Kidney International, Vol. 50 (1996), pp. 489–498

Altered expression of transforming growth factor-βs in chronic renal rejection

LASZLO ZS. HORVATH, HELMUT FRIESS, MARTIN SCHILLING, BETTINA BORISCH, JUDITH DEFLORIN, LESLIE I. GOLD, MURRAY KORC, and MARKUS W. BÜCHLER

Department of Visceral and Transplantation Surgery, and Department of Pathology, University of Berne, Inselspital, Berne, Switzerland; and Department of Pathology, New York University Medical Center, New York, and Division of Endocrinology, Diabetes and Metabolism, University of California, Irvine, Irvine, California, USA

Altered expression of transforming growth factor-βs in chronic renal rejection. We examined the altered expression of transforming growth factor-βs in chronic renal rejection in humans, including transforming growth factor beta-1 (TGF-β1), TGF-β2, TGF-β3 and their receptors, transforming growth factor beta receptor type I (TβR-I) and TβR-II. Using Northern blot analysis and immunohistochemistry, 10 specimens of chronically rejected and 8 normal kidney samples were analyzed. By Northern blot analysis the expression of mRNA encoding TGF-β1, TGF-β2, TGF-β3 ($P < 0.02$), TβR-I and TβR-II ($P < 0.02$) was decreased in chronically rejected renal cortex samples, compared to normal controls. Immunohistochemical analysis of the normal renal cortex showed strong immunostaining for TGF-β1 and TGF-β3, and mild immunostaining for TGF-β2 in the proximal and distal tubulointerstitium, but no signal for any of the TGF-β isoforms in the glomeruli or in the cortical vessels. In sharp contrast, the glomeruli and the cortical vessels of the rejected kidney specimens exhibited strong immunostaining for TGF-β1 and TGF-β3, whereas the tubules revealed a decrease in immunoreactivity. TβRI and TβRII immunostaining showed similar changes as observed with TGF-β1 and TGF-β3 antibodies. There was a concomitant increase in B-cell accumulation in the glomeruli, while T-cells and macrophages were diffusely abundant in the rejected samples. Since TGF-βs are potent inducers of extracellular matrix proteins and have been shown to be involved in fibrotic disease, the increase in TGF-β1 and TGF-β3 immunoreactivity in the glomeruli suggests that there is a redistribution in TGF-β expression in chronic renal allograft rejection. Together with changes affected by B-cell mediated immunity, the above alterations might contribute to the histopathological changes that occur in this disorder, such as intimal fibrosis, arteriosclerosis and glomerular and tubular sclerosis.

Chronic rejection is the most common cause of graft loss in renal transplantation. Despite this fact, research has mainly focused on the prevention of acute rejection with the development of powerful immunosuppressants. To date, no effective treatment has been developed for the irreversible damage associated with chronic rejection. The most characteristic histopathological features of chronic rejection in renal allografts are intimal fibrosis mainly in the cortical arteries, glomerular ischemic simplification, interstitial fibrosis and tubular atrophy.

Received for publication May 26, 1995
and in revised form March 18, 1996
Accepted for publication March 18, 1996

© 1996 by the International Society of Nephrology

T cell mediated immunity plays a critical role in allograft rejection [1]. A variety of interactions have been implicated in the rejection process, including cytokines such as IL-1, IL-2, transforming growth factor beta-1 (TGF-β1), TGF-β2 and TGF-β3 [2, 3]. The initial targets of rejection appear to be the graft endothelial structures. Interstitial mononuclear cells (lymphocytes, macrophages, plasma cells, eosinophil cells) infiltrate the rejected graft and serve as histological indicators of chronic cell-mediated rejection.

TGF-β1, TGF-β2 and TGF-β3 belong to a superfamily of multifunctional homologous polypetide growth factors, which act through pleiotropic effects in various cell types [4, 5]. They play a major role in the regulation of extracellular matrix formation and fibrosis, arteriosclerosis and angiogenesis, immunosuppression, carcinogenesis, inflammation, tissue repair and also in normal differentiation and growth [6–12]. TGF-β1, TGF-β2 and TGF-β3 are the mammalian isoforms of TGF-β. In addition cDNA clones have also been isolated for TGF-β4 from chicken [13], and TGF-β5 from frog [14], but these proteins have not been identified in mammalian cells.

TGF-βs bind to specific cell surface binding proteins with high affinity in many different cell types. Two receptors involved in signal transduction have been identified, a 53 kDa (type I TGF-β receptor, TβR-I), and a 70 kDa (type II TGF-β receptor, TβR-II), the cytoplasmic domaine have serine threonine kinase activity [15, 16]. In addition, a 200 to 400 kDa (type III TGF-β receptor, TβR-III) non-signaling proteoglycan has been described [15–21]. This receptor, also called betaglycan, is required for the presentation of TGF-βs to the TβR-I/TβR-II complex [16, 19–21]. Studies using deletion mutants have indicated that TβR-I and TβR-II co-operate to initiate a signal [15, 16]. TβR-II binds the ligands and sequestors TβR-I into a complex for signaling [16].

Recent studies indicate that the induction of growth factors may play a pathobiological role in experimental acute and chronic rejection [22–33]. TGF-β1 has been implicated as a critical regulatory molecule in the cause of renal fibrosis and arteriosclerosis [12, 34]. However, it is not known whether other TGF-β isoforms and their receptors contribute to the perturbations that occur in the renal rejection. Therefore, in the present study, we investigated the expression of all three mammalian TGF-β isoforms (TGF-β1, TGF-β2, TGF-β3) and their signaling receptors

Table 1. Monoclonal and polyclonal antibodies used in immunohistochemistry on paraffin-embedded material

Cluster	Antibody	Source	Lymphohistiocytic specificity
CD 45	LCA[a]	DAKO	Common lymphocyte marker
CD 20	L 26[a]	DAKO	B cells, follicular dendritic cells
CD 3	CD 3	DAKO	T cells
Ø	MAC 387[a]	DAKO	Macrophages, histiocytic cells

[a] Monoclonal antibodies DAKO, Dakopatts, Copenhagen, Denmark

(TβR-I, TβR-II) in human chronic renal allograft rejection by Northern blot analysis and immunohistochemical staining.

Methods

Patients

Kidney samples of 10 patients (6 female, 4 male; median age 52.7 years; range 45 to 63 years) with chronic renal rejection, established according to clinical records and histopathological analysis, were analyzed. Before these samples were used in our analysis the clinical diagnosis was verified by histopathological examination by two independent pathologists. Immediately after surgical removal of the kidney, the cortex was separated from the medulla in the operating room and a larger representative area of the separated cortex was cut approximately in half and used for both histological fixation and molecular analysis in order to ensure comparison. Tissues destined for histological analysis were fixed in Bouin solution for 12 to 24 hours, then dehydrated through graded alcohol and subsequently embedded in paraffin. For RNA extraction the tissues were snap frozen in liquid nitrogen immediately upon surgical removal, and maintained at −80°C until use. The median range of time between the transplantation and the episode of chronic kidney rejection was 51 months (range 5 months to 9 years). Patients were all nondiabetic adults. From the rejected group one patient was free of immunosuppressive drugs prior to renal explantation, three patients were administered prednisolone and salicylic acid and three patients had a combination of prednisolone and salicylic acid with immunosuppressive agents such as azathioprine and cyclosporine. Normal renal tissues were obtained from six patients undergoing surgery because of renal cell carcinoma and from two previously healthy individual through an organ donor program (4 female, 4 male; median age 54.75 years; range 28 to 80 years). The expression of TGF-β1, TGF-β2, TGF-β3, TβR-I and TβR-II was assessed by Northern blot analysis and immunostaining in all specimens.

Immunohistochemical staining

Paraffin-embedded sections (2 to 4 μm thick) were immunostained for TGF-β1, TGF-β2, TGF-β3, TβR-I, TβR-II and for lymphocyte and macrophage cell markers (Table 1) using the streptavidin-peroxidase/biotin technique (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). Tissue sections were submerged for 15 minutes in TBS buffer (10 mM Tris-HCl, 0.85% NaCl, pH 7.4) containing 0.1% (vol/vol) Triton X-100 and washed for five minutes in TBS solution, as previously reported [35, 36]. Endogenous peroxidase activity was quenched by incubating the slides in methanol and in methanol/0.6% hydrogen peroxide, followed by washing in methanol and TBS/0.1% BSA [35, 36]. Following treatment with hyaluronidase (1 mg/ml in 100 mM sodium acetate, 0.85% NaCl), the sections were blocked for 30 minutes at 37°C with 10% normal goat serum prior to overnight incubation at 4°C with isoform specific polyclonal TGF-β1, TGF-β2 and TGF-β3 antibodies (2.5 μg/ml) diluted in TBS containing 5% normal goat serum and 0.1% BSA [35, 36]. Bound antibody was detected with a biotinylated goat anti-rabbit IgG secondary antibody and streptavidin-peroxidase complex (Kirkegaard & Perry Laboratories), followed by incubation with diaminobenzidine tetrahydrochloride (0.05%) as the substrate and counterstained with Mayer's hematoxylin.

To ensure antibody specificity, control slides were either incubated in the absence of primary antibody or with an irrelevant IgG antibody. In both cases no immunostaining was detected. The isoform specific antibodies to mature TGF-β1, TGF-β2 and TGF-β3 were produced in the laboratory of Leslie Gold, and their preparation and characterization are described in detail by Pelton et al [36]. Briefly, the antibodies were raised in rabbits to synthetic peptides corresponding to amino acid residues 4-19 of TGF-β1 and TGF-β2 and 8-29 of TGF-β3. Each antiserum was purified by ammonium sulfate precipitation followed by peptide affinity chromatography using the respective peptide immunogen. The antisera were shown to be isoform-specific by Western blot analysis using mature recombinant TGF-β1 and TGF-β3 and porcine native TGF-β2, as illustrated [36]. No cross-reactivity among the antisera was observed. All tissue sections that were compared were treated at the same time using identical incubation times and dilutions of all reagents including the antisera. Antibody activity was previously shown to be absorbed with 33 Molar excess of each peptide used as immunogen [36, 37]. Each antiserum absorbed with peptide showed no immunoreactivity, proving antibody specificity. Antibodies against TβRI and TβRII were obtained from Santa Cruz Laboratories, California. Denaturation of the tissue by fixation in formalin exposes the epitopes so that immunoreactivity is enhanced. We have observed that frozen sections require ten times more antisera to achieve similar intensity of immunostaining (unpublished observation). This is consistent with the fact that ten times more antisera was required if native TGF-β was not reduced prior to SDS-PAGE in Western blot analysis (unpublished observation).

Both monoclonal and polyclonal antibodies to lymphocyte (CD 45) and macrophage cell markers were used as primary antibodies as detailed in Table 1. The individual antisera of T-cells (CD 3), B-cells (CD 20) and macrophages (MAC 387) showed no cross-reactivity. No immunostaining was detected when the slides were processed either in the absence of the primary antibody or in the presence of affinity purified non-immune rabbit or mouse anti-IgG.

Grading of the immunohistochemical staining was defined on the basis of the percentage of morphological changes occurring over an area of the cortex, and was quatified according to the number of positively stained cells: Ø, no staining; (+) < 10%; + 10 to 30%; ++ 30 to 50%; +++ > 50%.

Probe synthesis

The TGF-β1 cDNA probe consisted of a 280 bp *Eco*RI/*Xba*I fragment of human TGF-β1 cDNA [38, 39]. The TGF-β2 cRNA probe consisted of a 600 bp *Hin*dIII/*Pst*I fragment of human TGF-β2 cDNA [38], corresponding to nucleotides 253 to 853 [38,

39]. The TGF-β3 cRNA consisted of a 125 bp *Xba*I/*Bgl*II fragment of human TGF-β3 cDNA [38], corresponding to nucleotides 917-1042 [38, 39]. TβR-I and TβR-II cRNA probes were generated from a 377 bp and a 476 bp *Bam*HI fragment of human TβR-I and TβR-II cDNA [10]. The 7S cDNA probe consisted of a 190 bp fragment of the mouse 7S cytoplasmic cDNA which cross hybridizes with human 7S RNA [9, 10, 40]. The antisense cRNA probes used for Northern blot analysis were radiolabeled with [α-^{32}P] CTP (Du Pont, Boston, MA, USA). All the membranes were also hybridized with 7S in order to assess equivalent RNA loading [40–42]. [α-^{32}P] dCTP (Du Pont) was incorporated into TGF-β1 and 7S cDNA probes using a random primer labeling system (Boehringer-Mannheim, Germany) [43].

Northern blot analysis

Total RNA was extracted by the guanidine isothiocyanate method [44], size-fractionated on 1.2% agarose/1.8 M formaldehyde gels, and stained with ethidium bromide for verification of RNA integrity and loading equivalency [40, 45]. The RNA was electro-transferred onto Nylon membranes (GeneScreen; Du Pont) and cross-linked by UV irradiation [40, 45]. The blots were then prehybridized, hybridized and washed under conditions appropriate for antisense cRNA riboprobes (TGF-β2, TGF-β3, TβR-I, TβR-II) or cDNA probes (TGF-β1, 7S), as previously described [9, 10, 35].

For the antisense riboprobes the blots were prehybridized overnight at 65°C in 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 5 × SSC, 5 × Denhardt's solution (1 × Denhardt's = 0.02% ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 250 μg/ml salmon sperm DNA, and 50 mM Na_2PO_4, pH 6.5. The blots were then hybridized for 18 hours at 65°C in the presence of 1×10^6 cpm/ml of the labeled antisense riboprobe, washed twice at 65°C in a solution containing 1 × SSPE (150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA) and 0.5% SDS, and twice at 65°C in a solution containing 0.1 × SSPE and 0.5% SDS [40, 45].

For TGF-β1 and 7S cDNA probes the blots were prehybridized overnight at 42°C in a prehybridization buffer that contained 50% formamide, 1% SDS, 0.75 M NaCl, 5 mM EDTA, 5× Denhardt's solution, 100 μg/ml salmon sperm DNA, 10% Dextran sulfate and 50 mM Na_2PO_4, pH 7.4. The hybridization was carried out at 42°C for 18 hours with the labeled cDNA probe (1×10^6 cpm/ml), washed twice (23°C) in 2 × SSC and three times at 55°C in 0.2 × SSC and 2% SDS [40, 45].

Blots were then exposed at −80°C to Fuji X-ray film using intensifying screens, and the intensity of the radiographic bands was quantified by video densitometry (Biorad 620, USA), as previously reported [40].

Statistical analysis

Results were expressed as mean or as median and range. For statistical analysis the Wilcoxon-test was used [46]. Significance was defined as $P < 0.05$.

Results

Chronically rejected kidney shows increased and redistributed immunoreactivity for TGF-β isoforms compared to normals

TGF-β isoforms are secreted as latent molecules consisting of a precursor, termed latent associated peptide (LAP), covalently complexed to the mature potentially bioactive dimer (ie pro-TGF-β). Only the active mature dimer can bind to TGF-β receptors. Therefore, any antibodies produced to the mature amino acid sequences of TGF-β isoforms will presumably detect the mature molecules both, complexed to the LAP portion and separated from the complex in its activated form (unless the epitopes on the mature portion are masked by the LAP peptide). For this reason, the antibodies will not be able to distinguish between the latent and active molecules. Thus, the antisera detect potentially active or activated TGF-β isoforms (that may or may not bind to TGF-β receptors).

Figure 1 shows the differences among the immunostaining patterns obtained of TGF-β1, TGF-β2 and TGF-β3 in normal (A, C, E) and rejected (B, D, F) renal samples. Figure 2 shows a higher magnification to emphasize the major structures of immunohistochemical localization of TGF-β1 in the rejected cortex. Tables 3 and 4 summarize the pathological changes detected in the rejected and the normal group. Grading was defined on the basis of the percentage of morphological changes occurring over an area of the cortex, and was quantified according to the number of positively stained cells: Ø, no staining; (+) < 10%; + 10 to 30%; ++ 30 to 50%; +++ > 50%.

Normal kidney samples exhibited intense immunoreactivity for both TGF-β1 (Fig. 1A) and TGF-β3 (Fig. 1E) in the proximal and distal tubules in the renal cortex, whereas the interstitial space was devoid of immunoreactivity. Only a few glomeruli showed mild TGF-β1 immunostaining around the epithelial layer of the Bowman's capsule (Fig. 2 A, B). The endothelium and the mesangium of the normal glomeruli, as well as the large vessels and peritubular capillaries did not exhibit TGF-β1 immunoreactivity (Fig. 1A; Fig. 2 A, B). TGF-β2 immunoreactivity was slight in most of the normal tissues (Fig. 1C).

Rejected kidney samples exhibited a decrease in the intensity of TGF-β1 (Figs. 1B and Fig. 2 C, D) and TGF-β3 (Fig. 1F) immunoreactivity in the tubules by comparison with the normal specimens (Fig. 2 A, B, and Table 4). A minimal number of the tubules were positively stained, most notably the proximal ones. In contrast, immunoreactivity in the glomeruli for TGF-β1 (Fig. 1-B; Fig. 2-C,D) and TGF-β3 (Fig. 1-F) was markedly increased in the epithelial and intraglomerular cells in the chronically rejected kidneys. The peritubular capillaries and the characteristically thickened intimal layer of the cortical vessels in the rejected kidney were also positively immunostained for TGF-β1, TGF-β2 and for TGF-β3 in contrast to the normal tissues (Fig. 1 B, D, F). Moreover, strong immunoreactivity for TGF-β1 and TGF-β3 was detected among the infiltrating lymphocytes, macrophages and other inflammatory cells of the grafted kidney (Table 3). TGF-β2 immunostaining in the rejected kidney showed less differentiated changes compared to normal samples. The intensity of immunostaining was mild and more intense staining was only detected in 2 out of the 10 rejected specimens. As shown in Figure 3, TβRI and TβRII immunoreactivity exhibited the same pattern of distribution as TGF-β1 and TGF-β3 in the rejected renal tissue.

Figure 4 illustrates the immunostaining of the various immunocells infiltrating the graft. Chronically rejected samples were characterized by a strong infiltration of T-lymphocytes (CD 3) and macrophages (MAC 387) localized diffusely throughout the whole tissue (Fig. 4 C, E). In contrast, B-lymphocytes (CD 20) were

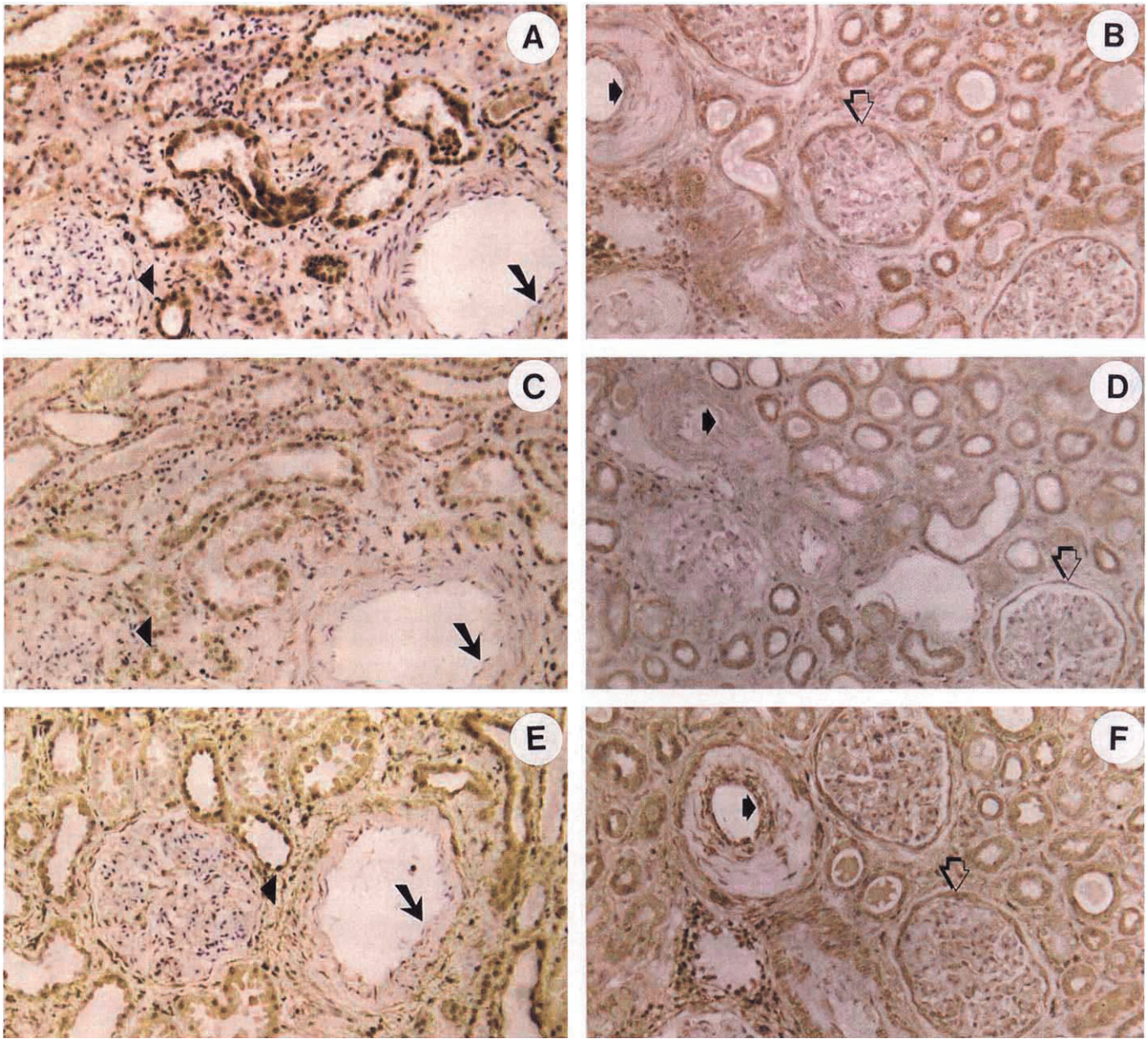

Fig. 1. *TGF-β immunostaining in normal (***A, C, E***) and rejected (***B, D, F***) renal tissues.* The same area is examined in consecutive slides. Symbols are: (black arrow) nonstaining arteries in normal renal tissue; (black triangle) intact glomeruli in normal cortex; (white arrowhead) positive staining in the epithelial and mesangial part of the rejected glomeruli; (black arrowhead) positive staining in the intimal layer and endothelial cells of the sclerotic arteries in rejected grafts. Original magnification is ×200.

found focally, accumulating around and in the damaged glomeruli but were not associating with the intact glomeruli of the rejected specimens, as shown in Figure 4D.

TGF-β isoform and receptor mRNA levels are decreased in rejected kidney specimens

Northern blot analysis of total renal RNA was performed to determine the levels of TGF-β1, TGF-β2, TGF-β3, TβR-I and TβR-II mRNA in both rejected and normal renal cortexes (Fig. 5). As shown in Figure 6, densitometric scanning of all the rejected tissues compared to normal samples indicated an overall decrease in mRNA for TGF-β1, TGF-β2, TGF-β3, TβR-I and TβR-II in the rejected specimens. Furthermore, this decrease tended to vary directly with the general fibrosis in rejection. In the rejected tissues there was a 1.41-, 1.95- and 2-fold decrease in TGF-β1, TGF-β2 and TGF-β3 mRNA levels respectively, compared to the corresponding levels in the normal controls (Fig. 6). TβR-I and TβR-II mRNA levels were 1.3-fold and 3.61-fold decreased in rejection. However, these differences were statistically significant only in the case of TGF-β3 ($P < 0.02$) and TβR-II ($P < 0.02$) mRNA levels.

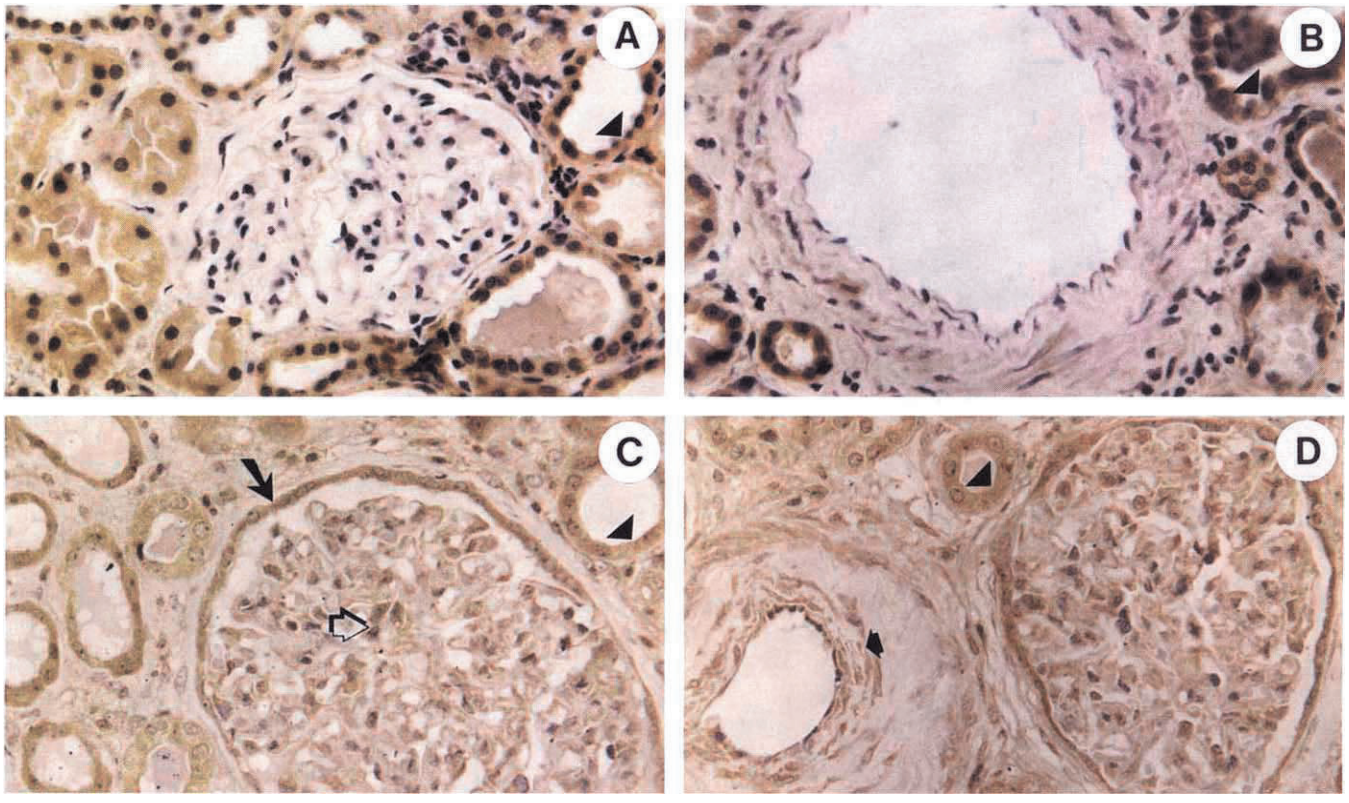

Fig. 2. *Significant characteristic localization of TGF-β1 in the normal kidney* (**A, B**) *compared to chronic allograft rejection* (**C, D**) *detected by immunohistochemical analysis.* A nonstaining intact glomeruli (A) and an intact artery in normal tissue (B). Symbols are: (black arrow) positive glomerular epithelial staining; (white arrowhead) positive intraglomerular mesangial staining; (black arrowhead) positive staining in the intimal layer of the arteries; (black triangle) positive tubular staining. Original magnification ×400.

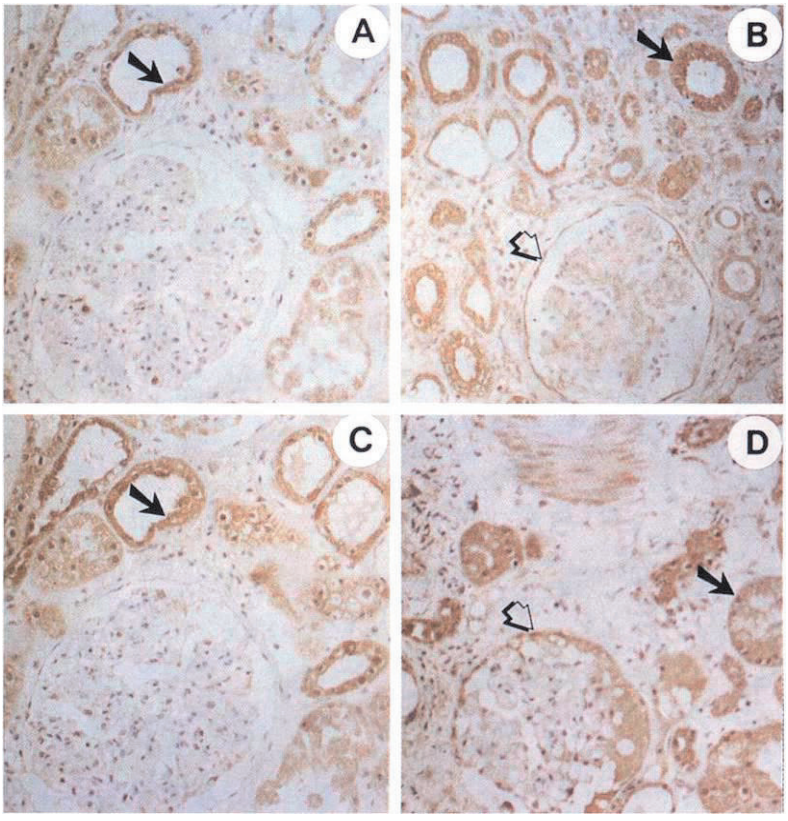

Fig. 3. *TβRI* (**A, B**) *and TβRII* (**C, D**) *immunostaining in normal (A, C) and rejected (B, D) renal tissues.* The same area is examined in consecutive slides. black arrow: positive tubular staining, white arrowhead: positive glomerular epithelial staining. Original magnification is ×200.

Table 2. Role of growth factors after different organ transplantations

Transplanted tissue	Growth factor	Correlation with rejection-CAV	Author, date, source, [Ref]
1. Cornea—human	EGF	*In vitro* pre-treated tissue prevents rejection	Lass, 1994 *Ophthalmology* [24]
2. Langerhans islet—mouse	TGF-β	Elevated. Prevention of rejection?	Gill, 1991 *Transplant Proc* [25]
3. Langerhans islet—rat to mouse	TGF-β	*In vitro* pre-treated tissue prevents rejection	Carel, 1993 *Transplantation* [26]
4. Liver—human	bFGF	Elevated. Chronic rejection identification?	Bishop, 1992 *Transplant Proc* [27]
5. Aorta—rat	PDGF, EGF, IGF-1	Elevated in CAV/chronic rejection	Häyry, 1993 *FASEB J* [28]
6. Heart—rat	TGF-β1,2,3, LTBP	Elevated. Responsible for rejection?	Waltenberger, 1993 *J Immunol* [29]
7. Heart & kidney—rat	PDGF-β	Elevated. Cause of CAV?	Higgy, 1991 *Transplant Proc* [30]
8. Heart—human	TGF-β, FGF	Elevated. Cause of CAV?	Zhao, 1993 *Clin Exp Immunol* [22] Zhao, 1993 *Transplant* [23]
9. Heart—human	PDGF	Elevated. Cause of CAV?	Shaddy, 1992 *J Heart Lung Trans* [31]
10. Renal—rat	EGF	Decreased in chronic rejection.	Stein, 1993 *Transplant Proc* [32]
11. Renal—human	LMW-GF serum ultrafiltrate	Decreased to 0 level in CRF or rejection, back to normal in good renal function	Jacob, 1993 *Clin Nephrol* [33]
12. Renal—human	TGF-β1	Increased TGF-β1, PAI-1 and fibronectin in glomeruli	Shihab, 1995 *J Am Soc Nephrol* [34]

Abbreviations are CRF, chronic renal failure; CAV, chronic arterial vasculopathy.

Table 3. Grading of the pathological changes and staining intensity of the graft infiltrating immune cells in renal cortex in the rejected and normal kidney specimens

	Normal	Rejected
Glomeruli	Ø	++
Arteriosclerosis	Ø; (+)	++
Fibrosis	Ø	++
Necrosis	Ø	+
Lymphocytes	+	++
Macrophages	(+)	+++

Grading was defined on the basis of the percentage of morphological changes occurring over an area of the cortex, and was quantified according to the number of positively stained cells: Ø, no staining; (+), <10%; +, 10–30%; ++, 30–50%; +++, > 50%.

Table 4. Staining intensity of TGF-βs and graft infiltrating cell markers in renal cortex

	Normal	Rejected
TGF-β1		
BC	Ø	++
TUB	++	+
TGF-β2		
BC	Ø	+
TUB	+	+
TGF-β3		
BC	Ø	+++
TUB	++	+
Lymphocytes (CD 45)	+	+++
T cells (CD 3)	(+); +	++
B cells (CD 20)	Ø; (+)	+++
Macrophages (MAC-387)	(+)	+++

Abbreviations are: BC, Bowman's capsule; TUB, tubules; CD 45, general lymphocyte marker; CD 3, T cell marker; CD 20, B cell marker; MAC 387, macrophage marker. Grading was defined on the basis of the percentage of morphological changes occurring over an area of the cortex, and was quantified according to the number of positively stained cells: Ø, no staining; (+), <10%; +, 10–30%; ++, 30–50%; +++, > 50%.

Discussion

Characteristic pathological changes of chronic renal allograft rejection include the presence of interstitial fibrosis, arteriosclerosis and necrosis, glomerular ischemic simplification and tubular atrophy. TGF-β isoforms especially TGF-β1 has been implicated as a critical regulatory molecule in the cause of fibrosis and arteriosclerosis [11, 12]. The role for TGF-β in the fibrotic response is due to its ability to stimulate the production of proteins that compose the extracellular matrix, including fibronectin, collagens and proteoglycans. Moreover, TGF-β stimulates the production of protease inhibitors to prevent breakdown of the deposited matrix components. The most significant studies indicating a causative role for TGF-β in an animal model of glomerulonephritis were those of Border et al [12]. In these studies neutralizing antibodies to TGF-β completely obviated the fibrotic response in experimentally induced glomerulonephritis in rats [12]. Similar pathological changes were observed in the glomerular epithelium and mesangium in glomerulonephritis and progressive kidney fibrosis [47, 48]. The increased expression of TGF-β1 and TGF-β3 in the vasculature most likely mediates the intimal proliferation, which causes arteriosclerosis, thereby accelerating the process of chronic renal allograft rejection.

An important function of TGF-βs which may effect chronic renal allograft rejection is their role in the regulation of the immune response [6–8]. TGF-βs furthermore have immunosuppressive effects upon various target cells within the immune system *in vivo* [49, 50], which would have an effect in preventing rejection. For example a possible mechanism could be the suppression of T-cell activation by antagonizing the function of IL-1 and IL-2 [2, 3]. TGF-βs can also inhibit B-lymphocyte differentiation and proliferation [8, 51]. However, TGF-βs also demonstrate other effects on immune cells, which would promote the process of rejection such as chemotactic effects on monocytes and macrophages [6–8].

Table 2 summarizes certain growth factors studied, and their possible or putative function in both ameliorating or causing rejection in the transplantation of various organs. Several studies reported upregulation of TGF-β, EGF, FGF or PDGF both in human and animal models of chronic rejection after cornea, Langerhans islet, liver, aorta or heart transplantation [24–31]. Only three have studied kidney allograft rejection, and in contrast to our studies, in chronic renal allograft rejection down-regulation of epidermal growth factor (EGF) and other growth factors such as IGF-I and IGF-II were found. Stein-Oakley et al reported lower EGF levels in a rat model of chronic kidney rejection, detected by Northern blot and immunohistochemical analysis [32]. In humans, Jacob et al measured decreased low molecular

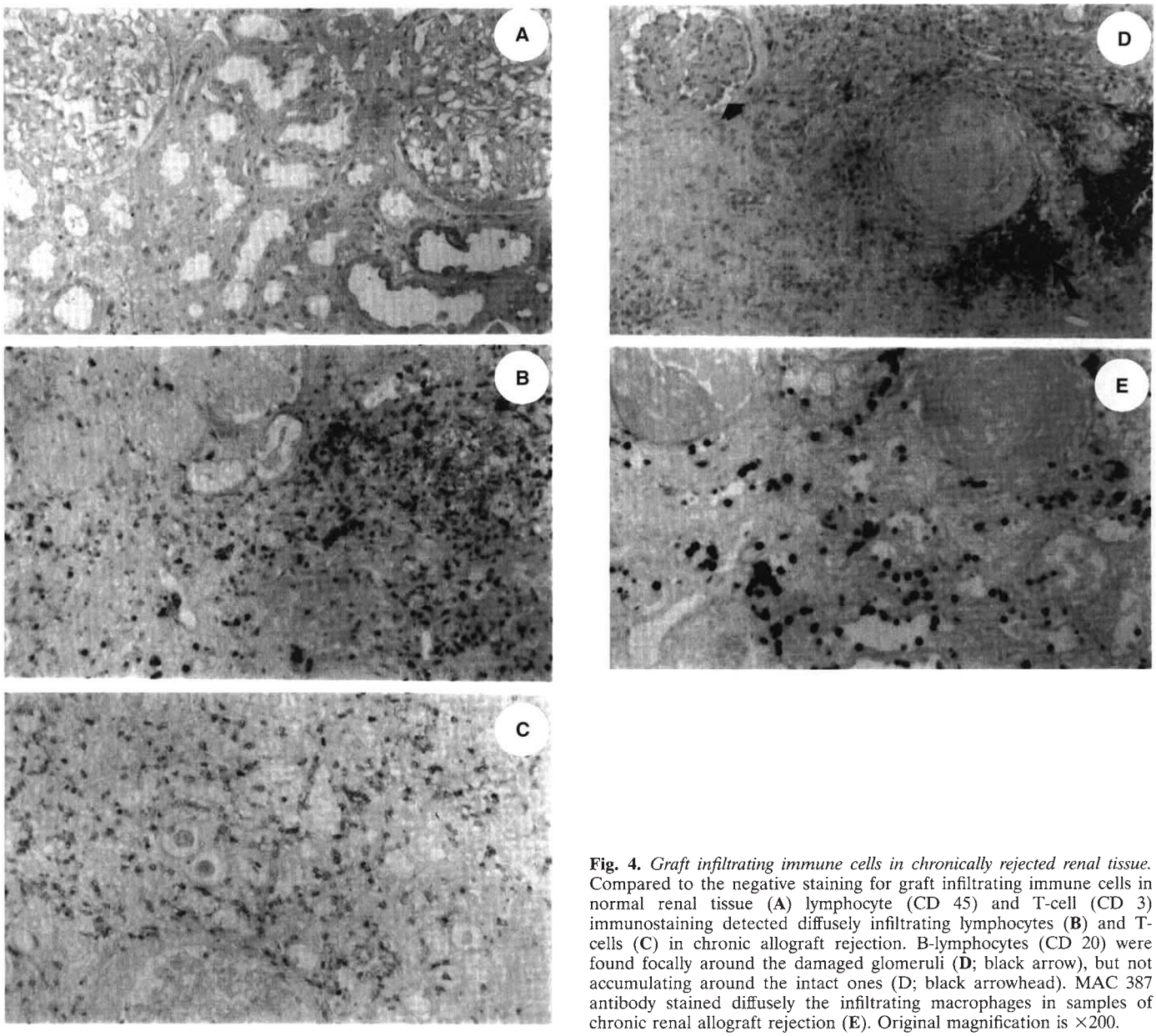

Fig. 4. *Graft infiltrating immune cells in chronically rejected renal tissue.* Compared to the negative staining for graft infiltrating immune cells in normal renal tissue (**A**) lymphocyte (CD 45) and T-cell (CD 3) immunostaining detected diffusely infiltrating lymphocytes (**B**) and T-cells (**C**) in chronic allograft rejection. B-lymphocytes (CD 20) were found focally around the damaged glomeruli (**D**; black arrow), but not accumulating around the intact ones (D; black arrowhead). MAC 387 antibody stained diffusely the infiltrating macrophages in samples of chronic renal allograft rejection (**E**). Original magnification is ×200.

weight growth factor levels (IGF-I, IGF-II) in serum ultrafiltrates with bioassay technique [33]. Shihab et al found an increase in TGF-β1 mRNA and protein in glomeruli of chronicly rejected human kidney allografts when needle bipsies were taken [34]. However, they did not examine the other TGF-β isoforms and their receptors and the concomitant localization of immune cells in kidney rejection in their studies.

The tendency for TGF-βs and TβRs mRNA levels to be decreased in the rejected kidneys was not statistically significant with the exception of TGF-β3 and TβR-II mRNA levels. This decreased expression paralleled the general cell and tissue destruction associated with chronic renal rejection, and was most likely due to the marked loss of tubular cells in the rejected samples. In contrast, Shihab et al found increased TGF-β1 mRNA (and protein) in the tissue specimens of chronicly rejected kidney allografts compared to normal. The difference between the two studies may be due to the presence of less necrosis in their samples which may have been derived from kidney with less severe disease [34]. In our study, the increase in TGF-β1 and TGF-β3 immunoreactivity was limited to the less abundant glomeruli and vascular structures. Thus, there was a redistribution of TGF-β1 and TGF-β3 expression in the rejected kidney from the tubular cells to the glomeruli. Since the glomerular epithelial and mesangial cells are most likely responsible for the extracellular matrix production in the kidney [47], our findings raise the possibility that enhanced expression of TGF-β1 and TGF-β3 within the glomeruli contribute to the fibrosis in chronically rejected renal allografts.

Immune cell staining (detected by markers) revealed the distribution pattern of lymphocytes (CD 45), T-cells (CD 3) and

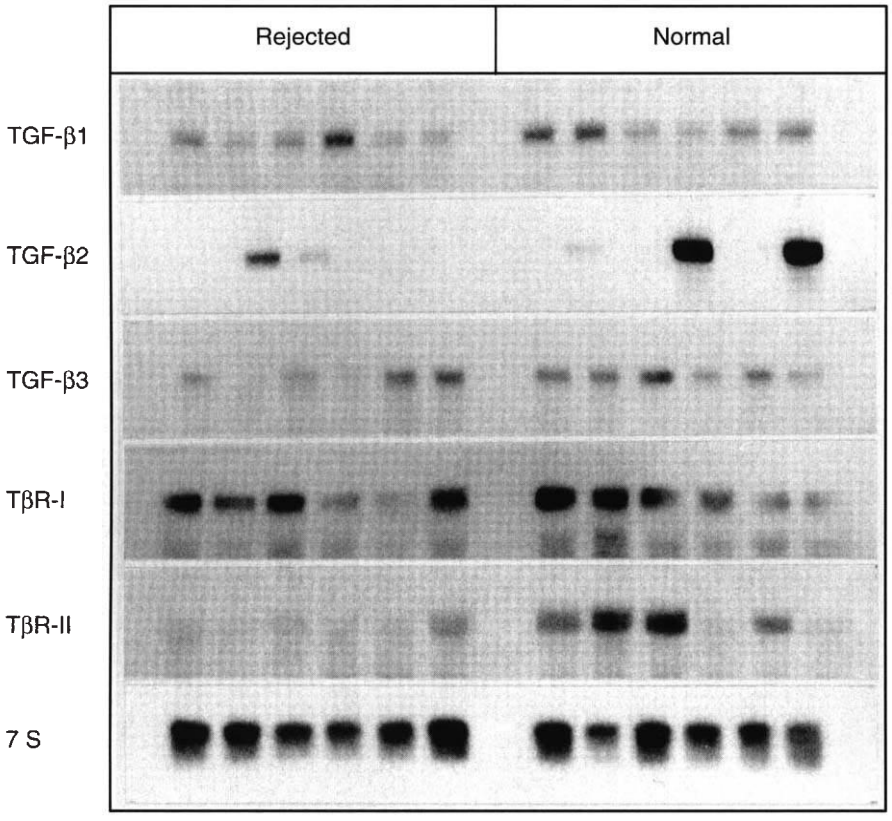

Fig. 5. *Northern blot analysis of TGF-β1, TGF-β2, TGF-β3 and TβR-I and TβR-II mRNA in 6 rejected and in 6 normal kidney samples.* The same samples were run in all blots in the same order. 7S RNA was used to verify equal RNA loading.

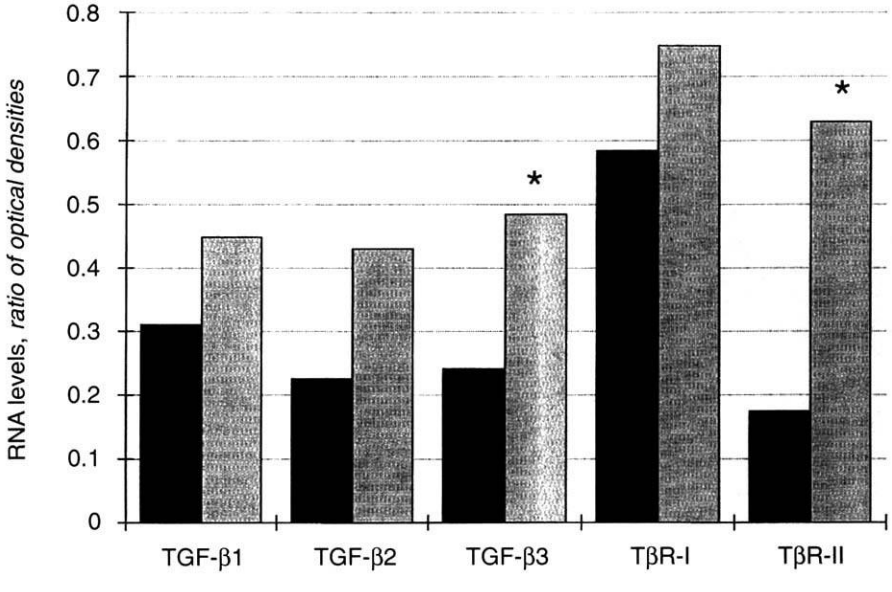

Fig. 6. *Densitometric analysis.* The ratio of optical density between TGF-β1, TGF-β2, TGF-β3, TβR-I, TβR-II and the corresponding 7S signals were calculated. Bars represent the mean values: (▨) rejected; (■) normal. $^*P <$ 0.02

macrophages (MAC-387) showed diffuse localization in the rejected kidney samples. Specific immunostaining for CD 20 detected B-lymphocytes accumulating focally around the glomeruli in the chronically rejected allografts, whereas in the normal tissues there was no immunoreactivity for TGF-βs in the glomerular structures, consistent with no evidence of B-cell infiltration.

Cell mediated immunity has been shown to be involved in host versus graft reaction of tissue rejection. Therefore, it is interesting that both TGF-β, a potent immunosuppressive agent, and B-cells co-localized to the glomeruli involved in apparent tissue destruction. This observation raises the possibility that TGF-β1 and TGF-β3 may also participate in the regulation of B-cell mediated immune responses involved in glomerular destruction. Further studies are necessary to evaluate this possibility.

Acknowledgments

The study was supported by the Swiss National Fonds SNF Grant 32-39529, by public Health Service Grants CA-40192 and DK-44948 (Murray Korc) and CA-49507 (Leslie I Gold) awarded by the National Institute of Health, USA. Part of this work was presented at the 23rd International Congress of Nephrology, Madrid, July, 1995. The authors thank Professor Urs Studer for his collaboration, and Ms. Véronique Gaschen and Mr. Kaspar Brönnimann for their technical assistance.

Reprint requests to Martin Schilling, M.D., Department of Visceral and Transplantation Surgery, University of Berne, Inselspital, CH-3010 Berne, Switzerland.

References

1. MASON D: The role of T cell subpopulation in allograft rejection. *Transplant Proc* 20:239–242, 1988
2. KEHR JH, WAKEFIELD LM, ROBERTS AB, JAKOWLEW S, ALVAREZ-MON M, DERYNCK R, SPORN MB, FAUCI AS: Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T-cell growth. *J Exp Med* 163:1037–1050, 1986
3. DUBOIS C, RUSCETTI F, PALASZYNSKI E, FALK L, OPPENHEIM J, KELLER J: Down modulation of the interleukin-1 receptor by transforming growth factor-β: Produced mechanism of inhibition of IL-1 action. *J Exp Med* 172:737, 1990
4. ROBERTS AB, SPORN MB: The transforming growth factor-βs, in Peptide growth factors and their receptors, in *Handbook of Experimental Pharmacology* (vol 95), edited by SPORN MB, ROBERTS AB, Heidelberg, Springer, 1990, pp 419–472
5. DERYNCK R, LINDQUIST PB, LEE A, WEN D, TAMM J, GRAYCAR JL, RHEE L, MASON AJ, MILLER DA, COFFEY RJ, MOSES HL, CHEN EY: A new type of transforming growth factor-β, TGF-β3. *EMBO J* 6:1633–1636, 1987
6. WAHL SM, MCCARTNEY-FRANCIS N, MERGENHAGEN SE: Inflammatory and immunomodulatory roles of TGF-β. (abstract) *Immunol Today* 10:258, 1989
7. KEHRL JH: Transforming growth factor-β: An important mediator of immunoregulation. *Int J Cell Cloning* 9:438, 1991
8. RUSCETTI FW, PALLADINO MA: Transforming growth factor-β and the immune system. *Prog Growth Factor Res* 3:159, 1991
9. FRIESS H, YAMANAKA Y, BÜCHLER M, EBERT M, BEGER HG, GOLD LI, KORC M: Enhanced expression of transforming growth factor-beta isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology* 105:1846–1856, 1993
10. FRIESS H, YAMANAKA Y, BÜCHLER M, BEGER HG, KOBRIN MS, BALDWIN RL, KORC M: Enhanced expression of the type II transforming growth factor-beta receptor in human pancreatic cancer cells without alteration of type III receptor expression. *Cancer Res* 53: 2704–2707, 1993
11. BORDER WA, NOBLE N: Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 331:1286–1292, 1994
12. BORDER WA, NOBLE NA, YAMAMOTO T, HARPER JR, YAMAGUCHI YU, PIERSCHBACHER MD, ROUSLAHTI E: Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. *Nature* 360:361–364, 1992
13. JAKOWLEW SB, DILLARD PJ, SPORN MB, ROBERTS AB: Complementary deoxyribonucleic acid encoding transforming growth factor-β4 from chicken embryo chondrocytes. *Mol Endocrinol* 2:1186–1195, 1988
14. KONDAIAH P, SANDS MJ, SMITH JM, FIELDS A, ROBERTS AB, SPORN MB, MELTON DA: Identification of a novel transforming growth factor-β (TGF-β5) mRNA in *Xenopus laevis*. *J Biol Chem* 265:1089–1093, 1990
15. LIN HY, WANG X-F, NG-EATON E, WEINBERG RA, LODISH HF: Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. *Cell* 68:775–785, 1992
16. WRANA JL, ATTISANO L, CARCAMO J, ZENTELLA A, DOODY J, LAIHO M, WANG X-F, MASSAGUE J: TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* 71:1003–1014, 1992
17. MASSAGUE J: The transforming growth factor-β family. *Annu Rev Cell Biol* 6:597–641, 1990
18. SPORN MB, ROBERTS AB: Transforming growth factor-β: Recent progress and new challenges. *J Cell Biol* 119:1017–1021, 1992
19. MASSAGUE J: Receptors for the TGF-beta family. *Cell* 69:1067–1070, 1992
20. LOPEZ-CASILLAS F, CHEIFETZ S, DOODY J, ANDRES JL, LANE WS, MASSAGUE J: Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-β receptor system. *Cell* 67:785–795, 1991
21. WANG X-F, LIN HY, NG-EATON E, DOWNWARD J, LODISH HF, WEINBERG RA: Expression cloning and characterization of the TGF-beta type III receptor. *Cell* 67:797–805, 1991
22. ZHAO XM, FRIST WH, YEOH TK, MILLER GG: Expression of cytokine genes in human cardiac allografts: Correlation of IL-6 and transforming growth factor-beta (TGF-β) with histological rejection. *Clin Exp Immunol* 93:448–451, 1993
23. ZHAO XM, YEOH TK, HIEBERT M, FRIST WH, MILLER GG: The expression of acidic fibroblast growth factor (heparin-binding growth factor-1) and cytokine genes in human cardiac allografts and T cells. *Transplantation* 56:1177–1182, 1993
24. LASS JH, MUSCH DC, GORDON JF, LAING RA: Epidermal growth factor and insulin use in corneal preservation. Results of a multicenter trial. *The Corneal Preservation Study Group Ophthalmology* 101:352–359, 1994
25. GILL RG: Transforming growth factor β prevents islet allograft rejection. *Transplant Proc* 23:747–748, 1991
26. CAREL JC, SHEEHAN KC, SCHREIBER RD, LACY PE: Prevention of rejection of transforming growth factor beta-treated rat-to-mouse islet xenografts by monoclonal antibody to tumor necrosis factor. *Transplantation* 55:456–458, 1993
27. BISHOP GA, MATSUMOTO GW, MCCAUGHAN, KENNEY JS: Identification of cytokine protein expression in human liver allograft rejection: Methods development and expression of IL-1β, IL-6, IFN-γ, and basic fibroblast growth factor. *Transplant Proc* 24:2310–2312, 1992
28. HÄYRY P, RÄISÄNEN A, USTINOV J, MENNANDER A, PAAVONEN T: Somatostatin analog Lanreotide inhibits myocyte replication and several growth factors in allograft arteriosclerosis. *FASEB J* 7:1055–1060, 1993
29. WALTENBERGER J, WANDERS A, FELLSTRÖM B, MIYAZONO K, HELDIN C-H, FUNA K: Induction of transforming growth factor-β during cardiac allograft rejection. *J Immunol* 151:1147–1157, 1993
30. HIGGY N, DAVIDOFF H, BENEDIKTSSON H, PAUL LC: Platelet-derived growth factor receptor expression in chronic rejection of cardiac and renal grafts in the rat. *Transplant Proc* 23:609–610, 1991
31. SHADDY RE, HANSEN JC, COWLEY CG: Effects of T cells on platelet-derived growth factor-like protein secretion from endothelial cells. *J Heart Lung Trans* 11:48–57, 1992
32. STEIN-OAKLEY AN, TZANIDIS A, FULLER PJ, JABLONSKI P, THOMPSON NM: Chronic renal allograft rejection: Altered distribution and reduced expression of epidermal growth factor in a rat model. *Transplant Proc* 25:2902–2903, 1993
33. JACOB C, MAACHI F, EL FARRICHA O, DOUSSET B, KESSLER M, BELLEVILLE F, NABET P: Role of the kidney in the expression of low molecular weight factors with growth factor activity. *Clin Nephrol* 39:327–334, 1993
34. SHIHAB FS, YAMAMOTO T, NAST CC, COHEN AH, NOBLE NA, GOLD LI, BORDER WA: Transforming growth factor-β and matrix protein expression in acute and chronic rejection of human renal allografts. *J Am Soc Nephrol* 6:286–294, 1995
35. YAMANAKA Y, FRIESS H, BÜCHLER M, BEGER HG, GOLD LI, KORC M: Synthesis and expression of transforming growth factor-beta1, beta2, and beta3 in the endocrine and exocrine pancreas. *Diabetes* 42:746–756, 1993
36. PELTON RW, SAXENA B, JONES M, MOSES HL, GOLD LI: Immunohistochemical localization of TGF-β1, TGF-β2, and TGF-β3 in the mouse embryo; Expression patterns suggest multiple roles during embryonic development. *J Cell Biol* 115:1091–1105, 1991
37. MCMULLEN H, LONGAKER MT, CABERA RC, SUNG JJ, CANETE J, SIEBERT JW, LORENZ HP, GOLD LI: Analysis of TGF-β1, TGF-β2, and TGF-β3 immunoreactivity during ovine wound repair. *Wound Repair Regen* 3:141–156, 1995
38. DERYNCK R, JARRETT JA, CHEN EY, EATON DH, BELL JR, ASSOIAN RK, ROBERTS AB, SPORN MB, GOEDDEL DV: Human transforming

growth factor-β complementary sequence and expression in normal and transformed cells. *Nature (Lond)* 316:701–705, 1985
39. ARRICK BA, KORC M, DERYNCK R: Differential regulation of expression of three transforming growth factor-β species in human breast cancer cell lines by estradiol. *Cancer Res* 50:299–303, 1990
40. KORC M, CHANDRASEKAR B, YAMANAKA Y, FRIESS H, BÜCHLER M, BEGER HG: Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant increase in the levels of epidermal growth factor and transforming growth factor alpha. *J Clin Invest* 90:1352–1360, 1992
41. BALDWIN RL, KORC M: Growth inhibition of human pancreatic carcinoma cells by transforming growth factor beta-1. *Growth Factors* 8:23–34, 1993
42. BALMAIN A, KRUMLAUF R, VASS JK, BIRNIE GD: Cloning and characterization of the abundant cytoplasmic 7S RNA from mouse cells. *Nucl Acids Res* 10:4259–4277, 1982
43. FEINBERG AP, VOGELSTEIN B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132:6–13, 1983
44. CHOMCZYNSKI P, SACCHI N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
45. KORC M, OWERBACH D, QUINTO C, RUTTER WJ: Pancreatic islet-acinar cell interaction: Amylase messenger RNA levels are determined by insulin. *Science* 213:351–353, 1981
46. SIEGEL S: *Nonparametric Statistics for Behavioral Sciences*. New York, McGraw-Hill Book Co., 1956
47. NAKAMURA T, MILLER D, ROUSHLAHTI E, BORDER WA: Production of extracellular matrix by glomerular epithelial cells is regulated by transforming growth factor-β1. *Kidney Int* 41:1213–1221, 1992
48. YAMAMOTO T, NOBLE NA, MILLER DE, BORDER WA: Sustained expression of TGF-beta 1 underlies development of progressive kidney fibrosis. *Kidney Int* 55:916–927, 1994
49. WALLICK SC, FIGARI IS, MORRIS RE, LEVINSON AD, PALLADINO MA: Immunoregulatory role of transforming growth factor-β (TGF-β) in development of killer cells: Comparison of active and latent TGF-β1. (abstract) *J Exp Med* 172:1777, 1990
50. BRANDES ME, ALLEN JB, OGAWA Y, WAHL SM: Transforming growth factor-β1 suppresses acute and chronic arthritis in experimental animals. (abstract) *J Clin Invest* 87:1108, 1991
51. JOHNS LD, FLANDERS KC, RANGES GE, SRIRAM S: Successful treatment of experimental allergic encephalomyelitis with transforming growth factor-β1. (abstract) *J Immunol* 147:1792, 1991