05 compared to PDGF-stimulated control.

MPA was still significantly lower than PDGF-induced p38 MAPK activation.

**The role of ERK and p38 MAPK on PDGF-induced proliferation and ECM production**

PD98059 (MEK inhibitor) and p38 MAPK inhibitor at concentrations which did not show any significant effect on
basal activity of MC blocked PDGF-induced \(^{3}\)H\)thymidine incorporation (Fig. 5A), fibronectin secretion (Fig. 5B), and total collagen synthesis (Fig. 5C).

**Discussion**

The significance of the present study is to provide the mechanisms involved in which MPA inhibits ECM synthesis as well as proliferation of MC. Specifically, MPA, at concentrations inhibiting p38 MAPK activation, inhibited PDGF-induced proliferation and ECM expression in rat MC, which provides p38 MAPK as a possible therapeutic target for treatment of CAN.

We first confirmed that PDGF significantly increased MC proliferation (Ziswiler et al., 1998; Hauser et al., 1999; Kawano et al., 2003) and fibronectin (Hayama et al., 2000) and collagen synthesis (Yamabe et al., 2000) compared to control. It is also well known that MPA inhibits MC proliferation and ECM synthesis (Ziswiler et al., 1998; Hauser et al., 1999; Dubus et al., 2002, 2003).

Depletion of guanosine nucleotide has been reported to be a mechanism involved in inhibitory effect of MPA on MC proliferation (Ziswiler et al., 1998; Hauser et al., 1999). Intracellular GTP contents were decreased as early as 30 min in hematopoietic cells (Gu et al., 2003), but a 48 h MPA treatment is necessary to achieve a 50% decrease in intracellular...
GTP in endothelial cells (Bertalanffy et al., 1999). Although there is no published data demonstrated kinetics of intracellular GTP in MC treated with MPA, our present data that MPA added into the media at 1 h before the addition of PDGF effectively inhibited fibronectin and collagen expression suggest that inhibitory effect of MPA may not depend on guanosine nucleotide depletion.

On the other hand, PDGF significantly increased ERK1/2 at 10 min after the addition of PDGF and p38 MAPK activation at 5 min (Fig. 2). MPA at concentration inhibiting proliferation and ECM expression effectively blocked PDGF-induced p38 MAPK phosphorylation, suggesting that p38 MAPK may be a key target with respect to the inhibitory mechanism of MPA on PDGF-induced MC activation. The observation that p38 MAPK inhibitor effectively inhibited PDGF-induced proliferation or fibronectin and collagen expression supports that the inhibitory effect of MPA would be partially due to the inhibition of p38 MAPK activation. However, MPA at 10 μM showed limited reduction of ERK1/2 activation, which remained significantly higher than the control. Partial inhibition of ERK1/2 activation by MPA together with that PD98059 prevented PDGF-induced proliferation or fibronectin and collagen expression suggests that inhibition of ERK1/2 phosphorylation may be necessary to prevent MC activation but may not be the target for inhibitory effect of MPA on MC activation. We, in contrast, observed MPA effectively inhibited both ERK1/2 and p38 MAPK activation in VSMC (Park et al., 2004). Different effects of MPA on ERK1/2 activation between MC and VSMC are in accordance with cell type-specific response of MAPK (Yang et al., 2004; Nicholl et al., 2005; Zhan et al., 2003; Kawano et al., 2000; Kimoto et al., 2004; Papakrivopoulou et al., 2004).

Small G proteins are upstream regulators of MAPK phosphorylation. It is, therefore, possible that MAPK activation would be regulated by IMPDH inhibition. In fact, MPA-induced hematopoietic cell apoptosis has been reported to be concerned with ERK and mammalian target of rapamycin (mTOR) activation through IMPDH inhibition (Gu et al., 2003). Tiazofurin (Olah et al., 1990) and ribavirin (Vallee et al., 2000), other IMPDH inhibitors, inhibit small G proteins in hepatoma and melanoma cell line, respectively. Exogenous guanosine partially rescued the inhibitory effects of MPA on p38 MAPK activation in the present study, suggesting that guanosine reduction is partially responsible for inhibitory effect of MPA on p38 MAPK activation in MC. The exact relationship between MAPK activation and IMPDH activity in MC is certainly an issue for further study.

It would be interesting to evaluate the effect of MPA on TGF-β1-induced MAPK activation and subsequent ECM protein secretion since TGF-β1 is considered as an important player in ECM synthesis (Bottinger and Bitzer, 2002), and MAPK as well as Smad play important roles in TGF-β1 signaling pathways (Rhyu et al., 2005).

The dose of MPA that inhibited PDGF-induced MC activation in the present study was at a clinically relevant concentration (Allison and Eugui, 1993) and it would be interesting to evaluate whether p38 MAPK may act as a possible therapeutic target of MPA in vivo. Recently, MPA has been used as an effective therapeutic strategy to prevent and treat proliferative glomerulonephritis in a clinical setting (Contreras et al., 2004; Briggs et al., 1998).

In conclusion, this study demonstrates that MPA may inhibit PDGF-induced MC proliferation and ECM synthesis partly through inhibiting p38 MAPK activation.

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References


