Expression of osteopontin in gentamicin-induced acute tubular necrosis and its recovery process

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Background. There is controversy regarding the exact localization and roles of osteopontin (OPN), a multipotential chemokine, in renal injury. There is little information on the expression and role of OPN in gentamicin-induced acute tubular necrosis (ATN) and its recovery process.

Methods. A severe ATN model was made using male Wistar rats by injecting gentamicin (150 mg/kg/day) for five days and limiting the provision of water. The expression and localization of OPN mRNA and protein, ED1 as a macrophage marker, proliferating cellular nuclear antigen (PCNA), CD44 as an OPN receptor, megalin as a proximal tubule marker, and their relationships to each other were examined from the early tubular necrotic period to the late recovery period by Northern blotting, in situ hybridization, and double immunohistochemical staining.

Results. In the gentamicin group, OPN mRNA and protein were expressed in only the PCNA-positive proliferating cortical distal tubules, not in the necrotic proximal tubules, until day 6 after the first administration, but were found markedly in PCNA-positive regenerative proximal and distal tubules on days 10, 15, and 30. The localization of PCNA-positive cells was almost always accompanied with the up-regulated expression of OPN using quantitative analysis ($P < 0.01$). CD44 expression was markedly up-regulated in the renal cortical tubular epithelium from days 6 to 30. In the control group, no expression of OPN and CD44 in the cortical area was found throughout the experimental period.

Conclusions. These results suggested that OPN is related to the proliferation and regeneration of tubular epithelial cells after tubular damage.

Osteopontin (OPN) is a 44 kD secreted glycosylated phosphoprotein. Originally isolated from bone, it has been shown to be expressed in a number of different tissues, including the kidney, lung, liver, bladder, pancreas, and breast [1–3]. Structurally, the OPN protein is roughly globular, contains an arginine-glycine-aspartic acid (RGD) sequence, and is highly phosphorylated on up to 28 serine residues [4].

Osteopontin is expressed in the normal kidney and induced under various experimental pathological conditions [5, 6], such as angiotensin II-induced tubulointerstitial nephritis [7], cyclosporine nephropathy [8], hydropnephrosis caused by unilateral ureteral ligation [9], renal ischemia [10, 11], cisplatin nephropathy (abstract; Iguchi et al, J Am Soc Nephrol 9:A3043, 1998), and crescentic glomerulonephritis [12, 13]. Despite this, little is known about the expression of OPN in gentamicin-induced acute tubular necrosis (ATN) and its recovery process, including regeneration of renal tubular epithelial cells following ATN. In addition, the exact localization of OPN in the kidney has been controversial because the discrimination between the proximal and distal tubules becomes difficult in pathological conditions [14].

Despite the significant progress in understanding the structure and topography of OPN, its functional role remains insufficiently understood [15]. Based on the known activities of OPN in vitro and in vivo, there are currently two hypotheses for its role in renal pathologies and pathophysiology [6]. One hypothesis is that OPN is not only produced by macrophages and activated T cells, but also modulates their activity, both in migrating to sites of injury and in responding to the activators present at such sites [6]. Another attractive hypothesis holds that in some settings, OPN may act as a cell survival factor for renal cells [15–17]. Recently, studies on the roles of OPN have focused on its renoprotective action [15–17], including repair and regeneration following renal injury, in addition to its chemoattractive function.

Gentamicin toxic nephropathy includes acute tubular necrosis in the early period, infiltration of monocytes/
Macrophages, and regeneration of renal tubular epithelial cells during the recovery process [18, 19]. The relationship between these toxic pathological and pathophysiological processes and OPN expression is not understood. In this study, we used a severe ATN model induced by gentamicin administration and dehydration and examined the expression and localization of OPN in cortical tubules by Northern blot analysis, in situ hybridization, immunohistochemistry, and double staining with antimegalin antibodies as a proximal tubule marker. Throughout the observation of the exact localization of OPN, ED-1 as a monocyte/macrophage marker, proliferating cellular nuclear antigen (PCNA), CD44 as an OPN receptor, and their relationship to each other from the early ATN period to the later recovery period, we tried to clarify OPN’s role in severe gentamicin-induced ATN and its recovery process.

METHODS

Animal experiments

Experiments were performed on 40 seven-week-old male Wistar rats (Charles River Japan, Yokohama, Japan), weighing between 230 to 270 g, that were divided into two groups: a gentamicin group and a control group. The rats in the gentamicin group were given 150 mg/kg/day of gentamicin sulfate solution (Sigma-Aldrich Co., St. Louis, MO, USA) by subcutaneous injection in the neck for five days, and the rats in the control group were given an equal volume of normal saline instead. All animals were fed a diet of standard laboratory chow and allowed free access to water, but were deprived of water for 24 hours before the first gentamicin administration and for 12 hours a day during gentamicin administration.

Five rats in each group were sacrificed under ether anesthesia on days 6, 10, 15, and 30 after the first gentamicin injection, respectively. Blood was taken from the abdominal aorta for the assay of urea nitrogen and creatinine via urease ultraviolet absorption spectrophotometry and alkali picric acid method, respectively. The bilateral kidneys of one rat were rapidly removed and bisected for Northern blot analysis and for 12 hours a day during gentamicin administration.

In this study, the previously mentioned digoxigenin (DIG)-labeled OPN cRNA probe (50 ng/mL) was used. Hybridization was performed at 68°C for 16 hours with shaking according to the standard protocol. After hybridization, the filter was stringently washed and then incubated at room temperature for 30 minutes in a buffer with 0.1 μL/mL of polyclonal sheep anti-DIG Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim). The excessive antibodies were washed off with the buffer. A color reaction was performed by incubating the filter with 0.1 μL/mL NBT/BCIP color-substrate solution at room temperature for two hours.

In situ hybridization

Renal tissues for in situ hybridization were fixed with 4% paraformaldehyde in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4), dehydrated in an ethanol series, and embedded in paraffin under RNase-free conditions. Serial 5 μm thick sections were cut. Details of in situ hybridization techniques used here were reported previously [20]. In this study, a DIG-labeled OPN cRNA probe (0.5 μg/mL) and an OPN sense probe as a negative control were used.
control were used. The hybridization for OPN mRNA was performed at 52°C for 16 hours. The alkaline phosphatase-conjugated anti-DIG-antibody was reacted with the sections at 37°C for 30 minutes, and a coloring reaction with NBT/BCIP was performed at room temperature for 12 hours. The sections obtained from the cisplatin nephropathy model (abstract; Iguchi et al, *J Am Soc Nephrol* 9:A3043, 1998) were used as a positive control.

**Immunohistochemistry**

Immunohistochemical staining was carried out on 5 µm wax sections. The sections were first dewaxed and dehydrated. Then they were incubated with 0.6% H₂O₂ in methanol for 30 minutes to eliminate endogenous peroxidase activity and were treated with normal goat serum (Chemicon, Temecula, CA, USA) at room temperature for 30 minutes. Subsequently, they were incubated at 4°C for 24 hours with primary antibodies, which included the following: mouse monoclonal anti-OPN antibody (1 µg/mL; ARP, Belmont, MA, USA), rabbit polyclonal antimegalin antibody (5 µg/mL; kindly provided by Drs. Robert A. Orlando and Marilyn G. Farquhar of the University of California, San Diego, CA, USA), mouse anti-ED1 antibody (10 µg/mL; Serotec, Oxford, UK), mouse monoclonal antiproliferating cell nuclear antigen antibody (1:20; Dako, Glostrup, Denmark), and mouse monoclonal anti-CD44 antibody (1:100; Pharmingen, San Diego, CA, USA). The sections were incubated with a secondary antibody, either goat anti-mouse IgG conjugated with the alkaline phosphatase or the peroxidase or anti-rabbit IgG conjugated with the peroxidase at room temperature for 30 minutes. After washing with PBS, the sections were incubated with a Fuchsin alkaline phosphatase substrate solution (Dako, Carpinteria, CA, USA) with an endogenous alkaline phosphatase inhibitor (Dako, USA), or a DAB peroxidase substrate solution (Nichirei, Tokyo, Japan). The cellular nuclei of the sections were counterstained with hematoxylin. The cisplatin nephropathy model sections (abstract; Iguchi et al, *J Am Soc Nephrol* 9:A3043, 1998) were used as a positive control.

**Double staining**

Double staining was performed on the same tissue section, which included a combination of in situ hybridization for OPN mRNA and immunohistochemistry for megalin as a proximal tubule marker, a combination of immunohistochemistry for OPN protein and megalin, a combination of OPN protein and ED-1 as a monocyte/macrophage marker, and a combination of the OPN protein and PCNA as a cell proliferation or regeneration marker. For the in situ hybridization and immunohistochemistry tests, the sections were washed in PBS for 3 × 5 minutes after in situ hybridization, and then immunohistochemical staining was performed as described previously in this article. In immunohistochemical double staining, the first immunohistochemical staining was performed with a DAB peroxidase substrate solution, which showed a brown color reaction, followed by washing in PBS for 3 × 5 minutes and treatment for 10 minutes in a microwave oven in a 0.01 mol/L citrate buffer (pH 6.0). Then the second immunohistochemical staining was performed with a Fuchsin alkaline phosphatase substrate solution, which showed a red color reaction.

**Quantitative analysis**

A point-counting method was employed to quantitate the OPN mRNA and OPN protein-positive fractional volume of renal cortical proximal (megalin-positive), nonproximal (megalin-negative), and total tubular epithelium on double-staining sections for OPN mRNA or OPN protein and megalin, respectively [21]. In each field, 121 points (intersections) were counted on a 1 cm² eyepiece graticule with 11 × 11 equidistant grid lines. Under high magnification (×400), 20 consecutive nonoverlapping fields per section, that is, a total of 2420 points of the renal cortex in each kidney, were observed. The percentages of OPN-positive proximal, nonproximal, and total tubules were calculated, while the points falling on glomeruli, Bowman’s capsules, or tubular lumens were excluded.

The quantitations for ED1-positive cells and PCNA-positive cells were undertaken under high magnification (×400) in which 20 randomly selected cortical graticule fields per section were examined. The ED1-positive cells located in the tubulointerstitial area, including renal tubular lumens and the PCNA-positive cells located in the renal tubular epithelium, were counted, and the numbers were averaged for each field.

The correlation analysis for OPN and PCNA was undertaken on double-staining immunohistochemistry sections for the OPN protein and PCNA.

**Statistical analysis**

Statistical analysis was performed using the statistical software StatView Version 5. A *P* value of less than 0.05 was considered significant. Data are expressed as mean ± SD.

**RESULTS**

**Biochemical data**

The level of blood urea nitrogen (BUN) and serum creatinine (S₉) in the gentamicin group rats increased significantly on day 6 after the first administration (*P* < 0.01), reached its peak on day 10 (*P* < 0.05), and then gradually decreased and almost returned to normal by day 30. Two rats in the gentamicin group died on days 13 and 15, respectively (Table 1).
Histologic changes

Periodic acid-Schiff (PAS) staining showed that renal tubular necrosis apparently developed on day 6 after the first administration in the gentamicin group. Superficial cortical tubules revealed severe degeneration or necrosis of epithelial cells. On day 10, there were lots of desquamated epithelial cell debris in the renal tubular lumens. Mononuclear cells infiltrated interstitial lesions from the superficial cortex to the corticomedullary zone. New cellular lines that were thought to be regenerative tubular epithelial cells appeared along the tubular basement membranes. The regenerative tubular epithelial cells became larger, and the renal tubular epithelium became thicker on day 15. On day 30, most of the cortical tubular structures almost returned to the normal architecture, but spotty infiltration of mononuclear cells, atrophy of renal tubules, and fibrosis of the interstitium remained. The control group showed no significant histologic changes throughout the experimental period.

Based on the immunohistochemical staining of megalin and cellular nuclear staining, it was evident that the necrotic tubules were proximal tubules. The distal tubules showed some proliferation changes on day 6. The regenerative changes occurred mainly in the proximal tubules, but also in the distal tubules from day 10 to day 15. By combination with PAS staining, it was shown that focal atrophic tubules existed in both the proximal and distal tubules on day 30 (Fig. 1).

Expression and localization of OPN mRNA and protein

Compared with the control group, Northern blot analysis showed markedly up-regulated expression of OPN mRNA in the kidneys of the gentamicin group from days 6 to 30 (Fig. 2).

In the gentamicin group, histologic localization of OPN mRNA was shown in the cortical tubules by in situ hybridization from the early ATN period on day 6 to the later recovery period on day 30. OPN mRNA expression was most widely distributed on day 10 and day 15, and decreased on day 30 in the cortical region. In the control group, OPN mRNA was not detected in the cortical region throughout the experimental period, except for a weak expression in the descending thin limb of the loop of Henle (not shown). The expression of OPN mRNA in the gentamicin group was also positive in the medullary tubules from days 6 to 30 and was stronger than that of the control. The OPN mRNA signals in the medulla were strongest on day 6 and then decreased gradually and almost returned to normal on day 30 in the gentamicin group. Intraglomerular expression of OPN mRNA was not detected in either group, and only parietal epithelial cells of Bowman’s capsule showed weak positive signals in the gentamicin group during the experimental period (Fig. 3). OPN mRNA expression was shown simultaneously in the renal tubules of the cisplatin nephropathy model as a positive control (figure not shown). The sense control for OPN mRNA was negative (figure not shown).

The expression of the OPN protein was recognized as a punctate distribution in the cortical tubular epithelium from days 6 to 30, with a peak on day 15 in the gentamicin group. In the control group, the expression of OPN protein was not detected in the cortical tubules during the experimental period (figure not shown). The localization of the OPN protein was positive in the medullary tubules in both groups from days 6 to 30, but its distribution in the gentamicin group was wider than the control on days 6, 10, and 15 except for day 30. Bowman’s capsule epithelial cells showed positive OPN protein expression in only the gentamicin group (Fig. 4). OPN protein expression was shown simultaneously in the renal tubules of the cisplatin nephropathy model as a positive control (figure not shown).

Double staining of in situ hybridization for OPN mRNA and immunohistochemistry for the megalin protein used as a proximal tubule marker clearly revealed that expression of OPN mRNA was present in only distal tubules, but not in the proximal tubules of the renal cortex during the ATN period on day 6 after the first administration. However, in the recovery period on days 10, 15, 30, the expression of OPN mRNA was present not only in the distal tubules, but also in the proximal tubules showing regenerating changes in the renal cortex. Double-staining immunohistochemistry for the OPN protein with megalin showed that the localization of the OPN protein was coincident with that of OPN mRNA in the renal cortex (Fig. 5).

Quantitative analysis showed that the percentage of OPN mRNA-positive tubules in the proximal (megalin-positive) tubules was highest on day 15 in the gentamicin group, while that in the nonproximal (megalin-negative) tubules, for example, the distal tubules, was highest on day 10 (Fig. 6). The location and level of OPN protein expression was almost the same as that of OPN mRNA in the renal cortical tubules (data not shown).

Demonstration of ED1-positive cells and the relationship between ED1 and OPN

To examine the relationship between OPN and monocye/macrophage infiltration, we performed immunohistochemistry for ED1 (a monocyte/macrophage marker) and double staining for OPN and ED1. In the gentamicin group, only a few ED1-positive cells were present in the cortical tubulointerstitium on day 6, but many were present in the cortical interstitium and within the tubular lumens on days 10 and 15, with the most on day 10 after the first administration. On day 30, they were focally present in an atrophic, thick tubular epithelium and its
Fig. 1. Renal histologic changes in gentamicin-induced acute tubular necrosis (ATN) and recovery. (A–E and F–J) Serial sections, respectively. (A–E) PAS staining. (F–J) Immunohistochemical staining of megalin (a proximal tubule marker, brown) and cellular nuclear staining. (A and F) Normal cortical renal tubules. Note that megalin is positive in only the proximal tubules (arrow), but not in other tubules (arrowhead, F). (B and G) ATN on day 6 after the beginning of gentamicin injection (150 mg/kg/day × 5). Note the flattened, desquamated epithelium, denuded basement membranes, and intraluminal cellular debris (arrow, B). Tubular necrosis occurs only in proximal tubules (arrow, G), and evidence of proliferation in distal tubules is shown (arrowhead, G) as compared with control (arrowhead, F). (C) Recovering renal cortical tubules on day 10. Renal tubules are lined by regenerating cells (arrow). Some tubules are filled with amorphous debris, while others have cleared. Mononuclear cells aggregate in the renal interstitium (arrowhead). (D) Recovering renal cortical tubules on day 15. The renal tubular epithelium becomes thick, but the microvilli are relatively short and simple (arrow). (H and I) Regenerating changes occur not only in proximal tubules (arrow), but also in distal tubules (arrowhead) on day 10 (H) and day 15 (I). (E) Cortical renal tubules on day 30. Most of the cell structure is comparable to controls, but a small focus of atrophic tubules (arrow), interstitial fibrosis, and mononuclear cells remained (arrowhead). (J) Focal atrophic tubules also include proximal (arrow) and distal tubules (arrowhead) on day 30. Original magnification ×100.
Fig. 2. Northern blot analysis in gentamicin-induced ATN and recovery.

Fig. 3. Osteopontin (OPN) mRNA expression from in situ hybridization in renal cortical tubules treated by gentamicin. (A) Up-regulation of OPN mRNA only in cortical tubules having no necrosis, but not in necrotic tubules on day 6 after the first gentamicin administration. (B and C) Marked up-regulation of OPN mRNA in regenerative renal tubules on day 10 (B) and day 15 (C). (D) Up-regulation of OPN mRNA focally in some areas of the renal cortex on day 30. Original magnification ×100.
Fig. 4. Expression of the OPN protein with immunohistochemistry in renal cortical tubules treated by gentamicin. (A) Up-regulation of OPN protein in cortical tubules having no necrosis, but not in necrotic tubules on day 6 after the first gentamicin administration. (B and C) Marked up-regulation of the OPN protein as a punctate distribution in regenerative renal tubules on day 10 (B) and day 15 (C). (D) Up-regulation of the OPN protein in the focal area of renal tubular atrophy and interstitial fibrosis on day 30. Original magnification ×100.

peripheral interstitium (Figs. 7 and 8). No distinct macrophage infiltration was found in the control group (not shown). Double staining showed that the location of OPN expression had no distinct relationship with the site of monocyte/macrophage accumulation in most areas of the cortex, but in some areas of the cortex, the location of OPN expression was almost coincident with the site of monocyte/macrophage infiltration in the gentamicin group (Fig. 9).

Demonstration of PCNA-positive cells and colocalization of PCNA and OPN

To clarify the relationship between OPN expression and cellular proliferation/regeneration, we carried out immunohistochemistry for PCNA and double staining for OPN and megalin, OPN and PCNA. There were few PCNA-positive cells in the rat kidneys of the control group. In contrast, in the gentamicin group, the PCNA-positive cells were observed in great numbers in the distal tubules
Fig. 5. Localization of OPN mRNA and the OPN protein in renal cortical tubules treated by gentamicin. OPN mRNA (blue) is shown by double staining of in situ hybridization for OPN and immunohistochemistry for megalin (a proximal tubule marker, brown; A–E). OPN protein (red) is shown by immunohistochemical staining of OPN and megalin (F–J). A–E and F–J are serial sections, respectively. (A and F) Normal renal tubules showing no OPN mRNA or OPN protein. (B and G) Up-regulation of OPN mRNA and the OPN protein in cortical distal tubules (arrow), but not in necrotic proximal tubules on day 6 after the first gentamicin administration. (C and H) Marked up-regulation of OPN mRNA and the OPN protein not only in cortical distal tubules (arrow), but also in proximal tubules (arrowhead) on day 10. (D, E, I, and J) Up-regulation of OPN mRNA and the OPN protein mainly in cortical proximal tubules (arrow), but also in distal tubules on day 15 (D and I) and on day 30 (E and J). Double staining showed that the localization of the OPN protein was coincident with that of OPN mRNA in the renal cortex (arrow). Original magnification ×100.
Table 1. Blood urea nitrogen (BUN) and serum creatinine (S_{cr}) levels in rats after gentamicin administration

<table>
<thead>
<tr>
<th>Group</th>
<th>BUN mg/dL</th>
<th>S_{cr} mg/dL</th>
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<tr>
<td></td>
<td>Day 6</td>
<td>Day 10</td>
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<tr>
<td>Gentamicin</td>
<td>112.78 ± 32.69^a</td>
<td>229.41 ± 134.4^a</td>
</tr>
<tr>
<td>Control</td>
<td>22.92 ± 1.39</td>
<td>23.16 ± 3.78</td>
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Data are means ± SD. Gentamicin group rats were given 150 mg/kg/day gentamicin sulfate solution for 5 days by subcutaneous injection. Control group rats received an equal volume of normal saline instead.

^a \( P < 0.01 \) compared with Control

^b \( P < 0.05 \) compared with Control

in the deep cortex, corticomedulla, and medulla in the acute proximal tubular necrotic period on day 6 and in the proximal and distal tubules of the cortex, as well as medullary tubules, in the recovery period on days 10, 15, and 30, with the most seen on day 10 after the first administration (Figs. 10 and 11; double staining for OPN and megalin not shown). The double staining for OPN and PCNA showed that PCNA-positive cells were mainly present in the tubular epithelium with up-regulated expression of OPN (Fig. 12). The correlation analysis showed that PCNA-positive tubular epithelial cells were associated with the expression of OPN in the gentamicin-induced ATN period and the following recovery period (\( r = 0.966, 0.913, 0.850, \) and \( 0.956 \) on days 6, 10, 15, and 30, respectively, \( P < 0.01 \)).

Expression of CD44

The immunohistochemistry for CD44 showed that expression of CD44 was markedly up-regulated in the cortical tubular epithelium and infiltrating cells in the cortical interstitium and tubular lumens from the tubular necrotic period on day 6 to the recovery period on days 10, 15, and 30 after the first administration in the gentamicin group. In the control group, no expression of CD44 in the cortical tubular epithelium was found throughout the experimental period (Fig. 13).

DISCUSSION

Osteopontin is a secreted glycoprotein that has broad tissue distribution and diverse biological functions [3, 6]. Its kidney expression has been demonstrated in normal and a number of renal injury models [5, 6]. There is one report regarding the expression of OPN in a gentamicin-induced acute renal failure model [22]. In that report, rats were given subcutaneous injections of gentamicin (40 mg/kg) daily for 10 days and were sacrificed for analysis on the 10th day of gentamicin administration. In the model, the serum creatinine and blood urea nitrogen levels revealed abnormal elevation from the eighth day. The expression of OPN mRNA was not enhanced in that mild model by Northern blot analysis or in situ hybridization. We successfully made a severe ATN model by injecting a high dose of gentamicin (150 mg/kg/day) for five days and limiting the provision of water to the
Fig. 7. Demonstration of ED1-positive cells in gentamicin-induced ATN and recovery. (A) A few ED1-positive cells are present in the cortical tubular epithelium and interstitium on day 6 after the first gentamicin administration. (B and C) Many ED1-positive cells are present in the cortical interstitium and within tubular lumens on day 10 (B) and day 15 (C). (D) Quite a few ED1-positive cells are focally present in some tubular epithelia and interstitia on day 30. Original magnification ×100.

Fig. 8. Quantitative analysis of cortical ED1-positive cells in gentamicin-induced ATN and recovery. ED1-positive cells in the tubules, interstitium, and tubular lumens of the renal cortex reached a peak on day 10. GM6, GM10, GM15, and GM30 show days 6, 10, 15, and 30 after the first administration in the gentamicin group, respectively. *P < 0.01; #P < 0.05.
experimenetal rats. In this study, the up-regulated expression of OPN mRNA and protein was shown distinctly by Northern blot analysis, in situ hybridization, and immunohistochemistry in the renal cortex from days 6 to 30 after the first gentamicin administration. The OPN mRNA levels of the whole kidney by Northern blot analysis on day 30 in the gentamicin group were almost the same as the control, possibly because the OPN mRNA signals in the medulla almost returned to normal and OPN expression was restricted only in the focal area of the cortex on day 30.

Studies of histologic localization in the normal kidney and sources of OPN have given conflicting results [14]. In an early immunohistochemical study in the rat, OPN was found only in the proximal tubules [23]. Because immunostaining was observed in components of the vascular-lysosomal system, it was concluded that OPN was absorbed from the tubule fluid [14]. Subsequent studies using both in situ hybridization and immunohistochemistry demonstrated OPN mRNA and protein expression in only the distal tubular cells of the normal rat kidney [24], whereas other investigators found that OPN was expressed in the descending thin limb of the loop of Henle and in the papillary and pelvic epithelium of the renal fornix in the normal rat kidney [25]. Under renal disease conditions, the OPN expression of some models, such as renal stone formation, was found mainly in the distal tubular cells [22], but that of other models, such as ischemia-induced acute renal failure, was noted not only in the distal tubular cells, but also in the proximal tubular cells [5]. These different results may be mainly due to the different causes and regions of damage in different disease models. On the other hand, they may also be due to different investigators having different opinions on the same renal tubule segment. In fact, it is very difficult to distinguish proximal tubules clearly from the loop of Henle, distal tubules, and collecting ducts in some pathological conditions, as shown in Figure 1. In this study, we confirmed the exact localization and source of OPN in renal cortical tubules using serial sections and double staining of OPN mRNA and megalin, as well as double staining of the OPN protein and megalin. Megalin served as a renal proximal tubule marker because it is thought to exist only in proximal tubular and glomerular epithelial cells, but not in distal tubules, the loop of Henle, or collecting ducts of the kidney [26–31]. Because OPN mRNA and OPN protein were expressed in the same region as renal cortical tubules, as shown in Figure 5, it was clear that the OPN protein of renal cortical tubules resulted from renal cortex tubular epithelial cell production, but not from resorption of renal tubular fluid. Several in vitro studies have demonstrated that cultured renal epithelial cells have the ability to secrete OPN [32–34].

The roles of OPN in renal pathology and pathophysiology have been insufficiently understood. In most studies, a correlation existed between the magnitude of OPN expression in areas of tubulointerstitial injury and infiltration with macrophages, suggesting that OPN could participate directly in renal macrophage infiltration in vivo [35–37]. In OPN knockout mice, it was reported that the macrophage influx after unilateral ureteral obstruction was significantly reduced as compared with control mice (abstract; Ophascharoensuk et al, J Am Soc Nephrol 8:A481, 1997). Further evidence that OPN may modulate macrophage function comes from wound-healing experiments done by Liaw et al, in which the increased cell debris in the healed wounds in OPN-deficient mice was thought to represent a reduction of macrophage activity [38]. However, there are some opposing reports. For example, in the renal cortex of streptozotocin-induced diabetic rats, increased levels of OPN were not associated with accumulation of monocyte/macrophages as identified by a cell type-specific monoclonal antibody ED1 [39]. In human crescentic glomerulonephritis, the majority of strongly OPN-positive cells were monocyte/macrophages in a number of crescents, but interstitial monocyte/macrophages did not express OPN [40]. In our current study, the location of OPN expression had no close relationship with the site of monocyte/macrophage accumulation in most areas. Only in some areas, the location of OPN expression was almost coincident with the site of monocyte/macrophage infiltration in the gentamicin group, as shown in Figure 9. These contrasting results suggest that OPN may work as a chemotactic protein in certain areas and time phases, and additionally may play other roles as a multifunctional chemokine in other areas and time phases in renal injury and its recovery process.

Recently, OPN has emerged as an important growth promoter of vascular smooth muscle cells and cardiac fibroblasts [41, 42]. In the kidney, OPN has been shown to play a role in the regeneration of renal proximal tubule cells after acute severe hypoxic or ischemic injury [43, 44]. In addition, OPN is up-regulated during the repair process of glomerular injury in the anti-Thy-1 model of glomerulonephritis (abstract; Prols et al, J Am Soc Nephrol 7:A2585, 1996). Hypoxia stimulated the release of OPN, and anti-OPN antibodies completely prevented hypoxia-induced proliferation in cultured mesangial cells and LLC-PK1 tubular epithelial cells (abstract; Sahai et al, J Am Soc Nephrol 8:A1983, 1997, and J Am Soc Nephrol 9:A2280, 1998). These results suggest that OPN may mediate the proliferation of mesangial and LLC-PK1 cells induced by hypoxia [45]. In our study, OPN mRNA and protein were only expressed in the proliferating distal tubules of the rat kidney, but not in necrotic proximal tubules in the gentamicin-induced ATN period on day 6 after the first of administration, whereas during the recovery process on days 10, 15,
Fig. 9. Relationship between ED1 and OPN in gentamicin-induced ATN and recovery. Double staining for OPN (brown) and ED1 (red) show the location of OPN expression had no distinct relationship with the site of ED1-positive cell accumulation in some areas of the cortex on day 10 (A) and day 15 (B and C). However, in some areas of the cortex on day 30 (D), the location of OPN expression was almost coincident with the site of ED1-positive cell infiltration in the gentamicin group. Original magnification ×100.

Fig. 10. Demonstration of proliferating cell nuclear antigen (PCNA)-positive cells in cortical tubules in gentamicin-induced ATN and recovery. (A) There are very few PCNA-positive cells in normal cortical tubules. (B) The PCNA-positive cells are present in tubules having no necrosis on day 6 after the first gentamicin administration (a lot of desquamated epithelial cell debris in the PCNA-positive tubular lumens can be seen). (C) There are many PCNA-positive cells in the cortical tubules on day 10. The new regenerative cells show flat cellular nuclei. (D) The PCNA-positive cellular nuclei become larger on day 15. (E) PCNA-positive cells are focally present in the cortical tubules and interstitium on day 30. Original magnification ×100.

Fig. 12. Colocalization of PCNA and OPN in gentamicin-induced ATN and recovery. Double staining for OPN (brown) and PCNA (red) shows that the PCNA-positive cells are mainly present in the tubular epithelium with up-regulated expression of OPN on days 6 (A), 10 (B), 15 (C), and 30 (D) after the first gentamicin administration, although not all cells in the OPN-positive area are PCNA-positive, especially on day 15 (C). Original magnification ×100.
Fig. 11. Quantitative analysis of cortical tubular PCNA-positive cells in gentamicin-induced ATN and recovery. The number of cortical tubular PCNA-positive cells reached a peak on day 10, but there was no significant difference compared with that on day 15. GM6, GM10, GM15, and GM30 show days 6, 10, 15, and 30 after the first administration in the gentamicin group, respectively. *P < 0.01. NS, no significant difference.
Fig. 13. Expression of CD44 in gentamicin-induced ATN and recovery. Immunohistochemistry for CD44 shows CD44 staining is negative in the cortical tubules in the control group (A), but CD44-positive staining is markedly present in the cortical tubular epithelium and infiltrating cells in the cortical interstitium and tubular lumens on days 6 (B), 10 (C), 15 (D), and 30 (E) after the first administration in the gentamicin group. Original magnification ×100.
and 30, OPN was found markedly in the regenerative proximal and distal tubules of the renal cortex. Double staining showed that PCNA-positive cells were mainly present in the tubular epithelium with up-regulated expression of OPN at any time during the necrotic period on day 6 or the recovery period on days 10, 15, and 30, and at any site in the proximal or the distal tubules. Correlation analysis showed that the number of PCNA-positive tubular epithelial cells was associated significantly with the expression of OPN. These results suggest that OPN may be related to the proliferation and regeneration of renal tubular epithelial cells following ATN induced by gentamicin and dehydration. The proliferation and regeneration of the distal tubules during the gentamicin-induced ATN period in this study may have resulted from ischemic injury induced by the limiting of water, because aminoglycoside therapy sensitizes the kidney to subsequent ischemic insults [46–48], although only limiting of water did not elicit renal injury to normal rats in the control group.

The mechanism by which OPN may play a role in cell proliferation and regeneration is presently little understood. It is possibly associated with the leukocyte antigen CD44, which is a widely distributed cell surface proteoglycan functioning as a receptor for a lot of matter, including the matrix constituent hyaluronan (HA) and OPN [49, 50]. In this study, CD44 expression was markedly up-regulated in the renal tubular epithelium from the necrotic period on day 6 to the recovery period on days 10, 15, and 30 after the first gentamicin administration. CD44 may trigger different cellular functions, depending on its ligand [51]. Oertli, Fan, and Wuthrich found that the binding of HA to CD44 could inhibit mouse cortical tubular cell proliferation markedly [50]. Furthermore, CD44 on mouse cortical tubular cells functions as an HA receptor, which has the capacity to bind its ligand and can be modulated with anti-CD44 antibodies [50]. Weber, Ashkar, and Cantor have identified the cytokine OPN as a ligand for CD44, and OPN binds to naturally expressed and stably transected CD44 in a manner that is specific, dose-dependent, inhabitable by anti-CD44 antibodies, and sensitive to competition by HA [52]. These results suggest that OPN interaction with CD44 may block the binding of CD44 to HA, thereby blocking the inhibition of HA on cell growth and other processes such as cell differentiation.

This study confirms the up-regulated expression of OPN in proximal and distal tubules in severe gentamicin-induced ATN from the early necrotic period to the later recovery period. OPN may be correlated with the proliferation and regeneration of renal tubular epithelial cells following renal toxic or ischemic injury.

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