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Original Article

Chronic allograft nephropathy: expression and localization of PAI-1 and PPAR-γ

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Abstract

Background. Chronic allograft nephropathy (CAN) is a major cause of loss of renal allografts. Mechanisms postulated to be involved include sequelae of rejection, warm ischaemia time, drug toxicity, ongoing hypertension and dyslipidaemia. Plasminogen activator inhibitor-1 (PAI-1) is implicated not only in thrombosis, but also in fibrosis, by inhibiting matrix degradation, and is expressed in renal parenchymal cells as well as in macrophages. Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the steroid receptor superfamily, and plays a major beneficial role in lipid regulation, insulin sensitivity and macrophage function, factors that may play a role in CAN. We therefore studied the expression of these molecules in CAN.

Methods. All renal biopsy/nephrectomy files from Vanderbilt and Nashville VAMC from a 6 year period were reviewed to identify all renal transplant biopsies or nephrectomies more than 6 months after transplant with CAN. CAN was defined as fibrosis in the graft, vascular, interstitial or glomerular. All cases were scored for severity of fibrosis in vasculature (0–3 scale), glomeruli (% affected with either segmental and/or global sclerosis) and interstitial fibrosis (% of sample affected). PAI-1 and PPAR-γ immunostaining was assessed on a 0–3 scale in glomeruli, vessels and tubules.

Results. Eighty-two patients with a total of 106 samples met entry criteria. The population consisted of 59 Caucasians and 23 African-Americans; 49 males, 33 females with average age 37.9 ± 1.7 years. Average time after transplant at time of biopsy was 60.5 ± 4.9 months (range 7–229). Glomerulosclerosis extent in CAN was on average 26.5 ± 2.4% compared with 3.6 ± 1.2% in normal control kidneys from native kidney cancer nephrectomies and 0% in transplanted kidney biopsies from patients obtained ≥6 months after transplantation without CAN. Native control kidneys showed mild interstitial fibrosis (8.0 ± 1.2%), whereas transplant controls showed very minimal fibrosis (2.0 ± 2.0%). Interstitial fibrosis in CAN kidneys was on average 47.9 ± 2.4%. Glomerular PAI-1 and PPAR-γ staining scores were markedly increased in CAN (1.8 ± 0.1, 2.3 ± 0.1, respectively) compared with normal control kidneys from native kidney cancer nephrectomies (PAI-1 0.2 ± 0.2 and PPAR-γ 0.4 ± 0.2, $P < 0.001$) and transplanted kidney biopsies from patients obtained ≥6 months after transplantation without CAN (PAI-1 0 and PPAR-γ 0, $P < 0.001$). Tubular PAI-1 and PPAR-γ staining scores were 1.9 ± 0.1 and 1.9 ± 0.1, respectively, and also increased over both native and transplant kidney controls (0.8 ± 0.2 for both categories for PAI-1, 1.2 ± 0.2 for both categories for PPAR-γ, respectively). Vascular sclerosis in CAN was 1.0 ± 0.1 with increased PAI-1 and PPAR-γ scores (1.7 ± 0.1, 1.2 ± 0.1, respectively) compared with controls. Infiltrating macrophages were increased in CAN, and were positive for both PAI-1 and PPAR-γ. Biopsies with less sclerosis overall showed a trend for less PAI-1 and PPAR-γ staining.

Conclusion. PAI-1 and PPAR-γ are both increased in CAN compared with non-scarred native or transplant control kidneys. We speculate that altered matrix metabolism and macrophage function might be involved in the development of CAN.

Keywords: chronic allograft nephropathy; macrophage; peroxisome proliferator-activated receptor-γ; plasminogen activator inhibitor-1

Introduction

Chronic allograft nephropathy (CAN) is a progressive, irreversible functional and morphological deterioration

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of the renal graft evolving months or years after transplantation and is the most common cause of allograft loss and proteinuria in long-lasting transplants [1–3]. The morphologic characteristics include vascular occlusive changes, interstitial fibrosis, mononuclear inflammatory cell infiltration, tubular atrophy and variable glomerular lesions [4]. Importantly, the transplant is not protected from factors, which cause progressive scarring in the native kidney. Thus, the mechanisms involved in CAN include both immune-mediated (e.g. HLA mismatch, acute rejection episodes) and non-immune mediated processes (e.g. reperfusion injury, prolonged ischaemia time, dysregulation of matrix and cell turn-over, macrophage infiltration, hypertension, hyperlipidaemia, drug toxicity) [3,5].

The common histological end point is the accumulation of extracellular matrix (ECM). ECM metabolism is regulated by a balance of matrix synthesis and degradation. Plasminogen activator inhibitor-1 (PAI-1) is now recognized to play a key role in ECM degradation [6,7]. PAI-1 is a 50 kDa glycoprotein member of the SERPIN family and is the major physiological inhibitor of tissue plasminogen activator and urokinase-like plasminogen activator. PAI-1 inhibits fibrinolysis and also inhibits extracellular matrix degradation, thus promoting fibrosis [6,7]. PAI-1 is increased in many settings of endothelial injury. Serum levels of PAI-1 are increased in transplanted patients and correlate with the rate of decline of glomerular filtration rate (GFR) [8]. PAI-1 mRNA is increased in glomerular, tubular and inflammatory interstitial cells, including macrophages, in both experimental and human chronic kidney diseases [6,7,9]. PAI-1 can be up-regulated by growth factors (transforming growth factor (TGF)-β, TGF-α, epidermal growth factor and tumour necrosis factor-α), coagulation factors (fibrin, tissue plasminogen activator), metabolic factors (glucose, lipoproteins), hormones (renin, angiotensin II, aldosterone) and tissue injury factors (radiation, hypoxia) [10,11]. The factors that can down-regulate PAI-1 are less well known and include nitric oxide, interferon-γ, natriuretic factors and lipid-lowering drugs, including the thiazolidinediones, agonists of PPAR-γ [6].

PPAR-γ is a member of the steroid receptor superfamily, and plays a major beneficial role in lipid regulation, insulin sensitivity and macrophage function, factors that may play a role in CAN [12]. PPAR-γ expression is highly up-regulated in macrophages and T-cells in inflammatory processes and can be induced by IL-4 and other immunoregulatory molecules. Activated macrophages release reactive oxygen species, proteolytic enzymes, growth factors, cytokines, eicosanoids, IL-6 and procoagulant factors. Persistent macrophage infiltration is linked to increased fibrosis, but macrophages also may have beneficial effects on remodelling and repair after tissue injury, depending on their state of activation [13,14]. Interestingly, PPAR-γ expression was up-regulated in interstitial, but not in glomerular macrophages in human diabetic nephropathy, where PAI-1 was also increased, and was also increased after injury in animal model of sclerosis [9,15]. Further, PPAR-γ agonism was protective against sclerosis in a nondiabetic rat model, suggesting that PPAR-γ expression could be a counter-regulatory response to injury [15].

Links between PAI-1 and PPAR-γ are thus suggested by their common expression in several tissues, including endothelium, macrophages and adipose tissue, and the regulation of PAI-1 by PPAR-γ in several settings [6,7,9,15,16]. In cultured human pre-adipocytes or human vein endothelial cells with tumour necrosis-α-induced elevated PAI-1, treatment with thiazolidinediones attenuated PAI-1 expression [16,17]. We therefore investigated the expressions of PAI-1 and PPAR-γ in patients with CAN to examine a possible role of these factors in allograft fibrosis.

Methods

Case selection

All cases with CAN, defined as the presence of fibrosis more than 6 months after transplant, and/or transplant glomerulopathy (TGP) diagnosed between 1994 and 1999 from Vanderbilt Medical Center and Nashville Veteran's Administration Medical Center were reviewed. TGP is a more specific lesion of chronic allograft rejection and is characterized by endothelial cell injury with accumulation of flocculent material in the subendothelial space, reduplication of glomerular basement membrane and mesangial interposition. Only cases with at least five glomeruli by light microscopy, and immunofluorescence and/or electron microscopy studies to rule out other concurrent renal disease were included. Patients with diabetes or *de novo* or recurrent disease were excluded. A total of 82 patients with 106 samples met entry criteria. Four patients (3 males and 1 female) had a second graft, and 20 patients had a second biopsy of the same graft. Twenty-three samples were from transplant nephrectomies.

Recipient age, gender, race, primary disease, HLA match, ischaemia time, number of acute rejection episodes, type of donor (living related, living non-related or cadaveric), and laboratory and clinical data (serum creatinine, glucose, triglycerides, cholesterol, proteinuria, blood pressure, serology for hepatitis B, C and cytomegalovirus) were determined from clinical records.

Histological assessment

All the glass slides available from the cases were reviewed (at least 9 stained slides). The light microscopic findings were assessed and scored semi-quantitatively, on a 0 to 3+ scale, (no lesion = 0, mild = 1+, moderate = 2+, severe = 3+). The morphological analysis included assessment of mesangial matrix, mesangial cellularity, splitting of glomerular basement membrane and vascular sclerosis (medial thickening and intimal fibrosis, for arterioles, interlobular and large arteries, averaged). Vascular scores were for some analyses normalized as a percent of possible maximum score based on the types of vessels sampled. Tubular atrophy, interstitial fibrosis and

interstitial macrophage infiltrate were assessed and extent semi-quantified as a percentage of the specimen involved. Glomerulosclerosis, either segmental or global, was assessed as percent of glomeruli involved.

We also defined our own histological severity index for biopsies using ranks for interstitial fibrosis, glomerulosclerosis, and vascular sclerosis, the variables most closely related to possible effects of PAI-1 and PPAR-γ based on previous findings. Rankings were used to provide a common metric for these histological variables. The index is a linear combination of weighted ranks with interstitial fibrosis ranks given a weight of six, as it has been most closely related to renal failure, glomerular sclerosis a weight of three, and vascular sclerosis a weight of one (the latter based on the relatively less well sampled vascular component). As each of these variables could maximally be 100%, the maximum total weighted injury score of 1000 is possible.

Normal native kidney control samples were obtained from adult patients with surgically removed kidneys diagnosed with malignancies, assessing the non-tumorous portions. Normal transplant control biopsies were obtained from patients with biopsies done ≥6 months after transplantation and without morphological evidence of CAN or TGP. These patients had no urinary abnormalities, and histopathological examination excluded any glomerular disease.

Immunohistochemical studies

Immunostaining

Formalin-fixed paraffin-embedded tissue was available in all cases for immunostaining for PAI-1 and 96 of 106 cases for PPAR-γ double-staining with the macrophage marker CD68. Immunohistochemical staining was performed using avidin–biotin peroxidase technique with the following antibodies, as previously described [9]: monoclonal human antibody to PAI-1 (1:25, American Diagnostica, Greenwich, CT) and monoclonal antibody to mouse PPAR-γ (1:10, Santa Cruz Biotechnology, Santa Cruz, CA). Double-staining for PPAR-γ/CD68 was performed using monoclonal mouse antibody to anti-human CD68 (1:100, Dako, Carpinteria, CA) with the alkaline phosphatase-streptavidin method following PPAR-γ staining. Briefly, 4 μm paraffin sections were deparaffinized in xylene, rehydrated in graded ethanols, and quenched in 3% H_2O_2/methanol solution for 10 min to block endogenous peroxidase. For PPAR-γ, sections were then pretreated with antigen retrieval by microwaving for 20 min in a 0.01 mol/ml citrate buffer (pH 6), followed by avidin–biotin block (InnoGenex, San Ramon, CA, USA). Both PAI-1 and PPAR-γ sections were treated with proteinaceous block for endogenous antigenic sites (Powerblock, InnoGenex) for 30 min at room temperature. Either PAI-1 or PPAR-γ antibody (overnight at 4°C) was then applied to the sections. Sections were washed 3× in phosphate-buffered saline (PBS) triton, and sequentially incubated with rabbit anti-mouse IgG (1:100 for 40 min) for PAI-1, and swine anti-rabbit IgG (1:50 for 30 min) for PPAR-γ (Dako) and finally developed with diaminobenzidine (DAB) as chromagen. Double-staining with monoclonal antibody CD68 (1:100 for 90 min) for identification of macrophages also was performed. Sections were rinsed 3× and sequentially incubated with secondary biotinylated goat anti-mouse IgG (InnoGenex) for 30 min and alkaline phosphatase–streptavidin conjugate (InnoGenex) for 30 min. Finally, sections were developed with Sigma fast red TR/Naphtol AS-MX for 5 min, counterstained with haematoxylin and coverslipped with aqueous-based permanent mounting media (PPAR-γ) (Innovex-Biosciences, Richmond, CA, USA) or cytoseal 60 mounting media (PAI-1) (Stephens Scientific, Kalamazoo, MI, USA). Biopsies from three adult patients with arterionephrosclerosis were used as positive controls. Negative controls were performed by substituting the primary antibody with normal serum, which showed no staining.

Immunohistochemical scoring

Glomerular PPAR-γ and PAI-1 were assessed as follows: 0 = no staining; 1+ = 2–5 positive cells/glomerulus; 2+ = 6–10 positive cells/glomerulus; 3+ = >10 positive cells/glomerulus. Vascular PPAR-γ and PAI-1 were scored: 0 = no staining; 1+ = occasional nuclear staining; 2+ = 6–10 positive cells; 3+ = >10 positive cells on average in each vessel profile. Tubular PPAR-γ and PAI-1 were scored: 0 = no staining; 1+ = weak, very focal; 2+ = mild/moderate intensity in >25% of tubules; 3+ = >50% of tubules. The intensity of interstitial macrophage infiltration (by CD68) and PAI-1 and PPAR-γ positivity were scored on a 0 to 3+ scale: 0 = no staining; 1+ = 2–5 positive cells/hpf; 2+ = 6–10 positive cells/hpf; 3+ = >10 positive cells/hpf on average. All scoring was done in a blinded fashion.

Statistical analysis

When mean values were given as descriptive statistics, a variation was expressed in terms of standard error. Non-parametric statistics were used for analyses. Comparisons between the cases with CAN and each of the control groups used Wilcoxon/Kruskal–Wallis significance tests. Preliminary assessment of relationships between variables used Spearman correlations. Analysis of disease progression among the subset of patients with multiple biopsies was conducted using logistic regression modelling. SAS software was used for all analyses.

Results

Eighty-two patients were studied. Their average age was 37.9 ± 1.7 years (range 2–70). The population

consisted of 59 Caucasians (33 males and 26 females) and 23 African-Americans (16 males and 7 females). Caucasians were younger than African-Americans. Gender differences in age were not significant. Table 1 gives the underlying disease of the 82 patients, and the clinical indications for the biopsies/nephrectomies. There were 46 primary cadaver donor transplants (27 males and 19 females) and 36 living-related donor transplants (22 males and 14 females). Table 2 provides data describing the clinical presentation at the time of biopsy or nephrectomy for the 86 transplants. The mean time from transplantation to biopsy or nephrectomy was 60.5 ± 4.9 months (range 7–229 months). The number of HLA mismatches is also shown in Table 1. About half (40 of 82) of the patients had not had a previous recognized episode of acute rejection. There was significant increase in interstitial fibrosis ($47.9 \pm 2.4\%$) in patients with CAN in contrast to normal controls ($8.0 \pm 1.2\%$) and non-scarred transplants ($2.0 \pm 2.0\%$). Glomerulosclerosis was also significantly increased in patients with CAN ($26.5 \pm 2.4\%$) in contrast to normal controls ($3.6 \pm 1.2\%$) and non-scarred transplants (0%). Vascular sclerosis in CAN was only 1.0 ± 0.1 slightly increased compared with normal controls (0.6 ± 0.4) and non-scarred transplants (0 ± 0). Interstitial macrophages were increased in CAN (1.5 ± 0.1), compared with normal and transplant control (no macrophages detected). The results of PAI-1 and PPAR-γ staining are presented in Table 3. All PAI-1 and PPAR-γ stainings in glomeruli, vessels, tubules and macrophages were significantly increased in CAN kidney *vs* normal and non-scarred transplant controls.

We next sought to determine whether total injury as calculated by a severity index correlated with specific clinical or immunostaining markers. The histological severity index in all CAN samples was on average 535 ± 24 (range 115–1009), and was less severe in females than males regardless of HLA mismatch or age. Overall, there was no correlation of the injury score with any clinical parameters except diastolic and systolic blood pressures and serum creatinine ($r = 0.22$, 036, 0.49, $P < 0.02$, $P = 0.0001$, $P < 0.0001$, respectively). Serum creatinine correlated with macrophage PAI-1 ($r = 0.51$) and PPAR-γ scores ($r = 0.50$) and PPAR-γ vascular scores ($r = 0.32$). Immunohistochemistry scores did not increase stepwise by serum creatinine quartiles for other variables (Table 4). The injury score correlated with PAI-1 and PPAR-γ macrophage scores ($r = 0.39$, 0.30, $P = 0.0001$, $P = 0.0031$, respectively). Individual elements of injury were then examined, revealing that interstitial fibrosis, a major component of the composite score, correlated with the presence of PAI-1 and PPAR-γ in macrophage ($r = 0.40$, 0.31, $P = 0.0001$, $P = 0.0018$, respectively). Macrophages also showed phenotypic heterogeneity for PPAR-γ expression. Macrophages were PPAR-γ positive when present in arteries and interstitium, in contrast to PPAR-γ negative macrophages in the glomeruli (Figure 1).

In the subset of 20 patients with a second biopsy, 11 had increased interstitial fibrosis with increasing

Table 1. Cause of ESRD and clinical presentation[a]

	Cadaveric donor	Living related	Total
Primary disease			
Glomerulonephritis	10	16	26
Diabetes	9	1	10
Hypertension	15	3	18
PCKD	2	1	3
Other	10	16	26
Unknown	2	1	3
Clinical presentation for biopsy/nephrectomy			
Increasing serum creatinine	32	34	66
Nephrotic syndrome	1	1	2
Isolated proteinuria	2	1	3
Haematuria	3	0	3
Acute rejection	4	0	4
Other	6	2	8
Number of rejection episodes			
None	20	20	40
1	19	11	30
2	6	5	11
>3	1	–	1
N/A[b]	2	2	4
HLA mismatch			
0	2	2	4
1	2	0	0
2	3	4	7
3	9	5	14
4	12	14	26
5	10	7	17
6	8	3	11
N/A[b]	2	3	5

[a]Data from 86 transplants in 82 patients.
[b]N/A, not available; PCKD, polycystic kidney disease.

Table 2. Laboratory data at the time of study[a]

Serum creatinine (mg/dl)	4.2 (1.4–15.1)
Triglycerides (mg/dl)	87.5 (63.0–551.0)
Cholesterol (mg/dl)	213.6 (88.0–428.0)
Glucose (mg/dl)	113.9 (55.0–350.0)
Proteinuria	
None	11
Trace	10
1+	11
2+	20
3+	18
4+	12
Not performed	4
CMV serology	
Positive/negative	66/12
Not performed	8
Hepatitis B serology	
Positive/negative	4/53
Not performed	29
Hepatitis C serology	
Positive/negative	19/56
Not performed	11
Blood pressure (mmHg)	
Systolic	149.5 (90–210)
Diastolic	86.8 (52–130)

[a]Data shown as mean and range from 86 transplants in 82 patients.

Table 3. Comparison of PAI-1 and PPAR-γ staining in CAN samples *vs* controls

	Glomerulus		Tubule		Vascular		Macrophage	
	PAI-1	PPAR-γ	PAI-1	PPAR-γ	PAI-1	PPAR-γ	PAI-1	PPAR-γ
CAN	1.8 ± 0.1	2.3 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.7 ± 0.1 (56.5 ± 2.1%)	1.2 ± 0.1 (41.5 ± 2.6%)	1.5 ± 0.1	1.7 ± 0.1
Native control	0.2 ± 0.2[a]	0.4 ± 0.2[a]	0.8 ± 0.2[a]	1.2 ± 0.2[a]	0.6 ± 0.4[a] (19.8 ± 13.2%)	0.2 ± 0.2[a] (6.6 ± 6.6%)	0[a]	0[a]
Tx control	0[a]	0[a]	0.8 ± 0.2	1.2 ± 0.2[a]	0.3 ± 1.5[a] (30.6 ± 7.1%)	0.1 ± 0.1[a] (10.8 ± 5.1%)	0[a]	0[a]

Tx, transplant.
[a] $P < 0.001$ control *vs* patient. Vascular scores are expressed also as percentage, to normalize for variable sampling of different size vessels.

Table 4. PAI-1 and PPAR-γ staining by quartile of severity of renal dysfunction

Serum creatinine	1st quartile	2nd quartile	3rd quartile	4th quartile
PAI-1				
Glomerulus	1.73 ± 0.09	1.58 ± 0.15	1.92 ± 0.08	2.04 ± 0.12
Macrophage	1.00 ± 0.16	1.04 ± 0.16	1.46 ± 0.19	2.44 ± 0.14
Tubular	1.70 ± 0.09	1.89 ± 0.09	1.80 ± 0.13	2.04 ± 0.16
Vascular	48.19 ± 3.62	54.70 ± 3.43	50.52 ± 4.00	71.85 ± 3.93
PPAR-γ				
Glomerulus	2.36 ± 0.17	2.44 ± 0.14	2.00 ± 0.18	2.38 ± 0.16
Macrophage	1.26 ± 0.13	1.38 ± 0.18	1.64 ± 0.17	2.46 ± 0.15
Tubular	1.77 ± 0.17	2.00 ± 0.15	1.78 ± 0.19	2.00 ± 0.17
Vascular	34.04 ± 5.18	39.08 ± 4.58	35.83 ± 5.27	57.35 ± 4.94

Patients were divided into quartiles based on serum creatinine at the time of biopsy, and average scoring for PAI-1 and PPAR-γ, on a 0–3+ scale for glomerular, macrophage and tubular, and as a percentage for vascular staining, calculated.

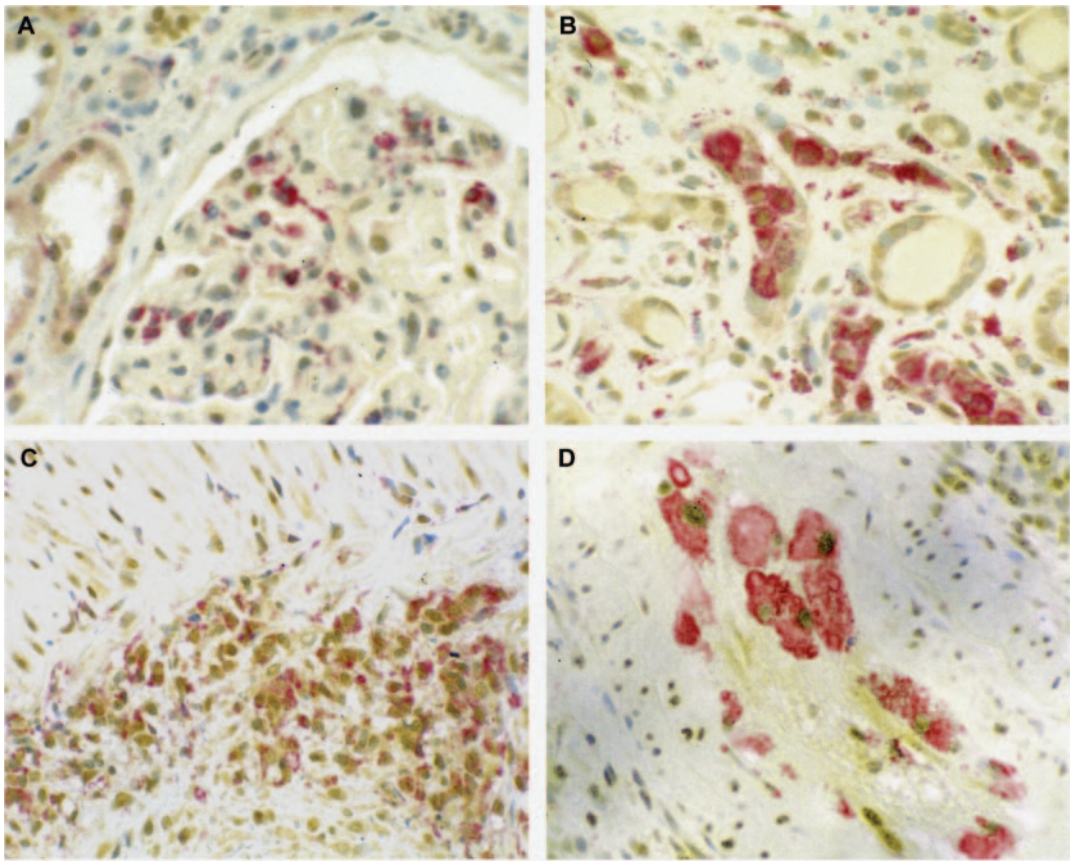

Fig. 1. Macrophage and PPAR-γ staining in glomeruli (**A**), interstitium (**B**) and vessels (**C** and **D**). Macrophages, identified by red CD68 positivity, showed nuclear PPAR-γ staining (brown) in vessels and interstitium. In contrast, glomerular macrophages were PPAR-γ negative (A,B,D, ×400, C, ×100, anti-PPAR-γ and CD68 immunostaining).

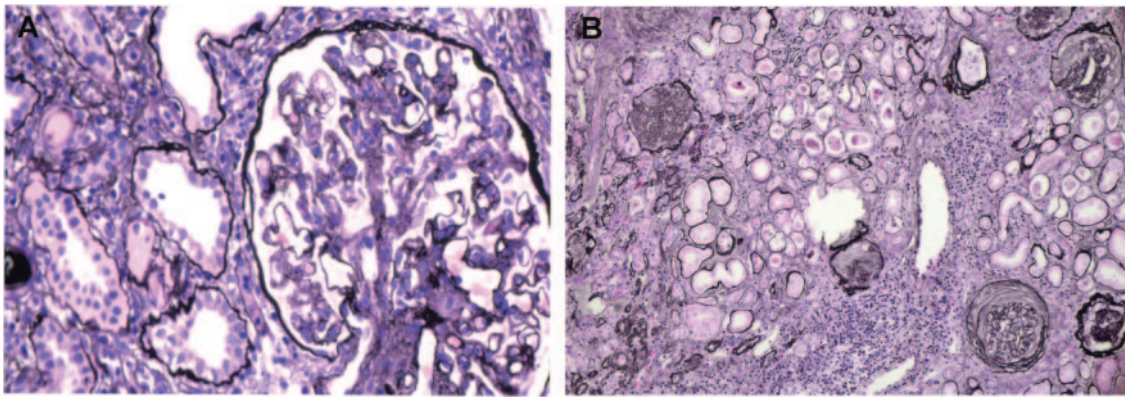

Fig. 2. Course of CAN lesions in first (**A**) and second (**B**) biopsies in one patient. There is marked interval progression over 19 months in glomerulosclerosis and interstitial fibrosis (×100, ×400, Jones' silver stain).

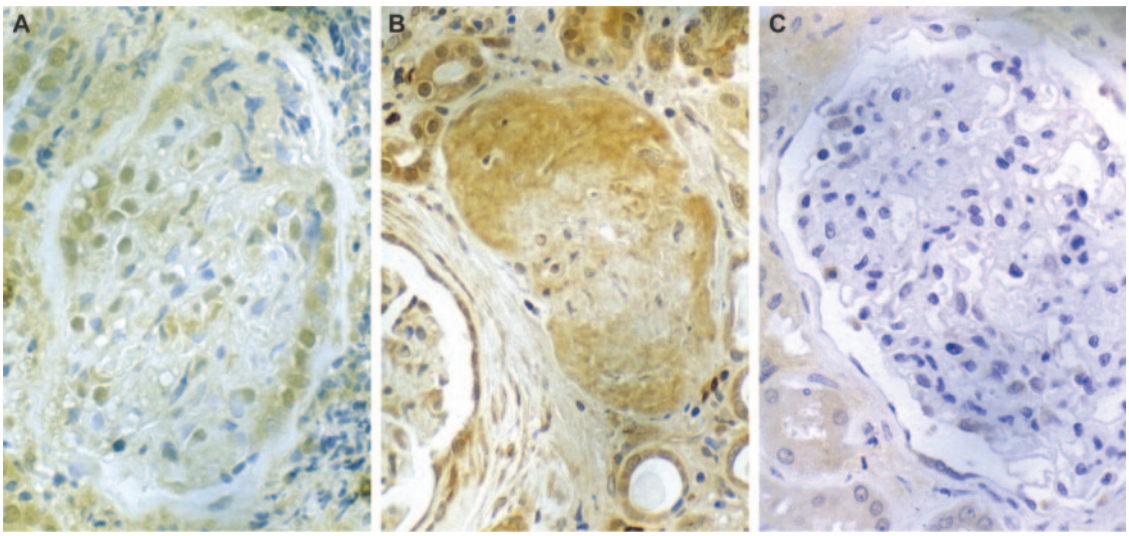

Fig. 3. Comparison of PAI-1 staining in first (**A**) and second (**B**) biopsies in one CAN patient compared with no staining in normal control (**C**) with significant interval increase in PAI-1 staining over 6.5 months from A to B (×400, anti-PAI-1 immunostaining).

biopsy interval (range 5 days to 47 months) (Figure 2). Nine of these 20 patients had no change or interval decrease in interstitial fibrosis (biopsy interval 6 days–14 months). Average PAI-1 staining in the first biopsy in progressors *vs* non-progressors was not different ($1.64 \pm 0.15, 1.91 \pm 0.16, 54 \pm 8.3\%$ *vs* $1.7 \pm 0.2, 1.7 \pm 0.2, 57 \pm 6.3\%$ for glomerular, tubular and vascular scores, respectively). Average PPAR-γ staining in the first biopsy in progressors *vs* non-progressors was not different (2.1 ± 0.2, 2.1 ± 0.3, $34 \pm 9.3\%$ *vs* 2.0 ± 0.2, 1.7 ± 0.3, $29 \pm 6.9\%$ for glomerular, tubular and vascular scores, respectively). However, patients with the most marked increase in interstitial fibrosis had an interval increase in PAI-1 and PPAR-γ staining, mostly in glomeruli (50% interval increase) (Figures 3 and 4).

Discussion

CAN is the most prevalent cause of renal allograft failure in long-lasting transplants. Clinically, CAN is characterized by variable loss of graft function, proteinuria and hypertension [1]. Several studies have demonstrated that CAN is a multifactorial process caused by immune and non-immune mechanisms that lead to nonspecific morphological changes in the graft, including interstitial fibrosis, tubular atrophy, vascular sclerosis and glomerulosclerosis [2,3]. Many factors have been implicated in CAN progression; however, the mechanism(s) responsible for the progressive fibrosis and tissue remodeling are not completely understood. In this study, we have focused on non-immune mechanisms of CAN. We found that PAI-1 and PPAR-γ expressions are increased in kidney transplants with CAN compared with normal native kidneys or transplants without CAN. These results mirror what we have previously observed in diabetic nephropathy, where these molecules were also over-expressed [9]. We postulate that increased PAI-1 promotes fibrosis, and that the increased PPAR-γ is a counter-regulatory response to injury. Indeed, *in vitro* evidence suggests that activation of PPAR-γ could contribute to downregulation of PAI-1 [16,17].

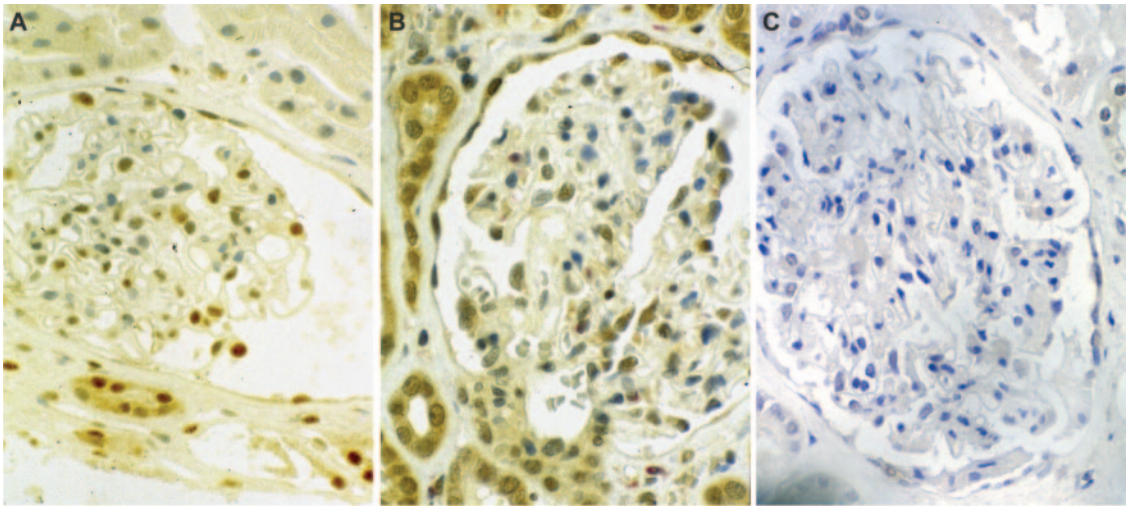

Fig. 4. Comparison of PPAR-γ staining in first (**A**) and second (**B**) biopsies in one CAN patient compared with no staining in normal control (**C**) with significant interval increase in PPAR-γ staining over 13.8 months from A to B (×400, red, anti-CD68; brown, anti-PPAR-γ immunostaining).

PAI-1 is a multifunctional protein with actions that may be dependent on or independent of its protease inhibitory effects [6]. The protease-inhibitory actions of PAI-1 extend beyond fibrinolysis and include modulation of extracellular matrix turnover, cell migration and activation of several pro-enzymes and latent growth factors. PAI-1 has been implicated in several renal pathogenetic processes including thrombotic microangiopathy, proliferative and crescentic glomerulonephritis, and diabetic nephropathy amongst others [6,9,10]. More recently, it has become evident that PAI-1 also plays a pivotal role in the progression of renal diseases, involving both glomerulosclerosis and tubulointerstitial fibrosis linked to its action to inhibit matrix degradation. PAI-1 is produced by different cells within the kidney, including the glomeruli, tubules and inflamed interstitium [6]. Interestingly, both angiotensin and aldosterone induce PAI-1 expression, and conversely, inhibition of either angiotensin or aldosterone decreased PAI-1 and sclerosis in experimental models of chronic kidney disease [6,10,11,15].

In the present study, PAI-1 in tubules, glomerular and vessels was increased in CAN biopsies compared with normal kidney controls and transplant biopsies without CAN. Those patients with most marked increase in interstitial fibrosis in repeat biopsies had increase in PAI-1 staining from first to second biopsies, particularly glomerular staining. These findings support a possible pathogenic role of PAI-1 in CAN, likely linked to its effects to inhibit ECM degradation. Importantly, the intact renal transplants without fibrosis did not show increased PAI-1, thus supporting that increased PAI-1 is specifically linked to the fibrosis of CAN, and not just upregulated by transplant *per se*. We also examined PPAR-γ staining and found it to be increased in tubules, glomeruli and vessels in CAN biopsies compared with controls, indicating a possible role of this molecule in response to injury. We have shown that a PPAR-γ agonist was protective in a non-diabetic model of progressive glomerulosclerosis in the rat associated with decreased PAI-1 expression [18]. Our recent *in vivo* and *in vitro* studies further demonstrated that PPAR-γ expression was increased in podocytes in puromycin aminonucleoside nephropathy (PAN), a model of podocyte toxicity [19]. Further, adding PPAR-γ agonist protected against PAN-induced injury, supporting that PPAR-γ increase in sclerosis is a counter-regulatory beneficial response [19]. This possibility is further supported by previous *in vitro* data demonstrating a transcriptional regulation of PAI-1 by PPAR-γ [16,17].

Of note, macrophages in glomeruli in CAN were PPAR-γ negative, contrasting PPAR-γ positive macrophages in interstitium and vessels. These findings indicate macrophage phenotypic heterogeneity, and may have important implications for the role of macrophages in renal injury. *In vitro* and *in vivo* animal studies have demonstrated that macrophage properties are determined by the first activating factor they encounter, and further that there is a hierarchy of responsiveness when macrophages are challenged simultaneously by two activating factors: for example IFN-γ effects are dominant in macrophage activation compared with those of IL-4 and TGF-β [13,20]. Macrophage differences in activation may also depend on time interval since recruitment and tissue infiltration. Thus, macrophage localization to glomerular segments at different stages of injury may be associated with exposure to a different microenvironment than that seen for interstitial macrophages. The activation programme depends in part on the infiltrating cell and in part on stimuli from the tissue where the macrophages become residents. Macrophage infiltration, especially in the interstitium, may have an early, beneficial role after injury, whereas persistent macrophage infiltration, whether in glomeruli or interstitium, typically is profibrotic [7]. The differential expression of PPAR-γ in varying sites in these CAN

kidneys further points to possible heterogeneous roles of macrophages. Further studies will be necessary to define the specific interaction of these PPAR-γ positive *vs* negative macrophages within renal parenchyma.

We conclude that PAI-1 and PPAR-γ are both increased in biopsies with CAN compared with control native and transplant kidneys. There are phenotypic differences in infiltrating macrophages in CAN, which may be important for macrophage-dependent mechanisms related to fibrosis. We speculate that altered matrix metabolism and macrophage function might be involved in the development of CAN.

Conflict of interest statement. All authors have signed the conflict of interest statement, indicating there is no conflict of interest.

References

1. Paul LC. Chronic allograft nephropathy: an update. *Kidney Int* 1999; 56: 783–793
2. Colvin RB. Chronic allograft nephropathy. *N Engl J Med* 2003; 349: 2288–2290
3. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. *N Engl J Med* 2003; 349: 2326–2333
4. Kasiske BL, Kalil RS, Lee HS *et al*. Histopathological findings associated with a chronic, progressive decline in renal allograft function. *Kidney Int* 1991; 40: 514–524
5. Krieger NR, Becker BN, Heisey DM *et al*. Chronic allograft nephropathy uniformly affects recipients of cadaveric, nonidentical living-related and living-unrelated grafts. *Transplantation* 2003; 75: 1677–1682
6. Eddy AA. Plasminogen activator inhibitor-1 and the kidney. *Am J Physiol Renal Physiol* 2002; 283: F209–F220
7. Fogo AB. Renal fibrosis: not just PAI-1 in the sky. *J Clin Invest* 2003; 112: 326–328
8. Lanhlou A, Peraldi MN, Thervet E *et al*. Chronic graft dysfunction in renal transplant patients: potential role of plasminogen activator inhibitor type 1. *Transplantation* 2002; 73: 1290–1295
9. Paukesakon P, Revelo MP, Marcantoni C, Fogo AB. Microangiopathic injury and augmented PAI-1 in human diabetic nephropathy. *Kidney Int* 2002; 61: 2142–2148
10. Fogo AB. The role of angiotensin II and plasminogen activator-inhibitor-1 in progressive glomerulosclerosis. *Am J Kidney Dis* 2000; 35: 179–188
11. Brown NJ, Vaughan DE, Fogo AB. Aldosterone and PAI-1: implications for renal injury. *J Nephrol* 2002; 15: 230–235
12. Guan YF, Breyer MD. Peroxisome proliferators-activated receptors (PPARs): novel therapeutic targets in renal disease. *Kidney Int* 2001; 60: 14–30
13. Welch JS, Ricote M, Akiyama TA *et al*. PPAR-γ and PPARδ negatively regulate specific subsets of lipopolysaccharide and INF-γ target genes in macrophages. *Proc Natl Acad Sci* 2003; 100: 6712–6717
14. Henson P. Suppression of macrophage inflammatory responses by PPARs. *Proc Natl Acad Sci* 2003; 100: 6295–6296
15. Huang Y, Haraguchi M, Lawrence DA, Border WA, Yu L, Noble NA. A mutant, noninhibitory plasminogen activator inhibitor type 1 decreases matrix accumulation in experimental glomerulonephritis. *J Clin Invest* 2003; 112: 379–388
16. Roberts AW, Thomas A, Rees A, Evans M: Peroxisome proliferator-activated receptor-gamma agonists in atherosclerosis: current evidence and future directions. *Curr Opin Lipidol* 2003; 14: 567–573
17. Zirlik A, Leugers A, Lohrmann J *et al*. Direct attenuation of plasminogen activator inhibitor type-1 expression in human adipose tissue by thiazolidinediones. *Thromb Haemost* 2004; 91: 674–682
18. Ma LJ, Marcantoni C, Linton MR *et al*. Peroxisome proliferators-activated receptor-γ agonist troglitazone protects against nondiabetic glomerulosclerosis in rats. *Kidney Int* 2001; 59: 1899–1910
19. Kanjanabuch T, Ma LJ, Guan YF *et al*. Protective effects of peroxisome proliferator-activated receptor-gamma (PPAR-γ) against puromycin aminonucleoside (PAN)-induced podocyte injury and death. *J Am Soc Nephrol* 2003; 14: 93A
20. Wallace A, Minto M, Erwig LP. Heterogeneity of macrophage activation in anti-Thy-1.1 nephritis. *Am J Pathol* 2003; 163: 2033–2041

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