Prevention of glomerular crescent formation in glomerulonephritis by mycophenolate mofetil in rats

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Abstract

Background. Glomerular crescent formation is a prominent feature of aggressive forms of glomerulonephritis (GN) and is associated with a poor prognosis. We investigated whether the potent immunosuppressive agent mycophenolate mofetil (MMF) could prevent crescent formation in a model of anti-glomerular basement membrane (GBM) GN in the rat.

Methods. GN with glomerular crescents was induced by the injection of anti-GBM antibody to female Wistar–Kyoto (WKY/NCrj) rats. The experimental rats were divided into two groups: rats received vehicle (0.5% carboxymethylcelrose) or MMF (20 mg/kg/day) orally. Body weight was measured and the urine and blood samples were evaluated. The rats were sacrificed at day 14, and histological analysis was performed. The mRNA expression of cytokines and adhesion molecules in the kidney was analysed by reverse transcription–polymerase chain reaction (RT–PCR).

Results. Marked proteinuria, glomerular crescent formation and glomerulosclerosis were observed in this model, and these were significantly reduced by MMF treatment. Marked glomerular macrophage and T-cell infiltration was also observed, and MMF treatment significantly inhibited macrophage but not T-cell infiltration. RT–PCR and immunohistochemical analysis revealed that mRNA and protein expression of osteopontin was decreased by the treatment with MMF. In addition, MMF treatment in the early stages of GN could inhibit proteinuria, glomerular crescent formation and glomerulosclerosis.

Conclusions. These findings suggest therapeutic potential for MMF in the inhibition of glomerular crescent formation in GN and provide new insights into the mechanism underlying the amelioration of crescentic GN by MMF treatment.

Keywords: glomerular crescent formation; glomerulonephritis; immunosuppressant; inflammatory cells; osteopontin

Introduction

Glomerular crescents are a feature of rapidly progressive glomerulonephritis (GN) and are associated with a poor prognosis [1]. Although the pathogenesis of glomerular crescent formation remains to be fully defined, it is likely to involve several coordinated events with cellular immune responses induced by glomerular macrophage and lymphocyte infiltration playing an important role. Indeed, depletion or blocking of these cells has been shown to reduce glomerular injury and proteinuria in experimental models of crescentic glomerulonephritis [2,3]. Furthermore, the presence of macrophages and lymphocytes within glomerular crescents suggests that these cells directly contribute to the development of irreversible scarring and subsequent end-stage renal failure.

Mycophenolate mofetil (MMF) is a potent immunosuppressive agent and has been used in the prevention of acute rejection after organ transplantation [4]. MMF is the morpholinoethyl ester of mycophenolic acid (MPA), which is a potent, non-competitive and reversible inhibitor of eukaryotic inosine monophosphate dehydrogenase, a key enzyme in de novo purine synthesis. This enzyme catalyses the conversion of inosine monophosphate into GMP and, since lymphocytes rely on the de novo pathway for production of nucleotides, MPA exerts an anti-proliferative effect on these cells by depleting the intracellular GTP pool.
In addition, MMF has been also shown to inhibit the proliferation of mesangial cells [5] as well as adhesion molecule expression by lymphocytes and endothelial cells [4]. Thus, MPA might also suppress the inflammatory cell infiltration and mesangial cell proliferation evident in the development of GN. Furthermore, recent clinical studies demonstrate that MMF has a renoprotective effect in transplanted patients with renal dysfunction induced by long-term cyclosporin A (CsA) treatment [6]. We therefore hypothesized that MMF could inhibit glomerular crescent formation and nephritic activity in an experimental model of crescentic GN.

In the present study, we investigated whether MMF could ameliorate proteinuria and glomerular crescent formation in a model of anti-glomerular basement membrane (GBM) GN in the rat. We further explored the effects of MMF on glomerular macrophage and T-cell infiltration, and on osteopontin expression. The findings obtained from this study suggest the therapeutic potential for MMF in crescentic GN and provide new insights into the mechanism underlying its action.

Materials and methods

**Experimental animals and reagents**

Twenty-five female WKY/NCrj rats (aged 7–8 weeks), purchased from Charles River Japan (Yokohama, Japan) and maintained at our animal centre, were used in this study. They had free access to standard chow and drinking water and were maintained on a 12 h light and dark cycle. All experiments in this study were performed in accordance with the Jichi Medical School Guide for Laboratory Animals.

MMF was kindly provided by Roche Bioscience (Palo Alto, CA). Monoclonal antibodies, W3/13 (T cells and granulocytes) and ED1 (macrophages) were purchased from Serotec (Oxford, UK). Polyclonal antibody raised against rat osteopontin was purchased from IBL Co. Ltd (Gunma, Japan).

**Production of monoclonal anti-GBM antibody (SR2)**

Anti-GBM antibody was prepared as described previously [7]. Briefly, WKY/NCrj rats were injected intraperitoneally (i.p.) with an emulsion of 100 μg of the non-collagenous (NC1) domain fraction of rat kidneys and Freund’s complete adjuvant (FCA), and boosted once. After development of GN, spleen cells from these rats were harvested and fused with mouse SP2/0-Ag14 melanoma cells. The monoclonal antibody, named SR2, was collected and purified from the supernatant of hybridoma cultures. The concentration of the antibody was measured by absorbance at 280 nm.

**Experimental protocol**

Anti-GBM rat GN was induced by i.p. injection of 50 μg of SR2 dissolved in GIT medium (Wako Pure Chemical Industries, Osaka, Japan). In the preliminary experiments, we observed that intravenous injection of SR2 reproducibly induced anti-GBM rat GN. In this model, glomerular crescents (both cellular and fibrous types) and glomerulosclerosis (both global and segmental types) were observed. MMF was given by gavage. Twelve rats with anti-GBM nephritis were divided into two groups just after the SR2 injection: (i) group C (n=6) rats received vehicle (0.5% carboxymethylcellulose) orally for 14 days; and (ii) group M (n=6) rats received MMF in 0.5% carboxymethylcellulose solution (20 mg/kg/day) orally for 14 days. To examine whether late MMF treatment could inhibit anti-GBM nephritis, additional groups were examined: (i) group M1 (n=3) in which MMF treatment (20 mg/kg/day) was started 1 day after the injection of SR2; and (ii) group M5 (n=4) in which MMF treatment (20 mg/kg/day) was started 5 days after the injection of SR2. Rats were sacrificed at day 14 and various parameters of the severity of nephritis were analysed together with histological changes, and compared between the two groups. The remaining rats received vehicle and were sacrificed at days 2, 4 and 7 (each, n=2) in order to evaluate the sequence of histological changes.

Body weight measurement and laboratory data analysis

Body weights were measured daily. Blood samples were collected from rat tail vein. Blood cell counts and serum levels of creatinine (Cr) were measured using an enzymatic method of SRL Inc. (Tokyo, Japan). For urinary analysis, 24 h urine samples were collected in metabolic cages (Natsume Seisakusho Co. Ltd, Tokyo, Japan), during which time rats had free access to standard chow and water. The samples were centrifuged at 3000 r.p.m. for 10 min and the urinary protein level was measured with the pyrogallol red-molybdate protein assay of SRL Inc.

**Histology**

The rats were sacrificed and perfused with saline. The kidney samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and embedded in paraffin. Sections were cut (2.5 μm) and stained with periodic acid-Schiff (PAS). Fifty glomeruli per animal were assessed, and the number of crescentic (including both cellular and fibrous types) and sclerotic glomeruli (including both global and segmental types) determined by blinded observers.

**Immunohistochemistry**

The kidney tissues perfused with saline were fixed with 4% paraformaldehyde solution in 0.1 M phosphate buffer containing 8% sucrose at 4°C for 4 h, washed with phosphate-buffered saline (PBS) containing 20 and 30% sucrose, embedded in OCT compound (Miles Laboratory, IN) and frozen in liquid nitrogen. Thin (8–10 μm) cryosections were cut for staining with ED1 and anti-osteopontin antibody. Paraffin-embedded sections were used for staining with W3/13, treated with microwaves while soaked in 10 mM citrate buffer (pH 6.0) for 10 min with the intention of antigen retrieval after dewaxing. Sections for ED1 and W3/13 were blocked with 10% bovine serum albumin (BSA)/10% goat serum and 3% H2O2 for 20 min each and incubated overnight at 4°C with primary antibody (ED1, dilution 1:500; W3/13, dilution 1:250), respectively, followed by peroxidase-conjugated anti-mouse immunoglobulin (Nichirei Corporation,
Using a standard avidin–biotin complex technique, sections for anti-osteopontin antibody were incubated overnight at 4°C with primary antibody (dilution 1:40), followed by biotin-conjugated anti-rabbit IgG (Rockland Inc., PA, dilution 1:250) for 1 h and horseradish peroxidase-labelled streptavidin (Vector Laboratories, CA, dilution 1:200) for 30 min. Immunoreactive cells were detected with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dojindo Laboratories, Kumamoto, Japan) and counterstained with haematoxylin. In this experiment, antibody W3/13-positive cells were considered as T cells because no granulocyte infiltration was observed in our anti-GBM nephritis models [7]. The number of positively stained cells in 50 glomeruli per animal was assessed by a blinded observer.

**RT–PCR analysis**

Total RNA was prepared from whole kidney tissues using the acid guanidine–phenol–chloroform method using RNA-Bee Isolation Reagent (Tel Test, Inc., Friendswood, TX). Reverse transcription–polymerase chain reactions (RT–PCRs) were performed using SuperScript One-Step with Platinum Taq (Invitrogen Co., Carlsbad, AL) and a thermal cycler (TP2000, Takara Biomedicals, Tokyo, Japan). During this analysis, the optimum number of cycles was used so as not to saturate the PCR products. The PCR conditions and primers used were as follows: forward and reverse primers for rat osteopontin were 5'-CTCGCGG TGAAAGTGGCTGA-3' and 5'-GACCTCAGAAGATGA CTCT-3', 28 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min; for monocyte chemoattractant protein-1 (MCP-1), 5'-TAT GCAGGTCTCTTGACG-3' and 5'-AAGTGTTGAACC AGGATTCAACA-3', 32 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 45 s and extension at 72°C for 45 s; for rat intercellular cell adhesion molecule-1 (ICAM-1), 5'-AGGATATCCATCCATCCCACA-3' and 5'-GCCACAG TTCTCAAAAGCACA-3', 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TATTGGGGCCGCTGGTCACA-3' and 5'-CCACCTTCTGTGTCATCA-3', 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. To determine the mRNA expression of interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12 and IL-13, reverse transcription was performed using the SuperScript First-Strand Synthesis System (Invitrogen Co.) and multiplex PCR was performed using the MPCR Kit for cytokines TH1/TH2 (Maxium Biotech Inc., CA). After PCR, an aliquot of each amplification mixture was subjected to electrophoresis on a 1.3–2.0% agarose gel, and DNA was stained with ethidium bromide. Bands were visualized and photographed by use of a Luminescent Image Analyzer (LAS-1000, Fuji Photo Film Co. Ltd) and quantified by NIH image 1.64 (Bethesda, MD).

**Statistical analysis**

Data were expressed as mean values ± SD and analysed using one-way analysis of variance (ANOVA) with Bonferroni-Dunn post hoc test and an unpaired t-test. The correlation was estimated by using simple regression analysis. Statistical analysis was performed using StatView software (Abacus Concepts Inc., CA). Differences with P < 0.05 were considered to be significant.

**Results**

**Effect of MMF treatment on urinary protein and serum Cr levels**

Figure 1 shows the urinary protein and serum Cr levels at days 0, 4 and 14 in each group. As expected, the level of proteinuria gradually increased after the injection of SR2 antibody. This increased urinary protein level was significantly decreased at day 4 (104.2 ± 45.8 vs 46.6 ± 37.0 mg/24 h, P < 0.05) and day 14 (132.1 ± 67.9 vs 51.4 ± 40.7 mg/24 h, P < 0.05) in group M compared with group C (Figure 1A). However, the serum Cr level was not increased in this model and MMF treatment showed no effect on the serum Cr level during this experiment (Figure 1B).

**Fig. 1.** Effect of MMF on urinary protein and serum Cr levels. Serial changes in 24 h urinary protein (A) and serum Cr (B) levels in group C (open bars) and group M rats (filled bars). *P < 0.05 vs group C by ANOVA.
**Effect of MMF treatment on histological change**

Representative photographs of kidney tissues in anti-GBM GN in group C and group M at day 14 are shown in Figure 2A–D. Glomerular crescent formation and glomerulosclerosis were observed as prominent features of the model. In group C, both glomerular crescent formation and glomerulosclerosis were observed frequently, whereas they were less marked in group M. As shown in Figure 2E and F, the ratios of both crescentic and sclerotic glomeruli were significantly reduced in group M (number of crescentic glomeruli, 22.33 ± 4.59 vs 9.67 ± 6.86/50 glomeruli, \( P < 0.005 \); number of sclerotic glomeruli, 19.67 ± 3.20 vs 10.33 ± 2.59/50 glomeruli, \( P < 0.01 \)). Furthermore, there was a significant correlation between the level of proteinuria and number of crescentic glomeruli (\( r = 0.653, P < 0.05 \)) or sclerotic glomeruli (\( r = 0.671, P < 0.05 \)).

**Fig. 2. Effect of MMF on glomerular crescent formation and glomerulosclerosis.** (A–D) The kidney sections in group C (A and B) and group M (C and D) were stained with PAS. Glomerular crescent formation (black arrows) and glomerulosclerosis (white arrows) were observed in group C. Bars indicate 50 μm. (E and F) The extent of glomerular crescent formation (E) and glomerulosclerosis (F) was quantified in group C (open bars) and group M (filled bars). *\( P < 0.01 \) and **\( P < 0.005 \) by unpaired t-test.
Effect of MMF treatment on expression of cytokines and adhesion molecules

To investigate the responsible factors involved in anti-GBM nephritis in the rat, we performed RT–PCR analysis to assess the expression of osteopontin, MCP-1, ICAM-1, IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12 and IL-13. As shown in Figure 4, reduced mRNA expression of osteopontin was detected in group M compared with group C. However, no significant difference was detected in the mRNA expression of other factors between the two groups (data not shown). Similarly, immunohistochemical analysis demonstrated that osteopontin expression was increased in both glomeruli and tubular epithelial cells in group C and this increase was completely prevented in group M (Figure 5A–F). In particular, osteopontin expression was detected at the site of glomerular crescents and tuft. As shown in Figure 5G, the number of glomerular osteopontin-positive cells was significantly reduced in group M compared with group C (86.3 ± 28.7 vs 47.2 ± 31.9/50 glomeruli, \( P < 0.05 \)).

Effect of late MMF treatment on urinary protein and histological change

To examine whether late MMF treatment could inhibit anti-GBM nephritis, MMF treatment was started after the injection of SR2. The urinary protein level was significantly inhibited at day 4 (104.2 ± 45.8 vs 40.7 ± 37.3 mg/24 h, \( P < 0.05 \)) and at day 14 (132.1 ± 67.9 vs 39.8 ± 31.9 mg/24 h, \( P < 0.05 \)) in group M1, compared with group C (Figure 6A). The protein level tended to decrease in group M5, but this was not significant. In addition, the ratios of both crescentic and sclerotic glomeruli were significantly reduced in group M1 (number of crescentic glomeruli, 22.33 ± 4.59 vs 12.00 ± 2.65/50 glomeruli, \( P < 0.01 \); number of sclerotic glomeruli, 19.67 ± 3.20 vs 9.33 ± 1.53/50 glomeruli,
P<0.005) (Figure 6B and C). Similarly, the number of macrophages and osteopontin-positive cells in the glomeruli was significantly reduced in group M1 compared with group C (number of macrophages, 165.0± 58.6 vs 331.2±93.1/50 glomeruli, P<0.05; number of osteopontin-positive cells, 44.0±16.6 vs 86.3±28.7/50 glomeruli, P<0.05), whereas there was no significant difference between group C and M5 (number of macrophages, 264.5±78.8; number of osteopontin-positive cells, 79.5±27.5/50 glomeruli).

Adverse effects of MMF

Several adverse effects of MMF were observed through this experiment. The haematocrit level and white blood cell counts were significantly lower in group M than group C (24.95±2.77 vs 41.55±1.20%, P<0.0001 and 4083.3±757.4 vs 9000.0±1052.6/ml, P<0.0001, respectively). The body weight increment tended to be lower in group M compared with group C, but there was no significant difference in the rate of body weight increase between the two groups (11.67± 10.79 vs 19.65± 6.40%, NS). Diarrhoea was observed in two of six rats in group M.

Discussion

The major findings of this study are that (i) MMF treatment reduced urinary proteinuria, glomerular crescent formation and glomerulosclerosis in a model of anti-GBM GN in the rat; (ii) marked glomerular infiltration of both macrophages and T cells was observed in this model, and treatment with MMF significantly inhibited macrophage infiltration but did not affect T-cell infiltration; (iii) renal expression of osteopontin mRNA and protein was increased in anti-GBM GN and this was inhibited by MMF treatment; and (iv) MMF treatment in the early stages of established GN could inhibit urinary proteinuria, glomerular crescent formation, glomerulosclerosis, macrophage infiltration and osteopontin expression. These findings suggest that MMF acts to inhibit glomerular crescent formation through the inhibition of macrophage infiltration and the therapeutic potential for MMF in crescentic GN.

Glomerular crescent formation is a prominent feature of aggressive forms of GN, and is usually associated with a poor prognosis [1]. In particular, glomerular crescent formation is often detected in patients with rapidly progressive GN. Indeed, those patients with the most severe and rapidly progressive disease typically exhibit the greatest frequency of glomerular crescent formation. Several therapeutic strategies to combat crescentic GN have been postulated. For instance, the inhibition of inflammatory cytokines such as IL-1 [8] or adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1)/leukocyte function antigen-1 (LFA-1) [9] recently have been demonstrated to be successful treatments for experimental crescentic GN. Furthermore, soluble forms of cytotoxic T lymphocyte-associated molecule-4 (CTLA4Ig) prevent the development of autoimmune glomerulonephritis [10]. These observations indicate that the cellular immune responses by macrophages and T cells contribute to the development of glomerular crescent formation. In the present study, we therefore hypothesized that the potent immunosuppressive agent MMF could prevent glomerular crescent formation.

MMF is a potent inhibitor of inosine monophosphate dehydrogenase, with a relatively selective effect on lymphocyte activation with subsequent inhibition of lymphocyte proliferation [4]. MMF has also been reported to attenuate the proliferation of mesangial cells [5] and adhesion molecule expression by lymphocytes and endothelial cells [4]. Interestingly, recent clinical studies demonstrated that conversion therapy from CsA to MMF prolongs graft survival and improves renal dysfunction in patients with organ transplantation [6]. Since nephrotoxicity is one of the major adverse effects of long-term treatment with calcineurin inhibitors such as CsA and tacrolimus (FK506), this conversion therapy has been proposed as an alternative strategy for immunosuppression in transplanted patients with renal dysfunction. In addition, it was reported that MMF significantly inhibited the proliferation of mesangial cells in vitro, and this inhibitory effect was prevented by addition of guanosine, suggesting the inhibition of inosine monophosphate dehydrogenase as a responsible mechanism [5]. Here, we demonstrated that MMF treatment diminished proteinuria, glomerular crescent formation,
glomerulosclerosis and glomerular macrophage infiltration in a model of anti-GBM GN in the rat. Consistent with our findings, there are several reports indicating that MMF treatment is of therapeutic benefit in various experimental models of kidney disease including Thy-1 nephritis [11], diabetic nephropathy [12] and remnant kidney [13]. However, the effects of MMF on glomerular crescent formation have not been investigated, and our findings suggest a new therapeutic target in renal disease for MMF treatment.

We demonstrated that MMF was effective in ameliorating established anti-GBM nephritis when MMF treatment was started 1 day, but not 5 days, after the anti-GBM antibody injection. In this regard,
Neito et al. [14] demonstrated that MMF treatment inhibited mercury-induced anti-GBM autoantibody production and prevented the development of autoimmune nephritis, but this inhibitory effect of MMF was ineffective when MMF treatment was started 9 days after the mercury injection. We further showed that late MMF treatment starting on day 1, but not on day 5, could inhibit proteinuria, glomerular crescent formation, glomerulosclerosis, macrophage infiltration and osteopontin expression, suggesting that early MMF treatment could be effective in established anti-GBM nephritis. Taken together, these findings suggest that MMF treatment in the early stages of established GN has a therapeutic potential in disease activity.

In this study, MMF treatment inhibited glomerular macrophage infiltration but did not affect glomerular infiltration by T cells in anti-GBM GN in the rat. Of note, the number of glomerular macrophages was positively correlated with the level of glomerular crescent formation, suggesting that inhibition of macrophage infiltration is the predominant mechanism underlying the prevention of glomerular crescent formation by MMF treatment. Because previous reports have shown that several factors such as adhesion molecules, chemoattractants and inflammatory cytokines play an important role in accumulation of macrophages within glomeruli [1,3], we used RT–PCR analysis to examine the expression of a number of factors including cytokines and adhesion molecules in the presence or absence of MMF treatment. Although analysis of whole kidney RNA is not sensitive to changes within the glomerulus, we found that osteopontin mRNA expression was significantly decreased in the MMF treatment group. To confirm whether mRNA expression reflects the protein expression, we analysed the protein expression by immunohistochemistry and showed that protein expression of osteopontin was also markedly decreased by the MMF treatment. Osteopontin is a secreted glycoprotein that contains an arginine–glycine–aspartate (RGD) cell adhesion sequence and is involved in the accumulation of intrarenal macrophages [15]. Consistent with our findings, several studies have demonstrated that osteopontin mediates the accumulation of the glomerular macrophages in a model of anti-GBM GN. Lan et al. [16] reported that glomerular osteopontin expression proceeded and correlated with macrophage infiltration in the development of glomerular crescent formation. Pichler et al. [17] demonstrated that osteopontin expression and macrophage infiltration were associated with interstitial fibrosis in cyclosporin nephropathy, and angiotensin II receptor antagonist inhibited these pathological changes, suggesting that angiotensin II might be one of the key regulators of osteopontin expression in the kidney. With respect to blocking experiments for osteopontin, Yu et al. [18] reported that treatment with neutralizing antibody against osteopontin resulted in a significant reduction of proteinuria and glomerular injury. In contrast, Bonvini et al. [19] demonstrated that glomerular macrophage infiltration and renal injury by anti-GBM GN in osteopontin-deficient mice were not less pronounced than those in wild-type mice. Because compensatory mechanism could participate in gene disruption mice, further investigations are required to elucidate the role of osteopontin in glomerular macrophage infiltration and the precise
mechanisms underlying the prevention of macrophage infiltration by MMF treatment.

Several clinical trials of MMF treatment in patients with nephritis have been reported recently. For instance, Choi et al. [20] evaluated the use of MMF in 46 patients with biopsy-proven primary glomerulopathies. They concluded that empirical MMF treatment in the majority of patients with primary glomerulopathies was well tolerated and achieved the goals of steroid withdrawal, improvement of the nephritic syndrome and stabilization of renal function. In this report, a small number of patients developed gastrointestinal symptoms (nausea, vomiting and diarrhoea) that resolved on dose reduction, and the development of progressive anaemia was not observed.

Since the adverse effects secondary to MMF treatment are dose dependent, the MMF dosage should be titrated carefully in order to avoid these adverse effects.

In summary, we have shown that MMF treatment reduces the level of proteinuria, glomerular crescent formation and glomerulosclerosis in a model of anti-GBM nephritis in the rat. We further demonstrated that MMF treatment inhibits glomerular macrophage infiltration and osteopontin expression. Taken together, these findings suggest the therapeutic potential of MMF in the inhibition of glomerular crescent formation in crescentic GN and provide new insights into the mechanism underlying the action of MMF treatment in crescentic GN.

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