Renoprotective effects of the AGE-inhibitor pyridoxamine in experimental chronic allograft nephropathy in rats

Femke Waanders1,2, Else van den Berg1,2, Ryoji Nagai3, Ingrid van Veen2, Gerjan Navis1 and Harry van Goor2

1Department of Nephrology, 2Department of Pathology and Laboratory Medicine, University Medical Centre Groningen, The Netherlands and 3Department of Biochemistry, Kumamoto University, School of Medicine, Kumamoto, Japan

Abstract

Background. Advanced glycation end products (AGEs) are involved in diabetic nephropathy (DN). The AGE formation inhibitor pyridoxamine (PM) is renoprotective in DN and in normoglycaemic obese Zucker rats. In chronic allograft nephropathy (CAN), renal AGE accumulation occurs as well.

Methods. To investigate whether inhibition of AGE formation is renoprotective in CAN, we studied the Fisher 344 to Lewis (F–L) allograft rat model of experimental CAN. Fisher to Fisher (F–F) isografts served as controls. Proteinuria, renal function and renal histology of untreated transplanted rats (F–L n = 8, F–F n = 8) were compared to rats receiving PM 2 g/l in drinking water for 20 weeks starting at transplantation (F–L n = 5, F–F n = 10). All rats received cyclosporin A (1.5 mg/kg/day) for 10 days after transplantation to prevent early acute rejection.

Results. Compared to untreated allografts, PM significantly decreased proteinuria (76 ± 18 vs 29 ± 3 mg/day), serum creatinine (130 ± 12 vs 98 ± 5 μmol/l), focal glomerulosclerosis (116 ± 27 vs 16 ± 5 AU), glomerular macrophage influx (5.6 ± 0.6 vs 3.3 ± 1.0), interstitial fibrosis (132 ± 24 vs 76 ± 2 AU) and interstitial macrophage influx (47.0 ± 8.7 vs 15.4 ± 5.0). Moreover, PM significantly ameliorated tubular accumulation of pentosidine, compared to untreated allografts (2.5 ± 0.6 vs 0.3 ± 0.3, all p < 0.05). In the isograft controls, these values did not differ between untreated and PM treated rats.

Conclusion. PM exerts renoprotective effects and decreases renal pentosidine accumulation in experimental CAN, suggesting a detrimental role for renal AGE accumulation in the pathogenesis of renal damage in this non-diabetic model. These results indicate that inhibition of AGE formation might be a useful adjunct therapy to attenuate CAN.

Keywords: advanced glycation end products; chronic allograft nephropathy; kidney transplantation; Maillard reaction; pentosidine; pyridoxamine

Introduction

Several lines of evidence support a pathophysiological role for advanced glycation end products (AGEs) in diabetic nephropathy. Accumulation of AGEs occurs in glomerular and tubulointerstitial compartments in proportion to the severity of renal damage [1] and pharmacological intervention in AGE formation with pyridoxamine (PM), and aminoguanidine protects against renal structural lesions, proteinuria and renal function loss in experimental diabetes [2,3]. Interestingly, PM not only provides renoprotection in diabetes, but also in normoglycaemic obese Zucker rats [2,4] and in angiotensin-II infused rats [5], demonstrating that its beneficial effects are not limited to hyperglycaemic models of renal damage.

Involvement of AGEs in chronic allograft nephropathy (CAN) is supported by several studies. First, in patients with biopsy-proven CAN, serum AGE levels are increased compared to renal transplant recipients with normal renal function and patients with chronic renal failure of their native kidneys, which suggests that the increased AGE levels in CAN cannot be attributed to decreased renal function alone [6]. Second, increased renal deposition of the AGE pentosidine in proximal tubular cells was previously shown to occur in renal transplant recipients, which was related to increased serum levels of pentosidine [7]. Finally, accumulation of AGEs in skin is associated with CAN [8].

Since AGEs are nephrotoxic in vitro and in vivo [9,10], renal AGE accumulation may contribute to...
progressive renal damage in CAN. Intervention in AGE formation by PM may thus have renoprotective potential in CAN. To test this hypothesis, we investigated whether PM is renoprotective in the experimental Fisher 344 to Lewis rat model of CAN.

Materials and methods

Animals, surgical procedures and treatment

Forty-three inbred adult male rats (±250 g) were studied (Harlan Inc., Zeist, TheNetherlands). Rats were housed in a temperature-controlled room of 18–20°C with a 12 h light/dark cycle. The rats had free access to standard chow and drinking water. The local animal ethics committee at the University of Groningen approved all experimental procedures and the Principles of Laboratory Animal Care (NIH publication no. 85-23) were followed.

Renal transplantation was performed according to standard procedures [11], in which Lewis rats (SnHsd) served as recipients and Fisher 344 rats (F344/NHsd) as donors. Donor rats were anaesthetized with isoflurane/N₂O/O₂, left kidneys were flushed in situ with saline and removed; donor rats were sacrificed. Kidneys were preserved in saline on ice for 20 min and transplanted orthotopically into the recipient rat under isoflurane/N₂O/O₂ anaesthesia. The left renal vessels and ureter were anastomosed end-to-end using 10–0 prolene sutures. Vascular clamps were released immediately after the vascular anastomosis was completed, with a warm ischaemic time of 27±3 min. Cyclosporin A (CsA; 1.5 mg/kg/day, Sandimmune, Sandoz Pharma AG, Basel, Switzerland) was daily administered subcutaneously to all rats for 10 days. Ten days after transplantation, the native right kidney was removed and the transplanted kidney was inspected for viability and hydronephrosis. Nine animals (eight Fisher to Lewis allografts and one Fisher to Fisher isograft) in whom surgical (ischaemia, n = 3) or urological complications (hydronephrosis, n = 6) were observed, were excluded from follow-up and sacrificed. Both in the Fisher to Lewis (F–L) vehicle group and in the F–L PM group, we preliminary sacrificed one rat at week 16 because of declined condition. In the Fisher to Fisher (F–F) vehicle group there was one premature death after 13 weeks. Those animals were excluded from analyses.

Accordingly, for the long-term follow-up the following groups were studied: eight F–L allografts treated with vehicle (VEH), five F–L allografts treated with PM [PM(HCl); 2 g/l in drinking water], eight F–F isograft controls treated with VEH and 10 F–F isografts treated with PM. Treatment with PM started directly after transplantation. Since PM is a photosensitive substance, it was supplied in drinking bottles that were covered with aluminium foil. The dose of PM (2 g/l) was based on previous studies in non-diabetic Sprague-Dawley rats [2] and obese Zucker rats [4]. Rats were sacrificed at week 20.

Clinical parameters

Body weight was measured weekly. Every other week, the rats stayed in individual metabolic cages (Bioquant™, Merck, Darmstadt, Germany) to collect 24 h urine samples. Urinary protein excretion was measured by the pyrogallol red molybdate method. Blood samples were collected at the end of the experiment, prior to sacrifice of the rats. Concentrations of creatinine, urea, sodium, potassium and cholesterol were all analysed on a multi-test analyzer system (Merck MEGA reagents (DiaSys Diagnostic Systems, Holzheim, Germany)). Creatinine concentrations in urine and serum were determined with the Jaffé method. Serum values of urea were determined with the urease-GLDH method and concentrations of potassium and sodium were measured with indirect potentiometry. Cholesterol was determined enzymatically.

Sacrification and assessment of renal morphologic damage

At the end of the study (week 20), rats were anaesthetized, kidneys were perfused with saline and rats were sacrificed. A coronal tissue slice through the mid-portion of the kidney was fixed in 4% paraformaldehyde and processed for paraffin embedding [11]. Paraffin embedded sections (4 μm) were stained with periodic acid-Schiff (PAS) and examined by light microscopy by a qualified and independent pathologist in a blinded fashion to evaluate focal glomerulosclerosis (FGS) and interstitial fibrosis (IF). To assess the degree of FGS, the blinded pathologist semi-quantitatively scored 50 glomeruli on a scale of 0–4. FGS was scored as present when collapse of capillary lumens, mesangial matrix expansion, hyalinosis and adhesion formation were present in the same quadrant. If 25% of the glomerulus was affected, a score of 1 was given, 50% was scored as 2, 75% as 3 and 100% as 4. The degree of IF was scored similarly in 30 consecutive visual fields. IF was defined as expansion of the interstitial space, with or without the presence of atrophied and dilated tubules and thickened tubular base- ment membranes. Medullary tissue, glomeruli and vessels were excluded from the calculated areas of fibrotic involvement. A score of 0 was given when no IF was present in a field, 1 for 0–25% with IF, 2 for 25–50%. 3 for 50–75% and 4 for 75–100% of the field showing IF. To obtain the final score, we multiplied the degree of injury by the percentage of glomeruli (for FGS) or visual fields (for IF) with the same degree of injury and added these scores, rendering a theoretical range of 0–400.

In addition, the histopathological findings were graded by a blinded qualified pathologist according to the Banff 97 working classification of renal allograft pathology [12]. In short, a score of I (mild) was given when mild fibrosis of the interstitium (6–25% of the cortical area) and mild atrophy of the tubules (up to 25% of the cortical area of the tubules) was present either with or without specific glomerular or vascular findings suggestive of CAN. A score of II (moderate) was given when moderate IF (25–50% of the cortical area) and moderate tubular atrophy (25–50% of the area of the cortical tubules) were present and a score of III (severe) was given when severe IF (affecting greater than 50% of the cortical area) and tubular atrophy (involving greater than 50% of the area of the cortical tubules) was present either with or without specific changes as in grade I.

Immunohistochemistry

Deparaffinized and rehydrated sections (4 μm) were subjected to heat-induced antigen retrieval by overnight
incubation in a 0.1 M Tris/HCl buffer (pH 9.0) at 80°C. Endogenous peroxidase was blocked with 0.3% H₂O₂ in phosphate-buffered saline (PBS) for 30 min and sections were incubated with an ED1-antibody (Sertec, Oxford, UK) or an anti-pentosidine antibody (33.8 µg/ml) [13] for 60 min at room temperature. Binding of the antibody was detected using sequential incubations with peroxidase (PO)-labelled rabbit anti-mouse and PO-labelled goat anti-rabbit antibodies; both for 30 min. Peroxidase activity was developed using 3,3′-diaminobenzidine tetrachloride (DAB) for 10 min. Sections were counterstained with haematoxylin. Macrophages were assessed in 50 glomeruli and in 30 interstitial fields per kidney. To assess differences in renal pentosidine accumulation, a pathologist performed pair-wise visual comparisons between randomly allocated pairs of VEH and PM sections in a blinded fashion. Moreover, staining intensity in dilated tubular structures was assessed by the same blinded pathologist on a scale of 0 to 4: 0 (absent), 1 (occasionally weak), 2 (weak), 3 (moderate) and 4 (strong staining).

**Data analysis**

Data are expressed as mean ± standard error. Statistical analysis of group differences was performed by a Kruskal-Wallis ANOVA on ranks and Mann-Whitney U-tests. Statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was assumed at the 5% level (two-tailed).

**Results**

**Body weight**

Throughout the experiment, food and water intake was similar in all groups. Weight gain did not differ between the PM and the VEH treated allografts. In the PM treated F–F isografts, weight gain was significantly less than in the VEH treated isografts (Table 1).

**Urinary protein excretion**

In the VEH treated F–L allografts, a gradual but significant increase in proteinuria was observed during the 20 weeks of the experiment, which was significantly ameliorated by PM in the F–L allografts. In isografts, urinary protein excretion was in the normal range; this was unaffected by PM (Figure 1).

**Creatinine, urea and electrolytes**

PM significantly inhibited the rise in serum creatinine and urea that was observed in the F–L allografts (Figure 2A). PM did not affect creatinine or urea in the F–F isografts (Figure 2B). No differences in sodium or potassium levels were observed between the groups (Table 1).

**Cholesterol**

Cholesterol was significantly elevated in F–L allografts treated with VEH, compared to the isografts, which was partly and non-significantly reduced by PM (Table 1).

**Renal structural changes**

FGS and IF were abundantly present in VEH treated F–L allografts, which was significantly ameliorated by PM (Figure 2C and D, respectively, microphotographs of renal histology are presented in Figure 3A).

In the VEH treated F–L allograft group, all kidney sections were classified as Banff grade II or III with a mean of 2.4 ± 0.2, whereas in the PM treated F–L allografts Banff grade was significantly lower; all kidney sections were classified as grade I. Both in the VEH and in the PM treated F–F isograft controls the Banff grade varied from 0 to 1 (0.3 ± 0.2 and 0.4 ± 0.2, respectively, Figure 2F).

**Renal pentosidine accumulation**

Pentosidine was found in the brush border and cytoplasm of dilated tubular structures. In agreement with less tubular damage in PM treated allografts, consistently less total immunoreactive tubular pentosidine was observed in PM treated F–L allografts vs

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**Table 1. Clinical characteristics**

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<tr>
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<tr>
<td></td>
<td>VEH (n = 8)</td>
<td>PM (n = 10)</td>
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<tr>
<td>Weight gain (%)</td>
<td>40 ± 3†</td>
<td>45 ± 3†</td>
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<tr>
<td>Sodium (mmol/l)</td>
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<td>138 ± 1</td>
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<td></td>
<td>138 ± 0</td>
<td>140 ± 1</td>
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<tr>
<td>Potassium (mmol/l)</td>
<td>5.5 ± 0.2</td>
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<td>4.8 ± 0.1</td>
<td>5.2 ± 0.2</td>
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<tr>
<td>Cholesterol (mmol/l)</td>
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<td>2.1 ± 0.2</td>
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Parameters at end of study are shown. All values are expressed as mean ± SE. VEH, vehicle treated; PM, pyridoxamine treated. *P < 0.05 vs F–F VEH and F–F PM, †P < 0.05 vs F–F VEH.

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**Fig. 1.** Time course of proteinuria development in VEH and PM treated allografts and isografts. Data are expressed as mean ± SE. F–L, Fisher 344 to Lewis allograft; F–F, Fisher 344 to Fisher 344 isograft; VEH, vehicle treated; PM, pyridoxamine treated.  *P < 0.05 vs all other groups.
Fig. 2. Graphs represent mean ± SE. (A) Plasma creatinine. (B) Plasma urea. (C) Focal glomerulosclerosis, semi-quantitative score (arbitrary units 0–400). (D) Interstitial fibrosis, semi-quantitative score (arbitrary units 0–400). (E) Scoring of histology according to the Banff criteria [12] (0–3). (F) Pentosidine staining in dilated tubular structures; semi-quantitative score (arbitrary units 0–4). VEH and PM treated Fisher–Fisher isograft controls were all negative. (G) Mean glomerular macrophages (mean number per glomerulus of 50 glomeruli scored). (H) Mean interstitial macrophages (mean number per interstitial field of 30 fields scored). VEH, vehicle treated; PM, pyridoxamine treated. *P < 0.05 vs all other groups, #P < 0.05 vs Fisher–Fisher VEH and PM.
VEH treated F–L allografts (Figure 2F, microphotographs are presented in Figure 3B). VEH and PM treated F–F isograft controls were all negative.

Renal macrophage influx

A vast influx of macrophages in the glomeruli and interstitium was seen in VEH treated F–L allografts, which was significantly inhibited in PM treated F–L allografts (Figure 2G and H, respectively, microphotographs are presented in Figure 3C).

Discussion

We demonstrated renoprotective effects of PM in experimental chronic allograft nephropathy. PM significantly ameliorated the development of proteinuria, renal function impairment, renal structural damage, glomerular and interstitial macrophage influx as well as tubular pentosidine accumulation. Our results provide proof of principle for the involvement of renal AGE-accumulation in the pathogenesis of CAN.

As anticipated, CAN was characterized by renal function impairment, as manifested by elevated serum creatinine concentrations, proteinuria, renal macrophage infiltration, focal and segmental glomerulosclerosis and IF with inflammatory infiltrates and tubular atrophy. A novel finding was that pentosidine accumulates in the kidney in this experimental model of CAN. In CAN, renal AGEs accumulated in dilated, diseased tubule, which is in accordance with our prior findings in proteinuria-induced renal damage [13]. PM considerably ameliorated all these renal effects. PM as such had no effect in the isograft controls without CAN.

In our experimental model of CAN, PM reduced renal damage and the inflammatory response. Renal damage was associated with a vast influx of macrophages in glomeruli as well as interstitium, which was significantly ameliorated by the AGE formation inhibitor PM. There are at least two mechanisms by which AGEs may contribute to tissue injury. First, AGEs can directly alter the structure and function of extracellular matrix proteins [14]. Inhibition of AGE formation by PM reduced renal damage in CAN. Renal macrophage influx can be reactive to damage and the reduction in renal macrophages could thus be a secondary effect. A second mechanism by which AGEs can induce end-organ injury is via a ligand–receptor interaction with the receptor for advanced glycation end products (RAGE), which is widely expressed in the kidney including in podocytes [1] and tubular epithelial cells [15]. Nuclear factor (NF)–κB sites are located on the RAGE promoter and control cellular expression of RAGE, linking RAGE to the inflammatory response [16]. RAGE knock-out mice are protected against macrophage influx, glomerulosclerosis and proteinuria in the adriamycin model (abstract JASN nov 2006, vol 17; 66A).
Binding of AGEs to their receptor can release mediators like MCP-1 [17], TGF-β [10] and NF-κB [15]. The induced inflammatory response may lead to tissue damage through extracellular matrix deposition, fibrosis, tubular atrophy and smooth muscle cell proliferation, which eventually may contribute to the progressive renal damage found in CAN.

PM ameliorated renal function impairment, as apparent from the lower creatinine and urea levels in our model of experimental CAN. Our study was not specifically designed to elucidate the mechanisms by which PM exerts its renoprotective effects, but several inferences can be made. PM was associated with less tubular pentosidine accumulation on immunohistochemistry. This localization of pentosidine accumulation is similar to that in another model of experimental renal damage, i.e. adriamycin nephropathy [13] and may be due to impaired renal clearance of AGEs. In uraemia, increased plasma and tissue AGE levels are independent of diabetes or hyperlipidaemia [18]. Under physiological conditions, AGEs are freely filtered by renal glomeruli, reabsorbed in the proximal tubule where they are degraded or modified and eventually excreted in the urine [19]. Since the kidney plays a key role in AGE disposal, protection of renal function by PM may lead to less renal AGE accumulation and consequently less subsequent renal damage. We suggest that AGEs can induce a vicious circle in which AGE accumulation leads to renal damage; the associated impaired renal function leads to higher AGE levels, leading to more renal AGE accumulation mediating further renal damage. This vicious circle may be a non-immunologic factor that contributes to the progressive renal damage in CAN.

PM is one of the three active metabolites of vitamin B6, which plays a role in many metabolic functions after having undergone phosphorylation. In tissues, the major vitamin B6 compounds are pyridoxamine phosphate and pyridoxal phosphate. In plasma, pyridoxal phosphate represents the main form of vitamin B6. Kidney transplant patients with normal or near normal renal function have marked decreased plasma pyridoxal phosphate levels, with 65% of the patients studied having a major deficit [20]. In renal transplant patients, this plasma deficiency could indicate decreased tissue pyridoxamine phosphate levels. In our experimental, renal transplant model, PM could have exerted its renoprotective effects through the supplementation of a tissue pyridoxamine phosphate deficit.

Because we excluded animals with observed urological (hydronephrosis) or surgical complications (ischaemia), we studied a relatively pure model of CAN. However, due to this exclusion of animals, we studied only small numbers. In spite of the small numbers, our results were statistically significant and showed the renoprotective effects of AGE formation inhibition, providing proof of principle for the involvement of renal AGE-accumulation in the pathogenesis of CAN.

Our results are in concordance with previous studies with PM in diabetic models [2,4]. In those studies, PM also decreased proteinuria and exhibited protective effects on renal morphology. Moreover, increased cortical expression of α-SMA and vimentin were reduced by PM in an angiotensin-II infusion model of renal damage [5]. In our study, weight gain was significantly lower in the PM treated isografts compared to the vehicle treated isografts. This finding is in concordance with others who observed a significant decrease in body weight in PM treated diabetic rats [2]. In that study, there were no nutritional deficiencies associated with the decrease in body weight of rats treated with PM.

In phase II clinical trials in diabetic patients with albuminuria, PM was safe and well-tolerated. Although not originally designed to test this endpoint, post hoc analyses of these studies showed that PM significantly reduced the rise in serum creatinine and urinary albumin excretion in these patients [21]. Future phase III studies of this agent in diabetic patients are keenly awaited.

In conclusion, renal accumulation of AGEs occurs in experimental chronic allograft nephropathy. PM exerts renoprotective effects and ameliorates renal pentosidine accumulation, suggesting a detrimental role for renal AGE accumulation in the pathogenesis of renal damage in this non-diabetic model. Since clinical studies using AGE inhibition in diabetic patients are currently under way, intervention in AGE formation in human transplantation may be feasible shortly. Our data suggest that inhibition of AGE formation by PM could be explored as an adjunct therapy in renal transplantation to prevent CAN.

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Conflict of interest statement. None declared.

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