

Transforming Growth Factor- β and Matrix Protein Expression in Acute and Chronic Rejection of Human Renal Allografts^{1,2}

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ABSTRACT

Because the increased tissue expression of TGF- β underlies fibrosis in many diseases, it was hypothesized that sustained elevated transforming growth factor (TGF)- β overexpression might be responsible for fibrosis in chronic rejection of the renal allograft. To test this hypothesis, biopsies were obtained from 5 patients with acute rejection, 5 patients with chronic rejection, 10 normal individuals, and 10 patients with kidney disease. The tissues were examined by immunofluorescence for the three TGF- β isoforms (1, 2, and 3) and the two matrix proteins induced by TGF- β that serve as markers of fibrosis: fibronectin extracellular domain A positive (EDA+) and plasminogen activator inhibitor-1 (PAI-1). The tubulointerstitium from all cases of acute rejection and chronic rejection showed highly significant increases in immunostaining for the three TGF- β isoforms ($P < 0.001$), fibronectin EDA+ ($P < 0.005$), and PAI-1 ($P < 0.001$). In the glomeruli, only TGF- β 1 expression achieved statistical significance ($P < 0.005$) in acute rejection, whereas in chronic rejection, all three TGF- β isoforms ($p < 0.001$) in addition to fibronectin EDA+ ($p < 0.001$) and PAI-1 ($p < 0.001$) were elevated. There was both cellular and matrix staining of the TGF- β isoforms. In striking contrast, control kidney tissues were negative or only weakly positive. Because TGF- β was present both in acute and in chronic

rejection but not in control tissues and because acute rejection episodes are a good predictor for chronic rejection, these results suggest that TGF- β may play a role in the pathogenesis of fibrosis in chronic rejection.

Key Words: Transforming growth factor- β , fibronectin extracellular domain A positive, plasminogen activator inhibitor-1, acute rejection, chronic rejection

The incidence of chronic rejection as a cause of graft failure has changed little with time. It remains the single most important cause of graft failure after the initial year (1,2). Renal transplants with chronic rejection exhibit a gradual and progressive deterioration in function in association with proteinuria and hypertension (3). The histopathology of chronic rejection in the renal allograft is characterized by fibrosis involving graft arteries and arterioles, glomeruli, and interstitium (4). However, the pathogenesis of chronic rejection remains entirely unknown. Immunologic mechanisms have been implicated by the fact that acute rejection episodes, especially in the early posttransplant period, correlate with the development of chronic rejection (5). However, although immunosuppressive drugs are useful in reversing acute rejection, they are ineffective in chronic rejection (3). Cellular infiltrates and antibody and complement deposits are observed, not only in rejection, but also in well-functioning grafts (6,7), indicating that other nonimmunologic mechanisms are also operating (8). Independent of the initiating events, the end result is a histology dominated by fibrosis (9). The accumulation of excessive extracellular matrix (ECM) leading to tissue destruction and loss of function is also a central feature of chronic diseases of the kidney and other organs (10,11).

Cellular infiltrates are seen variably over the lifetime of a renal allograft (6,7). Presumably derived from this infiltrate, a number of cytokines are expressed (12). Transforming growth factor- β (TGF- β) is a cytokine that mediates a number of biologic processes including inflammation and host defense, in addition to development, tissue repair, and tumorigenesis (13,14). TGF- β also provides a link between each of the processes by which cells respond to injury and initiate repair. By increasing adhesion molecules, generating a potent chemotactic gradient, and inducing itself and an array of other factors, TGF- β orchestrates leukocyte recruitment and activation (15,16). TGF- β then down-regulates these processes by inhibiting the

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functions of inflammatory cells and facilitates healing by promoting fibroblast recruitment and ECM synthesis. TGF- β directly stimulates the synthesis of individual ECM components (17–20). It also blocks ECM degradation by decreasing the synthesis of proteases and stimulating protease inhibitors like plasminogen activator inhibitor-1 (PAI-1) (21–23). In addition to its effect on matrix accumulation, TGF- β also promotes immune suppression (13,24–26).

In mammals, the TGF- β family consists of three isoforms, TGF- β 1, TGF- β 2, and TGF- β 3, that are structurally and functionally closely related (27). The conservation of different forms of TGF- β may reflect differential regulation under the control of unique promoters. TGF- β is secreted as a high-molecular-weight latent precursor molecule that is cleaved at a dibasic amino acid site. The bioactive molecule consists of a 25-kd covalently linked dimer (112 amino acids for each dimer) that can be released from the C terminus of the latent form by acidification, heat, and certain proteases *in vitro*, although it is still unknown how latent TGF- β is activated *in vivo*.

Recently, two components of the ECM, tenascin and fibronectin extradomain A positive (EDA+), were shown to be preferentially expressed in the interstitial and vascular compartments of human biopsies with acute and chronic rejection (28). In another study, tenascin was found to be present in the glomeruli of human biopsies of a number of glomerular diseases, including acute and chronic rejection (29). In addition, TGF- β is up-regulated during cardiac allograft rejection in the rat (30) and its expression was shown to correlate with interleukin-6 (IL-6) in human cardiac allografts with acute rejection (31).

We hypothesized that TGF- β overexpression might be responsible for the fibrosis of chronic rejection. To test this hypothesis, we studied the expression and distribution of the TGF- β isoforms in renal allograft biopsies with the separate clinical and histologic diagnosis of acute and chronic rejection. We also studied the expression and localization of two ECM components directly induced by TGF- β , fibronectin EDA+, and PAI-1. We report an elevated expression of the TGF- β isoforms, and concomitantly of fibronectin EDA+ and PAI-1, in both the tubulointerstitium and the glomeruli in chronic rejection. Similar findings were observed in acute rejection, except for the TGF- β 2 and TGF- β 3 isoforms, which were up-regulated in the interstitium but not in the glomeruli. Our findings implicate TGF- β in the pathogenesis of the fibrotic lesion of chronic rejection.

MATERIALS AND METHODS

Materials

Kidney biopsy samples were obtained from patients undergoing diagnostic evaluation and examined at Cedars-Sinai Medical Center. All kidney biopsies were done to provide diagnostic and therapeutic guidance for unexplained deterioration in renal function after kidney transplantation. Renal allograft biopsy tissue from five patients with acute rejection

and five patients with chronic rejection were obtained. Control tissues consisted of biopsy specimens from 10 normal kidneys and 10 kidneys from patients with diseases that normally do not cause glomerulosclerosis (5 with minimal change disease and 5 with thin basement membrane disease). Normal renal allograft controls were not used because it was hard to justify performing a biopsy on an allograft with a normal function. The diagnosis was established in each case by the use of conventional clinical and pathologic criteria by physicians not involved in this study. At least 20 glomeruli were present in each sample. All transplant patients were receiving cyclosporine as part of their protocol immunosuppressive regimen. The recipients were all nondiabetic adults over 18 yr old at the time of transplantation, and all grafts were cadaveric kidneys harvested from adult donors. The histologic diagnosis of acute and chronic rejection was made on the basis of the Banff working classification of kidney transplant pathology (4). Acute allograft rejection was defined by variable combinations of tubulointerstitial, vascular, and glomerular pathology. Although an interstitial cellular infiltrate was universally seen, the presence of tubulitis was a requirement for the diagnosis of acute allograft rejection. The acute histologic lesions of cyclosporine nephrotoxicity were not seen in this material, and we did not include any cases of recurrent or *de novo* glomerulonephritis. Other possible causes of acute allograft dysfunction were excluded on a clinical basis. Chronic allograft rejection was diagnosed in the appropriate clinical setting when the biopsy also showed the characteristic histologic findings including arterial fibrous intimal thickening, interstitial fibrosis, and tubular atrophy. Several cases of transplant glomerulopathy were also included and were characterized by duplication of the glomerular basement membrane and mesangial interposition. The changes of chronic rejection were accompanied by variable cellular infiltrates.

Tissue Preparation

Tissue blocks were embedded in paraffin for light microscopy studies and in OCT-mounting medium for immunofluorescence studies. The tissue blocks were then snap frozen in precooled isopentane and stored at -70°C until use. Four-micrometer sections were obtained with a cryostat (Tissue-Tek-II; Miles Laboratories, Elkhart, IN). For light microscopy studies, paraffin sections were stained with periodic acid-methenamine silver. For immunofluorescence studies, the tissue sections were fixed in acetone, washed in phosphate-buffered saline (PBS; pH 7.4), sealed, and stored with plastic film at -70°C until use. The sections were immunostained and compared with the control kidney tissue. Negative controls for staining were provided by either omitting the primary antibody or by using instead normal rabbit or mouse immunoglobulins. Immunofluorescence examination was performed with an epifluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany).

Antibodies

The primary antibodies to the three isoforms of TGF- β (TGF- β 1, TGF- β 2, and TGF- β 3) were produced in the laboratory of Dr. L. Gold and have been previously biochemically characterized (32). Briefly, peptides of each of the TGF- β isoforms were synthesized with residues 4 to 19 for TGF- β 1 and TGF- β 2 and residues 9 to 20 for TGF- β 3. Rabbits were immunized with the purified peptides. The resulting antisera did not cross-react with the TGF- β peptides that were not used as immunogen. Each antiserum was purified by ammonium sulfate precipitation, followed by affinity chromatogra-

phy with the respective immunogenic peptide. Each antipeptide antiserum was tested for both immunoreactivity with the corresponding mature isoform of the TGF- β molecule and cross-reactivity between the TGF- β isoforms by western blot analysis.

Mouse monoclonal antibody to human fibronectin EDA+ was provided by Dr. L. Zardi (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy) (20). Mouse monoclonal antibody to human PAI-1 was obtained from American Diagnostica, Inc. (Greenwich, CT). Tissue sections were incubated at 37°C in a moist chamber with the primary antibodies to TGF- β 1 at a concentration of 40 μ g/mL, TGF- β 2 at a concentration of 87 μ g/mL, TGF- β 3 at a concentration of 48 μ g/mL, fibronectin EDA+ undiluted, and PAI-1 at a concentration of 5 μ g/mL. The tissue sections were then washed with PBS three times at room temperature.

Fluorescein isothiocyanate-conjugated donkey F(ab')₂ anti-rabbit immunoglobulin G (Jackson Immunoresearch, West Grove, PA) diluted 1:300 with PBS was used as the secondary detecting antibody for the TGF- β isoforms, and fluorescein isothiocyanate-conjugated rat F(ab')₂ anti-mouse immunoglobulin G (Jackson Immunoresearch) diluted 1:30 with PBS was used as secondary antibody for the detection of fibronectin EDA+ and PAI-1. The slides were incubated for 60 to 90 min at 37°C in a moist chamber and washed three times in PBS at room temperature. The slides were then mounted with a cover glass with Bartels buffered glycerol mounting medium FA (Baxter, McGraw Park, IL).

Scoring of Tissue Sections

The intensity of glomerular matrix staining for TGF- β 1, TGF- β 2, TGF- β 3, fibronectin EDA+, and PAI-1 was evaluated semiquantitatively by scoring 20 randomly selected glomeruli per sample according to the following 0 to 4 scale in coded sections observed at \times 400 magnification: Grade 0 (negative) to Grade 4. The severity of tubulointerstitial matrix deposition was evaluated by scoring 20 randomly selected cortical areas per sample according to the following 0 to 4 scale in coded sections observed at \times 250 magnification: 0 = normal, 1 = deposition that involves less than 10% of the cortical area, 2 = involving 10 to 30%, 3 = involving 30 to 50%, and 4 = involving more than 50%. The photographs were taken under identical conditions with equal exposure times of 60 s. The mean value per biopsy was calculated.

Statistical Analysis

All values were expressed as mean \pm SD. Differences between groups in the immunofluorescence scoring of TGF- β and matrix components were analyzed by an unpaired *t* test.

RESULTS

Light Microscopy

Representative photomicrographs of control normal tissue, acute allograft rejection, and chronic allograft rejection are shown in Figure 1. In the normal control tissue, the glomeruli, tubules, interstitium, and arterioles were without morphologic abnormalities. In acute allograft rejection, the interstitium was diffusely edematous and infiltrated by numerous lymphocytes, some of which were in the walls of the tubules. There was swelling of the arterial endothelial cells. In chronic allograft rejection, there was arterial intimal fibrosis with luminal narrowing. The glomerular me-

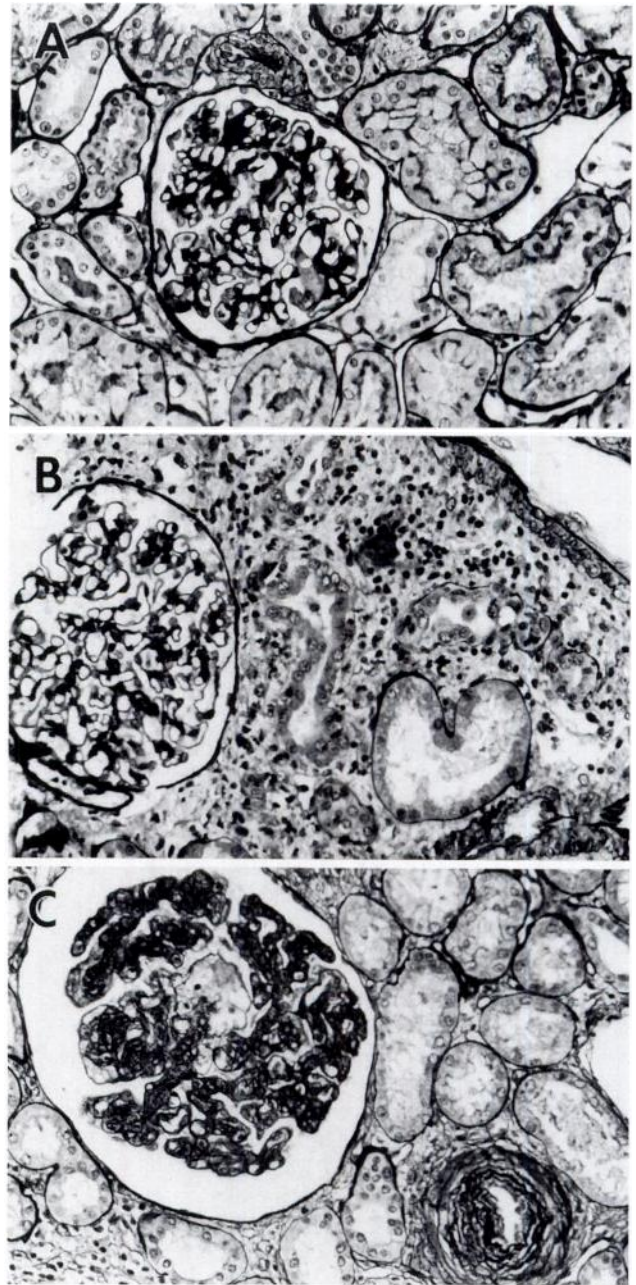


Figure 1. Photographs of renal tissue stained with periodic acid-methenamine silver. Sections from control normal kidney (A), acute renal allograft rejection (B), and chronic renal allograft rejection (C) are shown. Original magnification, \times 250.

sangium was widened with increased matrix. Many capillary walls were thickened, and some had double contours. Tubular atrophy and interstitial fibrosis were quite prevalent.

Immunofluorescence Studies: TGF- β Expression

Control Tissue. Representative immunofluorescence stainings for the three TGF- β isoforms (TGF- β 1,

TGF- β 2, and TGF- β 3) are shown in Figure 2, and quantitation of the staining is shown in Figure 3. The immunostaining in the 10 normal kidneys was similar to the staining observed in the 10 disease control kidneys. Staining was negative, or at most weakly positive, in the glomerular, interstitial, and vascular compartments. Infrequently, weak reactivity was noted around some Bowman capsules and in the immediate peritubular stroma.

Acute Allograft Rejection. The tubulointerstitium from all cases of acute rejection showed highly significant immunostaining for the three isoforms of TGF- β

($P < 0.001$). On the other hand, in the glomeruli, only TGF- β 1 immunostaining achieved statistical significance ($P < 0.005$). These findings correlate with the histology of acute rejection characterized predominantly by a heavy interstitial cellular infiltrate with usually little glomerular inflammation. There was evidence of both cellular and matrix immunostaining by the three isoforms of TGF- β .

Chronic Allograft Rejection. Glomerular immunostaining of all three isoforms of TGF- β achieved statistical significance ($P < 0.001$), as compared with only TGF- β 1 in acute rejection. Also, the tubulointersti-

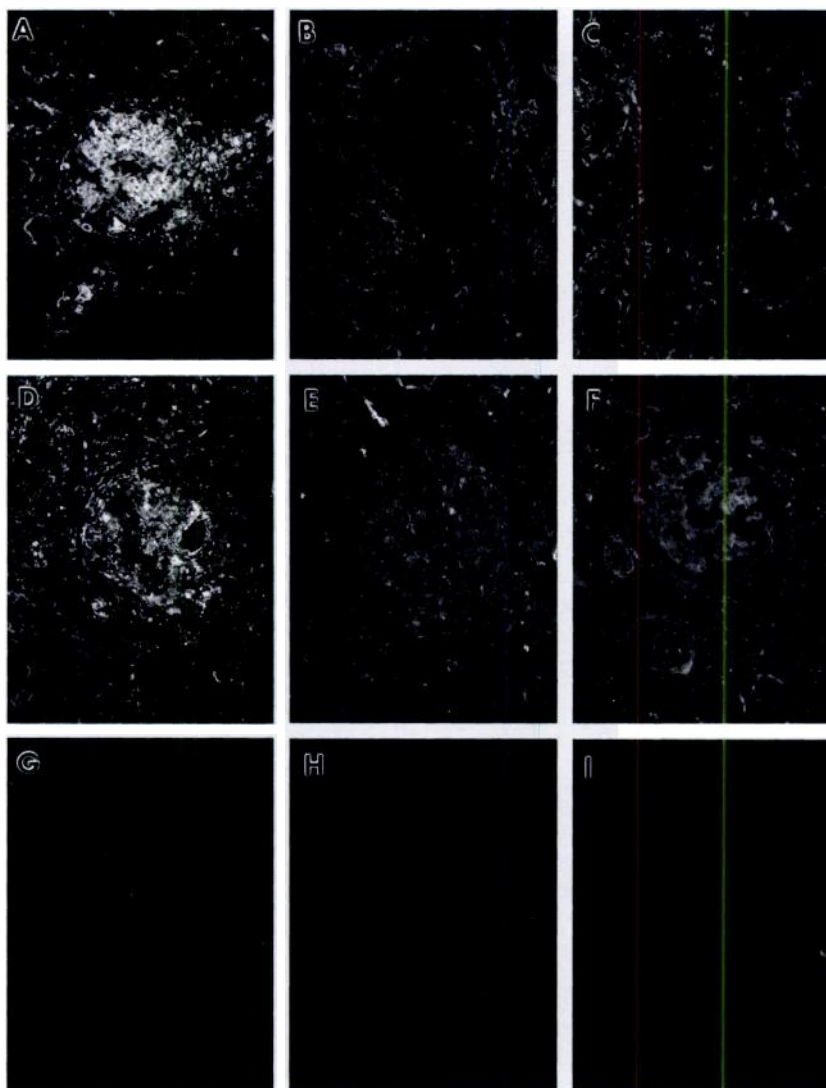


Figure 2. Immunofluorescence micrographs of the expression of the three isoforms of TGF- β in acute and chronic rejection and control kidneys (normal and disease control kidneys). Tissues from patients with acute rejection (A to C), chronic rejection (D to F), and control (G to I) were stained with antibodies to TGF- β 1 (A, D, and G), TGF- β 2 (B, E, and H), and TGF- β 3 (C, F, and I). In the glomeruli obtained from patients with acute rejection, as compared with control, only TGF- β 1 was increased, whereas in the tubulointerstitium, all three isoforms of TGF- β were increased. As compared with the control, all three isoforms of TGF- β were increased in both the glomeruli and the tubulointerstitium obtained from patients with chronic rejection. The photographs were taken under identical conditions with equal exposure times of 60 s. Original magnification, $\times 250$.

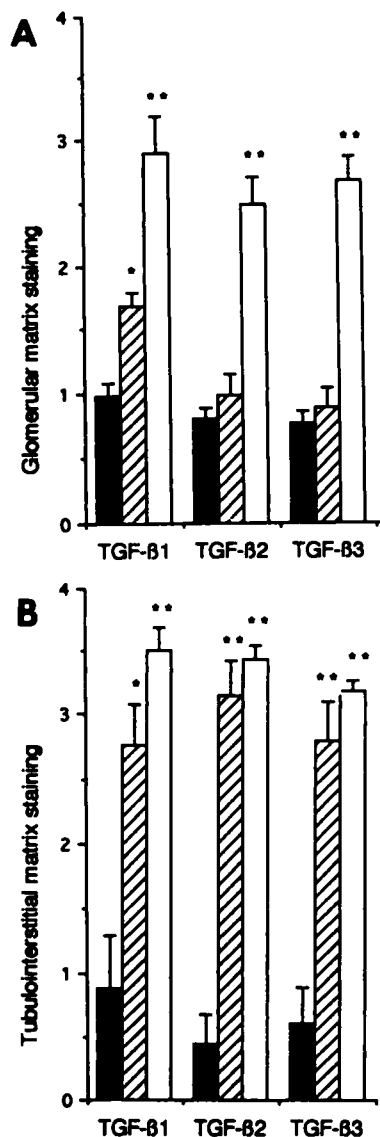


Figure 3. Quantitation ECM deposition for the three TGF- β isoforms. (A) Glomerular staining and (B) tubulointerstitial staining scores for (dark bars) control (normal and disease control kidney), (hatched bars) acute rejection, and (clear bars) chronic rejection. * $P < 0.005$; ** $P < 0.001$ as compared with control tissue. Values are mean \pm SD.

tium from all cases of chronic rejection showed highly significant immunostaining for all three isoforms of TGF- β ($P < 0.001$) and was more intense in chronic rejection as compared with acute rejection. These results are expected because fibrosis is almost universally presented in both the interstitium and the glomeruli of renal allografts with chronic rejection. The pattern of immunofluorescence staining for the three isoforms of TGF- β was also noted to be particularly intense in the vascular compartment, which is consistent with the characteristic histology of fibrous intimal thickening observed in chronic rejection.

Fibronectin EDA+ Expression

Control Tissue. Similar to the observation with TGF- β , the immunostaining in the 10 normal kidneys was similar to the staining observed in the 10 disease control kidneys and was negative or weakly positive in all of the renal tissue compartments.

Acute Allograft Rejection. The expression of fibronectin EDA+ in acute rejection was highly significantly increased in the tubulointerstitium ($P < 0.005$) but not in the glomeruli. These findings correlate with the characteristic histology of acute rejection in renal allografts and mirror the expression of TGF- β in this study, suggesting that TGF- β is inducing increased fibronectin EDA+ deposition.

Chronic Allograft Rejection. In contrast to acute allograft rejection, fibronectin EDA+ expression was highly significantly elevated not only in the tubulointerstitium ($P < 0.005$) but also in the glomeruli ($P < 0.001$). The pattern of immunofluorescence staining for fibronectin EDA+ in renal allograft tissues with chronic rejection was also noted to be intense in the vascular compartment in a pattern similar to that of the three isoforms of TGF- β , also suggesting that TGF- β is inducing matrix deposition. In addition, fibronectin EDA+ immunostaining was relatively more intense in chronic rejection as compared with that in acute rejection. Representative photomicrographs are shown in Figures 4A to C, and quantitation of the staining is shown in Figure 4D.

PAI-1 Expression

Control Tissue. Similar to the observations above, the immunoreactivity in the normal kidneys and control disease kidneys were similar and were negative or only weakly positive.

Acute Allograft Rejection. The expression of PAI-1 in acute rejection was highly significantly increased in the tubulointerstitium ($P < 0.001$) but not in the glomeruli. Thus, these findings are identical to our results for the TGF- β isoforms and fibronectin EDA+.

Chronic Allograft Rejection. Renal allografts with chronic rejection revealed PAI-1 expression to be highly significantly elevated not only in the tubulointerstitium ($P < 0.001$) but also in the glomeruli ($P < 0.001$). Additionally, the pattern of immunofluorescence staining for PAI-1 in the renal allograft tissue with chronic rejection was also noted to be intense in the vascular compartment in a manner similar to that of the three isoforms of TGF- β and fibronectin EDA+. Similar to the TGF- β isoforms and fibronectin EDA+, the immunostaining for PAI-1 was more intense in the setting of chronic rejection as compared with acute rejection. Representative photomicrographs are shown in Figure 5A to C, and quantitation of the staining is shown in Figure 5D.

DISCUSSION

The findings presented here correlate with the characteristic histology of acute and chronic rejection.

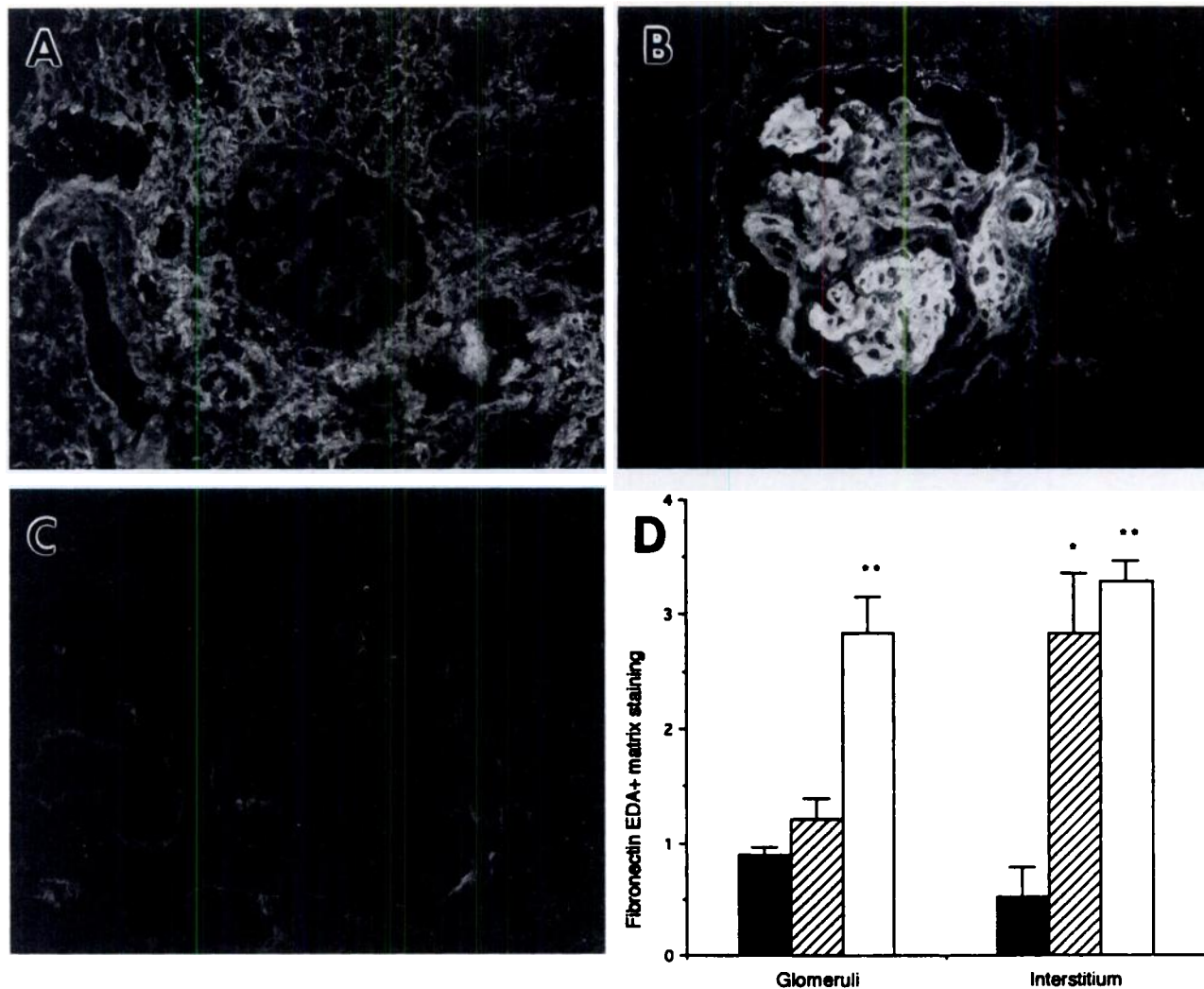


Figure 4. Immunofluorescence micrographs and quantitation of ECM deposition for fibronectin EDA⁺. Tissues from patients with acute rejection (A) and chronic rejection (B) were stained with an antibody to fibronectin EDA⁺. As compared with control (C), fibronectin EDA⁺ was increased in the tubulointerstitium of acutely rejecting allografts but not in the glomeruli. In chronic rejection, there was an increase in the expression of fibronectin EDA⁺ in both the glomeruli and the tubulointerstitium. The photographs were taken under identical conditions with equal exposure times of 60 s. Original magnification, $\times 250$. Glomerular and tubulointerstitial staining scores (D) for (dark bars) control (normal and disease control kidney), (hatched bars) acute rejection, and (clear bars) chronic rejection are shown. * $P < 0.005$; ** $P < 0.001$ as compared with control tissue. Values are mean \pm SD.

Acute rejection is characterized by a heavy interstitial cellular infiltrate and tubulitis. The glomerulus is not involved to the same extent the interstitium is affected. Thus, it is not surprising to find increased expression of all TGF- β isoforms in the tubulointerstitium, but not in the glomeruli, of renal allografts with acute rejection. It is probable that TGF- β is produced by this inflammatory infiltrate. Because the TGF- β induction of matrix synthesis is a key factor in tissue repair after injury, the expression of TGF- β may represent an attempt by the graft and the host for tissue healing after inflammation. Because of its chemotactic nature, the presence of TGF- β may also contribute to

the recruitment of the inflammatory components of tissue repair (33).

In chronic rejection, the three TGF- β isoforms were increased not only in the tubulointerstitium but also in the glomeruli. This is in accordance with the pathology of chronic rejection characterized by graft fibrosis in both the glomeruli and the tubulointerstitium. All of our patients were receiving cyclosporine as part of their immunosuppressive drug regimen. Cyclosporine can not only influence the character of the cellular infiltrate but can also cause interstitial fibrosis, which recent data have suggested may be mediated by TGF- β (34,35).

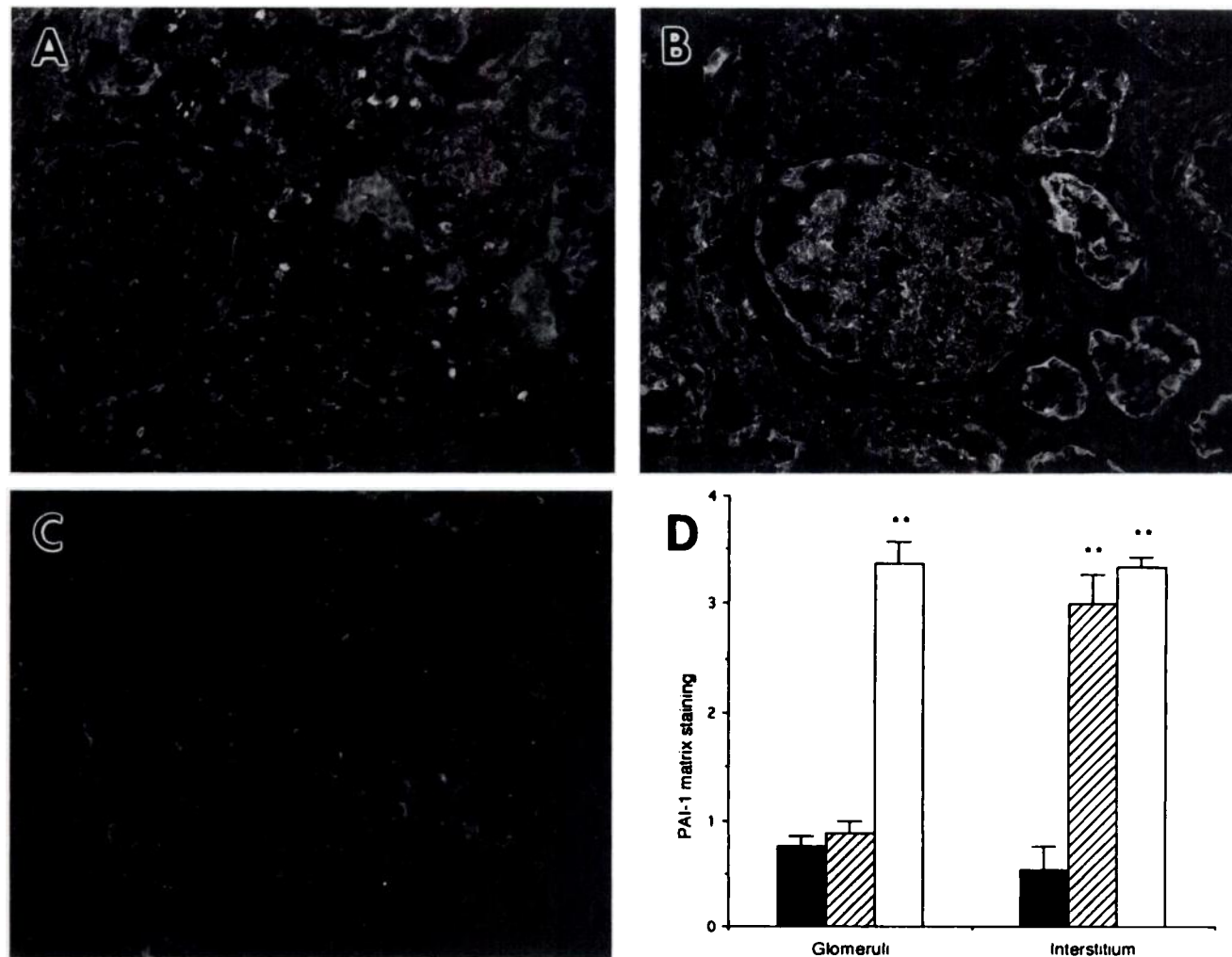


Figure 5. Immunofluorescence micrographs and quantitation of ECM deposition for PAI-1. Tissues from patients with acute rejection (A) and chronic rejection (B) were stained with an antibody to PAI-1. As compared with control (C), PAI-1 was increased in the tubulointerstitium of allografts with acute rejection but not in the glomeruli. In chronic rejection, there was an increase in the expression of PAI-1 in both the glomeruli and the tubulointerstitium. The photographs were taken under identical conditions with equal exposure times of 60 s. Original magnification, $\times 250$. Glomerular and tubulointerstitial staining scores (D) for (dark bars) control (normal and disease control kidney), (hatched bars) acute rejection, and (clear bars) chronic rejection are shown. $**P < 0.001$ as compared with control tissue. Values are mean \pm SD.

TGF- β is known to be produced by a variety of cells including macrophages. Previous studies have indicated that TGF- $\beta 1$ is produced by macrophages and alveolar epithelial cells in pulmonary fibrosis (33) and by resident glomerular cells and macrophages in chronic glomerulonephritis (36–38). In an animal model of chronic glomerulonephritis, glomerular TGF- $\beta 1$ remained elevated in association with glomerulosclerosis and tubulointerstitial fibrosis (36), suggesting that sustained TGF- $\beta 1$ expression contributed to the development of progressive kidney fibrosis.

TGF- β is a known chemoattractant for monocytes (16) and fibroblasts (39). In addition, it is known to induce its own production (40) and its persistent expression after repeated injuries can lead to a cycle of

continued TGF- β production (11). In this study, the acute rejection tissues demonstrated an up-regulated expression of all three TGF- β isoforms in the tubulointerstitium, whereas only the TGF- $\beta 1$ isoform was elevated in the glomeruli.

Clinically, acute rejection episode(s) correlate positively with the development of chronic rejection in the kidney allograft (5). Because we have shown elevated TGF- β expression in both acute and chronic rejection, it is possible that acute rejection episode(s) may be responsible for initiating a cycle of continued TGF- β production and resultant ECM production and deposition, thus resulting in the development of the lesion of chronic rejection characterized by excessive fibrosis. Our data suggest that increased expression of the

three TGF- β isoforms underlies the increased synthesis and deposition of the ECM.

Other cytokines are also implicated in tissue remodeling such as platelet-derived growth factor (PDGF), basic fibroblast growth factor, tumor necrosis factor, and IL-1. Studies using *in vivo* gene transfection, gene "knockout," and cytokine administration have shown that each cytokine has a characteristic role in tissue repair (25,26,41,42). PDGF stimulates cell proliferation and migration; fibroblast growth factor induces angiogenesis; and tumor necrosis factor and IL-1 promote inflammation, cell migration, and proliferation. TGF- β is unique in its widespread actions enhancing ECM deposition, and it also regulates other cytokines. There are several lines of evidence that directly link TGF- β to the initiation and termination of tissue repair, and the sustained production of TGF- β underlies the development of tissue fibrosis independently from other cytokines (14).

In transplantation, the PDGF receptor is up-regulated in the renal and cardiac allograft with chronic rejection (43). In addition, TGF- β was shown to be elevated in cardiac allografts with acute rejection (30,31). However, no previous report has provided evidence for the direct involvement of a cytokine in the deposition of ECM in the allograft. Because of the similarities between matrix accumulation in chronic rejection and matrix accumulation in tissue repair, where the role of TGF- β is well established, we believe that it is highly likely that TGF- β plays a causative role in ECM accumulation in the acute and chronic rejection of kidney allografts. It is unknown whether blocking TGF- β expression with specific antagonists can ameliorate the fibrotic lesion of chronic rejection. Future studies in animal models may help elucidate this important question.

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