Transpl Int (2003) 16: 681–688
DOI 10.1007/s00147-003-0593-3

ORIGINAL ARTICLE

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Expression of growth arrest-specific gene 6 and its receptors in dysfunctional human renal allografts

Received: 15 August 2002
Revised: 19 November 2002
Accepted: 28 January 2003
Published online: 27 May 2003

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Abstract Growth arrest-specific gene 6 (Gas6) and its receptors Rse, Axl and Mer have recently been found to be involved in a rat model of chronic allograft nephropathy (CAN). Thus, in this study we investigated the function of Gas6 and its receptors in human renal allograft dysfunction. Expression of Gas6 and its receptors was detected by immunohistochemical staining. Gas6 and its receptors were widely expressed in glomeruli, tubules and vessels of renal allografts. Gas6 expression was detected in normal-functioning allografts and was increased in acute rejection ($P < 0.05$), acute tubular necrosis ($P < 0.05$) and CAN ($P < 0.01$). Gas6 receptors were not upregulated in any of the allograft groups, except for the Axl receptor, which increased only in acute tubular necrosis ($P < 0.01$). Gas6 expression was also found to correspond with the expression of α-smooth muscle actin, a general marker of CAN ($r^2 = 0.21$, $P < 0.01$). These findings suggest that Gas6, acting as a growth factor, is increased in the process of kidney allograft dysfunction and in CAN.

Keywords Renal transplantation · Allograft dysfunction · Gas6 · Rse · Axl · Mer

Introduction

The growth arrest-specific gene 6 (Gas6) protein has been identified as a new member of the vitamin K-dependent family of proteins, with a significant degree of amino acid identity to protein S, a serum protein that negatively regulates blood coagulation [1]. It acts as the ligand for three members of the family of tyrosine kinases: Axl, Mer and Rse [2, 3, 4]. Gas6 is ubiquitously expressed and has been implicated as a mitogen for endothelial cells [5], neural cells [6] and vascular smooth muscle cells [7, 8, 9, 10]. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis [11]. Furthermore, Gas6 has also been confirmed to be an endogenous mitogen for glomerular mesangial cells [12, 13] and is upregulated in a rat model of chronic allograft nephropathy (CAN, also known as chronic rejection) [14]. However, the expression of Gas6 and its receptors in human allograft kidneys has not been investigated.

Allograft dysfunction after renal transplantation is generally associated with one or more of the following pathological diagnoses: acute rejection (AR), acute tubular necrosis (ATN), calcineurin inhibitor toxicity (CIT) and CAN. Among these, CAN is the most common cause of allograft loss in transplant patients.

Many growth factors are involved in kidney allograft dysfunction, especially in CAN [15, 16]. AR is mainly

induced by immunological mechanisms, but there is also upregulation of growth factors, such as TGF-β, EGF and IGF, which may contribute to the subsequent development of CAN [16, 17, 18]. The upregulation of some growth factors also plays an important role during the recovery of the kidney from ATN [15]. Thus, the study of the function of growth factors in kidney allografts is important to delineate the mechanisms underlying allograft damage and the relationships between CAN and AR, ATN or CIT. Gas6 has been identified as a growth factor that can stimulate the proliferation of vascular smooth muscle cells [7, 10], mesangial cells [12, 13] and fibroblasts [19]. Proliferation of these cell types is a hallmark of CAN and other types of kidney dysfunction, suggesting that Gas6 may be involved in the pathogenesis of renal allograft dysfunction.

In this study we examined the expression of Gas6 and its receptors in four clinical entities associated with allograft dysfunction after renal transplantation, compared with the normal-functioning allograft (control normal, Cn). The results were compared with the expression of CD68 (a macrophage marker) and α-smooth muscle actin (α-SMA), both of which are involved in the pathogenesis of CAN [20, 21].

Materials and methods

Renal transplant patients

This study of human kidney transplant patients was approved by the ethics review committee of the Royal Prince Alfred Hospital, New South Wales, Australia (approval reference X99-0148).

The patients received a variety of immunosuppressive combinations as per unit standard at the time of transplantation. All patients received prednisone. The majority were on triple-drug therapy with a calcineurin inhibitor and purine antagonist. Twelve of 32 patients received a signal 3 inhibitor in combination with cyclosporine and prednisone in the context of clinical trials. The latter were spread out through the five study groups. There were no obvious differences in immunosuppressive-treatment regimes between groups.

A total of 38 renal allograft biopsy tissue samples from 32 patients who received their renal allograft between 1997 and 2000 was retrospectively analysed. Twenty-seven patients underwent one biopsy each, five patients had two biopsies and one patient had three biopsies. The biopsies were performed on clinical indication to determine the basis for graft dysfunction as marked by a rise in baseline creatinine or by significant delayed graft function after transplantation. None were protocol biopsies. The 38 biopsies were selected from a total of 500 performed in the study period and based on the pathological diagnosis, where the selection criteria required that there was nil or minimal pathological overlap between the dysfunction diagnoses under study.

The biopsy tissues included in this study were classified on the basis of the Banff working classification standard defined in 1997 [22]. AR was characterised by significant lymphocyte interstitial infiltration and invasion of tubules or the walls of blood vessels. CIT was characterised by arterial and arteriolar hyalinosis as well as interstitial fibrosis. ATN was diagnosed by the absence of lymphocytic infiltration, the loss or flattening of tubular epithelium and tubular dilatation with epithelial cell regeneration and mitotic figures. CAN was based on the presence of interstitial fibrosis, tubular atrophy and arterial fibrous intimal thickening.

Immunohistochemical staining

Renal biopsy tissue was routinely fixed in formalin and embedded in paraffin blocks. Sections were cut from paraffin-embedded kidney tissue at 2-μm thickness onto gelatine-coated slides. Xylene was used to remove the paraffin, and the tissue sections were rehydrated in graded ethanol to water. Slides stained with haematoxylin and eosin (H&E) were used for histopathological evaluation of the tissue.

The avidin–biotin complex technique was used to detect the binding of specific antibodies to Gas6, its receptors, CD68 and α-SMA. Endogenous peroxidase activity was blocked with 1% H_2O2 in methanol for 15 min, followed by antigen retrieval with protease (P4630, Sigma) at 0.5 mg/ml for 5 min. Non-specific protein binding was blocked with 20% normal horse serum for 20 min at room temperature. The sections were then incubated for 1 h with primary antibodies including (1) anti-CD68 and anti-α-SMA mouse monoclonal antibodies (DAKO, Carpinteria, Calif., USA); (2) anti-Gas6, anti-Axl, anti-Rse and anti-Mer goat polyclonal antibodies (Santa Cruz, Santa Cruz, Calif., USA). The same concentrations of mouse and goat IgG were used separately as a negative control for antibodies raised in mouse or goat, respectively. Antibody dilutions were CD68 and α-SMA (1:100) and Gas6, Axl, Mer and Rse (1:80), diluted in Tris buffer (100 mmol/l NaCl and 50 mmol/l Tris-HCl, pH 7.4) containing 1% normal horse serum. Primary antibody binding was detected with the DAKO LABS+ Kit, Peroxidase (K0690, DAKO). Sections were incubated with biotinylated universal secondary antibody for 15 min, followed by streptavidin peroxidase for another 15 min. Staining was visualised with DAB solution (K3468, DAKO Liquid DAB+ substrate-chromogen solution) for 8 min. The slides were counterstained with haematoxylin followed by dehydration in graded ethanol to xylene and mounted with DPX for evaluation. To ensure uniformity between experiments, we included samples from all five groups in each individual immunohistochemical staining experiment.

We analysed the antibody-stained sections for Gas6 and its receptors using the Image pro Plus 4 (Media, Cybermedic) analysis system for the light microscope, as described previously [23, 24]. Positive targets of these respective antibody-staining cells, either in the whole kidney biopsy or within the glomeruli, were identified and designated on the displayed image. This definition of positivity was used for analysis of all subsequent samples. Ten randomly selected fields per section at 400× magnification were analysed. All the glomeruli in each slide were selected when expression of Gas6 and its receptor proteins was detected in glomeruli. The image analysis results are presented as image analysis units (percentage of positive staining area).

Cells with positive staining for CD68 in the tubulo-interstitium were counted with an ocular grid at 400× magnification. α-SMC staining was assessed by morphometric analysis using point counting with an ocular grid at 400× magnification [25]. Using ten such fields we analysed both antibodies and then averaged the results.

Statistics

Results are reported as mean ± SE. For statistical analysis, an unpaired Student's t-test was used for each antibody staining in the different groups. Correlation coefficients were analysed with Statview (Abacus Concepts, Berkeley, Calif., USA). A P value of less than 0.05 was considered to be statistically significant.

Results

Clinical parameters of patient groups

Thirty-eight allograft kidney biopsies obtained from 32 patients were assigned to five groups, depending on their histopathological diagnosis (Table 1). The diagnoses were: seven patients with Cn, seven in AR, nine in CAN, seven in CIT and eight in ATN. Clinical parameters for the patients are listed in Table 1. The transplantation-to-biopsy interval was significantly longer only between CAN and ATN or CIT. There were no differences in the baseline level of plasma creatinine among the five groups, except that the plasma creatinine level obtained at the time of biopsy in the Cn group was lower than in the other four groups ($P < 0.05$).

Expression of Gas6 and its three receptors in renal allografts after transplantation

The morphology of antibody staining of Gas6 and its receptor proteins in human transplanted kidneys is shown in Fig. 1. The slides that were stained with the negative control goat IgG isotype showed no staining. Positive staining for Gas6 was observed in the glomeruli, tubules and vessels of all groups, although the level of expression varied. A baseline level of expression of Gas6 was observed in the Cn group (Fig. 2). There was a widespread increase in expression of Gas6 in the biopsies of CAN and ATN. Compared with the control allografts, the level of expression was increased threefold for the CAN ($P < 0.01$) and twofold for the ATN ($P < 0.01$).

The staining of receptors for all five diagnostic groups is also shown in Fig. 2. The distribution of staining in the glomeruli, tubules and vessels for each receptor followed a similar pattern to the Gas6 staining. The overall staining intensity for each of the receptors was approximately 2- to 10-times lower than the staining for Gas6 ligand. The Axl receptor was found to be upregulated in the ATN group compared with the Cn ($P < 0.01$). Notably, Mer and Rse were not increased in any of the dysfunction groups studied (Fig. 2).

Expression of Gas6 and its three receptors in the glomerulus after renal transplantation

Gas6 has been reported to be involved in the proliferation of mesangial cells in vitro [13]. Thus, the expression of Gas6 and its receptors was specifically analysed within the glomeruli. The results were generally similar to those obtained from whole kidney. Gas6 was significantly increased in glomeruli in the AR group ($P < 0.05$), the CAN group ($P < 0.01$) and the ATN group ($P < 0.05$) compared with the Cn group (Fig. 2).

The overall staining intensity in the glomeruli for each of the receptors was approximately 3- to 40-times lower than the staining for Gas6 ligand. Notably, there was no increase in the intensity of staining for any of the receptors in the four dysfunction groups studied.

Expression of CD68 and α-SMA protein, compared with Gas6 and its receptors

When the immunohistochemical staining with anti-CD68 and α-SMA antibodies was compared in the five groups, the CD68 staining level in the AR and CAN groups was significantly higher than Cn ($P < 0.05$ for AR and $P < 0.01$ for CAN) (Fig. 3). $CD68^+$ cells were mainly present within inflammatory infiltrates in the interstitium between tubules and glomeruli for the AR group, but in the CAN group $CD68^+$ cells were generally increased in scar tissue, the interstitium and glomeruli.

α-SMA expression was significantly increased in the CAN group ($P < 0.01$) (Fig. 3). Cytoplasmic staining for α-SMA was present in vascular smooth muscle cells of vessels, interstitial myofibroblasts and glomerular mesangial cells, consistent with previous observations [14].

Pooled data of the staining of CD68 and α-SMA were compared by linear regression analysis with the expression of Gas6 and its receptor proteins. There was no correlation between the staining of CD68 and Gas6 or its receptors ($P > 0.05$). The α-SMA staining was found to be correlated with Gas6 expression ($r^2 = 0.21$, $P < 0.01$), but not with Gas6 receptor expression ($P > 0.05$) (Table 2).

Table 1 Clinical characteristics of the five patient groups

Characteristic	Control (normal)	Acute rejection	Chronic allograft nephropathy	Acute tubular necrosis	Calcineurin inhibitor toxicity
No. of male patients (%)	7 (57.1)	7 (42.9)	9 (22.2)	8 (75.0)	8 (75.0)
Age (years)	50.4 ± 10.5	52.0 ± 8.7	44.7 ± 12.0	39.7 ± 11.2	42.5 ± 8.8
Days post-transplantation at biopsy	160.0 ± 252.2	68.4 ± 74.1	313.2 ± 242.1	28.4 ± 40.9	34.3 ± 37.3
Plasma creatinine at baseline (μmol/l)	118.1 ± 8.9	120.0 ± 47.1	143.3 ± 29.4	104.2 ± 21.2	134.2 ± 34.5
Plasma creatinine at biopsy date (μmol/l)	125.0 ± 8.4	156.3 ± 41.0	232.8 ± 45.1	240.4 ± 208.2	379.0 ± 194.3
Delayed graft function (%)	25	33.3	77.8	42.8	20

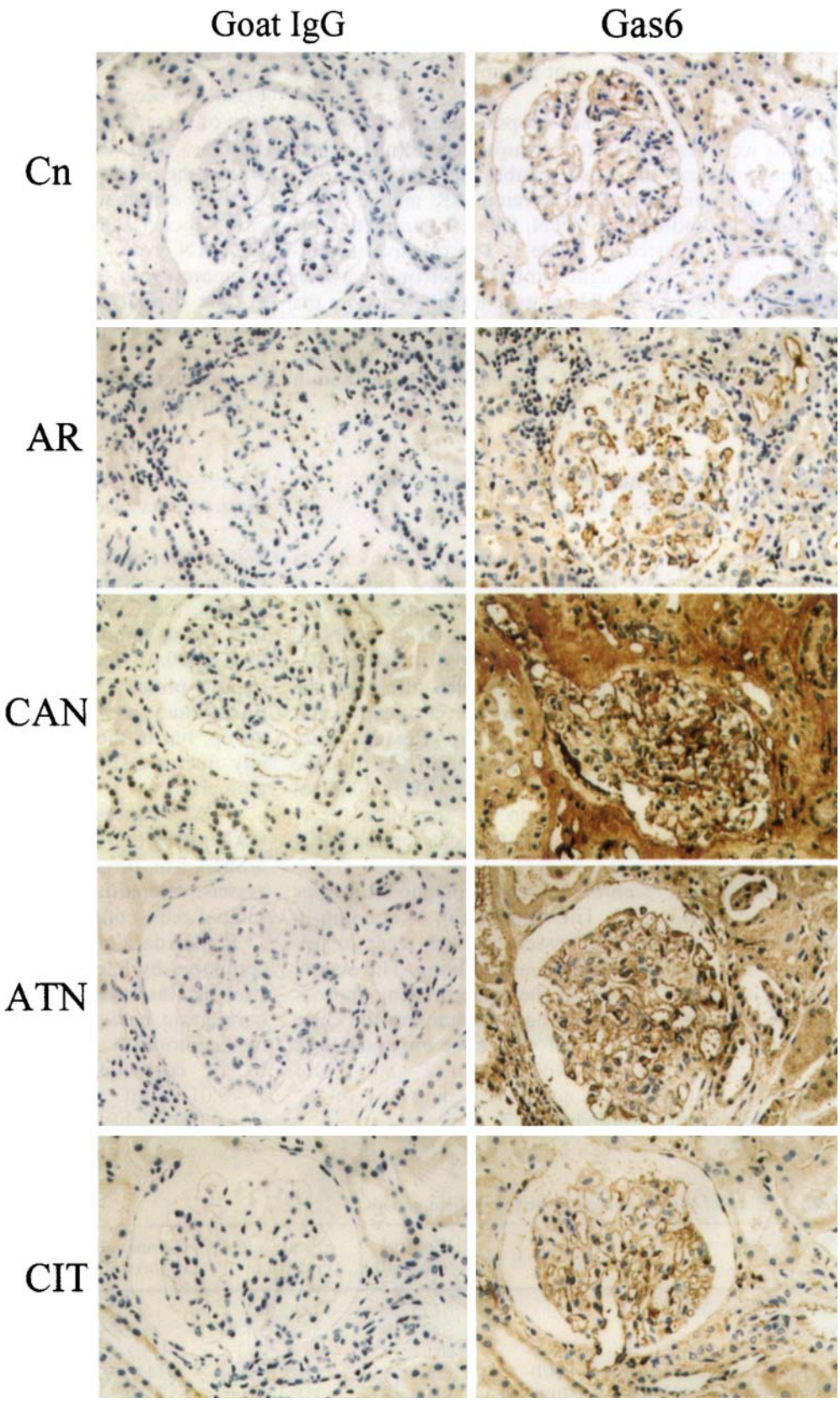

Fig. 1 Immunohistochemical staining of anti-Gas6 antibody in five groups of dysfunctional human allografts. The goat IgG group was used as a negative control, at the same concentrations as the antibody groups (*left column*)

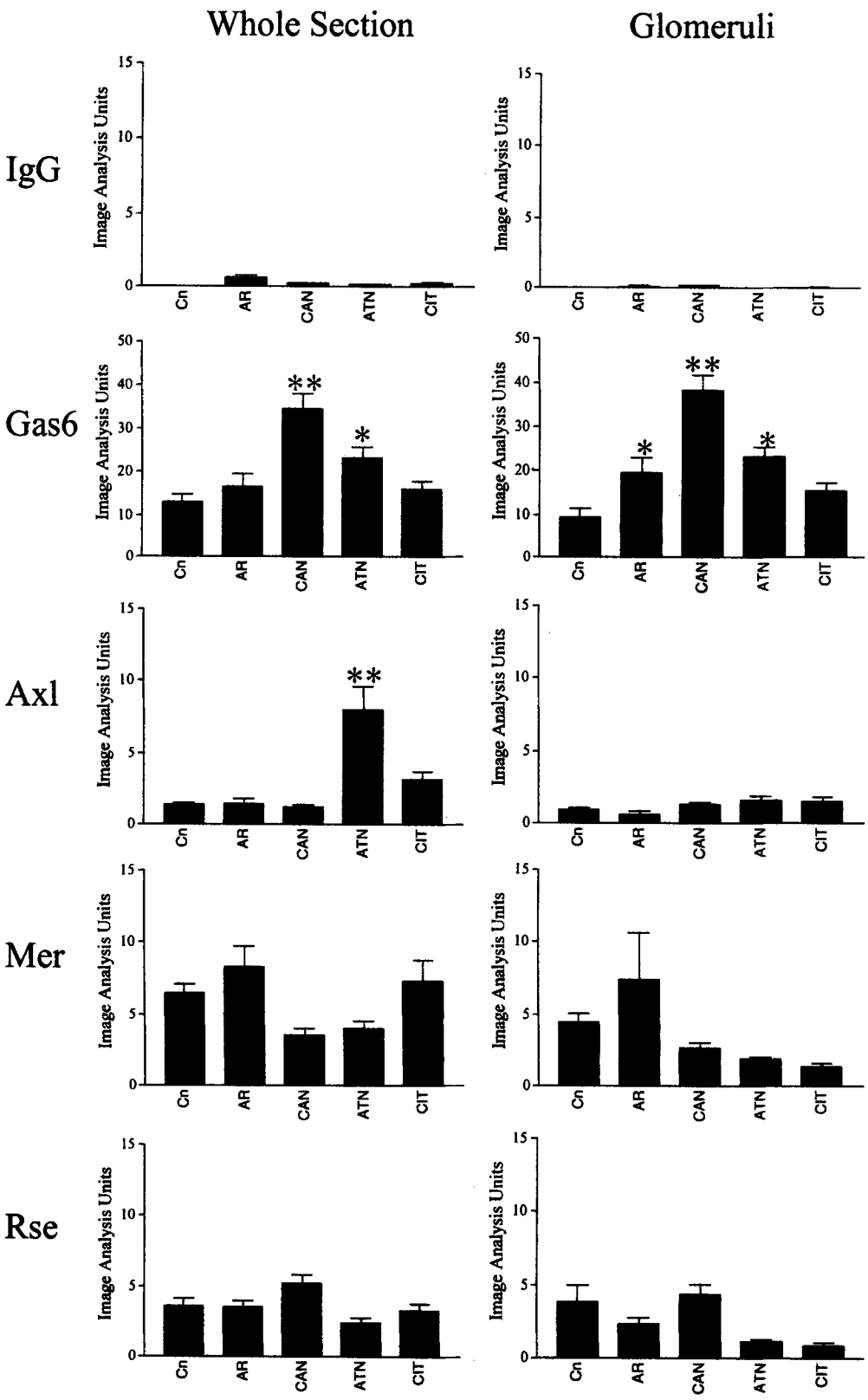

Fig. 2 Expression levels of Gas6 and its receptor proteins in human dysfunctional allograft groups obtained by analysis of immunohistochemical staining with a computerised image system. *Left* the results of expression in the whole kidney, *right* staining only in the glomeruli. The image analysis unit was the positive percentage in the analysed tissue. The *P* value is the comparison of different dysfunction allograft with normal functioning allograft ($*P < 0.05$, $**P < 0.01$)

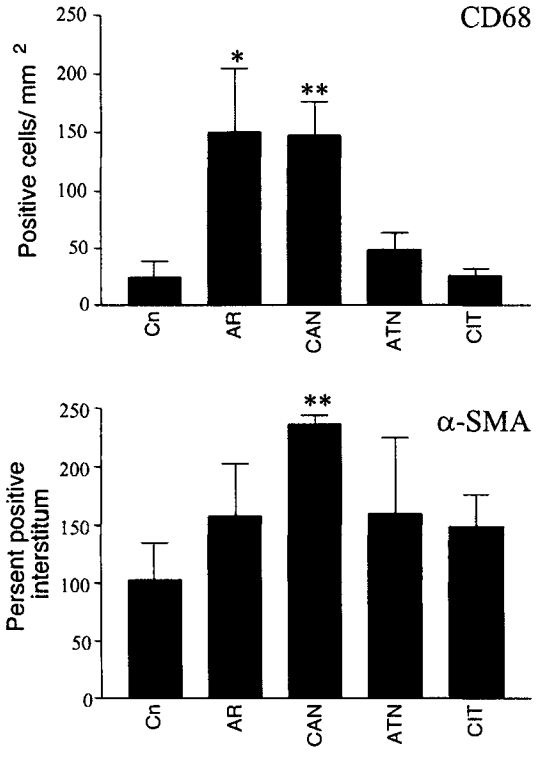

Fig. 3 Positive staining of CD68 (*top*) and α-SMA (*bottom*) protein was detected in all five groups of allograft biopsies. The *P* value is the comparison of different dysfunction allograft groups with the normal functioning allograft group (*$P < 0.05$, **$P < 0.01$)

Table 2 Analysis of correlation between staining of CD68, α-SMC and Gas6 or its receptors (*P* value)

Parameter	Gas6	Axl	Mer	Rse
CD68	0.3997	0.7911	0.3569	0.5435
α-SMC	0.0084	0.6363	0.6223	0.5526

Discussion

In this study we have shown for the first time that expression of Gas6 and its receptors is increased in human allograft dysfunction, due to AR, ATN and, especially, CAN. We have previously shown that Gas6 mRNA is extensively expressed in rat kidney and is increased in the early stages of CAN [14]. Gas6 has been implicated in reversible cell growth arrest [1], survival [19], proliferation [6, 7, 19] and cell adhesion [10, 26, 27] through a phosphotyrosine kinase signal pathway. These various functions may be tissue-specific. Gas6 has been shown to be a potentiating growth factor for many types of cell, including fibroblasts [19], smooth muscle cells [7, 10] and mesangial cells [12, 13]. All these cell types have been implicated in allograft dysfunction. Thus, Gas6 may play a role in the development of allograft dysfunction, especially in the pathogenesis of CAN.

We investigated the expression of Gas6 and its receptors in human renal allografts that were exhibiting either AR, CAN, ATN or CIT. Our results showed that Gas6 gene expression was substantially higher in the CAN and ATN groups, but was also significantly elevated in the AR group within the glomerulus. The three Gas6 receptors were found to be expressed in kidney tissue, although the expression was generally lower than Gas6 itself. Compared with control non-dysfunctional allografts, the Axl receptor was significantly upregulated in the ATN group. Mer and Rse receptor expression was similar in all five groups, including control allografts. These results show that differential upregulation of Gas6 protein expression is consistent with its possible role in the pathogenesis of allograft dysfunction, especially in CAN after renal transplantation.

Gas6 has been previously identified as a ligand for members of the subfamily of receptor phosphotyrosine kinases and can activate all three subfamily members, Axl, Mer and Rse [2, 3, 4]. Receptors in the Axl/Mer/Rse family share high-sequence homology with each other. As Gas6 can stimulate each of these different receptors, it is possible that it may activate more than one signalling pathway in cells that express multiple receptors. Gas6 receptors have been found in cells from various tissues: the Axl receptor is expressed in 3T3 fibroblasts [28], vascular smooth muscle cells [10] and mesangial cells [12]. Both Rse and Mer are found in testicular somatic cells [29], and Axl and Rse are present in human Schwann cells [6]. All three receptors can be detected in Sertoli cells [30], neurones [31] and platelets [11]. Notably, available studies have not examined all receptors in each tissue.

To date, there has been only one study of the relationship between the Gas6/Axl pathway and mesangial cell proliferation. Substantial upregulation of Gas6/Axl was observed in a rat model of experimental glomerulonephritis [13], although this study did not examine Rse and Mer receptor expression in the kidney. In general, the finding of differential expression of a ligand associated with a pathological process requires the discovery of its putative receptors in order for the relevant signalling interactions to be identified. In the present study, all three receptors were found in the kidney allografts, with upregulation of Axl in acute tubular necrosis. This may reflect the differential involvement of various cell types, expressing different receptors, in acute tubular necrosis compared with acute or chronic rejection after kidney transplantation. The finding that Gas6 but not its receptors was increased in CAN suggests that if Gas6 is involved in CAN, this is controlled by the level of Gas6 production, rather than the level of its receptors.

The glomerulus, which includes mesangial cells, capillary endothelial cells and visceral and parietal epithelial cells, plays a central role in kidney structure and function. An increase in mesangial matrix in the

glomerulus is a notable feature in CAN. In the experimental rat glomerulonephritis model, Gas 6 and Axl receptor expression was mainly co-localised in mesangial cells [13], although Mer and Rse receptors were not examined. We selectively examined human allograft glomeruli for the expression of Gas6 and Axl, Mer and Rse receptors. Expression of Gas 6 and its receptors was seen in most cell types, including endothelial, mesangial and epithelial cells. More detailed studies will be required to quantitate more accurately the localisation of Gas6 and its receptors within the glomerulus. Notably, previous studies have shown that Gas6 and its receptors are present on endothelial cells [5, 29] and mesangial cells [13]. Our results show that many cell types within the glomerulus appear to secrete Gas6, and that most cell types within the glomerulus stain for one or more Gas6 receptors. Thus, Gas6 may act in an autocrine and/or paracrine manner in vivo.

The expression of CD68 and α-SMA has been reported to be upregulated in dysfunctional allograft groups [20, 21, 32]. The increase in CD68 is due to macrophage cell infiltration in AR and CAN [32]. α-SMA is mainly increased due to proliferation and phenotypic change of interstitial myofibroblasts, vascular smooth muscle cells and mesangial cells in CAN [20]. In this study we demonstrated a correlation between the expression of α-SMA and Gas6. However, there was no correlation between CD68 and Gas6 expression. These data are consistent with the notion that Gas6 acts as a growth factor for α-SMA positive-staining myofibroblasts, vascular smooth muscle cells and mesangial cells in allograft dysfunction.

In summary, this is the first study to investigate the expression of Gas6 and its receptors in human allografts after renal transplantation. It shows that Gas6 and its receptors are extensively expressed in human kidney tissue, including glomeruli, vessels and tubules. Gas6 was significantly increased in the dysfunctional groups, especially in the CAN group. Our results support the hypothesis that Gas6, acting as a growth factor, is involved in the pathogenesis of allograft dysfunction, especially in the development of CAN. Gas6 may be a possible new target for the overcoming of dysfunctional pathological conditions in kidney allografts.

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