Detection of Osteopontin mRNA in Epithelial Cells of Bowman's Capsule as Revealed by *in situ* Hybridization

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ABSTRACT. Recent Studies have revealed that tubulointerstitial damage with infiltration of macrophage occurs in the interstitium adjacent to tubules producing osteopontin mRNA and proteins. In this study, we examined the expression of the osteopontin gene in epithelial cells of Bowman's capsule in various glomerular injuring models, because these cells exist between the glomerular capillary tufts and interstitium. This result demonsrated that the osteopontin gene was expressed in the damaged tubular epithelial cells and epithelial cells of Bowman's capsule in three different models of glomerular diseases. These lesions were followed by a monocyte-macrophage influx. Given the evidence that osteopontin expression of the epithelial cells of Bowman's capsule appears with glomerular damage, and that it may be a monocyte-macrophage adhesive/chemotactic factor, our dara are consistent with the hypothesis that osteopontin may play an important role in monocyte-macrophage accumulation and glomerular damage, mainly extracapillary lesions.

Key words : osteopontin — monocyte-macrophage — epithelial cells of Bowman's capsule — tubulointerstitial injury

Osteopontin is a highly acidic, phosphorylated, secreted glycoprotein, also known as uropontin.[1] Originally isolated as a matrix molecule in bone,[2] osteopontin is now known to be produced by a variety of cell types including renal tubular epithelial cells,[3] macrophages[4] and smooth muscle cells.[5] Thus far, only a single gene for osteopontin has been identified[6] and the gene encodes a protein containing 317 amino acids in the rat.[5] Osteopontin from various species contains highly conserved regions that have homology to calcium-, heparin-, and cell-binding motifs of other proteins.[5] Of particular interest, osteopontin contains an arg-gly-asp motif that has been shown to promote arg-gly-asp-dependent adhesion of osteoblasts, osteoclasts, kidney, and smooth muscle.[7-9] Besides the promotion of adhesion, osteopontin has been shown to be a potent inhibitor of calcium oxalate formation *in vitro*,[1] and a stimulator of bone resorption *in vitro*.[10] Recently, evidence supporting the possibility that osteopontin functions as a chemotactic molecule has been reported. In addition, recent studies have reported that the elevated expression of osteopontin occured early and that the expression was followed by a monocyte-macrophage influx in tubulointerstital injury.[11,12] In this study, we

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examined expression of the osteopontin gene in the epithelial cells of Bowman's capsule in various glomerular injury models, because these cells exist between the glomerular capillary tufts and interstitium. Our study showed that the epithelial cells of Bowman's capsule play a significant role in progressive glomerular damage, such as in the promotion of adhesive lesions.[13]

MATERIALS AND METHODS

Animals : Female WKY rats (Charles River Japan Co, Yokohama, Japan) and male Wistar rats (Clea Japan Co, Osaka, Japan), which were housed in metabolic cages and given food and water *ad libitum*, were used in this study. All surgery and all infusions were performed under general anesthesia with ether and additional injections of phenobarbital when necessary. This experiment was approved by the Animal Research Committee of Kawasaki Medical School (No. 96-074, 1996) and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

Disease models : Anti-Thy1. 1 glomerulonephritis (Thy1 GN) was induced by intravenous injection of anti- thymocyte plasma (clone : OX - 7, Cedarlane Laboratories, Ontario, Canada) into Wistar rats (N=4), as described[1] by Johnson *et al*.[14] Puromycin aminoclesodide nephrosis (PAN nephrosis) was also induced by intracutaneous injection of puromycin (1.5 mg/100 g body weight per day) (Sigma Chemical Co, St Louis, Mo, USA) into Wistar rats (N=4) for five days.[15] Finally, crescentic glomerulonephritis (Crescentic GN) produced by glomerular basement membrane (GBM) antigen (emulsified with an equal volume of Freund's complete adjuvant, provided by Dr Y. Sado) injection into footpads of WKY rats (N=4).[16] These models rats were sacrificed at days 4, 7, 14 (Thy1 GN), 25 (PAN nephrosis), and 12, 16, 21 (Crescentic GN) following induction of disease.

Renal morphology and immunohistochemistry : For the morphological study, rats were perfused with 4% paraformaldehyde (PFA) via the left ventricle, and both kidneys were fixed. After dehydration, the kidneys were embedded in paraffin and sections of 5 μm were prepared. After deparaffinization, Periodic Acid-Schiff (PAS) staining of these sections was performed. The sections were also treated with 0.3% hydrogen peroxidase in methanol for 30 min at room temperature to inactivate endogenous peroxidase. Then they were immersed in non-immunohorse serum for 30 min, washed in 0.02 M phosphate-buffered saline (pH 7.2), and reacted with anti-ED1 monoclonal antibody (Serotec, Oxford, UK) for 2 hrs at room temperature. After washing in phosphate-buffered saline, bound antibodies were also detected using the avidin-Biotin-complex (ABC) kit (Vector Co, Barlingame, USA). The bound antibodies were detected using 3,3 - diaminobenzidine tetrahydrochloride (DAB) in Tris-buffered saline containing 0.02% hydrogen peroxide for 5-10 min.

In situ hybridization : In situ hybridization was performed as described by Tsukamoto *et al*[17] and Yoshimura *et al*.[18]

Probe preparation : Murine osteopontin cDNA (provided by Dr S. Nomura) was cloned into the *Hind* III sites of the transcription vector pBluescriptSK (−) at a site between the T3 and T7 promoters. The template was linearized with the restriction enzyme EcoR I (anti-sense) or Xho I

(sense probe) and labeled RNA probes were synthesized with T7 RNA polymerase (anti-sense probe) or T3 RNA polymerase (sense probe) using digoxigenin-labeled uridine-triphosphate (DIG-UTP) as the substrate according to the manufacturer's instructions (Boehringer-Mannheim, Mannheim, Germany).

Tissue preparation : The sections used were cut from the paraffin block prepared for renal morphology and immunohistochemistry. After deparaffinization through conventional xylene and ethanol steps, the sections were treated with glycine (2mg/ml in PBS for 10 min) to quench the fixative and with acetic anhydride (0.25% v/v in 0.1 M triethanolamine at pH 7 for 15 min) to reduce non-specific binding.

Hybridization : For hybridization, 20 μl of a hybridization mixture containing 50% formamide, 2X SSC (1X SSC=0.15 M NaCl and 0.015 M Na citrate), 10% dextran sulphate, 0.25% bovine serum albumin, 1 mg/ml yeast tRNA, 1 mg/ml denatured salmon sperm DNA, and the RNA probe (500 ng/ml) was applied to the sections. Then they were covered with 25 X 50 mm Parafilm and incubated in a moisturized chamber at 50°C for 15-16 hrs. The Parafilm on a slide was then floated off by immersion in 2 X SSC, 50% formamide and 10 mM DTT (Dithiothreitol). The sections were washed three times with 2 X SSC, 50% formamide, 10 mM DTT at 50°C for 1 hr with agitation, and then were treated with 20 μg/ml RNase A in 10 mM Tris (pH 8.0) containing 0.5 M NaCl, 1 mM EDTA at 37°C for 30 min. Then they were washed three times with 0.1 X SSC containing 10 mM DTT at 50°C for 1 hr.

Colorimetic detection of mRNA following hybridization : Detection was accomplished with the Genius Nonradioactive Nucleic Acid Detection Kit (Boehringer-Mannheim). Slides were washed for 1 min in Buffer 1 (100 mM Tris-HCl, 150 mM NaCl : pH 7.5) and then incubated in Buffer 1 containing 2% normal sheep serum and 0.3% Triton X-100 at room temperature for 30 min. Next, 100 μl of anti-digoxigenin antibody conjugated to alkaline phosphatase (1 : 500 dilution) was applied to the sections, and they were incubated in a humid chamber overnight at 4°C. Following washing in Buffer 1 and Buffer 2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$; pH 9.5), 100 μl of color solution was applied to the slides and incubation was carried out at room temperature in a dark, humid chamber. The color solution was made by adding 45 μl NBT (nitroblue tetrazolium salt, 75 mg/ml in dimethyformamide, 70% (v/v)), 35 μl X-phosphate solution (5-bromo-4-chloro-3-indolyl phosphate toluidium salt, 50 mg/ml in dimethylformamide) and 2.4 mg levamisole (Sigma) to 10 ml Buffer 3 (10 mM Tris-HCl, 1 mM EDTA ; pH 8.0).

Control : Control experiments were performed to confirm the specificity of hybridization between the probes and targer mRNAs. The sense strand probes were used as a control.

RESULTS

Renal morphology and immunohistochemistry :

Thy1 BN : This model rat showed acute mesangiolysis on days 1 and 2. Significant glomerular cell proliferation was observed between days 2 and 6. On day 14, glomerular cell proliferation had returned to the normal range. Marked ED1 positive cell infiltration was noted on day 1 (mesangiolytic phase), after which it decreased, but still remained presence in comparison with

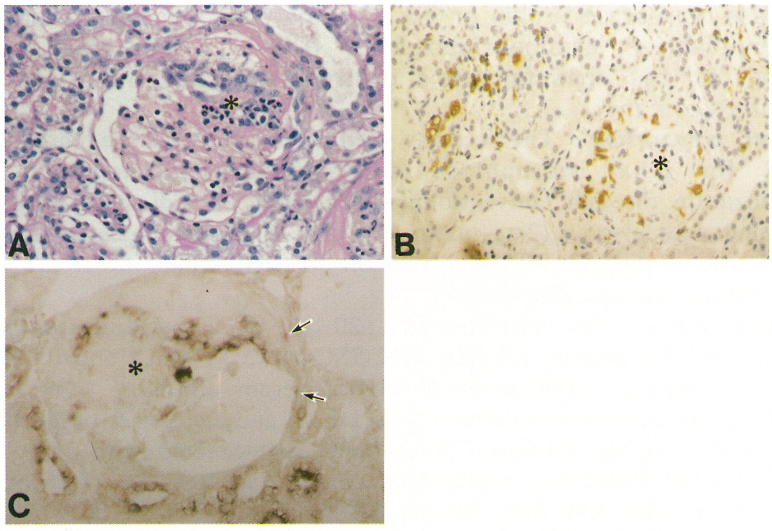

Fig 1. Mesangilproliferative nephritis (Thy1 GN)
(A) Mesangiolytic lesion (asterisk) is shown at day 2. PAS Original Magnification×400
(B) Marked ED1 postive cells infiltrate in to the glomerulus with mesangiolytic lesion (asterisk). Day 2. Original Magnification×400
(C) Osteopontin mRNA is localized to the epithelial cells of Bowman' capsule (arrows) during the mesangiolytic phase. Asterisk : mesangiolytic lesion. Day 2. Original Magnification×400

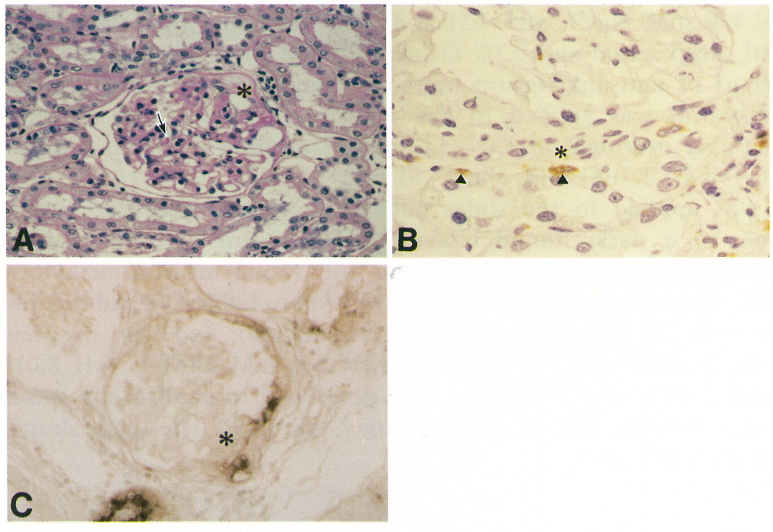

Fig 2. Puromycin aminoclesodide neghrosis (PAN nephrosis)
(A) Cytoplasmic granules that stained positive with PAS are present in podocytes (arrow). Proliferation of glomerular epithelial cells can also be seen in the adhesive lesion. PAS Original Magnification×400
(B) Site of adhesive lesion (asterisk) showing no participation of ED1 (+) cells. However, ED1 (+) cells (arrowheads) are present in the periglomerular interstitium. Original Magnification×400
(C) Osteopontin mRNA is localized to the epithelial cells of Bowman's capsule near an adhesive lesion (asterisk). Original

normal rats on days 2, 4, 6 (Fig 1A, B).

PAN nephrosis: PAS-positive granules (absorption droplets) and vacuolar changes were detected in degenerative podocytes. The most obvious abnormality was the appearance of local lesions that consisted of a cluster of vacuolar and often hypertrophic epithelial cells in the urinary space. Adhesive lesions were frequently associated with segmental mesangial expansion with a slight hypercellularity or hyalinosis. ED1 positive cells were found exclusively in the mesangial areas and capillary lumens. ED1 positive cells were also present in the periglomerular interstitium (Fig 2A, B).

Crescentic GN: Severe necrotizing and mesangioly glomerular damage was observed from day 16. After glomerular damage, mesangial hypercellularity with mensangial cell proliferation and extracellular matrix accumulation began with crescent formation. ED1 positive cells were detected in mesangiolytic, crescentic and periglomerular areas from days 12 to 21 (Fig 3A, B).

In situ hybridization: In normal rat renal cortex, osteopontin mRNA localized to distal tubular epithelium. In all the models, osteopontin expression was up-regulated cortical tubular epithelial cells during the course of the diseases (Fig 4).

Thy1 GN: When Thy1 GN was induced with anit-thymocyte plasma, the expression of osteopontin mRNA remarkably increased in the epithelial cells of Bowman's capsule (Fig 1C), proximal and distal tubular regions (Fig 4) without any histological damage by day 4 post-injetion. However, on day 14, the mRNA expression was seen focally in many cortical tubules far from the glomeruli.

PAN nephrosis: The expression of the mRNA was detected in the

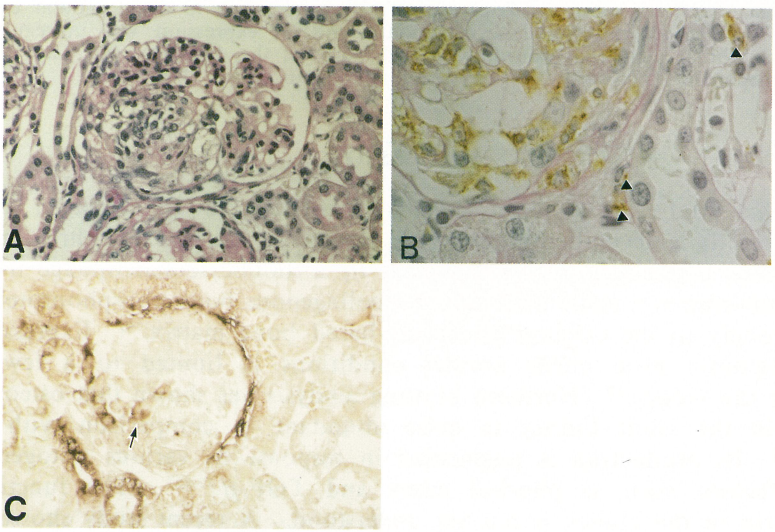

Fig 3. Crescentic glomerulonephritis (crescentic GN)
(A) A segmental necrotizing and mesangiolytic lesion is found with exudative changes. Day 16, PAS Original Magnification×400
(B) ED1 positive cells are detected in mesagiolytic, crescentic and periglomerular areas (arrowheads). Original Magnification×1000
(C) The expression of osteopontin mRNA is detected at Bowman's capsule and crescent lesion (arrow). Day 16, Original Magnification×400

epithelial cells of Bowman's capsule close to adhesive lesions (Fig 2C). As with Thy1 GN, osteopontin mRNA was detected in proximal and distal tubules, and collecting ducts (Fig 4C).

Crescentic GN: When a crescent was observed, the expression of osteopontin mRNA was localized in Bowman's capsules and crescent lesions (Fig 3C). Osteopontin mRNA was also detected in proximal and distal tubules, and collecting ducts (Fig 4D).

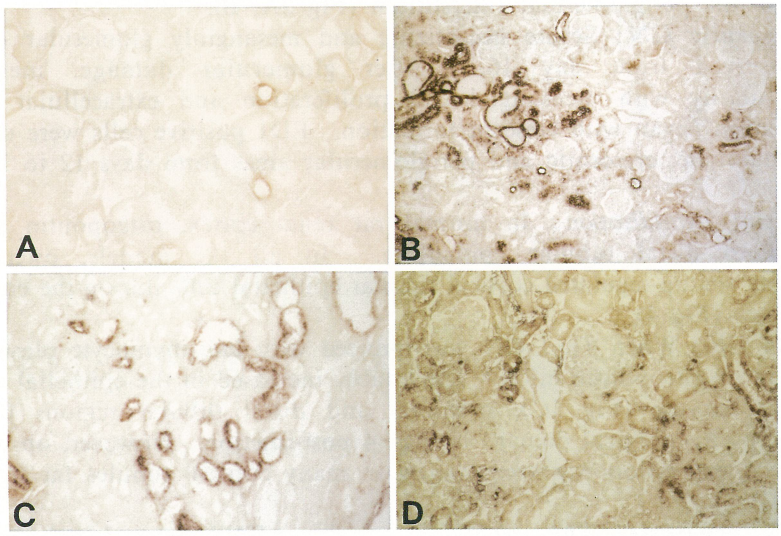

Fig 4. Osteopontin gene expression of up-regulated in tubules in all three models. In normal rat renal cortex, osteopontin mRNA is localized to distal tubular epithelial cells (A). In all three models, osteopontin mRNA expression was up-regulated in cortical tubular epithelial cells duming the course of the diseases. (B: Thy1 GN, C: PAN nephrosis, D: Crescentic GN) Original Magnification×200

DISCUSSION

In this study, osteopontin mRNA was detected in damaged tubular epithelial cells and epithelial cells of Bowman's capsule in three models. Furthermore, these lesions were followed by a monocyte-macrophage influx.

Osteopontin is a sialic acid-rich, noncollagenous bone phosphoprotein that binds strongly to the calcium phosphate-based bone matrix.[19] Expression of the osteopontin gene under normal conditions is limited to a few sites, including the kidney.[19] Northern blotting analysis has shown that osteopontin mRNA in the adult kidney is quite abundant in comparison with other tissues.[20] Its production is augmented in response to various mitogens and growth factors, such as phorbol esters and TGF-β.[21,22] The function of osteopontin in the kidney is not yet understood. A recent study showed that osteopontin was markedly up-regulated in cortical distal tubular epithelium in rats following infusion of angiotensin II and that the sites of osteopontin expression correlated with the sites of monocyte-macrophage infiltration and tubular injury.[11] These data suggest that osteopontin may act as a marker of tubular injury and that it may function as a chemotactic or adhesive factor in the recruitment of a monocyte-macrophages to these sites. Although it is

possible that osteopontin may play a role in the interstitial monocyte-macrophage recruitment that occurs in tubulointerstitial disease, one can not exclude the possibility that it may have other functions in the kidney or other tissues.[23–26] For example, we have noted that high levels of osteopontin are found in the medullary tubules, and others have also reported osteopontin mRNA, osteopontin protein, or a related 30-kD protein fragment in the tubular fluid, whereas in diseased kidneys the protein may be released into the extracellular space. Other studies have noted that osteopontin may be found in normal urine.[1] Indeed, Hoyer *et al* have suggested that osteopontin may function as an endogenous inhibitor of calcium crystal formation.

The epithelial cells of Bowman's capsule seen between the glomerulus and interstitium, however, have received little attention in studies of various renal disease. Previously, Gaffney and Panner[27] described yet another type of abnormal epithelial cell of Bowman's capsule-prominent parietal epithelium (PPE) in patiens with membranous nephropathy. They also reported that PPE cells have the ultrastructural characteristics of actively proliferating cells and damaged cells. Furthermore, patients with membranous nephropathy with PPE have had, on average, proteinuria of longer duration and greater severity than have patients with membranous nephropathy with normal epithelial cells of Bowman's capsule. In addition, we[28] and others[29–31] previously reported that the adhesive lesion included a complex series of changes in both the podocytes and epithelial cells of Bowman's capsule. These findings suggest that the epithelial cells of Bowman's capsule play an important role in progressive glomerular damage. In this study, osteopontin mRNA was detected in the epithelial cells of Bowman's capsule in three models of glomerular diseases. Furthermore, these lesions (periglomerular areas) were followed by a monocyte-macrophage influx. We have constructed a cell-mediated paradigm for progressive nonimmune renal injury involving macrophages, up-regulated TGF-β expression, extracellular matrix accumulation, and eventual scarring.[32] This putative process appears to be operant in a number of glomerulopathic and tubulointerstitial models of renal injury including the experimental nephrotic syndrome produced by puromycin aminonucleoside and adriamycin, renal ablation, and protein overload proteinuria.[33,34]

In conclusion, this study provides evidence that osteopontin is expressed by the epithelial cells of Bowman's capsule in glomerular injury. In addition, given the evidence that osteopontin expression of epithelial cells of Bowman's capsule appears with glomerular damage, and that it may be a monocyte-macrophage adhesive or chemotactic factor, our data are consistent the hypothesis that osteopontin may play an important role in monocyte-macrophage accumulation and glomerular damage (mainly extracapillary lesions). However, further studies are necessary for a clearer and more detailed understanding of the role of osteopontin in the epithelial cells of Bowman's capsule at the site of glomerular damage.

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