Endothelin-Mediated Oncofetal Fibronectin Expression in Chronic Allograft Nephropathy

Iram Siddiqui,[1] Zia A. Khan,[1,2] Dameng Lian,[3] Jifu Jiang,[3] Robert Zhong,[1,3] Bertha Garcia,[1] and Subrata Chakrabarti[1,4]

Background. Chronic allograft nephropathy is a sclerotic process characterized by an increased extracellular matrix (ECM) protein deposition. Fibronectin (FN) is a major component of ECM. FN has been reported to undergo alternative splicing and produce several isoforms including the extra domain-B (ED-B) containing embryonic isoform. In the present study, we investigated ED-B$^+$ FN expression in chronic allograft nephropathy and its relationship with endothelins (ET).

Methods. To establish chronic allograft nephropathy, allografts were performed between Fisher 344 → Lewis rats. Allograft recipients were then randomly divided into two groups, allografts and allografts treated with ET receptor antagonist bosentan. Lewis → Lewis recipients were used as isograft controls. Grafts were harvested at 30, 90 and 140 days for histological and gene expression analyses with respect to ED-B$^+$ FN, ET-1 and transforming growth factor-β1 (TGF-β1) mRNA. ED-B$^+$ FN protein levels were assessed by immunohistochemical analysis. Additionally, we analyzed human renal allograft biopsies.

Results. Our data demonstrates that rat chronic allograft nephropathy is associated with progressive upregulation of ED-B$^+$ FN mRNA and protein. ET-1 and TGF-β1 mRNA were also upregulated. Treatment of allograft recipient rats with bosentan prevented upregulation of ED-B$^+$ FN and TGF-β1. We further show that total FN, ED-B$^+$ FN, ET-1 and TGF-β1 mRNA expression were upregulated in human chronic allograft nephropathy specimens.

Conclusion. Results obtained from both human and rat renal allograft tissues suggest that expression of ED-B$^+$ FN is upregulated in chronic allograft nephropathy and such upregulation is mediated via ET-1 and its interaction with TGF-β1.

Keywords: Fibronectin, Chronic rejection, Endothelins, Fibrosis.

(*Transplantation* 2006;82: 406–414)

Long-term graft dysfunction is recognized as the predominant cause of irreversible posttransplantation graft loss. Incidentally, 30–50% chronic allograft nephropathy occurs five years after transplantation (*1*, *2*). Alloreactivity of host against the graft appears to be an important factor in the etiopathogenesis of chronic allograft nephropathy (*3*). However, alloantigen-independent factors are also associated with the pathogenesis of chronic transplant rejection (*4*). Irrespective of the etiology, replacement of the tissue with fibrosis and subsequent graft dysfunction is the eventual outcome. Both experimental and clinical evidence indicate that extracellular matrix (ECM) proteins, vasoactive peptides and cytokines play a major role in causing architectural remodeling in chronic allograft nephropathy (*5–8*).

From the perspective of organ rejection, allogenic solid organs may undergo three different pathological entities: hyperacute, acute, and chronic rejection. Testing of potential transplant recipients for circulating anti-HLA antibodies prior to transplantation has minimized the risk of hyperacute rejection. Similarly, the use of certain immunosuppressive agents, such as cyclosporine A (CsA), tacrolimus and rapamycin, effectively prevents acute rejection (*3*). However, long-term graft survival has not been consistently achieved in renal transplant recipients. Obliterative vasculopathy (*9*, *10*) leading to the development of glomerulosclerosis and interstitial fibrosis is a characteristic feature of chronic renal rejection now referred to as chronic allograft nephropathy. This phenomenon is thought to be initiated by graft vascular injury by both immunological and nonimmunological factors. Histologically, chronic allograft nephropathy is a sclerotic process characterized by progressive deposition of the ECM proteins throughout the renal architecture.

Part of this study was presented at the 93rd Annual Meeting of the United States and Canadian Academy of Pathology, Vancouver, BC, Canada, March 6 to 12, 2004.

[1] Department of Pathology, University of Western Ontario, London, Ontario, Canada.

[2] Vascular Biology Research Program and Department of Surgery, Children's Hospital Boston, Harvard Medical School, Boston, MA.

[3] Department of Surgery, University of Western Ontario, London, Ontario, Canada.

[4] Address correspondence to: Subrata Chakrabarti, M.D., Ph.D., Department of Pathology, 4011 Dental Sciences Building, University of Western Ontario, London, Ontario, Canada N6A 5C1.

E-mail: subrata.chakrabarti@schulich.uwo.ca

Received 12 December 2005. Revision requested 24 March 2006.

Accepted 24 March 2006.

ISSN 0041-1337/06/8203-406

DOI: 10.1097/01.tp.0000228905.44649.06

One of the most specialized proteins in the ECM, fibronectin (FN), may regulate various cellular and extracellular events and may play a key role in the host immune cascade triggered by organ transplantation (*5*). Interestingly, FN molecules are shown to be different in normal tissues as compared to pathological tissues. FN molecules undergo alternative splicing to produce embryonic isoforms triggered by various pathological conditions (*11*). Premessenger RNA of FN, encoded by a single gene with 50 exons, is processed by alternative splicing. Three sites for premRNA processing have been identified: extra domain A (ED-A), extra domain B (ED-B), and type three homology segment (IIICS). FN molecules containing the ED-B domain, also known as oncofetal FN, has been shown to be almost exclusively expressed during embryogenesis, in some disease processes and tumorigenesis (*12–14*). Normally in adult tissues, it is only expressed in the blood vessels of the functional layer of the endometrium (*15*). FN has been implicated in a number of vascular disorders including diabetic complications and cancer (*12*, *16*). We have demonstrated upregulation of ED-B$^+$ FN via endothelin-1 (ET-1) and transforming growth factor-β1 (TGF-β1) in several organs exhibiting chronic diabetic complications which are manifested as sclerotic process similar to chronic rejection (*16*, *17*). We and others have also shown increased expression of ED-B$^+$ FN in various malignancies (*13*, *18*). ED-B$^+$ FN has also been reported to regulate vascular endothelial proliferation (*19*). Hence, it is plausible that chronic allograft nephropathy, essentially a vascular disorder, is associated with the preferential ED-B$^+$ FN expression. The possible mechanism that may underlie increased FN and ED-B$^+$ FN expression may be the alteration of fibrogenic proteins. We have previously shown significant upregulation of potent fibrogenic proteins, ET-1 and ET-3, in rat chronic renal allograft rejection (*6*). Furthermore ET-inhibition prevented chronic rejection in these renal allografts which suggests a potential role of ETs in the pathogenesis of chronic allograft nephropathy (*6*). This postulation was further supported by others demonstrating ET-inhibition prevented chronic rejection in rat cardiac allografts (*20*).

Our study was, therefore, designed to investigate the expression of ED-B$^+$ oncofetal FN in chronic allograft nephropathy. We have also investigated the mechanistic basis of ED-B$^+$ oncofetal FN expression by ET-1 and the possible involvement of TGF-β1.

MATERIALS AND METHODS

Animal Model of Chronic Allograft Nephropathy

We used a well characterized model of chronic allograft nephropathy of Fisher 344-to-LEWIS rats (Jackson laboratories, Bar Harbor, Maine USA) (*6*, *21*, *22*). The animals were housed at the Animal Care Facility of University of Western Ontario, London, Ontario, in accordance with guidelines by the Canadian Council on Animal Care (1984). Age matched, male rats weighing 250–300 g, were used for the surgeries. Three groups of animals were included in this study: a) isografts (LEWIS to LEWIS; n=12), b) allografts (FISHER 344 to LEWIS; n=12), and c) allografts treated with ET antagonist bosentan (Ro 47-0203, 100 mg/kg/day; n=12). Bosentan, an orally active dual ET receptor antagonist, was kindly provided by Dr. Martine Clozel, Actelion Pharmaceuticals Ltd., Allschwil, Switzerland (*23*). To investigate the temporal alterations in the expression of ED-B$^+$ oncofetal FN mRNA and protein and the interacting peptides, ET-1 and TGF-β1, we sacrificed animals at three time points, 30, 90, and 140 days (n=4/group).

Transplantation

Orthotopic renal transplantation was used as the surgical model with end-to-end anastomosis between donor and recipient vessels and ureter. All procedures were performed in accordance with the guidelines of Standard Operating Procedures of University of Western Ontario. London, Ontario. All Lewis recipients received CsA (1.5 mg/kg/day s.c.) for 10 days posttransplantation to prevent acute rejection. One group of allograft recipients was treated with bosentan by daily gavage. As noted previously (*24*), there are no known side effects of the daily gavage. The dose is based on multiple previous studies (*6*, *25*, *26*) and shows no nephrotoxicity.

Animal Monitoring and Tissue Collection

Ninety percent of the animals survived till the end point. Two animals died earlier and were excluded from the study. Minimum dose of CsA (1.5 mg/kg/d for 10 days) successfully prevented the development of acute rejection and early graft loss in Lewis recipients. Chronic allograft nephropathy was assessed by both functional as well as histological assessments. Each transplanted kidney cortex was snap-frozen in liquid nitrogen and then stored at −80°C. The remaining tissues were divided and fixed in 10% neutral buffered formalin and then paraffin embedded.

Functional Analysis

Urine collected at the time of sacrifice was used for assessment of urinary albumin (Nephrat, Exocell, Philadelphia, PA) and creatinine levels (Creatinine Companion, Exocell).

Blood was collected by intracardiac route and the serum layer was separated and stored at −80°C. Serum creatinine levels were measured using a commercial kit following the instructions provided by the manufacturer (Sigma Chemical Company, St. Louis, MO).

Human Biopsy Samples

Human renal allograft biopsy specimen were obtained from the London Health Sciences Center, London, Ontario, after approval by the Ethical Committee at University of Western Ontario in accordance with the guidelines of the Declaration of Helsinki for research involving human tissues. Samples were divided into two groups: one group comprised of samples from established cases of chronic allograft nephropathy (n=7), second group consisting of allografts with no evidence of chronic allograft nephropathy (n=8), and nontransplant renal biopsy specimens from patients with renal disease and no evidence of fibrosis (n=2). Detailed clinical information with respect to the renal biopsies included in this study are outlined in Table 1. The samples were subjected to RNA isolation and real-time quantitative RT-PCR for ED-B$^+$ oncofetal FN mRNA levels. ET-1 and TGF-β1 mRNA expression were also quantified.

TABLE 1. Clinical information of human renal allografts

Patients	Age	Sex	Time after transplantation	Original disease	Immunosuppressive regimen	Acute rejection episode	Prior episode of infection
With CAN							
1	40	Male	5 yr	IgA nephropathy	Prednisone/FK/MMF	None	None
2	72	Male	5 yr	IgA nephropathy	CsA/Prednisone/FK/MMF	None	None
3	63	Female	12 yr	Analgesic nephropathy	CsA/Prednisone/FK/MMF	None	CMV-Herpes zoster
4	25	Male	2 yr	Failed kidney transplant	CsA/Prednisone/FK/MMF	None	None
5	30	Female	14 yr	ESRD/Reflux nephropathy	Prednisone/FK/MMF	None	None
6	43	Male	6 yr	Alport's syndrome	Prednisone/FK/MMF	None	None
7	22	Female	4 yr	Reflux nephropathy	Prednisone/FK/MMF	None	None
Without CAN							
1	51	Male	1 mo	Liver Tx-CsA toxicity/ESRD	CsA/Prednisone/FK/MMF	None	None
2	10	Male	3 mo	Obstructive uropathy	Prednisone/FK/MMF	None	None
3	58	Female	3 yr	Polycystic kidneys	Prednisone/FK/MMF	None	None
4	47	Female	2 yr	Reflux nephropathy	Prednisone/FK/MMF	None	None
5	71	Male	1 mo	Focal glomerulosclerosis	Prednisone/FK/MMF	None	None
6	41	Male	2 yr	Diabetes-IDDM/ESRD	Prednisone/FK/MMF	None	None
7	71	Male	3 mo	Hypertensive nephrosclerosis	Prednisone/FK/MMF	None	None
8	74	Female	3 mo	Hepatitis C cirrhosis	CsA/Prednisone/FK/MMF	None	None
9	18	Male	Nontransplant	Hematuria			
10	75	Male	Nontransplant	Renal failure			

CAN, chronic allograft nephropathy; CsA, cyclosporine A; FK, tacrolimus; MMF, mycophenolate mofetil; ESRD, end-stage renal disease; IgA, immunoglobulin A; Tx, transplant; CMV, cytomegalovirus.

Histological Analysis

All paraffin embedded tissues were cut into 5-μm thick consecutive sections and were subsequently stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS), and Mason's trichrome. We determined interstitial fibrosis, tubular atrophy, glomerular mesangial proliferation, arterial intimal proliferation, and arterial hyalinization according to the Banff 1997 working classification of renal allograft pathology (*27*, *28*). The additional feature of glomerular sclerosis was assessed by chronic allograft damage index CADI (*29*). Degree of rejection was graded on a scale of 0–3, as described in Banff 1997 (0=none, 1=mild <25%, 2=moderate 25–50%, 3=severe >50%) and CADI (0=no sclerotic glomeruli, 1=<15% of sclerotic glomeruli, 2=16–50% of sclerotic glomeruli, 3=>50% of sclerotic glomeruli). In this study, we also developed a scoring method to determine the grade of cortical scars as number of scars present from 0–3 (0=none, 1=mild 1–2 scars, 2=moderate 3–5 scars, 3=severe >5 scars). All sections were assessed by two independent investigators unaware of the animal groups.

Immunohistochemical Localization

ED-B$^+$ FN antibody was generated by immunization of BALB/c mice. Synthetic human ED-B$^+$ peptide (ITDSSIGLRWTPLNSSTIIGY) conjugated to keyhole limpet hemocyanin (KLH) was injected (100 mg/animal) in complete Freund's adjuvant. This antibody has previously been characterized (*19*). For ED-A$^+$ domain of FN, commercially available monoclonal mouse anti-cellular fibronectin antibody (clone IST-4) was used (Sigma-Aldrich Ltd. Canada).

Rat kidney tissues fixed in 10% neutral buffered formalin were processed for paraffin embedding. The blocks were cut into 5-μm thick sections and subsequently processed for immunostaining. Envision+System-HRP (DAB) (DakoCytomation, CA, USA) was used for immunostaining using the manufacturer's protocol. Three percent H_2O_2 (DakoCytomation, CA, USA) was used to block endogenous peroxidase. Incubation in primary antibodies, mouse anti-ED-B$^+$ FN and mouse anti-cellular fibronectin (ED-A$^+$ FN) was done at 1:800 and 1:1000 dilutions, respectively. Primary antibody was omitted for the negative control. Labeled polymer-HRP conjugated with secondary anti-mouse antibodies were used to incubate the slides. Visualization was performed using Substrate-DAB+Chromogen solution (DakoCytomation, CA, USA). Sections were counterstained with Mayer's Hematoxilin. Specimens were then analyzed by two independent investigators, unaware of the study groups.

RNA Isolation and cDNA Synthesis

Total RNA isolation from the human renal allograft biopsy specimens and rat kidney tissues was performed us-

ing TRIzol reagent (TRIzol; Invitrogen, Burlington, Ontario, Canada). RNA was extracted and cDNA was synthesized using 6 μg of total RNA with oligo-(dT) primers (Invitrogen) as described previously (*30*, *31*).

Real-time Reverse Transcriptase Polymerase Chain Reaction

mRNA levels for total FN, ED-B$^+$ FN, ET-1 and TGF-β1 were quantified. Primer sequences for real-time RT-PCR are as follows; β-actin; human/rat, 5′-CCTCTATGCCAACACAGTGC-3′, 5′-CATCGTACTCCTGCTTGCTG-3′, ED-B$^+$ FN; human/rat, 5′-CCGCCATTAATGAGAGTGAT-3′, 5′-AGTTAGTTGCGGCAGGAGAAG-3′, total FN; human, 5′-GATAAATCAACAGTGGGAGC-3′, 5′-CCCAGATCATGGAGTCTTTA-3′, rat, 5′-CCAGGCACTGACTACAAGAT-3′, 5′-CATGATACCAGCAAGGACTT-3′, ET-1; human, 5′-AAGCCCTCCMAGAGAGCGTTAT-3′, 5′-CCGAAGGTCTGTCACCAATGT-3′, 6FAM-TGACCCACAACCGAG-MGBNFQ, rat, 5′-GCTCCTGCTCCTCCTTGATG-3′, 5′-CTCGCTCTATGTAAGTCATGG-3′, TGF-β1; human, 5′-GCCCACTGCTCCTGTGACA-3′, 5′-CGGTAGTGAACCCGTTGATGT-3′, 6FAM-CAGGGATAACACACTGC-MGBNFQ, rat, 5′-GTAGCTCTTGCCATCGGG-3′, 5′-GAACGTCCCGTCAACTCG-3′. Real-time quantitative RT-PCR was performed in a real-time thermocycler (LightCycler; (Roche Diagnostics Canada, Montreal, Quebec, Canada) using the Readymix SYBR Green Taq (Sigma-Genosys, Canada) detection method. The primer sequences and reaction profiles have previously been described (*16*, *30*). All samples were normalized to a housekeeping gene (β-actin). All PCR reactions demonstrated specificity of amplification as determined by the melting curve analysis.

Statistical Evaluation

Histopathologic data were tested using Kruskal-Wallis one-way analysis of variance on ranks. The remaining data were analyzed using unpaired ANOVA and two-tailed Student's *t* test. Data are expressed as mean±standard error of mean (SEM). A value of $P=0.05$ or less is considered significant.

RESULTS

Functional Features of Chronic Allograft Nephropathy

Allograft recipients not treated with bosentan demonstrated features of chronic allograft nephropathy. Allografted rats showed significant progressive increase in proteinuria (Fig. 1A). Such increase was significantly blocked by ET receptor antagonist bosentan at all time points (at day 30; allografts 6.34±2.11 mg/dl vs. isografts 1.66±0.66 mg/dl, $^*P=0.046$, allografts vs. treated allografts 2.22±0.9 mg/dl, $^\dagger P=0.038$. At day 90; allografts 12.7±5.4 mg/dl vs. isografts 2.5±1.5 mg/dl, $^*P=0.045$, allografts vs. treated allografts 3.68±1.4 mg/dl, $^\dagger P=0.001$. At day 140; allografts 20.2±3.06 mg/dl vs. isografts 1.33±0.33 mg/dl, $^*P=0.003$, allografts vs. treated allografts 11.7±2.5 mg/dl, $^\dagger P=0.033$). In association with proteinuria, allograft recipients demonstrated significantly high serum creatinine levels at day 140 as compared to isografts (allografts 0.61±0.02 mg/dl vs. isografts 0.3±0.01 mg/dl, $^*P=0.007$). Increase in serum creatinine levels was prevented in allografts with bosentan treatment (treated allografts 0.49±0.03 mg/dl, $^\dagger P=0.007$). Similarly, urine creatinine levels were significantly low in allografts as compared to isografts (allografts 123.3±17.4 mg/dl vs. isografts 330.7±59 mg/dl, $^*P=0.007$) while treated allografts had significantly high urine creatinine levels (treated allografts 192.6±25.3 mg/dl, $^\dagger P=0.02$). However at days 30 and 90, no differences in serum and urine creatinine concentrations were noted among the study groups (data not shown).

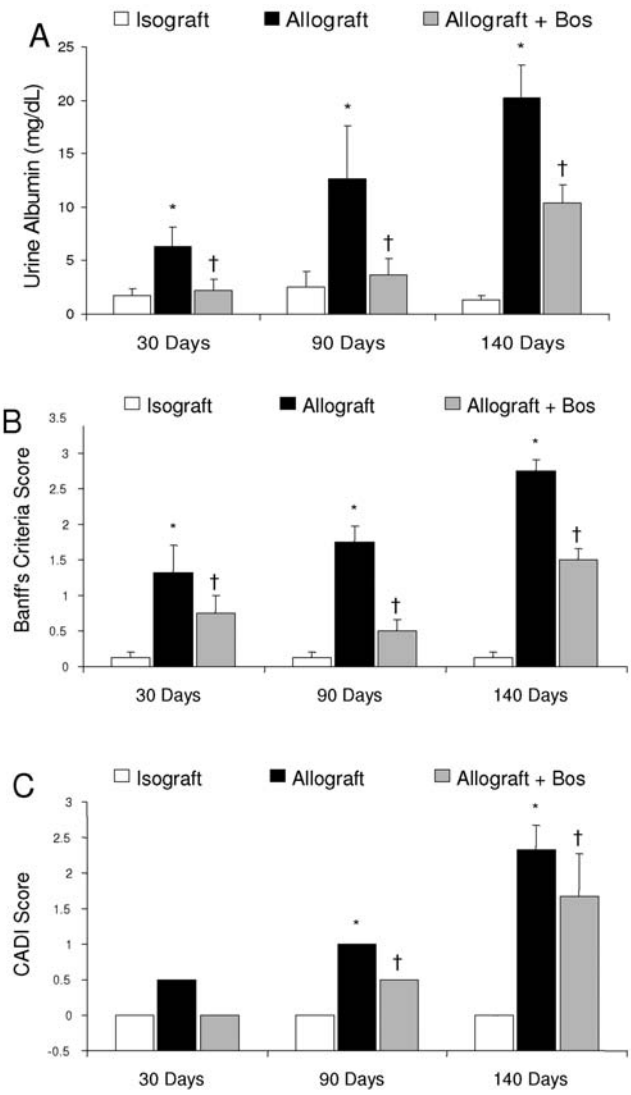

FIGURE 1. Functional and histopathological analyses for chronic allograft nephropathy in rats. (A) Concentration (mg/dl) of albumin in rat urine samples at various time points posttransplantation. (B) Histology scoring using indices as established by Banff's criteria of allograft nephropathy. (C) Chronic allograft damage index (CADI) showing isograft, allograft and allograft treated with bosentan. Data are expressed as the mean ± SEM. $^*P<0.05$ compared to isografts. $^\dagger P<0.05$ compared to allografts. Please see text for specific *P* values.

Histological Features of Chronic Allograft Nephropathy

We then sought to determine whether our animal model developed morphological features of chronic allograft nephropathy and if these features were affected by adminis-

tration of bosentan in allografts. Untreated allografts showed evidence of chronic progressive allograft nephropathy at all time points. Such progression was consistently prevented in bosentan-treated allografts (Fig. 1, B and C). Allografts showed features of transplant nephropathy (at day 30 $^{*}P=0.024$, at day 90 $^{*}P=<0.001$ and at day 140 $^{*}P=<0.001$) while allografts treated with bosentan (Bos) showed significant reduction in such changes (at day 30 $^{\dagger}P=0.03$, at day 90 $^{\dagger}p=<0.001$ and at day 140 $^{\dagger}P=0.001$). Allografts developed interstitial fibrosis, tubular atrophy and cortical scars as early as at day 30. At this stage although some features of transplant nephropathy were apparent, advanced lesions such as intimal proliferation and glomerulosclerosis were not observed. Interestingly, mRNA expression of ED-B^{+} FN and total FN were not upregulated at day 30 (see below). Mesangial proliferation started to occur at day 90 along with only mild intimal proliferation in large sized vessels. Intimal proliferation and obliterative vaculopathy was pronounced at day 140, where it was apparent in medium and large sized blood vessels in allografts (Fig. 2, B and C). However, allografts treated with bosentan showed persistent reduction in vascular changes and showed only mild intimal proliferation (Fig. 2D). Interstitial fibrosis developed in allografts at day 30 and was pronounced at day 140 in allografts (Fig. 2E). Allografts treated with bosentan showed less interstitial fibrosis at all time points (Fig. 2F). CADI score demonstrated features of glomerulosclerosis in allografts (at day 90 $^{*}P=0.01$ and at day 140 $^{*}P=0.02$) while treated allografts showed mild glomerulosclerosis with segmental lesions in the affected glomeruli (at day 90 $^{\dagger}P=0.035$ and at day 140 $^{\dagger}P=0.037$) (Fig. 2, G and H). Such features were only minimal at day 30 in allografts. Isograft controls had no significant signs of chronic allograft nephropathy at any time point.

FN and ED-B^{+} FN mRNA Expression in Chronic Allograft Nephropathy

In our animal model of chronic allograft nephropathy, we show that both total FN and relative ED-B^{+} FN (ED-B^{+} FN: total FN) mRNA and total FN mRNA expression were not altered at day 30 (Fig. 3, A and B). These results parallel the absence of advanced lesions such as intimal proliferation and glomerulosclerosis. At day 90 posttransplantation, total FN ($^{*}P=0.001$) and ED-B^{+} FN ($^{*}P=0.005$) mRNA expressions were significantly higher in the untreated allografts as compared to isografts. This expression increased to six ($^{*}P=0.025$) and nine ($^{*}P=0.0007$) folds respectively in untreated allografts at day 140 (Fig. 3, A and B). Total FN ($^{\dagger}P=0.001$ at day 90, $^{\dagger}P=<0.001$ at day 140) and ED-B^{+} FN ($^{\dagger}P=0.004$ at day 90, $^{\dagger}P=0.001$ at day 140) mRNA levels downregulated significantly in allografts treated with bosentan when compared to untreated allografts (Fig. 3, A and B).

ET-1 and TGF-β1 mRNA Expression in Chronic Allograft Nephropathy and the Effect of Bosentan

We carried out quantification of ET-1 and TGF-β1 mRNA in the kidney tissues from various groups. Our data showed that both ET-1 ($^{*}P=0.01$ at day 30, $^{*}P=0.001$ at day 90 and $^{*}P=0.001$ at day 140) and TGF-β1 ($^{*}P=0.024$ at day 30, $^{*}P=0.004$ at day 90 and $^{*}P=0.005$ at day 140) mRNA were increased in allografts when compared to isografts at all time points (Fig. 3, C and D). Furthermore, bosentan treatment prevented such upregulation of both ET-1 ($^{\dagger}P=0.001$ at day 30, $^{\dagger}P=0.024$ at day 90 and $^{\dagger}P=0.0128$ at day 140) and TGF-β1 ($^{\dagger}P=<0.001$ at day 30, $^{\dagger}P=0.004$ at day 90 and $^{\dagger}P=0.011$ at day 140).

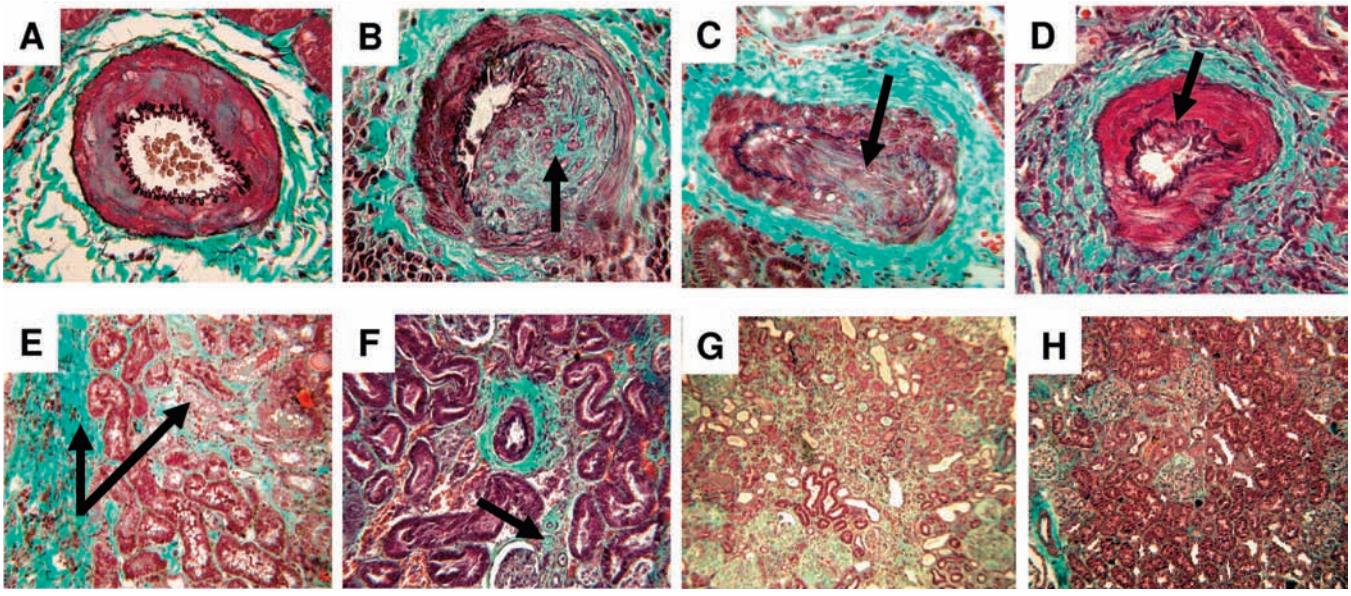

FIGURE 2. Histopathological features of chronic allograft nephropathy in rat kidney. Sections of rat renal cortical arteries stained with elastic trichrome showing (A) isografts at day 140 with no intimal proliferation, (B) allograft at day 140 with severe intimal proliferation (arrow), (C) allograft at day 140 with obliterative vasculopathy (arrow), (D) allograft treated with bosentan at day 140 with reduced intimal proliferation (arrow). Sections of kidney from allograft stained with elastic trichrome showing (E) severe interstitial fibrosis at day 140 (arrows), (F) mild interstitial fibrosis in allografts treated with bosentan at day 140, (G) severe glomerulosclerosis in allograft at day 140, and (H) mild segmental glomerulosclerosis in allograft treated with bosentan at day 140.

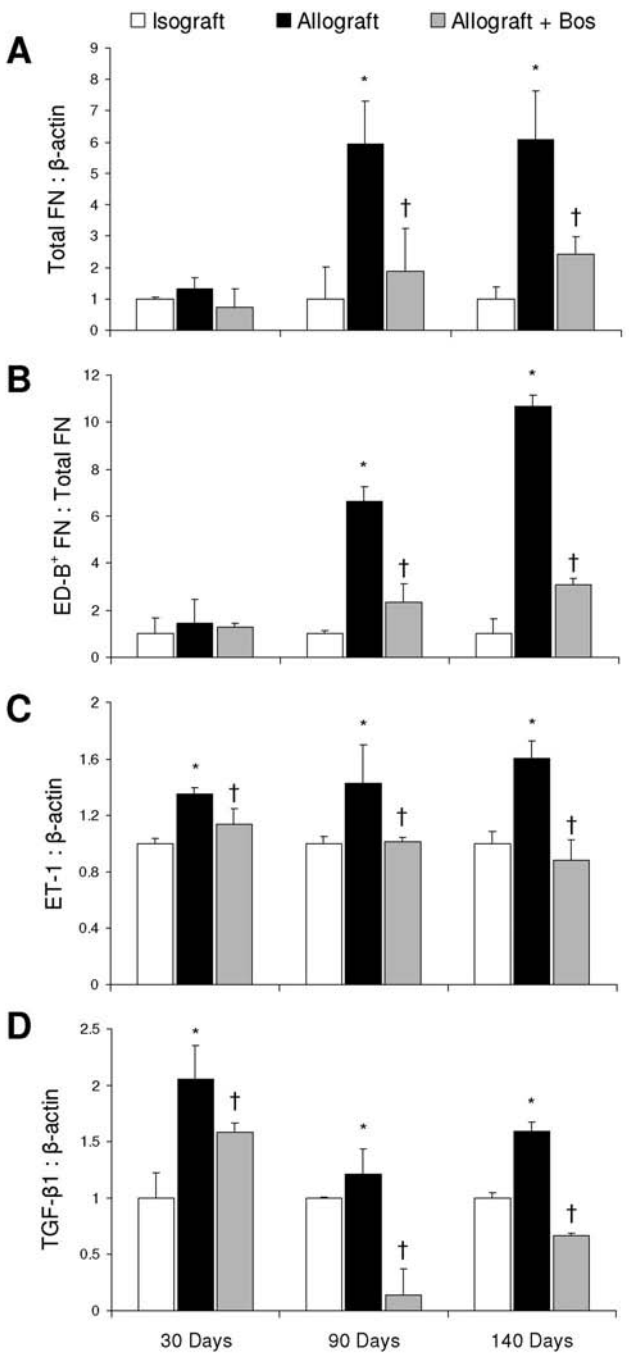

FIGURE 3. ED-B$^+$ FN mRNA expression in rat kidney. Rat kidney tissues at days 30, 90, and 140 after transplantation were assessed by real time RT-PCR for (A) total FN, (B) relative ED-B$^+$ FN, (C) ET-1, and (D) TGF-β1 transcript levels. Bosentan prevented such upregulation in treated allografts mRNA levels are expressed as ratio of target mRNA to β-actin mRNA; ED-B$^+$ FN is expressed as ratio of ED-B$^+$ FN to total FN. *$P<0.05$ compared to isografts. $^\dagger P<0.05$ compared to allografts. Please see text for specific P values.

ED-B$^+$ FN Protein Expression

In order to determine the protein expression and localization, we performed immunohistochemical analysis of the rat renal grafts. The expression of ED-B$^+$ FN was distributed along the areas of fibrosis typically seen in chronic allograft nephropathy (Fig. 4, B and F). Allografts had a strong positive staining present in the endothelium and smooth muscle cells of the large arteries (Fig. 4F) in comparison to treated allografts (Fig. 4G). Arteries with maximum obliterative vasculopathy showed more immunoreactivity for ED-B$^+$ FN (Fig. 4F). It is of interest that ED-B$^+$ FN expression was also found in the perivascular cellular infiltrate in untreated allografts (Fig. 4F). Glomerular epithelial and endothelial cells in the allografts showed intense immunoreactivity of ED-B$^+$ FN (Fig. 4B). We further examined the renal allograft sections for ED-A$^+$ FN immunoreactivity. Although the distribution of both ED-B$^+$ and ED-A$^+$ FN staining was similar in the untreated allografts, the later was low in intensity (Fig. 4, D and H). Untreated allografts sacrificed at day 140 expressed most intense immunoreactivity for ED-B$^+$ FN (Fig. 4, B and F) while treated allografts showed reduced intensity of staining (Fig. 4, C and G). Isografts showed low ED-B$^+$ FN expression in the arterial intima, glomeruli and interstitium (Fig. 4, A and E).

Increased ED-B$^+$ FN Expression in Human Renal Biopsy Specimens

In human chronic allograft nephropathy specimens, mRNA analysis showed upregulation of ED-B$^+$ FN by quantitative real time RT-PCR. Expression of total FN (*$P=0.01$) and relative ED-B$^+$ FN (*$P=0.001$) mRNA in biopsy samples taken from patients with established chronic allograft nephropathy was significantly higher than in the samples with no evidence of chronic allograft nephropathy (Fig. 5, A and B). Our next objective was to investigate whether the increased ED-B$^+$ FN expression in human allograft nephropathy was also associated with increased levels of ET-1 and TGF-β1. In parallel to the results of rat renal tissues, our human data also showed that both ET-1 (*$P=0.04$) and TGF-β1 (*$P=0.001$) transcripts were upregulated in patients with established chronic allograft nephropathy (Fig. 5, C and D).

DISCUSSION

We have, for the first time, demonstrated an upregulation of ED-B$^+$ FN in an animal model of chronic allograft nephropathy and in human renal biopsy tissues with chronic allograft nephropathy. We have also demonstrated that the increased expression of ED-B$^+$ FN is associated with upregulated ET-1 and TGF-β1 expression.

ECM proteins, vasoactive peptides and cytokines play a major role in chronic allograft nephropathy. ETs are the most potent vasoactive peptides, which play a role in hypertension, ischemia-reperfusion injury and in pathological changes associated with increased vascular cell proliferation (*32*). We and others have demonstrated increased ET-1 expression in chronic renal transplant rejection (*6*, *33*). It has also been demonstrated that ET-1 regulates expression of ECM protein (*34*, *35*). FN is an adhesive macroglycoprotein which is abundantly distributed in the ECM. FN is involved in regulating various cellular and extracellular events and may play a key role in the host immune cascade triggered by organ transplantation (*5*). Pathological conditions of liver, lung and kidney which result in fibrosis may lead to upregulation of FN and its isoforms (*36*). We have previously demonstrated in-

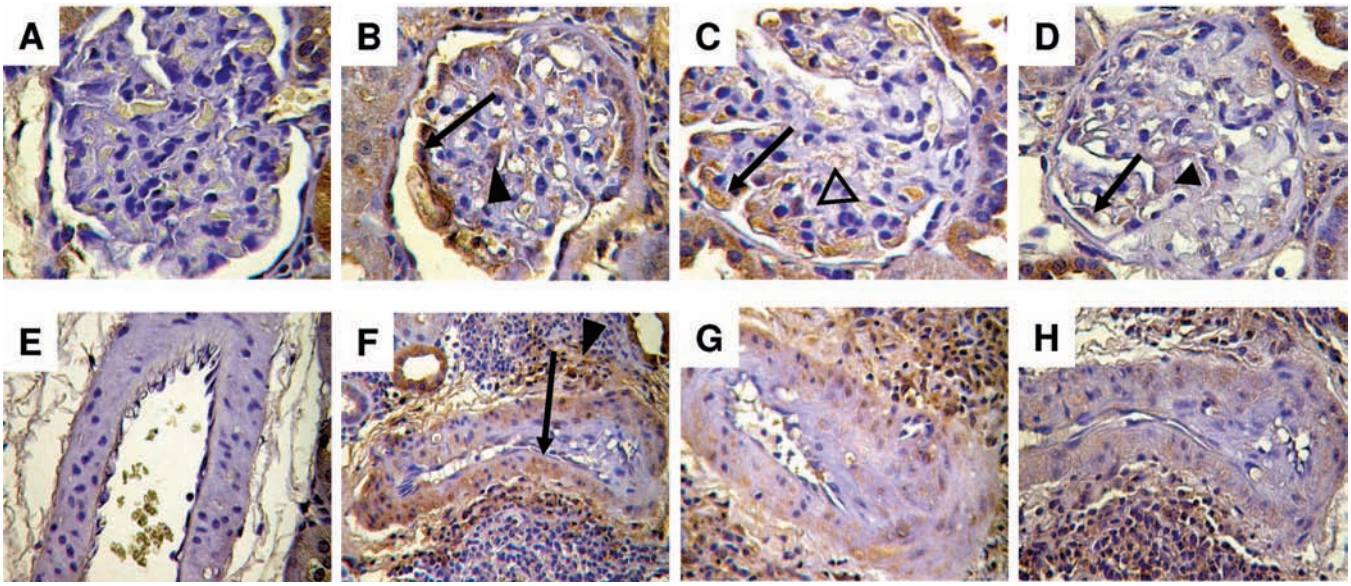

FIGURE 4. ED-B$^+$ FN protein expression in rat kidney. Immunohistochemical staining of rat renal cortical tissue showing (A) no immuno-reactivity for EDB$^+$ FN in the glomerulus of an isograft at day 140, (B) section from an allograft with intense EDB$^+$ FN immunoreactivity in the glomerular endothelium (arrow head) and epithelium (arrow) at day 140, (C) section from an allograft treated with bosentan showing low intensity for EDB$^+$ FN immunostaining in the endothelium (open arrow head) and epithelium (arrow) at day 140, (D) section from an allograft showing low positivity for EDA$^+$ FN immunostaining in glomerular endothelium (arrow head) and epithelium (arrow) at day 140, (E) an arterial section from an isograft with no EDB$^+$ FN immunoreactivity at day 140, (F) an arterial section from an allograft showing strong EDB$^+$ FN immunoreactivity in the neointima (arrow) and perivascular infiltrate (arrow head) at day 140, (G) an arterial section from an allograft treated with bosentan showing low EDB$^+$ FN immunostaining at day 140, and (H) an arterial section from an allograft showing low EDA$^+$ FN immunostaining at day 140.

creased ED-B$^+$ FN expression regulated by ET-1 and TGF-β1 in chronic diabetic complications (*16*, *17*, *37*).

Accumulating evidence suggest that profibrogenic peptides such as ETs induce fibroblast proliferation via ET receptors and result in FN production (*38*). These fibroblasts may undergo differentiation to form myofibroblasts which primarily cause alpha-actin positive vascular smooth muscle cell proliferation, obliterative vasculopathy and down stream ischemic injury (*39*, *40*, *41*). In this study, arterial intimal proliferation was pronounced in allografts sacrificed at day 140 and was reduced in allografts treated with bosentan, suggesting the role of ET induced cell proliferation and differentiation. We further showed that ET blockade prevented expression of total FN, ED-B$^+$ FN, and TGF-β1 mRNA.

Exact significance of ED-B$^+$ FN is not known. However, ED-B$^+$ FN has been shown to provide "outside-in" signaling in endothelial cells which leads to endothelial cell proliferation and differentiation (*19*). Our data indicate significant alteration of ED-B$^+$ oncofetal FN mRNA expression late in the process. Such upregulation was not seen early when intimal proliferation and glomerulosclerosis were absent. This finding suggests that ED-B$^+$ FN expression may be of more significance in the production of advanced fibrotic lesions. Along with mRNA upregulation, increased intensity of ED-B$^+$ FN staining was seen in the endothelium, intima and media of the walls of the arteries and in glomerular endothelium and epithelium suggesting that this protein may be associated with cellular proliferation and differentiation. We have previously demonstrated the role of this splice variant in endothelial proliferation (*19*). In keeping with our study, role of splice variants of cellular FN in remodeling during proliferative glomerular diseases and in rat cardiac allografts have been demonstrated (*42*, *43*).

In this study ET-blockade reduced both TGF-β1 and ED-B$^+$ FN. TGF-β1 is a key regulator of fibrogenesis in various diseases including chronic allograft nephropathy (*44*, *45*). TGF-β1 has also been shown to increase ECM protein synthesis in a number of cell types including endothelial and glomerular epithelial cells (*46*). We found that TGF-β1 and ET-1 expression increased early and remained elevated throughout the study period. On the other hand, although FN and ED-B$^+$ FN expression increased later, they were prevented by ET-1 blockade. Early upregulation of TGF-β1, in association with increased inflammatory cell infiltrate, has been demonstrated (*46*). Later, with the reduction of inflammatory infiltrate, other cells such as endothelial or glomerular epithelial cells may act as its source (*46*). Exact significance for the lack of correlation with ET-1 and TGF-β1 with the levels of ED-B$^+$ FN expression is not clear. These data suggest that these peptides may perform various functions at different time points in the course of chronic allograft nephropathy, which however, needs further characterization. Furthermore, prevention of TGF-β1 mRNA expression by ET blockade at all time points may suggest the role of ETs in the regulation of TGF-β1 in this process. In endothelial cells, TGF-β1 blockade reduces ED-B$^+$ FN expression (*17*). Hence, it is possible that ET-blockade may act via TGF-β1 to prevent ED-B$^+$ FN expression. However, such notion in the context of chronic allograft nephropathy has to be established by conducting specific experiments.

In conclusion, we have demonstrated the upregulation of ED-B$^+$ containing splice variant of FN both in animal and

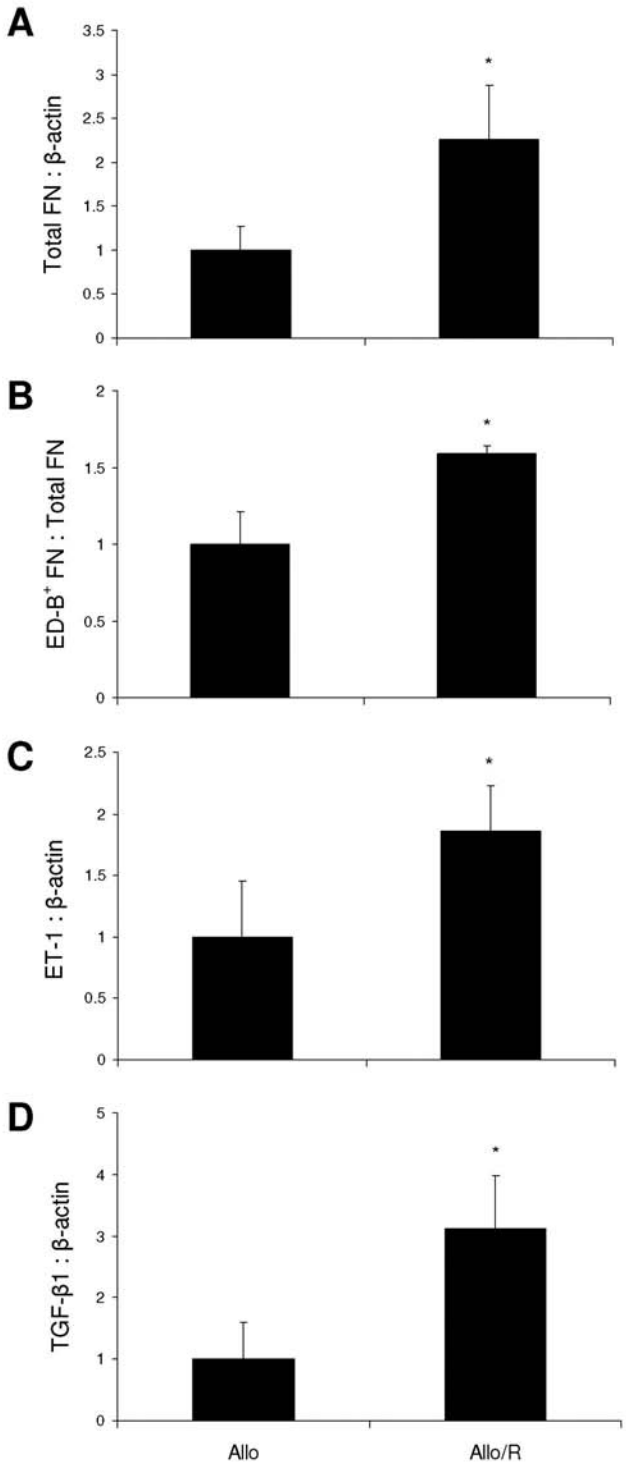

FIGURE 5. ED-B$^+$ FN mRNA expression in human kidney. Quantitative analysis of mRNA expression showing (A) total FN, (B) ED-B$^+$ FN, (C) ET-1, and (D) TGF-β1 in chronic allograft rejection. mRNA levels are expressed as ratio of target mRNA to β-actin mRNA; ED-B$^+$ FN is expressed as ratio of ED-B$^+$ FN to total FN. Allo, allografts with no signs of chronic allograft nephropathy (n=10); Allo/R, allografts with clinical and morphological features of chronic allograft nephropathy (n=7). *$P<0.05$ compared to Allo. Please see text for specific P values.

in human chronic allograft nephropathy. Our results indicate that chronic allograft nephropathy is characterized by ET-1 and possibly TGF-β1 mediated re-expression of ED-B$^+$ FN. The significance of ED-B$^+$ FN in transplant nephropathy remains to be fully elucidated. These findings suggest that ED-B$^+$ FN may represent a novel target for the development of diagnostic and therapeutic modalities for chronic allograft nephropathy.

ACKNOWLEDGMENTS

The authors acknowledge grant support from the Multiple Organ Transplant Program (MOTP) and Lawson Health Research Institute (LHRI).

REFERENCES

1. Hayry P, Isoniemi H, Yilmaz S, et al. Chronic allograft rejection. *Immun Rev* 1993; 134: 33.
2. Hosenpud JD, Bennett LE, Keck BM, et al. The Registry of the International Society for Heart and Lung Transplantation: Seventeenth official report 2000. *J Heart Lung Transplant* 2000; 19: 909.
3. Hillebrands JL, Rozing J. Chronic transplant dysfunction and transplant arteriosclerosis: new insights into underlying mechanisms. *Expert Rev Mol Med* 2003: 1.
4. Tullius SG, Heemann U, Hancock WW, et al. Long-term kidney isografts develop functional and morphologic changes that mimic those of chronic allograft rejection. *Ann Surg* 1994; 220: 425.
5. Coito AJ, Kupiec-Weglinski JW. Extracellular matrix proteins: bystanders or active participants in the allograft rejection cascade? *Ann Transplant* 1996; 1: 14.
6. Deng DX, Jiang J, Garcia B, et al. Endothelin-1, endothelin-3 and their receptors (endothelin (A) and endothelin (B)) in chronic renal transplant rejection in rats. *Transpl Int* 2000; 13: 175.
7. Watschinger B, Sayegh MH, Hancock WW, Russell ME. Upregulation of endothelin-1 mRNA and peptide expression in rat cardiac allografts with rejection and arteriosclerosis. *Am J Pathol* 1995; 146: 1065.
8. El-Gamel A, Sim E, Hasleton P, et al. Transforming growth factor beta (TGF-beta) and obliterative bronchiolitis following pulmonary transplantation. *J Heart Lung Transplant* 1999; 18: 828.
9. Hayry P, Paavonen T, Mennander A, et al. Pathophysiology of allograft arteriosclerosis. *Transplant Proc* 1993; 25(2): 2070.
10. Orosz CG, Pelletier RP. Chronic remodeling pathology in grafts. *Curr Opin Immunol* 1997; 9: 676.
11. Gould VE, Martinez-Lacabe V, Virtanen I, et al. Differential distribution of tenascin and cellular fibronectins in acute and chronic renal allograft rejection. *Lab Invest* 1992; 67: 71.
12. Castellani P, Dorcaratto A, Pau A, et al. The angiogenesis marker ED-B+ fibronectin isoform in intracranial meningiomas. *Acta Neurochir (Wien)* 2000; 142: 277.
13. Nanki N, Fujita J, Yang Y, et al. Expression of oncofetal fibronectin and syndecan-1 mRNA in 18 human lung cancer cell lines. *Tumour Biol* 2001; 22: 390.
14. Sakai M, Sasaki Y, Yamagishi N, et al. The preterm labor index and fetal fibronectin for prediction of preterm delivery with intact membranes. *Obstet Gynecol* 2003; 101: 123.
15. Castellani P, Viale G, Dorcaratto A, et al. The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis. *Int J Cancer* 1994; 59(5): 612.
16. Khan ZA, Cukiernik M, Gonder JR, Chakrabarti S. Oncofetal fibronectin in diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2004; 45: 287.
17. Khan ZA, Barbin YP, Farhangkhoee H, et al. Glucose-induced serum and glucocorticoid-regulated kinase activation in oncofetal fibronectin expression. *Biochem Biophys Res Commun* 2005; 329: 275.
18. Khan ZA, Caurtero J, Barbin YP, et al. ED-B fibronectin in non-small cell lung carcinoma. *Exp Lung Res* 2005; 31: 701.
19. Khan ZA, Chan BM, Uniyal S, et al. EDB fibronectin and angiogenesis - a novel mechanistic pathway. *Angiogenesis* 2005; 8: 183.
20. Simonson MS, Herman WH, Robinson A, et al. Inhibition of endothelin-converting enzyme attenuates transplant vasculopathy and rejection in rat cardiac allografts. *Transplantation* 1999; 67: 1542.
21. Diamond JR, Tilney NL, Frye J, et al. Progressive albuminuria and

glomerulosclerosis in a rat model of chronic renal allograft rejection. *Transplantation* 1992; 54: 710.
22. Chandraker A, Azuma H, Nadeau K, et al. Late blockade of T cell costimulation interrupts progression of experimental chronic allograft rejection. *J Clin Invest* 1998; 101: 2309.
23. Clozel M, Breu V, Gray GA, et al. Pharmacological characterization of bosentan, a new potent orally active non-peptide endothelin receptor antagonist. *J Pharmacol Exp Ther* 1994; 270: 228.
24. Grant D, Zhong R, Stiller C, et al. A comparison of cyclosporine A and Nva2-cyclosporine (cyclosporine G) in a rat renal allograft model. *Transplantation* 1987; 44: 9.
25. Evans T, Deng DX, Chen S, Chakrabarti S. Endothelin receptor blockade prevents augmented extracellular matrix component mRNA expression and capillary basement membrane thickening in the retina of diabetic and galactose-fed rats. *Diabetes* 2000; 49: 662.
26. Deng D, Evans T, Mukherjee K, et al. Diabetes-induced vascular dysfunction in the retina: role of endothelins. *Diabetologia* 1999; 42: 1228.
27. Racusen LC. The Banff 97 working classification of renal allograft pathology. *Kidney Int* 1999; 55: 713.
28. Solez K, Axelsen RA, Benediktsson H, et al. International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. *Kidney Int* 1993; 44: 411.
29. Isoniemi H, Taskinen E, Hayry P. Histological chronic allograft damage index accurately predicts chronic renal allograft rejection. *Transplantation* 1994; 58: 1195.
30. Chen S, Mukherjee S, Chakraborty C, Chakrabarti S. High glucose induced, endothelin-dependent fibronectin synthesis is mediated via NF-kappa B and AP-1. *Am J Physiol* 2003; 284: C263.
31. Chen S, Evans T, Deng D, et al. Hyperhexosemia induced functional and structural changes in the kidneys: role of endothelins. *Nephron* 2002; 90: 86.
32. King AJ, Brenner BM, Anderson S. Endothelin: a potent renal and systemic vasoconstrictor peptide. *Am J Physiol* 1989; 256: F1051.
33. Watschinger B, Sayegh MH. Endothelin in organ transplantation. *Am J Kidney Dis* 1996; 27: 151.
34. Clozel M, Salloukh H. Role of endothelin in fibrosis and anti-fibrotic potential of bosentan. *Ann Med* 2005; 37: 2.
35. Muldoon LL, Rodland KD, Forsythe ML, Magun BE. Stimulation of phosphatidylinositol hydrolysis, diacylglycerol release and gene expression in response to endothelin, a potent new agonist for fibroblasts and smooth muscle cells. *J Biol Chem* 1989; 264: 8529.
36. Jarnagin WR, Rockey DC, Koteliansky VE, et al. Expression of variant fibronectins in wound healing: Cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis. *J Cell Biol* 1994; 127: 2037.
37. Chen S, Khan ZA, Cukiernik M, Chakrabarti S. Differential activation of NF-kappa B and AP-1 in increased fibronectin synthesis in target organs of diabetic complications. *Am J Physiol Endocrinol Metab* 2003; 284: E1089.
38. Clozel M. Effects of bosentan on cellular processes involved in pulmonary arterial hypertension: do they explain the long-term benefit? *Ann Med* 2003; 35: 605.
39. Zhang K, Rekhter MD, Gordon D, Phan SH. Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and in situ hybridization study. *Am J Pathol* 1994; 145: 114.
40. Hayry P, Paavonen T, Mennander A, et al. Pathophysiology of allograft arteriosclerosis. *Transplant Proc* 1993; 25: 2070.
41. Orosz CG, Pelletier RP. Chronic remodeling pathology in grafts. *Curr Opin Immunol* 1997; 9(5): 676.
42. Barnes JL, Torres ES, Mitchell RJ, Peters JH. Expression of alternatively spliced fibronectin variants during remodeling in proliferative glomerulonephritis. *Am J Pathol* 1995; 147: 1361.
43. Coito AJ, Brown LF, Peters JH, et al. Expression of fibronectin splicing variants in organ transplantation: a differential pattern between rat cardiac allografts and isografts. *Am J Pathol* 1997; 150: 1757.
44. Baczkowska T. Serum TGF-beta1 correlates with chronic histopathological lesions in protocol biopsies of kidney allograft recipients. *Transplant Proc* 2005; 37: 773.
45. Matl I, Viklicky O. Transforming growth factor (TGF-beta) and kidney transplantation. *Cas Lek Cesk* 2002; 141: 601.
46. Baczkowska T. Serum TGF-beta1 correlates with chronic histopathological lesions in protocol biopsies of kidney allograft recipients. *Transplant Proc* 2005; 37(2): 773.