To examine whether the hMSC-positive nephrons are viable, hMSCs were developed in the Fabry mouse (22), which does not express the gene encoding α-gal A enzyme. This defect leads to the abnormal accumulation of glycosphinogolipid mainly in glomerular podocytes and tubular epithelial cells, leading to renal failure after birth. The hMSCs were injected into E9.5 Fabry mouse embryos and subjected to relay culturing to regenerate the kidney. Compared with the wild-type mouse (655.0 ± 199.6 nmol per mg per hour), the basal level of α-gal A bioactivity in the metanephros from the Fabry mouse is low (19.7 ± 5.5 nmol per mg per hour), whereas chimeric kidney primordia expressed significantly higher amounts of α-gal A bioactivity (204.2 ± 98.8 nmol per mg per hour, $P < 0.05$, Fig. 5*a*). Furthermore, accumulation of the glycosphinogolipid Gb3 within the ureteric buds and S-shaped bodies (Fig. 5*b Right*) in the metanephros of the Fabry mouse was markedly resolved by integration of the hMSC-derived nephrons that possess α-gal A and act to normalize substrate metabolism in the surrounding host cells (Fig. 5*b Center*). This result indicates that the neogenerated nephrons were biologically viable.

In this study, we show that allowing hMSCs to grow in a specific organ location in whole-embryo culture can commit them to the fate of that organ. Injection of GDNF-transfected hMSCs into embryos followed by relay culture enables the generation of chimeric kidneys. In some cases, entire nephrons are hMSC-derived. These hMSC-derived cells are functional as tested by their ability to metabolize Gb3.

Thus, hMSCs could be reprogrammed for other fates and organ structures, depending on the embryonic environment into which they are. An added advantage of using hMSCs is that although they are mesodermal in origin, they have the potential to differentiate into cell types that are normally derived from ectoderm or endoderm (23). Thus, it might be possible to reconstitute organs such as liver and pancreas that, unlike the kidney, are derived from the endodermal germ layer. Furthermore, by changing the conditions of organ culture after the initiation of organ development during whole-embryo culture, a specific cell or simple-structured tissue, such as an endocrine gland, may be generated from autologous MSCs. Importantly, the host immune system is not sufficiently developed during this stage of whole-embryo culture, thus facilitating the tolerance to xenogenic cells. Here, we have demonstrated a system that might provide the means to generate self-organs from autologous MSCs by using the inherent developmental system of an immunocompromised xenogeneic host.

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# Leukemia inhibitory factor induces multi-lineage differentiation of adult stem-like cells in kidney via kidney-specific cadherin 16

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**Abstract**

Side population (SP) is reported to be a stem cell-rich population. In the presence of leukemia inhibitory factor (LIF), cultured kidney SP cells differentiated into multi-lineage in collagen gel but not in synthesized polymer that has no cell adhesion factor. In cultured kidney SP cells, gene expression of kidney-specific cadherin 16 was specifically upregulated in collagen gel but not in synthesized polymer. Moreover, decreasing cadherin 16 expression using siRNA abolished LIF-induced multi-lineage differentiation of kidney SP in collagen gel. These results indicated that LIF induced multi-lineage differentiation of adult stem-like cells in kidney via cadherin 16.


*Keywords:* Stem cell; Regenerative medicine; Cadherin 16

Existence of adult stem cells is reported in various kinds of tissues, and the so-called side population (SP) is reported to be a stem cell-rich population [1–4]. SP cells exist in various kinds of tissues [5], but the mechanism of multi-lineage differentiation has not been clarified for each type of tissue-derived SP cell, especially in the kidney [6]. In this study, we cultured kidney SP cells both in type I collagen gel and thermoreversible gelation polymer (TGP) that has no cell adhesion factor, and compared differentiation of the cells. Leukemia inhibitory factor (LIF) induced multi-lineage differentiation of kidney SP cells in collagen gel but not in TGP. To clarify the key adhesion factor that determined LIF-induced multi-lineage differentiation of kidney SP cells, DNA microarray analysis was performed. Microarray analysis clarified that kidney-specific cadherin 16 [7] was specifically upregulated in collagen gel but not in TGP. Finally, we examined the role of cadherin 16 in LIF-induced multi-lineage differentiation of kidney SP cells by decreasing cadherin 16 expression using siRNA. Our results showed that cell adhesion factor such as cadherin 16 is required to induce multi-lineage differentiation of adult stem-like cells such as SP cells in the kidney.

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## Materials and methods

*SP cell sorting and culture.* C57/B6 mice (8 weeks of age) were purchased from Clea Japan (Tokyo, Japan). All the procedures described here were approved by the Animal Committee of the University of Tokyo. Mice were anesthetized and perfused via the abdominal aorta with normal saline. The kidneys were harvested and the tissue was minced with a razor blade and digested with collagenase. The cell suspensions were filtered through a cell-strainer (Falcon 2350) to remove debris. The filtrates were analyzed as previously described [4]. Briefly, reserpine was added at a final concentration of 50 μM [4], and cells were then incubated at 37 °C for 15 min and Hoechst dye was added. Kidney SP cells were isolated using a FACS Vantage (BD Biosciences) for flow cytometric sorting. After FACS sorting, kidney SP cells were cultured on MEF feeder cells (RPMEF-N: Dainippon Pharmaceutical, Osaka, Japan) for 7 days with RES101 medium (Dainippon Pharmaceutical, Osaka, Japan) containing leukemia inhibitory factor (LIF: 10 ng/ml). After pre-culture on MEF feeder cells for 7 days, the kidney SP cells were re-seeded in type I collagen gel (Koken, Tokyo, Japan) or thermoreversible gelation polymer (TGP) with RES101 medium until day 28. TGP was purchased from Mebiol (Tokyo, Japan). In Figs. 2B–D, cells just after FACS sorting were cultured in collagen gel or TGP gel for 48 h in the presence or absence of LIF.

*Microarray and real-time PCR analysis.* DNA microarray hybridization experiments were performed using Clontech Atlas glass Mouse 3.8 I microarray (BD Biosciences) according to the manufacturer's protocol. The protocol and a complete list of genes can be viewed at http://www.bdbiosciences.com/clontech/techinfo/manuals/index.shtml. The DNA arrays were scanned using GenePix4000A [8]. Quantitative real-time PCR was performed using commercially available TaqMan probes (AQP2: Mm00437575 m1, CD31: Mm00476702 m1, neurofilament: Mm00456201 m1, and GATA4: Mm00464689 m1), and analyzed on an ABI PRISM 7000 sequence detector system (BD Biosciences). Quantitative values were obtained from the threshold PCR cycle number at which an increase in signal associated with exponential growth of the PCR product started to be detected. The relative mRNA levels in each sample were normalized to its GAPDH content.

*RNA interference.* The siRNA for cadherin 16 (pre-designed siRNA, ID#60533) was purchased from Ambion. Transfection was carried out using Qiagen RNAi Starter Kit. 14.5 ml of 20 mM siRNA and 33.75 ml of transfection reagent were added to a medium in a total volume of 250 ml, and left for 15 min at room temperature to allow the formation of transfection complexes. Subsequently, the mixture was diluted with a type I collagen gel to a total volume of 1 ml, and the FACS sorted kidney SP cells were incubated in the gel with LIF-containing culture medium for 48 h.

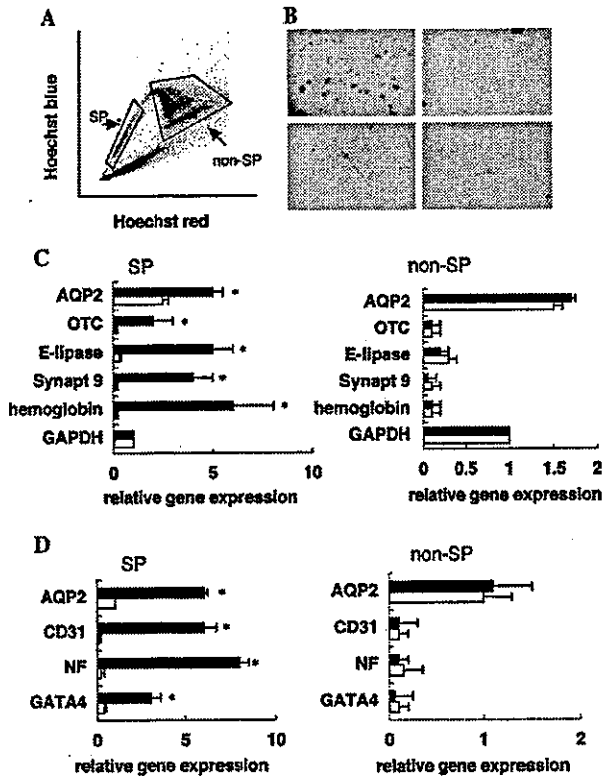


Fig. 1. LIF-induced multi-lineage differentiation of kidney SP cells in collagen gel. (A) Representative FACS profile of SP cells and non-SP cells isolated from the kidney. (B) Representative photographs of cultured kidney SP cells in collagen gel (left upper and lower panels) and TGP gel (right upper) on day 28. Upper panels are low power (40×: phase contrast) and others are high power (400×: phase contrast) photographs. (C) Results of microarray analysis of several lineage-specific genes in cultured kidney SP cells and non-SP cells on day 28 (black bars) compared to day 7 (white bars). Hemoglobin: hemoglobin β-chain, Synapt 9: synaptotagmin, E-lipase: endothelial lipase, OTC: ornithine decarbamylase, and AQP2: aquaporin 2. Expression was normalized to that of GAPDH. Values represent means ± SEM. $^*p < 0.05$ vs. day 7. (D) Results of quantitative PCR of representative genes of three germ layers (ectoderm: NF; mesoderm: CD31, AQP2; and endoderm: GATA4). Closed bars are day 28 and open bars are day 7. Values represent means ± SEM ($n = 4$). $^*p < 0.05$ vs. day 7.

## Results

### *LIF-induced multi-lineage differentiation of kidney SP cells in collagen gel*

We isolated whole kidney cells from C57/B6 mice and stained them with Hoechst 33342 dye [9]. The isolated cells were subjected to FACS analysis and immediately used for cell culture (Fig. 1A). Kidney SP cells attached and formed colonies on a mouse MEF feeder layer, and the colonies increased in size until around day 7 but for some reason stopped growing, and then we re-seeded the cells in type I collagen or TGP gel on day 8. On day 28, kidney SP cells formed island-like colonies and tube-like structures in collagen gel (Fig. 1B, left upper panel and lower panels). On the other hand, kidney SP cells never formed such structures in TGP gel not containing cell adhesion factor (Fig. 1B, upper right panel). To examine the multi-lineage differentiation of kidney SP cell in collagen gel and TGP gel, we compared the gene expression of culture SP cells on day 28 with that on day 7 by microarray analysis. Gene expression of several lineage-specific genes such as hemoglobin β-chain [10] (erythrocyte), endothelial lipase [11] (endothelial cell), synaptotagmin [12] (neuron), and ornithine decarbamylase [13] (liver) was very low on day 7, but was significantly upregulated in collagen gel culture on day 28 (Fig. 1C). On the other hand, these genes showed no change in TGP gel culture (Fig. 1C). To examine the

differentiation of kidney SP cells into three germ layers, we performed quantitative real-time PCR analysis of representative genes of the three germ layers (ectoderm: neurofilament (NF); mesoderm: CD31, aquaporin 2 (AQP2); and endoderm: GATA4). In collagen gel culture on day 28, all genes were significantly upregulated as compared to day 7 (Fig. 1D). As in the case with microarray analysis, there was no significant change in gene expression of these genes in TGP gel culture (Fig. 1D).

### *LIF-induced multi-lineage differentiation was mediated via cadherin 16*

To clarify the mechanism of LIF-induced multi-lineage differentiation of kidney SP cells in collagen gel, but not in TGP gel, we tried to clarify the contribution of cell adhesion factors such as cadherins. Compared to TGP gel culture, cadherin 16 was specifically upregulated in collagen gel with LIF on day 28 (Fig. 2A). Next, we examined the effect of LIF on cadherin 16 expression in cultured kidney SP cells by real-time PCR. Treatment with LIF significantly upregulated cadherin 16 in kidney SP cells cultured in collagen gel but not in TGP gel (Fig. 2B). On the other hand, treatment with LIF showed no effect on kidney non-SP cells (Fig. 2B). Finally, we examined whether LIF-induced multi-lineage differentiation is mediated by cadherin 16, we treated kidney SP cells and non-SP cells with siRNA for cadherin 16 and cultured the cells in collagen gel in the presence of LIF. As shown in Fig. 2C, LIF-induced multi-lineage differentiation was significantly inhibited by pre-treatment with siRNA in SP cells. In non-SP cells, LIF alone showed minimum effect and siRNA showed no significant effect (Fig. 2C).

## Discussion

Our present results demonstrated that LIF induced multi-lineage differentiation of adult stem-like cells such as SP cells in the kidney via kidney-specific cadherin 16. Cadherin 16 is a unique, tissue-specific member of the cadherin family of cell adhesion proteins that is expressed in the adult kidney and developing genitourinary tract [7,14]. During embryonic development, cadherin 16 is expressed in developing renal tubules in the metanephros and the expression of cadherin 16 is developmentally regulated as well as tissue-specific. Recently, a gene that is homologous to cadherin 16 has been identified in zebrafish, and several studies suggested that cadherin 16 may function as a cell adhesion protein that is required for maintaining tubular integrity [15]. Moreover, Yoshino et al. [16] reported that leukemia inhibitory factor (LIF) is upregulated in ARF, and is considered to play a role for regeneration process. Although the precise functional role of cadherin 16 remains to be determined, our results strongly suggest that cadherin 16 may play an important role in LIF-induced regenerative processes as in developing processes.

TGP gel is a chemically synthesized hydrophilic 3D culture material, which is composed of poly(*N*-isopropylacrylamide-co-*n*-butyl methacrylate) and polyethylene glycol (PEG), and acts as a hydrophilic 3D scaffold without adhesion factors. Recently, we have found that osteogenic differentiation of human mesen-

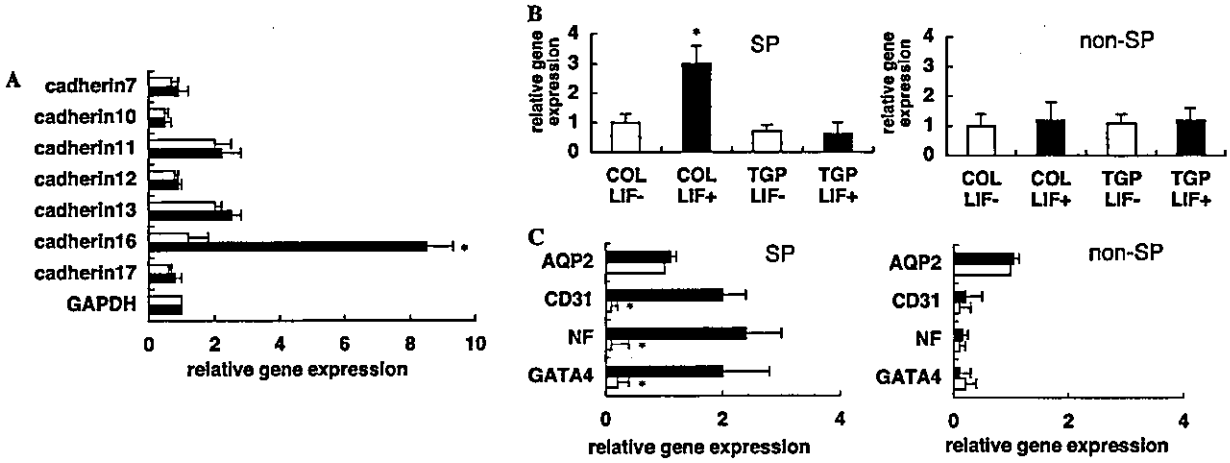


Fig. 2. (A) Microarray analysis of cadherins in cultured kidney SP cells in collagen gel (closed bars) and TGP gel (open bars) on day 28. Average expression levels of each gene were calculated from three independent hybridizations. Expression was normalized to that of GAPDH. Values represent means ± SEM. *$p < 0.05$ vs. TGP gel. (B) Results of quantitative PCR analysis of cadherin 16 in kidney SP cells and non-SP cells cultured in collagen gel or TGP gel. Values represent means ± SEM ($n = 4$). *$p < 0.05$ vs. COL LIF. (C) Results of quantitative PCR analysis of representative genes of three germ layers (ectoderm: NF; mesoderm: CD31, AQP2; and endoderm: GATA4). Closed bars are LIF alone and open bars are LIF and siRNA. Values represent means ± SEM ($n = 4$). *$p < 0.05$ vs. LIF alone.

chymal stem cells (HMSC) was augmented in TGP culture but not in collagen gel [17]. Compared to bone marrow, kidney tissue is rich in extracellular matrix, and this may explain why kidney SP cells required adhesion factors such as cadherin 16 for multi-lineage differentiation. The structure of TGP makes it possible to vary the diffusive speed according to the character of the molecules [17]. TGP may be able to maintain a high local concentration of hydrophobic molecules and hydrophilic large molecules, and this creates a concentration gradient of each molecule. Our results showed that cadherin 16 played key roles in LIF-induced multi-lineage differentiation but it may be possible that TGP may keep high local concentration of LIF-induced unknown factors that can promote multi-lineage differentiation.

Although the physiological function of cadherin 16 is still unclear, the promoter of the mouse cadherin 16 is recently clarified [14,18]. The promoter is TATA-less but contains other consensus promoter elements including an initiator element, GC boxes, and a variant CAAT box as well as potential binding sites for activator protein (AP)-2, hepatocyte nuclear factor (HNF)-1, HNF-3, basic helix–loop–helix (bHLH) proteins, and GATA factors. LIF is a member of the interleukin 6 (IL-6) family and IL-6 induces AP-2 in kidney mesangial cells [19]. These results suggest that LIF may induce cadherin 16 via induction of AP-2 in cultured kidney SP cells in collagen gel.

In summary, our results demonstrated that LIF induced multi-lineage differentiation of adult stem-like cells such as SP cells in the kidney via kidney-specific cadherin 16. These results suggest a new functional role of kidney-specific cadherin 16 in regenerative processes of kidney disease.

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ORIGINAL ARTICLE

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# Association of a single-nucleotide polymorphism in the immunoglobulin $\mu$-binding protein 2 gene with immunoglobulin A nephropathy

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**Abstract** Immunoglobulin A (IgA) nephropathy is the most common form of primary glomerulonephritis worldwide. The pathogenesis of IgA nephropathy is unknown, but it is certain that some genetic factors are involved in susceptibility to the disease. Employing a large-scale, case-control association study using gene-based single-nucleotide polymorphism (SNP) markers, we previously reported four candidate genes. We report here an additional significant association between IgA nephropathy and an SNP located in the gene encoding immunoglobulin $\mu$-binding protein 2 (IGHMBP2) at chromosome 11q13.2–q13.4. The association ($\chi^2 = 17.1$, $p = 0.00003$; odds ratio of 1.85 with 95% confidence interval of 1.39–2.50 in a dominant association model) was found using DNA from 465 affected individuals and 634 controls. The SNP (G34448A) caused an amino acid substitution from glutamine to lysine (E928K). As the gene product is involved in immunoglobulin-class switching and patients with the A allele revealed higher serum levels of IgA ($p = 0.048$), the amino acid change might influence a class switch to increase serum IgA levels, resulting in a higher risk of IgA nephropathy.

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## Introduction

Immunoglobulin A (IgA) nephropathy, originally described by Berger and Hinglasis (1968), is now recognized as the most common form of primary glomerulonephritis worldwide. This disease is defined as a process of proliferative changes in glomerular mesangial cells and increases in mesangial matrices associated with deposition of IgA. At first, IgA nephropathy was considered to be a mild clinical manifestation with a relatively good prognosis. However, since recent reports have indicated that 20–30% of patients develop end-stage renal disease within 10–30 years after the onset of the disease (Koyama et al. 1997), its prognosis is now regarded as more serious than previously thought.

The pathogenesis of IgA nephropathy is unknown, but accumulating evidence suggests that genetic factors are involved in disease susceptibility (Donadio and Grande 2002). For example, the prevalence of IgA nephropathy seems to reflect demographic and ethnic characteristics of the populations studied; furthermore, several cases of familial IgA nephropathy have been reported (Scolari 2003).

Recent approaches to identify disease-susceptibility genes have focused on genome-wide analysis of associations using single-nucleotide polymorphisms (SNPs). SNPs are likely to be useful as markers for identification of genes involved in complex diseases because they are the most common type of genetic variation in human DNA (Kruglyak 1999). We have been screening gene-based SNPs on a genome-wide scale to detect possible associations with susceptibility to IgA nephropathy and have already reported that specific SNP alleles in the L- and E-selectin (*SELL* and *SELE*) genes on chromosome 1q24–25 (Takei et al. 2002), the *HLA-DRA* locus at 6p21.3 (Akiyama et al. 2002), and the polymeric immunoglobulin receptor (*PIGR*) gene at 1q31–q41 (Obara et al. 2003) were candidate susceptibility factors for IgA nephropathy in the Japanese population.

Through an extension of those genome-wide analyses, we have now identified an additional association with IgA nephropathy in the Japanese population involving SNPs in the immunoglobulin μ-binding protein 2 (*IGHMBP2*) gene.

## Materials and methods

### DNA samples

This study enrolled 465 Japanese individuals with IgA nephropathy who were referred to several hospitals in Japan (Department of Medicine, Kidney Center, Tokyo Women's Medical University; Department of Urology, Iwate Medical University; Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences; Iwate Prefectural Ofunato Hospital; Sanai Hospital; Osaka City General Hospital; Toride Kyodo General Hospital; and Osaka Rosai Hospital). IgA nephropathy was diagnosed on the basis of clinical manifestations as well as renal-biopsy findings. Patients with Henoch–Schönlein purpura and secondary IgA nephropathy, such as hepatic glomerulosclerosis, were excluded from the analysis. Peripheral blood samples were obtained for DNA extraction. As controls, we analyzed DNA from 634 volunteers from the general Japanese population. Written informed consent was obtained from all participants, and DNA was prepared from each blood sample according to standard protocols. We are given approval for the study using human materials from our committee dealing with ethics for medical research.

### Multiplexed polymerase chain reactions

We amplified multiple genomic fragments using 20 ng of genomic DNA for each polymerase chain reaction (PCR), as described previously (Ohnishi et al. 2001). Sequences of the primers used in this study are available from the JSNP database (http://snp.ims.u-tokyo.ac.jp/). Each PCR was performed in a 20-μl solution containing 50 pmol of each primer, 10U Ex-*Taq* DNA polymerase (TaKaRa), and 0.55 μg *Taq*Start (Clontech Laboratories) in a GeneAmp PCR system 9700 (Applied Biosystems). Initial denaturation was performed at 94°C for 2 min, followed by 37 cycles of amplification at 94°C for 15 s and annealing at 60°C for 45 s, with final extension for 2 min at 72°C.

### Genotyping

For a new genome-wide association study, we selected 88,148 SNPs described in our JSNP database (Haga et al. 2002). All genotyping was done by the Invader assay, which combines a structure-specific cleavage enzyme with a universal fluorescent resonance energy transfer (FRET) system (Mein et al. 2000). FRET probes were labeled with either FAM or VIC corresponding to each allele. Signal intensity was indicated as the ratio of FAM or VIC to ROX, an internal reference. Each 5-μl reaction contained 0.25 μl of signal buffer, 0.25 μl of each FRET probe, 0.25 μl of the structure-specific cleavage enzyme, 0.25 μl of allele-specific probe mix, and 2 μl of the PCR product diluted 1:10. Samples were incubated in a GeneAmp PCR system 9700 (95°C for 5 min and then at 63°C for 15 min) and were analyzed on an ABI Prism 7700 sequence detector.

### Statistical analysis

The genotype distribution and allele frequencies of each selected SNP were compared between cases and controls by means of chi$^2$ tests. Significance was judged according to the guidelines of Lander and Kruglyak (1995). Fisher's exact test was used when criteria for the chi-squared test could not be applied. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated by Woolf's method. Hardy–Weinberg equilibrium was assessed by chi-squared statistics (Nielsen et al. 1998). Linkage disequilibrium (LD) coefficients were calculated and expressed as $D' = D/D_{max}$ (Devlin and Risch 1995). Clinical data were recorded as means ± SD. Statistically significant differences were assessed with Student's *t* tests using Stat View SE.

## Results

We performed this genome-wide association study in a stepwise manner. First, we genotyped 94 Japanese patients with IgA nephropathy for 88,148 gene-based SNPs selected from our database (Haga et al. 2002) by means of the high-throughput multiplex PCR-Invader assay method (Ohnishi et al. 2001). A comparison of the allelic frequencies among these 94 patients with frequencies calculated for 634 members of the general Japanese population identified 2,109 SNPs that revealed *p* values of 0.01 or smaller. We then genotyped these 2,109 SNPs using DNAs of 94 additional patients (Fig. 1) and found that 581 of them still revealed *P* values of 0.01 or smaller.

When these 581 polymorphisms were analyzed for all 465 cases, 13 of the SNPs, including those reported previously in the HLA-DRA gene, revealed *p* values of 0.0001 or smaller. One of the SNPs showing relatively more significant associations was located in exon 14 of the immunoglobulin $\mu$-binding protein 2 (IGHMBP2) locus on chromosome 11q13.2–q13.4. This SNP, G34448A, was associated with IgA nephropathy with $\chi^2 = 17.1$, $P = 0.00003$ (odds ratio = 1.85, 95% CI = 1.39–2.50) by a dominant association model (Table 1). The G to A substitution at nucleotide 34448 changed glutamine to lysine (E928K) (Fig. 2).

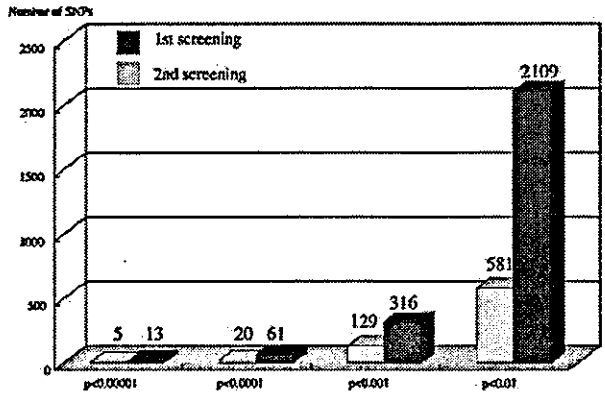


Fig. 1 Results of the first and second screening experiments. First screening (94 cases), $P < 0.01$: 2,109/88,148 SNPs; second screening (188 cases), $P < 0.01$: 581/2,109 SNPs. By the increase of the number of the cases examined, most of the SNPs lost their association

We subsequently carried out LD mapping of 21 SNPs with minor allele frequencies of > 20% that were located in the *IGHMBP2* region; this genomic fragment included six other genes (*MTL5*, *CPT1A*, *MRPL21*, *MRGPRD*, *MRGPRF*, *and TPCN2*). Mapping procedures separated these 21 SNPs into three LD blocks; the candidate SNP was located in the LD block containing two genes, *MRPL21* and *IGHMBP2*. When we genotyped patients with IgA nephropathy for the eight SNPs present in that LD block, G34448A in exon 14 of *IGHMBP2* showed the most significant association with the disease (Fig. 3).

Since individuals having the AA or GA genotype (homozygotes for the minor allele, or heterozygotes) of G34448A were likely to have a higher risk of IgA nephropathy ($P = 0.00003$) in a dominant manner, we compared available clinical data at the time of biopsy for patients with A alleles (AA and GA genotype) with those with the GG genotype (Table 2). We found no significant differences with respect to gender, age, levels of serum creatinine or 24-h urinary excretion of protein, but observed higher serum IgA levels in patients with AA and GA genotypes than in subjects with the GG genotype ($P = 0.048$).

Table 1 Genotype frequencies and association tests of the SNP in the *IGHMBP2* gene (465 cases of IgA versus 634 control)

| | IgA number (%) | Control number (%) | |
|---|---|---|---|
| Major allele | 798 (85.8%) | 1162 (91.6%) | |
| Minor allele | 132 (14.2%) | 106 (8.3%) | |
| Total | 930 (100%) | 1268 (100%) | |
| Major homozygous | 342 (73.5%) | 531 (83.8%) | |
| Heterozygous | 114 (24.5%) | 100 (15.8%) | |
| Minor homozygous | 9 (1.9%) | 3 (0.47%) | |
| Total | 465 (100%) | 634 (100%) | |
| | $\chi^2$ | *P* | Odds ratio (95%CI) |
| Allele frequency (minor versus major) | 18.9 | 0.00001 | 1.81 (1.38–2.38) |
| Other versus major homozygous | 17.1 | 0.00003 | 1.85 (1.39–2.50) |
| Minor homozygous versus other | 5.3 | 0.0353[a] | 3.90 (1.03–14.77) |

[a]Fisher's exact test

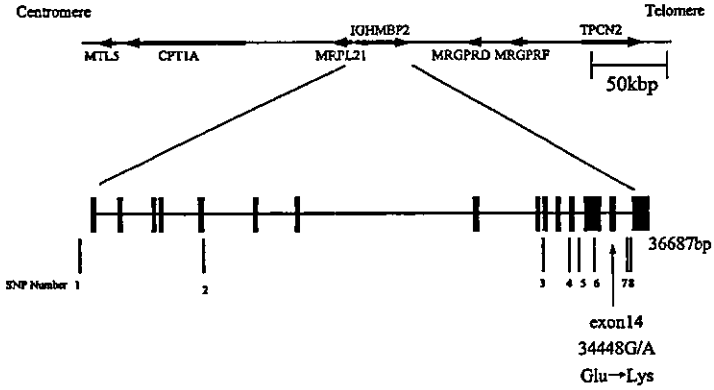


**Fig. 2** Genomic structure of chromosome 11q13.2–q13.4 around the *IGHMBP2* gene. *IGHMBP2* gene contains 36687 bp and 15 exons. The 34448G/A SNP lies in exon 14 of *IGHMBP2* (*upward arrow*). The G to A substitution at nucleotide 34448 changed an amino acid from glutamine to lysine (E928K); exons are indicated by *black rectangles*. The SNP number is the same as Fig. 3

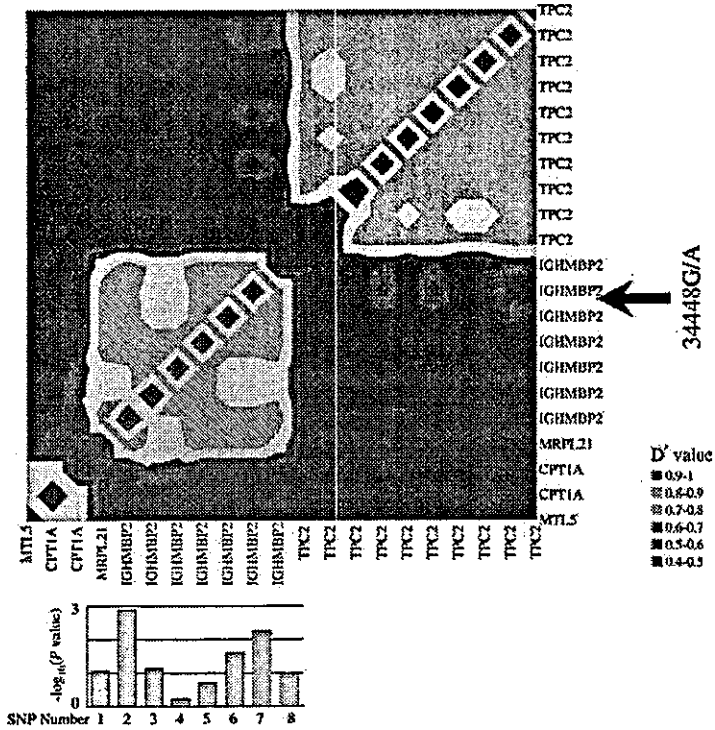


**Fig. 3** Pairwise linkage disequilibrium between SNPs in the *IGHMBP2* region, as measured by *D'* in the case population and case–control association plots [$-\log_{10}(P$ value)]. The candidate SNP was located in the LD block containing two genes, *MRPL21* and *IGHMBP2*. G34448A in exon 14 of *IGHMBP2* showed the most significant association with the disease in this block. The SNP number is the same as Fig. 2

**Table 2** Clinical data at the time of biopsy. Mean ± SD, *NS* not significant

| | GG | GA + AA | *P* value |
|---|---|---|---|
| Gender (M/F) | 140/122 | 60/38 | NS |
| Age (years) | 35.9 ± 14.2 | 38.9 ± 14.9 | NS |
| Serum IgA (mg/dl) | 319.4 ± 117.6 | 352.4 ± 162.9 | 0.048 |
| Serum creatinine (mg/dl) | 1.06 ± 0.66 | 1.08 ± 0.67 | NS |
| Proteinuria (g/day) | 1.16 ± 1.33 | 1.04 ± 1.33 | NS |

## Discussion

Our genome-wide SNP system offers at least two advantages. First, our SNP database is an extensive catalog of gene-based SNPs located within exons, introns, and promoter regions (Haga et al. 2002). Second, using a combination of multiplex PCR and the Invader assay, our method allows high-throughput, low-cost screening that requires as little as 0.1 ng of genomic DNA per single SNP. Through a genome-wide SNP analysis, we found a significant association between IgA nephropathy and an SNP in the *IGHMBP2* gene. This polymorphism, G34448A, substitutes lysine for glutamine in the gene product; the allele encoding lysine was more frequently observed in individuals with IgA nephropathy than in the general Japanese population. *IGHMBP2* is ubiquitously expressed and consists of 15 exons encoding a protein of 993 amino acids that possesses a putative DNA helicase region, an R3H motif, and a zinc-finger AN1-like domain.

Although the physiological function of IGHMBP2 is not known in detail, the protein is suspected to be involved in immunoglobulin-class switching, pre-mRNA processing, and regulation of transcription through DNA-binding or interaction with TATA-binding proteins (Fukita et al. 1993; Molnar et al. 1997; Zhang et al. 1999; Miao et al. 2000; Liepinsh 2003). Fukita et al. (1993) first showed that IGHMBP2 binds to single-stranded DNA with 5′-phosphorylated guanine-rich sequences related to the immunoglobulin $\mu$-chain switch (Sm) region. Immunoglobulin switch (S) regions are essential targets of S–S recombination, which changes the expressed class (isotype) of the immunoglobulin heavy chain (H) constant region (C) from $\mu$ to $\gamma$, $\varepsilon$, or $\alpha$ while maintaining the antigen specificity determined by DNA sequences in the variable region. S regions are located 5′ to each $C_H$ gene, except for $C_\delta$.

Increases in serum IgA levels have been observed in 50–70% of patients with IgA nephropathy, along with selective deposition of IgA1 in the kidney (Conley et al. 1980; Trascasa et al. 1980). We found that patients with the A allele of G34448A had higher serum IgA levels ($P=0.048$) and suspected that the E928K amino acid substitution might affect the class-switch event thereby increasing serum IgA levels, elevating the risk of IgA deposition, and causing the disease. However, the biological role of the zinc-finger AN1-like domain, where the E928K substitution lies, is still unknown. To clarify the relationship between E928K amino acid substitution and mesangial IgA deposition, examination of the frequency of this SNP in another nephritis with the same deposition, such as Henoch–Schönlein purpura, might be effective, but we do not have any available sample.

Gharavi et al. (2000) reported a genome-wide analysis of familial cases, but no familial history of the disease was observed in most Japanese patients with IgA nephropathy. This disease is a complex disorder, the etiology of which involves immunological, environmental, and genetic factors (Hsu et al. 2000). The fact that mesangial IgA deposition has been observed in 16% of allografts in non-blood-related Japanese kidney donors (Suzuki et al. 2003) indicates that latent mesangial IgA deposition is a relatively common phenomenon. Furthermore, patients with IgA nephropathy vary significantly with respect to progression and prognosis; some have a relatively good prognosis while others lose renal function, sometimes very rapidly. Thus, a variety of mechanisms may lay down deposits of IgA in glomerular mesangial areas, causing subsequent glomerular injuries and clinically active renal disease.

So far, we have identified five candidate genes that may be related to susceptibility to IgA nephropathy. On the basis of that information, we propose the potential mechanisms of IgA nephropathy indicated in Fig. 4, although it might be too preliminary, and more extensive biological and clinicopathological analysis would be required to substantiate this hypothesis. The onset of IgA nephropathy could be associated with antigens such as viruses, fungus, bacteria, or foods that are processed

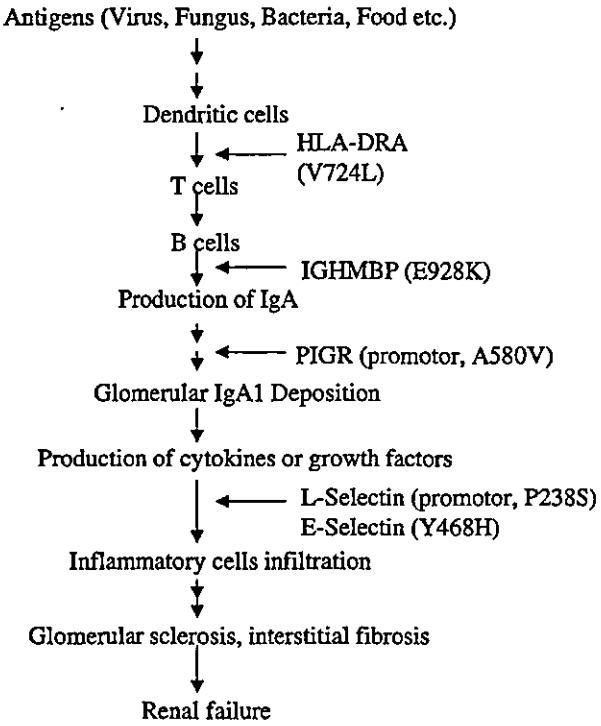


Fig. 4 Potential mechanism of IgA nephropathy based on results of case–control association studies using SNP markers for four candidate genes

and presented to T cells. HLA-DR, which regulates immune responses against protein antigens, is of great importance in the selection and activation of CD4-positive T cells; we identified the gene encoding HLA-DR earlier as a candidate susceptibility gene (Akiyama et al. 2002). HLA-DR molecules with the V724L substitution might account for individual differences in immune responses of T cells, which activate antibody-producing B cells. For its part, as noted above, the E928K variant of IGHMBP2 might influence a class-switch leading to increased serum IgA levels.

The third of our candidates, PIGR, is an integral membrane secretory component localized on the basolateral surface of secretory epithelial cells where it is thought to mediate the transepithelial transport of polymeric IgA. We showed earlier that a genetic variation in the promoter region of the PIGR gene caused an A580V substitution associated with IgA nephropathy and suggested that the V allele might affect binding of polymeric IgA to PIGR and cause deposition of mesangial IgA (Obara et al. 2003). IgA deposits in the kidney can trigger production of a variety of cytokines and growth factors by renal cells and by circulating inflammatory cells, leading to the characteristic histopathological features of mesangial-cell proliferation and depositions of immunoglobulin and complement in mesangial regions.

*SELL* and *SELE* genes encode cell–cell adhesion molecules involved in the leukocyte–endothelial cell

interaction required for extravasation at sites of tissue injury. SELE is expressed predominantly in cytokine-activated endothelium, and SELL is present in circulating leukocytes. We reported that Y468H in the *SELE* gene, as well as P238S-*SELL* and an SNP in the promoter region of *SELL*, were strongly associated with IgA and suggested that these substitutions could affect the quality and/or quantity of gene products and possibly play a significant role in inflammatory changes leading to renal fibrosis and ultimately renal failure (Takei et al. 2002).

Although functional studies must be undertaken to determine how these genetic variations, now including E928K-IGHMBP2, can affect the onset and development of IgA nephropathy, the results of our genetic studies have suggested several potential mechanisms for investigation.

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# Osteopontin expression in acute renal allograft rejection

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**Osteopontin expression in acute renal allograft rejection.**

*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

**Key words:** osteopontin, acute rejection, renal allograft, immunohistochemistry, in situ hybridization, donor, protocol, interstitial inflammation, regeneration, apoptosis.

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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

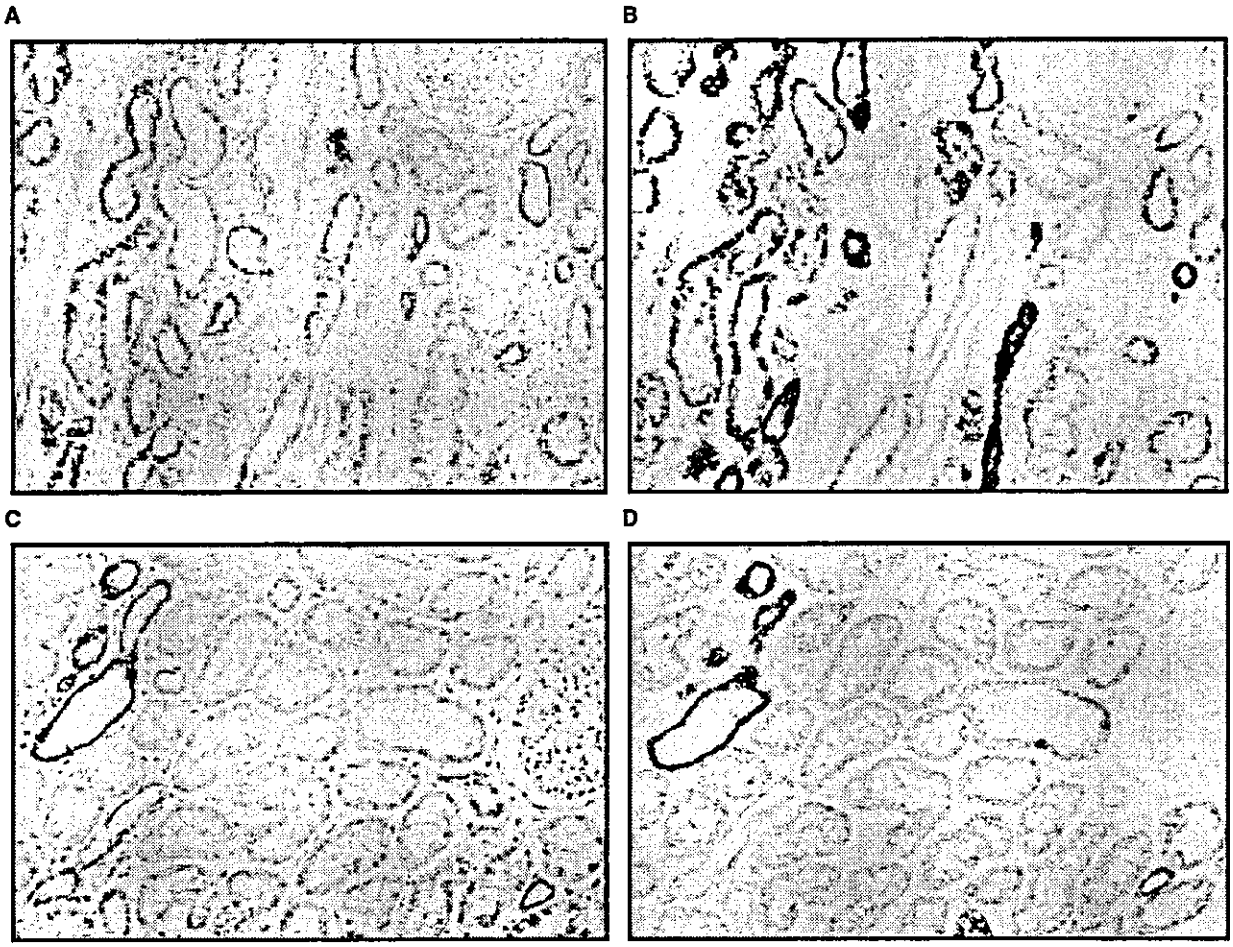


**Fig. 1. Replicate tissue sections.** (*A* and *B*) Sections with acute rejection demonstrating the colocalization of osteopontin (OPN) protein and mRNA by immunohistochemistry and in situ hybridization. OPN protein is visualized by alkaline phosphatase (red) and OPN mRNA is hybridized with digoxigenin-labeled 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).

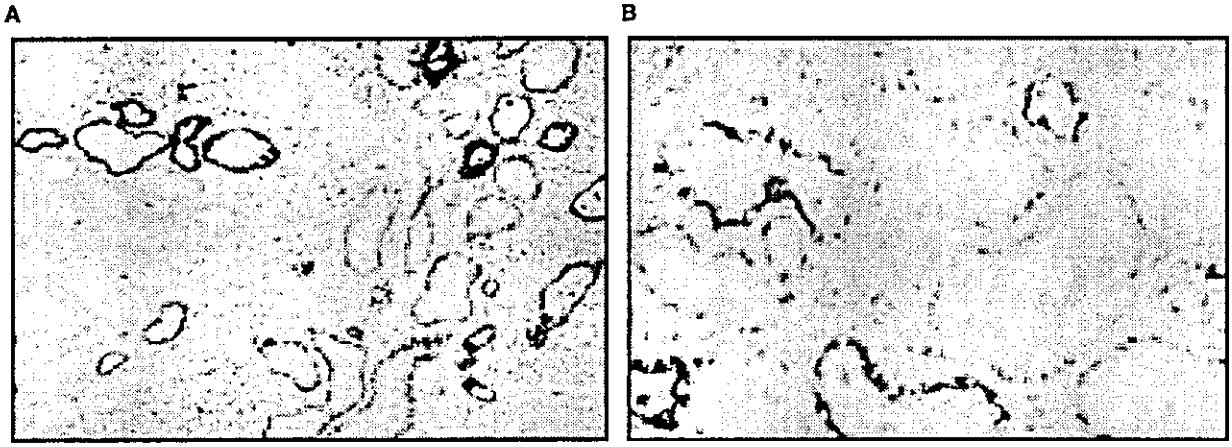


**Fig. 2. Double immunohistochemistry.** (*A*) Osteopontin (OPN) (red), and epithelial membrane antigen (EMA) (brown) in a rejection specimen, demonstrating strong OPN expression in the tubular segments surrounded by numerous inflammatory cells. (*B*) Higher power view of the same kidney (A), demonstrating the distinct perinuclear staining pattern of OPN [magnification ×66 (A) and ×160 (B)].

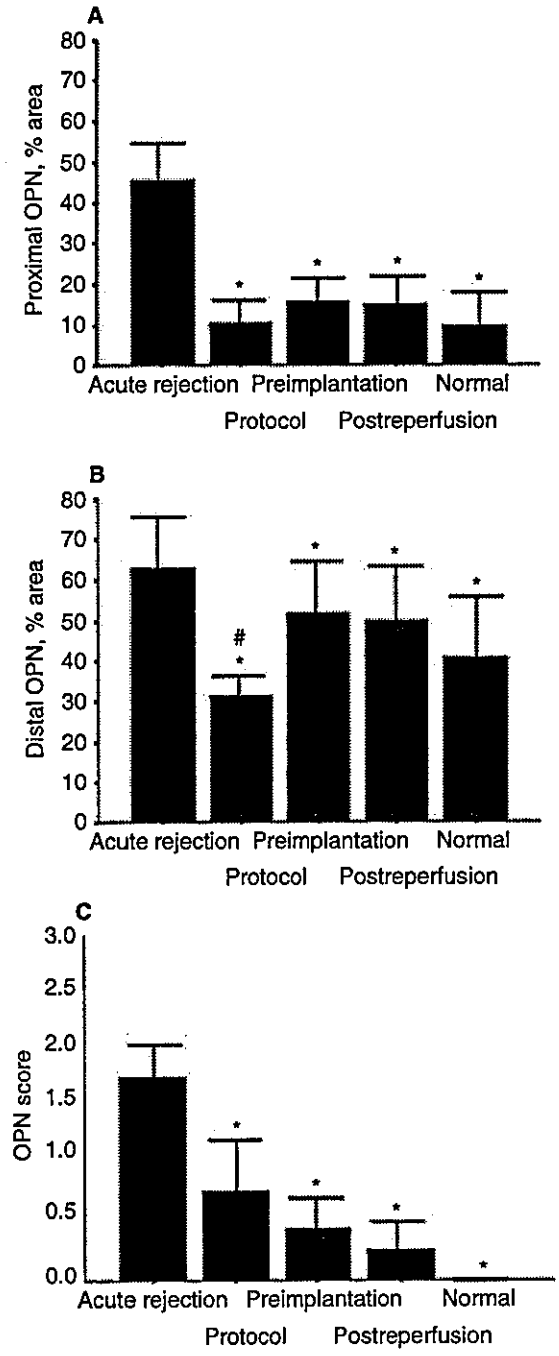


**Fig. 3. Quantitative analysis of osteopontin (OPN) expression.** (*A*) Proximal [epithelial membrane antigen (EMA)-negative] tubules. (*B*) Distal (EMA-positive) tubules. (*C*) OPN score in acute rejection ($N = 22$), protocol ($N = 9$), preimplantation ($N = 20$), postreperfusion ($N = 15$), and normal biopsies ($N = 4$). *$P < 0.05$ versus acute rejection; #$P < 0.05$ versus preimplantation and postreperfusion.

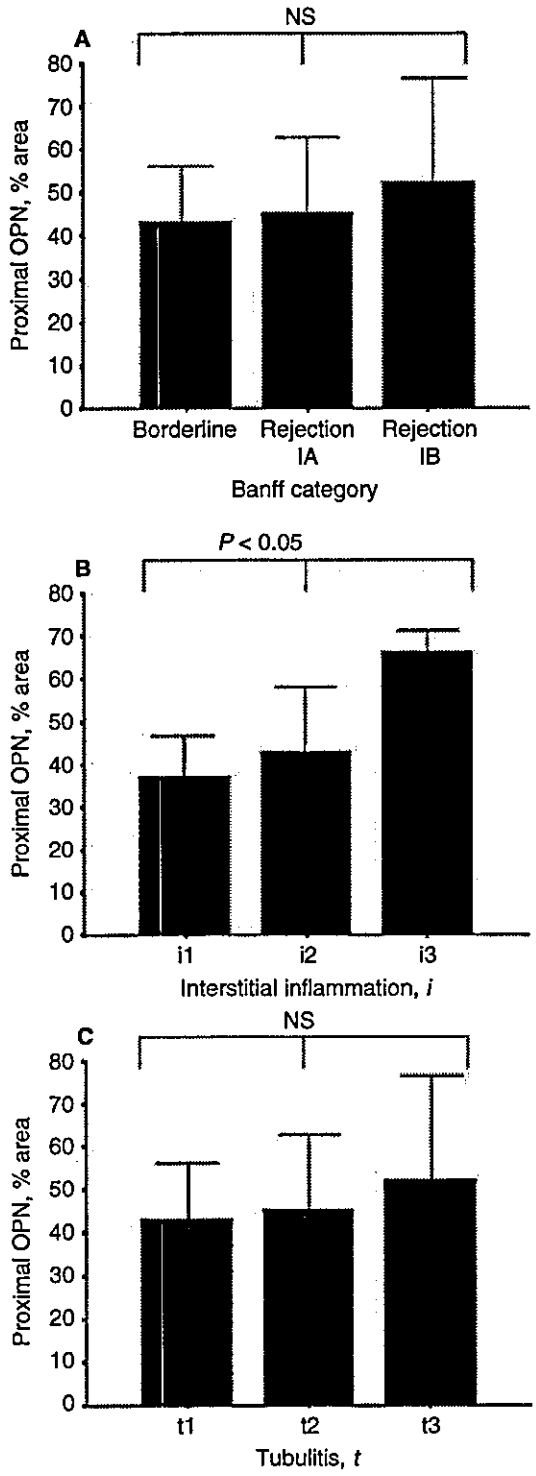


**Fig. 4. Quantitative analysis shows OPN expression.** (*A*) Expression is not associated with the pathological grade of rejection. (*B*) Expression is significantly correlated with the degree of interstitial inflammation. (*C*) Expression is not correlated with tubulitis. NS is not significant.

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 observed 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

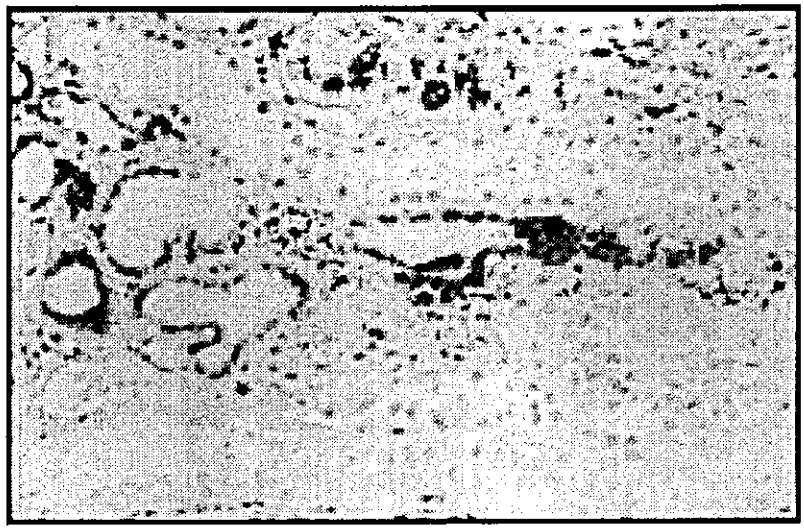

**Fig. 5. Double immunohistochemistry.** (*A*) Osteopontin (OPN) (red) and CD68+ macrophage (brown) in renal graft with acute rejection, demonstrating the CD68+ macrophages in close proximity with tubules expressing OPN (magnification ×100). (*B*) Regression analysis showed OPN expression in patients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

C

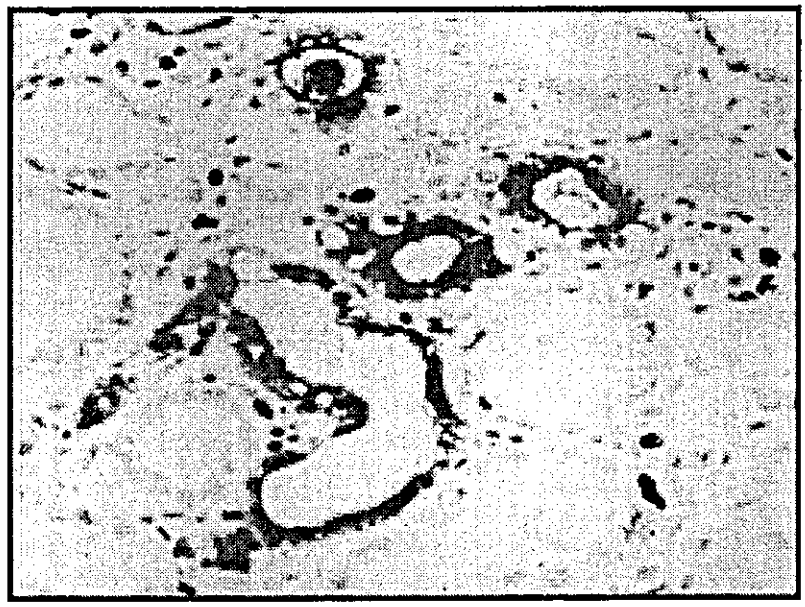

**Fig. 6. Regression analysis shows significant correlation between osteopontin (OPN) expression.** (*A*) Ki-67+ tubular cells, (*B*) Ki-67+ interstitial cells in patients with acute rejection. (*C*) Demonstrative case of acute rejection showing the location Ki-67+ cells (brown) was related to tubular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

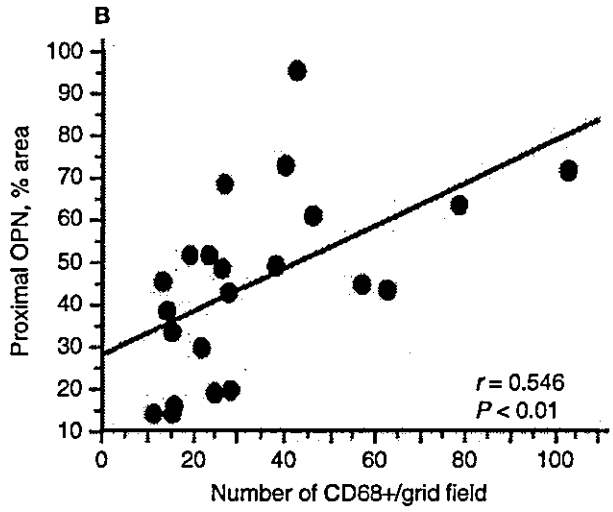


Fig. 5. (continued.)

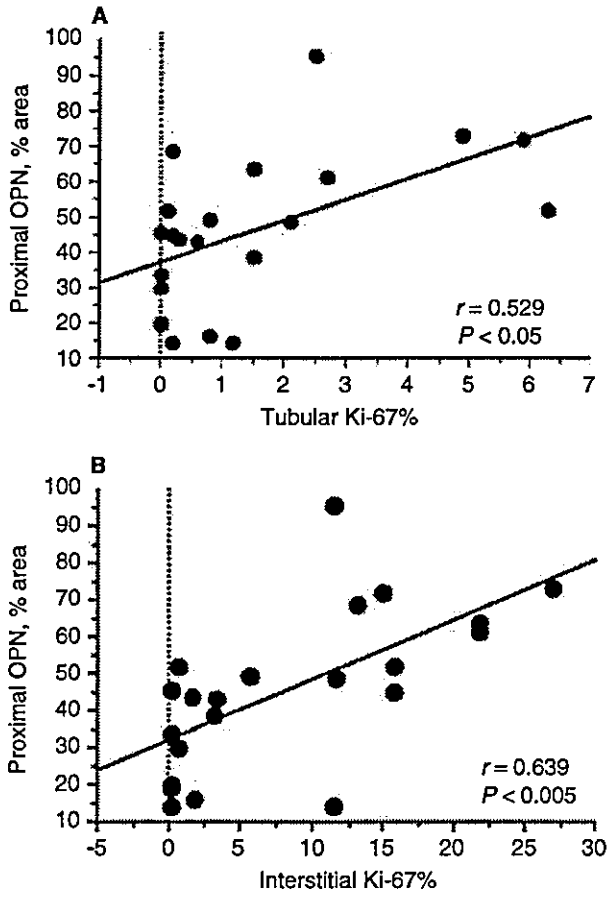


Fig. 6. (continued.)

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