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ORIGINAL ARTICLE

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# De-novo expression of vascular ecto-5′-nucleotidase and down-regulation of glomerular ecto-ATPase in experimental chronic renal transplant failure

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**Abstract** Ischemic injury plays an important role in chronic renal transplant failure (CRTF). Down-regulation of ecto-adenosine triphosphatase (ATPase) in combination with up-regulation of ecto-5′-nucleotidase is a hallmark of ischemic injury. We studied the expression of renal ecto-5′-nucleotidase and ecto-ATPase in experimental renal transplantation. Fisher 344-to-Lewis allografted rats were either treated with an angiotensin-converting enzyme inhibitor (ACEi) or left untreated. Lewis-to-Lewis syngrafted rats served as controls. Untreated allografted rats developed proteinuria, glomerulosclerosis, and mild intimal hyperplasia. ACEi completely prevented focal and segmental glomerulosclerosis (FGS) and proteinuria, but significantly enhanced intimal hyperplasia. Untreated allografted rats revealed marked vascular ecto-5′-nucleotidase activity, which increased with ACEi. Vascular ecto-5′-nucleotidase activity was absent in syngrafted animals. Ecto-5′-nucleotidase activity correlated well with intimal hyperplasia. Glomerular ecto-ATPase expression was significantly reduced in untreated allografted rats compared to syngrafted rats and correlated well with the extent of FGS. ACEi prevented reduction in glomerular ecto-ATPase. We found de-novo expression of ecto-5′-nucleotidase at sites of renal intimal hyperplasia. Glomerular ecto-ATPase expression was markedly reduced in allografted rats and was prevented by ACEi. These enzyme expression patterns suggest local ischemic damage in experimental CRTF.

**Keywords** Kidney transplantation · Rat · Ecto-adenosinetriphosphatase · Ecto-5′-nucleotidase · Ischemia

## Introduction

In human renal transplantation, a significant proportion of grafts is lost due to chronic renal transplant failure (CRTF). More than 50% of the grafts that do survive for 1 year fail within 10 years through progressive loss of renal function and histopathological structures, i.e., interstitial fibrosis, intimal thickening of the arteries, and focal and segmental glomerulosclerosis (FGS). The vascular changes consist of dense intimal fibrosis mainly in the cortical arteries and result in renal ischemia manifested by glomerular ischemic simplification and obsolescence, interstitial fibrosis, and tubule atrophy. The latter process results in a decline in renal function and slowly rising serum creatinine levels.

Ischemic injury may play a significant role in the pathogenesis of CRTF since it causes local production of reactive oxygen species (ROS), which may subsequently damage the graft. Ischemia also leads to cellular adenosine triphosphate (ATP) release [13], which can be degraded by ecto-adenosinetriphosphatase (ATPase). It has been shown that glomerular

ecto-ATPase is extremely sensitive for oxygen stress, resulting in down-regulation of this enzyme after exposure to ROS [2, 19]. Glomerular ecto-ATPase is markedly decreased in patients with acute and chronic rejection [3] as well as in subjects with delayed diuresis following renal transplantation [19] and after renal xenotransplantation [17].

Further degradation of nucleotides by ecto-5′-nucleotidase promotes the formation of adenosine, which exerts potent vaso-active, anti-inflammatory, and anti-thrombotic activity [16]. In vivo, glomerular expression of ecto-5′-nucleotidase is found in human CRTF and in subjects with glomerular ischemia due to malignant hypertension [3] as well as in experimental glomerulosclerosis [16]. Therefore, we feel that down-regulation of local ecto-ATPase expression concomitant with up-regulation of ecto-5′-nucleotidase is characteristic of ischemic injury [3].

In the Fisher-to-Lewis rat model for human CRTF, structural changes such as FGS, interstitial fibrosis and inflammation, and vascular intimal fibrosis develop in the transplanted organ. Treatment with angiotensin-converting enzyme inhibitor (ACEi) in this model prevents FGS and proteinuria without affecting interstitial changes [4, 18]. However, this treatment regimen causes massive increments in vascular intimal hyperplasia when used for more than 20 weeks [18].

In the present study, we document the presence of local ischemic damage in the renal vasculature of transplanted rats with CRTF, using specific staining methods for ecto-ATPase and ecto-5′-nucleotidase. Furthermore, we assessed whether intervention with ACE inhibition modulates these expression patterns.

## Materials and methods

### Experimental techniques

Inbred male rats, weighing 215 ± 9 g were used (Harlan, Horst, The Netherlands). Lewis SsN rats (Lew) served as recipients, Fisher 344 NHsd rats (F344) as donors. Lew kidneys placed in Lew recipients served as controls. The rats were anesthetized with isoflurane; left donor kidneys were flushed with saline, preserved in saline on ice for approximately 20 min, and transplanted orthotopically. The right donor kidney was also removed and used to determine basal expression of ecto-ATPase and ecto-5′-nucleotidase. The recipient rat underwent left-sided nephrectomy, and subsequently, the left renal vessels and ureter were anastomosed end-to-end to the vessels and ureter of the donor kidney with 10-0 Prolene sutures (Johnson & Johnson, Brussels, Belgium). Vascular clamps were released after the vascular anastomosis was completed, with a warm ischemia time of 15 to 18 min. The right native kidney of the recipient was removed 10 days after transplantation, and the transplanted kidney was checked at that time. One out of ten transplanted kidneys was considered a technical failure due to hydronephrosis. These rats were excluded from the study. A short course of cyclosporin A (1.5 mg/kg per day; Sandimmun, Sandoz Pharma, Basle, Switzerland) was administered subcutaneously to allografted and syngrafted rats over the first 10 post-operative days to reverse acute rejection. Principles of laboratory animal care were followed; all animal procedures were approved by the animal research ethics committee of the Faculty of Medical Sciences of the University of Groningen.

### Experimental groups

After cessation of cyclosporine therapy, allografted rats received either no treatment and served as controls (F-L, $n=9$) or received the ACEi lisinopril (Merck, Sharp & Dohme Research Laboratories, Rahway, N.J., USA) (F-L + ACEi, $n=8$, 75 mg/l drinking water). This regimen prevents clinical signs of CRTF and preserves glomerular morphology, although long-term treatment induces massive vascular thickening [18]. Syngrafted rats were not treated (L-L, $n=8$). Lisinopril was dissolved in drinking water. Seventy-five milligrams of lisinopril were dissolved in 100 ml of a saturated NaHCO3 solution (Merck, Darmstadt, Germany) and then diluted in 3.9 l 0.5% (w/v) methylcellulose suspension (Sigma-Aldrich Chemie, Steinheim, Germany). Untreated allografted and syngeneically grafted rats received only the methylcellulose suspension with NaHCO3 in the same concentration. The rats were housed under standard conditions with free access to drinking solution and standard rat chow.

### Clinicopathological parameters

The total observation period was 34 weeks. Body weight was determined every 4 weeks. We determined urinary protein excretion, measured by the pyrogallol red-molybdate method, every 4 weeks and at the end of the study by placing the rats for 24 h in metabolic cages with access to medication solution only. At the end of the observation period, rats were anesthetized with isoflurane, and systolic blood pressure (SBP) was measured by the tail-cuff method. Subsequently, the aorta was cannulated and the kidney graft was perfused in situ for 1 min with saline.

### Tissue processing and histochemical staining procedures

After perfusion, two coronal tissue slices of the kidney were obtained. One slice was snap-frozen in liquid isopentane (–80 °C) and stored at –80 °C. The other specimen was fixed in 4% paraformaldehyde and processed for paraffin embedding. The paraffin sections were stained with periodic acid-Schiff (PAS) reagent and Verhoeff stain for assessment of glomerular and vascular damage.

### Immunohistochemistry and enzyme histochemistry

We performed immunohistological examination for rat ecto-ATPase on frozen sections using a monoclonal anti-ecto-ATPase antibody [5] followed by peroxidase-conjugated rabbit anti-mouse antibody and peroxidase-conjugated goat anti-rabbit antibody. Peroxidase activity was visualized with 3-amino-9-ethylcarbazole (AEC). For the demonstration of renal ecto-5′-nucleotidase activity, we used conventional enzyme histochemistry [21] with adenosine monophosphate (AMP) as a substrate and lead as capture ion. The precipitated reaction product (lead phosphate) was visualized with sodium disulfide, showing a dark-brown reaction product [21]. Since macrophage-derived cytokines can up-regulate the expression of 5′-nucleotidase, we immunostained sections with the ED1 antibody [9] to determine whether these cells were present at sites of increased ecto-5′-nucleotidase. We performed additional immunostaining according to standard procedures for smooth muscle α-actin and endothelial cells to determine the cellular composition of vascular intimal hyperplasia. Sections in which the first antibody was replaced with phosphate-buffered saline served as controls and were consistently negative.

### Analysis of structural, immunohistochemical, and enzyme histochemical changes

Morphological changes, i.e., severity of FGS, were scored semi-quantitatively on a scale of 0 to 4+. FGS was scored positive if mesangial matrix expansion and adhesion formation were present in the same quadrant. If 25% of the glomerulus was affected, a score of 1+ was adjudged; 50% was scored as 2+; 75% as 3+; and 100% as 4+. The ultimate score was obtained by multiplication of the degree of change by the percentage of glomeruli with the same degree of injury, and addition of these scores (maximum score 400). A total number of 40–50 glomeruli per animal were scored moving from cortex to medulla and vice versa.

Arterial intimal surface area was measured by morphometry. Verhoeff-stained paraffin-embedded sections were screened at a magnification of 200× by a light microscope with a camera attached and a drawing prism. The image of a given transversally cut artery present on a computer screen was traced over the surface of a graphic tablet connected to a computer. The image was traced along the lamina elastica and the lumen. The surface area of the arterial intima was calculated by computer analysis. To compare larger and smaller vessels, we calculated the ratio between intimal surface area and intimal circumference.

Vascular ecto-5′-nucleotidase staining was scored semi-quantitatively. When staining was present in one quadrant of the vessel, a score of 1+ was adjudged; staining in 2 quadrants was scored as 2+; in 3 quadrants as 3+; and when staining was present in 4 quadrants, a 4+ score was given. Final scores were determined as described above for FGS. All visible vessels present in the section were scored.

Glomerular ecto-ATPase staining was measured by computer-assisted morphometry. Thirty glomeruli were screened with a light microscope equipped with a camera device connected to a video screen. The image of a given glomerulus present on the screen was traced with a cursor along the glomerular tuft over the surface of a graphic tablet connected to the computer. Subsequently, the total surface with red precipitate was measured and divided by the total surface of the glomerulus. An average score was calculated per section.

### Statistical analysis

Reported values are the group mean ± standard deviation. Differences between untreated allografted and syngrafted rats were tested for significance with the Mann-Whitney U-test or the *t*-test. Differences were considered statistically significant with a $P$ value of less than 0.05. We log-transformed the values for vascular ecto-5′-nucleotidase activity and FGS to normalize their distribution. The Pearson correlation coefficient was calculated between FGS score and ecto-ATPase staining for untreated allografts, and correlation between ecto-5′-nucleotidase and intimal hyperplasia was calculated for all groups.

## Results

### Clinical and structural changes

No differences in body weight were observed between the experimental groups throughout the study. In the untreated allografted rats, proteinuria was significantly elevated at 12 weeks (31 ± 16 mg/24 h) compared with the syngrafted animals (13 ± 3 mg/24 h, $P < 0.01$), further increasing to 93 ± 75 mg/24 h at 34 weeks. The syngrafted rats did not develop proteinuria (22 ± 13 vs 93 ± 75 mg/24 h, $P < 0.01$) at 34 weeks. Treatment of the allografted group with lisinopril completely prevented proteinuria at the end of the study (8 ± 4 vs 93 ± 75 mg/24 h, $P < 0.0001$).

SBP was increased in the untreated allograft group (145 ± 19), although not significantly different from the syngrafted rats (125 ± 30). ACE inhibition resulted in a significantly lower SBP than in untreated allografts (91 ± 10 vs 145 ± 19 mg/24 h, $P < 0.0001$). FGS was prominent in the untreated allografted rats, whereas syngrafts revealed no signs of FGS (137 ± 138 vs 6 ± 11 mg/24 h, $P < 0.01$). ACE inhibition completely prevented the development of FGS when compared with the untreated allografted animals (2 ± 3 vs 137 ± 138 mg/24 h, $P < 0.001$).

Data on intimal surface area are shown in Fig. 1. Intimal surface area ($\mu m^2/\mu m$ circumference) was significantly lower in syngrafted rats compared with allografted rats ($P < 0.01$). ACE inhibition for 34 weeks dramatically increased intimal hyperplasia compared with untreated allografted rats ($P < 0.0001$).

### Ecto-5′-nucleotidase activity

No differences were observed in ecto-5′-nucleotidase activity between non-transplanted F344 and Lew control kidneys. Both in syngrafted and allografted rats, ecto-5′-nucleotidase activity was predominantly localized on the brush border of the renal tubules (Fig. 2a and c).

Except for strong staining in the tunica media of larger renal arteries, ecto-5′-nucleotidase was absent in glomeruli and arteries from syngrafted rats (Fig. 2a and

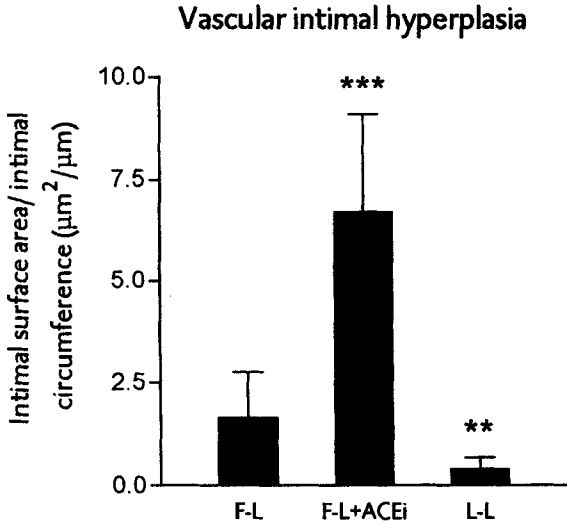


**Fig. 1** The presence of vascular intimal hyperplasia in untreated allografted rats (F-L), allografted rats receiving ACEi (F-L+ACEi) and syngrafted rats (L-L). Values are presented as mean ± SD. $^{**}P < 0.01$, L-L vs F-L; $^{***}P < 0.001$, F-L+ACEi vs F-L

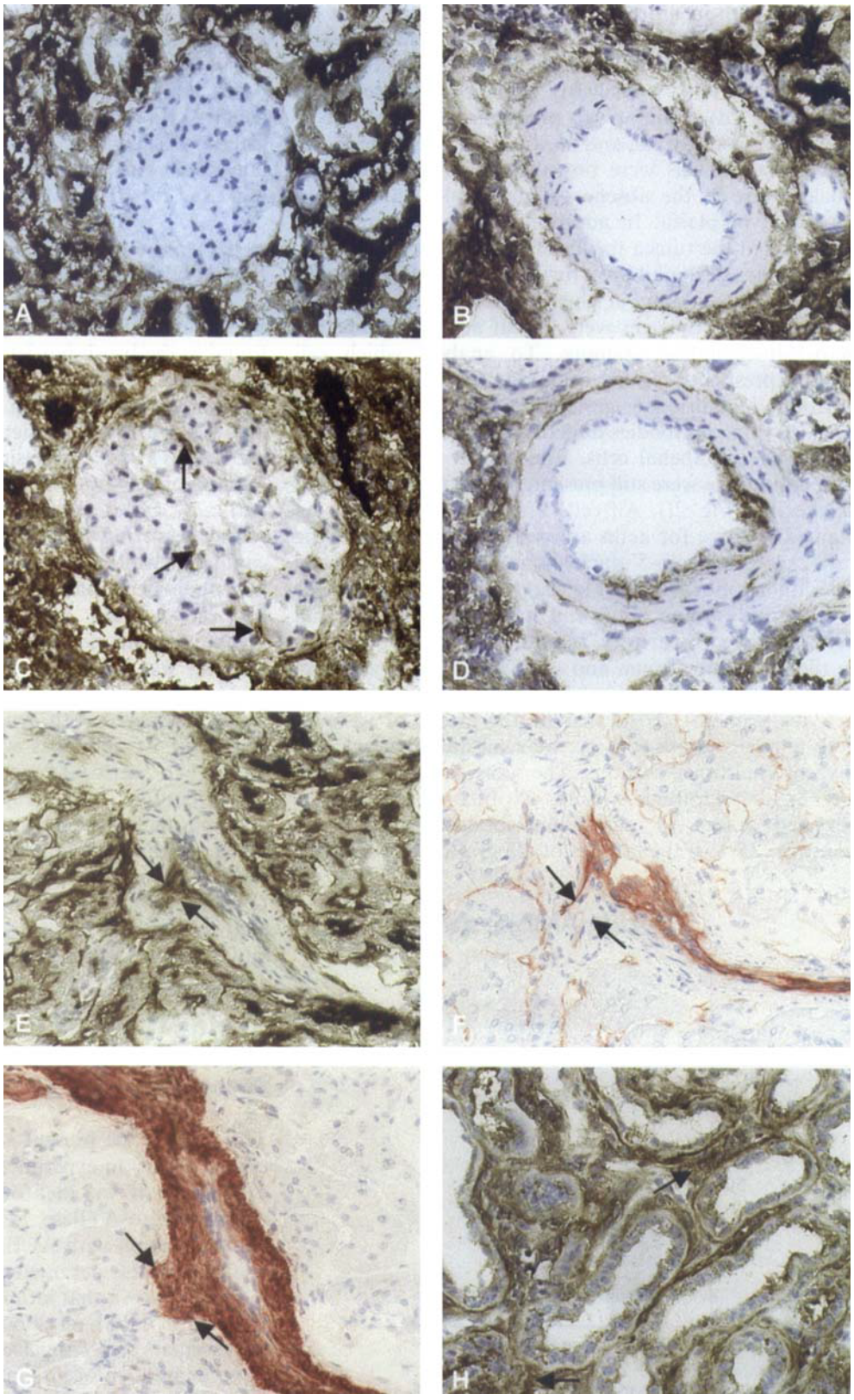


**Fig. 2** Representative micrographs of renal sections stained for ecto-5′-nucleotidase (**a**, **b**, **c**, **d**, **e**, **h**), actin (**f**), and RECA-1 (**g**). **a** Section of a glomerulus from a syngrafted rat, stained for ecto-5′-nucleotidase. Tubular enzyme activity is clearly present, while the glomerulus is negative. **b** Renal artery from a syngrafted animal, stained for ecto-5′-nucleotidase. No enzyme activity is found except for in the tunica media. **c** Glomerulus from an allografted rat, with extensive fibrotic lesions stained for ecto-5′-nucleotidase. Glomerular enzyme activity is clearly present (*arrows*). **d** Renal artery from an allografted rat, stained for ecto-5′-nucleotidase. This artery was scored as 2+, since staining is present in two quadrants. **e**, **f**, **g** Three consecutive sections stained for ecto-5′-nucleotidase (**e**), endothelial cells (**f**), and actin (**g**). The enzyme activity in the neointima (**e**, *arrows*) is present mainly in cells positively stained for actin (**g**, *arrows*), while endothelial cells are not present at the site of enzyme activity (**f**, *arrows*). **h** Renal section from an allografted rat, with interstitial fibrosis. Enzyme activity is present in tubular cells, but also at sites of interstitial fibrosis (*arrows*). Final magnification 200×

b). Glomeruli in untreated allografted animals with severe FGS did reveal some ecto-5′-nucleotidase activity (Fig. 2c). In the untreated allograft group, ecto-5′-nucleotidase activity was clearly present in the renal arteries (Fig. 2d). Staining was predominantly localized in the hyperplastic intima and occasionally in the media. In some cases vessels were positively stained for ecto-5′-nucleotidase in the absence of morphological signs of intimal hyperplasia. In addition, marked staining was observed in the tunica media of larger arteries.

Chronic ACE inhibition further increased the ecto-5′-nucleotidase staining in the thickened vascular areas (Fig. 2e). Macrophages were absent at sites of de-novo ecto-5′-nucleotidase staining. To analyze which cell types express ecto-5′-nucleotidase in the vasculature, we performed additional immunostaining on consecutive sections using antibodies directed against smooth muscle actin and endothelial cells. Endothelial cells lining the luminal surface were still present, despite marked intimal thickening (Fig. 2f). All cells in the intima and media stained positive for actin antibody, suggesting that the cells expressing ecto-5′-nucleotidase were smooth muscle cells or myofibroblasts (Fig. 2g). The area positive for 5′-nucleotidase and actin and negative for endothelial cells is marked by arrows. In addition, the fibrotic areas in the renal interstitium and some inflammatory cells in all allografted rats revealed marked ecto-5′-nucleotidase activity (Fig. 2h). To determine the extent of vascular ecto-5′-nucleotidase activity, we semi-quantitatively analyzed renal tissue sections. The scores are presented in Fig. 3. In syngrafted animals, 4% of the renal arteries revealed traces of ecto-5′-nucleotidase activity. In contrast, 25% of the arteries of allografted rats revealed intensive ecto-5′-nucleotidase activity, which was significantly increased when compared with syngrafted rats ($P < 0.01$). Treatment of the allograft group with an ACE inhibitor further increased ecto-5′-nucleotidase activity ($P < 0.02$ vs untreated allografts) in 40% of the arteries. Vascular ecto-5′-nucleotidase expression correlated well with the extent of intimal hyperplasia in allografted rats ($r = 0.71$, $P < 0.01$).

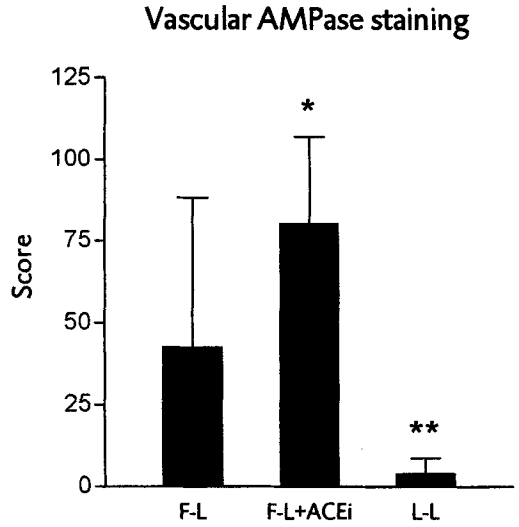


**Fig. 3** The presence of ecto-5′-nucleotidase activity in untreated allografted rats (F-L), allografted animals receiving ACEi (F-L + ACEi), and syngrafts (L-L). Values are presented as mean ± SD. *$P < 0.02$, F-L + ACEi vs F-L; **$P < 0.01$, L-L vs F-L

### Ecto-ATPase expression

By immunohistochemistry, ecto-ATPase staining was found in the brush border of the tubuli, the peri-tubular areas, and the renal arteries in both syngrafted and allografted animals (Fig. 4a). Using morphometrical analysis, we found no significant difference in glomerular ecto-ATPase expression between non-transplanted kidneys of F344 ($n = 8$) and Lew ($n = 8$) rats (42% ± 6% and 37% ± 3%, respectively, $P$ = n.s.). Syngrafted rats revealed strong ecto-ATPase expression along the capillary wall of the glomeruli (Fig. 4b, Fig. 5). Morphological analysis, however, revealed lower values for ecto-ATPase expression in syngrafted animals compared with non-transplanted controls. Glomerular staining was markedly reduced in the untreated allograft group (Fig. 4c, Fig. 5) and was significantly lower compared with syngrafts ($P < 0.02$). In contrast, treatment of allografts with ACE inhibitor preserved glomerular ecto-ATPase staining at the level seen in syngrafted rats (Fig. 4d, Fig. 5). Ecto-ATPase staining in untreated rats was significantly reduced when compared with ACEi-treated rats ($P < 0.05$). Ecto-ATPase staining strongly correlated with FGS ($r = 0.87$, $P < 0.01$) in the untreated allograft group. Both in treated and untreated allografts, areas of interstitial fibrosis were ecto-ATPase-positive. These interstitial cells had the morphological characteristics of interstitial fibroblasts.

## Discussion

The goal of the present study was to localize renal ischemic injury in experimental CRTF as reflected by the expression of vascular or glomerular ecto-5′-nucleotidase and ecto-ATPase. Secondly, we assessed the effect of intervention with ACE inhibition in this model with respect to these parameters.

It was shown that intimal thickening, which is known to occur in CRTF, is associated with de-novo expression of vascular ecto-5′-nucleotidase. In addition, this de-novo expression occurs in localized foci of intimal cells and enhances significantly with the increase in intimal thickening following ACE inhibition. The presence of ecto-5′-nucleotidase in vessels that do not have the

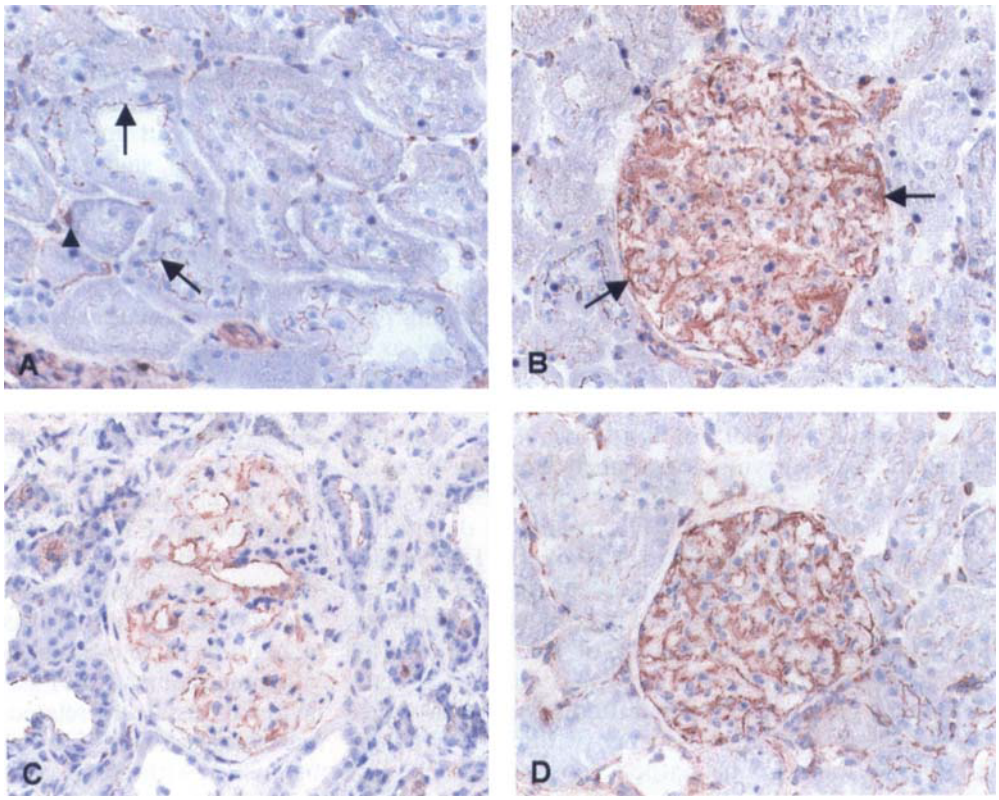


**Fig. 4** Representative micrographs of renal sections from syngrafted (**a**, **b**), untreated allografted (**c**), and ACEi-treated allografted rats (**d**), stained for ecto-ATPase. **a** Ecto-ATPase staining is clearly present on the tubular brush border (*arrows*) and also in the peri-tubular areas (*arrowhead*). **b** Ecto-ATPase staining is clearly present along the glomerular capillary wall of kidneys from syngrafted rats (*arrows*) and from allografted rats after ACE treatment (**d**). **c** Allografted rats showed diminished glomerular ecto-ATPase staining when compared with syngrafted rats. Final magnification 200×

morphological characteristics of intimal hyperplasia does suggest that enzyme expression occurs early in the disease. The marked aggravation of intimal thickening caused by ACE inhibition in this model has been described previously and is believed to be the result of long-term ( > 20 weeks) treatment [18]. Since all the cells present in the thickened intima express smooth muscle cell α-actin, the expression of ecto-5′-nucleotidase most probably originates from smooth muscle cells. It is likely that local up-regulation of ecto-5′-nucleotidase relates to the production of adenosine at sites of ischemic damage, as is described in experimental hypoxia [12]. Thus, a hallmark of cellular ischemic tissue damage is release of ATP and extracellular hydrolysis of ATP to AMP. Conversion of AMP to adenosine by ecto-5′-nucleotidase may serve as a counter-mechanism to ischemia producing adenosine, which exerts various beneficial functions including anti-inflammatory and anti-thrombotic activities, vasodilatation, and scavenging of ROS [7, 8, 14, 16]. It is obvious that obliteration of renal arteries promoting an ischemic micro-environment in this model may enhance hypoxia. Although it is tempting to ascribe an anti-ischemic function to ecto-5′-nucleotidase in proliferating intimal cells, as similarly described for the renal interstitium by Le Hir et al. [12], such a putative function remains to be proven in the present model. The function of ecto-5′-nucleotidase expression in the neointima is unknown. Studies with chicken gizzard ecto-5′-nucleotidase suggest that it also functions as a potent ligand of fibronectin and laminin [20] and that it is involved in the spreading of various mesenchyme-derived cells on a laminin substrate [6].

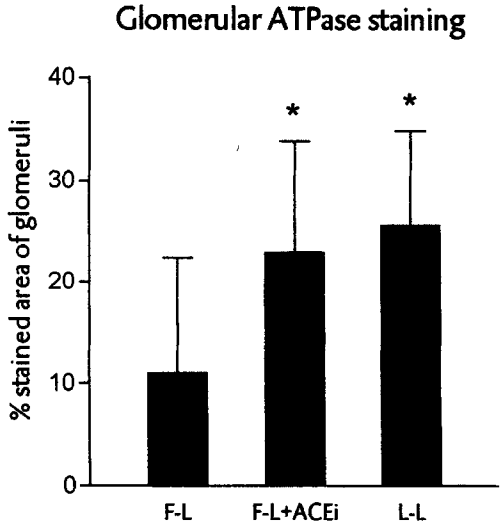


**Fig. 5** The presence of glomerular ecto-ATPase staining in untreated allografted rats (F-L), allografted animals receiving ACEi (F-L + ACEi), and syngrafted rats (L-L). Values are presented as mean ± SD. $*P < 0.02$, F-L vs L-L; $*P < 0.05$, F-L + ACEi vs F-L

Thus, in addition to its anti-inflammatory and anti-thrombotic properties, ecto-5′-nucleotidase may also be involved in the spreading of cells in the neointima of the obliterated arteries in our model.

With respect to glomerular expression of ecto-5′-nucleotidase, we noted that focal expression of this ecto-enzyme occurs exclusively in severely damaged glomeruli of allografted rats. This observation in combination with diminished expression of glomerular ecto-ATPase in allografted rats may point to ischemic injury of the glomerular microvasculature. An identical staining pattern has been observed in human CRTF as well as in ischemic glomerular injury due to malignant hypertension [3]. Down-regulation of glomerular ecto-ATPase expression may be mediated by the local ROS, since this glomerular ecto-enzyme is extremely sensitive to toxic oxygen products [15]. X-irradiation of rat kidneys, giving rise to local release of ROS, results in diminished expression of glomerular ecto-ATPase [1, 15]. Similar oxidant-mediated loss of glomerular ecto-ATPase expression has been demonstrated in adriamycin nephrosis [15]. The significant correlation of the magnitude of sclerotic lesions on the one hand and diminished ecto-ATPase in glomeruli of allografted rats on the other hand suggests that local ischemia within the capillary tuft plays a role in the pathogenesis of FGS in this model.

The pathogenesis of the glomerular lesions in CRTF is unknown. Following an acute inflammatory allograft response in the initial phase after graft rejection, it seems obvious that intimal hyperplasia of intra-renal arteries may contribute to downstream ischemia, giving rise to local release of ROS as well as subsequent down-regulation of glomerular ecto-ATPase and collapse of glomerular capillaries. In other experimental models of glomerulosclerosis, up-regulation of ecto-5′-nucleotidase was found as well [16].

Interestingly, long-term ACE inhibition enhanced vascular intimal proliferation and intimal ecto-5′-nucleotidase activity, whereas this treatment significantly protected the glomerular microvasculature, showing normal glomerular ecto-ATPase expression concomitant with a significant reduction of glomerulosclerosis in allografted rats. This is in line with evidence obtained from other investigators, suggesting that the reno-protective action of ACE inhibition depends on improving renal interstitial oxygenation [11]. Interstitial oxygenation may counteract local ischemic injury in the present model, explaining the reno-protective effect of ACE inhibition on the glomerular microvasculature as reflected by normal expression of glomerular ecto-ATPase. In contrast, ACE inhibition is known to impair reparative angiogenesis in murine limb ischemia, which may explain the absence of beneficial effects of ACE inhibition on vascular lesions in the present study. Repair of ischemia-reperfusion injury and allo-related damage to the vasculature may be inhibited by long-term ACE inhibition [10].

In summary, in the present allograft model, we localized ischemic-like injury in renal arteries as well as in the glomerular microvasculature, as reflected by up-regulation of ecto-5′-nucleotidase and down-regulation of glomerular ecto-ATPase. Increased intimal thickening in renal vessels correlated well with enhanced ecto-5′-nucleotidase expression, whereas glomerulosclerosis was associated with decreased expression of glomerular ecto-ATPase. Finally, while long-term ACE inhibition caused increased intimal thickening with signs of ischemia in renal vessels, this treatment caused protection of glomerular ecto-ATPase, which correlated with fewer glomerular lesions in these animals. Further studies are needed to analyze the mechanisms underlying the enzyme changes observed in this model.

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