Treatment of the post-ischaemic inflammatory syndrome of diabetic nephropathy

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Abstract

**Background.** Diabetes mellitus and its complications are a public health problem of epidemic proportions. Both diabetes and chronic kidney disease (CKD) increase the risk of acute kidney injury (AKI). Months after a single episode of acute ischaemia to the diabetic kidney, we have found an accelerated progression of nephropathy, with impaired function, severe renal inflammation, microvascular dysfunction, fibrosis and apoptotic cell death. We termed this entity the post-ischaemic inflammatory syndrome. We now test the hypothesis that blocking inflammation ameliorates the post-ischaemic inflammatory syndrome.

**Methods.** Obese–diabetic ZS rats (F\(_1\) hybrids of spontaneously hypertensive heart failure and Zucker fatty diabetic rats) were treated with mycophenolate mofetil (MMF), subjected to renal ischaemia or sham surgery, and monitored via the powerful technique of intravital microscopy.

**Results.** Amelioration of post-ischaemia inflammation with MMF therapy improved long-term renal function, microvascular dysfunction, fibrosis and apoptosis.

**Conclusion.** These data support the hypothesis that the post-ischaemic inflammatory syndrome accelerates diabetic CKD, is a critical determinant of injury, and can be successfully treated.

**Keywords:** acute kidney failure; anti-inflammatory agents; diabetic nephropathies; inflammation; ischaemia

Introduction

Diabetes and obesity are highly prevalent [1], and complicating chronic kidney disease (CKD) is now the most frequent cause of end-stage renal disease (ESRD) [2]. Tragically, nearly one-quarter of these patients die in their first year of ESRD treatment [2]. Diabetics with CKD are also at a high risk for severe acute kidney injury (AKI)/acute renal failure [3]. AKI, in turn, can accelerate CKD deterioration to ESRD [4–6].

Renal inflammation is prominent in diabetic nephropathy, and is positively correlated with functional impairment [7]. The inflammatory response includes upregulation of the leucocyte adhesion receptors intercellular adhesion molecule-1 (ICAM-1) and selectins [8]. In both humans [9] and animals [10,11], these adhesion receptors are critical in renal interstitial leucocytic infiltration. In experimental diabetic CKD, ICAM-1 is strongly expressed in the apical aspects of proximal tubules and in peritubular capillaries [12,13]. LOX-1, a multi-functional receptor for oxidized lipids and leucocytes, is also upregulated in diabetic CKD [14]. Our recent work in experimental diabetic nephropathy confirmed that this pro-inflammatory mediator contributes to renal inflammation, tubular injury and interstitial fibrosis [12]. Moreover, acute ischaemia is a critical factor in the pathophysiology of diabetic nephropathy. We have shown an acceleration of functional and structural deficits and renal inflammation long after an episode of acute renal ischaemia [15]. We named this striking long-term consequence of superimposed acute ischaemia the post-ischaemic inflammatory syndrome. Although the syndrome is not fully recognized in clinical practice, the finding of accelerated renal apoptosis in diabetic nephropathy is entirely consistent with its expression in humans [16].

We tested the hypothesis that treatment with the anti-inflammatory agent mycophenolate mofetil (MMF) would ameliorate the functional and histologic deficits of the post-ischaemic inflammatory syndrome in experimental diabetic nephropathy. We used the ZS model of metabolic syndrome: F\(_1\) hybrid rats derived from the Zucker diabetic (ZDF) and the spontaneously hypertensive heart failure (SHHF) rat. Obese–diabetic, but not lean, ZS rats develop albuminuria, glomerulosclerosis, interstitial fibrosis and renal failure, and the nephropathy is accelerated post-ischaemia [12,15,17]. We found that attenuating the post-ischaemic inflammatory syndrome with MMF improved long-term renal function and limited microvascular dysfunction, fibrosis, and apoptotic cell death after acute renal ischaemia in obese–diabetic rats. These findings underscore the critical role of inflammation in diabetic nephropathy as well as the potential for effective intervention.

Materials and methods

Animal protocols

All experiments were conducted in conformity with the ‘Guide for the Care and Use of Laboratory Animals’. Obese–diabetic male ZSF\(_1\) rats (ZS, Charles River, Wilmington, MA, USA), 8–24 weeks old, were
Post-ischaemia renal inflammation in diabetes

Fed with Purina diet 5008 with 27% protein, 17% animal fat and 56% carbohydrate. Body weights were recorded, and sera plus urine samples were collected biweekly for chemical analyses [17]. Treatment with i.p. MMF (CellCept, Roche Pharmaceuticals, Nutley, NJ, 20 mg/kg on Day 1 then 10 mg/kg/day) was initiated 2 days prior to renal ischaemia and continued for 2 weeks. Rats were anaesthetized with intraperitoneal (i.p.) pentobarbital (50 mg/kg) and placed on a homeothermic table to maintain core body temperature at ∼37°C. Renal ischaemia was induced by occluding both renal pedicles for 25 min with microaneurysm clamps as described [10]. Sham surgery was an identical surgical procedure: kidneys were exposed, but ischaemia was not induced. For intravital imaging, a small flank incision was made to expose the kidney. Systolic blood pressure was measured by tail cuff or femoral artery catheter prior to imaging.

Intravital multi-photon fluorescence microscopy

Intravital imaging was performed with a Bio-Rad MRC-1024 confocal/multi-photon microscope (Hercules, CA, USA) equipped with a titanium–sapphire laser (Spectraphysics, Mountain View, CA, USA). Rats were placed on the warmed (37°C) microscope stage. General anaesthesia was accomplished with pentobarbital (50 mg/kg) or thiobarbital (80 mg/kg) i.p. The left kidney was surgically exposed, placed in a glass bottom culture dish (Warner Instruments, Hamden, CT, USA) and bathed in warm 0.9% NaCl. Hoescht 33342 (250 μg in 0.5 mL 0.9% NaCl; Molecular Probes, Eugene, OR) was injected intravenously (i.v.) immediately prior to imaging to identify nuclei and the focal plane. Renal microvascular flow was visualized using fluorescein isothiocyanate (FITC)-conjugated 100 000 Da (large) dextran (400 μg in 0.5 mL 0.9% NaCl; Molecular Probes) injected i.v. immediately prior to imaging. A smaller (20 000 Da) Texas Red-conjugated dextran (2 mg in 0.9% NaCl i.v.) was used to assess microvascular integrity [15].

Immunohistochemistry

Sections were stained as described [18] using rabbit anti-rat LOX-1 [14] and mouse anti-rat ICAM-1 [10] followed by Texas Red-conjugated donkey anti-rabbit immunoglobulin G (IgG) and FITC-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) and the nuclear dye Dapi (Molecular Probes). Images were collected with a Zeiss anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and the nuke anti-rabbit immunoglobulin G (IgG) and FITC-conjugated donkey anti-rabbit immunoglobulin G (IgG) (Jackson Immunoresearch, West Grove, PA) and the nuclear dye Dapi (Molecular Probes). Images were collected with a Zeiss microscope equipped with a filter set appropriate for the fluorophores used. Images were analysed with MetaMorph software (Universal Imaging Corporation, Downingtown, PA).

Statistics

Data were expressed as means ± 1 standard error. Analysis of variance was used to determine if the differences among mean values reached statistical significance. Tukey’s test was used to correct for multiple comparisons. Correlations were determined using non-parametric (Spearman’s) correlation coefficient. The null hypothesis was rejected at P < 0.05.

Results

Immunosuppression limits functional impairment in the post-ischaemic inflammatory syndrome of diabetic nephropathy

Obese–diabetic ZS rats were randomly divided into four study groups: Obese–diabetic MMF-treated controls (OS MMF) underwent sham surgery. The surgically exposed kidneys in the obese–diabetic MMF-treated ischaemia group (OI MMF) were subjected to 25 min of ischaemia. These two groups were compared to their littermates, obese–diabetic sham surgery (OS) and obese–diabetic ischaemia (OI) rats given vehicle (saline) described previously [15]. Initial values for serum creatinine and blood urea nitrogen (BUN) were within normal limits for all rats at 8 weeks of age, the point of entry to the study, and 2 weeks before surgery, a laparotomy

Fig. 1. Amelioration of progressive renal dysfunction post-ischaemia with MMF. Mean serum creatinine, urea nitrogen (BUN) and urinary albumin/creatinine were significantly higher by 14 weeks post-ischaemia (24 weeks old) in obese–diabetic/ischaemia (OI) [compared to obese–diabetic/sham surgery (OS)] rats. Treatment with MMF (OI MMF) resulted in amelioration of these functional deficits. *P < 0.05; **P < 0.01 vs obese–diabetic/sham; †P < 0.05; ††P < 0.01 vs obese–diabetic/ischaemia.

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Microvascular flow/permeability/density

Renal capillary red blood cell velocity (RBCV) was measured to estimate renal capillary plasma flow rates. We used MetaMorph software to determine the displacement of intracapillary erythrocytes in sequential images with correction for microvascular angle. Vascular leak was quantified by analysing initial intravital images in 4 × 4 grids, with each grid scored for the presence or absence of small and large dextran extravasation, expressed as fractions of total grid segments [15]. Renal capillary density was quantified as pixel density representing intravascular large dextran in the initial images obtained after dextran injection.

Image scoring

All quantification was performed on coded images. Intravascular leukocytes, identified as nucleated cells within the microvasculature in intravital images, were classified as free flowing (non-adherent to vessel wall) or adherent (adherent to vessel wall for >10 s). Erythrocyte aggregates were identified as shadows of stacked red blood cells moving in unison, and were recorded as either present or absent in each quadrant of coded images. Fluorescence corresponding to immunoreactive LOX-1 and ICAM-1, fibrosis, capillary area, fraction of abnormal tubules and glomerular area were quantified using MetaMorph. Fibrosis is expressed as the fraction of the tissue area imaged as collagen. Tubules with areas of denudation, shrunken cells or intraluminal casts were classified as abnormal. Apoptotic cells were identified as those with condensed and fragmented nuclei, and expressed as fraction of total nuclei in the image.
In OI and OI MMF rats, mean serum creatinine levels increased progressively from 18 to 24 weeks of age, well after renal ischaemia. MMF provided unambiguous protection from renal failure: At 24 weeks of age, mean serum creatinine was significantly lower (P < 0.01) in the OI MMF than in the OI group. Urinary protein excretion was improved in the OI MMF as compared to the OI and in the OS MMF as compared to the OS group. Proteinuria at 12 weeks was predictive of both serum creatinine and fibrosis at study termination (r = 0.95). Body weights increased comparably in all obese–diabetic rats. Mean serum glucose was markedly elevated throughout the study with no statistically significant differences among groups. Systolic blood pressure was also higher in OI and OI MMF rats when compared to sham-operated obese–diabetic rats (Figure 2).

**Immunosuppressive effect of MMF in the diabetic kidney**

Multiple abnormalities were found in the kidneys of obese–diabetic rats using the powerful technique of intravital microscopy (Figure 3). Renal intravascular leucocytes were quantified in intravital images, expressed as leucocytes number/μ63 microscopic field (Figure 4) and confirmed in post-mortem sections. Mean number of intravascular...
renal leucocytes/field at 12 weeks of age was increased after ischaemia in the obese–diabetic rats, and the increase was ameliorated by 26% with MMF therapy. At 20 weeks of age, treatment with MMF decreased capillary leucocyte content by 75% and 53%, respectively, in the sham and ischaemia groups. Furthermore, leucocytes that adhered to vascular endothelium in vivo were identified and quantified. The number of adherent leucocytes was decreased with MMF in both the OS MMF and OI MMF rats. Consistent with in vivo data, the neutrophil number in fixed sections at study termination was also lower in the OI MMF group than in the OI group: 19.2 ± 1.4 vs 25.8 ± 3.2 cells/high-power field (hpf), respectively, P < 0.03. Total intravascular leucocyte number was highly correlated with fibrosis (r = 0.99) and serum creatinine (r = 0.96).

**Amelioration of erythrocyte aggregation with immunosuppression**

Renal capillary blood flow in rats with obesity–diabetes was distorted by the increased numbers of circulating red blood cell (RBC) aggregates. These aggregates, easily identified by intravital microscopy, were virtually nonexistent in normal (lean) rats, were increased in the
obese–diabetic rats and were markedly augmented after ischaemia, Figure 4. The number of RBC aggregates was significantly decreased with MMF in the ischaemia group.

Improvement in microvascular blood velocity with immunosuppression

To examine the consequences of inflammation and RBC aggregation, renal blood flow in peritubular capillaries was determined from direct measurements of RBC velocity (RBCV) by intravital imaging, Figures 3, 5 and 6. Initial RBCV, at 12 weeks of age and 2 weeks after surgery, was markedly depressed in both groups of post-ischaemia rats (vs sham groups). At 20 weeks of age (10 weeks after the ischaemia), RBCV decreased in all groups. However, the mean plasma flow was greater in both sham and post-ischaemia groups treated with MMF.

Improved microvascular density with immunosuppression

Impaired renal capillary RBCV was accompanied by renal microvascular attenuation in obesity–diabetes. Accordingly, the collected intravital two-photon images were used to quantify fractional intravascular fluorescence and the effect of ischaemia and immunosuppression on the integrity of the renal microvascular network (Figures 3, 5 and 6). Renal ischaemia caused an early attenuation of the microvasculature which worsened with time. This microvascular rarification post-ischaemia was prevented by treatment with MMF. The intravital data are consistent with the fractional area of capillaries in histologic sections at study termination: 65% of sham (OS) values in OI which was ameliorated with MMF (104% in OS MMF and 86% in OI MMF, all \( P < 0.01 \)). As shown in the following section, the significant pruning of the renal peritubular vasculature was associated with the markedly abnormal capillary permeability.

Reduction in abnormal microvascular integrity (permeability) with immunosuppression

Representative two-photon intravital kidney images of OS, OI, OS MMF and OI MMF are shown in Figures 3 and 5, and the data summary is in Figure 6. Prior to imaging, rats were injected with three fluorophors: Hoechst, a blue fluorescing dye to label nuclei; a 20 000-Da (small) Texas Red-conjugated dextran, a red fluorescing dye to track capillary leakage; and a 100 000-Da (large) FITC-labelled dextran, a green fluorescing dye to label intravascular spaces. Sequential images were obtained beginning \( \sim 20 \) min after injection of Hoechst and 2 min after injection of both dextrans (Figure 5). Specific fluorescence intensities for both small and large dextrans in the interstitial space were quantified with MetaMorph. The fraction of grids with interstitial leaked dextran is in Figure 6. Two weeks post-ischaemia, abnormal permeability to the smaller (red) dextran was markedly increased post-ischaemia and improved with MMF treatment. In contrast, interstitial leak of dextran was minimal in normal rats [15,20]. The leakage of the larger dextran was similarly distributed, albeit at a lower rate. Two weeks following surgery
(after recovery of renal function), leaked interstitial fluorescence of larger FITC–dextran was markedly increased in the two post-ischaemia groups. Abnormal microvascular permeability persisted throughout the study. These data demonstrate a generalized renal capillary leakage in obesity–diabetes which was further aggravated by ischaemia. The changes were prevented, in part, with MMF.

**Decreased expression of the pro-inflammatory receptors ICAM-1 and LOX-1 with immunosuppression**

The pro-inflammatory adhesion receptors ICAM-1 and LOX-1 are critical to leucocyte recruitment in tissue injury and inflammation. Immunoreactive ICAM-1 and LOX-1 were barely detectable in control (lean rat) kidneys, but both were markedly increased in obese–diabetic rats [12]. Both pro-inflammatory receptors (Figure 7) were further increased months after ischaemia in obese–diabetic rats (OI, 2.02 ± 0.26- and 2.05 ± 0.11-fold increase over OS in ICAM-1 and LOX-1 respectively, both P < 0.01). However, post-ischaemia expression of ICAM-1 and LOX-1 were both significantly attenuated in the MMF-treated rats (OI MMF 1.12 ± 0.12- and 1.47 ± 0.08-fold over OS, P < 0.001 vs OI). LOX-1 and ICAM-1 expression were each positively correlated with fibrosis, creatinine and proteinuria (Table 1).

**Improvement in fibrosis with immunosuppression**

In both humans [21–23] and animal models [14], renal interstitial fibrosis is closely associated with loss of renal function and progressive diabetic nephropathy, and can serve as an early indicator of response to therapy [14]. We followed interstitial fibrosis in vivo via second harmonic imaging of renal collagen (Figure 8). Interstitial fibrosis was also quantified in trichrome-stained sections obtained at study termination. At 20 weeks of age in the post-ischaemia rats, renal fibrosis was significantly attenuated by MMF treatment. Fibrosis at study termination, consistent with quantification by second harmonic imaging, was significantly less in MMF-treated groups.

![Fig. 8. Reduced fibrosis with immunosuppression. Trichrome staining of kidney sections removed 22 weeks after surgery shows peritubular fibrosis (dark) with increased interstitial cellularity in OS rats (A) with further increases in the OI group (B). Improvement was seen with MMF in the sham surgery (OS MMF, C) and ischaemia (OI MMF, D) groups. Lower panels show second harmonic imaging of collagen in the same groups. Quantification of fractional area representing fibrosis is shown in E. Arrow indicates the degree of fibrosis in lean, non-diabetic controls. *P < 0.05, **P < 0.01 vs obese–diabetes/sham; ‡‡P < 0.01, ‡‡‡P < 0.001 vs obese–diabetes/ischaemia. Please see supplementary material (http://ndt.oxfordjournals.org) for colour figure.](image-url)
In addition, the fraction of abnormal tubules and glomerular hypertrophy were decreased in the groups treated with MMF (Table 2).

Reduction in cell death with immunosuppression

Renal tubular cell apoptosis is thought to be a key factor in tubular atrophy and tubulointerstitial fibrosis, and thus renal failure in diabetic nephropathy [16,24–26]. Elevated glucose, oxidized lipids and inflammation result in apoptosis in cultured cells [27–29]. Clinically, renal tubular apoptosis in biopsy specimens is correlated with later functional loss [16]. Thus, apoptosis was quantified in OS, OI, OS MMF and OI MMF kidneys. In intravital renal images (Figure 9), marked increases in condensed fragmented nuclei consistent with apoptosis in obesity–diabetes were observed 2 weeks after ischaemia and increased with time. MMF dramatically limited apoptotic cell death in the diabetic kidney, so that, at 20 weeks of age, apoptosis in the OI MMF group was not only significantly less frequent than in the OI group but also less frequent than in the untreated OS group.

Discussion

Inflammation and ischaemia are critical components of progressive diabetic nephropathy; superimposed acute renal injury can result in CKD or ESRD [4–6,30]. The mechanism of this progressive and accelerated renal decline is unknown. We have demonstrated that a single episode of acute renal ischaemia causes long-term self-sustained renal inflammation and fibrosis, key features of diabetic kidney disease [15]. This unique model is characterized by renal failure, the hallmark of human diabetic nephropathy.

We hypothesized that anti-inflammatory treatment would limit renal injury post-ischaemia. Accordingly, we examined the effect of the immunosuppressive agent MMF on renal function, inflammation, microvascular dysfunction, apoptosis and fibrosis in obese–diabetic rats with nephropathy. We focused on four groups of rats at risk: obese–diabetic/sham (OS), obese–diabetic/ischaemia (OI), obese–diabetic/sham MMF (OS MMF) and obese–diabetic/ischaemia MMF (OI MMF). We have included the OS and OI rat littermates [15] for straightforward comparisons with the MMF-treated obese–diabetic rats. Body weights and serum glucose levels were markedly elevated and comparable in the four obese–diabetic groups. Serum creatinine and urea increased progressively weeks after the single ischaemic episode in OI rats; MMF therapy blunted

![Figure 9](http://ndt.oxfordjournals.org)
their elevation. Proteinuria was highest in OI; MMF also limited proteinuria post-ischaemia and in OS rats. Renal inflammation was prominent in obese–diabetic rats, increased further weeks after ischaemia, and was improved significantly by MMF treatment. Increases in the pro-inflammatory receptors, ICAM-1 and LOX-1, were also more pronounced in the post-ischaemia group; LOX-1 expression was significantly attenuated by MMF therapy in the post-ischaemia group.

Leucocyte–endothelial adhesion and mediators released from WBC can cause microvascular abnormalities thought critical in diabetic nephropathy. Renal microvascular dysfunction in obese–diabetic rats was manifested by microvascular leak and reduced peritubular capillary blood flow. These critical abnormalities were exacerbated post-ischaemia. The renal peritubular capillary network was also attenuated, and fibrosis increased post-ischaemia. Microvascular abnormalities and fibrosis were improved by MMF. Tubular cell apoptosis was markedly increased after ischaemia, and MMF dramatically decreased apoptosis by ~50%. Peritubular capillary loss and interstitial fibrosis can result in an ongoing cycle of hypoxia/ischaemia. Interstitial hypoxia is one final pathway of renal failure in both tubular and glomerular diseases [31].

MMF has significant anti-inflammatory effects and is protective in experimental diabetic nephropathy. Renal injury in diabetes results from the interaction of multiple factors including metabolic abnormalities, the renin–angiotensin system (RAS) and other mediators. Despite important therapeutic advances in blood glucose control and RAS blockade, diabetic nephropathy remains the leading cause of ESRD in many countries. Thus, slowing of progressive diabetic nephropathy with MMF would have therapeutic implications. We previously documented that the renal protective anti-inflammatory dose of MMF employed herein did not alter the key parameters of the metabolic syndrome [17]. We suggest, based on that earlier work and the current data, that the renal-protective actions of MMF post-ischaemia were likely systemic and local. For example, lower leucocyte counts and lower levels of the chemotactic factor CINC-1 [17], as well as interference with T and B lymphocytes [32], were likely beneficial. In addition, it is likely that MMF acted directly on renal cells [33] or on inflammatory cells within the kidney. These conclusions are supported by our previous report of a complex renal post-ischaemia syndrome that involves production of several cytokines, including interleukin (IL)-1 and tumour necrosis factor-α [11]. The chemotactic cytokine KC [34], the mouse version of rat CINC-1 [35], is also increased post-ischaemia.

In the nephropathy of obesity–diabetes complicated by ischaemia, i.e. in ‘acute on chronic’ renal failure [15], synergistic and complex inflammatory responses, perhaps emanating from chronic and acute ischaemia, converge and promote a self-sustained degrading inflammatory/fibrotic process that results in progressive renal failure and organ loss. Microvascular injury is characterized by leakage from renal capillaries and rarefaction. A single episode of renal ischaemia in obesity/diabetes results in accelerating inflammation and has long-term consequences, mainly a more aggressive form of nephropathy. Decreasing inflammation with MMF improves multiple parameters, including renal function, in the post-ischaemia inflammatory syndrome of diabetic nephropathy. These data support the hypothesis that inflammation is critical in renal dysfunction in experimental diabetes post-ischaemia. The longevity of the process and the sensitivity to anti-inflammatory therapy create therapeutic opportunities that may favourably modify an otherwise dismal outcome.

**Abbreviations**

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<tr>
<td>AKI</td>
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<td>CKD</td>
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<td>ESRD</td>
<td>end-stage renal disease</td>
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<td>FITC</td>
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<td>ICAM-1</td>
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<td>ZS</td>
<td>Zucker/spontaneously hypertensive heart failure rat hybrids (F1)</td>
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**Conflict of interest statement.** None declared.

**Supplementary Data**

Supplementary data is available online at http://ndt.oxfordjournals.org.

**References**

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