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GENETIC DISORDERS – DEVELOPMENT

Aprt/*Opn* double knockout mice: Osteopontin is a modifier of kidney stone disease severity

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***Aprt*/*Opn* double knockout mice: Osteopontin is a modifier of kidney stone disease severity.**

Background. Osteopontin (OPN) is reported to have two distinct functions in kidney disease: Promotion of inflammation at sites of tissue injury, and inhibition of calcium oxalate monohydrate stone formation. However, many of the studies supporting these functions were carried out in animal models of acute renal injury or in cultured cells; thus, the role of OPN in chronic renal disease is not well defined. We examined the role of OPN in adenine phosphoribosyltransferase (*Aprt*) knockout mice, in which inflammation and formation of 2,8-dihydroxyadenine (DHA) kidney stones are prominent features, by generating *Aprt*/*Opn* double knockout mice.

Methods. We characterized the phenotypes of six- and 12-week-old *Aprt*−/− *Opn*−/−, *Aprt*−/− *Opn*+/+, *Aprt*+/+ *Opn*−/−, and *Aprt*+/+ *Opn*+/+ male and female mice using biochemical, histologic, immunohistochemical, and in situ hybridization techniques.

Results. At 6 weeks of age, there was no difference in phenotype between double knockout and *Aprt* knockout mice. At 12 weeks, there was increased adenine and DHA excretion, renal crystal deposition, and inflammation in double knockout versus *Aprt* knockout male mice. Double knockout and *Aprt* knockout female mice at 12 weeks had less pathology than their male counterparts, but kidneys from double knockout females showed more inflammation compared with *Aprt* knockout females; both genotypes had similar levels of DHA crystal deposition.

Conclusion. We conclude that (*1*) OPN is a major inhibitor of DHA crystal deposition and inflammation in male mice; and (*2*) OPN is a major modifier of the inflammatory response but not of crystal deposition in female mice. Thus, separate mechanisms appear responsible for the tissue changes seen in DKO males versus females.

Key words: adenine phosphoribosyltransferase, 2,8-dihydroxyadenine, inflammation, kidney stone disease, osteopontin, xanthine dehydrogenase.

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Many natural inhibitors of kidney stone formation have been identified in urine, but inhibitors affecting calcium oxalate monohydrate stone formation have been the most widely studied [1]. Urinary proteins with inhibitory activity include bikunin, osteopontin (OPN), prothrombin fragments, and Tamm-Horsfall protein [2–8]. Most of these proteins are anionic, with many acidic amino acid residues, and they frequently contain post-translational modifications such as phosphorylation or glycosylation. The inhibitors appear to bind to calcium oxalate surfaces [9], but the structural features that favor crystal binding and subsequent inhibition of stone formation are not fully understood. The inhibitory effects of OPN on stone formation are likely to be dependent on the phosphorylation state of OPN, with higher levels causing more inhibition of crystal nucleation and aggregation [10]. In normal mouse kidney, OPN expression was observed primarily in the thick ascending limbs of the loop of Henle and in distal convoluted tubules [11]. In human kidney, OPN expression has been observed in the descending loop of Henle, collecting ducts, and the renal papilla [12], sites that are considered to be associated with the onset of stone formation. An increase in OPN expression has been demonstrated in proximal tubular epithelium in humans following cyclosporine toxicity and in rodent models of renal injury [13], including kidneys from calcium oxalate monohydrate stone forming rats [14, 15]. OPN also is reported to be a promoter of tissue inflammation [13, 16–21]. OPN likely promotes inflammation via interaction with the CD44 receptor on macrophages and induction of chemotaxis, and via attachment to the β_3 integrin receptors and induction of cell spreading and activation [16, 21, 22].

Two knockout mouse models for OPN have been developed, including the one at our institution [23, 24]. *Opn* knockout mice appear anatomically normal, are fertile, show no size differences from their wild-type littermates,

and have histologically normal kidneys [13, 25]. However, they do show some phenotypic differences from wild-type mice, including resistance to ovariectomy-induced bone resorption [26] and parathyroid hormone (PTH)-induced enhancement of cortical bone formation [27]. Several models of acute renal injury, including ischemia [28], ureteral ligation [29], and cyclosporine toxicity [30], have been created in these mice. These models all demonstrated more macrophage infiltration in wild-type mice compared with *Opn* knockout mice, suggesting that OPN is a promoter of inflammation in the kidney. OPN also has renoprotective effects, as demonstrated by significantly more blood urea nitrogen (BUN) and creatinine retention and increased structural renal damage following ischemia in *Opn* knockout mice compared with wild-type mice [25]. Following ethylene glycol–induced hyperoxaluria, there was significant intratubular deposition of calcium oxalate monohydrate crystals in *Opn* knockout mice, whereas wild-type mice were unaffected, further supporting a renoprotective role for OPN [31]. In a mouse model of mycobacterial infection, *Opn* knockout mice had more severe infections and greater granuloma burden, suggesting that the protective effects of OPN are not limited to the kidney [32].

Many of the studies supporting the proinflammatory and the stone-inhibiting properties of OPN have been carried out in animal models of acute renal injury or in cultured cells. These models are not appropriate for chronic kidney diseases, such as hereditary stone disease, where the disease process may have been active since birth. Over 30 hereditary stone diseases have been recognized [33], including kidney stones associated with hypercalciuria, hypophosphatemia, hyperoxaluria, cystinuria, hyperuricosuria, and xanthinuria [34]. Our laboratory has been investigating another genetic disorder—adenine phosphoribosyltransferase (APRT) deficiency—in which kidney stone disease is a major phenotype [35]. APRT catalyzes the synthesis of adenosine monophosphate (AMP) from adenine, but in APRT deficiency adenine is oxidized by xanthine dehydrogenase (XDH)—via the intermediate 8-hydroxyadeine (HA)—to 2,8-dihydroxyadenine (DHA). DHA is highly insoluble at normal urine pH and this can lead to crystal aggregation and stone formation in many APRT-deficient patients [36]. We previously generated *Aprt* knockout mice [37, 38], and we have used these mice to identify some of the early cellular and molecular events involved in kidney stone formation [39–42]. These mice show DHA crystals in the collecting ducts as early as 2 days of age, and older mice have crystal deposition throughout the renal system as well as high levels of inflammation and tissue fibrosis [43]. We also observed increased OPN mRNA expression in kidney sections from these mice [Tzortzaki et al, unpublished data], suggesting that OPN may be one of the initial protective responses of the kidney to the presence of crystals.

In this study, we examined the role of OPN in DHA kidney stone formation by creating *Aprt*/*Opn* double knockout mice. We hypothesized that if one of the major roles of OPN is the inhibition of crystal aggregation and stone formation in the urinary tract, then the onset of stone disease would be earlier or the phenotype more severe in double knockout mice compared with *Aprt*−/− mice. The increased crystal burden in the double knockout mice would also be expected to lead to increased inflammation in these mice. We studied both male and female mice, given the gender differences in the prevalence of kidney stones [44–46]. Mice of all four genotypes (*Aprt*−/− *Opn*−/−, *Aprt*−/− *Opn*+/+, *Aprt*+/+ *Opn*−/−, and *Aprt*+/+ *Opn*+/+) were examined at 6 and 12 weeks of age using biochemical, histologic, immunohistochemical, and in situ hybridization techniques. These age groups were selected because at 6 weeks inflammation is first seen to develop in Aprt-deficient male mice, and at 12 weeks inflammation and fibrosis are severe in male mice and also evident in female mice [37, 43].

METHODS

Mouse breeding, DNA extraction, and genotyping

Animal studies were approved by the Rutgers University Institutional Animal Care and Use Committee. Strain 129 mice of genotypes *Aprt*−/− *Opn*+/+ [37] and *Aprt*+/+ *Opn*−/− [24] were mated to generate *Aprt*+/− *Opn*+/− mice, which were then interbred to create *Aprt*−/− *Opn*−/− mice. DNA was extracted from mice tails at weaning and the genotypes at the *Aprt* and *Opn* loci determined using allele-specific polymerase chain reaction (PCR) [24, 47]. To examine the effects of *Opn* background on mouse survival, pups born to *Aprt* heterozygous parents that were homozygous +/+ or −/− for *Opn* were genotyped, and the observed and expected genotype frequencies compared using chi square.

Weight and growth analysis

Male and female mice of all four genotypes were weighed on a weekly basis from weaning to 12 weeks of age. Each genotype included at least six mice of each gender. Data were analyzed for rate of weight change, range of change (maximum minus minimum weight), and maximum achieved weight using growth curves fitted to Gompert's growth formula:

$$\text{Weight} = \text{Asymptote}^{-b_2 b_3 \wedge \text{age}}$$

where b_2 and b_3 are the rate and range of weight change, respectively, and the asymptote is the maximum weight.

Urine collection and analysis

Urine samples were collected in individual metabolic cages (Braintree Scientific, Braintree, MA, USA); mice