Renal osteopontin protein and mRNA upregulation during acute nephrotoxicity in the rat

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Abstract
Background. The effect of segment-specific proximal tubular injury on spatio-temporal osteopontin (OPN) distribution was determined in two different nephrotoxic rat models to evaluate its conceivability with a possible role for OPN in acute renal failure (ARF). OPN gene expression was further determined in proximal and distal tubular cells to investigate the origin of increased renal OPN.

Methods. Renal OPN protein and mRNA expression were compared in the rat during mercuric-chloride- vs gentamicin-induced ARF using immunohistochemistry and in situ hybridization.

Results. Mercuric chloride primarily induced tubular injury and subsequent cell proliferation in proximal straight tubules (PST), whereas gentamicin predominantly injured proximal convoluted tubules (PCT). In both models, the distribution of OPN protein was associated with increased OPN mRNA levels in proximal as well as distal tubular cells. However, upregulation was delayed in the proximal tubular segment suffering most from injury, i.e. PST in gentamicin ARF vs PCT in mercuric-chloride ARF. OPN immunostaining at the apical cell membrane from distal tubules was in contrast to perinuclear vesicular staining in proximal tubular cells.

Conclusions. OPN gene and protein expression is induced in both proximal and distal tubular cells during rat toxic ARF. The distinct subcellular localization in proximal vs distal tubular cells indicates differences in OPN processing and/or handling. The spatio-temporal distribution is consistent with a possible role in renal injury and regeneration.

Keywords: acute renal failure; gentamicin; kidney; mercuric chloride

Introduction

Acute renal failure (ARF) due to ischaemia-reperfusion injury or toxic exposure still has a high mortality rate, despite major improvements in dialysis treatment and in clinical practice in general. Extensive tubular lesions including cell necrosis and shedding are accompanied by transient impairment of renal function and interstitial leukocyte accumulation [1]. Proliferation of surviving tubular cells marks the onset of subsequent renal regeneration. Migration, spreading, and firm attachment of immature tubular cells to the denuded basement membrane precedes cell polarization and differentiation [2]. Eventually, renal function and tissue integrity can be restored in a limited time. New rational interventions may accelerate this regeneration process, increase its efficiency, and improve the outcome of the syndrome.

Experimental and structural data suggest that osteopontin (OPN) has the potential to influence this remodelling process. OPN is a phosphorylated glycoprotein, originally isolated from bone but produced by a variety of cell types, including renal tubular epithelium [3]. Considerable amounts are found in urine, where it presumably inhibits the formation of calcium oxalate crystals [4]. However, the abundant expression of OPN after renal injury, but also in other organs and tissues in conditions of high cell turnover such as vascular wall, heart, skin, lung, skeletal muscle, and bone, suggests that this protein may be important in tissue remodelling [5].

A protective role in the kidney was assumed from the reduced tolerance of OPN knockout mice to renal ischaemia, possibly by inhibition of inducible nitric oxide synthase (iNOS) [6]. By means of its interaction with a variety of integrins, the arginine-glycine-aspartic acid (RGD) sequence of OPN may promote cell attachment, mediate cell migration [7], and decrease tubular cast formation [8]. Furthermore, OPN reduces apoptotic cell death, and its macrophage-attractant properties as well as its stimulation of TGF-β activity and collagen I and IV deposition suggest that it has profibrogenic properties [9].
In rat kidney, increased OPN expression was reported in angiotensin II hypertension [10], after unilateral ureteral obstruction [11], in various experimental models of glomerulonephritis and tubulointerstitial nephritis [12–15], and after renal ischaemia [16–18]. We have previously demonstrated that immunoreactive OPN appears in distal tubular and regenerating proximal tubular cells during renal ischaemia–reperfusion injury, but not in severely injured proximal tubular cells [18]. Assuming that OPN participates in the tissue remodelling process after renal injury in general, this relationship should also be present in other, unrelated ARF models such as toxic ARF, where alteration of haemodynamic parameters does not seem to play a central role in the initiation of renal injury [19]. Therefore, in the present study, in two different models of toxic ARF, we investigated whether segment-specific proximal tubular injury affects renal OPN distribution. A second important issue is the origin of immunoreactive OPN in proximal tubular cells. The only description of OPN mRNA in rat proximal tubular cells to suggest that proximal tubular cells are able to produce OPN [17] is contradicted by Madsen and colleagues [20], who found OPN after lipopolysaccharide (LPS) induction only in the vacuolar–lysosomal system of rat proximal tubular cells and concluded that proximal tubular OPN is reabsorbed from the tubular fluid. To determine in the present study whether the presence of immunoreactive OPN in proximal tubular cells is the consequence of increased OPN gene expression rather than endocytotic uptake, the distribution of OPN mRNA was compared with the localization of OPN protein.

Two nephrotoxic rat models with a comparable time course but a different proximal tubular target were chosen. Gentamicin induces acute tubular injury to the cortical proximal convoluted tubules (PCT) in particular, whereas the major target of mercuric-chloride (HgCl₂) nephrotoxicity are the proximal straight tubules (PST) in the outer stripe of the outer medulla (OSOM) and medullary rays.

Subjects and methods

Animal treatment

Gentamicin or mercuric chloride was administered to female Wistar rats (200–220 g). A first group (n = 28) received 400 mg/kg gentamicin intraperitoneally (i.p.) (40 mg/ml in 0.9% NaCl) divided over three daily injections at days 1 and 2. A second group (n = 28) received a single subcutaneous (s.c.) injection of 2.5 mg/kg mercuric chloride (1 mg/ml in 0.9% NaCl) at day 1. Control rats (n = 7 and n = 14 for each treatment respectively) received vehicle under the same regimen. During the following 2 weeks, four gentamicin or mercuric-chloride-treated animals and at least one control animal for each treatment were sacrificed at different time intervals. All procedures were performed in accordance with the NIH guidelines for the care and use of laboratory animals (85–23).

Serum creatinine

Serum samples from blood drawn at sacrifice were analysed in duplicate using the Jaffé colorimetric test.

Immunohistochemical stainings

Stainings were performed on 4-μm paraffin sections from methacarn-fixed renal tissue. Proliferating cells were immunostained with a rabbit anti-mouse monoclonal antibody (PC10 from Dako, Denmark) directed to proliferating cell nuclear antigen (PCNA). After blocking with normal goat serum and incubation with the primary antibody, the sections were incubated with biotinylated goat anti-rabbit antisem in the presence of normal rat serum, and stained by the avidin–biotinylated horseradish peroxidase complex (Vectastain from Vector Laboratories, USA) using 3,3’-diaminobenzidine as the chromogen. Sections were counterstained with methyl green and periodic acid–Schiff reagent (PAS). No staining was observed when the primary antibody was omitted or replaced by an irrelevant mouse antibody of the same isotype.

Double immunostainings for OPN and macrophages were performed sequentially. After blocking with normal horse serum and incubation with ED-1 mouse anti-rat macrophage monoclonal antibody (Serotec, UK), sections were incubated with biotinylated horse anti-mouse antisem in the presence of normal rat serum, followed by the avidin–biotinylated alkaline phosphatase complex (Vectastain). A blue colour was developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoxylphosphate (BCIP) in the presence of 1-bromotetramisole. In a next step, the sections were washed and blocked with normal rabbit serum, and incubated with affinity purified goat anti-OPN antiserum (OP199). The sections were then incubated with biotinylated rabbit anti-goat antisem in the presence of normal rat serum. Peroxidase staining was performed as for PCNA except for the colour development, where 3-amino-9-ethylcarbazole was used as the chromogen. Nuclei were counterstained with methyl green. No staining was observed when ED-1 or OP199 were omitted or replaced with an irrelevant mouse antibody of the same isotype, or with pre-immune goat serum respectively.

In situ hybridization

Hybridizations were performed on 4-μm paraffin sections from Dubosq-Brasil-fixed renal tissue. After proteinase K treatment and postfixation in 4% paraformaldehyde, tissue sections were hybridized to either antisense or sense fluorescein-labelled RNA probe (RNA colour kit, Amersham, UK), generated by run-off transcription of the Eco RI (antisense) or Cla I (sense) linearized rOPN plasmid. This construct contains the Eco RI–Cla I fragment (563 bp) from the 2B7 rat OPN cDNA clone. Background was reduced by RNase A treatment and washes at high stringency. Incubation with an anti-fluorescein antibody conjugated to alkaline phosphatase was performed according to the manufacturer’s instructions. A dark purple colour was developed during overnight incubation in NBT and BCIP. No staining was observed when the OPN sense probe was used or when the sections were pre-treated with RNase A.
Analysis methods

Cell proliferation and tubular injury were scored on the same PCNA/PAS sections. Proliferating cells were counted in 10 randomly chosen microscopic fields (total area = 3.10 mm²) in cortex and OSOM respectively, with distinction made between proximal and distal tubular cells. A total of 100 tubules per renal section were scored for tubular injury: 25 proximal and 25 distal tubules in cortex and in OSOM respectively. A proximal tubule was considered as injured if its brush border was disrupted or lost, or if it contained vacuolized cells or necrotic cells. A distal tubule was considered as injured if it contained disrupted, vacuolized, or necrotic cells. Even when injury was severe, proximal tubules could still be distinguished from distal tubules by at least one of the following criteria: topographical localization, tubular perimeter, cytoplasm density and nucleus position, absence or presence of brush border, cell height, and basolateral appearance. OPN-positive tubules and ED-1-positive macrophages were counted simultaneously in 10 randomly chosen microscope fields (total area 0.78 mm²) in cortex and OSOM respectively.

Statistics

Statistical analyses were performed using SPSS statistical software. Means and standard deviations of at least four animals were compared by one-way ANOVA, and differences further isolated with the Student–Newman–Keuls post-hoc test. Percentages of injured tubules were compared with the Pearson chi-square test.

Results

Serum creatinine

Transiently increased serum creatinine values indicated a substantial decrease of the glomerular filtration rate and the development of ARF in both models (Figure 1).

Controls. Normal serum creatinine values were found throughout the entire observation period in control animals of both models.

Gentamicin ARF. Serum creatinine values started to rise at day 4 and were maximal at day 6 (5.8 ± 1.2 mg/dl), while normal values were again reached at day 12.

Mercuric-chloride ARF. Serum creatinine values increased more rapidly and peaked earlier than in gentamicin ARF. Maximal values were reached at day 2 (3.9 ± 1.4 mg/dl), while values were again normal at day 6.

Tubular injury and cell proliferation

The localization of tubular injury and cell proliferation reflected the segment-specific nephrotoxicity of gentamicin and mercuric chloride (Figures 2–4).

Controls. Renal morphology was normal in control animals while proliferating cells were scarce (Figure 2A and 2E). Less than 10 cells with PCNA-positive nuclei were found in all tubular segments (Figure 4).

Gentamicin ARF. Tubular injury was most prominent in the cortical PCT (P < 0.001 vs PST). The first signs of injury were already seen 24 h after the first gentamicin administration (day 1) and included lysosomal swelling, brush border loss, and cell vacuolization (Figure 2C). This was followed by extensive cell necrosis and detachment from the basement membrane, often resulting in completely desquamated tubules. Almost all PCT (99%) were damaged or necrotic at day 4 (Figure 3). Denuded tubular basement membranes became gradually repopulated with regenerating, flattened epithelial cells, of which most showed prominent PCNA immunostaining.

Fig. 1. Evolution of serum creatinine values after gentamicin and mercuric-chloride administration (*P < 0.05 vs controls).
Fig. 2. Proximal tubular injury and cell proliferation in the corticomedullary junction after gentamicin (left column) and mercuric-chloride administration (right column). PAS/PCNA staining (magnification 220x) of (A) control rat, (B) gentamicin-treated rat at day 4, (C) at day 8, and (D) at day 12. (E) Control rat, (F) mercuric-chloride-treated rat at day 2, (G) at day 6, and (H) at day 14. Gentamicin strongly injures cortical PCT (□) whereas mercuric chloride mainly damages PST in the OSOM and medullary rays (*). A few PCNA-positive cells (arrows) can be found in control animals and at the end of the observations.
Fig. 3. Evolution of tubular injury in renal cortex and OSOM from gentamicin- or mercuric-chloride-treated rats (*P < 0.05 vs controls).
Fig. 4. Evolution of cell proliferation in renal cortex and OSOM from gentamicin- or mercuric-chloride-treated rats (*$P < 0.05$ vs controls).
Consequently, cell proliferation was maximal in the cortical PCT at day 8 (Figure 4). This led to restoration of normal tissue architecture at day 12 (Figure 2G).

**Mercuric-chloride ARF.** In contrast to gentamicin ARF, tubular injury was most pronounced in the PST of OSOM and medullary rays (P < 0.001 vs PCT) (Figure 2D). In agreement with the rapid increase in serum creatinine values, impressive numbers of necrotic PST could already be seen at day 1, reaching a maximum of 93% of all PST at day 2 (Figure 3). In analogy to gentamicin ARF, proliferating cells started to cover the denuded basement membranes after maximal injury (Figure 2F). Consistent with the localization of the most severe tubular injury, cell proliferation was highest in the PST and maximal at day 4 (Figure 4). Normal tissue architecture was restored at day 14 (Figure 2H), except for some focal areas in the medullary rays that still contained regenerating PST.

**OPN immunostaining**

OPN immunostaining in both models rapidly increased in proximal and distal tubular cells, but was delayed in the proximal tubular segment that was most severely injured (Figures 5 and 6).

**Controls.** OPN was almost exclusively located in the medulla, where most thin limbs and the papillary uroepithelium were stained (Figure 6A). Only a small number of thick ascending limbs and proximal tubular cells contained OPN, whereas OPN immunostaining was absent in glomeruli and interstitium.

**Gentamicin ARF.** OPN immunostaining was rapidly induced in the PST (Figure 6B), but remained absent in the severely injured PCT during the first 2 days (Figure 5). Thereafter, the number of OPN-positive proximal tubules gradually increased in cortex and OSOM. OPN immunostaining in distal tubules also started to increase early and was comparable in cortex and OSOM.

**Mercuric-chloride ARF.** Many PCT and glomerular parietal epithelial cells already contained OPN within 24 h after the treatment (Figure 6C). In analogy to gentamicin, OPN immunostaining was absent until day 2 in the proximal tubular segment suffering most from injury, which were now the PST in the OSOM (Figure 5). However, the number of OPN positive PCT in the cortex gradually increased starting at day 1. OPN expression was also induced at day 1 in most distal tubules in cortex and OSOM (Figure 6D), to decrease gradually towards day 14. Near the end of the observation period, those tubules still expressing OPN were located in some focal areas where regeneration was still ongoing (Figure 6E).

The subcellular OPN immunostaining pattern was very peculiar in both models. In most proximal tubular cells, OPN was present in only a few perinuclear vesicles (Figure 6F), but in some tubules a punctate pattern could be seen just below the apical cell surface (Figure 6B). In contrast, distal tubular cells showed prominent OPN immunostaining at the apical cell surface, whereas in only a few cells perinuclear staining was noticeable (Figure 6G). Immunostaining was also most intense at the apical cell surface of immature, regenerating proximal tubular cells (Figure 6H).

**OPN in situ hybridization**

The localization of OPN mRNA (Figure 7) closely reflected the OPN immunostaining pattern (Figure 6).

**Controls.** OPN mRNA was detected in most medullary thin limbs of Henle and in papillary uroepithelial cells (Figure 7A), and was in every aspect consistent with the localization of immunoreactive OPN.

**Gentamicin ARF.** OPN gene expression was strongly upregulated in distal tubules in cortex and OSOM. OPN mRNA was not detected in severely injured PCT in the cortex, but was present in PST (Figure 7B).

**Mercuric-chloride ARF.** In analogy to gentamicin ARF, OPN mRNA expression reflected increased OPN immunostaining. OPN mRNA was not found in severely injured PST but was induced in the PCT, in distal tubules from cortex and OSOM and in glomerular parietal cells (Figure 7C, D). OPN mRNA was also found in the focal areas with immature proximal tubular cells that were present near the end of the observations (Figure 7E). The absence of the proximal tubular brush border was often associated with the presence of OPN mRNA (Figure 7F). In general, distal tubular cells showed a stronger signal than proximal tubular cells (Figure 7G). In addition, in further agreement with the immunostaining results was the strong OPN mRNA expression found in regenerating proximal tubular cells (Figure 7H).

**Interstitial macrophage accumulation**

Macrophages accumulated in the renal interstitium after both treatments (Figure 8) but no consistent association with OPN expression was observed.

**Controls.** Only few macrophages were found in the renal interstitium of control rats.

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*Fig. 5.* Evolution of OPN immunoreactivity in renal cortex and OSOM from gentamicin- or mercuric-chloride-treated rats (*P* < 0.05 vs controls).
Gentamicin ARF. Interstitial macrophages accumulated diffusely in both cortex and OSOM and persisted until the end of the observations (Figure 8). A clear colocalization with tubular OPN expression was never observed.

Mercuric-chloride ARF. Interstitial macrophage numbers increased to impressive numbers at day 4 in the OSOM (Figure 8), whereas in the cortical interstitium no significant increase in macrophages was observed. A colocalization with tubular OPN expression was only seen in focal areas at the end of the observations, when macrophages surrounded the immature proximal tubules with increased OPN expression (Figure 5E).

Discussion

We investigated renal OPN expression in two rat models with a distinct localization of acute tubular injury. We confirmed that OPN immunostaining in normal rat kidney is primarily located in the renal papillary epithelium and the thin limbs of the loop of Henle [11,18]. Furthermore, we demonstrated for the first time that renal OPN protein and mRNA expression is concomitantly upregulated in proximal and distal tubular cells during toxic ARF.

The vesicular OPN immunostaining in proximal tubular cells vs the apical staining in distal tubular cells also agrees with our findings in ischaemic ARF [18] and with the vesicular pattern previously observed in regenerating vascular endothelial cells [21]. The identity of these vesicles in renal cells is still unknown. They may correspond to the Golgi apparatus, where the protein is glycosylated and phosphorylated before secretion. Madsen and colleagues [20] found OPN in the Golgi apparatus from distal tubular cells by immuno-electron microscopy. However, in proximal tubular cells the authors detected OPN only in the endosomal–lysosomal system. In proximal tubules from embryonic chicken kidney, OPN was located in the apical tubulo-vesicular system and in some polymorphic lysosome-like structures [22]. These locations suggest that immunoreactive OPN accumulates in proximal tubular cells after endocytotic internalization. However, we have now demonstrated in two different models of segment-specific nephrotic injury that increased OPN immunostaining was accompanied by a concomitant OPN mRNA upregulation, not only in distal but also in proximal tubular cells.

These data indicate that the presence of immunoreactive OPN in proximal tubular cells is secondary to OPN gene induction in these cells. Being directed to the apical cell surface after synthesis, OPN is secreted into the tubular lumen, but like in distal tubular cells, at least a part of it may also become linked with the apical cell surface. It has also been demonstrated that especially in proximal tubular cells membrane proteins recycle between the plasma membrane and an endosomal compartment [23]. Such a mechanism would explain why OPN accumulates on cells with a poorly developed endocytotic mechanism such as distal tubular cells and regenerating proximal tubular cells, but becomes internalized in more mature proximal tubular cells having a well developed endocytotic mechanism, and would also reconcile our present findings with those of Madsen and colleagues [20]. An alternative explanation for the absence of OPN at the apical cell surface of mature proximal tubular cells is provided by the heterogeneous glycoalyx composition along the nephron. This is also in line with apical OPN staining in immature, regenerating proximal tubular cells, which exhibit loss of cell polarization and changes in cell membrane composition [2,24]. Finally, proximal tubular cells may produce OPN isoforms with biological properties different from OPN produced by distal tubular cells. Both phosphorylated and non-phosphorylated OPN are secreted by normal rat kidney (NRK) cells in vitro; the phosphorylated form binds cell surface fibronectin, whereas the non-phosphorylated form is associated with soluble fibronectin [25]. Further studies investigating the renal processing and trafficking of OPN should be conducted to explain this peculiar subcellular staining pattern.

The previously reported macrophage-attractant properties of OPN were not confirmed in the present study [9,11]. A spatial association between tubular OPN expression and interstitial macrophage accumulation was only found near the end of the observations in focal areas with regenerating proximal tubules. The association may be more apparent in experimental models where injury is essentially focal.

The exact role of renal OPN in ARF remains to be defined but the spatiotemporal pattern of OPN upregulation in injured and regenerating tubular cells is consistent with a role in the tissue remodelling process during ARF. Liaw et al. [21] suggested a similar role for OPN in the remodelling process after vascular injury by promoting cell attachment and cell spreading. Migration and spreading of regenerating tubular cells after gentamicin treatment was previously suggested by the induction of vimentin expression in rat proximal tubular cells [26], while a partial colocalization of vimentin and OPN was observed in protein-overload proteinuria [14]. Tubular cells may upregulate OPN to reduce apoptotic cell death [9], inhibit iNOS activity, and decrease tubular cast formation [6]. OPN upregulation after renal injury may therefore represent a survival strategy of both injured and regenerating tubular cells, the former contributing to the maintenance of tubular integrity, and the latter aiming at the repopulation of the tubular basement membrane.

In conclusion, OPN gene and protein expression is induced in both proximal and distal tubular cells during toxic ARF. The subcellular localization suggests differences in OPN processing and/or trafficking in proximal vs distal tubular cells. The spatio-temporal
Fig. 6. Double immunostaining of ED-1+ cells (macrophages; blue) and osteopontin (red). (A) OPN in cells of the papillary epithelium (arrowheads) and the thin limb of Henle’s loop (arrows) in a control kidney (400 ×). Thick ascending limb (TAL) cells are mostly negative (asterisks). (B) Strong intracellular OPN staining in most tubules 6 days after gentamicin treatment (400 ×). Punctate staining can be seen just below the apical cell surface of some tubules (arrows) (C) Strong OPN immunostaining 1 day after mercuric-chloride treatment (260 ×). Some tubules show apical staining, whereas others have only vesicular intracellular staining. Distinct intracellular staining can be seen in glomerular parietal epithelial cells (arrows) and in the proximal tubule that originates from the glomerulus (arrowhead). (D) Low magnification overview (32 ×) of OPN immunostaining and interstitial macrophage accumulation in renal OSOM (left side) and cortex (right side) 1 day after mercuric-chloride treatment. (E) Colocalization of interstitial macrophages and OPN immunostaining 14 days after mercuric-chloride treatment in a focal area where regeneration is still ongoing (160 ×). (F) Typical perinuclear staining pattern in proximal tubular cells (1000 ×). (G) Prominent apical staining in distal tubular cells (1000 ×). Perinuclear staining can also be seen in some cells (arrows). (H) Apical staining on immature, regenerating proximal tubular cells (asterisks) 6 days after mercuric-chloride treatment (260 ×).
Fig. 7. *In situ* hybridization of OPN mRNA. (A) Strong OPN mRNA signal in thin limbs ofHenle (arrows) anduroepithelial cells (arrowheads) in a control rat (350×). No OPN mRNA is found in thick ascending limbs (asterisks). (B) OSOM of gentamicin-treated rat at day 1 (350×). Strong OPN mRNA signal in distal tubules and moderate expression in PST. (C) Cortex of mercuric-chloride-treated rat at day 1 (200×). Strong OPN mRNA signal in distal tubules (d) and moderate expression in PCT (p). Distinct staining can also be seen in glomerular parietal epithelial cells (arrows) and in the proximal tubule that originates from the glomerulus (arrowhead). (D) Low magnification overview (40×) of OPN mRNA localization in renal OSOM (left side) and cortex (right side) 1 day after mercuric-chloride treatment. (E) OPN mRNA 14 days after mercuric-chloride treatment in a focal area where regeneration is still ongoing (90×). (F) OPN mRNA signal in proximal tubular cells (350×) is much lower or absent when the brush border is present (arrows). (G) Strong OPN mRNA signal in distal tubular cells (200×). (H) Strong OPN mRNA signal can also be found in immature, regenerating proximal tubular cells (asterisks) (200×).
distribution pattern is consistent with a possible role as a protective factor in tubular injury and regeneration.

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