Effects of Mycophenolic Acid on the Proliferation and Endothelin-1 and Interleukin-6 Secretion of Rat Pulmonary Microvascular Endothelial Cells

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Key Words
Mycophenolic acid • Pulmonary arterial hypertension • Rat • Pulmonary microvascular endothelial cells • Endothelin-1 and interleukin-6

Abstract
Objective: To investigate the effect of mycophenolic acid (MPA) on the proliferation of rat pulmonary microvascular endothelial cells (PMVECs) and on the secretion of endothelin-1 (ET-1) and interleukin-6 (IL-6) by these cells. Methods: Rat PMVECs were treated with five different final concentrations of MPA (0, 0.1, 1, 10 and 100 μM). The fetal bovine serum-induced proliferation of the PMVECs was detected using the Cell Counting Kit-8 (CCK-8). The levels of ET-1 and IL-6 secretion were assessed by radioimmunoassay. Results: At the 24 h time point, the optical density (OD) values at 450 nm for the five groups showed that there were no significant differences between the groups treated with 0 and 0.1 μM MPA (P=0.388) or between the groups treated with 10 and 100 μM MPA (P=0.292), but the OD values were significantly different between all other pairs of groups (P<0.001). At the 48 h time point, the OD values at 450 nm for the five groups showed that there was no significant difference between the groups treated with 0 and 0.1 μM MPA (P=0.094), but the OD values were significantly different between all the other groups (P<0.001). At the 72 h time point, the OD values at 450 nm for the five groups showed that there was no significant difference between the groups treated with the final concentrations of 0 and 0.1 μM MPA (P=0.931), but the OD values were significantly different between all other pairs of groups (10 μM MPA vs 100 μM MPA: P=0.037; 0 μM MPA vs 1 μM MPA: P=0.006; 1 μM MPA vs 10 μM MPA: P=0.005; all other comparisons: P<0.001). There were no significant differences in ET-1 concentration between the groups treated with 0 and 0.1 μM MPA (P=0.156) or the groups treated with 10 and 100 μM MPA (P=0.262), but there were significant differences between all other pairs of groups (P<0.001). By contrast, there were no significant differences in IL-6 concentrations between any pair of groups (P>0.05). Conclusions: MPA inhibits fetal bovine serum-induced rat PMVEC proliferation and ET-1 secretion in a dose-dependent manner.
Introduction

Pulmonary arterial hypertension (PAH) is characterized by a progressive increase in pulmonary vascular resistance, leading to right ventricular failure and ultimately death. Vasoconstriction, remodeling of the pulmonary vessel wall, and thrombosis contribute to increased pulmonary vascular resistance in PAH [1]. The remodeling of small arteries is the main pathological finding associated with PAH, with marked proliferation of pulmonary artery smooth muscle cells (SMCs) and pulmonary endothelial cells resulting in the obstruction of pulmonary arteries [1].

Mycophenolate mofetil (MMF), the prodrug of mycophenolic acid (MPA), is an immunosuppressive agent used for treating transplant recipients and individuals with several types of connective tissue diseases. MMF may inhibit the activity of inosine monophosphate dehydrogenase (IMPDH) and interfere with de novo guanosine nucleotide synthesis, thereby suppressing lymphocyte proliferation [2]. Research on transplantation and arteriosclerosis has revealed that MPA functions effectively to inhibit the proliferation of vascular endothelial cells, vascular SMCs and fibroblasts, in addition to lymphocytes. Our previous study [3] showed that MPA can effectively inhibit the development of monocrotaline-induced PAH in rats and can alleviate thickening of the pulmonary arteriolar wall and inhibit abnormal vascular remodeling.

Because pulmonary microvascular endothelial cells (PMVECs) play a crucial role in the pathogenesis of PAH [4], we sought to investigate the effect of MPA on the proliferation of rat PMVECs and the secretion of endothelin-1 (ET-1) and interleukin-6 (IL-6).

Materials and Methods

Animals, reagents, and equipment

The animals used in this study were male Wistar rats (180-220 g body weight, Grade II). MPA was purchased from Sigma-Aldrich, Inc. The Cell Counting Kit-8 (CCK-8) was purchased from DOJINDO. The equipment used in this study included a Bio-Rad680 Enzyme mark instrument, a Leica CM2 inverted microscope, and an RMI3000S-T-VBA CO2 incubator.

PMVEC culture and identification

PMVECs were isolated and cultured from the marginal lung of Wistar rats using the tissue-sticking method. The cells were incubated at 37 °C under 5% CO₂ in DMEM-Ham's F12 medium (M&C Gene Technology) containing 20% heat-inactivated fetal bovine serum (FBS) (Sijiqing, China) supplemented with 2 mM glutamine and 1.5 g/l sodium carbonate. Cells were identified as PMVECs if they presented the typical “cobblestone morphology” and expressed factor VIII associated antigen. All of the experiments were performed using cells between passages 3 and 5.

Proliferation of PMVECs (cell counting)

The PMVECs were inoculated in 24-well plates at 20,000 cells per well and incubated in DMEM-Ham's F12/20% FBS for 24 hours. After the cells had been incubated in serum-free medium for 24 hours, we added MPA at the final concentrations of 0 and 10 μM. Each group occupied 6 wells. The number of PMVECs was counted at 0 hours (0 h), 24 hours (24 h), 48 hours (48 h) and 72 hours (72 h). The growth curves were drawn based on these data. The experiments were repeated three times, and the average growth curve was calculated.

Proliferation of PMVECs (MTT)

PMVECs were incubated in DMEM-Ham's F12/20% FBS in 24-well plates for 24 hours at 20,000 cells per well. After the cells had been incubated in serum-free medium for 24 hours, the culture medium was changed to DMEM-Ham's F12/20% FBS containing 0, 0.1, 1, 10 or 100 μM MPA. Each group occupied 16 wells. After 48 hours, the optical density (OD) was read at 490 nm using an Enzyme mark instrument.
Proliferation of PMVECs (CCK-8)

PMVECs were incubated in DMEM-Ham’s F12/20% FBS in 24-well plates for 24 hours at 20,000 cells per well. After the cells had been incubated in serum-free medium for 24 hours, the culture medium was changed to DMEM-Ham’s F12/20% FBS containing 0, 0.1, 1, 10 or 100 μM MPA. After 24 h (16 wells), 48 h (24 wells) and 72 h (16 wells), the culture medium was changed to 200 μl of serum-free medium containing 20 μl of the CCK-8 reagent for 2 hours. Then, the OD was read at 450 nm on an Enzyme mark instrument.

Assessment of ET-1 secretion

PMVECs were incubated in DMEM-Ham’s F12/20% FBS in 24-well plates for 24 hours at 20,000 cells per well. Each group occupied 16 wells. After the cells had been incubated in serum-free medium for 24 hours, the culture medium was changed to DMEM-Ham’s F12/20% FBS containing 0, 0.1, 1, 10 or 100 μM MPA, and the plates were incubated for 48 hours. Supernatant samples were collected and stored at −20°C until analysis of ET-1 secretion using a radioimmunoassay.

Assessment of IL-6 secretion

PMVECs were incubated in DMEM-Ham’s F12/20% FBS in 24-well plates for 24 hours at 20,000 cells per well. Each group occupied 16 wells. After the cells had been incubated in serum-free medium for 24 hours, the culture medium was changed to DMEM-Ham’s F12/20% FBS containing 0, 0.1, 1, 10 or 100 μM MPA for 48 hours. Supernatant samples were collected and stored at −20°C until analysis of IL-6 secretion using a radioimmunoassay.

Cytotoxic effects of MPA

The activity of lactate dehydrogenase (LDH) in the supernatants of PMVEC cultures after different treatments was measured by automated sample processing.

Statistics

Using SPSS (version 11.5), all statistical data were reported as the mean ± SD. One-way analysis-of-variance was used to detect differences between the group means for each time point. If the one-way analysis-of-variance showed a significant difference, the Least Significant Difference Procedure was used for multiple comparisons. Two-tailed p values < 0.05 were considered significant.

Results

Rat PMVEC culture and identification

The cultured rat PMVECs showed a fusiform or polygon shape in vitro, and the monolayer cultures displayed the typical cobblestone morphology (Fig. 1). The PMVECs expressed factor VIII associated antigen (Fig. 2). The primary culture of PMVECs was established successfully.

The effect of MPA on the inhibition of the PMVEC growth curves (cell counting)

The differences between PMVEC growth curves (MPA concentrations: 0 and 10 μM) based on cell counting are shown in Fig. 3.

The effect of MPA on the inhibition of the proliferation of PMVECs (MTT)

The OD values at 490 nm for the five groups (MPA concentrations: 0-100 μM) were 0.397±0.052, 0.397±0.049, 0.339±0.047, 0.206±0.030 and 0.179±0.024, respectively. There were no significant differences between the groups treated with 0 μM and 0.1 μM MPA (P=0.993) or the groups treated with 10 μM and 100 μM MPA (P=0.079), but the OD values were significantly different between all other pairs of groups (P<0.001) (Fig. 4).

The effect of MPA on the inhibition of the proliferation of PMVECs (CCK-8)

At every time point that we investigated (24 h, 48 h and 72 h) with 1-100 μM MPA, the OD values for the three groups were significantly different from the control.
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The data and statistical results are described in Fig. 5. The growth curves based on these data are shown in Fig. 6.

At the 24 h time point, the OD values at 450 nm for the five groups treated with 0-100 μM MPA were 1.151±0.200, 1.196±0.129, 0.875±0.200, 0.615±0.048 and 0.560±0.098, respectively. The statistical results showed that there were no significant differences between the groups treated with 0 μM and 0.1 μM MPA (P=0.388) or the groups treated with 10 μM and 100 μM MPA (P=0.292), but the OD values were significantly different between all other pairs of groups (P<0.001) (Fig. 5-A).

At the 48 h time point, the OD values at 450 nm for the five groups treated with 0-100 μM MPA were 2.611±0.208, 2.520±0.234, 1.989±0.166, 0.845±0.164 and 0.651±0.120, respectively. The statistical results showed that there was no significant difference between the groups treated with 0 μM and 0.1 μM MPA (P=0.094), but the OD values were significantly different between all other pairs of groups (P<0.001) (Fig. 5-B).

At the 72 h time point, the OD values at 450 nm for the five groups treated with 0-100 μM MPA were 2.617±0.237, 2.624±0.190, 2.373±0.382, 0.791±0.174 and 0.609±0.166, respectively. The statistical results showed that there was no significant difference between the groups treated with 0 μM and 0.1 μM MPA (P=0.931), but the OD values were significantly
MPA can inhibit ET-1 secretion by PMVECs

The ET-1 concentrations for the five groups treated with 0-100 μM MPA were 259.98±36.83 pg/ml, 244.65±32.65 pg/ml, 115.75±13.41 pg/ml, 58.31±20.04 pg/ml and 46.21±30.10 pg/ml, respectively. With 1-100 μM MPA, the concentrations of ET-1 were significantly different from the control group (P<0.001) and had the tendency to decrease...
with increasing MPA concentration. There were no significant differences between the groups treated with 0 μM and 0.1 μM MPA (P=0.156) or the groups treated with 10 μM and 100 μM MPA (P=0.262), but the ET-1 concentrations were significantly different between all other pairs of groups (P<0.001) (Fig. 7).

**MPA cannot inhibit IL-6 secretion in PMVECs**

The IL-6 concentrations for the five groups treated with 0-100 μM MPA were 106.60±32.41 pg/ml, 96.73±24.20 pg/ml, 96.70±30.01 pg/ml, 103.20±29.27 pg/ml and 120.70±35.88 pg/ml, respectively. There were no significant differences between any pair of groups (P>0.05) (Fig. 8).

**LDH Test**

The LDH activity remained in the normal range in all experiments, suggesting that MPA does not have a cytotoxic effect on endothelial cells (data not shown).

**Discussion**

PAH remains a difficult to treat disease. It is widely accepted that immune and/or inflammatory mechanisms could play a significant role in PAH genesis or progression, especially in patients with connective tissue diseases, including systemic scleroderma, systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), polymyositis, Sjögren syndrome, and rheumatoid arthritis [5]. More than 20% of patients with idiopathic PAH have either circulating anti-nuclear antibodies or elevated serum levels of proinflammatory cytokines (interleukin-1 and interleukin-6) [5]. Research has shown that PAH associated with SLE might respond to a treatment combining glucocorticosteroids and cyclophosphamide [5, 6] or MMF [3]. In addition to its anti-inflammatory function, MMF can attenuate the development of PAH through its anti-proliferative properties [7]. These findings provide new insight into the potential role of MMF in the treatment of PAH.

The pathogenesis of PAH involves three major processes that contribute to the narrowing of the pulmonary artery. First, vasoconstriction occurs as a result of an imbalance between vasodilators and vasoconstrictors in the pulmonary circulation. Second, vascular smooth muscle and endothelial cell proliferation results in vascular remodeling. Finally, coagulation abnormalities result in thrombosis in situ, which contributes to elevated pulmonary vascular resistance [8]. Animal experiments [3, 7] have shown that MPA effectively inhibits the development of monocrotaline-induced rat PAH and inhibits abnormal pulmonary vascular remodeling. *In vitro* experiments have revealed that MPA inhibits the proliferation of human pulmonary arterial SMCs in a dose-dependent manner. Because the endothelium also plays a crucial role in the pathogenesis of PAH, we considered that MPA might have multiple effects on endothelial cells, thus contributing to the potential efficacy of MPA as a treatment for PAH.

Abnormal cellular proliferation has been described in PAH and is likely the principal abnormality leading to occlusion of the pulmonary microvasculature and to increased pulmonary vascular resistance [4]. Pathologic features in patients with PAH are characterized by the muscularization of distal pulmonary arterioles, concentric thickening of the intima, and obstruction of the vascular lumen by proliferating endothelial cells forming plexiform lesions [9]. In this study, we used the simple method of cell counting to assay the effects of MPA on proliferation. The results showed that 10 μM MPA inhibited the growth of PMVECs. Using MTT and CCK-8 assays, we showed that MPA inhibited fetal bovine serum-induced rat PMVEC proliferation in a dose-dependent manner. No anti-proliferative properties were observed when using an MPA concentration as low as 0.1 μM. The anti-proliferative properties of MPA gradually became apparent by increasing the MPA concentration, using 1 μM, 10 μM and 100 μM MPA. To better characterize the clinical relevance, we calculated the simple SI/MPL ratio between the concentration with a significant inhibition *in vitro* (SI) and the maximum systemic plasma level (MPL) *in vivo*. Ratios below 1 indicate clinical relevance.
of the data [10]. The SI/MPL ratios from our study are all smaller than 1 (the MPL for MPA was 34 μg/ml [10]; the ratios were 0.0094, 0.094 and 0.94 when the concentrations of MPA were 1, 10 and 100 μM, respectively), indicating that the antiproliferative effects can, at least from a theoretical point of view, be achieved in a clinical setting in vivo.

Incubation with MPA at the concentrations of 0.1 μM to 100 μM had no effect on the LDH activity in the supernatants of PMVECs, indicating that the effects of MPA observed in this study were not attributable to nonspecific toxicity.

Advances in the understanding the molecular mechanisms of PAH pathogenesis suggest that microvascular endothelial dysfunction is a prominent feature of PAH [4]. Chronically impaired production of vasoactive mediators, such as nitric oxide and prostacyclin, along with prolonged overexpression of vasoconstrictors, such as ET-1, not only affect vascular tone but also promote vascular remodeling [5]. The remodeled pulmonary microvessels and plexiform lesions have increased ET-1 propeptide expression, and plasma ET-1 levels are elevated, likely from pulmonary spillover into the systemic circulation [4]. In the past two decades, novel therapies have been developed, including several endothelin receptor antagonists that offered clinical benefits [4]. In this study, MPA exposure led to a dose-dependent decrease in ET-1 levels in supernatants from cultured PMVECs, which may partially explain how MPA attenuates the development of PAH.

IL-6 was originally identified as a B-cell differentiation factor, but it is now known to be a multifunctional cytokine that regulates immune responses and inflammation [2]. Recent accumulating evidence indicates a pathologic role for IL-6 in promoting the proliferation of both smooth muscle cells and endothelial cells in the pulmonary arterioles, resulting in the development of PAH [11]. Patients with idiopathic PAH are consistently found to have an increased level of IL-6 in the circulation [12, 13] and the lung tissue [14]. Moreover, daily subcutaneous injection of rats with recombinant IL-6 increased the medial thickness of the small pulmonary arteries, leading to PAH [15]. Together, these findings suggest that PAH development is associated with IL-6 overproduction; however, in our study MPA did not inhibit fetal bovine serum-induced IL-6 secretion by PMVECs.

In previous studies, the anti-proliferative effects of MMF on human coronary endothelial cells [16] and human umbilical vein endothelial cells have been described [2]. This is the first time that the effects of MPA, an active immunosuppressive agent, on PMVECs have been investigated. The data demonstrated that clinically relevant concentrations of MPA (1 μM, 10 μM and 100 μM) have a significant antiproliferative effect and showed apparent inhibition of the secretion of ET-1 but not IL-6. IL-6 is an important inflammatory factor in PAH, and we propose that MPA could attenuate the development of PAH through its ability to inhibit vascular remodeling rather than its anti-inflammatory function. However, more experiments need to be performed to test this hypothesis.

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