Osteopontin expression in acute renal allograft rejection

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Background. Osteopontin (OPN) is a potent chemoattractant for mononuclear cells that is up-regulated in various inflammatory states of the kidney. The role of OPN and its expression in human renal allograft rejection are unknown.

Methods. We examined by immunohistochemistry and in situ hybridization, renal biopsies from patients with acute rejection ($N = 22$), protocol biopsies without rejection ($N = 9$), and perioperative donor biopsies ($N = 35$) for intrarenal expression of OPN, and its correlation with clinical, laboratory, and histopathologic parameters. In the rejection biopsies, interstitial monocyte/macrophage infiltration, tubulointerstitial cell proliferation/regeneration and apoptosis were investigated.

Results. In the majority of rejection biopsies, OPN expression by proximal tubular epithelium was widespread, and tended to be enhanced in the tubules surrounded by numerous inflammatory cells. Conversely, in patients that did not experience episodes of rejection and in donor biopsies, OPN expression by proximal tubules was nil or weak. OPN mRNA was colocalized with its translated protein in the renal tubular epithelium. OPN expression positively correlated with the degree of interstitial inflammation ($P < 0.05$), CD68+ monocyte infiltration ($P < 0.01$), Ki-67+ regenerating tubular and interstitial cells ($P < 0.05$ and $P < 0.005$, respectively), but not with terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL)-positive apoptotic tubular cells.

Conclusion. These data suggest that inducible expression of OPN in the tubular epithelium may have a pathogenic role in acute renal allograft rejection by mediating interstitial monocyte infiltration and possibly tubular regeneration.

Acute rejection produces significant monocytes accumulation and activation in the graft, which is supposed to be initiated by chemoattractants, including osteopontin

(OPN), and has a pivotal role in the pathologic process of rejection, acting directly or in concert with other arms of the immune system.

OPN is a secreted phosphoprotein that has a number of diverse biologic functions, including cell adhesion, migration, and signaling [1–3]. Originally isolated from bone, but is also produced in the kidney, hence, its alternative name uropontin. In rodent and human kidney, OPN is constitutively expressed by distal tubular epithelium [4–6]. OPN is a potent chemotactic molecule for macrophages in vivo [7], and its up-regulated expression by proximal tubular epithelial cells in association with monocyte/macrophage infiltrates has been described in a number of rodent models of renal injury [8–13], and in human renal diseases [14–16]. These studies have suggested that OPN is likely to be a critical mediator of inflammation in specific diseases and injury states.

OPN may also function as a cell survival factor, and may protect cells from undergoing apoptosis [17]. We have previously demonstrated a correlation between up-regulated OPN expression in proximal tubular epithelium and the proliferation and regeneration of tubular epithelial cells during the recovery phase of gentamicin-induced acute tubular necrosis [18]. Similar results have been shown in other toxic models [19] and ischemic/reperfusion models of renal injury [20–22], thereby lending further support for its renoprotective role.

To elucidate the pathogenic significance of OPN and its expression in acute renal allograft rejection, we examined by immunohistochemistry and in situ hybridization, renal biopsies from patients with acute rejection, protocol biopsies without rejection, and perioperative donor biopsies for intrarenal expression of OPN, and its correlation with clinical, laboratory, and histopathologic parameters.

METHODS

Patients

Of 90 consecutive renal transplants performed at Niigata University Hospital over the period (between

May 1996 and August 2002), 32 patients suffered at least one episode of clinical acute rejection in the first year after transplantation. Clinical acute rejection was suspected in cases with acute allograft dysfunction with normal or subtherapeutic levels of calcineurin inhibitors and normal findings by renal ultrasound. Of these, all patients who had undergone a renal biopsy within 7 days of the onset of acute allograft dysfunction with (1) pathologically confirmed acute rejection or borderline rejection according to the standardized criteria of Banff working classification of kidney transplant pathology and (2) adequate formalin-fixed tissue available for immunohistochemistry were included in this study ($N = 20$). The remaining cases (12 of 32) with clinical acute rejection were excluded because six patients had not undergone a renal allograft biopsy, three patients lacked the minimum criteria for borderline rejection or in whom cyclosporine toxicity was suspected, and three patients lacked adequate tissue to allow the studies described below. At the time of biopsy, 15 out of 20 patients were on triple-drug regimen, including cyclosporine or tacrolimus with mycophenolate mofetil, azathioprine or mizoribine and prednisolone. The other five patients were on dual therapy with tacrolimus and prednisolone. In 11 rejection cases, antirejection treatment was initiated before graft biopsy based on clinical suspicion.

In an attempt to stop steroid in renal transplant recipients with early uncomplicated clinical course, the practice to obtain protocol biopsies was introduced at Niigata University Hospital in January 2003. Until March 2004, 16 protocol biopsies were done. Among them, nine lacked the minimum criteria for borderline rejection, and served as a control group. The immunosuppressive regimen in the control group was comprised of cyclosporine or tacrolimus, mycophenolate mofetil, and prednisolone.

The study also included the perioperative biopsies of transplanted kidneys ($N = 35$) of the same patients with acute rejection, including 15 pairs of preimplantation biopsies and 1-hour postreperfusion biopsies of the same grafts and preimplantation biopsies of five additional grafts. Normal human kidney specimens ($N = 4$) were obtained from normal portions of kidneys resected for localized neoplasms. Biopsies were performed only after obtaining written informed consent from the patients.

Clinical data were gathered from our patient and pathology databases and review of medical records (e.g., age, warm ischemic time, total ischemic time, and immunosuppressive therapy). Serum creatinine level, and urinary protein excretion, of each patient were obtained at the time of biopsy. Additionally, serum creatinine level was obtained at two other points; the maximum serum creatinine within 1 week of renal biopsy, and at 3 months after the biopsy, an arbitrary date set up to signify a stable outcome.

Antibodies

To examine the hypothesis that different molecular forms of OPN, which may have diverse or even contrary functions in normal or pathologic conditions, may be detected by antibodies against different epitopes of OPN, we tried two monoclonal antibodies which recognize different epitopes of human OPN (IBL, Fujioka, Japan). O-17 is a rabbit IgG affinity-purified antibody directed against the N-terminal of human OPN. 10A16 is a mouse IgG1 antibody directed against a mid part of human OPN. Their specific recognition of OPN has been characterized by Western blotting [23]. Both antibodies demonstrated identical patterns of staining, we therefore chose one of these reagents, O-17, because of its specificity against the most active N-terminal fragment which contains the cell-binding arginine-glycine-aspartate (RGD) sequences to perform the immunohistochemical staining in this article.

E29 (Dako, Glostrup, Denmark) is a murine monoclonal IgG2a that is specific for epithelial membrane antigen (EMA). EMA is known to be expressed by distal convoluted tubules, collecting ducts, and the thick ascending limb of the loop of Henle, and was shown to be colocalized with OPN in human adult kidney [4].

PG-M1 (Dako) is a well-characterized murine monoclonal antibody directed against the CD68 epitope present on human monocytes and macrophages.

MIB-1 (Dako) is a well-established murine monoclonal antibody for the demonstration of Ki-67 antigen, a nuclear protein preferentially expressed during cell proliferation [24].

Immunohistochemistry

Serial sections of formalin-fixed, paraffin-embedded biopsies of 3 μm thickness were prepared. For immunohistochemistry to detect OPN, the sections were first deparaffinized and rehydrated, they were then heated in a 0.01 mol/L citrate buffer (pH 6) under microwave (5 minutes × 2) to unmask antigenicity. Subsequently, they were treated with normal goat serum (Chemicon, Temecula, CA, USA) at room temperature for 30 minutes to block nonspecific binding, and incubated with the primary antibody, O-17 at a dilution of 1:50 over night at 4°C. After washing with phosphate-buffered saline (PBS), OPN was detected using alkaline phosphatase enhanced detection kit (red) (Ventana Medical Systems, Tucson, AZ, USA).

Double immunohistochemistry was performed to detect OPN in combination with EMA, Ki-67 and CD68. Briefly, the sections were treated once more in a microwave oven, immersed in 3% H_2O_2 in methanol to block the residual endogenous peroxidase, and in case of CD68, incubated with trypsin (Wako Pure Chemical Industries, Osaka, Japan) for 30 minutes at 37°C. They were sequentially incubated with normal goat serum,

the primary antibody; either E29 (1:100), MIB-1 (1:50), or PG-M1 (1:1) overnight at 4°C, biotinylated goat antimouse secondary antibody, and the avidin-biotin-peroxidase [horseradish peroxidase (HRP)] complex (Ventana Medical Systems). The sections were then visualized with 3, 3′- diaminobenzidine (DAB) Dako, Carpinteria, CA, USA) to give a brown reaction product. The cellular nuclei of the sections were counterstained with hematoxylin, overslipped, and examined under light microscopy.

Detection of apoptotic cells

Rejection specimens were examined for apoptosis using terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) of fragmented DNA as described by Gavrieli, Sherman, and Ben-Sasson [25]. DNA labeling was performed using the TACS (Trevigen Apoptotic Cell System) 2 TdT/DAB kit for apoptosis detection in situ. Details are found in manufacturer's instructions (Trevigen, Gaithersburg, MD, USA). Briefly, paraffin-embedded sections were deparaffinized in xylene, rehydrated through graded concentration of ethanol, and washed with PBS for 10 minutes. To facilitate the penetration of enzymes and biotinylated deoxyuridine, the slides were subjected to 30 minutes of proteinase K (10 µg/mL) digestion, and washed in deionized water two times for 2 minutes. Endogenous peroxide was quenched by immersion in 3% H_2O_2 in 40% methanol for 5 minutes. Then, the sections were rinsed in TdT labeling buffer, incubated in the TdT labeling mixture at 37°C for 1 hour. The reaction was stopped and the sections were washed with PBS two times for 2 minutes. They were subsequently covered with peroxidase-labeled streptoavidin for 15 minutes, washed in PBS to remove unbound conjugate, and finally stained with DAB-H_2O_2 solution. Sections were counterstained with methyl-green for 1 minutes, and coverslips were mounted.

To confirm the staining specificity, the TUNEL procedure was modified as follows; for the positive controls, TACS-Nuclease was added to the labeling mix to generate DNA break in every cell. Negative controls included omission of TdT from buffer solution.

In situ hybridization

To amplify cDNA of human OPN coding region (903 bp) by polymerase chain reaction (PCR), two primers, 5′-ATGAGAATTGCAGTGATTTGC-3′ as a forward primer and 5′-CGTAGAAGACTCCAGTTAA TT-3′ as a reverse primer, were used. The PCR product was cloned into a pGEM-T cloning vector (Promega, Madison, WI, USA). The template was subsequently digested with *Nde*I, and ligated with T4-ligase to obtain OPN cDNA of 481 bp (from 422 to 903 bp).

The plasmid sample with the OPN cDNA (481 bp) was linearized with *Nco*I as a sense probe and *Nde*I as an antisense probe, respectively. In vitro transcription of the cDNA was done using a digoxigenin (DIG) RNA labeling kit (SP6/T7) (Roche Diagnostics, Penzberg, Germany). 500 ng of linearized plasmid was used as a template and incubated with T7 DNA-dependent RNA polymerase for 2 hours to obtain an antisense probe. A SP6 promoter was used to produce a sense probe, which was used as a negative control.

In situ hybridization was done by the automated mRNA in situ hybridization application (Ventana Medical System) as described in [26]. Briefly, serial sections were automatically deparaffinized, fixed, and acid treated. Then, the tissue sections were subjected to cell conditioning and protease digestion. Hybridization was performed with DIG-labeled OPN antisense probe (30 ng/slide) at 60 for 6 hours, followed by incubation with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) at 37°C for 1 hour, and the signal was detected using a nitro blue tetrazolium chloride 5-bromo-4-cloro-3-indolyl phosphate toluidine salt (NBT/BCIP) substrate solution for 3 hours.

Quantitative analysis

All counts and pathologic evaluations were performed on coded slides without prior knowledge of the clinical or histologic diagnosis. In each section, all fields of the renal cortex were counted on a 1 cm^2 eyepiece graticule with 10 × 10 equidistant squares. Under high magnification (×400), a minimum of 10 and a maximum of 20 consecutive nonoverlapping fields per section were counted (average measured area, 3.5 ± 0.75 mm^2). On double-staining sections, the percentage area of OPN-positive proximal (EMA-negative) and nonproximal (EMA-positive) tubular cell segments in the total area of proximal and nonproximal tubules were calculated, while the squares falling on glomeruli, Bowman's capsules, interstitium, or tubular lumen were excluded. In addition, the intensity of staining in proximal tubular segments was graded semiquantitatively, as described previously [27], with a scale of 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining, comparable to the observed intensity of staining in distal tubular segments.

On separate serial sections, the quantification for CD68-positive, Ki-67–positive, and apoptotic cells was undertaken. Under high magnification (×400) equivalent numbers of cortical graticule fields per section were examined, and the data are presented as the average number of CD68-positive cells per field; the percentage of Ki-67–positive tubular and interstitial cells relative to the total number of tubular and interstitial nuclei, respectively, and as the percentage of apoptosis-positive

Table 1. Baseline characteristics of the transplant recipients[a]

	Acute rejection (N = 20)	No rejection (N = 9)
Age *years*	34.7 ± 12.5	41.6 ± 13.2
Gender *Male/female*	15/5	5/4
Donor *living/cadaver*	19/1	9/0
Warm ischemic time *minutes*	6.7 ± 3.1	5.0 ± 1.3
Total ischemic time *minutes*	111.3 ± 101.1	94.9 ± 39.8
Number of biopsies/patients	22/20	9/9
Biopsy time *days after transplantation*	68.0 ± 83.2	55.0 ± 71.9
Serum creatinine at biopsy *mg/dL*	2.1 ± 1.9	1.2 ± 0.7
Maximum serum creatinine within a week of renal biopsy *mg/dL*	2.5 ± 2.3	NA
Serum creatinine 3 months after biopsy *mg/dL*	1.6 ± 0.8	1.1 ± 0.9
Urine protein at biopsy *g/day*	0.3 ± 0.3	0.2 ± 0.2
Banff category	Borderline = 11	<Borderline = 9
IA	7	
IB	4	

NA is non applicable.

[a]Plus-minus values stand for means ± SD were not significantly different between the two groups.

tubular cells. The correlation analysis for OPN and CD68 or Ki-67 was undertaken on double-staining immunohistochemistry sections for OPN protein and either CD68 or Ki-67 antigen.

Statistical analysis

Results were analyzed by a commercial software package, SPSS 11.5 for Windows. We used unpaired Student *t* test, Mann-Whitney U test, Kruskal-Wallis H test, Wilcoxon single-rank test, and linear regression analysis, as appropriate. *P* values less than 0.05 were used as the criteria of statistically significant differences.

RESULTS

Table 1 shows the clinical profile of patients with acute rejection versus control group. There was no significant difference between the two groups with regards to age, duration of ischemia, time of biopsy, serum creatinine, and urinary protein. As for the donors, there were five males and 15 females, aged 55.6 ± 10.8 years.

Expression of OPN protein and mRNA

To characterize the renal tubular epithelium, we carried out double immunohistochemistry for OPN and EMA as a marker of distal tubule. All renal biopsies (*N* = 70), including acute rejection, protocol, perioperative and normal biopsies, were scored for both the percentage of OPN-positive tubular area and the intensity of OPN immunostaining. Distal tubules served as an internal control for OPN immunostaining, as they constitutively express OPN protein, and did so in many, but not all, segments of the distal nephron in every tissue studied.

In our study, all of the 22 rejection biopsies showed tubulitis with interstitial infiltration but no vasculitis. The high number of borderline rejections (*N* = 11) is probably because many of those biopsies were obtained after antirejection treatment was initiated so that inflammatory changes may have diminished in individuals that did indeed have a significant rejection episodes [28].

In the majority of rejection biopsies that we examined, OPN expression by the proximal tubular epithelium was widespread, exhibited low-to-moderate signal intensity, and was predominantly observed in a distinct perinuclear pattern. The intensity of OPN immunostaining was remarkably high in the degenerated proximal tubular cell segments and in the tubules surrounded by numerous inflammatory cells. In addition, a few number of the infiltrating cells within the interstitium demonstrated positive OPN expression. Glomerular OPN expression was occasionally observed within the glomerular tuft and in the parietal epithelial cells lining Bowman's capsule. Two representative cases are shown in Figures 1A, and 2.

Quantitative analysis showed that OPN-positive area in the rejection biopsies was significantly higher than that of protocol, preimplantaion, postreperfusion, and normal biopsies, in both proximal and distal tubules. The signal intensity of OPN expression by proximal tubules, a rough measure of the amount of protein present, was significantly higher in the rejection biopsies when compared with that of the other groups (Fig. 3), suggesting that OPN expression was induced in the proximal and distal tubular epithelium in acute rejection.

Regression analysis showed no significant correlation between OPN expression and any of the three levels of serum creatinine, as outlined in the **Methods** section, or with the level of urinary protein excretion (rho = 0.05; *P* > 0.05). Although no correlation could be found between OPN expression and the pathologic grade of rejection, OPN expression by proximal tubules significantly correlated with the extent of interstitial inflammation but not with tubulitis (Fig. 4). There were no significant differences in the tubular expression of OPN, between biopsies from patients receiving cyclosporine (*N* = 8) or tacrolimus (*N* = 12) as a maintenance immunosuppressive therapy, or between biopsies from those with and without prior antirejection treatment.

The protocol biopsies used in this study had no prominent mononuclear inflammatory cell infiltrate or tubulitis. The perioperative biopsies generally had no specific pathologic abnormality except for the cadavaric biopsy specimen which showed features of acute tubular necrosis.

In the protocol and perioperative biopsies, OPN was uniformly expressed at high intensity by a subset of distal tubules, whereas no or weak expression of OPN by proximal tubules (Fig. 1C). Quantitative analysis showed
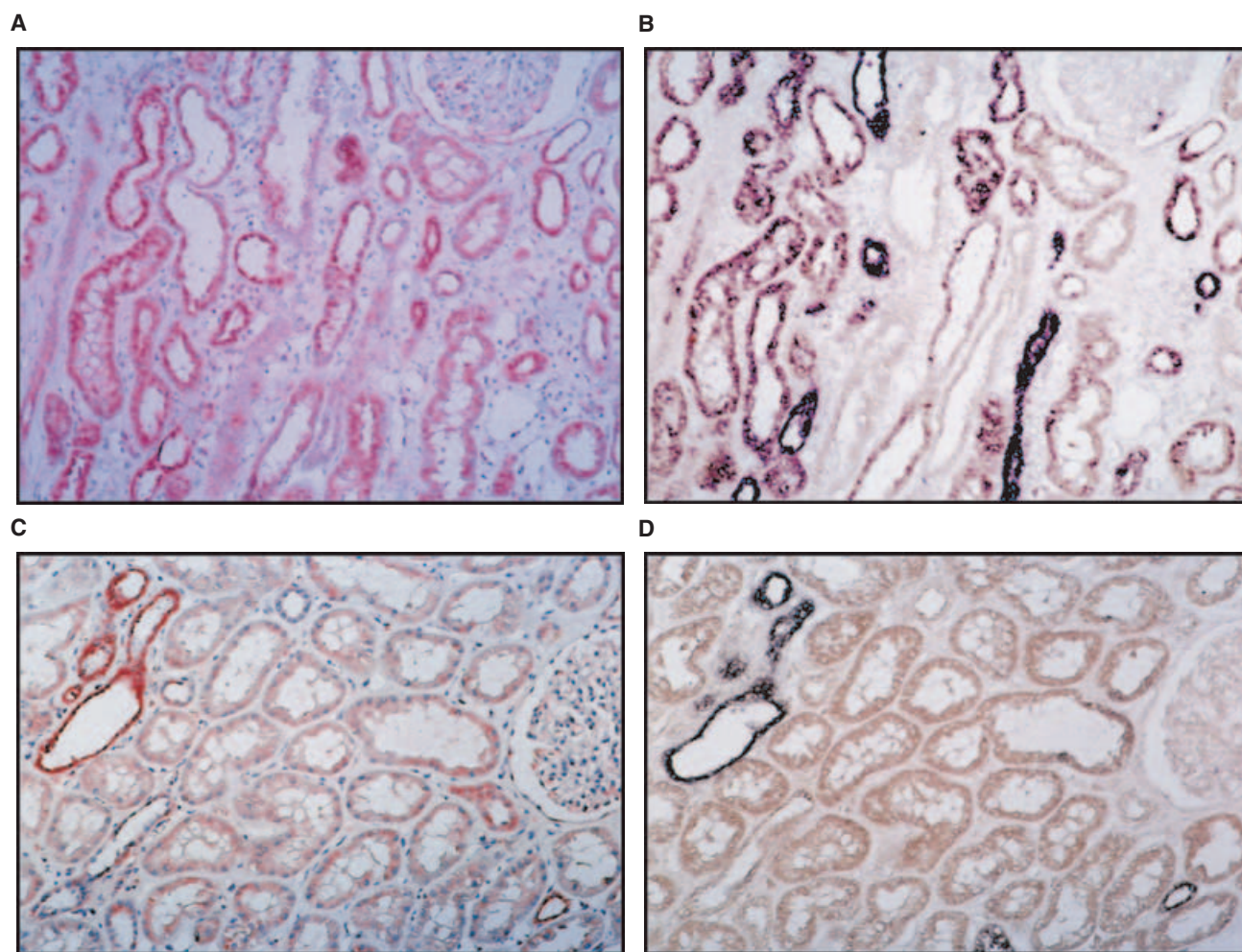
Fig. 1. Replicate tissue sections. (*A* and *B*) Sections with acute rejection demonstrating the colocalization of osteopontin (OPN) protein and mRNA by immunohistochemistry and in situ hybridization. OPN protein is visualized by alkaline phosphatase (red) and OPN mRNA is hybridized with digoxigenin-labeled OPN antisense probe. There is widespread expression of OPN by both proximal and distal tubules. Glomerular OPN expression can be seen within the glomerular tuft and in the parietal epithelial cells lining Bowman's capsule. (*C* and *D*) Sections from a protocol biopsy without rejection showing OPN protein expression generally confined to the distal tubules, corresponding to the patterns of OPN synthesis by in situ hybridization (magnification ×66).
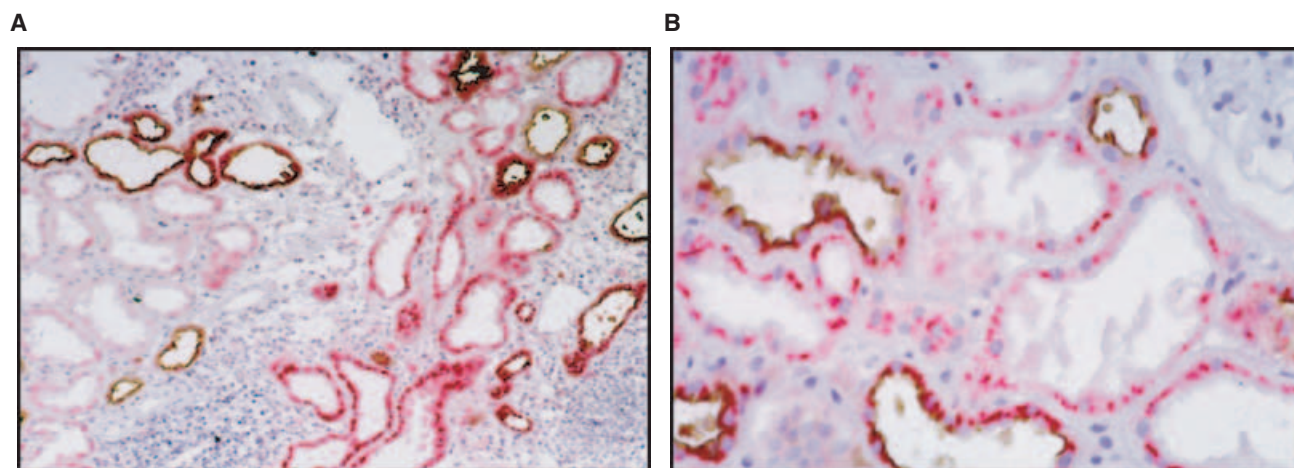

Fig. 2. Double immunohistochemistry. (*A*) Osteopontin (OPN) (red), and epithelial membrane antigen (EMA) (brown) in a rejection specimen, demonstrating strong OPN expression in the tubular segments surrounded by numerous inflammatory cells. (*B*) Higher power view of the same kidney (A), demonstrating the distinct perinuclear staining pattern of OPN [magnification ×66 (A) and ×160 (B)].
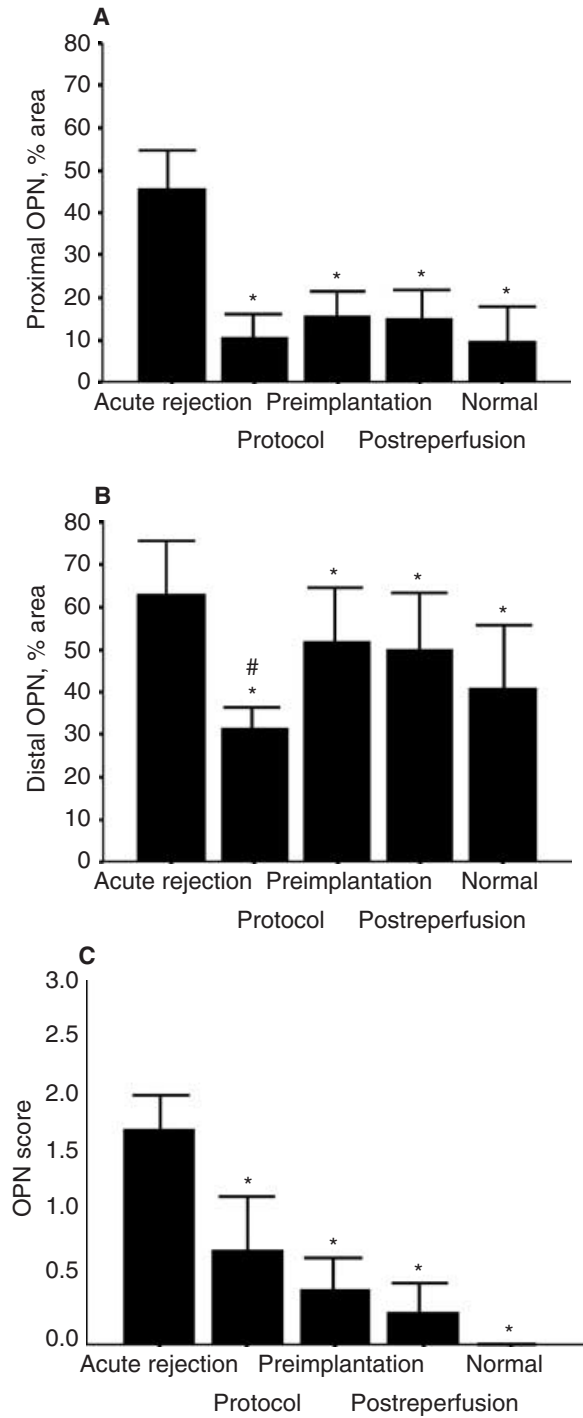
Fig. 3. Quantitative analysis of osteopontin (OPN) expression. (*A*) Proximal [epithelial membrane antigen (EMA)-negative] tubules. (*B*) Distal (EMA-positive) tubules. (*C*) OPN score in acute rejection (*N* = 22), protocol (*N* = 9), preimplantation (*N* = 20), postreperfusion (*N* = 15), and normal biopsies (*N* = 4). **P* < 0.05 versus acute rejection; #*P* < 0.05 versus preimplantation and postreperfusion.

Fig. 4. Quantitative analysis shows OPN expression. (*A*) Expression is not associated with the pathological grade of rejection. (*B*) Expression is significantly correlated with the degree of interstitial inflammation. (*C*) Expression is not correlated with tubulitis. NS is not significant.

that OPN-positive area in proximal tubules was not significantly different between protocol, perioperative, or normal biopsies. OPN-positive area in distal tubules was indeed significantly lower in the protocol biopsies when
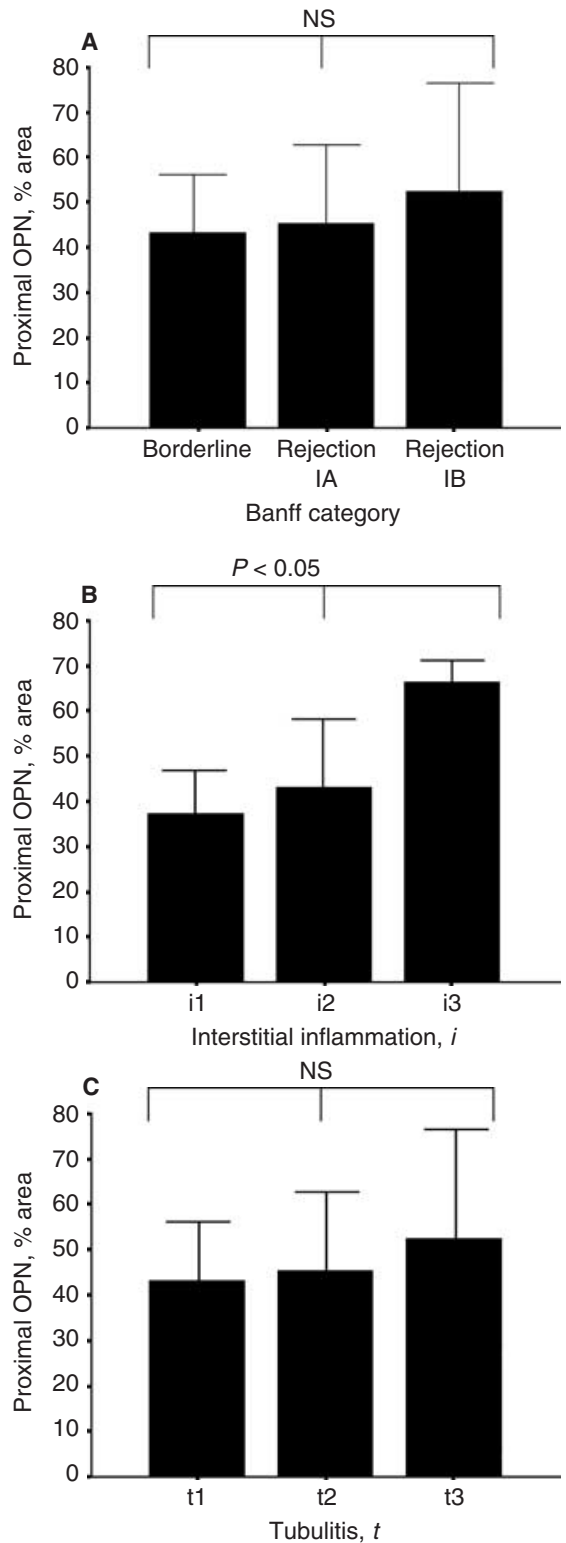
compared with that of perioperative biopsies but not with normal biopsies (Fig. 3B).

In the perioperative biopsies, regression analysis between OPN expression and donor age did not show a significant correlation. There was also no correlation between warm ischemic time and OPN expression in preimplantation biopsies or between total ischemic time and OPN expression in postreperfusion biopsies.

To confirm the colocalization of OPN, protein and mRNA replicate sections for immunohistochemistry and in situ hybridization were used. In all sections that we examined, results of in situ hybridization for OPN mRNA expression closely mirrored those seen by immunohistochemistry. As can be seen in Figure 1, the patterns of localization of OPN protein and mRNA were very similar. All sections examined with a sense probe were negative.

Correlation of OPN expression and macrophage infiltrate

To examine the relationship between OPN and monocyte/macrophage infiltration in acute rejection, we performed immunohistochemistry for CD68 and double-staining for OPN and CD68. In many cortical areas CD68-positive cells tended to be localized in close proximity with tubular segments that demonstrated positive OPN expression (Fig. 5A). The quantitative data on the extent of interstitial macrophage infiltration and OPN expression present in the rejection specimens used for this study are presented as a scattergram in Figure 5B. In individual tissue section, the area of OPN-positive tubular segments positively correlated with the degree of interstitial macrophage accumulation (rho = 0.546; $P < 0.01$).

Demonstration of Ki-67–positive cellular nuclei and its relationship with OPN

To clarify the relationship between OPN expression and cellular proliferation/regeneration, we carried out immunohistochemistry for Ki-67 and double-staining for OPN protein and Ki-67 antigen. Rejection biopsies were scored independently for the percentage of Ki-67–positive tubular and interstitial cells. In almost all acute rejection specimens that we examined, Ki-67–positive tubular cells were rare (mean 1.4%; range 0% to 6.3%), whereas Ki-67–positive interstitial cells varied considerably from 0.2% to 27% (mean 8.3%). Regression analysis showed that the number of Ki-67–positive tubular epithelial and interstitial cells was associated significantly with OPN expression by proximal tubules (rho = 0.529, 0.639; and $P < 0.05$, < 0.005, respectively) (Fig. 6A and B). The result of double-staining, however, demonstrated that the location of OPN expression had no distinct relationship with that of tubular or interstitial cell proliferation in most areas, but in some areas; the Ki-67–positive cells were ob-

served within or in the vicinity of OPN-positive tubules (Fig. 6C).

Demonstration of cellular apoptosis and its relationship with OPN

Apoptotic cells were detected in the tubular epithelia in 18 cases of acute rejection (82%), with an overall rate of 2.5%, and range of 0% to 10.6%. TUNEL-positive cells were almost exclusively observed in the distal tubular epithelial cells (Fig. 7), in contrast apoptosis was very rare in the proximal epithelial cells. Apoptosis was occasionally seen in the interstitial and glomerular compartment. There was no significant correlation between TUNEL-positive tubular cells and the extent of OPN expression by either proximal or distal tubules. No correlation could be found between TUNEL-positive tubular cells and the extent of allograft dysfunction or the pathologic grade of rejection.

DISCUSSION

OPN is well-known as a mediator of tubulointerstitial injury that accompanies glomerulonephritis [7, 8, 14–16]; whereas its significance in renal allograft rejection remains elusive. The present study clearly indicated that OPN (protein and mRNA) expression significantly enhanced in acute rejection, and was correlated with interstitial inflammation, macrophage infiltration, and cellular proliferation but not with apoptosis.

Hudkins et al [27] [abstract; Hudkins KL, *J Am Soc Nephrol* 11:A3497, 2000] found strong OPN protein and mRNA expression by tubular epithelium in pretransplant biopsies and in biopsies with cyclosporine toxicity without an inflammatory cell infiltration, though the number of donor biopsies available in their study was too small to calculate a correlation.

The strong OPN expression in donor biopsies has been assumed to be caused by ischemia, a known factor to induce OPN expression in renal proximal tubular epithelium [21, 29]. However, no data were available on the association between the duration of ischemia and the level of OPN expression.

In this study, we found no or weak expression of OPN protein and mRNA by proximal tubules in the majority of perioperative donor biopsies, and was independent to the ischemic time. This is simply because almost all of the studied donor biopsies, in contrast with those by Hudkins et al [27], were from living donors; therefore, the ischemic time was generally short. Constitutive expression of OPN by distal tubules was significantly higher in the perioperative donor biopsies as compared with that of protocol biopsies. This finding is unlikely to be attributable solely to the fact that constitutive OPN expression by distal tubules varies widely in human adult
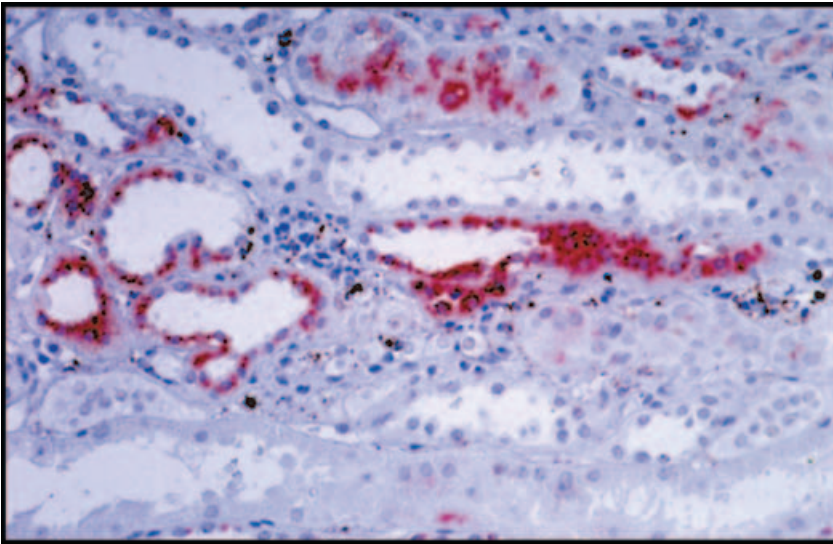
A

Fig. 5. Double immunohistochemistry. (*A*) Osteopontin (OPN) (red) and CD68+ macrophage (brown) in renal graft with acute rejection, demonstrating the CD68+ macrophages in close proximity with tubules expressing OPN (magnification ×100). (*B*) Regression analysis showed OPN expression in patients with acute rejection significantly correlated with CD68+ monocyte infiltration of adjacent interstitium.
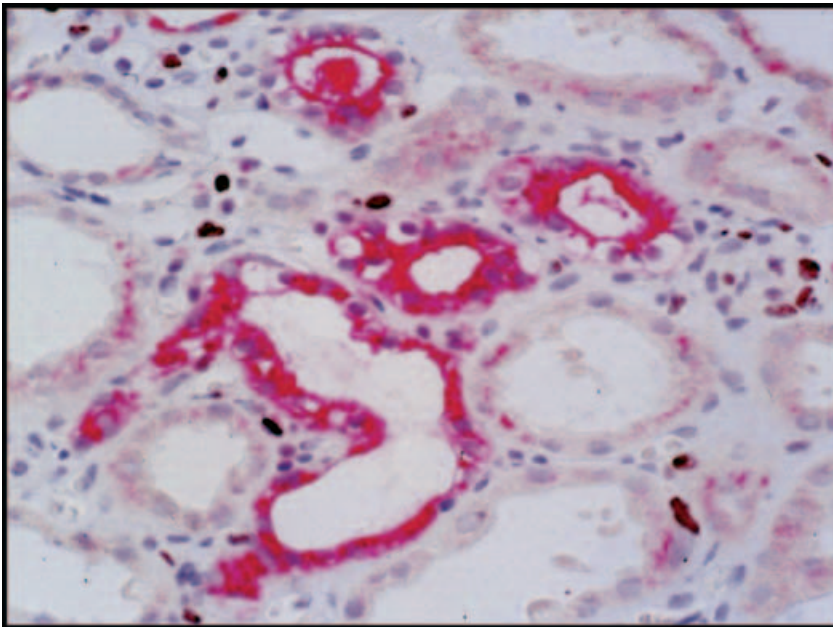
C

Fig. 6. Regression analysis shows significant correlation between osteopontin (OPN) expression. (*A*) Ki-67+ tubular cells, (*B*) Ki-67+ interstitial cells in patients with acute rejection. (*C*) Demonstrative case of acute rejection showing the location Ki-67+ cells (brown) was related to tubular OPN expression (red) in some areas (magnification ×100).

kidney [4]. Although no significant correlation could be found between ischemia time and the level of OPN expression by distal tubules in the donor biopsies, it is likely that the ischemia/reperfusion which might preferentially induce OPN in the distal tubules. In a rat model of renal ischemia, it has been shown that OPN expression by distal tubules rapidly increased, as it was already highly significant 12 hours after reperfusion, whereas proximal tubules showed a delayed response [21].

In the majority of rejection biopsies that we examined, the presence of increased OPN immunostaining that was accompanied by a concomitant OPN mRNA up-regulation, not only in distal but also in proximal tubular

cells indicate OPN gene induction in these cells with a possible role of OPN in acute rejection.

As the hallmark of acute rejection is tubulointerstitial inflammation, we hypothesize that some intrarenal proinflammatory cytokines act via autocrine/paracrine mechanism to stimulate OPN gene transcription and expression. The consistently observed up-regulation of OPN in areas of cellular infiltrate supports this hypothesis. Classic mediators of acute inflammation such as tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) strongly induce OPN expression [30, 31]. Cytokine mRNA analysis on human renal allograft biopsies by a PCR-based assay confirmed the presence of
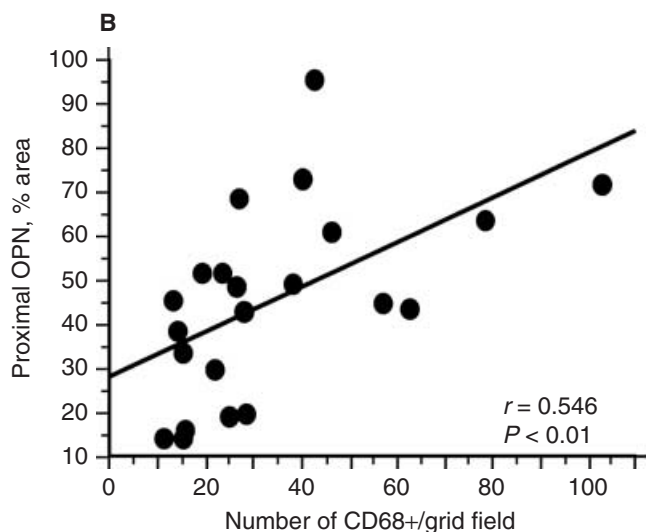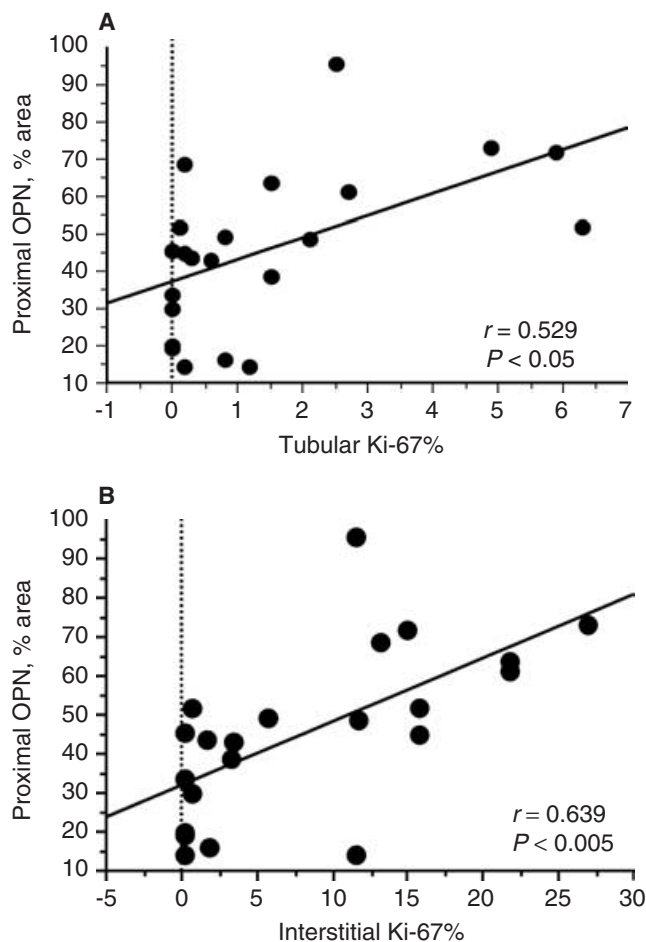
Fig. 5. (continued.)

Fig. 6. (continued.)

these two mediators in acute cellular rejection [32], while immunohistochemical studies demonstrate a marked TNF-α expression by infiltrating inflammatory cells and adjacent tubular cells in acute cellular rejection but not in normal kidney [33, 34]. Other potential culprit that can induce OPN up-regulation is endothelin-1 [35], which was shown to be up-regulated on the tubular epithelial cells in acute rejection [36, 37]. Taken together, these observations may explain the high level of OPN expression in acute rejection. Nonetheless, other factors such as ischemia may also be involved.

The Banff working classification of renal transplant pathology only focuses on the cellular inflammatory reactions, including interstitial cell infiltration, tubulitis, and arteritis. However, the extent of tubular cell injury is not fully addressed in this classification. In this study, the degenerated proximal tubular epithelial cells consistently showed strong expression of OPN, suggesting that OPN may have a complementary diagnostic value in assessing the tubular cell injury in acute rejection.

Acute rejection is predominantly a cell mediated process with CD4+ T lymphocytes playing a central role. CD4+ T cells differentiate into two distinct T-helper cell subsets, Th1 or Th2 cells, which have distinct profile of cytokine production and thus mediate distinct functions. Th1 cells are mainly involved in cell-mediated immunity, whereas Th2 cells are associated with humoral immunity [38]. OPN, by reacting with its receptors, influences the polarization of T-helper cells to the Th1 or Th2 phenotypes. OPN integrins interaction enhances Th1 whereas OPN-CD44 interaction inhibits Th2 cytokines expression [39, 40]. Cytokines secreted by the Th1 cells, such as interferon-γ (IFN-γ) and IL-2 play a critical role in graft rejection. Whether OPN directly affects those cytokines in acute rejection in vivo remains to be determined.

The function of OPN in acute rejection appears to extend beyond being merely proinflammatory. Our data showed significant relationship between OPN expression and tubulointerstitial cell proliferation/regeneration, suggesting a possible role of OPN in the repair of tubular injury. However, in contrast with our group's previous experimental model of acute tubular injury [18], double-staining for OPN and Ki-67 antigen did not show a close relationship in most areas. These contrasting results may be explained by the different models and phases of renal injury. In this study, biopsies were taken during the early phase of acute rejection; therefore most of the tubular cells have not yet been regenerating. Indeed most of the Ki-67–positive cells were in the interstitial compartment.

Apoptosis is a cellular phenomenon generally found within rejecting transplant [41]. Although the role and mechanisms of apoptosis during rejection of allograft kidneys are not known, it appears likely that some apoptotic effectors regulate the rejection process. The regulation of antiapoptotic and proapoptotic oncogenes may vary with
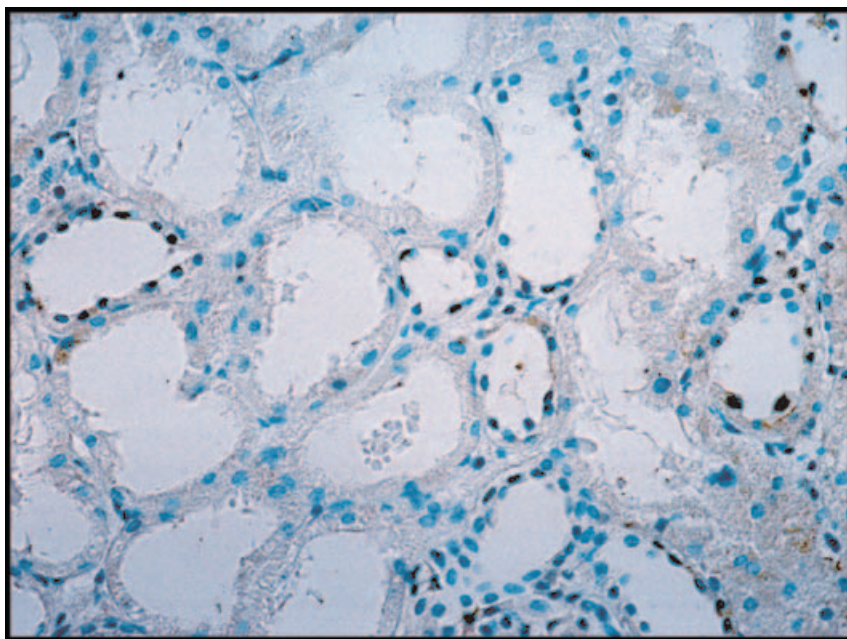
Fig. 7. A tissue section with acute rejection stained with a 2 terminal deoxynucleotidyl transferase (TdT)/3, 3′- diaminobenzidine (DAB) kit for apoptosis detection in situ, and methylene green counterstaining. Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) + signals (brown) were almost confined to the distal tubules, and were unrelated to interstitial cell infiltration (magnification ×100).

cell types during the acute and recovery phase of acute renal failure [42]. This may account for the frequently observed apoptosis in the distal but not the proximal tubules in acute rejection. OPN has antiapoptotic effect. In a recent study, ischemic kidneys from OPN knockout mice showed significantly enhanced apoptosis [43]. OPN expression did not correlate with the observed number of apoptotic cells, indicating that OPN is probably an irrelevant regulator of apoptosis in acute rejection.

The available data on the significance of OPN expression in human renal diseases are still limited. In IgA nephropathy, OPN has been shown to have a negative impact on the prognosis [16]. In membranous nephropathy, tubular expression of OPN was significantly higher in patients with progressive disease [15]. In these two kinds of chronic glomerulonephritis, the adverse prognostic significance of OPN expression is probably caused by its significant association with interstitial fibrosis. Overload of tubular cells with filtered proteins has been shown to induce OPN expression in the proximal tubular epithelium in rat remnant kidney model in vivo, suggesting that proteinuria may be a strong inducer of tubular OPN expression [44]. However, the correlation of proteinuria with OPN expression in human renal diseases is rather conflicting [16, 45]. We could not find significant correlation between OPN expression and renal function or urinary protein excretion. This discrepancy between the clinical and pathogenic significance of OPN expression may be resulted from the antirejection treatment, the biopsy timing, or because of the diverse biologic functions of OPN. The diversity of its function may limit the prospect of being a promising target for immunosuppressive therapy.

In the present study, we demonstrated the up-regulated tubular expression of OPN at both the protein and mRNA levels in biopsies from renal allograft with acute rejection. OPN expression was correlated with interstitial macrophage infiltration and cell proliferation in both the tubular and interstitial compartments, supporting the role of OPN in macrophage recruitment and the subsequent proliferation and regeneration of tubulointerstitial cells in acute rejection.

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