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Effects of mycophenolate mofetil on acute ischaemia–reperfusion injury in rats and its consequences in the long term

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Abstract

Background. Renal ischaemia–reperfusion injury (IRI) acutely decreases glomerular filtration rate (GFR) and impairs kidney function in the long term. Pre-treatment with chaetomellic acid (KM), an inhibitor of membrane-bound Ha-Ras, has demonstrated beneficial effects on acute renal ischaemia.

Methods. We tested whether mycophenolate mofetil (MMF, 20 mg/day for 4 days before IRI), an immunosuppressor with anti-inflammatory properties, improved renal outcome in uninephrectomized rats after IRI (45 min of renal ischaemia), alone or in combination with KM.

Results. One day after ischaemia, GFR was markedly depressed in untreated rats (−75% *vs.* normal rats, $P < 0.001$), and pre-treatment with MMF did not modify this fall (−75%, $P < 0.001$ *vs.* normal). KM (0.23 μg/kg before IRI) greatly prevented GFR loss (−39% *vs.* normal, $P < 0.05$), but its action was not further improved by the combined administration with MMF (GFR, −45% *vs.* normal, $P < 0.05$). MMF significantly reduced ICAM-1 expression and monocyte recruitment ($P < 0.05$ *vs.* untreated rats);

nevertheless, renal histology of MMF rats was similar to that of untreated rats. Additional rats were examined 6 months after IRI: untreated rats with IRI showed reduced renal function (−42% *vs.* normal, $P < 0.01$) and proteinuria ($P < 0.001$ *vs.* normal); rats pre-treated with MMF showed a similar pattern, whereas rats treated with KM before IRI presented a better GFR (−20% *vs.* normal, not significant) and near-normal values of proteinuria. The combination of KM + MMF gained the same results.

Conclusions. Pre-treatment with MMF before IRI does not confer functional or morphological protection to the kidney, despite the reduced expression of some inflammatory markers. The combination of MMF + KM does not offer additional advantages to solitary KM treatment.

Keywords: chaetomellic acid; ischaemia–reperfusion injury; mycophenolate mofetil; Ras; rat

Introduction

Renal ischaemia–reperfusion injury (IRI) represents the main determinant of delayed graft function (DGF) in kidney transplantation, which occurs in ~25% of kidneys from deceased donors. IRI triggers an intense inflammatory response mediated by leukocyte recruitment, increased expression of adhesion molecules and synthesis of cytokines that cause cellular damage [1], and may increase the immunogenicity of exposed tissues [2].

Substantial evidence has accumulated from both experimental studies and clinical outcome analyses that IRI *per se* can have an important negative impact on long-term graft function, also in living donor transplants, when the surgical procedures can be planned with attention; indeed, although it is commonly believed that an acute ischaemic insult to the kidney results in complete recovery of renal function, there is increasing experimental evidence that the early deregulation of some genes at the time of ischaemia may determine deep modifications in renal function and morphology a long time after ischaemia [3], resulting in proteinuria, progressive reduction in glomerular filtration rate (GFR) and glomerular sclerosis [4–7], the clinical setting that defines 'chronic allograft dysfunction'. These changes represent the late consequences of the primitive ischaemic insult, depending on the activation of the above-mentioned gene deregulation.

Despite the great clinical relevance of this problem, few therapeutic and experimental options have evolved over the years to enhance the recovery of acute ischaemic renal injury or to successfully prevent it. We have recently shown that the administration of a selective inhibitor of membrane-bound Ha-Ras (chaetomellic acid, KM) reduced to a great extent both functional and histological damage in uninephrectomized rats subjected to warm IRI compared to untreated controls [8]. This approach, in fact, limited the apoptosis and necrosis of tubular cells in ischaemic kidneys resulting in significant, although partial, preservation of renal function. Since Ras inhibition has only a limited effect on leukocyte-mediated inflammation, it is possible to hypothesize that the association of chaetomellic acid with an anti-inflammatory drug could further limit the acute decline in glomerular filtration rate, thus preserving renal function in the long term.

According to this hypothesis, we have tested the effects on IRI of mycophenolate mofetil (MMF), an immunosuppressor with anti-inflammatory properties. Beyond its specific action on leukocyte depletion, in fact, MMF inhibits cytokine-induced nitric oxide biosynthesis both *in vitro* [9] and *in vivo* [10], and limits the expression of some adhesion molecules involved in maintaining and amplifying inflammation [11].

MMF, the morpholinoethyl ester of mycophenolic acid (MPA), is currently used for the prevention of acute graft rejection [12], and due to its particular properties, in the treatment of immune-mediated inflammatory diseases, like systemic lupus erythematosus [13]. *In vivo*, it inhibits the enzyme inosine-5'-monophosphate dehydrogenase, the rate-controlling enzyme in the *de novo* biosynthesis of guanosine and deoxyguanosine nucleotides [14]. Since proliferating B and T lymphocytes are strictly dependent on this *de novo* pathway for purine biosynthesis, MPA effectively inhibits lymphocyte proliferation [15]. An additional metabolic effect of guanosine depletion is the inhibition of the transfer of mannose and fucose residues to glycoproteins [16], including cell adhesion molecules, like intercellular adhesion molecules (ICAM-1), involved in mediating the inflammatory response. Therefore, MMF could have a beneficial effect on renal IRI through the reduced recruitment of leukocytes and macrophages at the time of ischaemic damage, and in combination with KM, it could further preserve renal function after IRI.

The aim of the present experimental study was to clarify the effects of MMF, alone or in combination with KM, on renal IRI; additional rats were also studied after a prolonged follow-up (6 months) to ascertain whether these effects still persisted in the long term. IRI was performed in uninephrectomized rats, to mimic the period of the warm ischaemia of renal transplantation.

Materials and methods

The study was carried out on 59 male Sprague Dawley rats, 4 months old, fed a standard diet and tap water *ad libitum*, kept at constant temperature and humidity in a 12-hour dark/light cycle. All the rats underwent right nephrectomy, under light anaesthesia with sodium pentobarbital (Nembutal®, 50 mg/kg i.p.), and were randomly assigned to the experimental groups. Seven days later, the rats were prepared for the experimental protocol (left renal ischaemia or sham operation). Ischaemic damage was determined by clamping the left renal artery for 45 min with a smooth vascular clamp under anaesthesia (Nembutal®, 50 mg/kg i.p.). The reperfusion of the kidney was confirmed visually before suturing the abdominal wall; during the sham operation, the renal artery was not closed. At completion of surgery, the rats were restrained in their cages.

Acute studies

The acute studies were performed 24 hours after IRI. Rats were divided into five groups: Group CON, untreated rats with IRI ($n = 8$); Group MMF, rats treated with MMF for 4 days before IRI (20 mg/kg/day by gavage, $n = 8$); Group KM, rats treated with chaetomellic acid 4 hours before ischaemia (0.23 μg/kg dissolved in 0.25 ml of saline, i.p., $n = 7$); Group MMF + KM, rats treated with both drugs at the same dosage ($n = 8$); Group NOR, sham-operated rats without IRI ($n = 8$); this last group included four rats subjected to gavage with the vehicles of MMF

(olive oil and DMSO 0.5%) for 4 days: since there was no difference in body weight gain or in GFR compared to normal rats, their data were pooled together. Rats of Group CON received an i.p. injection of 0.25 ml of saline 4 hours before IRI induction.

The haemodynamic study was carried out under Inactin® anaesthesia (100 mg/kg i.p.); the details of such a procedure are described elsewhere [17,18]. After a 60-min stabilization period, three to five clearance periods were performed with collection of blood samples from the femoral artery and renal vein (through sharpened glass micropipettes), to assess the arteriovenous gradient of inulin, an estimate of renal filtration fraction.

At the end of the experiment, the kidney was removed and weighed under sterile conditions, and fragments of renal tissue were immediately stored (−80°C) for immunoblotting and immunohistochemical studies; further specimens of renal parenchyma for histological studies were fixed and paraffin embedded.

Long-term studies

Twenty additional rats from Group NOR, CON, MMF, KM and KM + MMF ($n = 4$ in each group) were followed up after ischaemia–reperfusion or sham operation for 6 months. The rats were then subjected to the haemodynamic study, with the same modalities of acute studies; monthly control of 24-hour proteinuria was performed in individual metabolic cages.

The employed dosage of KM acid (0.23 μg/kg) is associated with the best functional and histological renal outcome, as previously shown in our laboratory [21]. The dosage of MMF by gavage (20 mg/kg/day) is that commonly used in rats for its biological activity and the low incidence of side effects [11,19,20]. Animal care and use were carried out in accordance with Italian laws.

Immunoblot analysis on kidney tissues

Kidney fragments from rats used in the acute haemodynamic studies were homogenized in 250 mM sucrose, 5 mM imidazole (pH 6.5), 0.5 mM dithiothreitol (1:4 w/vol), using a glass Teflon potter. Samples were then centrifuged at 800 *g* at 4° for 10 min, and supernatants were centrifuged at 100 000 *g* (4°C) for 45 min in a 70.1 Ti rotor (Beckman). Supernatants (cytosols) were collected, and membrane pellets were suspended in radio immunoprecipitation assay buffer. Fifty microgram of membrane and cytosolic fractions were then subjected to immunoblot analysis for Pan-Ras and Ki-Ras protein, following the procedure described elsewhere [8,21].

Histological studies

The specimens of renal parenchyma, fixed in activated Bouin's solution, post-fixed in 10% formalin and embedded in paraffin (4-μm sections), were stained with haematoxylin–eosin, periodic acid–Schiff (PAS), Jones staining and observed in a blinded fashion. Fifty proximal tubules from the outer stripe of the outer medulla (OSOM) were examined in each rat at ×400 magnification and assigned to three categories: (i) tubules with normal appearance; (ii) tubules with signs of moderate to sublethal injury (loss of apical brush border); (iii) tubules with signs of acute tubular necrosis (from a few sloughed epithelial cells to tubules with a complete naked basal membrane). Proximal tubules were distinguished from distal tubules on the basis of morphological criteria [21]. Histological assessment was performed in a blinded fashion.

Immunohistochemical analysis

Samples were collected and harvested at the end of the haemodynamic studies. Four micrometre paraffin-embedded sections were dewaxed in xylene and alcohol-rehydrated; they were then placed in Coplin jars filled with a 0.01 M tri-sodium citrate solution and subjected to microwave irradiation. After heating, slides were thoroughly rinsed in cool running water for 5 min. They were then washed in tris buffer saline (TBS) (pH 7.4) and incubated with the anti-ED-1 mAb for 60 min, followed by specific biotinylated secondary antibodies and by peroxidase-labelled streptavidin (LSAB Dako); the signal was developed with 3,3-diaminobenzidine chromogen (Dako) as substrate. For all the sections, negative controls consisted of incubation with unrelated antibodies, or with omission of the first layer. Immunohistochemical staining of ICAM-1 was performed on the acetone-fixed frozen sections that were incubated with mouse anti-CD54 mAb for 60 min at room temperature, followed by secondary antibody and the same detection system (LSAB Dako). The sections were examined using standard light microscopy.

The number of ED1-positive cells in each case was calculated by counting the number of stained cells in 30 randomly selected high-power fields (400×) and is expressed as mean ± SE of cells per field. The expression of ICAM-1 was determined using a semi-quantitative grading (0–3) in at least 15 randomly selected high-power fields (400×) of each section, according to the extension of the staining in renal parenchyma (0% to >75%) [11].

Evaluation of all slides was performed in a blinded fashion by two pathologists who were unaware of the origin of the slides (G.T. and V.V.).

Analytical determinations

Urinary volume was measured gravimetrically in pre-weighed vials. The concentrations of inulin in plasma and urine were measured by the diphenylamine method [22]. Proteinuria was determined by a commercial kit (pyrogallol, Sigma). Filtration fraction was measured by renal extraction of inulin [(A − V / A) × 100]; the other determinants of renal dynamics were calculated according to standard formulae.

Statistics

The one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test, was used to compare the mean values and to find significant differences among the groups under study. Histological data were analysed by the Kruskal–Wallis one-way ANOVA followed by the Dunn's multiple comparison test. A P-value < 0.05 was considered statistically significant. Data are expressed as mean ± SD, unless otherwise specified.

Results

Displacement of Ras from cell membrane by KM acid and MMF in whole kidney

Sections of kidneys derived from rats undergoing the acute haemodynamic study were homogenized, and membrane and cytosolic fractions were subjected to immunoblotting with anti-pan-Ras and anti-Ki-Ras antibodies, as described in Materials and Methods (Figure 1).

Pre-treatment with Ras inhibitor in Group KM determined a significant decrease of membrane-bound active Ras proteins (−30% *vs.* CON), which represented variations

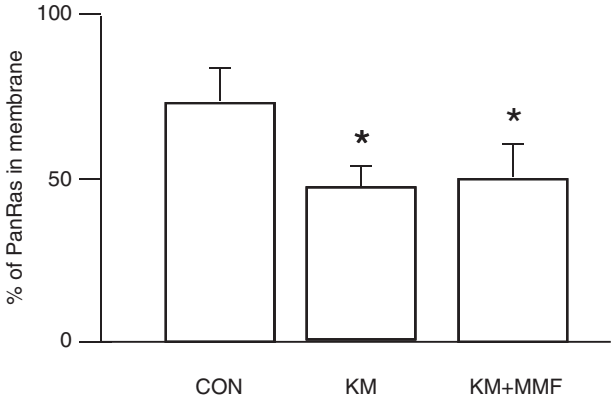

Fig. 1. Displacement of Ras protein from membrane of kidney tissue by KM and KM + MMF 24 hours after ischaemia. Rats were subjected to immunoblotting with anti-Pan-Ras and anti-Ki-Ras antibodies (data of Ki-Ras are not shown since they were similar in all the groups). The histograms show the percentages of membrane-bound Pan-Ras (HaRas + KiRas) in the different groups ($n = 6$). *$P < 0.05$ *vs.* CON.

Table 1. Renal dynamics in the five groups of rats under study, 24 hours after acute IRI

	BW	GFR	RPF	FF	HT	BP	RBF	RVR
NOR	402	0.85*	3.23	26.0	44***	115	5.81	21.7
(n = 8)	±15	±0.25	±0.96	±2.1	±2	±9	±1.84	±7.4
CON	383	0.20	NA	NA	43	114	NA	NA
(n = 8)	±14	±0.05			±3	±11		
MMF	360	0.21	NA	NA	40	119	NA	NA
(n = 8)	±42	±0.13			±3	±16		
KM	390	0.52**	2.56	20.3	40	123	4.41	29.6
(n = 7)	±18	±0.06	±0.74	±2.6	±3	±8	±1.21	±7.5
KM + MMF	364	0.46**	2.65	18.1	38	112	4.28	28.7
(n = 8)	±46	±0.15	±1.08	±4.1	±2	±13	±1.56	±9.2

NOR = normal uninephrectomized rats; CON = untreated rats with IRI; MMF: rats pre-treated with MMF before IRI; KM = rats pre-treated with KM before IRI; KM + MMF = rats pre-treated with both MMF and KM before IRI. Abbreviations and units of measurement: BW, body weight (g); GFR, glomerular filtration rate (ml/min/100 g b.w.); RPF and RBF, renal plasma and blood flow, respectively (ml/min/100 g b.w.); FF, filtration fraction (%); Ht, haematocrit (%); BP, mean blood pressure (mm Hg); RVR, renal vascular resistance (mm Hg ml^{-1} min). NA = not available.
*= $P < 0.05$ (minimum value) *vs.* other groups.
**= $P < 0.05$ *vs.* CON and MMF.
***= $P < 0.05$ *vs.* KM + MMF.
One-way ANOVA followed by Student–Newman–Keuls test.

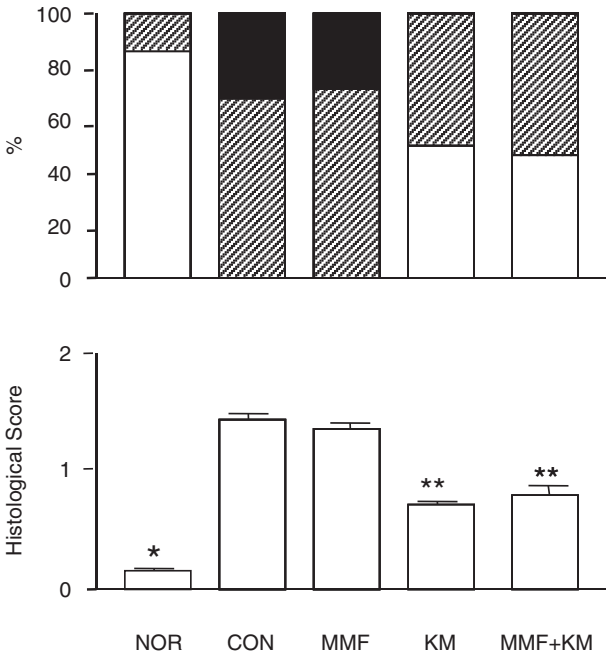

Fig. 2. Histological evaluation of renal tubular damage 24 hours after ischaemia. Upper panel shows the percentages of proximal tubules in the OSOM, categorized as tubules with a normal appearance (blank), tubules with signs of sublethal injury (dashed lines) and tubules with signs of acute tubular necrosis (black columns). Lower panel: tubules with a normal appearance, tubules with signs of sublethal injury and tubules with signs of acute tubular necrosis were scored 0, 1 and 2, respectively. Columns represent the mean score (± SEM). For abbreviations of groups, see text. *$P < 0.01$ *vs.* other groups; **$P < 0.05$ *vs.* CON and MMF.

in Ha-Ras, since Ki-Ras membrane binding was not affected. The results were not influenced by the concomitant administration of MMF (Group KM + MMF, −32%).

Acute renal haemodynamic studies

The data of the renal haemodynamic studies are reported in Table 1. There was no difference in body weight (b.w.), measured at the time of the haemodynamic study, among the five groups of rats under observation. Glomerular filtration rate averaged 0.85 ± 0.25 ml/min/100 g b.w. in sham-operated rats (Group NOR), and was equally depressed, on the average by 75%, in rats of Group CON and MMF (both $P < 0.0001$ *vs.* NOR); in these latter groups, an arteriovenous gradient of inulin could not be demonstrated in most rats, following the great functional damage: this precluded the evaluation of their renal dynamics. Pre-treatment with chaetomellic acid determined a consistent preservation of GFR after ischaemia in Group KM (0.52 ± 0.06 ml/min/100 g b.w., −39% *vs.* CON, $P < 0.05$), which was maintained also in rats of Group KM + MMF (−46% *vs.* CON, $P < 0.05$). In both of these groups, GFR results were significantly higher than in CON and MMF rats ($P < 0.05$).

Filtration fraction averaged 26.0 ± 2.1% in normal rats and was significantly depressed in both Group KM and KM + MMF (−22% and −30% *vs.* NOR, respectively, $P < 0.01$). In these groups, the decrease in GFR was mainly secondary to a rise in renal vascular resistances [+36% and +32%, respectively *vs*. NOR, not significant (NS)], and the consequent 25% average decrease observed both in renal plasma and blood flow, compared to normal rats of Group NOR.

Histological studies

Figure 2 describes the biopsy findings in the five groups of rats. Nearly 90% of tubular cells in OSOM appeared normal in rats of Group NOR. Rats of Group CON showed a consistent percentage of necrotic cells, averaging 34%, although signs of tubular damage were almost universally present. Pre-treatment with MMF did not modify the histological picture, with necrotic cells averaging 28%, whereas a consistent improvement was observed in Group KM, in which cellular necrosis was virtually absent, and the number of injured cells was consistently reduced (52%). The histological appearance of rats of Group KM + MMF overlapped that of Group KM.

Immunohistochemical analysis

The number of ED-1-positive cells and the expression of ICAM-1 24 hours after the acute IRI are represented in Figure 3. Untreated rats of Group CON showed an increased staining for ICAM-1 and ED1, both on endothelial and tubular cells. This staining was significantly decreased in the kidneys of animals treated with MMF, alone and with KM acid, reaching values statistically similar to those in the control rats.

In Group KM, treatment with chaetomellic acid slightly decreased the staining for ED1 and ICAM on endothelial and tubular cells, but did not reach the values of MMF treatment.

Long-term renal haemodynamic studies

The long-term effects on renal function of the pre-treatment with MMF (Group MMF), chaetomellic acid (Group

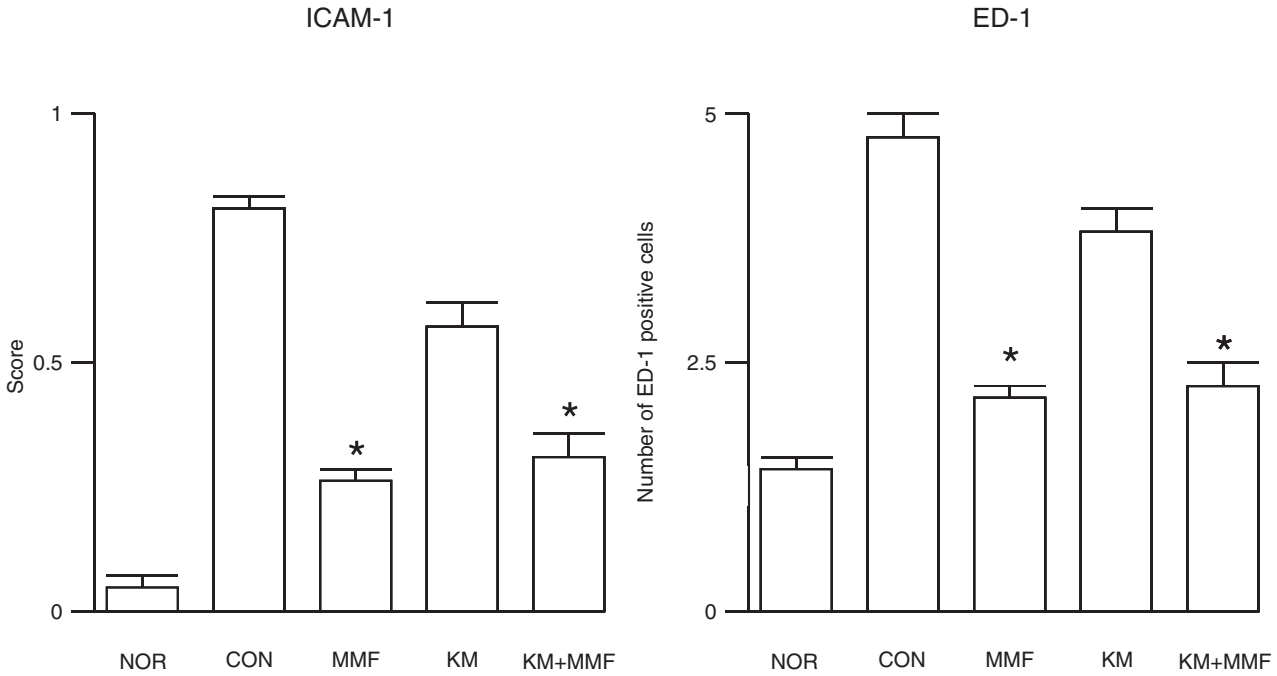

Fig. 3. Expression of ICAM-1 (left) and presence of ED-1-positive cells (right) 24 hours after ischaemia in the groups under study. The expression of ICAM-1 was determined using a semi-quantitative grading (0–3) according to the extension of the staining in renal parenchyma (0% to >75%). The number of ED1-positive cells in each case was calculated by counting the number of stained cells in 30 randomly selected high-power fields (400×). Values are expressed as means ± SEM. For abbreviations of groups, see text. *$P < 0.05$ *vs.* CON.

KM) and their combination (Group KM + MMF) on acute IRI were studied after a 6-month follow-up period and compared to the renal outcome of sham-operated rats (Group NOR) and ischaemic untreated rats (Group CON).

There was a tendency towards increasing values of 24-hour proteinuria in both CON and MMF group during the entire observation period compared to the other groups; the difference became statistically significant at the end of the third month of follow-up, and further increased until the end of the observation period (Figure 4). Rats of Group KM and Group KM + MMF were partially protected: their proteinuria values, in fact, remained significantly lower than those observed in Group CON and MMF in the last months of observation, and did not statistically differ from those of Group NOR, even resulting in numerically higher values (Group KM, +50.4% and Group KM + MMF, +85% *vs.* Group NOR).

Similar modifications were observed in renal haemodynamic studies (Table 2). Six months after the acute ischaemic injury, in fact, GFR was markedly depressed both in

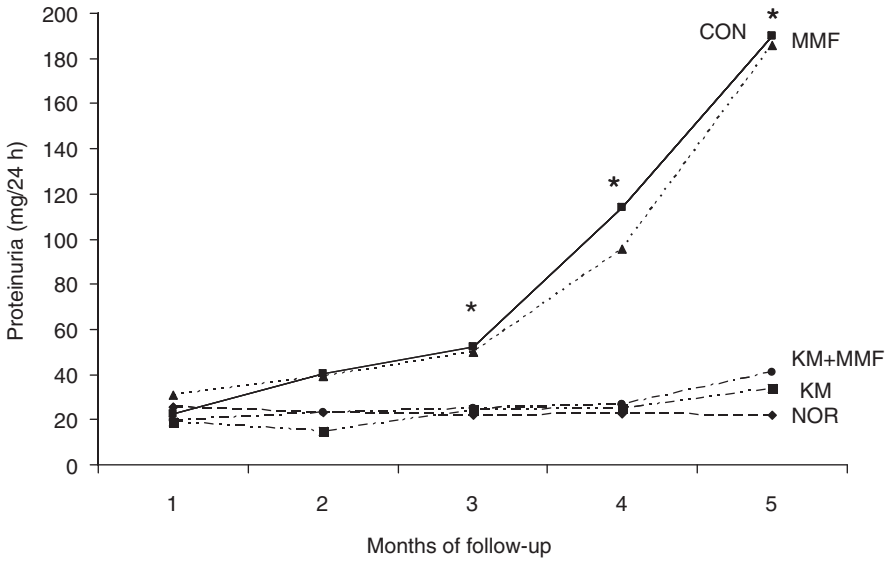

Fig. 4. Values of 24-hour proteinuria during the follow-up period in the different groups under study. For abbreviations of groups, see text. *$P < 0.05$ Group CON and Group MMF *vs.* other groups (minimum value).

Table 2. Renal dynamics in the five groups under study after 6-month follow-up

	BW	GFR	RPF	FF	HT	BP	RBF	RVR
NOR	611	0.87**	3.18*	28	45	125	5.65*	22.1
	±62	±0.06	±0.10	±2	±1.1	±5.7	±0.24	±2.77
CON	635	0.50	1.95	26	39.5	142 *	3.24	46.9*****
	±76	±0.12	±0.52	±2	±2	±9.6	±0.92	±14.5
MMF	617	0.49	1.70	29	42	133	2.94	45.8*****
	±34	±0.13	±0.13	±5	±1.5	±2.2	±0.28	±3.85
KM	638	0.69	2.24	31	42	122	4.10***	29.8
	±85	±0.12	0.42	±2	±2.3	±2.9	±0.56	±4.13
KM + MMF	642	0.68	2.50***	28	44	125	4.43****	28.5
	±40	±0.08	±0.41	±3	±3.0	±4.4	±0.63	±3.12

NOR = normal uninephrectomized rats; CON = untreated rats with IRI; MMF = rats pre-treated with MMF before IRI; KM = rats pre-treated with KM acid before IRI; KM + MMF = rats pre-treated with both MMF and KM before IRI; $n = 4$ in each group. Abbreviations and units of measurement: BW, body weight (g); GFR, glomerular filtration rate (ml/min/100 g b.w.); RPF and RBF, renal plasma and blood flow, respectively (ml/min/100 g b.w.); FF, filtration fraction (%); Ht, haematocrit (%); BP, mean blood pressure (mm Hg); RVR, renal vascular resistance (mm Hg ml^{-1} min)Statistical significance (one-way ANOVA followed by Student–Newman–Keuls test; $P < 0.05$, minimum value):* = *vs.* all the other groups.
** = *vs.* CON and MMF.
*** = *vs.* MMF.
**** = *vs.* CON and MMF.
***** = *vs.* NOR, KM and KM + MMF.

Group CON and MMF, when compared to Group NOR (−43%, $P < 0.001$), but was significantly preserved in Groups KM and KM + MMF (−20% *vs.* NOR, NS). The decline in GFR of the CON and MMF groups was mediated by the significant rise in renal vascular resistances compared to the other groups, and the consequent decline in renal plasma and blood flow. Again, both groups pre-treated with KM acid (KM and KM + MMF groups) showed a consistent protection in these determinants of renal dynamics.

Discussion

The treatment of renal transplant living donors with MMF before surgery could have a beneficial impact on graft outcome, both by reducing the inflammatory processes that occur in the kidney during the ischaemia/reperfusion phase and by determining a relative depletion of donor leukocytes, with potential benefits at the immunological level [23].

At present, clinical data are missing, and unfortunately, previous experimental data on MMF treatment of acute kidney injury have provided conflicting and inconclusive results. In fact, administration of MMF (20 mg/kg/day for 2 days) before renal ischaemia in rats did not improve renal function, compared to untreated animals, after 24 hours of reperfusion, although treated rats showed a significant improvement of GFR after 48 hours [11]; when administered after the ischaemic insult, MMF (20 mg/kg/day for 5 days) even had harmful results since it worsened both renal function and cellular damage [19,20]. Other experimental models of nephrotoxicity do not favour the use of MMF: administration of MMF after uranyl acetate injection, in fact, delayed the reparative processes in tubular cells compared to untreated rats [24].

A different behaviour of MMF has been described in a milder model of IRI (30 min of ischaemia), if combined with antioxidant bioflavonoids: while the prolonged administration of MMF (40 mg/kg/day for 5 to 7 days) had no effect on creatinine levels compared to control rats, the concomitant administration of MMF and flavonoids conferred a significant protection, suggesting that the anti-inflammatory properties of the drug can influence renal function [25].

Indeed, the anti-inflammatory effects of the drug are widely demonstrated. Treatment with different doses of MMF (40, 80 and 120 mg/kg/day) before IRI inhibited, in a dose-dependent fashion, the renal expression of the iNOS gene in mice, with a consistent amelioration of renal inflammation, although with no effect on renal function [10]. Moreover, the same dose of MMF employed in the present study decreased ICAM-1 expression and monocyte recruitment in IRI [11].

The possibility of combining the anti-inflammatory properties of MMF with the anti-apoptotic effects of KM, previously demonstrated in IRI [8], prompted us to carry out the present study, in the attempt to minimize the acute kidney damage occurring after its reperfusion. The data of the present study showed that treatment with MMF before the onset of IRI did not affect renal outcome, despite a 4-day treatment: after 24 hours of reperfusion, in fact, GFR and renal histology did not reveal differences between MMF-treated rats and the untreated control animals; this occurred in the presence of a significantly lower expression of ICAM-1 and the reduced presence of monocytes in treated rats. As expected [8], the inhibition of Ha-Ras in Group KM was largely beneficial: the administration of KM, in fact, resulted in significant functional and morphological protection to treated animals but, quite unexpectedly, the concomitant administration of MMF and KM did not offer additional protection to the kidney compared to KM alone. It is noteworthy that these treatments did not affect animal well-being, nor induce diarrhoea or weight loss.

We have subsequently hypothesized that the positive effects of MMF on inflammation detected in the acute phase of renal injury could influence renal outcome in the long

term, when the ischaemic rats develop hypertension, proteinuria and renal failure [4,6,7]. The results of the extended follow-up were particularly interesting. In fact, control untreated rats showed progressively increasing proteinuria 3 months after IRI and a significant decline in GFR after 6 months (−43% *vs.* untreated rats). In rats pre-treated with MMF, neither the development of proteinuria nor the decline in renal function was influenced at all. Protein urinary excretion, conversely, was significantly prevented in rats treated with the single dose of KM, denoting that the protection of renal outcome by KM persisted, as also witnessed by a well-preserved GFR (−20% *vs.* untreated rats).

Unfortunately, the concomitant administration of MMF with KM did not further ameliorate any of the considered parameters, indicating that the positive impact on inflammation at the time of ischaemia did not affect the onset of IRI sequels. It is possible to postulate that, in this setting, a continuous and prolonged treatment with MMF is advisable to maintain a low degree of inflammation. In fact, a 12-week administration of MMF after IRI in transgenic hypertensive rats significantly reduced proteinuria and glomerular sclerosis compared to untreated rats, with just a mild improvement in renal function [26]. In remnant rats, the prolonged administration of MMF attenuated renal histological damage and hypertension, but did not improve proteinuria, or GFR [27]. Recently, Pechman *et al.* have shown that prolonged treatment with MMF in a specific model of hypertension induced by high-salt intake 1 month after IRI could prevent the fall in GFR observed in control untreated rats [28]; taken together, these studies suggest that the chronic depletion of inflammatory cells is necessary to obtain any positive results on renal function.

Conversely, the persistence of the beneficial effect of the single dose of KM in the long term is certainly secondary to the better degree of renal function after IRI, as a possible consequence of some gene deregulation observed at the time of ischaemia.

In conclusion, our study suggests that the administration of MMF before IRI did not confer any specific protection to the kidney, nor did it have any effect in preventing the renal damage commonly observed after an acute kidney injury. At present, the possibility of reducing the damage by IRI through pre-treatment with MMF does not seem convincing, and further studies are necessary to elucidate the role of KM in preventing IRI in order to understand whether this drug maintains its beneficial effects also when administered after ischaemia. The search for new strategies aimed at minimizing IRI must, in fact, concentrate on the reperfusion phase of renal damage, which is more accessible to clinical intervention since it does not require pre-treatment of the kidney donor. A desirable exception could be represented by KM, since the donor kidney could be selectively perfused with this drug, with no peculiar risk for the recipient.

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Omacor®, *n*-3 polyunsaturated fatty acid, attenuated albuminuria and renal dysfunction with decrease of SREBP-1 expression and triglyceride amount in the kidneys of type II diabetic animals

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Abstract

Background. We assumed that *n*-3 polyunsaturated fatty acid (*n*-3 PUFA) would attenuate the tissue dyslipidemic condition through suppression of sterol regulatory element-binding protein (SREBP-1) in the kidney and would prevent renal progression in diabetic animals.

Methods. We gavaged Omacor®, composed of docosahexaenoic acid and eicosapentaenoic acid, to db/db mice for 2 weeks (0.2 g/100 g/day). We measured the markers of renal function, triglyceride amount and expressions of SREBP-1, liver X-activated receptor alpha (LXRα), collagen IV and TGFβ-1 in kidney lysate, and performed immunohistochemical staining for SREBP-1, desmin and WT-1 in the renal sections. We measured collagen IV in primary mesangial cells cultured with high glucose media (25 mM), both with and without a transient transfection of small interfering RNA (siRNA) SREBP-1.

Results. Omacor® decreased the concentration of serum free fatty acid, and the amount of renal triglyceride, which was associated with decreased expression of SREBP-1 in the kidney, albuminuria and renal dysfunction in db/db mice. Omacor® attenuated the expansion of mesangial matrix and the expression of desmin, preserved the WT-1 positive cells, and inhibited the phosphorylation of nuclear factor κB in renal tissue. In mesangial cells cultured in high glucose media, the suppression of SREBP-1 expression decreased the collagen IV in the cells.

Conclusions. Our study results demonstrated that *n*-3 PUFA prevented renal progression with attenuation of SREBP-1 and reduction of triglyceride in the diabetic kidney. This suggests that the regulation of dyslipidemic signals in the kidney could be a possible mechanism by which PUFA preserves renal function in the diabetic condition.

Keywords: diabetic nephropathy; hypertriglyceridaemia; *n*-3 PUFA; SREBP-1

Introduction

Diabetic nephropathy has become the most common single cause (41.5%) of end-stage renal disease and cardiovascular events in Korea [1]. In addition to the prominent roles played by hypertension, hyperglycaemia, growth factors, inflammatory cytokines, oxidative stress and advanced glycation end products [2], abnormal lipid metabolism and renal accumulation of lipids have also been proposed as an important mechanism in the pathogenesis of diabetic nephropathy [3,4]. Recently, increased renal expression of