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Decreased Osteopontin Expression in the Rat Kidney on a Sodium Deficient Diet

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Abstract

Osteopontin (OPN) is a secreted phosphoprotein that is constitutively expressed in the normal kidney and is induced by various experimental and pathologic conditions. Several possible functions of OPN have been suggested, however the mechanism and significance of OPN expression are still uncertain. Since high salt concentration or salt crystal have been known to enhance OPN expression in intact kidney or cultured renal cells, in the present study we examined whether or not a low salt condition had an effect on OPN expression in the kidney. Adult male Sprague-Dawley rats were fed either a normal sodium or a sodium deficient diet for 1 week. Kidneys were processed for *in situ* hybridization using a digoxigenin-labeled riboprobe and for immunohistochemistry using antibodies to OPN, renin, and Na-K-ATPase. In rats fed a normal sodium diet, OPN mRNA and protein were expressed only in the descending thin limbs of Henle's loop (DTL) and in the papillary and pelvic surface epithelium (PSE). In rats fed a sodium deficient diet, there was a marked decrease in OPN immunoreactivity in the DTL, but no changes in PSE. In contrast, no changes were observed in OPN mRNA expression in the DTL by *in situ* hybridization, indicating that decreased OPN protein expression was a result of translational regulation. As expected, rats fed a sodium deficient diet were associated with increased immunoreactivity for Na-K-ATPase and renin compatible with activation of the renin-angiotensin system. These results suggest that dietary sodium may be involved in the regulation of OPN expression in the DTL of the rat kidney.

Key Words: Osteopontin, kidney, sodium-restricted diet, immunohistochemistry, *in situ* hybridization, Sprague-Dawley rats

INTRODUCTION

OPN, originally considered to be primarily a bone protein containing a cell-adhesion arginine-glycine-aspartate amino acid sequence, has been known to be expressed in other tissues, notably the kidney.[1-3] Although there have been a few conflicting reports, OPN has been reported to be expressed in the DTL and PSE in the kidney of normal adult rats by immunohistochemistry and *in situ* hybridization.[4,5] Interestingly, OPN expression in renal tubular cells has been reported only to increase under various experimental or pathological conditions *in vivo* or *in vitro*.[5-12] These facts implied that increased expression of OPN may be a response to a kind of abnormal state of the cells, while the role of OPN in the pathogenesis of experimental renal disease has been the subject of a number of recent investigations, such as the roles in the processes of cell adhesion, renal stone formation, gene expression, immune response, and apoptosis.[6,9-11,13-15]

Although renal tubular fluid is frequently supersaturated in kidney stone mineral constituents, especially calcium salts, it is known to have inhibitory factors also for stone mineral crystal formation and growth which is considered to be originally secreted from kidney tubule cells.[16,17] OPN has been suggested to be a very important one among these factors. After OPN was identified in human and mouse urine, it has been demonstrated that OPN inhibited stone mineral crystal formation and growth *in vitro*, and that the addition of calcium salt to the culture media enhanced OPN expression levels in cultured cells.[11,18-20] For *in vivo* study about the renal stone inhibitory function of OPN, there have been only a very few

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reports. Gokhale et al. and Kohri et al. reported that OPN expression level was enhanced in a rat model of nephrolithiasis by feeding ethylene glycol, which is known to induce hyperoxaluria, a prerequisite for the formation of crystal in the kidney.[13,21] All these studies about the renal stone inhibitory function of OPN were designed to show the increase of OPN expression induced by increasing the salt concentration. This is, however, not enough to give a reason for the constitutive expression of OPN in the normal adult kidney as an inhibitory factor for renal stone formation and growth because OPN expression level has until now been known to be likely to increase in any experimental conditions.

In the present study, we examined OPN expression in the kidney of rats fed a sodium deficient diet to determine if a low salt condition has an effect on OPN expression in the kidney.

MATERIALS AND METHODS

Animals

Ten male Sprague-Dawley rats weighing approximately 200 g were housed in individual cages in a temperature- and light-controlled environment. Five rats in each group were allowed water *ad libitum* and fed either a sodium deficient (TD90228, Harlan Teklad, Madison, WI, USA; Na^+ content, 0.01–0.02%) or a normal sodium (Sam Yang Co., Wonju, Korea; Na^+ content, 0.3%) diet for 1 week.

Preservation of tissue

Animals were anesthetized with an intraperitoneal injection of urethane, 1.6 g/kg body weight. The kidneys were perfused briefly through the abdominal aorta with phosphate-buffered saline (PBS) to rinse out the blood and subsequently preserved by *in vivo* perfusion with a 4% paraformaldehyde (PFA) solution for 4 min. The kidneys were cut into sagittal slices that were immersed in PFA for 2 hours at 4°C. After being rinsed in PBS, tissue was dehydrated in a graded series of ethanol and embedded in wax (polyethylene glycol 400 disterate; Polysciences, Inc., Warrington, PA, USA). Four-micrometer wax sections were prepared for immunohistochemistry and *in situ* hybridization.

Antibodies

The primary antibodies used in the present study: Mouse monoclonal antibodies (MPIIIB10) to OPN from rat bone (diluted to 1 : 3,000) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA. Rabbit polyclonal antibodies to renin (diluted to 1 : 8,000), and mouse monoclonal antibodies to alpha 1-subunit of Na-K-ATPase (diluted to 1 : 1,500), were purchased at Upstate Biotechnology Inc. (Lake Placid, NY, USA).

Immunohistochemistry

Four-micrometer wax sections were processed for immunohistochemistry using the avidin-biotin-horseradish peroxidase technique (Elite Vectastain ABC kit for mouse IgG/rabbit IgG; Vector Laboratories, Burlington, CA, USA). In brief, the sections were dewaxed, rehydrated, and incubated with 1.4% H_2O_2 in methyl alcohol for 30 min to eliminate endogenous peroxidase activity. After treatment with blocking serum for 30 min, the sections were incubated with the primary antibodies overnight at 4°C. The sections were rinsed in PBS, incubated with the biotinylated secondary antibody for 60 min, and subsequently with the Vectastain ABC reagent for 60 min. After being rinsed with 0.1 M Tris buffer (pH 7.5), the sections were incubated with the 0.05% diaminobenzidine tetrahydrochloride and 0.033% H_2O_2 mixture in Tris buffer. Negative controls were performed following the same procedure, but without primary antisera.

In situ hybridization

The riboprobe for OPN used in this study was prepared as previously described.[22] After dewaxing, the sections were postfixed in 4% paraformaldehyde for 10 min, and treated in 0.2 N HCL for 20 min. Deproteination with proteinase-K (5 μg/ml) was applied for 10 min at room temperature. Prehybridization and hybridization steps were carried out at 53°C for 1 h and 15 h, respectively. The prehybridization buffer was composed of 50% formamide, 4 X SSC, 10% dextran sulfate, 1 X Denhardt's solu-

tion, and 1 $\mu g/\mu l$ of salmon sperm DNA. The hybridization buffer was identical with the prehybridization buffer except that salmon sperm DNA was substituted with OPN riboprobe (150 $ng/\mu l$). After post-hybridization washes, sections were incubated with anti-digoxigenin antiserum conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany), and histochemical detection was then performed using NBT/BCIP mixture (Boehringer Mannheim). The negative controls were run following the same procedure, but without OPN antisense probe, or with OPN sense probe instead of antisense probe.

RESULTS

By light microscopy, there were no apparent differences in kidney morphology between rats fed a normal sodium diet and rats fed a sodium deficient diet. The negative controls for immunohistochemistry

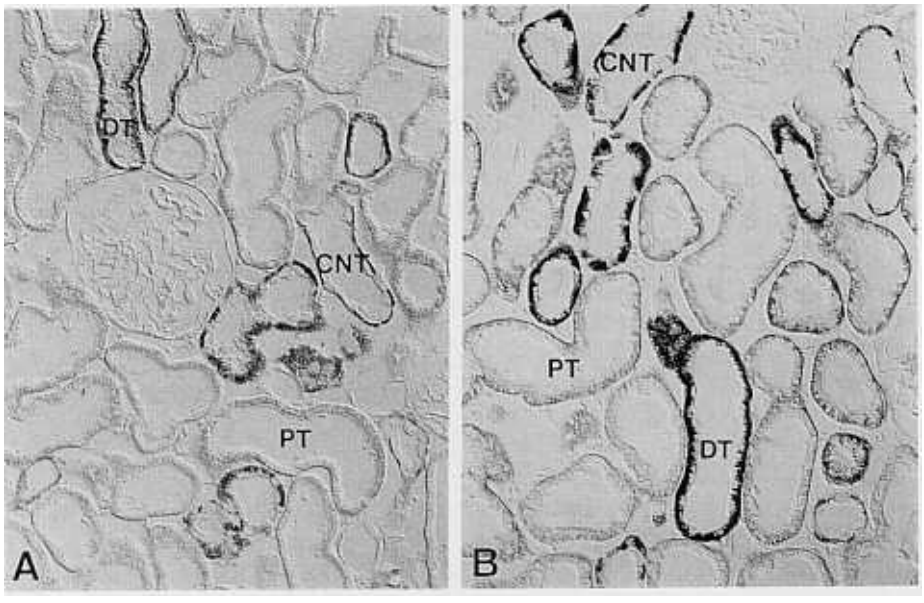

Fig. 1. Light micrographs illustrating alpha 1-subunit of Na-K-ATPase immunostaining in the renal cortex of rats fed either a normal sodium (A) or a sodium deficient (B) diet for 1 week. Note the increased immunoreactivities in the renal tubules of B compared with those of A. PT, proximal convoluted tubule; DT, distal convoluted tubule; CNT, connecting segment (Magnification, ×225).

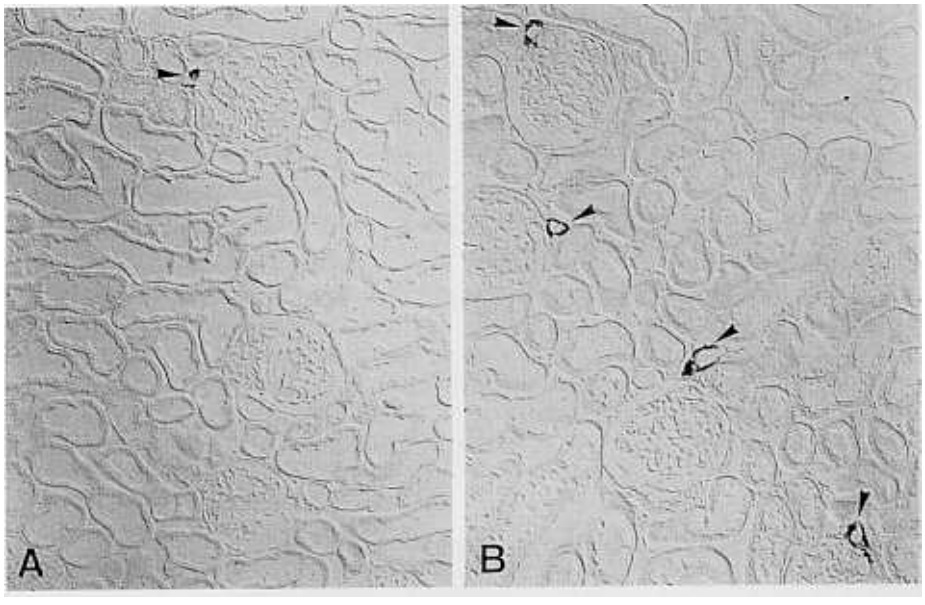

Fig. 2. Light micrographs illustrating renin immunostaining in the renal cortex of rats fed either a normal sodium (A) or a sodium deficient (B) diet for 1 week. Arrowheads indicate the immunostained afferent arterioles. The immunostained arterioles of B were much more numerous and showed stronger immunoreactivity than those of A (Magnification, ×150).

and *in situ* hybridization showed no positive signal (Fig. 5).

Immunohistochemistry

Alpha 1-subunit of Na-K-ATPase: In rats fed a normal sodium diet, moderate to strong immunostaining was detected in the basolateral membranes of the proximal tubule, thick ascending limb of Henle's loop, distal tubule, connecting tubule and collecting ducts (Fig. 1A). In rats fed a sodium deficient diet, there was no difference in labeled pattern, but the immunoreactivity increased in intensity compared with rats fed a normal sodium diet, especially in the cortical region (Fig. 1B).

Renin: In rats fed a normal sodium diet, strong immunostaining was detected in the afferent arterioles. The immunostained areas of afferent arterioles were small, and a great number of afferent arterioles on the sectioned preparation were shown negatively stained (Fig. 2A). In rats fed a sodium deficient diet, strong immunostaining was observed in almost all the afferent arterioles adjoining the vascular pole of glomeruli. The immunostained areas of afferent arterioles were shown to be more expanded compared with those of rats fed a normal sodium diet (Fig. 2B).

OPN: In rats fed a normal sodium diet, strong immunostaining was observed in the DTL in the outer medulla and in the PSE, especially corresponding to the fornix area of the renal pelvis. Immuno-

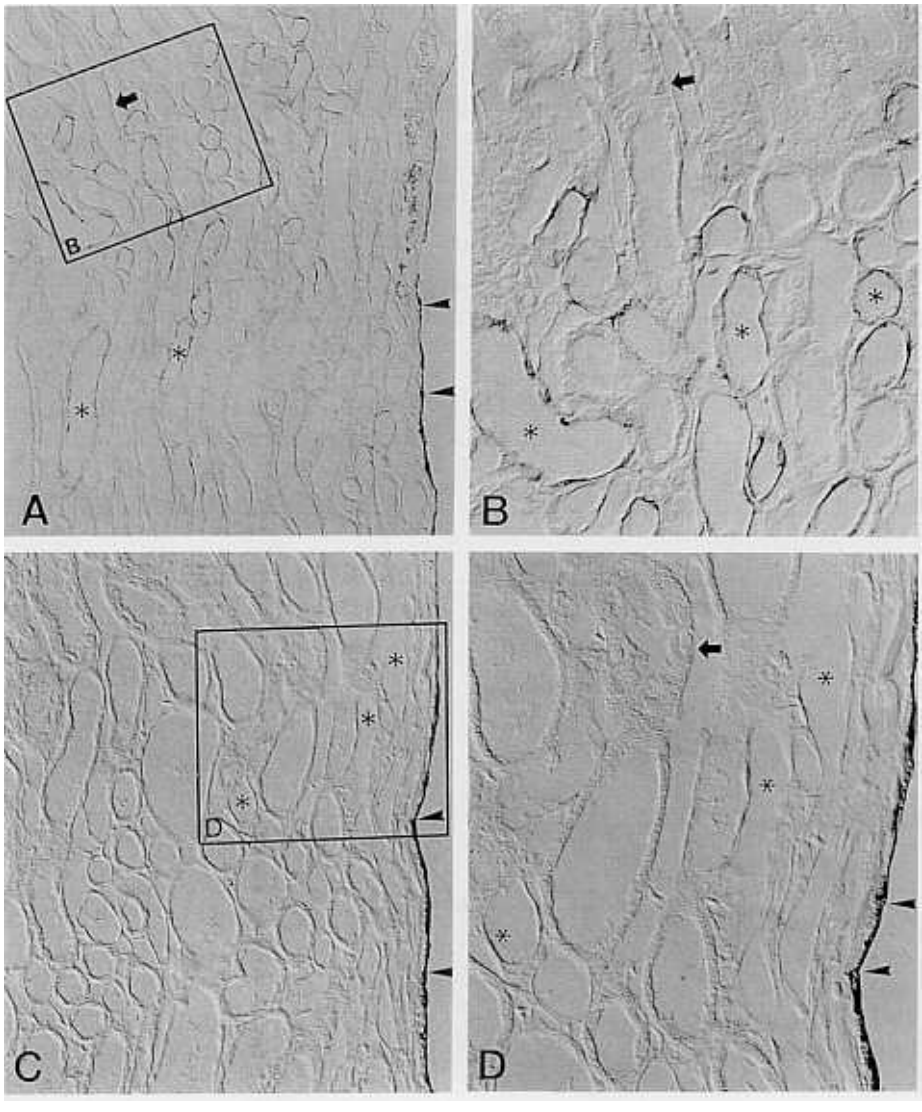

Fig. 3. Light micrographs illustrating osteopontin immunostaining in the renal outer medulla of rats fed a normal sodium diet (A & B) and a sodium deficient diet (C & D). A. The immunostained descending thin limbs (asterisks) and pelvic surface epithelium (arrowheads) are shown. B is the magnified micrograph of the area outlined by rectangular box in A. C & D. Note the pelvic surface epithelium (arrowheads) showing strong immunoreactivity and the descending thin limbs (asterisks) showing very weak to almost no immunoreactivity. The arrows indicate the straight part of proximal tubule showing no immunoreactivity (Magnifications, A & C, ×225; B & D, × 500).

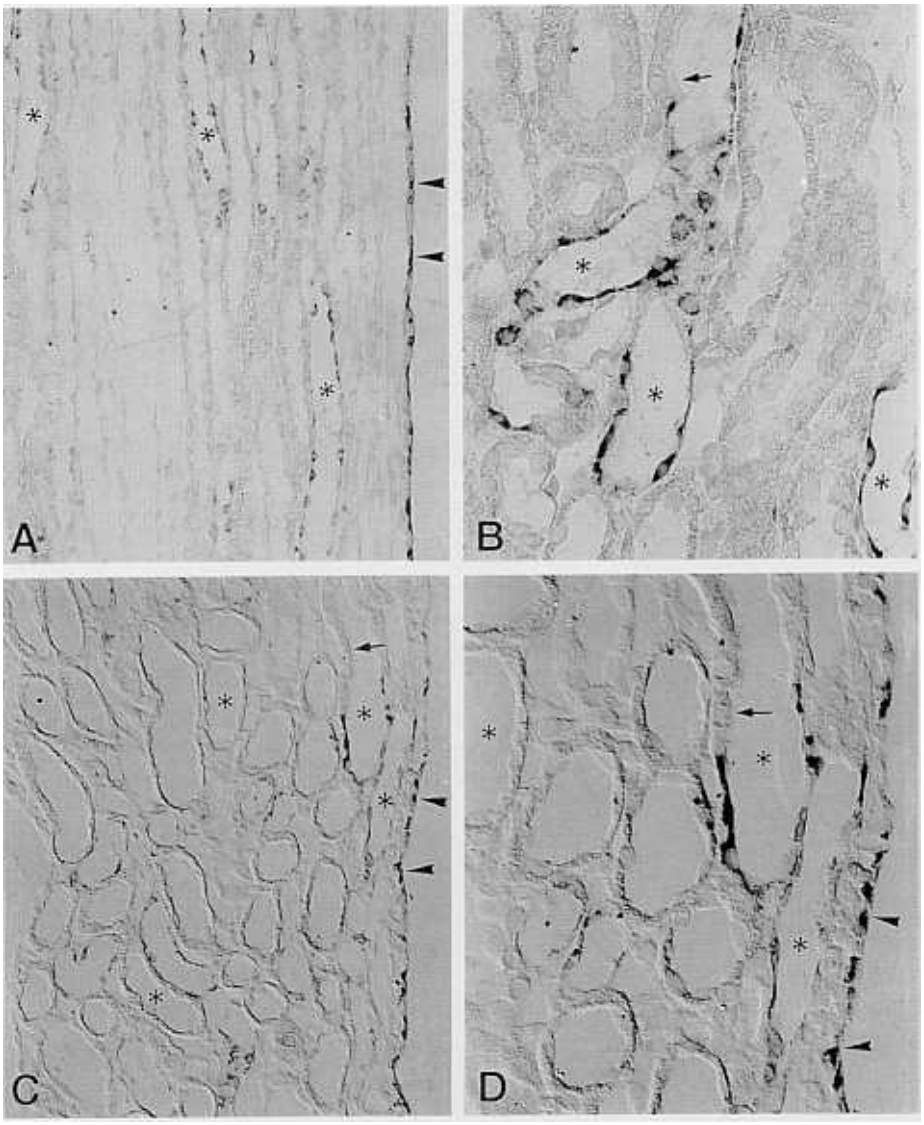

Fig. 4. In situ hybridization localization of osteopontin in the renal outer medulla of rats fed a normal sodium diet (A & B) and a sodium deficient diet (C & D). Note the hybridization signal in the descending thin limbs (asterisks) and the pelvic surface epithelium (arrowheads). The arrows indicate the straight part of proximal tubule showing no hybridization signal. There was no difference in labeled pattern and labeling intensity between the two animal groups (Magnifications, A & C, ×225; B & D, ×500).

staining of the DTL was most pronounced in the initial part that is continuous with the straight part of the proximal tubule (Fig. 3, A and B). Occasionally, a few thick ascending limbs of Henle's loop and/or distal tubules were sporadically stained (data not shown). In rats fed a sodium deficient diet, there was a marked decrease in immunoreactivity in the DTL. Although a few DTL were stained moderately, most DTL were stained very weakly or almost negatively. However, there was no change in strong immunostaining of PSE (Fig. 3, C and D).

***In situ* Hybridization for OPN:** In rats fed a normal sodium diet, there was a strong hybridization signal in the DTL in the outer medulla and PSE, especially corresponding to the fornix area of the renal pelvis (Fig. 4, A and B). Similar to the results for OPN immunostaining, a few thick ascending limbs of Henle's loop and/or distal tubules were sporadically stained (data not shown). In rats fed a sodium deficient diet, there was no difference in labeled pattern and labeling intensity compared with rats fed a normal sodium diet (Fig. 4, C and D).

DISCUSSION

OPN is a phosphoprotein with diverse functions.[23] Although several functions of renal OPN were suggested relating to tissue regeneration, renal stone formation, gene expression, chemoattraction and apo-

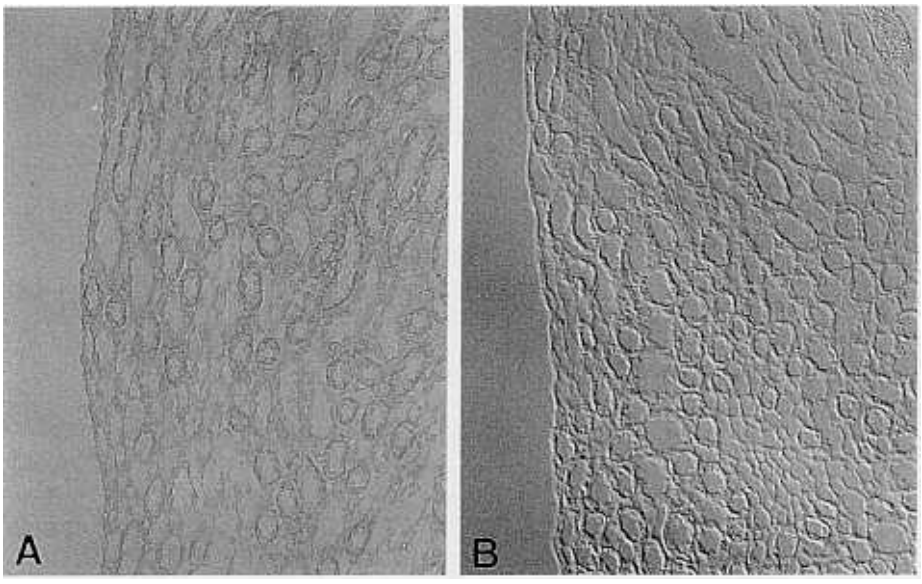

Fig. 5. Light micrographs showing renal outer medulla of rats fed a sodium deficient diet of negative controls for in situ hybridization of osteopontin (A) using sense probe and for osteopontin-immunostaining (B) without primary antibody incubation. No detectable signals were found in both preparations (Magnification, ×200).

ptosis, these still remain poorly understood.[6,9-11,13-15] One of the problems in studying renal OPN is that renal OPN is increased without exception and with almost the same pattern under all the experimental and pathological conditions performed untill now. Almost all *in vitro* studies have shown an increased OPN level compared with basal level, and *in vivo* studies have resulted in a marked increase of OPN expression in distal nephron including the thick ascending limb of Henle's loop and in proximal tubules with punctate pattern.[5,8,11,12] In contrast, the results of this study demonstrated that the expression of OPN protein in the DTL decreased without any change of OPN mRNA level in rats fed a sodium deficient diet. To our knowledge, these results are the first showing decreased expression of OPN protein in DTL in which OPN is known to be expressed constitutively.

One of the most likely functions of OPN in the kidney is the inhibition of the renal stone formation. This has been supported in part by reports which showed that an increased concentration of calcium salt was associated with the upregulation of OPN expression and that OPN expression was increased in the animal model of nephrolithiasis by feeding ethylene glycol which is known to induce hyperoxaluria.[11,13,21] There have been, however, no reports about the reduced constitutive expression of renal OPN. We assumed that if OPN has a role in the inhibition of renal stone formation, and if *in vitro* study that increased the concentration of calcium salt associated with the upregulation of OPN expression was also true *in vivo*, then the reduction of urinary salt may bring about the change of OPN expression. Although the molecular biological mechanism of OPN expression is still unknown, we considered that OPN expression may be changed by receptor-mediated regulation on the basis that calcium oxalate crystal bind to some specific receptors on renal cells and that bound calcium oxalate crystals are internalized and stimulate gene expression in renal cells.[24,25]

In this study, we used the animal model fed a sodium deficient diet because many papers have reported that a low sodium diet reduces urinary calcium and the risk of renal stone formation.[26-29] We first examined Na-K-ATPase and renin immunoreactivity in rats fed a sodium deficient diet because it is well known that during dietary salt deprivation, the renin-angiotensin system is stimulated.[30] This system is thought to be essential for maximal salt conservation by the kidney. A low-sodium diet is known to lead to a rise in renin expression and serum aldosterone, and then induce an increase in Na-K-ATPase activity.[31-33] The increased immunostaining of renin and Na-K-ATPase in kidney of rats fed a sodium deficient diet proved that our animal model was acceptable.

There are few reports about the effects of a high salt diet on OPN expression in the kidney. However, a high salt diet may cause upregulation of renal OPN expression, considering that a high salt diet increased

urinary calcium.[26,34] A recent report showed the upregulation of renal OPN after a high salt diet in the control animals and this result was also in agreement with our results in other ways.[35]

In this study, we used a specific antibody and riboprobe to OPN. From many reports, OPN antibody we used in this study proved to be very specific (for review, visit internet site. http://www.uiowa.edu/~dshbwww/mpiii.html). The OPN riboprobe used in this study was prepared as previously described.[22] The probe was designed from the sequence of mouse OPN cDNA (nucleotides 244−823) and prepared by reverse-transcription of an mRNA from mouse medullary thick ascending limb cells, followed by polymerase chain reaction. To determine the degree of identities of the amplified cDNA and Rat OPN mRNA, we used the BLAST program at the Advanced BLAST internet site (http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=1) which is a service of the National Center for Biotechnology Information. The percentage identity of the amplified cDNA vs. corresponding region of rat OPN mRNA was 89%. To test the specificity of the riboprobe, Northern blot with total RNA of normal rat kidney was performed, and a specific band corresponding to OPN mRNA was observed.

Together, the present study demonstrated that OPN protein expression in the DTL was markedly decreased without any change of OPN mRNA in rats fed a sodium deficient diet for 1 week. These observations may provide a clue to elucidate the reason OPN is constitutively expressed in the DTL.

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