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ORIGINAL ARTICLE

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Sequential protocol biopsies from renal transplant recipients show an increasing expression of active TGF β

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Abstract Chronic allograft nephropathy (CAN) is a major cause of graft loss after renal transplantation. Implicated in the pathogenesis of this complication is overproduction of the cytokine transforming growth factor beta (TGF β). In this study we measured changes in CAN's expression in stable patients early after transplantation, and studied links with established risk factors for CAN, such as delayed graft function, acute rejection, and cyclosporine exposure. We took biopsies from 40 renal allografts at time of transplantation (pre-perfusion), and then, using ultrasound guidance, at 1 week and 6 months after transplantation. An immunofluorescence technique was used to stain sections for active TGF β. These were then assessed by semi-quantitative scanning laser confocal microscopy. There was very little variation in active TGF-β expression among patients in their pre-perfusion biopsies. Expression had increased by 1 week and then very significantly by 6 months ($P < 0.0001$). Patients who suffered delayed graft function had increased TGF-β expression at both time points. There was no difference regarding donor type, acute rejection, and immunosuppressive drug (cyclosporine or tacrolimus). There was no correlation between the amount of TGF-β expression at any time-point and isotope glomerular filtration rate (GFR) at 12 months. This study demonstrated that in a group of stable renal allograft recipients, TGF-β expression in the kidney increased after transplantation. As the study used protocol biopsies, this increase is unlikely to be due to acute events, and probably represents a genuine increase.

Keywords Chronic allograft nephropathy · Transforming growth factor beta · Protocol biopsies

Introduction

As the use of modern immunosuppressives has resulted in less early graft loss due to acute rejection, many renal transplants succumb to a gradual deterioration several years later. This is characterised histologically by fibrosis affecting all compartments of the kidney, and is most commonly described as chronic allograft nephropathy (CAN). While the aetiology is incompletely understood, evidence from other forms of renal fibrosis suggests that a crucial common pathway is a disturbance in the control of extracellular matrix (ECM) turnover. While this control is complex, one cytokine, transforming growth factor beta (TGF β), has been consistently shown to play a vital role.

TGF β has been studied extensively in experimental models of renal disease and increased expression and shown to be associated with histological changes of fibrosis [22]. In transplantation, there is much circumstantial evidence to support its role in CAN [8], however,

much about its overall significance in the process remains unanswered. One of the reasons for this is that it does play an immunosuppressive role, and, therefore, some of the up-regulation after transplantation may be beneficial [12].

As yet there have been no reports examining TGF-β expression in the same kidney sequentially from before transplantation and further into the early post-transplant period. This is of some importance, because it is likely that genotypic differences mean that individual recipients have different baseline levels that are related to their susceptibility to fibrosis [1]. Comparison between patients may, therefore, be unreliable. A greater understanding of the natural history of TGF-β expression after renal transplantation would put into a better context the changes that are found during disease processes such as CAN.

We previously studied the expression of active TGF β after renal transplantation using an immunofluorescence technique [14]. The aim of the present study is to quantify the changes in TGF-β expression after transplantation using protocol biopsies at pre-perfusion, at 1 week and at 6 months after transplantation.

Patients and methods

A consecutive series of 40 patients undergoing renal transplantation underwent protocol biopsies. Baseline biopsies (pre-perfusion) were taken from each kidney at the time of transplantation, and subsequently tru-cut biopsies were taken using ultrasound guidance at 1 week and at 6 months after transplantation. Patients whose grafts had failed prior to a 6-month protocol biopsy were not included in the study. All patients underwent regular clinic follow-up, and renal function was quantified by use of the isotope glomerular filtration rate (GFR) measured by ^{51}Cr-EDTA at 6 and 12 months after transplantation. For immunosuppression we implemented a dual therapy with calcineurin inhibitor (cyclosporine or tacrolimus) and prednisolone. Patients with kidneys from non-heart-beating donors received reduced doses of calcineurin inhibitors and azathioprine (triple therapy). No patients were administered angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptor antagonists. Biopsy specimens were all assessed by an experienced histopathologist (PNF) and graded according to Banff criteria [16].

TGF-β immunofluorescence staining

Paraffin-processed tissue sections were de-waxed and re-hydrated before being incubated with 'blocking' serum (normal human serum 1/15 and normal rabbit serum 1/5 in Tris-buffered saline (TBS) pH 7.6) for 2 h at 4 °C. This was followed by incubation with the primary antibody, chicken anti-human TGF β1 (RD systems) at 1/100 in blocking serum, overnight at 4 °C. The specificity of this primary antibody had previously been shown by its capacity to block the biological activity of TGF β in an epithelial-cell proliferation assay [13]. After having been washed in TBS for 10 min, sections were incubated for 2 h at 37 °C with rabbit anti-chicken IgG conjugated to fluorescein isothiocyanate at 1/400 in blocking serum. Sections were then washed for a further 10 min in TBS and mounted in fluorescence mounting medium (DAKO, Ely, Cambs., UK). Matched negative controls were prepared by replacement of the primary antibody with normal anti-chicken IgG.

Sections were analysed by semi-quantitative scanning laser confocal microscopy as described previously [17]. Data were expressed as the ratio of mean fluorescence over the selected area of experimentally stained tissue (excluding the tubule lumen) to the corresponding value in control sections.

Statistics

Values of TGF-β staining intensity over time were compared using the Wilcoxon signed rank test. Comparisons between different groups were made with the Kruskal-Wallis test for comparison of non-parametrically distributed variables.

Results

Details of the patients studied are shown in Table 1; those of the histological findings in the protocol biopsies in Table 2. Histological results were mostly normal, however occasional sub-clinical rejection was found. Over 25% of the 6-month biopsies showed evidence of fibrosis.

The most striking finding was the highly significant increase in active TGF-β staining 6 months after transplantation (Fig. 1). When the levels of the individual patients were plotted, 34 showed an increase in TGF β over time and only six a drop (data not shown). The TGF-β levels both at pre-perfusion and 1 week after transplantation were similar and did not show a large range. TGF-β levels did not seem to be affected by donor type (Fig. 2). Although there was only a small number of patients in this study, an attempt was made to determine whether any of the established prognostic factors for graft outcome were related to the rise in TGF β. Only delayed graft function (DGF) seemed to be relevant; patients with DGF displayed increased TGF-β expression at both 1 week and at 6 months, when compared with those who did not, however, neither of these results quite reached significance (Fig. 3, 4). Six patients developed acute rejection in the first 6 months after transplantation; they did not have higher levels of TGF β at 6 months (Fig. 5). The type of immunosuppressive drug administered did not affect 6-month

Table 1 Patient details ($n=40$) (*CAD* cadaveric donor, *NHBD* non-heart-beating donor, *LRD* living related donor, *CIT* cold ischaemia time)

Characteristic	
Mean donor age in years	42.5 (14.2)
Mean recipient age in years	44 (12.7)
Gender ratio (M:F)	24:16
Donor type (CAD; NHBD; LRD)	17; 14; 9
Mean CIT	12.3 (8.0)
DGF	14/40 (35%)
AR by 6 months	6/40 (15%)

Table 2 Histological findings in protocol biopsies (according to the Banff 1997 classification)

Diagnosis	Biopsy		
	Pre-perfusion	1 week	6 months
Normal	36	21	26
Acute tubule necrosis	0	13	0
Borderline rejection	0	4	3
AR	0	2	0
CAN	4	0	11

TGF-β levels either (Fig. 6), and there was no correlation with drug levels for either agent (data not shown). There was no correlation between TGF-β levels at any time-point and GFR at 6 or 12 months after transplantation.

Discussion

This study demonstrates increased levels of active TGF β in renal transplants at 6 months when compared with baseline pre-perfusion levels. As the study was carried out using protocol biopsies, changes were likely to be due to transplantation rather than to any particular condition for which the biopsies had been taken. This study comprises a relatively small number of patients and so does not have the power to demonstrate differences between patient groups after transplantation. Furthermore, it does not show any influence of TGF-β levels on prognosis. This will need larger studies.

There have been problems with the study of TGF-β expression in biopsy specimens because of uncertainties about whether staining distinguished between active and latent forms. The antibody used in this study has been shown to react only with active TGF β; this study was therefore carried out optimally [13].

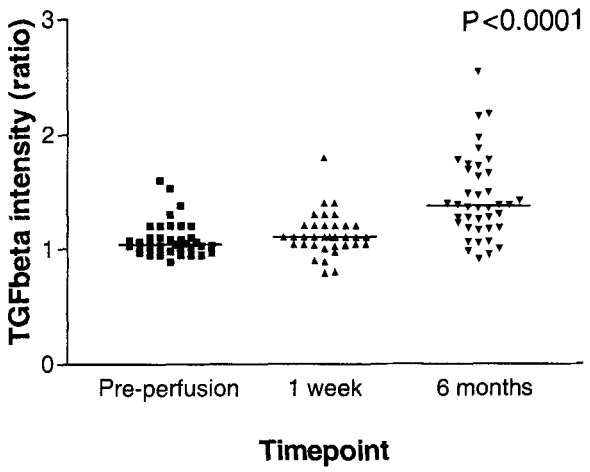

Fig. 1 Changes in TGF-β expression after transplantation

Although several human studies have reported increases in the expression of TGF β in CAN [15, 18, 19], none of these drew comparisons with stable post-transplant patients. Hence, it is not clear whether TGF β is truly involved in the process, or if it is an innocent bystander.

There have been few studies of renal TGF-β expression after transplantation in stable recipients. Lantz et al. included the biopsies of seven stable transplant recipients 6–24 months after transplantation in their series of 28 biopsies stained for TGF β [10]. Those showed definitely increased staining when compared with normal controls, measured in a semi-quantitative manner on a scale of + to + + +. No difference was shown between stable transplants and those with acute rejection (AR) or CAN. The only other study of TGF-β expression in stable patients after transplantation measured its levels in plasma. [5] There was a definite increase in the 17 transplant patients when compared with 43 healthy controls ($P=0.0004$). Again, no difference was determined between the stable transplants and those with AR or CAN. These findings are in agreement with those in the current study, which shows that while there was a definite increase in TGF β at 6 months, levels were not affected by established risk factors for CAN.

There is a very good reason for increased TGF-β levels in post-transplant patients, as it has an immunomodulatory role and is likely to be significant for reducing the extent of the reaction against the allograft [12]. It also seems to be up-regulated by ischaemia and to have a protective role [11]; indeed this may explain the increased levels found in patients with DGF in this series. As well as potential beneficial actions however, TGF β does play a role in fibrosis, which has been described as its dark side [2]. Possibly, certain patients are more prone to the unwanted effects of TGF β, and an interesting finding with regard to this has been that

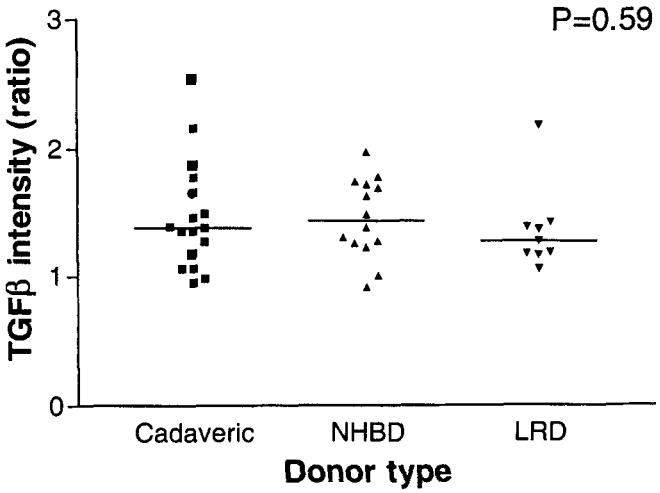

Fig. 2 Active TGF-β expression 6 months after transplantation, according to donor type

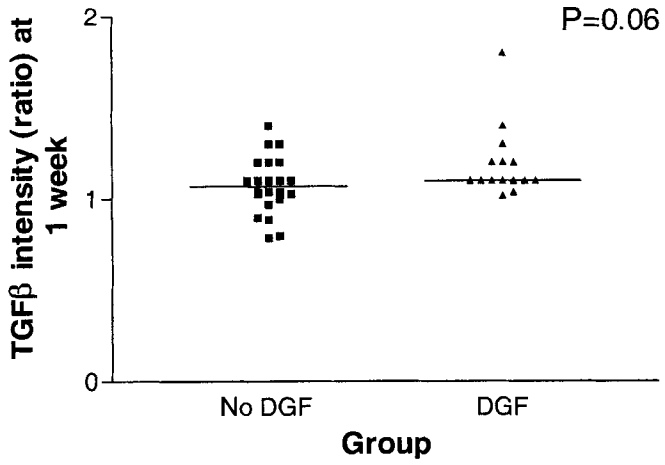

Fig. 3 Effect of DGF on active TGF-β expression 1 week after transplantation

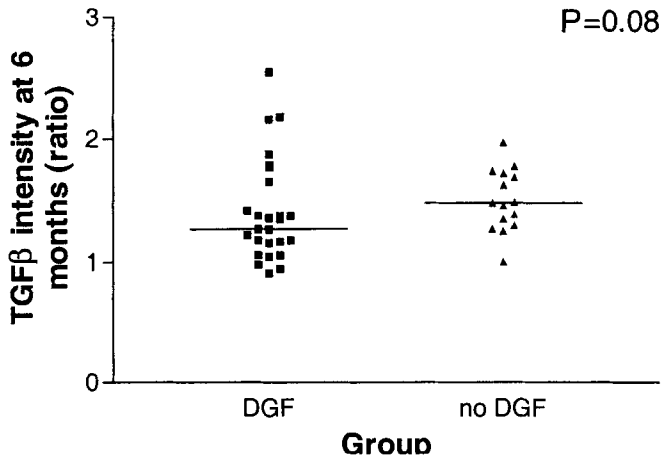

Fig. 4 Effect of DGF on active TGF-β expression 6 months after transplantation

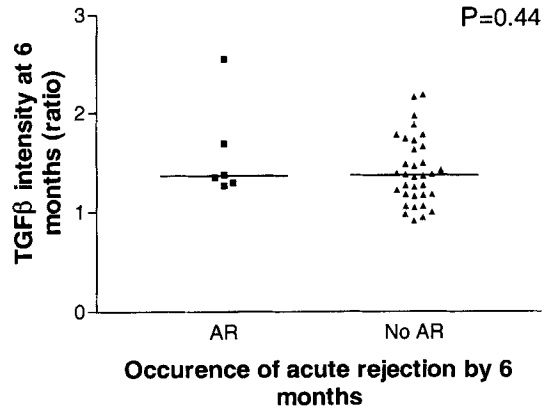

Fig. 5 Effect of AR on active TGF-β expression 6 months after transplantation

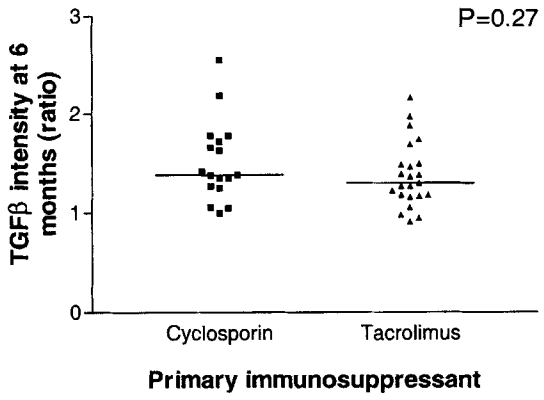

Fig. 6 Active TGF-β expression 6 months after transplantation, according to primary immunosuppressant drug

there are various genotypes of TGF β, with differing propensities for developing fibrosis [1].

The calcineurin inhibitors cyclosporine and tacrolimus induce TGF β [20, 21], and this has been proposed as a mechanism of action [9]. There has been much interest in differences between the two drugs, and indeed, we have previously shown that cyclosporine induced TGF β to a greater extent in biopsies taken for diagnostic purposes. A study using protocol biopsies after liver transplantation also showed this, and thus it is surprising that there was no difference found in the current study. However, all these studies comprise relatively small numbers, and so they are probably not conclusive. There have certainly been other studies that could not find a difference in the effects of the two drugs on TGF β, either [7]. ACE inhibitors have been shown to have a down-regulatory effect on TGF-β expression [3]. While there were no patients on these drugs in this study, they may have had a confounding effect when drug groups in other studies were compared. The use of angiotensin II receptor blockers therapeutically to reduce TGF-β levels after renal transplantation is an interesting concept that is being explored [4].

A correlation between increased TGF-β levels and rate of decline in renal function has been shown by Cuhaci et al. [6]. In this study of forty patients, TGF-β levels were graded as low or high in a qualitative manner. There was no correlation between TGF-β levels at 1 week and at 6 months and renal function at 6 months and at 1 year in this study. Obviously longer follow-up will allow a more detailed analysis to be made.

In conclusion, there still remains much to be learned about the importance of TGF β after renal transplantation, and its role needs to be defined more clearly before its manipulation for potential therapeutic gain is a practical option.

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