Differences in osteopontin up-regulation between proximal and distal tubules after renal ischemia/reperfusion

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Differences in osteopontin up-regulation between proximal and distal tubules after renal ischemia/reperfusion.

Background. Osteopontin (OPN) is a highly acidic phosphoprotein containing an arginine-glycine-aspartic acid (RGD) cell adhesion motif. High OPN expression has been found in tissues with high cell turnover, and OPN up-regulation has been demonstrated in several models of renal injury, suggesting a possible role in tissue remodeling and repair. However, its exact function in the kidney remains unknown. In this study, the possible contribution of OPN to regeneration and repair in the kidney was explored by studying the time course and subcellular localization of OPN up-regulation after renal ischemia/reperfusion injury in different nephron segments and by investigating its relationship with tubular morphology.

Methods. Rats that underwent 60 minutes of left renal ischemia and a right nephrectomy sacrificed at 10 different time points (from 1 hr to 10 days after reperfusion) were compared with uninephrectomized rats at each time point. In renal tissue sections immunostained for OPN, proximal (PTs) and distal tubules (DTs) in both the renal cortex and outer stripe of the outer medulla (OSOM) were scored for the degree of OPN expression and tubular morphology.

Results. Kidneys of uninephrectomized rats showed no injury, and the localization and intensity of their OPN expression remained unaltered compared with normal rats. After ischemia/reperfusion, morphological damage was most severe in PTs of the OSOM, but all examined nephron segments showed a significant increase in OPN expression. The time course of OPN up-regulation was different in PTs and DTs. DTs in both cortex and OSOM rapidly increased their OPN expression, with a maximum at 24 hours after reperfusion followed by a slow decrease. In contrast, PTs showed a delayed increase in OPN staining, with a maximum after five to seven days, higher in the OSOM than in the cortex. In OSOM PTs, OPN expression was predominantly associated with morphological regeneration, whereas DTs showed a substantial OPN up-regulation without major morphological damage. PTs and DTs displayed a different subcellular OPN staining pattern: OPN staining in DTs was located to the apical side of the cell; PTs, however, presented a vesicular, perinuclear staining pattern.

Conclusions. Our study found a different pattern of OPN up-regulation after renal ischemia/reperfusion in PTs versus DTs, both with regard to time course and subcellular localization. DTs show an early and persistent increase in OPN staining in the absence of major morphological injury, whereas OPN staining in PTs is delayed and is mostly associated with morphological regeneration. PTs show a vesicular, perinuclear OPN staining pattern, whereas DTs show OPN staining at the apical cell side.

Acute ischemic renal failure is a frequent clinical event, affecting as many as 5% of all hospitalized patients [1], with a high mortality rate [2] and no causal therapy yet available [3]. Renal ischemia and subsequent reperfusion induce acute tubular necrosis (ATN), characterized by desquamation of necrotic and sublethally damaged tubular cells into the tubular lumen. Ischemia/reperfusion (I/R) damage is primarily located in the outer stripe of the outer medulla (OSOM), with the S3 segment of the proximal tubule (PT) showing the highest susceptibility to I/R injury [4]. Through the release of various chemokines, damaged cells may induce leukocyte infiltration, consisting mainly of macrophages and T cells [5, 6].

Its remarkable regeneration potential enables the kidney to replace damaged cells and to restore epithelial continuity in order to recover its function. Surviving tubular cells re-enter the cell cycle and replicate [7], probably after partial dedifferentiation [8], and then migrate to fill the epithelial defect, spread out, become firmly attached to the tubular basement membrane, and re-establish their polarized and differentiated structure. However, the factors that trigger and control this repair process are poorly understood. A better understanding of renal regeneration may, in time, give rise to therapeutic interventions that accelerate recovery from ATN [3].

Osteopontin (OPN) is a highly acidic phosphoprotein that was first isolated from bone but was also produced in other tissues, including kidney tubular epithelium. OPN is implicated in cell adhesion. It can bind to differ-

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ent integrins through an arginine-glycine-aspartic acid (RGD) cell adhesion sequence, but possibly also through non-RGD domains [9]. OPN is thought to play a role in a wide array of pathophysiological processes, including bone remodeling [10, 11], tumor metastasis [12], vascular pathology [13, 14], and wound healing [15].

Osteopontin is secreted into urine and may prevent urinary stone formation [16–18]. Up-regulation of OPN expression has been demonstrated in different experimental models of renal injury [19–23], including I/R injury. Kleinman et al showed an up-regulation of OPN expression after 40 minutes of unilateral renal ischemia over a five day observation period, with a delayed onset of OPN expression in the S3 segment of the PT [24]. Padanilam, Martin, and Hammerman demonstrated an 18-fold increase in OPN mRNA 24 hours after 60 minutes of bilateral ischemia, and an increased staining for OPN protein and mRNA in distal tubules (DTs) and medullary thick ascending limb (mTAL) 24 hours and 5 days after reperfusion, and in PTs of the OSOM after 5 days [25]. However, to gain insight into the pathophysiological relevance of renal OPN up-regulation, there is still the need for more extensive information about the relationship between cell damage, necrosis, and regeneration of tubular epithelium after I/R on one hand and tubular OPN expression on the other. This study elaborates on our previous observations of a different subcellular pattern of OPN up-regulation in PTs and DTs in two models of toxic ATN, namely after administration of mercuric chloride and gentamicin (abstract; Verstrepen et al, *J Am Soc Nephrol* 7:2604, 1996).

This study aims to describe and compare OPN expression in PTs and DTs and to determine whether increased OPN expression after I/R of the kidney is predominantly associated with normal, damaged, or regenerating tubules. To this end, the time course of OPN up-regulation after kidney I/R in PTs and DTs of cortex and OSOM was investigated. The subcellular localization of OPN at a light microscopic level was studied, and the relationship between the morphology of kidney tubules and their OPN expression was analyzed.

METHODS
Animals and experimental design

Male inbred Lewis rats weighing 200 to 250 g were randomly assigned to two groups: (*a*) rats that underwent a right nephrectomy ($N = 33$) and (*b*) rats subjected to 60 minutes of left renal ischemia, immediately followed by right nephrectomy ($N = 43$). Prior investigation has shown that contralateral nephrectomy enhances functional and morphological recovery of the postischemic kidney [26, 27]. To evaluate the effect of uninephrectomy on tubular morphology and OPN expression, the left

kidneys of three normal controls were also examined (Fig. 1A).

Rats were anesthetized with sodium pentobarbital (Nembutal, Sanofi, 65 mg/kg intraperitoneally). After opening the abdomen via a long midline incision, 50 IU of heparin were instilled in the peritoneum. After careful dissection, sparing the blood supply to the adrenal gland, the left renal pedicle of rats in the ischemia group was clamped for 60 minutes with a microvascular clamp, after which a right nephrectomy was performed, with cauterization of the ureter stump, likewise sparing the adrenal gland vessels. In uninephrectomized animals, the left renal pedicle was dissected, but not clamped.

Postoperatively, rats were housed in separate cages on a 12-hour light-dark cycle; they were allowed free access to drinking water. As postischemic rats show more postoperative anorexia, rats in both experimental groups were pair fed with standard rat chow and weighed daily. Animals were sacrificed 1, 2, 6, 12, and 24 hours and 2, 3, 5, 7, and 10 days after reperfusion (ischemia group) or the end of the right nephrectomy (uninephrectomy group), respectively. After explorative analysis, the data of several time points were grouped, and the following five time groups were maintained: ≤12 hours, 24 hours, 2 to 3 days, 5 to 7 days, and 10 days.

All procedures were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals no. 85–23 (1985) and with approval of the Ethical Committee of the University of Antwerp.

Determination of serum creatinine

At the time of sacrifice, blood samples for determination of serum creatinine were obtained via cardiac puncture. Blood samples were allowed to clot and were centrifuged for 15 minutes at high speed. Serum creatinine was determined using a colorimetric method (Creatinine Merckotest; Diagnostica Merck, Darmstadt, Germany).

Tissue collection and immunohistochemical staining

Left kidneys were harvested immediately after sacrifice. Sagittal slices were fixed in methacarn for four hours and were subsequently processed for paraffin embedding. After blocking with normal rabbit serum, 4 μm thick sections were incubated overnight with OP199, an affinity-purified goat antirat OPN antiserum [28]. After washing, sections were incubated with a biotinylated rabbit antigoat IgG antibody (Vector Laboratories, Burlingame, CA, USA) and subsequently with avidin and biotinylated horseradish peroxidase (AB-complex; Vector Laboratories). A dark brown color was developed with diaminobenzidine in the presence of 0.03% H_2O_2. Sections were counterstained with methylgreen and periodic acid-Schiff.
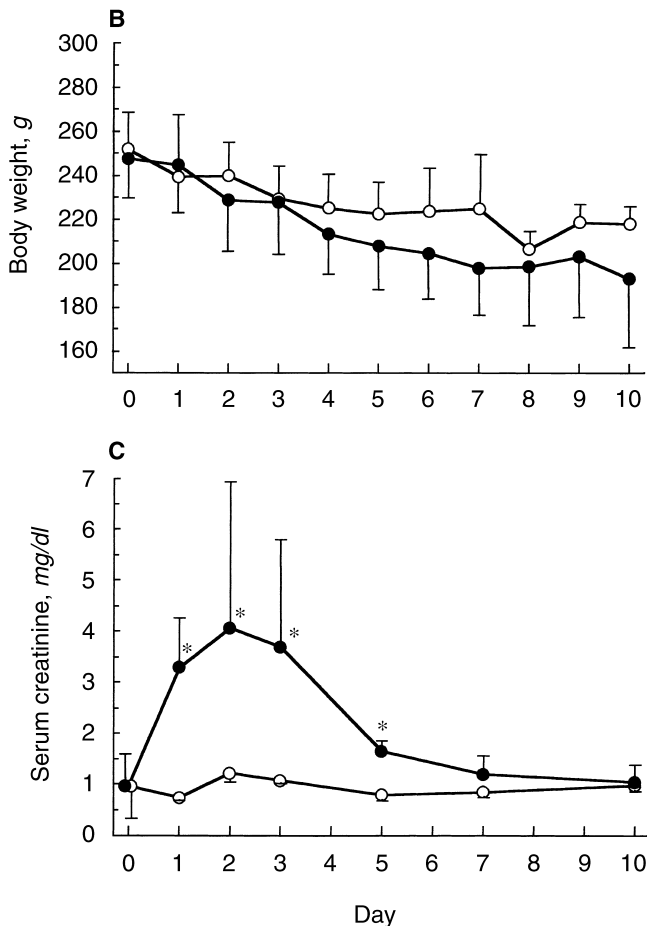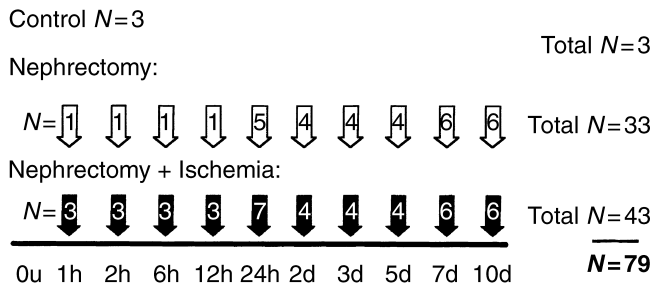
A Study set-up

Control *N*=3

Nephrectomy:

Total *N*=3

N= 1 1 1 1 5 4 4 4 6 6 Total *N*=33

Nephrectomy + Ischemia:

N= 3 3 3 3 7 4 4 4 6 6 Total *N*=43

0u 1h 2h 6h 12h 24h 2d 3d 5d 7d 10d $\overline{N=79}$

Scoring systems for morphology and osteopontin expression

In every tissue section, 50 PTs and 25 DTs in the cortex as well as OSOM were selected following a reproducible procedure, which comprised at random selection of the first tubular cross-section, followed by shifting the microscopic field over a fixed distance according to a standardized pattern. Each selected tubular cross section was scored for OPN staining and for tubular morphology (at magnification ×300).

The semiquantitative scoring system for OPN is based on the relative number of OPN-positive cells in a tubular cross section, with 0 = no OPN positive cells; 1 = <50% OPN positive cells; 2 = ≥50% OPN positive cells; and 3 = 100% OPN positive cells in the tubular cross section.

To evaluate tubular morphology, separate scoring systems for PTs and DTs were used. The term DT was used *sensu strictu*, with DT of the cortex standing for distal convoluted tubules (DCTs) and OSOM DT designating the mTAL. Even in severely damaged kidneys, it was possible to distinguish between PT, and DT, according to at least one of the following morphological criteria: topographical localization, tubular size and form, cytoplasmic density and position of the nuclei, and presence or absence of brush border and basolateral cell aspect [29].

Proximal tubule cross-sections were classified into three categories: normal, damaged, and regenerating. Normal PT cross sections showed a continuous epithelium, with an intact periodic acid-Schiff–positive brush border. Damaged PTs were PT cross sections showing no brush border observed within 24 hours after reperfusion and PT cross sections with a discontinuous epithelium, ranging from loss of a few cells to a totally naked basement membrane. Regenerating PTs were PT cross-sections without brush border observed more than 24 hours after reperfusion or those showing a flat epithelium containing cells with large nuclei and a very narrow cytoplasmic rim or showing some restoration of cytoplasmic volume with still large nuclei protruding into the tubular lumen.

Scoring for DT morphology was restricted to "normal" or "damaged" tubules according to the following criteria: Normal DT cross-sections had a continuous epithelium with cells showing basal striping. Damaged DT cross sections contained either cells that showed vacuolization or necrotic cells ranging from a few necrotic cells to total tubular necrosis. Because the number of damaged tubules and the extent of DT damage were limited, it was not possible to define morphological criteria to assess distal tubular regeneration by light microscopy.

Fig. 1. Animal data. (*A*) Experimental setup. Rats were randomly assigned to either the uninephrectomy or the ischemia group. Figures represent the number of animals used in each time group. Additionally, the left kidneys of three normal rats were examined to evaluate the effects of uninephrectomy on tubular morphology and OPN expression of the contralateral kidney. (*B*) Body weight in nephrectomized control (○) and nephrectomy + ischemia (●) rats. The animals in the ischemia group showed postoperative anorexia during approximately three days. They lost weight throughout the study period, but because of the method of paired feeding, no significant differences in body weight occurred between both groups. (*C*) Serum creatinine. Uninephrectomy resulted in a slight, but not significant, rise in serum creatinine on day 2. Kidney ischemia/reperfusion caused a significant increase in serum creatinine, already present 24 hours after reperfusion and lasting for several days, with serum creatinine returning to normal on day 7.

Statistics

Data are presented as mean ± SD or as percentages, as indicated. Data were statistically analyzed using *Systat*

7.0 for Windows software. For the animal data, we used a one-way analysis of variance test with a Newman–Keuls post hoc test. Data from the scoring systems for OPN expression and morphology were analyzed with Pearson's χ^2 test and Cochran's linear trend test when applicable. A $P < 0.05$ was considered significant.

RESULTS

Animals

Mortality. Nine out of 79 animals died prior to planned sacrifice, corresponding with an overall mortality rate of 12%. In the nephrectomy group, only one animal died. From the eight animals that died in the ischemia group, one animal died of postoperative bleeding. Another died with severe hemolysis after two days. The others mostly died after three to five days, probably because of severe acute renal failure. Data of these animals were not used.

Body weight. Animals in both experimental groups suffered a comparable postoperative weight loss, which persisted throughout the 10-day study period. Because of the method of paired feeding, no significant differences in body weight between ischemia and nephrectomy groups occurred (Fig. 1B).

Kidney function. As shown in Figure 1C, uninephrectomy gave rise to a slight, not statistically significant, rise in serum creatinine after two days (1.23 ± 0.19 mg/dl). However, a combination of uninephrectomy with 60 minutes of contralateral renal ischemia resulted in a sharp rise in serum creatinine, already present 24 hours after reperfusion (3.30 ± 0.97 mg/dl) and increased further toward a maximum after two days (4.07 ± 2.86 mg/dl), reflecting acute renal failure. Subsequently, serum creatinine levels decreased, again becoming normal after seven days (1.20 ± 0.36 mg/dl).

Tubular morphology

Throughout the study period, no signs of morphological renal injury or regeneration were observed in kidneys of uninephrectomized rats (Fig. 2). No significant differences in morphology were noticed between the kidneys of uninephrectomized rats and those of normal controls ($P = 0.31$; data not shown). Hence, uninephrectomized rats were used as a control group.

Proximal tubules of the OSOM corresponding with the S3 segment of the PT (proximal straight tubule) suffered the most severe morphological damage after renal I/R (Fig. 2). Tubular cells lost their brush border, became necrotic, and/or detached from the tubular basement membrane (Fig. 4B). Compared with uninephrectomized animals, the number of damaged tubules in postischemic kidneys had already significantly increased after 12 hours ($P < 0.001$), and became maximal after 24 hours, when only 5.1% of the tubules were still normal

(Fig. 2). Over the following days, the number of tubules with normal morphology increased again, reflecting ongoing morphological regeneration. During regeneration, the tubular basement membrane became lined with a flat epithelium, showing large nuclei protruding into the tubular lumen; later on, tubular epithelium in varying stages of restoration of cytoplasmic volume and cells that restored a brush border were seen (Fig. 4C). Morphological repair started at 24 hours after reperfusion. After two to three days, a large number of PT cross sections were regenerating (41.6% of OSOM PTs), reaching a maximum after five to seven days (67.7% of all PTs in the OSOM), and diminishing slightly on day 10 (55.5% of OSOM PTs). Morphological restoration was not completed by the end of the study period, with only 41.5% of PT cross sections in the OSOM showing normal morphology on day 10, but as stated earlier, regeneration was still ongoing (Fig. 4D).

Proximal tubules in the cortex showed the same time course of morphological alterations in that the maximum number of damaged tubular cross sections appeared after 24 hours (Fig. 2). However, injury to the PTs in the cortex was much less severe. After 24 hours, 60.6% of all tubules still displayed a normal morphology, and over the whole study period, the OSOM showed significantly more PTs with aberrant morphology than the cortex ($P < 0.001$). Not only was the number of damaged tubules lower in the cortex, but also the extent of morphological damage in PTs of the cortex was less. Most damaged PTs in the cortex only showed a loss of brush border or desquamation of a limited number of cells. Complete tubular necrosis was very seldom seen, except for some triangular zones in the outer cortex. In keeping with the limited morphological damage, the fraction of regenerating tubular cross sections was also lower than in the OSOM, reaching a maximum of 15.7% after five to seven days.

Distal tubules in both cortex and OSOM showed significant ($P < 0.001$) though limited morphological damage in postischemic animals compared with uninephrectomized rats, which was significantly greater in the OSOM than in the cortex ($P < 0.001$). However, morphological damage was limited with respect to both the number of damaged tubules and the severity of tubular injury. After 24 hours, a maximum of 16 and 11.7% tubules with damaged morphological appearance was seen in the OSOM and cortex, respectively. This was significantly less than the number of damaged PTs in both OSOM and cortex (both $P < 0.001$). Most damaged DT cross-sections showed vacuolization of some cells; only a limited number contained necrotic cells (maximum 7% of DTs in the OSOM after 24 hr), whereas complete tubular necrosis was never observed.

Osteopontin expression

Osteopontin expression in uninephrectomized rats, as assessed by our scoring system, was not significantly dif-
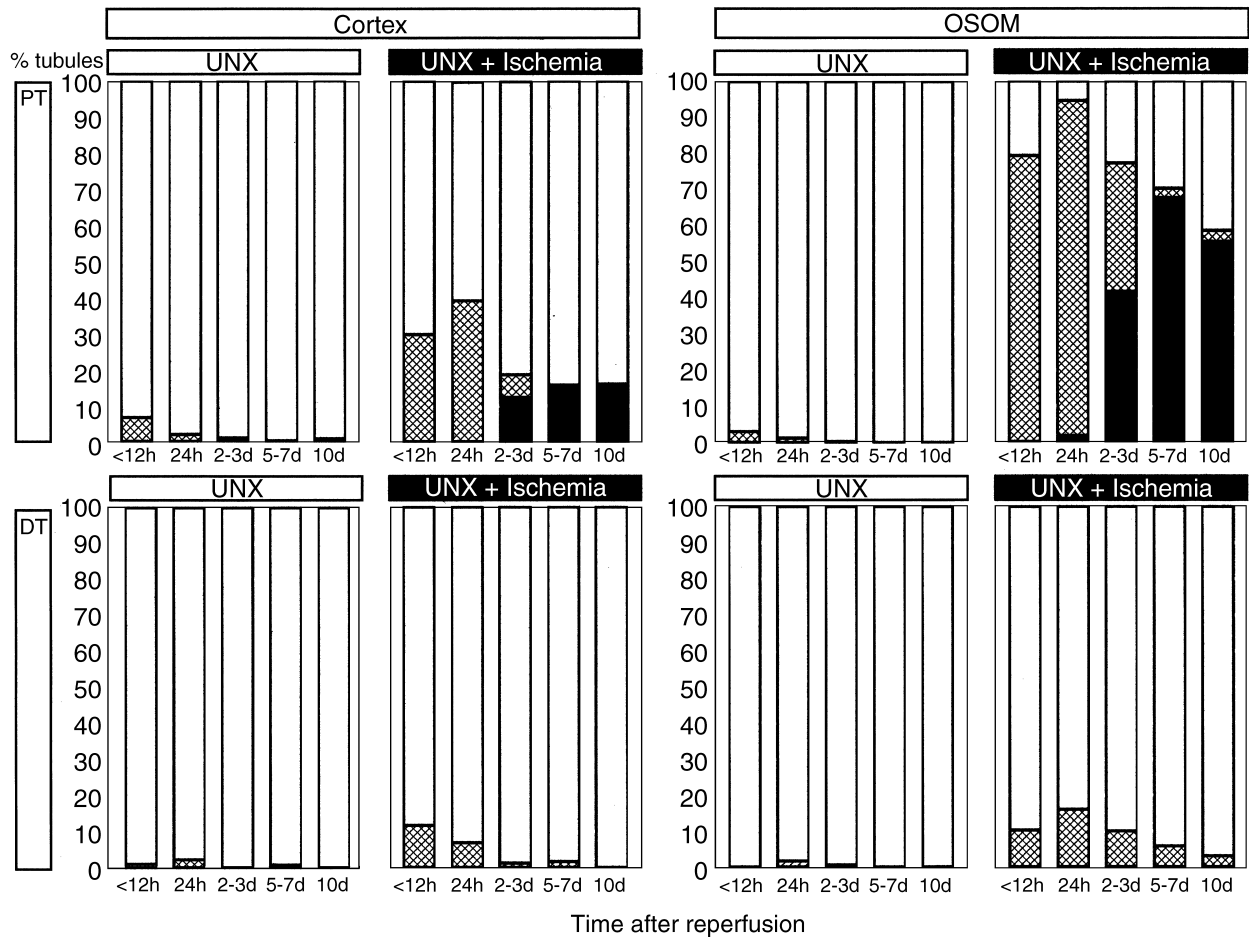
Fig. 2. Tubular morphology showing (□) normal, (⊠) damaged or (■) regenerating tubules. Uninephrectomy does not induce morphological signs of damage or regeneration. Kidney ischemia/reperfusion (I/R) causes the most severe injury in proximal tubules (PTs) of the outer stripe of the outer medulla (OSOM), with maximal damage 24 hours after reperfusion, followed by a regeneration process, which is not yet complete at the end of the study period. PTs of the cortex are also maximally damaged 24 hours after reperfusion, but the extent of the damage is significantly less than in PTs of the OSOM. Distal tubules (DTs) show very limited damage after kidney I/R, and morphological signs of regeneration in DTs could not be assessed (for statistical significances, see text).

ferent from OPN expression in normal controls ($P = 0.6$, data not shown). OPN expression was seen in thin limbs of Henle loop and in papillary epithelium (data not shown), which corresponds with previous reports [19, 30, 31]. In DTs, a basal OPN expression was found that remained constant over the study period; 43.3% of DTs in the cortex were OPN positive versus 11.7% in the OSOM, but the majority of these OPN-positive DTs show only OPN staining in a limited number of cells (score 1). PTs, on the other hand, showed almost no OPN staining in basal circumstances. In the cortex as well as OSOM, less than 2% of PTs were OPN positive (Fig. 3).

After kidney I/R, PTs of the OSOM showed no significant rise in OPN immunostaining up to 12 hours after reperfusion, and the increase became significant after 24 hours ($P < 0.001$) and reached a maximum after five to seven days, followed by a limited decrease on day 10

(Fig. 3). Likewise, PTs in the cortex showed a significant increase of OPN staining after 24 hours ($P < 0.001$), again with a similar time course as in the OSOM, with also a maximum after five to seven days. The maximum percentage of OPN-positive tubules was higher in the OSOM than in the cortex: 95.7 vs. 59.6%, respectively (Fig. 3).

Distal tubules showed a rapid increase in OPN staining after I/R of the kidney, which was already highly significant 12 hours after reperfusion ($P < 0.001$) and reached a peak after 24 hours. At this moment, virtually all DT cross sections stained positively for OPN. Subsequently, there was a slow decrease in OPN positivity of DTs toward the end of the study period, with globally still 64% of DTs showing OPN staining at day 10, which was still significantly higher than in the control group ($P < 0.001$). The time course and the extent of OPN up-regulation after I/R were comparable in DTs of cortex and
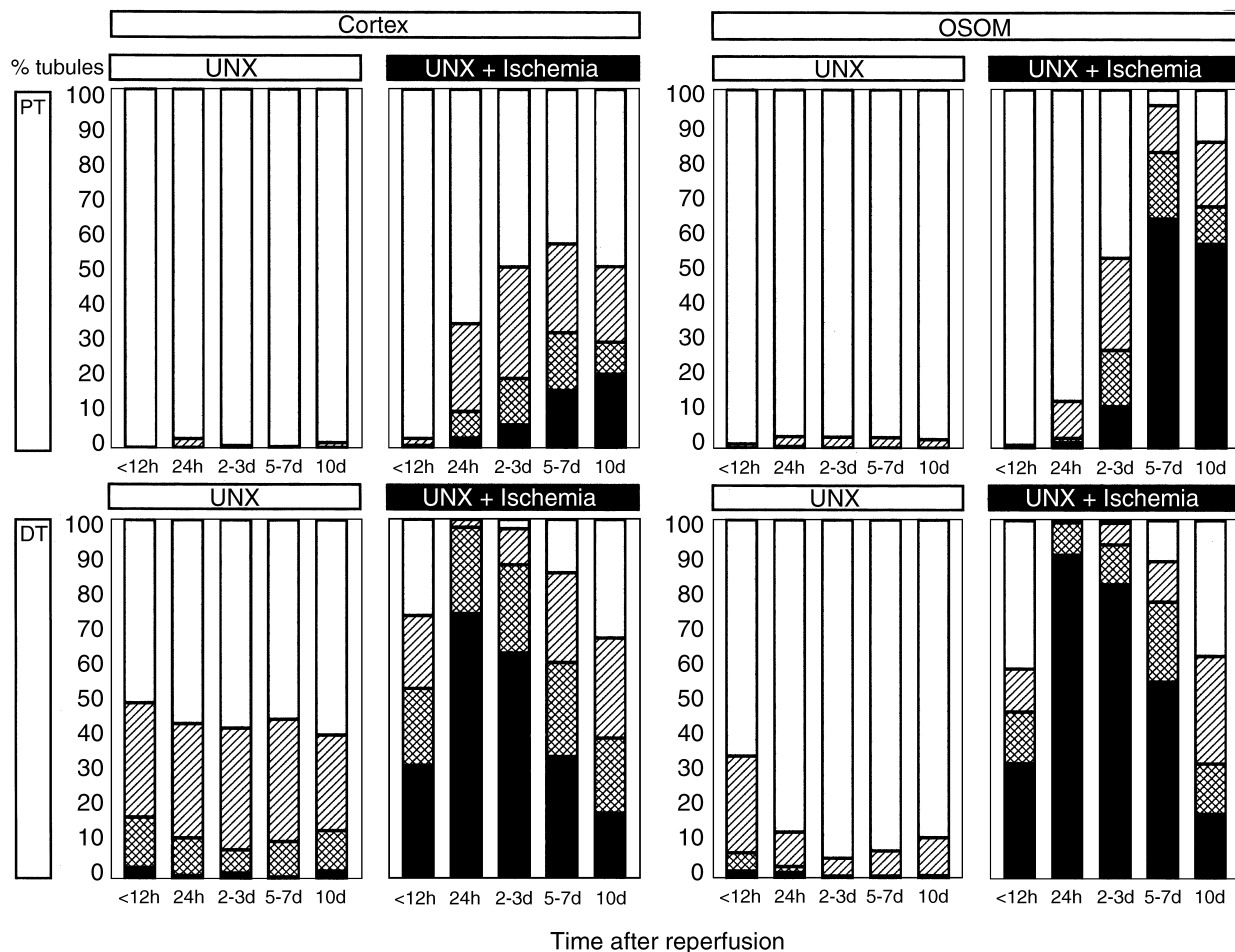
Fig. 3. Osteopontin (OPN) expression. OPN scores are: (□) 0 (negative); (▨) 1 (<50%); (⊠) 2 (>50%); (■) 3 (100%). In the nephrectomy group, a fraction of distal tubules (DTs), not significantly different of that in normal rats (data not shown), show OPN expression, whereas proximal tubules (PTs) in this group show almost no OPN expression. After I/R, DTs show a rapid and sustained increase in OPN expression. PTs in the outer stripe of the outer medulla (OSOM) show a delayed onset of OPN expression, remaining largely OPN negative during kidney injury and becoming highly OPN positive during morphological regeneration. PTs of the cortex show the same delay in OPN expression as PTs of the OSOM, but the fraction of OPN-positive tubules remains much lower (for statistical significances, see text).

OSOM, but the OSOM showed more tubules where all cells stained OPN positive (score 3): 90.3 vs. 73.7% in the cortex after 24 hours.

A striking observation is that the subcellular OPN staining pattern in PTs and DTs greatly differed. PTs with normal morphology showed a perinuclear, vesicular staining pattern (Fig. 4E). Remarkably, regenerating PTs in the OSOM additionally showed a transient OPN staining at the luminal cell side in combination with the perinuclear staining when the cytoplasm volume was par-

Fig. 4. Photomicrographs. (*A*) Normal outer stripe of the outer medulla (OSOM). Proximal tubules (PTs) showing a continuous epithelium with a prominent PAS-positive brush border (normal control, magnification ×250). (*B*) Damaged OSOM. OSOM at the moment of maximal morphological damage, 24 hours after reperfusion, with PTs showing desquamation of cells (1) and total tubular necrosis (2). Distal tubules (DTs) are mostly undamaged (3) and show prominent osteopontin (OPN) expression. The arrow indicates a damaged DT (magnification ×250). (*C*) Regenerating OSOM. At the moment of maximal morphological regeneration, five days after reperfusion, regenerating PTs show a flat epithelium with large nuclei protruding into the lumen (1) or display various stages of restoration of cytoplasmic volume (2). Notice the expression pattern of OPN, which is vesicular in the center of the cell in PT with normal morphology (arrow) and shows an additional apical staining in regenerating tubules and tubules that are restoring their brush border (arrowhead; magnification ×250). (*D*) Morphology at day 10. At the end of the study period, normal morphology is partially restored (1; PT with normal morphology), but regeneration is still ongoing (2; regenerating tubules). OPN expression is still high in regenerating PT (magnification ×250). (*E*) OPN expression in PT. OPN expression in PT is confined to a number of perinuclear, vesicle-like structures (arrows; magnification ×1000). (*F*) OPN expression in DTs. OPN staining in DTs is prominent at the apical cell side (arrows; magnification ×800).
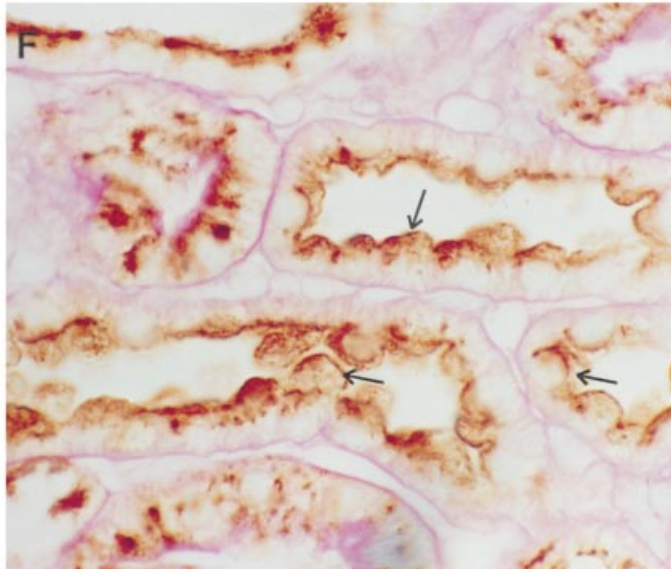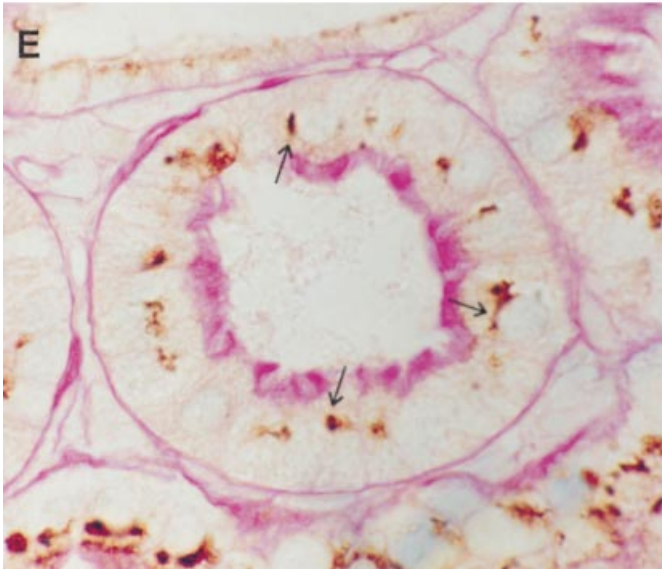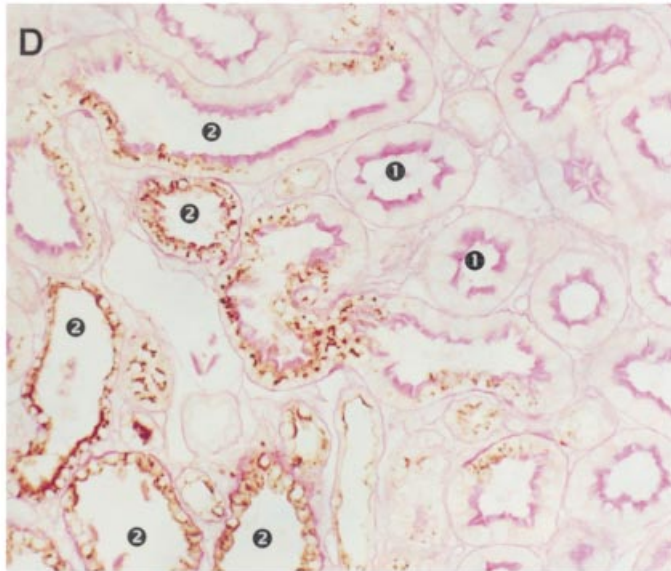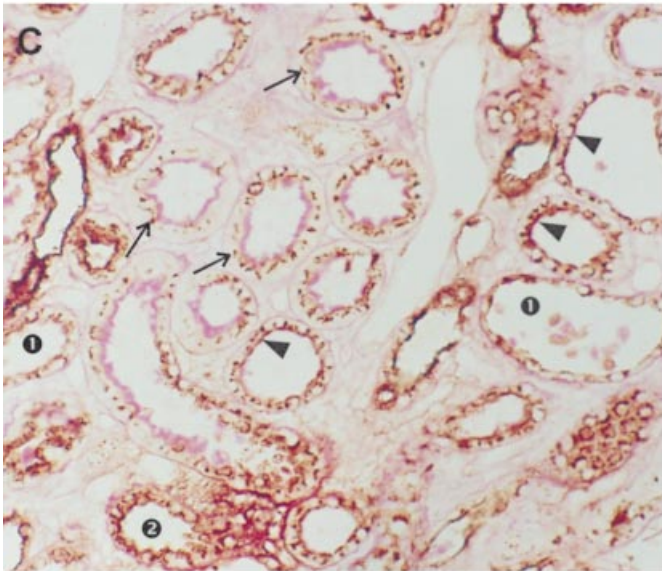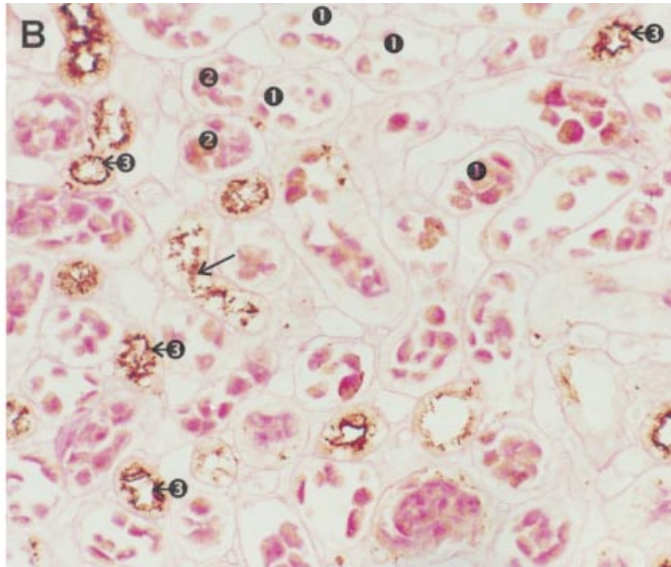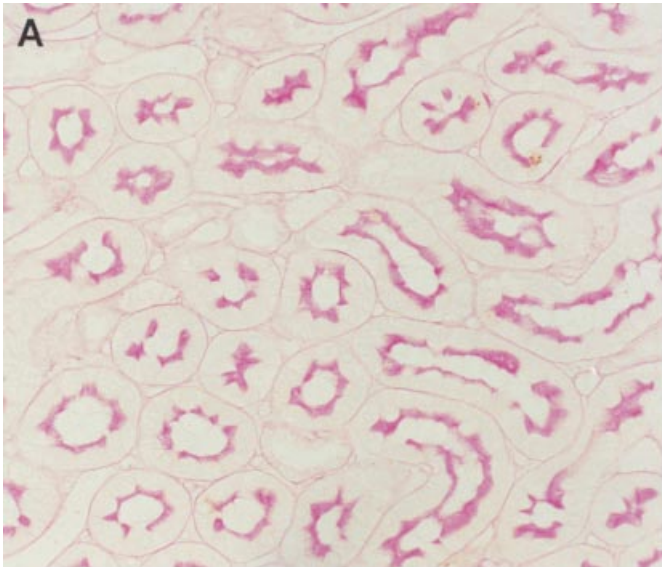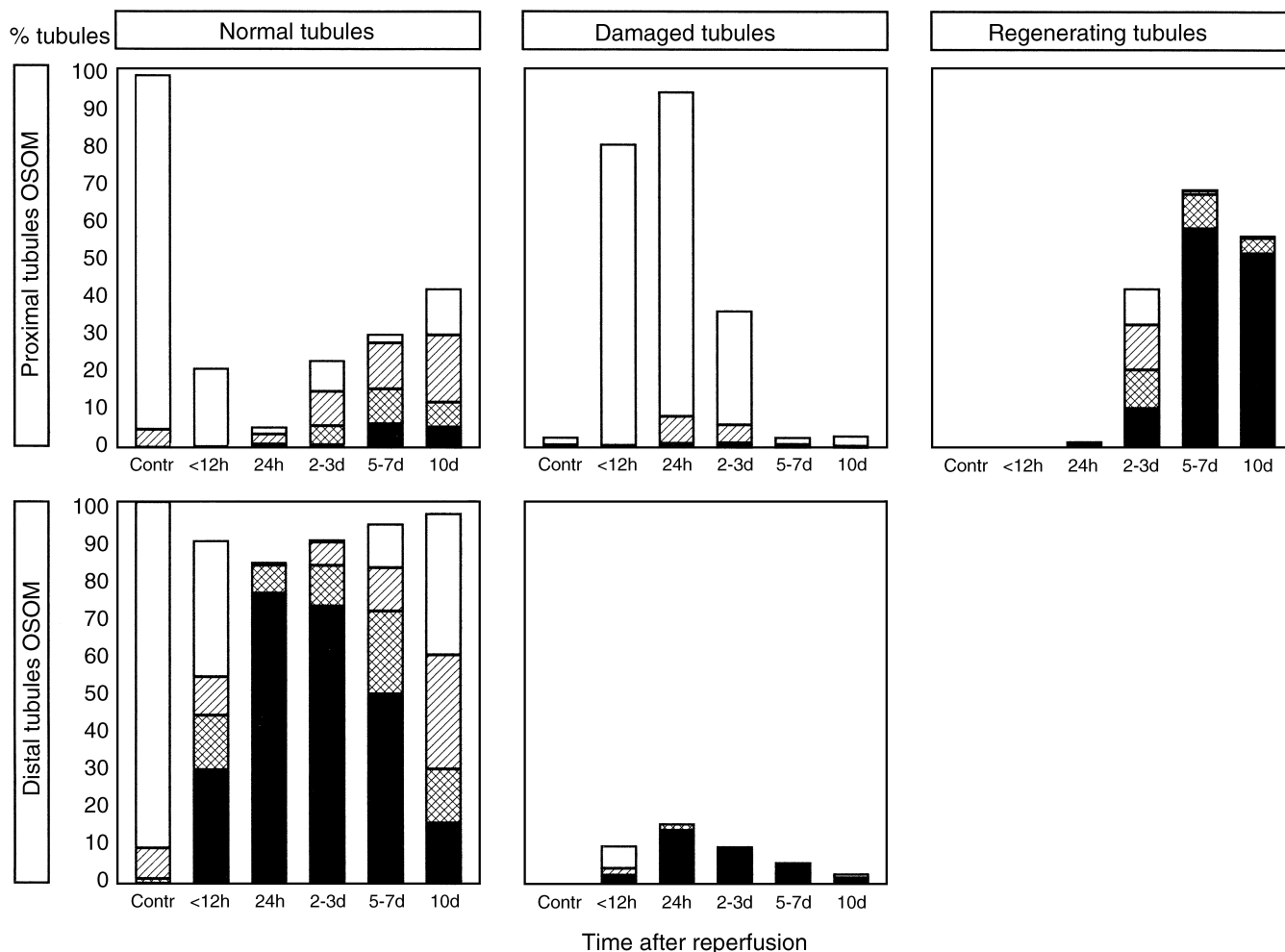
Fig. 5. Osteopontin (OPN) scores to determine the function level of tubular morphology. OPN scores are: (□) 0 (negative); (▨) 1 (<50%); (⊠) 2 (>50%); (■) 3 (100%). Proximal tubules (PTs) of the outer stripe of the outer medulla (OSOM) mostly remain OPN negative when morphologically damaged, but become highly OPN positive when showing morphological signs of regeneration. Thus, OPN expression in PTs of the OSOM is associated with morphological regeneration. Distal tubules (DTs) show substantially increased OPN expression without major morphological damage.

tially restored (Fig. 4C). In contrast, DTs showed a continuous layer of OPN positivity at the apical cell side (Fig. 4F).

Relationship between tubular morphology and osteopontin expression

The relationship between OPN expression and morphology of tubular cross sections after kidney I/R was studied by analyzing the OPN scores of normal, damaged, and regenerating tubules in the OSOM, as shown in Figure 5.

The PTs of the OSOM in uninephrectomized animals predominantly showed normal morphology combined with OPN negativity (92.7% of all tubules). In the first 24 hours after I/R, there was a shift toward damaged morphology (93% of the PT cross-sections in the OSOM at 24 hr), but OPN expression in damaged tubules stayed

very low throughout the study period. After 24 hours, when regenerating tubules started to appear, the large majority of these tubules displayed strong OPN positivity. After five to seven days, when regeneration was maximal, 92% of regenerating PT cross sections in the OSOM showed OPN staining in all cells (score 3). OPN staining in normal PTs increased after 24 hours but was much less prominent than in regenerating tubules. These data demonstrate that PTs in the OSOM are predominantly OPN negative when damaged and become highly OPN positive when regenerating.

In contrast, a rapid increase of OPN expression in normal DTs of the OSOM was seen over the first 24 hours toward a high percentage of tubules with OPN staining in all cells, which persisted throughout the study period. The limited number of damaged tubules was also strongly OPN positive. Thus, DTs in the OSOM showed

a fast and sustained up-regulation of OPN expression, regardless of their morphological appearance.

DISCUSSION

The aim of this study was to compare OPN expression in PTs and DTs in relationship to tubular morphology during postischemic injury and subsequent regeneration. After I/R of rat kidney, up-regulated OPN expression was found in both PTs and DTs of cortex and OSOM.

A single kidney ischemia model was used in this experiment, in the context of a program studying the effects of renal ischemia in transplantation. Moreover, the presence of the contralateral normal kidney after severe ischemic injury strongly impairs renal regeneration, even resulting in pronounced atrophy of the injured kidney [26, 27]. Several morphological criteria were used to obtain a classification of tubules into normal, damaged, or regenerating, based on the studies of Venkatachalam et al, whereas the sequence of events after renal I/R is described morphologically [4].

Up-regulation of OPN expression after I/R of the kidney has been demonstrated in previous studies. Kleinman et al found enhanced OPN expression after 40 minutes of unilateral kidney ischemia, without uninephrectomy [24]. Postischemic kidneys were compared with the contralateral normal ones. This study also observed a delayed onset of OPN up-regulation in the S3 segment, that is, at 24 hours no OPN staining was found in the S3 segment; by 48 hours, some OPN was visible in this segment, and the expression reached a maximum on day 5, which was the end of the study period.

Padanilam, Martin, and Hammerman determined total kidney OPN mRNA levels in a rat model with 60 minutes of bilateral kidney ischemia [25]. No increase in OPN mRNA was found 12 hours after reperfusion, but OPN mRNA levels had increased 18-fold by 24 hours. *In situ* hybridization and immunohistochemistry showed OPN staining in DTs and mTAL 24 hours postischemically and additional OPN expression in PTs after five days, when regeneration is in progress.

Our study presents morphological data, obtained over an extended study period ranging from 1 hour to 10 days after reperfusion, giving detailed information regarding the time course of OPN staining in relation to morphological injury and repair of the kidney after I/R.

Furthermore, we demonstrated a different subcellular staining pattern in PTs and DTs after I/R, in agreement with our previous findings in toxic acute renal failure (abstract; ibid). In PTs, a punctiform, perinuclear OPN staining was found, whereas DTs display OPN staining predominantly located to the apical cell side. The mechanism of this different expression pattern in PTs and DTs is still unclear. It might be related to the subcellular synthesis site and the destination of the protein. Madsen

et al demonstrated OPN up-regulation in a punctate pattern in PTs after the administration of the bacterial endotoxin lipopolysaccharide (LPS), which the authors interpreted as OPN uptake into the vacuolar-lysosomal system, whereas DTs showed enhanced OPN staining presumably in the Golgi apparatus and in small cytoplasmic vesicles [30]. However, OPN mRNA has been previously demonstrated in proximal cells (as well as in DTs) by *in situ* hybridization in models of ischemic [25] and toxic renal injury (abstract; ibid), indicating OPN transcription by PTs.

Another hypothesis, accounting for the difference in OPN staining pattern between PTs and DTs observed in this study, is that PTs and DTs may produce different molecular forms of OPN protein after renal I/R. Differential phosphorylation, glycosylation, alternative mRNA splicing, and proteolytic cleavage of OPN by thrombin may result in different molecular forms of OPN protein [32–35]. It is possible that the polyclonal antibody OP199 used in this study would detect more molecular forms than a monoclonal antibody. However, no differences in OPN staining pattern were found by us or others [22], when comparing OP199 with MPIIIB10, a mouse monoclonal against rat OPN, on serial sections. Which OPN form(s) that is actually produced in the kidney *in vivo* is not exactly known.

The pathophysiological relevance of OPN up-regulation after renal ischemia and reperfusion remains unknown. Our study demonstrates that OPN up-regulation is different in PTs versus DTs. In PTs, the delayed increase in OPN expression after kidney I/R is related to morphological repair. However, early and pronounced OPN up-regulation is present in DT cells, which predominantly retain normal morphology.

Osteopontin positivity is seldom found in severely damaged PTs of the OSOM. The absence of OPN up-regulation in such tubules can be expected, given the presence of extensive cell loss or even complete tubular necrosis. The small number of OSOM PTs that are OPN positive during the injury phase is morphologically normal, or suffer only slight morphological damage limited to the loss of brush border or loss of a few cells. Later on, during morphological restoration, a large number of PT, mostly with the morphology of regenerating tubules, display OPN staining.

The striking association of increased OPN staining with regenerating PTs suggests that OPN may have some function in the regeneration process of the kidney. The time course of OPN up-regulation in PTs of the OSOM, with a maximum after five to seven days, is parallel but delayed to that of proliferating cell nuclear antigen (PCNA) positivity in this segment, which reaches its maximum three days after reperfusion in this model [5]. Through its adhesive and migratory properties [14, 35–37], OPN may be important in the re-epithelialization

of the tubular basement membrane. Also, through interaction with cell surface integrins, which become redistributed over the entire cell surface in injured cells [38, 39], OPN could influence cell attachment and prevent desquamation of cells and intratubular cast formation. Experimental evidence pointing in this direction is that the *in vivo* administration of RGD containing peptides, which also interact with integrins, results in less severe acute renal failure and less cast formation after kidney I/R in rats [40]. It is possible that chemoattraction of macrophages [19, 20, 22, 41] (abstract; Ophascharoensuk et al, *J Am Soc Nephrol* 8:481A, 1997) and inhibition of inducible nitric oxide synthase (iNOS) induction [42] could limit the extent of the injury, because OPN inhibits NO-production and cytotoxicity of activated macrophages [43], and inhibition of iNOS expression results in higher survival rates of cells exposed to oxidative stress [44].

In the distal nephron, mTALs and distal convoluted tubules show rapid, prominent, and sustained up-regulation of OPN expression throughout the study period, in the absence of major morphological damage. This possibly reflects phenotypical alterations in the distal nephron as an adaptation to loss of proximal tubular function, causing water and solute overload to the DTs [45, 46]. Because I/R also injures the PTs, OPN up-regulation could be part of the hypertrophic and hyperplastic reaction of the distal nephron to PT cell loss. Alternatives are that OPN expression in DTs is induced by cytokines released from adjacent injured PTs [42] or that increased OPN expression reflects a minor injury not visible by light microscopy. The triggers for OPN up-regulation and the possible role of this protein in the regeneration process of the kidney remain subjects for further investigation.

In summary, our study reports a substantial increase in OPN expression after I/R of the rat kidney, in PTs and DTs of both cortex and OSOM. PTs and DTs show a different subcellular OPN expression pattern, with a vesicular, perinuclear immunostaining in PTs and OPN positivity at the luminal cell side in DTs. DTs show an early and persistent increase of OPN expression after I/R, whereas in PTs of the OSOM, the most severely damaged segment, up-regulation of OPN expression is delayed and associated with morphological regeneration. These findings suggest a function of OPN up-regulation in the regeneration process after kidney I/R, but its possible contribution to renal regeneration remains unclear and will be the subject of further investigation.

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APPENDIX

Abbreviations used in this article are: ATN, acute tubular necrosis; DCT, distal convoluted tubule; DT, distal tubule; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion; mTAL, medullary thick ascending limb; NO, nitric oxide; OPN, osteopontin; OSOM, outer stripe of outer medulla; PCNA, proliferating cell nuclear antigen; PT, proximal tubule; RGD, arginine-glycine-aspartic acid.

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