



## Effects of mycophenolate mofetil on the expression of monocyte chemoattractant protein-1 and fibronectin in high glucose cultured human mesangial cells

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**ABSTRACT.** The effects of high glucose on the expression of monocyte chemoattractant protein-1 (MCP-1) and the main component of the extracellular matrix, fibronectin (FN), were explored in human mesangial cells (HMCs), along with the intervention effects of mycophenolate mofetil (MMF) on these indicators. Cultured HMCs were divided into five groups: 1) normal control group (5 mM glucose); 2) high glucose group (30 mM glucose); 3) mannitol osmotic pressure control group (5 mM glucose + 25 mM mannitol); 4) high glucose + MMF-10 group (30 mM glucose + 10 µg/mL MMF); 5) high glucose + MMF-100 group (30 mM glucose + 100 µg/mL MMF). At 24, 48, and 72 h, reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay methods were used to detect the effects of MMF on MCP-1 mRNA and protein and FN expression in HMCs under

high glucose conditions. MCP-1 mRNA and protein expressions and FN secretion significantly increased in HMCs of the high glucose group compared with the normal control group ( $P < 0.01$ ), with the highest expression observed at 48 h. MMF could reduce the MCP-1 mRNA and protein and FN expression levels ( $P < 0.01$ ), and the inhibition occurred in a dose- and time-dependent manner ( $P < 0.05$ ). In conclusion, MMF could inhibit MCP-1 expression and the secretion of FN, indicating that it may delay the progression of glomerulosclerosis and interstitial fibrosis in diabetic nephropathy to ultimately achieve protective effects on the kidney.

**Key words:** Diabetic nephropathy; Human mesangial cells; Fibronectin; Monocyte chemoattractant protein-1; Mycophenolate mofetil

## INTRODUCTION

In recent years, more and more studies have shown that inflammation is involved in the development and progression of diabetic nephropathy (DN), and that the monocyte/macrophage infiltration-mediated renal inflammatory mechanism is one of the key factors involved in the sustained development of the initial injury (Wu et al., 2011). Monocyte chemoattractant protein-1 (MCP-1) is a cytokine that has a monocyte-specific chemotactic function; it is involved in the regulation of monocyte/macrophage infiltration, and plays an important role in the pathogenesis of DN. Mycophenolate mofetil (MMF) is an immunosuppressant that was first used in the intervention of diabetes mellitus models in 2003, where it was shown to effectively suppress renal macrophage infiltration; however, the exact mechanisms are not fully understood (Utamura et al., 2003). In the present study, we evaluated the effects of high glucose on MCP-1 and fibronectin (FN) expression in human mesangial cells (HMCs). We further examined the inhibition effects of MMF on MCP-1 and FN to explore the mechanisms underlying its role in kidney protection.

## MATERIAL AND METHODS

HMCs (ID 4200) and mesangial cell medium were purchased from Science Cell (USA). Trypsin was purchased from Sigma (USA). Fetal bovine serum was purchased from Hangzhou Si Ji Qing Biological Engineering Materials Co., Ltd. The RNA extract TRIzol was obtained from Invitrogen (USA). Primers, GAPDH, markers, and the reverse transcription-polymerase chain reaction (RT-PCR) kit were purchased from Dalian Takara Biotechnology Co., Ltd. The MCP-1 and FN enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai Sen Xiong Science and Technology Industrial Co., Ltd. MMF was purchased from Shenzhen Hong Cai Xiang Gen Bio-Technology Co., Ltd.

### HMC culture

The HMCs were cultured in mesangial cell medium containing 100 mL/L fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin, and placed in a 37°C CO<sub>2</sub>-humidified incubator. The medium was changed every 2 days, and the cells were passaged every 3 to 4 days.

## Grouping

At the logarithmic growth phase, HMCs were divided into five groups: 1) normal control group (NG; 5 mM glucose); 2) high glucose group (HG; 30 mM glucose); 3) mannitol osmotic pressure control group (Man; 5 mM glucose + 25 mM mannitol); 4) high glucose + MMF-10 group (M1; 30 mM glucose + 10 µg/mL MMF); 5) high glucose + MMF-100 group (M2; 30 mM glucose + 100 µg/mL MMF). Under each culture condition, the cell supernatants and cells were collected at 24, 48, and 72 h for further use. A total of  $2 \times 10^5$  HMCs were inoculated into each well of a 6-well culture plate with three replicate wells ( $N = 3$ ) for each group.

## Total cellular RNA extraction and detection

According to the TRIzol reagent product instructions, the total cellular RNA was extracted from samples of each group at 24, 48, and 72 h. The purity and content of RNA extraction were detected with an ultraviolet spectrophotometer.

## Detection of MCP-1 mRNA expression with RT-PCR

The nucleotide sequence of the primer was based on the reference sequence from Banba et al. (2000): MCP-1 primer upstream, 5'-TCGCTCAGCCAGATGCAATCAATGC-3' and downstream, 5'-CCCAGGGGTAGAACTGTGGTTCAA-3', and the amplified fragment length was 479 bp. The GAPDH (internal reference) primers were as follows: upstream, 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and downstream, 5'-CATGTGGGCCATGAGGTCCACCAG-3', and the amplified fragment length was 983 bp. The reaction conditions of the reverse transcribed cDNA with AMV reverse transcriptase were as follows: 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min. The synthetic cDNA was MCP-1-amplified using GAPDH primers as the internal reference. The reaction conditions were as follows: 94°C pre-denaturation for 5 min, 94°C denaturation for 0.5 min, 62.2°C annealing for 0.5 min, 72°C extension for 2 min, for a total of 32 cycles, followed by extension at 72°C for 10 min. The PCR products were subjected to 20 g/L agarose gel electrophoresis, and were then absorbance scanned in the gel image analysis system (UVP; USA). The housekeeping gene GAPDH was taken as the internal reference gene, and the ratio of the target gene absorbance to GAPDH absorbance represented the relative expression levels of the target gene.

## Cell supernatant detection

MCP-1 and FN levels were determined using corresponding ELISA kits, and all protocols were in strict accordance with the kit instructions.

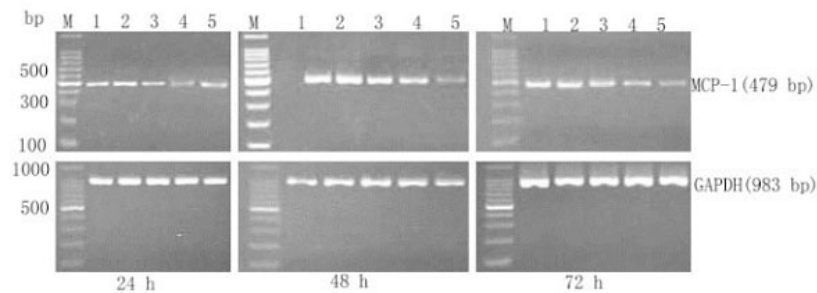
## Statistical analysis

Statistical analysis was performed using the SPSS15.0 software. The continuous variables are reported as means  $\pm$  SD (standard deviation). Analysis of variance was used for multiple group comparisons. Comparisons between two groups were conducted with the SNK-q method, and  $P < 0.05$  represented a statistically significant difference.

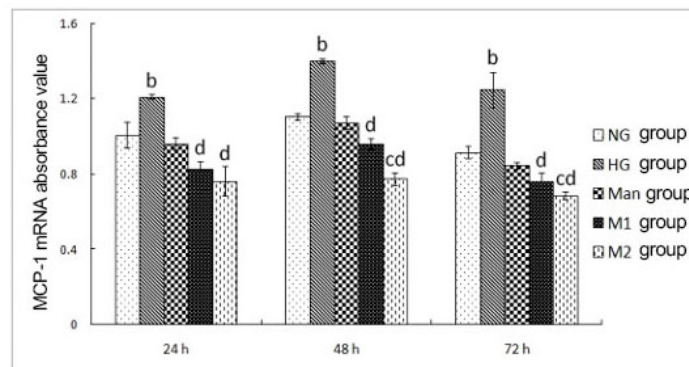
## RESULTS

### Expression of MCP-1 mRNA in HMCs

As shown in Figures 1 and 2, there was a small amount of MCP-1 mRNA expression in the NG group, whereas the expression was significantly up-regulated in the HG group. The difference between the two groups was statistically significant at each time point, with the largest difference observed at 48 h ( $P < 0.01$ ). No statistically significant difference in expression was found between the Man group and the NG group ( $P > 0.05$ ). Compared with the HG group, each MMF (M1 and M2) group showed significantly reduced expression of MCP-1 mRNA in HMCs ( $P < 0.01$ ). After 10 and 100  $\mu\text{g/mL}$  MMF were added to the cultured HMCs, the absorbance values of MCP-1 mRNA expression were as follows: after 24 h stimulation,  $0.8271 \pm 0.0432$  and  $0.7656 \pm 0.0778$ , respectively; after 48 h,  $0.9625 \pm 0.0291$  and  $0.7768 \pm 0.0336$ , respectively; after 72 h,  $0.7642 \pm 0.0431$  and  $0.6873 \pm 0.0192$ , respectively. These results showed that with increasing MMF concentrations and time, the MCP-1 mRNA expression inhibition effects were gradually strengthened; therefore, the inhibition effects showed to be significant in time- and dose-dependence ( $P < 0.05$ ).



**Figure 1.** Effects of mycophenolate mofetil on monocyte chemoattractant protein-1 (MCP-1) mRNA expression in human mesangial cells at 24, 48, and 72 h. Lane M = marker; lane 1 = NG group; lane 2 = HG group; lane 3 = Man group; lane 4 = M1 group; lane 5 = M2 group.



**Figure 2.** Absorbance values of monocyte chemoattractant protein-1 (MCP-1) mRNA at different time points. b =  $P < 0.01$  vs NG group; d =  $P < 0.01$  vs HG group; c =  $P < 0.05$  vs M1 group. NG = normal control group (5 mM glucose); HG = high glucose group (30 mM glucose); Man = mannitol osmotic pressure control group (5 mM glucose + 25 mM mannitol); M1 = high glucose + MMF-10 group (30 mM glucose + 10  $\mu\text{g/mL}$  MMF); M2 = high glucose + MMF-100 group (30 mM glucose + 100  $\mu\text{g/mL}$  MMF).

## Expression of MCP-1 protein and FN

As shown in Tables 1 and 2, after high glucose stimulation for 24, 48, and 72 h, the levels of MCP-1 protein and FN secretion increased in the HMC supernatants, and the difference was statistically significant when compared with the NG group ( $P < 0.01$ ), with the highest difference observed at 48 h. There was no statistically significant difference between the Man group and the NG group ( $P > 0.05$ ). Compared with the HG group, MCP-1 protein and FN secretion levels in each MMF group were significantly reduced ( $P < 0.01$ ). At the 48 and 72 h, the inhibition effects increased significantly with increasing MMF concentrations ( $P < 0.05$ ).

**Table 1.** Supernatant monocyte chemoattractant protein-1 (MCP-1) protein content in human mesangial cells at 24, 48, 72 h.

Group	MCP-1 protein content at different times (ng/mL)		
	24 h	48 h	72 h
NG	0.262 ± 0.028	0.341 ± 0.012	0.301 ± 0.036
HG	0.402 ± 0.031 <sup>b</sup>	0.582 ± 0.076 <sup>b</sup>	0.463 ± 0.038 <sup>b</sup>
Man	0.249 ± 0.018	0.327 ± 0.023	0.297 ± 0.043
M1	0.227 ± 0.079 <sup>d</sup>	0.303 ± 0.045 <sup>d</sup>	0.279 ± 0.037 <sup>d</sup>
M2	0.193 ± 0.033 <sup>d</sup>	0.281 ± 0.031 <sup>cd</sup>	0.237 ± 0.045 <sup>cd</sup>

Data are reported as means ± SD. <sup>b</sup> $P < 0.01$  vs NG group; <sup>d</sup> $P < 0.01$  vs HG group; <sup>c</sup> $P < 0.05$  vs M1 group. For group abbreviations, see legend to Figure 2.

**Table 2.** Supernatant FN protein content in mesangial cells of each group at 24, 48, 72 h.

Group	FN protein content at different times (ng/mL)		
	24 h	48 h	72 h
NG	72.698 ± 5.687	98.575 ± 8.246	95.020 ± 4.986
HG	120.360 ± 11.229 <sup>b</sup>	138.120 ± 5.012 <sup>b</sup>	129.173 ± 9.067 <sup>b</sup>
Man	71.017 ± 9.298	97.168 ± 9.632	93.641 ± 3.282
M1	70.187 ± 7.024 <sup>d</sup>	85.429 ± 5.201 <sup>d</sup>	79.176 ± 9.446 <sup>d</sup>
M2	63.717 ± 3.155 <sup>d</sup>	72.947 ± 9.924 <sup>cd</sup>	70.706 ± 5.747 <sup>cd</sup>

Data are reported as means ± SD. <sup>b</sup> $P < 0.01$  vs NG group; <sup>d</sup> $P < 0.01$  vs HG group; <sup>c</sup> $P < 0.05$  vs M1 group. For group abbreviations, see legend to Figure 2.

## DISCUSSION

DN is one of the most common and serious chronic complications in diabetes mellitus. A variety of pathological mechanisms are involved in the course of the disease, such as high blood glucose, lipid metabolism disorders, hemodynamic abnormalities, oxidative stress increase, and the participation of inflammatory factors. The pathology is characterized by the thickening of the basement membrane and by extracellular matrix (ECM) accumulation in the glomerular mesangial base. HMCs are the main target cells of DN pathogenic factors. Therefore, the study of changes in biological activity under high glucose is crucial for investigating the pathogenesis of DN.

MCP-1 is a member of the CC chemokine family, and can induce a large number of monocytes/macrophage cells to infiltrate in the glomeruli and renal interstitium. It can also promote the accumulation of ECM components to mediate glomerular sclerosis and interstitial fibrosis, and to promote the development of DN (Tam et al., 2009). Previous studies

confirmed that high glucose could stimulate MCP-1 expression in HMCs *in vitro*. The increase of MCP-1 would cause the aggregation and activation of macrophages, the increased release of inflammatory mediators, cytokines, and the ECM, thus accelerating glomerulosclerosis and tubule interstitial fibrosis. Furthermore, MCP-1 can promote mesangial cells to secrete FN, which is induced by nuclear factor- $\kappa$ B (NF- $\kappa$ B)- and transforming growth factor- $\beta$  (TGF- $\beta$ )-dependent mechanisms (Mezzano et al., 2004). Indeed, recent studies have shown that MCP-1 up-regulation in many kidney diseases, including DN, was activated by NF- $\kappa$ B in specific cases. High glucose can rapidly activate NF- $\kappa$ B through the generation of reactive oxygen species and protein kinase C, thereby up-regulating MCP-1 mRNA and protein expression to participate in DN injury. The inhibition of NF- $\kappa$ B activity can down-regulate the expression of MCP-1 (Soetikno et al., 2011). In experimental glomerulonephritis, TGF- $\beta$  could increase MCP-1 levels, which increases collagen deposition. Inhibition of the TGF- $\beta$  pathway could down-regulate the expression of MCP-1 mRNA to reduce fibrosis and play a role in kidney protection (Li et al., 2011).

In this study, under normal circumstances, the HMCs expressed a moderate level of MCP-1 and FN, whereas under the stimulation of high glucose, MCP-1 mRNA and protein expressions and FN secretion increased significantly, indicating that high glucose could stimulate MCP-1 expression in HMCs. Such increased expression of MCP-1 would then cause macrophage activation and aggregation, which would release the inflammatory mediators and cytokines to increase the ECM. Our results were consistent with the studies of Ide et al. (2010) and Ma et al. (2011). However, we here found that the MCP-1 mRNA and protein expressions and the FN secretion reached peak levels at 48 h, and then showed a downward trend at 72 h. The reason may be that when the HMCs were exposed to high glucose for a long period, the glucose had toxic effects on HMC metabolism, which might have induced apoptosis in some HMCs, thereby decreasing the MCP-1 mRNA and protein expressions and the FN secretion. The MCP-1 expression and FN secretion levels did not differ between the Man group and the NG group, indicating that the effects of high glucose in HMCs were not associated with osmotic pressure changes. We also found that in the normal glucose medium, the expression of MCP-1 protein and FN in HMC supernatants increased with prolonged incubation time. The effects may be associated with the increase of HMC culture time and the continuing proliferation of cells.

In this study, the *in vitro* culture of HMCs confirmed that high glucose could lead to the increased expression of the inflammation factor MCP-1 and the fibrosing factor FN, which would result in a series of inflammatory responses, glomerulosclerosis, and interstitial fibrosis to accelerate the occurrence and development of DN. These results are consistent with previous similar studies (Min et al., 2009; Li et al., 2011). We further observed the effects of MMF on the HMCs' MCP-1 expression and secretion of FN under high glucose conditions to reveal the possible mechanisms of MMF in the prevention and treatment of DN. MMF is the 2-ethyl ester derivative of mycophenolic acid, and can non-competitively and reversibly inhibit inosine monophosphate dehydrogenase and block the guanine nucleotide synthesis pathway to further block the synthesis of DNA and RNA. Its major pharmacological effects are the inhibition of lymphocyte and mononuclear cell proliferation, the reduction of antibody production, and the inhibition of cell surface adhesion molecules to achieve anti-inflammatory effects. MMF was originally used for anti-rejection treatment after organ transplantation. In recent years, MMF was also shown to play a significant role in the prevention and treatment of DN. Numerous animal experiments showed that after receiving MMF treatment, no signifi-

cant changes in blood pressure, blood glucose, or renal hemodynamics were observed in DN models, although the urinary albumin excretion and creatinine clearance rates significantly decreased, monocyte/macrophage infiltration and the ECM in renal tissues significantly decreased, and the kidney was effectively protected (Wu et al., 2006a, 2008).

This study demonstrated that MMF could effectively inhibit the MCP-1 mRNA and protein expressions in high glucose culture, which is consistent with the results of animal experiments by Wu et al. (2006b). However, our study compared different concentrations of MMF; we found that the low concentration of 10 µg/mL MMF alone could significantly inhibit MCP-1 mRNA and protein expression in high glucose culture, and could also inhibit the secretion of FN. With the high concentration of 100 µg/mL MMF and increased exposure time, the inhibition effects were significantly strengthened. MMF reduced the MCP-1 mRNA and protein expression and FN secretion in a dose- and time-dependent manner. The results of the present study suggested that MMF could inhibit the expression of MCP-1 and reduce the secretion of FN at the RNA and protein levels to ultimately reduce the inflammation and renal lesions and delay the progress of DN.

In summary, MMF could inhibit the high glucose-induced MCP-1 mRNA and protein expression in HMCs and reduce the secretion of FN from HMCs. These results indicated that MMF may reduce the accumulation of the ECM through the inhibition of MCP-1 mRNA and protein expression. It could also delay the progression of glomerulosclerosis and interstitial fibrosis in the DN to achieve the protective effects on the kidneys. The results of this study also suggested that even small doses of MMF might effectively protect the kidneys, but that the effects would be further strengthened with increasing dose and time of treatment. Our study therefore provides the basis for clinical practices, but because this treatment might increase side effects further, the relative benefits and disadvantages of the treatment should be considered.

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