Clinical relevance of immunohistochemical staining for ecto-AMPase and ecto-ATPase in chronic allograft nephropathy (CAN)

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Abstract

Introduction. Chronic allograft nephropathy (CAN) is a major cause of deterioration of kidney function in transplanted patients. It is thought that glomerular ischaemia may contribute to glomerular dysfunction and proteinuria in these subjects. As reduced expression of glomerular ecto-ATPase concurrent with upregulation of glomerular ecto-AMPase activity is associated with local ischaemia, we compared the expression of these glomerular ecto-enzymes in kidney biopsies from subjects with CAN or with acute rejection vs normal human kidney tissue.

Methods. Kidney biopsies in comparison with normal kidney tissue samples (n = 10) were studied from subjects with CAN (n = 6), acute interstitial rejection (n = 13), acute vascular rejection (n = 3), or subjects whose biopsies were histologically difficult to classify (n = 8). Cryostat sections (4 μm) were stained for ecto-ATPase using standard procedures. For the demonstration of ecto-AMPase activity, conventional enzyme histochemistry was used. Reaction product of individual sections was quantified using computerized image analysis.

Results. The results clearly show decreased expression of glomerular ecto-ATPase in combination with increased glomerular ecto-AMPase in all biopsies from subjects with CAN vs normal kidney tissue (P < 0.001). Although to a lesser extent, this staining pattern was also observed in patients with vascular rejection as well as in subjects whose biopsies were histologically difficult to classify (P < 0.01), while biopsies from subjects with interstitial rejection and normal control tissue stained negative for glomerular ecto-AMPase.

Conclusion. In CAN diminished glomerular ecto-ATPase expression occurs in association with significantly enhanced activity of glomerular ecto-AMPase. This is a strong indication for ischaemic injury of the glomerular microvasculature. As positive staining for ecto-AMPase in acute rejection episodes may be an important sign for long-term prognosis, we feel that screening of biopsies from individual subjects for glomerular ecto-AMPase activity should be considered.

Keywords: allograft nephropathy; ecto-AMPase; ecto-ATPase; glomerular dysfunction; ischaemia; proteinuria

Introduction

Chronic allograft nephropathy (CAN) is clinically characterized by a slowly progressive decline in glomerular filtration rate, usually in conjunction with proteinuria and arterial hypertension [1]. Histologically the renal tissue is characterized by non-specific lesions, i.e. tubular atrophy, interstitial fibrosis and fibrous intimal thickening in the arteries, with variable glomerular lesions. For this reason, it is not possible to diagnose CAN by histopathological criteria alone.

CAN has been extensively reviewed in the last decennia, since this disorder is a major cause of late kidney allograft failure [2–4]. First-year graft survival following transplantation has increased considerably during the past decades. This is due to various improvements including introduction of novel immunosuppressive drugs such as cyclosporin A (CsA). Recently, long-term improvement in graft survival has been demonstrated. Hariharan et al. [5] showed that from 1988 to 1996 the half-life of cadaveric grafts increased from 7.9 to 13.8 years. When censored for patients who died with a functioning graft the improvement was even greater—from 11 to 19 years. However, many kidneys are still lost in the long term, usually due to death with a functioning graft, 'chronic rejection', or perhaps more accurately designated as CAN, and recurrent or de novo glomerular disease.

Several clinical and experimental studies have shown that acute vascular rejection episodes are a major risk...
factor for the development of CAN [6,7]; however, their causal relationship with CAN is currently unknown. Additional risk factors associated with CAN comprise HLA mismatches, pre-transplant hyperlipidaemia, post-transplant infections, donor age, female gender, race of the donor and recipient obesity.

Although immunological mechanisms are important in graft rejection, increasing evidence supports the hypothesis that non-immunological mechanisms, such as ischaemia, may play a role in CAN [4,8]. Delayed graft function (DGF) has been studied with respect to glomerular ischaemic injury, as DGF is associated with decreased graft survival [9–11]. It is assumed that reactive oxygen species (ROS) resulting from reperfusion injury and/or ischaemia may cause local graft injury [12,13], reflected for instance by the downregulation of vascular and glomerular ecto-diphosphohydrolase (ecto-ATPase).

It has been shown experimentally that this ecto-enzyme is highly sensitive to ROS-mediated tissue injury [12]. In subjects with DGF, decrease of glomerular ecto-ATPase expression in kidney biopsies was shown, irrespective of the duration of ischaemia [11], suggesting again an oxidant-mediated mechanism.

In contrast to ecto-ATPase, upregulation of glomerular ecto-5′-nucleotidase (ecto-AMPase) was shown in these subjects (unpublished observations). Upregulation of ecto-AMPase is related to the conversion of extracellular AMP to adenosine, a reaction associated with the ischaemic micro-environment [14]. For instance, Le Hir et al. [14,15] have demonstrated enhanced activity of glomerular ecto-AMPase in the hypoxic rat kidney. Significant upregulation of ecto-AMPase was also demonstrated in subjects with glomerular ischaemia due to malignant hypertension [16]. As adenosine behaves as a regulatory nucleoside (inhibiting platelet aggregation, promoting vasodilatation, and scavenging ROS), upregulation of ecto-AMPase may function as a mechanism counteracting the features of ischaemia in the glomerular microcirculation [14]. Thus, decreased glomerular ecto-ATPase expression concomitant with enhanced ecto-AMPase activity can be considered as a characteristic of glomerular ischaemia. In the present study we applied this dual immunohistochemical staining to examine ischaemic injury in kidney biopsies from transplanted subjects in comparison with normal human kidney tissue.

Subjects and methods

As stated, diagnosis of CAN is based predominantly upon clinical criteria and to a lesser extent upon histopathological parameters. Patients with CAN show deterioration of renal function, without signs of overt rejection, recurrence of original disease or de novo glomerular disease. Although chronic CsA toxicity is a well-known factor causing deterioration of renal function, specific histological signs of this condition are often lacking. Furthermore, Furness et al. [17] showed that considerable over- or underscoring exists for different abnormalities (e.g. tubulitis, interstitial fibrosis or glomerulitis) when different pathologists examine the same biopsies (very poor ‘kappa scores’). In our study, no case with a biopsy classified as CAN produced a significant improvement of renal function after tapering and cessation of CsA, making chronic CsA toxicity as a major factor of the decline of renal function less likely.

Biopsies of patients transplanted after 1990 were studied. Kidney tissue fragments were obtained by a standard percutaneous biopsy and were snap frozen in isopentane and kept at −80°C until use. Cryostat sections (4 μm) were prepared according to standard protocols [18]. Biopsies (n = 30) each containing at least six glomeruli were studied. Light-microscope diagnosis was made according to the Banff criteria [19] and in none of the biopsies was disappearance of endothelium observed; CAN (n = 6), acute interstitial rejection (n = 13), acute vascular rejection (n = 3) and a group of subjects with acute rejection difficult to classify (DC) (n = 8).

All biopsies of subjects with CAN showed moderate or severe areas with interstitial fibrosis and tubular atrophy associated with interstitial aggregates of mononuclear cells. In addition, mild signs of chronic transplant glomerulopathy were observed with basement membrane thickening and double contours in up to 25% of peripheral capillary loops. Ischaemic alterations were also found, as well as focal global glomerulosclerosis, signs of collapse of capillary loops in non-sclerotic glomeruli, mild increases of mesangial matrix and reduplication of parietal layers in Bowman’s capsule. Mild vascular changes were observed, characterized by arterial fibrous intimal thickening. No signs of acute vascular or interstitial rejection were present and arteriolar hyalinosis and other tubulo-interstitial changes indicative of CsA-associated nephropathy were not seen. Biopsies of the DC subjects showed, in addition to interstitial alterations, non-specific vascular changes, which however lacked the characteristics to justify the diagnosis of vascular rejection.

Normal kidney tissue samples (n = 10) were taken from extirpated kidneys from subjects with Grawitz tumour (n = 7) and in three cases from donated organs unsuitable for transplantation because of injured vessels. Parts of these kidneys used for preparing control sections in the present study had previously been evaluated for morphological alterations by conventional staining, and no such alterations had been found. There were no significant differences between the groups in age (mean age: CAN, 42.5 ± 6.8 years; acute rejection, 38.6 ± 10.7 years) or number of HLA mismatches. All patients had received cadaveric renal transplants for the first time. Basic immunosuppression consisted of low-dose prednisolone, CsA and azathioprine. Concentrations of CsA in whole blood drawn prior to the biopsy did not differ significantly between the different groups (measured by radioimmunoassay, range 175–210 mg/l). Donor and recipient data are summarized in Table 1.

Immunohistochemistry and quantification of reaction product

Cryostat sections (4 μm) prepared from kidney biopsies of subjects with CAN as well as with acute rejection were stained for glomerular ecto-ATPase according to standard procedures [20], using monoclonal anti-ecto-ATPase antibody [21] and peroxidase-conjugated goat anti-mouse antibody as a second step, and subsequently visualized by 3-aminomethylcarbazole (AEC). Incubation steps were carried out at room temperature.
Table 1. Characteristics of graft donors and recipients with CAN or acute rejection

<table>
<thead>
<tr>
<th></th>
<th>CAN (n = 6)</th>
<th>Int (n = 13)</th>
<th>DC (n = 8)</th>
<th>Vasc (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient sex</td>
<td>3 male, 3 female</td>
<td>8 male, 5 female</td>
<td>4 male, 4 female</td>
<td>1 male, 2 female</td>
</tr>
<tr>
<td>Mean recipient agea</td>
<td>42.5 (31–49)</td>
<td>36.3 (21–48)</td>
<td>45.0 (19–61)</td>
<td>32.3 (25–37)</td>
</tr>
<tr>
<td>Donor sex</td>
<td>2 male, 4 female</td>
<td>8 male, 5 female</td>
<td>7 male, 1 female</td>
<td>1 male, 2 female</td>
</tr>
<tr>
<td>Mean donor agec</td>
<td>45.2 (28–65)</td>
<td>35.4 (12–52)</td>
<td>47.8 (16–67)</td>
<td>37.7 (18–55)</td>
</tr>
<tr>
<td>Mean proteinuria at biopsyf</td>
<td>5.1</td>
<td>0.49</td>
<td>0.47</td>
<td>0.50</td>
</tr>
<tr>
<td>Mean time Tx to biopsye</td>
<td>43.5</td>
<td>1.9</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Delayed graft function</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mean WIT 1b</td>
<td>0.58 (0–3.5)</td>
<td>0.88 (0–5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mean CITc</td>
<td>1265 (445–1500)</td>
<td>1598 (810–2220)</td>
<td>1412 (1380–1437)</td>
<td>1412 (1380–1437)</td>
</tr>
<tr>
<td>Mean WIT 2d</td>
<td>35 (30–42)</td>
<td>41 (33–53)</td>
<td>41 (32–64)</td>
<td>30 (25–39)</td>
</tr>
<tr>
<td>Mean proteinuria at biopsyf</td>
<td>43.5</td>
<td>1.9</td>
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<td>1412 (1380–1437)</td>
</tr>
</tbody>
</table>

WIT 1, first warm ischaemia time; WIT 2, second warm ischaemia time; CIT, cold ischaemia time; Tx, transplantation.
aMean age and range (in parentheses) in years.
bMean WIT 1 and range (in parentheses) in min.
cMean CIT and range (in parentheses) in min.
dMean WIT 2 and range (in parentheses) in min.
eMean time between Tx and biopsy in months.
fMean proteinuria at time of biopsy in g/24 h.

For demonstration of glomerular ecto-AMPase activity, conventional enzyme histochemistry was used according to the Wachstein–Meisel method [22], using AMP as a substrate and lead as capture ion. The precipitated reaction product (lead phosphate) was visualized with sodium disulphide showing a dark brown reaction product.

Quantification of the reaction product following either immunostaining or histochemistry was performed by computerized image analysis as described elsewhere [20]. Six to 10 glomeruli of each patient were scored using a digitizing tablet (mm1812; Summagraphics), and both the intensity of the reaction product as well as the area of the glomerulus were measured. Mean glomerular stainability was defined as mean optical density divided by the mean glomerular area, and was expressed in arbitrary units.

Statistical evaluation was performed using the Mann–Whitney test; P-values < 0.05 were considered as statistically significant. The results are expressed as arithmetic mean ± SD.

Results

The results show a significant decrease of glomerular ecto-AMPase expression in biopsies from subjects with CAN (Figure 1B) compared with the biopsies without rejection (Figures 1A and 2). A similar decrease of expression of glomerular ecto-AMPase, although to a lesser extent, is also seen in biopsies from acute rejection vs the control group (Figure 3). No significant differences could be detected between the mean amounts of reaction product of biopsies from patients with interstitial vs vascular or DC acute rejection (Figure 3). One of eight subjects of the DC group showed no decreased staining of glomerular ecto-AMPase compared with the control group (Figure 3).

As illustrated in Figures 1D and 4, biopsies from subjects with CAN show a clear amount of reaction product after staining for glomerular ecto-AMPase activity (in contrast to the control kidney samples, which stain negative; Figure 1C). Biopsies from subjects with acute interstitial rejection also stain negative for ecto-AMPase (Figure 4). The mean amount of reaction product in glomeruli from subjects with acute vascular rejection or those with DC is significantly increased compared with control kidney tissue (Figure 5). Only four of eight DC subjects showed significantly increased glomerular ecto-AMPase activity, whereas the other four cases were not significant compared with the control group. Using conventional staining, all glomeruli were equally affected and no relationship of altered staining for glomerular AMPase or ATPase with intimal proliferation could be detected.

Discussion

Decreased expression of glomerular ecto-ATPase in combination with increased glomerular ecto-AMPase activity occurs most prominent in subjects with CAN (Figures 1, 2 and 4). This staining pattern was also observed, although to a lesser extent, in subjects with vascular type and in DC type patients with acute rejection (Figures 3 and 5). Interestingly, patients with acute interstitial rejection did not show this particular staining pattern (Figures 3 and 4). These findings may fit well with the hypothesis that glomerular ischaemia is immunohistochemically characterized by diminished expression of glomerular ecto-ATPase on the one hand and upregulation of glomerular ecto-AMPase on the other [14,15,23,24]. Experimental studies in rats also support the notion that oxygen-free radicals affect the expression of glomerular ecto-ATPase due to the sensitivity of this ecto-enzyme for oxidant stress [12,23–25]. This may explain why, in the inflammatory microenvironment, in ischaemic conditions, and/or after reperfusion injury, ecto-ATPase may be affected. Although the exact mechanism of upregulation of glomerular ecto-AMPase activity in this condition remains to be elucidated, this feature is clearly associated with an ischaemic microenvironment [14,15].
Diminished expression of glomerular ecto-ATPase has also been described in subjects with DGF [11]. Thus, 1-h biopsies from subjects with delayed diuresis also showed decreased glomerular ecto-ATPase and increased ecto-AMPase activity similar to that observed in CAN. In contrast, in subjects with immediate diuresis, positive glomerular ecto-ATPase and negative ecto-AMPase staining occurred, similar to

Fig. 1. Photomicrographs showing glomeruli from either normal human kidney tissue (A and C) or kidney biopsies from patients with CAN (B and D). Kidney sections were stained immunohistologically for glomerular ecto-ATPase (A and B) or histochemically for glomerular ecto-AMPase activity (C and D). In CAN (B), a clear reduction of the amount of reaction product (glomerular ecto-ATPase) can be seen as compared with normal kidney tissue (A). Clear reaction product of glomerular ecto-AMPase is seen in subjects with CAN (D), in contrast to glomeruli (arrows) of control kidney tissue (C).

Fig. 2. Evaluation of glomerular ecto-ATPase expression in kidney biopsies from subjects with CAN or control kidney tissue. Dots represent mean glomerular staining in individual patients. A significant decrease of reaction product can be seen in CAN compared with control kidney tissue. CAN: n = 6 (mean 5.10); Control: normal kidney tissue, n = 10 (mean 38.76); P ≤ 0.001 (Mann–Whitney), CAN vs control.

Diminished expression of glomerular ecto-ATPase has also been described in subjects with DGF [11]. Thus, 1-h biopsies from subjects with delayed diuresis also showed decreased glomerular ecto-ATPase and increased ecto-AMPase activity similar to that observed in CAN. In contrast, in subjects with immediate diuresis, positive glomerular ecto-ATPase and negative ecto-AMPase staining occurred, similar to
that observed in the normal human kidney. The impairment of glomerular ecto-ATPase expression in DGF occurred irrespective of the duration of the ischaemia, suggesting the ischaemic injury of the glomerular tuft may be due to other factors such as ROS in association with reperfusion damage [13,26]. As oxidant-free radicals are important in downregulation of glomerular ecto-ATPase, it is likely that in acute inflammation also (i.e. during acute rejection), decreased expression of glomerular ecto-ATPase can be observed. Indeed, significant decrease of glomerular ecto-ATPase occurred in most of the subjects with acute rejection (Figure 3). A single DC patient showed normal glomerular ecto-ATPase staining. The reason for this is not clear; this individual belonged to the group with low AMPase staining in the DC group in Figure 5. Therefore we feel that staining for glomerular ecto-AMPase, rather than for ecto-ATPase, seems to be the major marker for glomerular ischaemia.

As shown in Figure 4, glomeruli from patients with acute interstitial rejection do not show significant upregulation of ecto-AMPase in contrast to CAN. This may indicate that upregulation of glomerular ecto-AMPase is not due to renal inflammatory response per se. Decreased expression of glomerular ecto-ATPase without concomitant increase of glomerular ecto-AMPase activity has also been described in human glomerular disorders, such as minimal-change disease [21]. In this type of glomerulopathy, there are no histopathological signs of glomerular ischaemia, whereas in subjects with clear-cut glomerular ischaemia due to malignant hypertension, significant activity of glomerular ecto-AMPase occurs [16].

Interestingly, as clinical evidence indicates that vascular acute rejection is the major predicting factor of late graft loss [7], our observation in biopsies from these subjects and in subjects with a diagnosis of DC showing significant upregulation of glomerular ecto-AMPase (Figure 5) may be of clinical relevance. Since the characteristic immunohistochemical staining pattern (i.e. ecto-ATPase down and ecto-AMPase up) in the latter group of subjects is similar to that in the vascular rejection subjects, it is possible that the initial histopathological diagnosis requires re-evaluation. In other words, positive glomerular ecto-AMPase staining in individuals with acute rejection may point to a less favourable prognosis in the long term. Follow-up studies are necessary to examine this issue. Preliminary data showing that only those DC subjects staining positive for glomerular ecto-AMPase (n = 4) were unresponsive to corticosteroid treatment, in contrast to the other DC subjects, may support this notion. So like vascular regression, having a biopsy positive for ecto-AMPase with abnormalities designated as DC may also be a risk factor for CAN. This, however, has to be confirmed in a prospective study.

In summary, we conclude from the present observations that reduced expression of glomerular ecto-ATPase in combination with increased activity of glomerular ecto-AMPase in subjects with CAN may point to ischaemic injury to the renal allograft. Early ischaemic damage in acute rejection, as reflected by enhanced glomerular ecto-AMPase activity, may lead to persistent glomerular ischaemia associated with CAN. We feel that screening of biopsies for ecto-AMPase activity may help to identify patients at risk for CAN, not only in individuals with DC acute rejection, but potentially also in those patients who have been under-diagnosed as interstitial rejection, due to the absence of vessels in the tissue samples obtained by percutaneous renal biopsy.
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References


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