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PDF issue: 2025-12-04

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(Citation)
Clinical Transplantation,20(1):26-29

(Issue Date)
2006-01

(Resource Type)
journal article

(Version)
Accepted Manuscript

(Rights)
The definitive version is available at www.blackwell-synergy.com.

(URL)
https://hdl.handle.net/20.500.14094/90000300

Title

CORRELATION BETWEEN ENDOTHELIN EXPRESSION IN EARLY POST-TRANSPLANT BIOPSY SPECIMENS AND LONG-TERM ALLOGRAFT FUNCTION IN LIVING RELATED RENAL TRANSPLANTATION

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Abstract

Objective. We investigated whether degree of immunohistochemically evident endothelin (ET) expression in early post-transplant biopsy specimens could predict long-term allograft function in living related renal transplantation.

Methods. Allograft biopsy specimens obtained from 40 patients with living related transplants were studied. Cases with episodes of acute rejection or calcineurin inhibitor toxicity were excluded. We immunostained graft biopsy specimens obtained before transplantation (PRE) and at 3 months afterward (3M) with anti-ET antibody. The number of stained tubular epithelial cells per 1000 tubular cells was defined as the staining index (S.I.). In the 21 patients whom we could assess at 3 years after transplantation, the correlation between ET expression and long-term graft function was examined.

Results. Anti-ET antibody staining was appreciable in tubular epithelium but not in glomeruli. Tubular S.I. at PRE and at 3M were 10.6± 15.3 and 32.0 ± 35.6 (mean ±SD) respectively ($p<0.01$). When patients were classified according to S.I. (group A, S.I.<25; group B, S.I.>25), declining ratio in Ccr at 3

years after transplantation for groups A and B with respect was 21.8 ± 15.4% and 41.9 ± 21.6% ($p<0.05$).

Conclusion. High ET expression in early posttransplantion, biopsy specimens was related to poor long-term allograft function following living related renal transplantation.

Keywords. renal transplantation, living related donors, endothelin, chronic allograft nephropathy

Introduction

Development of immunosuppressive agents has improved graft survival after transplantation. However chronic allograft nephropathy (CAN) remains as the most common cause of graft loss in renal transplantation 【1,2,3】. In CAN, so-called chronic rejection is characterlized by parenchymal ischemia 【4】. Since endothelin-1 (ET-1) was isolated from supernatants of cultured endothelial cells 【6】, two more isopeptides (ET-2 and ET-3) have been characterlized 【7】. All isoforms are products of cleavage of specific prohormones. Prepro-ET is cleaved to form big-ET , which in turn is converted by proteases into peptides 21 amino acids in length ; the most abundant of these is ET-1 【8,9,10,11】. Synthesized by vascular endothelial cells 【6,12】, renal mesangial cells 【13,14】, glomerular endothelial cells 【15】 and tubular epithelial cells 【16,17】, ET-1 is well positioned to cause changes in renal vascular tone, particularly cortical vasoconstriction causing reduced renal blood flow and glomerular filtration rate 【5,15】.

In this study we used anti-ET antibody to immunohistochemically examine ET expression in allograft biopsy specimens obtained for routine evaluation 3

months after living related renal transplantation using anti-ET antibody to stain ET. We also examined the relationship between ET expression and long-term graft function.

Materials and Methods

Patients. Between 1986 and 2002 , living related renal transplantation was performed for 71 patients at Kobe Graduate School of Medicine. Allograft biopsy specimens obtained at 3 months after transplantation (3M) were studied immunohistochemically in 40 of these patients. Immunosuppressive therapy included continuous intravenous infusion of calcineurin inhibitor (CNI , cyclosporine or tacrolimus) for 3 or 4 days , followed by oral CNI. The daily CNI dose was adjusted according to the trough concentration measured in whole blood just before the next dose. For induction , mizoribine (2 to 4 mg/kg/day) or mycophenolate mofetil (1.5g/day), methylpredonisolone (1mg/kg/day) , and antilymphocyte globulin was used in addition to CNI.

Shortly before transplantation , cortical wedge biopsy of the graft was performed. At approximately 3M and also at 3 years after transplantation, core

needle biopsy was performed using a Biopty gun (16G ; CR Bard , Covington , GA) under ultrasonographic guidance, with the patient's or parent's informed consent as appropriate for age. Biopsy specimens were evaluated by two observers according to the Banff working classification in a blinded fashion.

We excluded samples from cases with an episode of acute rejection or CNI toxicity. Acute rejection was diagnosed by an increase in serum creatinine exceeding 30% ; whenever possible , an additional core needle biopsy specimen was obtained to confirm the diagnosis. Of 40 patients 21 could be followed up from 3 months to 3 years after transplantation to study the relationship between ET expression and renal function according to creatinine clearance (Ccr). Patient demographics were shown (Table. 1). As an index of long-term change in graft function, Ccr declining ratio was determined as Ccr at 3M or 3Y –Ccr at PRE per Ccr at PRE.

Immunohistochemistry. Paraffin-embedded sections 2 μ m in thickness were cut from pre-transplantation specimens (PRE) , and 3M specimens , and 3Y specimens. These were subjected to ET immunostaining by an indirect immunoperoxidase method using an avidin-biotin-peroxidase kit (Vector

Laboratories , Burlingame , CA) with a mouse monoclonal primary antibody against human ET-1 (QED Bioscienc, San Diego, CA ; dilution 1:100).

Tissue sections were deparaffinized in xylene, rehydrated in graded ethanol Solutions, and washed in phosphate-buffered saline (PBS). Endogenous peroxidase was inactivated by incubation for 30 min at 37℃ in a methanol/peroxide solution (0.03%). After nonspecific binding was blocked with 1.5% normal horse serum in 0.5% PBS , sections were incubated overnight at 4 ℃ with the primary antibody. Bound antibody was detected using biotinylated horse anti-mouse IgG and avidin-peroxidase complex. Sections were stained with 3,3'-diaminobenzidine (Sigma Chemical, St. Louis, MO), and counterstained with 1% methyl green.

To investigate the effect of ET expression at 3M on subsequent clinical outcome and pathologic change in allografts , cases were classified into two groups according to number of stained tubular epithelial cells per 1000 tubules (staining index, or S.I.). Group A was defined by an S.I. under 25 , while group B cases had an over 25. Ccr declining ratio at 3M and at 3Y were compared

between groups A and B. S.I. was compared between PRE and 3M specimens.

Statistical analysis. Values are presented as mean±SD. The Mann-Whitney U test was used to compare Ccr declining ratio between groups A and B, and also to compare ET S.I. between PRE and 3M specimens.

Results

Immunohistochemical staining. Anti-ET antibody staining was seen in tubular epithelium, but little staining was seen in glomeruli (Fig. 1).

ET expression in grafts before and after transplantation. The S.I. for PRE and at 3M respectively was 10.6 ± 15.3 and 32.0 ±35.6 (Table .2), ($p<0.01$). Staining with anti-ET antibody at 3Y was very slight.

ET expression and graft function. Declining ratio of Ccr (Δ Ccr) during the 3 years following transplantation for groups A and B was 21.8 ± 15.4% and 41.9 ± 21.6% respectively (Table. 3). Thus, graft function worsed significantly more when ET immunoreactivity was high($p<0.05$).

Discussion

Recently, graft survival after transplantation has improved steadily as a result of refinements in immunosuppressive regimens and tissue typing. But chronic

allograft nephropathy (CAN) remains as the most common cause of graft loss in renal transplantation 【1,2,3】 . Histologically, chronic rejection is characterlized by parenchymal ischemia caused by gradual vascular obliteration. ET, a potent vasoactive and mitogenic peptide , is pathophysiologically important in events following transplantation 【5,16】 . During kidney allograft rejection, plasma concentrations of ET-1 are elevated 【17,18,19,20】 . Once tubulitis occurs, intracellular ET is released from tubular epithelial cells as a result of hyperpermeability or cell necrosis. When vascular rejection begins, ET is released into the vascular lumen, causing increased vascular resistance , decreased renal plasma flow, and decreased glomerular filtration rate. We therefore postulated that ET expression in early biopsy specimens could be used to assess risk of later functional decline in the graft.

We examined ET immunoreactivity in biopsy specimens at 3M after transplantation, correlating ET expression with change in graft function between 3M and 3Y after transplantation. Since a caderveric donor kidney is subjected to

varying degrees of warm and cold ischemia causing ischemia – reperfusion injury that may lead to acute tubular necrosis【9】, we excluded caderveric grafts from study.

We first compared ET expression, pretransplantation allograft specimens with expression in 3M allograft specimens. We found ET expression at 3M to be significantly higher than during the pretransplantion period. In addition to ischemia and rejection , immunosuppressive therapy can contribute to allograft injury ; CNI is reported to stimulate ET expression 【4,10,11】 . However , we found no significant differences in trough CNI concentration between groups with minimal and strong ET expression.

Group B grafts, with strong ET expression, showed significantly more rapid decline in graft function at 3Y. In 10 of 13 cases(group A), and 5 of 8 cases(group B), protocol biopsy at 3Y were performed. So 1 case(10%) in group A and 4 cases(80%) in group B showed biopsyproven CAN. Our findings suggest that ET expression in early posttransplantion biopsy specimens is related to long-term allograft function in living related renal transplantation.

We also examined relationships between immunohistochemically evident ET

expression and hypertension at 3Y after transplantation, finding no association between ET expression at 3M and at 3Y in ET expression , or between ET expression at 3M and subsequent hypertension. Uninary ET-1 concentration at 10 days after transplantation or later have been reported to be similar to findings in a the control group 【11】 . Thus urinary ET-1 excretion is regulated as opposed to being simply flow dependent. In a study in rats, an oral ET receptor antagonist was found to prevent CAN 【 3 】 . Our results suggest that amount of ET expression in an early biopsy specimen from the graft has prognostic significance, and that modifying ET effects might indeed represent a new strategy for preventing CAN.

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Figure legends

Table 1. Patient characterlistics. Characterlistics according to antibody staining pattern variables did not differ between groups A and B. Abbreviations : ALG, antilymphocyte globulin; WIT, warm ischemic time; FSGS, focal segmental glomerulosclerosis; IgA, IgA nephropathy; Alport, Alport syndrome; MPGN, mesangial proliferative glomerulonephritis; Hypo, hypoplastic kidney; HUS, hemorrhagic uremic syndrome; CNS, congenital nephrotic syndrome; RN, reflux

nephropathy.

Figure 1. Localization of immunostaining for endothelin in an allograft biopsy specimen. Immunoreactivity for endothelin was seen in tubular epithelium and glomeruli , Methylgreen ; magnification × 100 and × 400.

Table 2. Staining Index (S.I.) : stained tubular cells per 1000 tubular cells

Tubular endothelin expression at three months after transplantation was significantly higher than that at pre-transplant period ($p<0.01$).

Table 3. Change in creatinine clearance declining ratio in group A and B at three years after transplantation ($p<0.05$, group A & B)

	Group A (n=8)	Group B (n=13)
Age(yr)	12.1±3.6	9.8±5.9
Sex(M:F)	6:2	9:4
HLA mismatch		
AB	1.75 ±0.43	1.62±0.74
DR	0.75± 0.43	0.77±0.42
Donor Age(yr)	46.6±8.2	40.8±6.5
WIT(mm)	4.3±3.4	2.6±1.5
Rejection	0.4±0.5	0.8±1.0
Mean CyA trough level(ng/ml)	144.0±23.4	180.2±38.7
ESRD (FSGS:RN:MPGN :Alport:IgA:CNS: HUS:Hypo)	3:1:1:1:1:1:0:0	8:0:0:1:0:1:1:2

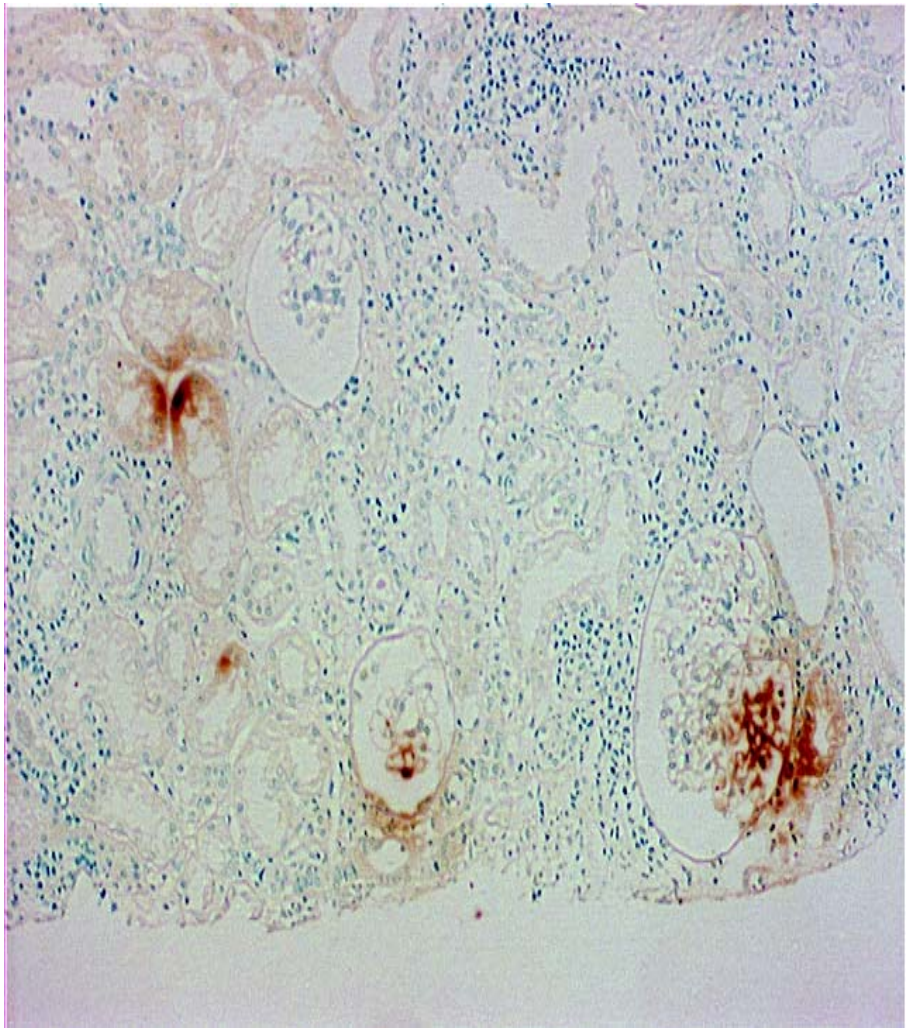
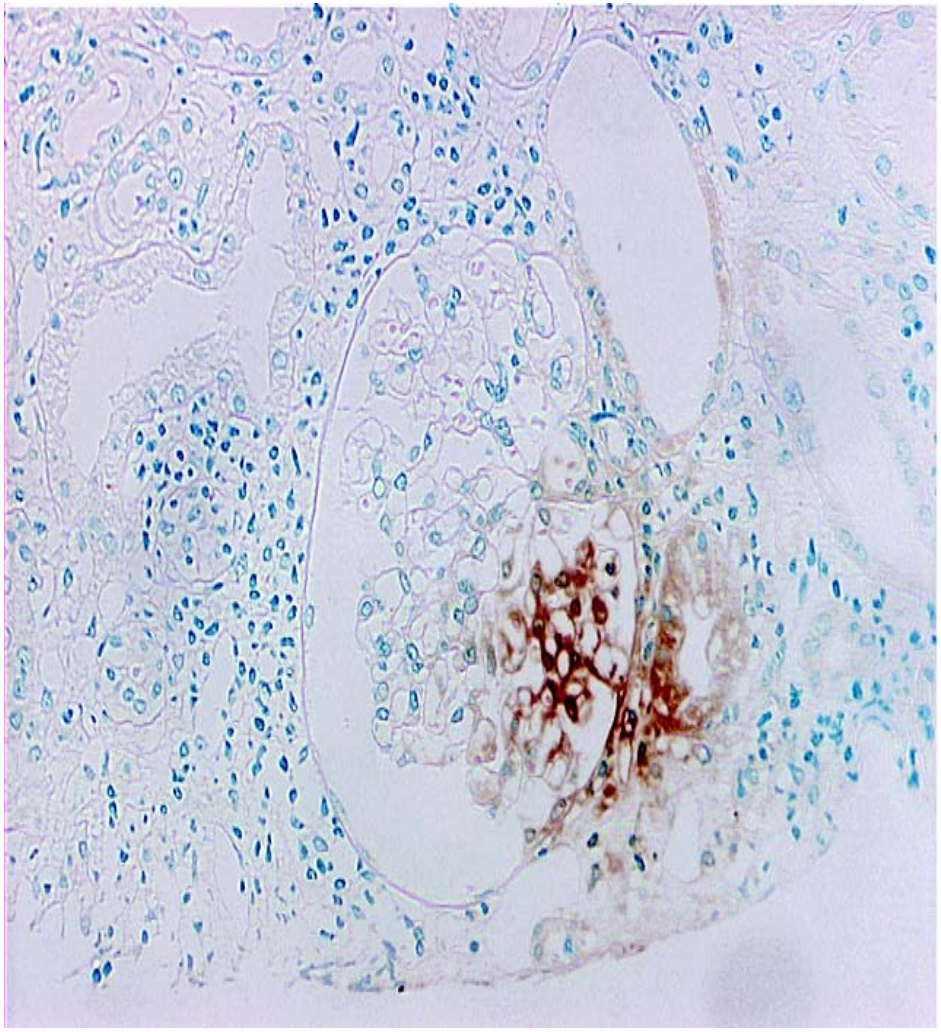
ET expression in specimens of PRE and 3M

	S.I.
PRE	10.6±15.3
	($p<0.01$)
3M	32.0±35.6

Change in Ccr in groups A&B

Declining ratio

Group A
41.9±21.6 %

(p<0.05)

Group B
21.8±15.4 %