

## TGF- $\beta$ 1 Expression and Chronic Allograft Nephropathy in Protocol Kidney Graft Biopsy

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### Summary

Chronic allograft nephropathy (CAN) represents a frequent and irreversible cause of long-term renal graft loss. TGF- $\beta$ 1 is a key profibrogenic cytokine associated with CAN pathogenesis. Because of clinical diagnostic inaccuracy, protocol biopsy has been suggested to be a beneficial method for early CAN detection. Protocol core biopsy was carried out in 67 consecutive cyclosporine-based immunosuppression-treated kidney transplant recipients with stable renal function 12 months after renal transplantation. Biopsy specimens were analyzed morphologically according to Banff-97' criteria and immunohistologically for TGF- $\beta$ 1 staining. The data obtained were correlated with plasma TGF- $\beta$ 1 levels and clinical data. CAN (grade I-III) was found in 51 patients (76 %). CAN grade I was found to be the most frequent one (44 %). A normal finding within the graft was made in only 12 patients (18 %). Clinically silent acute rejection Banff IA was present in 4 patients (6 %). In 8 patients (12 %) with CAN, borderline changes were present. We found a significant correlation between CAN grade and creatinine clearance, as measured by the Cockcroft-Gault formula ( $p < 0.01$ ) as well as body mass index ( $p < 0.01$ ). There was a significant correlation between chronic vasculopathy (Banff cv) and creatinine clearance, and between the degree of TGF- $\beta$ 1 staining and chronic vasculopathy ( $p < 0.01$ ). There were no relations between morphological findings and TGF- $\beta$ 1 plasma levels, cyclosporine levels, plasma lipids, HLA-mismatches, panel reactive antibodies (PRA), proteinuria, and the donor's age. In conclusion, CAN is a frequent finding in protocol kidney graft biopsies 12 months after transplantation. TGF- $\beta$ 1 tissue expression is linked with chronic vasculopathy.

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### Key words

Chronic rejection • Kidney transplantation • Biopsy • Fibrogenesis

### Introduction

Chronic allograft nephropathy (CAN) has lately emerged as a prominent problem in transplantology. One of the reasons is that, in spite of increasing and improving immunosuppressive treatment, the graft half-time has remained unchanged throughout the past several decades

(Paul *et al.* 2001). This is generally due to CAN, thus indicating a lack of our medical influence on this process. CAN has been described as histological lesions, frequently of unclear origin, accompanied with progressive deterioration of graft function and often with mild proteinuria. While the mechanisms of acute rejection have been considered as a predominately

immunological event, the mechanisms of CAN pathogenesis are not so clear, partly due to the fact that this process is apparently multifactorial. The main alloantigen-independent risk factors suggested to play a role in CAN pathogenesis are ischemia/reperfusion injury, hypertension, dyslipidemia, nephron underdosing, infection, and drug nephrotoxicity (Fellström 2001). At the same time, alloantigen-dependent risk factors including HLA match, level of pre-transplant panel-reactive antibodies, history of acute rejection, inadequate immunosuppression, and humoral response based on indirect alloantigen immune recognition have a major influence on the fate of the transplanted organ (Paul 2001).

The main histopathological features of CAN are vascular intimal hyperplasia, tubular atrophy, interstitial fibrosis, and various glomerular changes that sometimes develop into chronic transplant glomerulopathy (Furness 2001). Endothelial injury is the starting point of the pathogenetic pathway that terminates in tissue fibrosis. A cytokine that plays an important role in fibrogenesis has been suggested to be the transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1). TGF- $\beta$ 1 is produced by a large number of different cells including cells of the immune system that are reversibly responsive to its action (Waltenberg *et al.* 1993). TGF- $\beta$ 1 production is dependent on a genetic sequence polymorphism in the regulatory regions of the TGF- $\beta$ 1 gene (Awad *et al.* 1998).

As regards fibrogenesis, TGF- $\beta$ 1 stimulates the synthesis of most matrix molecules, blocks the degradation of matrix, and modulates the expression of integrin matrix receptors on cells (Border and Noble 1997). Interstitial fibrosis in CAN can be characterized by intragraft TGF- $\beta$ 1 (active form) expression (Sharma *et al.* 1988). An association of plasma TGF- $\beta$ 1 levels with different causes of graft dysfunction was found to be rather doubtful (Coupes *et al.* 1994).

After kidney transplantation, biopsies are occasionally carried out in patients in order to determine the reason for graft dysfunction. Protocol biopsies are performed according to their scheduled timing without any regard to graft function. The main aim of "early" biopsies in the course of the first three post-transplant months is to detect subclinical rejection, though CAN may already be present, too. Biopsies performed later (at month 6-12 or even later) may also reveal subclinical rejection, but more often signs of CAN are found (Legendre *et al.* 1998, Rush *et al.* 2000).

The aim of this study was to analyze protocol

kidney graft biopsies performed one year after transplantation and to demonstrate a role of active TGF- $\beta$ 1 expression within the graft and in the plasma.

## Methods

### Patients

A total of 67 patients (mean age  $52.1 \pm 11.9$  years, 47 males and 20 females) were enrolled into the study. Sixty patients received primary kidney grafts, 7 had undergone secondary transplantation. Three patients received their grafts from living donors. The common causes of end-stage renal disease were chronic glomerulonephritis (23 cases), interstitial nephritis (18 cases), polycystic kidney disease (13 cases) and diabetic nephropathy (6 cases). The mean number of mismatches between donor and recipient in HLA A, B, and DR loci was  $3.2 \pm 1.1$  and the mean % of panel-reactive antibodies (PRA) was  $27.7 \pm 24.5$ . The mean donor age was  $38.4 \pm 14.6$  years. All patients received triple immunosuppression based on cyclosporine A with first-year target trough plasma levels between 150-250 ng/ml (evaluated by monoclonal antibody and RIA) in combination with an antiproliferative agent, either mycophenolate mofetil or a rapamycin derivative and tapered steroid dose. Patients with PRA > 50 % received anti-rejection prophylaxis consisting of 10 doses of 2.5 mg of monoclonal antibody against CD-3+ lymphocytes. All acute rejections were biopsy-proved, and treated by methylprednisolone pulses and, in the case of resistance, by OKT3. At one year after transplantation ( $12.7 \pm 1.5$  months), protocol kidney graft biopsies were performed using a 16-gauge gun under ultrasound guidance. Patients with serum creatinine over  $250 \mu\text{mol/l}$  were excluded from the study. All patients gave their informed consent, and signed their agreement with protocol biopsy. The study protocol was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine. In the event of presence of clinically silent acute rejection, patients received a total of 1.5 g of methylprednisolone over the next 7 days after protocol biopsy.

### Clinical data

Following variables were recorded in the patients: age, gender, body mass index (BMI), number of HLA mismatches, maximal panel-reactive antibodies (PRA), etiology of end-stage renal disease, blood pressure, serum creatinine, glomerular filtration rate estimated using the Cockcroft-Gault formula (Cockcroft and Gault 1976), proteinuria, plasma cholesterol and

triglycerides levels, and acute rejection incidence. Blood samples were obtained on the day of protocol biopsy.

#### *Histomorphology*

Tissues were fixed in 10 % formalin for 15-30 min and then processed in a TPC 15 tissue processor (MEDITE Histotechnic, Germany). Four  $\mu$ m thick paraffin-embedded tissue sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), aldehyde-fuchsin orange G (AFOG), Sirius red with elastic stain and periodic acid silver-methenamine (PASM). Biopsy tissues were scored on the basis of the Banff 97 working classification (Racusen *et al.* 1999). Histological classification was made without information about the immunohistological results.

#### *Immunohistology*

Fifty-eight kidney graft tissue samples were considered suitable for immunohistological evaluation. After deparaffinization and rehydration, slides were cooked in a microwave oven (twice for 2 min and then twice every 5 min for 1 min at 750 W) using 0.01 M citrate buffer pH 6.0 for target retrieval. Endogenous peroxidase was blocked for 30 min in 1 % H<sub>2</sub>O<sub>2</sub> in 70 % methanol. Endogenous biotin was blocked using a Biotin blocking system (Dako, Glostrup, Denmark). The tissues were then preincubated in a protein mixture to prevent unspecific binding and FcR binding (10 % bovine serum with 20 % pig serum). Primary antibody (anti-human TGF- $\beta$  Biosource Int., CA, USA) was applied for 30 min diluted at a concentration of 0.5  $\mu$ g/100  $\mu$ l. On negative control slides, the step with monoclonal antibody was omitted. Detection of monoclonal antibody was done using a horseradish peroxidase labeled biotin streptavidin system and diaminobenzidine (LSAB Plus Kit, Dako, Glostrup, Denmark) following the company protocol. Immunostained slides were graded on the basis of a previously described scheme (Horvath *et al.* 1996).

#### *Plasma TGF- $\beta$ 1 levels*

TGF- $\beta$ 1 levels in EDTA-plasma were measured using a Human TGF- $\beta$ 1 Immunoassay (R&D Systems, Abingdon, UK). The assay employs the quantitative sandwich enzyme immunoassay technique. TGF- $\beta$ 1 soluble receptor Type II, which binds TGF- $\beta$ 1, was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any TGF- $\beta$ 1 present was bound by the immobilized receptor. After washing away all unbound substances, an enzyme-linked polyclonal antibody specific for TGF- $\beta$ 1 was added to the wells to

sandwich the TGF- $\beta$ 1 immobilized during the first incubation. Following washing to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of TGF- $\beta$ 1 bound in the initial step. The color development was stopped and the intensity of the color was measured. Detection limit of the method was 7 pg/ml.

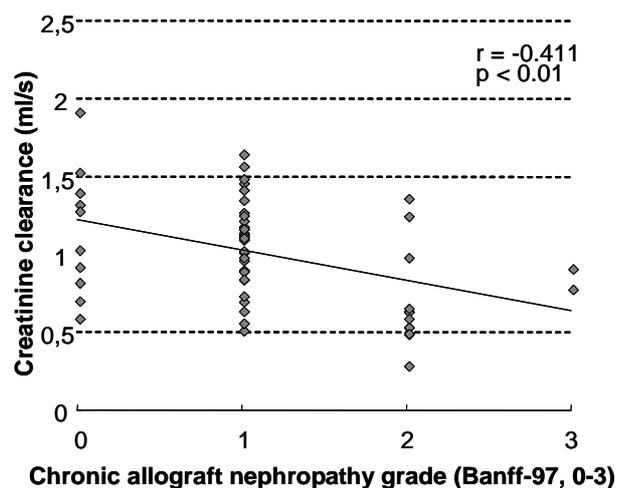
#### *Statistics*

The clinical and histomorphological data obtained were correlated (Spearman-Rank correlation) with immunohistological data, and plasma TGF- $\beta$ 1 levels. For comparison of data obtained in different groups, the Kruskal-Wallis test and the one-way ANOVA test were used. A contrast test was used for proving the trends. Data are expressed as means  $\pm$  S.D. and  $p < 0.05$  value was considered to be statistically significant.

## **Results**

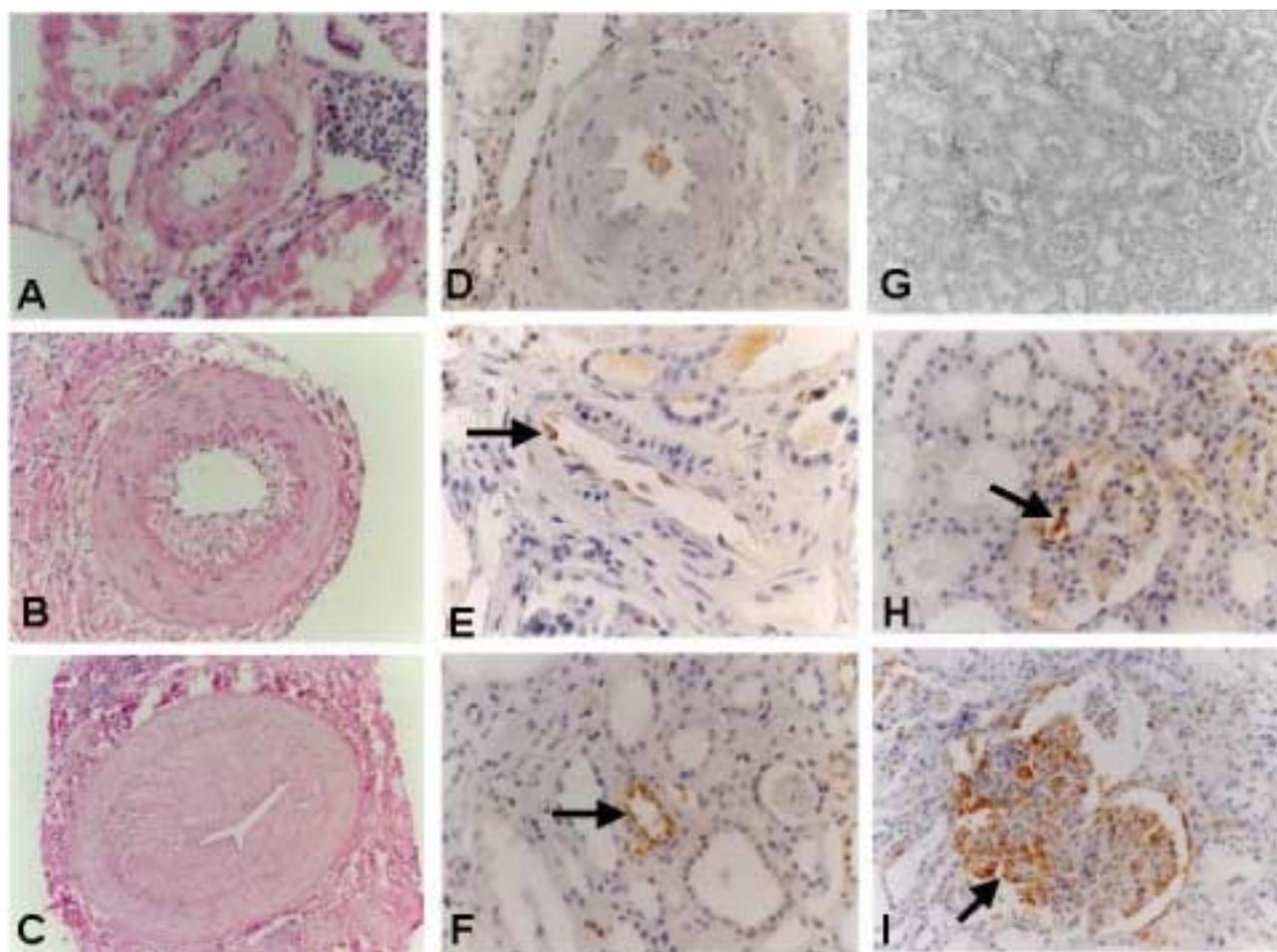
Chronic allograft nephropathy alone was found in 43 samples (64 %) out of 67 protocol biopsies in the 67 patients. The most common finding was mild CAN grade I (n=30) and moderate CAN grade II (n=12). A serious finding of CAN grade III was in 3 samples. In 8 patients, CAN grade I and II was combined with acute rejection Banff-IA or Banff borderline changes and, in 4 patients, acute rejection Banff-IA was a solitary finding. Only 12 patients had normal finding in protocol biopsy. A significant correlation was found between the grade of CAN based on the Banff-97' criteria and kidney graft function, as estimated by creatinine clearance calculated using the Cockcroft-Gault formula ( $p < 0.01$ ) (Fig. 1). There was a similar trend between chronic vasculopathy (Banff cv) and kidney graft function ( $p < 0.05$ ). We found a significant negative correlation between the body mass index and the grade of CAN ( $p < 0.01$ ). However, there was no correlation between the body mass index and chronic vasculopathy.

Forty-eight patients were free of acute rejection episodes during first 12 months after kidney transplantation, 17 patients experienced one acute rejection episode, and two patients two acute rejections. We found a significant correlation between the incidence of acute rejection episodes and the grade of CAN based on the Banff-97' criteria ( $p < 0.01$ ), and a significant correlation between acute rejection incidence and chronic vasculopathy ( $p < 0.05$ ).



**Fig. 1.** The degree of chronic allograft nephropathy (Banff-97 0-III) correlates significantly with kidney graft function as estimated by calculated creatinine clearance using the Cockcroft-Gault formula.

In our study, we analyzed the intensity of TGF- $\beta$ 1 staining within kidney graft structures. TGF- $\beta$ 1 was expressed predominantly within the peritubular capillaries, endothelium, glomerular epithelium and mesangium. We demonstrated a significant correlation between the intensity of TGF- $\beta$ 1 staining within the peritubular capillaries, interstitium and chronic vasculopathy. Major differences were observed between the sum of detail structure TGF- $\beta$ 1 staining and chronic vascular changes based on the Banff-97' classification. These differences reached a high statistical significance when analyzed by the linear trend test (Table 1, Fig. 2). We found no significant relation between the intensity of TGF- $\beta$ 1 staining in different renal structures and the grade of CAN (Table 2). We also showed that there was no significant relationship between kidney graft function and the intensity of TGF- $\beta$ 1 staining in particular structures, nor in the sum of these items.



**Fig. 2.** TGF- $\beta$ 1 kidney graft expression correlates with chronic vasculopathy. Typical chronic vascular changes characterized by concentric intimal proliferation and luminal narrowing (Banff-97' cv I: A, cv II: B, cv III: C) and examples of TGF- $\beta$ 1 immunohistochemistry within the vasculature (D negative, E mild, F dense) and glomeruli (G negative, H mild, I dense); arrows show positive TGF- $\beta$ 1 cell staining.

**Table 1.** TGF- $\beta$ 1 staining in different renal structures according to chronic vasculopathy.

Chronic vasculopathy (available samples)	0 (n=11)	I (n=27)	II (n=17)	III (n=3)
Glomerular epithelium	1.54 $\pm$ 0.78	1.78 $\pm$ 0.50	1.59 $\pm$ 0.60	1.67 $\pm$ 0.47
Mesangium	1.63 $\pm$ 0.64	1.85 $\pm$ 0.35	1.76 $\pm$ 0.42	1.67 $\pm$ 0.47
Endothelium	1.44 $\pm$ 0.83	1.96 $\pm$ 0.19	1.94 $\pm$ 0.23	1.33 $\pm$ 0.94
Intima	0	0.28 $\pm$ 0.60	0.35 $\pm$ 0.68	0
Peritubular capillaries	1.73 $\pm$ 0.44	1.93 $\pm$ 0.26	2.00 $\pm$ 0 <sup>a</sup>	1.67 $\pm$ 0.47
Proximal tubuli	0.18 $\pm$ 0.38	0.41 $\pm$ 0.62	0.59 $\pm$ 0.69	0.33 $\pm$ 0.47
Distal tubuli	1.36 $\pm$ 0.77	1.67 $\pm$ 0.67	1.76 $\pm$ 0.54	1.33 $\pm$ 0.94
Mononuclear cells	0.91 $\pm$ 0.90	1.26 $\pm$ 0.74	1.17 $\pm$ 0.71	1.33 $\pm$ 0.94
Instertitium	0.27 $\pm$ 0.61	0.77 $\pm$ 0.89	1.06 $\pm$ 0.94 <sup>a</sup>	1.33 $\pm$ 0.94
TGF- $\beta$ 1 total	8.81 $\pm$ 3.58	11.7 $\pm$ 2.35 <sup>a</sup>	12.2 $\pm$ 3.20 <sup>a,b</sup>	10.7 $\pm$ 4.78

<sup>a</sup>:  $p < 0.05$  vs. vasculopathy 0, <sup>b</sup>:  $p < 0.01$  vs. vasculopathy 0 and I (linear trend). Intensity of TGF- $\beta$ 1 staining in different renal structures was scored semiquantitatively (0-II). Chronic vasculopathy was defined according to the Banff-97' criteria (Banff cv 0-III).

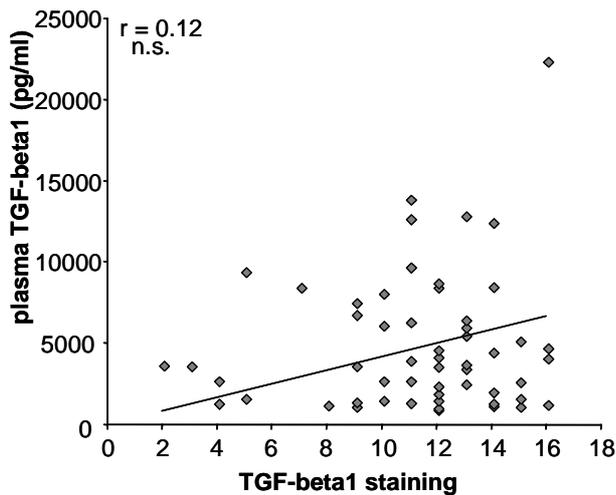
**Table 2.** TGF- $\beta$ 1 staining in different renal structures according to CAN grade.

CAN grade (available samples)	0 (n=11)	I (n=33)	II (n=12)	III (n=2)
Glomerular epithelium	1.70 $\pm$ 0.64	1.70 $\pm$ 0.59	1.42 $\pm$ 0.64	2.00 $\pm$ 0
Mesangium	1.70 $\pm$ 0.64	1.76 $\pm$ 0.42	1.83 $\pm$ 0.37	2.00 $\pm$ 0
Endothelium	1.77 $\pm$ 0.63	1.85 $\pm$ 0.45	1.75 $\pm$ 0.59	2.00 $\pm$ 0
Intima	0.33 $\pm$ 0.67	0.11 $\pm$ 0.31	0.42 $\pm$ 0.76	0
Peritubular capillaries	1.90 $\pm$ 0.30	1.87 $\pm$ 0.33	2.00 $\pm$ 0	2.00 $\pm$ 0
Proximal tubuli	0.10 $\pm$ 0.30	0.50 $\pm$ 0.62	0.50 $\pm$ 0.76	0.50 $\pm$ 0.50
Distal tubuli	1.60 $\pm$ 0.80	1.57 $\pm$ 0.67	1.58 $\pm$ 0.75	2.00 $\pm$ 0
Mononuclear cells	0.90 $\pm$ 0.83	1.23 $\pm$ 0.76	1.08 $\pm$ 0.75	2.00 $\pm$ 0
Instertitium	0.60 $\pm$ 0.83	0.76 $\pm$ 0.90	1.00 $\pm$ 0.91	1.00 $\pm$ 1.00
TGF- $\beta$ 1 Sum	10.4 $\pm$ 2.83	11.13 $\pm$ 3.22	11.58 $\pm$ 4.0	13.5 $\pm$ 1.5

The intensity of TGF- $\beta$ 1 staining in different renal structures was scored semiquantitatively (0-II). TGF- $\beta$ 1 is expressed predominantly within peritubular capillaries, endothelium, glomerular epithelium and mesangium. There was no significant correlation between the intensity of TGF- $\beta$ 1 staining in different renal structures and the grade of chronic allograft nephropathy according to Banff-97' criteria.

In the present study, we measured TGF- $\beta$ 1 plasma concentrations using a commercial ELISA test. Despite the non-significant trend towards higher TGF- $\beta$ 1 concentrations in patients with dense TGF- $\beta$ 1 staining within the kidney graft (Fig. 3), we found no relation between plasma levels of TGF- $\beta$ 1 and kidney graft function, the grade of CAN, chronic vasculopathy and

trough cyclosporine A levels. There was no correlation between the grade of CAN or chronic vasculopathy and variables such as the patient's age, gender, proteinuria, plasma cholesterol and triglycerides levels, blood cyclosporine levels, number of HLA mismatches, panel-reactive antibodies (PRA) and blood pressure.



**Fig. 3.** Trend towards higher plasma TGF- $\beta$ 1 levels in patients with higher TGF- $\beta$ 1 expression within the transplanted kidney.

## Discussion

In our study, protocol biopsy revealed CAN, either alone or in a combination with acute rejection changes, in 76 % of patients. Data about the prevalence of CAN show that it is detected in 22-25 % of patients, as early as 3 months after transplantation (Legendre *et al.* 1998, Serón *et al.* 2000, Nankivell *et al.* 2001). Subsequently, there is an increase not only in the prevalence of CAN but also in the severity of histological alterations (Legendre *et al.* 1998, Lehtonen *et al.* 2001). Nevertheless, the prevalence of CAN later after transplantation may vary from 19 % (Rush *et al.* 1998) to 85 % (Lehtonen *et al.* 2001). The variability may be due to different factors including selection of the patients studied or donors, whose age may affect the severity of histological alterations. Patients in present study were also selected according to their serum creatinine values. Although patients with serum creatinine over 250  $\mu$ mol/l were excluded, the prevalence of CAN was as high as 70 %. This prevalence corresponds to the data obtained in a group of patients treated with cyclosporine in a U.S. multicenter kidney transplant trial where protocol biopsies were performed 2 years after transplantation (Solez *et al.* 1998). Moreover, borderline changes or mild acute rejection were detected either in combination with chronic allograft nephropathy or alone in 12 patients (18 %) of our study group. Such rejection episodes are usually referred to as “subclinical rejection”, because the clinical signs of rejection are absent. The proportion of subclinical rejection cases in protocol biopsies is high at

three months after transplantation (Nankivell *et al.* 2001) and decreases over time. Subclinical rejection found at three months may lead to subsequent chronic damage, but our study showed that it might be present even later. The subclinical rejection prevalence of 18 % at one year, as demonstrated in our study, was comparable with the data in another study (Rush *et al.* 1998).

The presence of CAN with vasculopathy in protocol biopsies at 3 months after transplantation is associated with poor long-term graft survival rates (Serón *et al.* 2000). In our study, data of long-term follow-up are not available yet, but a correlation between chronic vascular changes and kidney graft function at one year is consistent with results of the above study.

TGF- $\beta$ 1 intragraft expression is believed to be closely related to CAN because TGF- $\beta$ 1 is a powerful stimulant of fibrosis. Moreover, the principal immunosuppressant cyclosporine used in our study has been shown to enhance both TGF- $\beta$ 1 mRNA (Khanna *et al.* 1997) and protein expression (Mohamed *et al.* 2000). As a result, cyclosporine has been implicated in CAN development. A correlation of TGF- $\beta$ 1 intragraft expression and a decline in renal function has been demonstrated (Cuhaci *et al.* 1999). In that study, the renal tubular cells were the dominant site of expression. In another study, tubules were spared and strong TGF- $\beta$ 1 staining was noted in the epithelial and intraglomerular cells, and in peritubular capillaries (Horvath *et al.* 1996). In our study, TGF- $\beta$ 1 staining appeared in different renal structures. A correlation was demonstrated between TGF- $\beta$ 1 staining within peritubular capillaries and chronic vascular changes, which correlated with kidney graft function.

The absence of a correlation between TGF- $\beta$ 1 concentrations and renal graft function was not surprising, as this correlation was not reported by other authors (Coupes *et al.* 1994). A significant correlation between TGF- $\beta$ 1 intragraft staining and plasma levels was not demonstrated, but the obtained non-significant result is encouraging and justifies the extension of our study group for further evaluation.

We demonstrated a significant negative correlation between the body mass index (BMI) and the degree of chronic allograft nephropathy according to the Banff classification. Patients with a lower BMI had worse morphological finding within the kidney graft tissue. A large study has recently shown that when looking at death censored graft survival, there was a tendency towards a higher risk for graft loss with low BMI values (Meier-Kriesche *et al.* 2002).

In conclusion, chronic allograft nephropathy occurs frequently in kidney grafts one year post-

transplant despite stable renal function. Subclinical rejection episodes do not seem to be rare events contributing to the poorer long-term outcome of kidney transplantation. TGF- $\beta$ 1 predominantly expresses within peritubular capillaries, endothelium and mesangium of the kidney graft. Its tissue expression correlates with chronic transplant vasculopathy more closely than with the grade of chronic allograft nephropathy.

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