

Calcium oxalate crystal localization and osteopontin immunostaining in genetic hypercalciuric stone-forming rats

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Background. The inbred genetic hypercalciuric stone-forming (GHS) rats develop calcium phosphate (apatite) stones when fed a normal 1.2% calcium diet. The addition of 1% hydroxyproline to this diet does not alter the type of stone formed, while rats fed this diet with 3% hydroxyproline form mixed apatite and calcium oxalate stones and those with 5% hydroxyproline added form only calcium oxalate stones. The present study was designed to determine the localization of stone formation and if this solid phase resulted in pathologic changes to the kidneys.

Methods. GHS rats were fed 15 g of the standard diet or the diet supplemented with 1%, 3%, or 5% hydroxyproline for 18 weeks. A separate group of Sprague-Dawley rats (the parental strain of the GHS rats), fed the standard diet for a similar duration, served as an additional control. At 18 weeks, all kidneys were perfusion-fixed for structural analysis, detection of crystal deposits using the Yasue silver substitution method, and osteopontin immunostaining.

Results. There were no crystal deposits found in the kidneys of Sprague-Dawley rats. Crystal deposits were found in the kidneys of all GHS rats and this Yasue-stained material was detected only in the urinary space. No crystal deposits were noted within the cortical or medullary segments of the nephron and there was no evidence for tubular damage in any group. The only pathologic changes occurred in 3% and 5% hydroxyproline groups with the 5% group showing the most severe changes. In these rats, which form only calcium oxalate stones, focal sites along the urothelial lining of the papilla and fornix of the urinary space demonstrated a proliferative response characterized by increased density of urothelial cells that surrounded the crystal deposits. At the fornix, some crystals were lodged within the interstitium, deep to the proliferative urothelium. There was increased osteopontin immunostaining in the proliferating urothelium.

Conclusion. Thus in the GHS rat, the initial stone formation occurred solely in the urinary space. Tubular damage was not observed with either apatite or calcium oxalate stones. The

apatite stones do not appear to cause any pathological change while those rats forming calcium oxalate stones have a proliferative response of the urothelium, with increased osteopontin immunostaining, around the crystal deposits in the fornix.

Hypercalciuria is the most common metabolic abnormality in patients with nephrolithiasis [1–7]. Hypercalciuria raises urine supersaturation with respect to the solid phases of calcium oxalate and calcium phosphate enhancing the probability of nucleation and growth of crystals into clinically significant stones [3]. Reversal of the hypercalciuria with thiazide diuretics, resulting in lowering of supersaturation, has been shown to reduce clinical stone formation in two long-term studies [8, 9].

To establish an animal model of hypercalciuria, we have successively inbred 56 generations of the most hypercalciuric progeny of hypercalciuric Sprague-Dawley rats each of which now excretes eight to ten times as much urinary calcium as similarly fed controls [10–23]. The hypercalciuria is due to increased intestinal calcium absorption [22] coupled to a defect in renal calcium reabsorption [16, 20] and enhanced bone mineral resorption [17], suggesting a systemic dysregulation of calcium homeostasis [21]. After eating standard rat chow (1.2% calcium) for 18 weeks, virtually all of these hypercalciuric rats form kidney stones while there was no evidence of stone formation in Sprague-Dawley controls [18]. Because of their hypercalciuria and stone formation we have termed the rats genetic hypercalciuric stone-forming (GHS) rats [10–13, 15, 18, 23]. We have shown that the stones formed by the GHS rats fed standard rat chow contain only calcium and phosphate, without oxalate, and by x-ray diffraction the stones are exclusively poorly crystalline apatite [11, 13, 15, 18].

We reasoned that the dietary provision of an oxalate precursor would produce an increase in urinary oxalate. We wished to avoid the commonly used oxalate precursor ethylene glycol [24–27] as it results in severe, life-threatening metabolic acidosis in mammals [28–31] and appears to be nephrotoxic [32–34]. Instead, we fed the GHS rats additional hydroxyproline, a common amino

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acid which is metabolized to oxalate [35]. We found that an increase in dietary hydroxyproline increased urinary oxalate excretion, supersaturation with respect to the calcium oxalate solid phase, the ratio of calcium oxalate to calcium phosphate supersaturation and led to the formation of calcium oxalate kidney stones [23].

In other rat models of kidney stone disease, especially those rats given ethylene glycol, the crystal deposition was associated with extensive tubular toxicity [24–27]. Thus, in the current study, we wished to determine the site of initial stone formation and questioned whether the calcium phosphate or calcium oxalate crystal deposition caused tubular injury. We found that the calcium phosphate crystals were found exclusively in the urinary space and caused only indentation of the urothelium; there was no tubular injury or proliferation. The rats given the larger amounts of hydroxyproline (3% and 5%) had pathologic changes in their kidneys. In these rats, which form calcium oxalate stones, focal sites along the urothelial lining of the papilla and fornix of the urinary space demonstrated a proliferative response that always surrounded the crystal deposits. At the fornix, some crystals appeared to be lodged within the interstitium, deep to the proliferative urothelium and there was increased osteopontin immunostaining in these areas.

METHODS

Establishment of hypercalciuric rats

Adult Sprague-Dawley rats (Charles River Laboratories, Kingston, NY, USA) were screened for hypercalciuria by placing the rats in individual metabolic cages, feeding them a constant amount of a standard calcium diet, and measuring urine calcium excretion. The most hypercalciuric male and female rats were used to breed the next generation. A similar protocol was used for screening and inbreeding of subsequent generations as described previously [10–23].

Study protocol

Twenty-nine 56th generation male GHS rats, initially weighing on average 315 g, were placed in metabolic cages for 18 weeks. Each rat was initially provided with 13 g/day of food, an amount that we have previously shown is completely consumed by a rat of this size [36]. At 12 weeks, the amount of food was increased to 15 g/day to account for the increased dietary needs of the now larger rats [23]. Any rat that ate less than 12 g of food per day until week 12 or ate less than 14 g of food per day from week 12 until the conclusion of the study or drank less than 15 mL of water on any day would have been excluded from the remainder of the study; however, all rats met these prospective criteria throughout the study.

The male GHS rats were randomly divided into four groups. As the GHS control, some GHS rats were fed

a standard 1.2% calcium, 0.65% phosphorus diet without modification, while other rats were fed the same diet supplemented with 1%, 3%, or 5% trans-4-hydroxy-L-proline (hydroxyproline) (ICN Biomedicals, Aurora, OH, USA). Male rats, as opposed to the female rats that were used in a number of previous studies [10–13, 15, 16, 18], were used because of their greater baseline oxalate excretion when fed this standard calcium diet [19]. As an additional control, we fed Sprague-Dawley rats, the strain from which the GHS rats were derived, the standard 1.2% calcium, 0.65% phosphorus diet without modification (Sprague-Dawley control). Previously we have shown that GHS rats develop calcium phosphate (apatite) stones when fed a normal 1.2% calcium diet [11, 13, 15, 18, 23]. The addition of 1% hydroxyproline to this diet does not alter the type of stone formed, while rats fed this diet with 3% hydroxyproline form mixed apatite and calcium oxalate stones and those with 5% hydroxyproline added form only calcium oxalate stones [23]. Given the consistency of stone type found in our prior study [23] and the parallel design of the current study, we did not analyze any stones formed by rats in the current study. Rather, we assumed that the GHS rats fed the identical diet, with or without an identical amount of hydroxyproline, for an identical period of time would form identical types of stones as demonstrated previously [23]. Each of the GHS groups consisted of seven rats (one rat initially placed in the 5% hydroxyproline group was sacrificed during the study due to an eye infection). The Sprague-Dawley control group consisted of five rats.

At the end of 18 weeks, each rat received an intraperitoneal injection of sodium pentobarbital (Nembutal) to induce complete anesthesia. The aorta was cannulated and the kidneys were perfused with 0.9% saline irrigation solution for ~30 seconds resulting in the kidneys lightening in color and the outflow from the vena cava becoming clear. Then a buffered fixation solution of 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate at pH 7.4 was perfused through the kidneys for ~20 minutes (~100 mL) via a mechanical perfusion pump set at a flow rate of 5 mL/min. Following perfusion, each kidney was resected, placed in a labeled 15 mL Falcon tube filled with the fixative buffer and refrigerated. Three kidneys, approximately equally divided between left and right, were selected from each diet group for histologic analysis. The kidneys were coded in Rochester and sent to Indianapolis for histologic evaluation where they were analyzed in a blinded manner. Only when the histologic analysis was complete, and all photographs taken, was the code broken.

Histologic analysis

Following 2 weeks of fixation, the upper and lower poles of each kidney were removed and the kidney was placed in an embedment cassette such that perfect cross

sections of the entire kidney (including the renal papilla) could routinely be obtained. All kidney specimens were dehydrated through a series of graded ethanol concentrations to 100% ethanol before embedment in a 50/50 mixture of Paraplast Xtra (Fisher Scientific, Itasca, IL, USA) and Pell-Away MicropCut (Polysciences, Inc., Warrington, PA, USA). Serial 4 μ m sections were cut on a standard microtome and stained for histologic analysis with hematoxylin and eosin (H&E), periodic acid-Schiff hematoxylin (PASH) or picro sirius red and for calcium deposits using the Yasue method [37]. The Yasue method uses 5% aqueous silver nitrate and rubeanic acid to generate a dark brown to black histochemical staining of calcium deposits. Sites of crystal location were identified in the Yasue stained sections, then correlated with regions of renal injury observed in the H&E, PASH or picro sirius red-stained sections.

Another series of sections were cut at 4 μ m on a standard microtome and placed on charged slides (Fisher Scientific, Itasca, IL, USA) for osteopontin localization by immunohistochemistry performed according to the peroxidase-antiperoxidase (PAP) method of Sternburger [38]. Subsequently, these slides were deparaffinized and rehydrated through graded alcohols and blocked for non-specific protein binding using 20% normal goat serum in 0.1 mol/L phosphate-buffered saline (PBS) at room temperature for 2 hours. The primary antibody, LF-123, was a rabbit antihuman osteopontin antibody [39] obtained as a gift from Dr. Larry Fisher at NIH and was used at 1:150 dilution. The secondary antibody was a goat antirabbit IgG (1:100) while the tertiary antibody was a rabbit-PAP (1:600). Both the secondary and tertiary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). The diluant for all antibodies was a 2% solution of normal goat serum in 0.1 mol/L PBS. Slides were incubated with the primary antibody overnight at room temperature followed by several rinses in PBS. Slides were subsequently incubated with secondary and tertiary antibodies at room temperature for 1 hour each, with several PBS rinses between each change of antibody. DAB (3,3-diaminobenzidine tetrahydrochloride) in Tris-HCl buffer was used as the chromogen. All sections were covered with DAB solution (0.05 mol/L Tris-HCl, 0.001% hydrogen peroxide, and 0.10% DAB), and incubated for approximately 10 minutes. Finally, each slide was rinsed well with buffer, dehydrated and mounted with Permount (Fisher Scientific) for viewing.

RESULTS

Sprague-Dawley control

This group of male animals is the parental strain of the GHS rats. At time of sacrifice no stones or crystals were found in the kidney or the urinary space (Fig. 1A to C). By

light microscopic examination no evidence of tubular injury was found in any segments of the nephron, or the adjoining vasculature. The urothelium appeared of uniform thickness. Osteopontin immunostaining was localized to cells of the thick ascending limbs, distal tubules, cortical collecting tubules, inner medullary collecting ducts, and urothelium (Fig. 1A).

GHS control

This group of male animals was fed the standard diet without any modifications. At the time of sacrifice stones of varying sizes were only found in the urinary space (Fig. 1D and E). Fed the standard calcium diet, the GHS rats form only poorly crystalline apatite (calcium phosphate) stones [10–13, 18, 23]. No stones or crystals were identified in cortical or medullary tubular lumens or the interstitium. Occasional sites along the urothelial lining of the renal papilla showed evidence of indentation from adjacent crystals (Fig. 1E) but the urothelium appeared uniform in thickness. Like the Sprague-Dawley controls, using light microscopy, no tubular, vascular, or urothelial injury was detected (Fig. 1E). The osteopontin immunostaining pattern was identical to that seen in the Sprague-Dawley controls, which included cells of the thick ascending limbs, distal tubules, cortical collecting tubules, inner medullary collecting ducts, and urothelium (Fig. 1D).

GHS animals fed hydroxyproline

The animals fed the 1% hydroxyproline supplemented diet formed stones only in the urinary space, just as the GHS control animals, and all stones were composed of apatite (Fig. 2A) [23]. No stones or crystals were identified in tubular lumens or the interstitium. An occasional site of crystal indentation of the urothelium from nearby crystals was present (Fig. 2B), another observation similar to the GHS controls. In addition, using light microscopy, no evidence of injury to cells of nephron, vasculature, or urothelium was detected. Osteopontin immunostaining was identical to that seen in the Sprague-Dawley controls, which was localized to cells in the thick ascending limbs, distal tubules, inner medullary collecting ducts, and urothelium (Fig. 2A).

All animals fed the 3% hydroxyproline supplemented diet formed stones only in the urinary space with no evidence of stones or crystals in tubular lumens or the interstitium (Fig. 2C). Stone composition changed to a mixture of calcium oxalate dihydrate, calcium oxalate monohydrate, and apatite [23]. Using light microscopy, no changes were noted in cells of the nephron; however, the urothelial lining of the renal papilla and renal pelvis demonstrated focal sites of proliferation noted as an increased thickness (Fig. 2D). Additional changes observed in the urothelium included indentation from adjacent crystals and entrapment of crystals primarily in the fornix

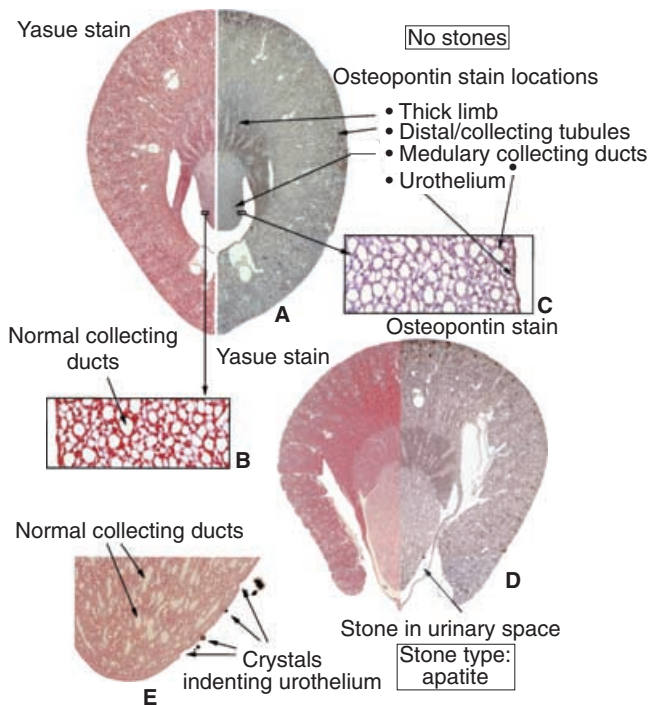


Fig. 1. Midtransverse paraffin sections through a kidney from a Sprague-Dawley control rat fed the standard diet (A, B, and C) and a genetic hypercalciuric stone-forming (GHS) control rat on the standard diet (D and E). Each midtransverse kidney section is shown with half of the kidney stained by the Yasue method and the other half with an immunohistochemical stain for osteopontin. No tissue injury was noted in either of the kidneys (B, C, and E). No stones were found in the kidneys of the Sprague-Dawley controls while apatite stones were only found in the renal pelvis of the GHS control. An identical pattern of osteopontin immunostaining, localized to cells of the thick ascending limbs, distal tubules, inner medullary collecting ducts and urothelium, was observed in both control kidneys [magnification $\times 10$ (A); $\times 40$ (B); $\times 40$ (C); $\times 10$ (D); and $\times 20$ (E)].

of the renal pelvis (Fig. 2D). Crystal entrapment was confirmed by observing a series of serial sections of these kidneys. Clearly, the proliferating urothelial cells surrounded the crystalline deposits, isolating the crystals within or beneath the urothelium. The osteopontin immunostaining of the nephron segments was identical to that seen in the Sprague-Dawley controls; however, those regions of urothelial proliferation showed increased staining (Fig. 2C).

Animals fed the 5% hydroxyproline supplemented diet also formed stones only in the urinary space (Fig. 3). All stones were calcium oxalate dihydrate and calcium oxalate monohydrate [23]. Using light microscopy, no crystals or cellular injury was noted along any segments of the nephron. However, these animals demonstrated more extensive urothelial proliferation that occurred along the entire length of the renal papilla to the fornix. Because of the greater amount of urothelial proliferation, crystal entrapment was seen more frequently and gave the appearance of crystals lodged within the interstitium, deep to the proliferative urothelium. While the pattern of osteopon-

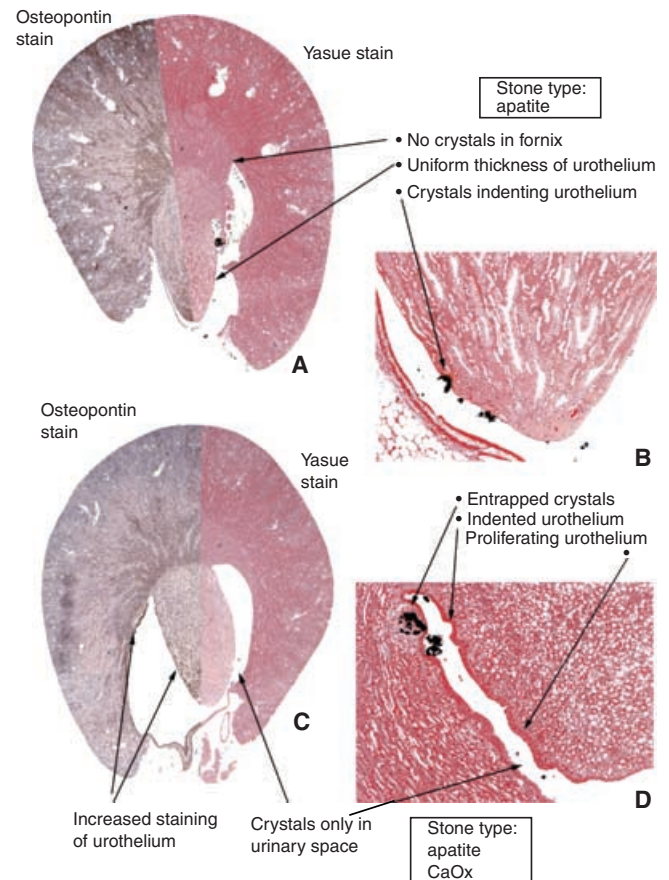


Fig. 2. Midtransverse paraffin sections through a kidney from a GHS rat fed the 1% hydroxyproline supplemented diet (A and B) and a genetic hypercalciuric stone-forming (GHS) rat fed the 3% hydroxyproline supplemented diet (C and D). Animals in both groups had stones only in the urinary space. No crystals or cell injury was identified in any cortical or medullary tubules. Urothelial changes were noted in both groups. The rats that ate the 1% hydroxyproline supplemented diet showed indentation of the urothelium by adjacent stones while the urothelium of the rats that ate the 3% hydroxyproline supplemented diet showed urothelial proliferation, indentation and crystal entrapment (D). Osteopontin immunostaining was unchanged in the 1% hydroxyproline group while the 3% hydroxyproline group showed increased osteopontin staining of the urothelium at those sites of proliferation [magnification $\times 10$ (A); $\times 20$ (B); $\times 10$ (C); and $\times 20$ (D)].

tin immunostaining of cortical and medullary nephron segments was identical to the Sprague-Dawley controls, sites of urothelial proliferation and crystal entrapment showed intense staining (Figs. 3A and 4). Osteopontin staining was also observed surrounding the stones.

DISCUSSION

Selecting for hypercalciuria, we have inbred Sprague-Dawley rats to establish a strain of animals whose urine calcium excretion is eight- to tenfold greater than that of similarly fed noninbred Sprague-Dawley rats [10–23]. While consuming a standard calcium diet, these hypercalciuric rats spontaneously form large numbers of poorly crystalline apatite (calcium phosphate) kidney stones [10–13, 18]. However, when the diet is supplemented with

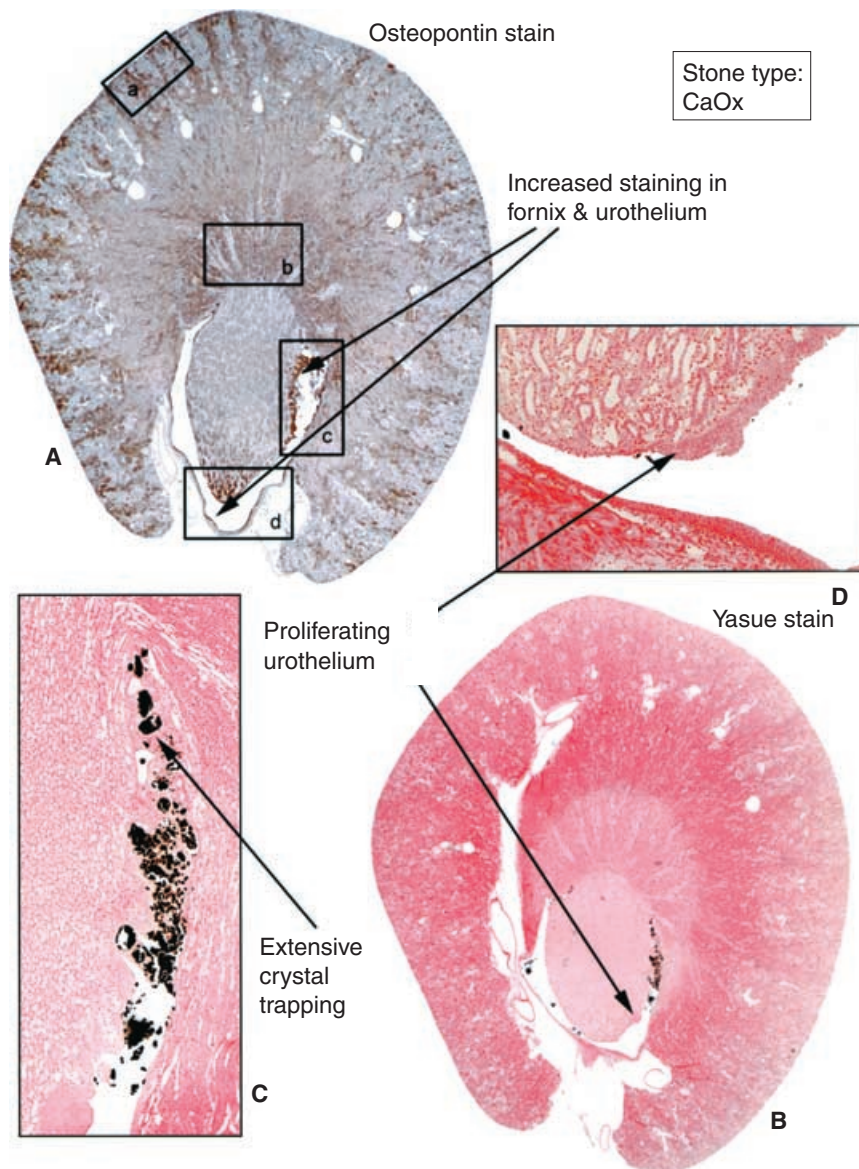


Fig. 3. Midtransverse paraffin sections through a kidney from a genetic hypercalciuric stone-forming (GHS) rat fed the 5% hydroxyproline supplemented diet (A, B, C, and D). These animals consistently possessed stones only in the urinary space but not in any nephron segments. No tubular injury was detected. However, extensive urothelial proliferation was noted along the side of the renal papilla extending to the fornix. Crystal entrapment was common at those sites of proliferation leading to isolation of some crystal from the urinary space. While osteopontin immunostaining of cortical and medullary nephron segments was identical to that described for the Sprague-Dawley controls, sites of urothelial proliferation and crystal entrapment showed intense staining [magnification $\times 10$ (A); $\times 10$ (B); $\times 25$ (C); and $\times 20$ (D)].

5% hydroxyproline, a common amino acid that is metabolized to oxalate, the inbred hypercalciuric rats form calcium oxalate stones [23]. The addition of 3% dietary hydroxyproline leads to the production of both apatite and calcium oxalate stones while 1% added hydroxyproline does not change the character of the stones. The purpose of the current study was to determine the localization of the initial stone formation and ascertain if the renal parenchyma exhibited a reaction to the stones. We found that all stones were located in the urinary space and the calcium oxalate, but not calcium phosphate, crystals induced cellular proliferation resulting in stone sequestration and were associated with increased osteopontin staining.

Osteopontin is an acidic, glycosylated, phosphoprotein that is secreted by renal tubular cells [40–42] and has been detected in regions of stone formation in both

humans and rats [43, 44]. Osteopontin has been shown to inhibit both calcium oxalate and calcium phosphate crystallization in vitro and is also thought to inhibit crystal formation in vivo [40, 41, 45–47]. In this study, intense staining for osteopontin was associated with the proliferative urothelial cells surrounding the calcium oxalate crystals. However, there did not appear to be increased osteopontin staining in the urothelium of rats that formed calcium phosphate stones. In these rats, the crystals did not generate the proliferative response that was observed with calcium oxalate crystals. Prior studies have demonstrated an association of osteopontin with calcium phosphate crystallization [45, 47]. Perhaps the lack of entrapment of the calcium phosphate stones by the urothelium, resulting in less crystal-cell interaction, explains the lack of up-regulation of osteopontin in this study.

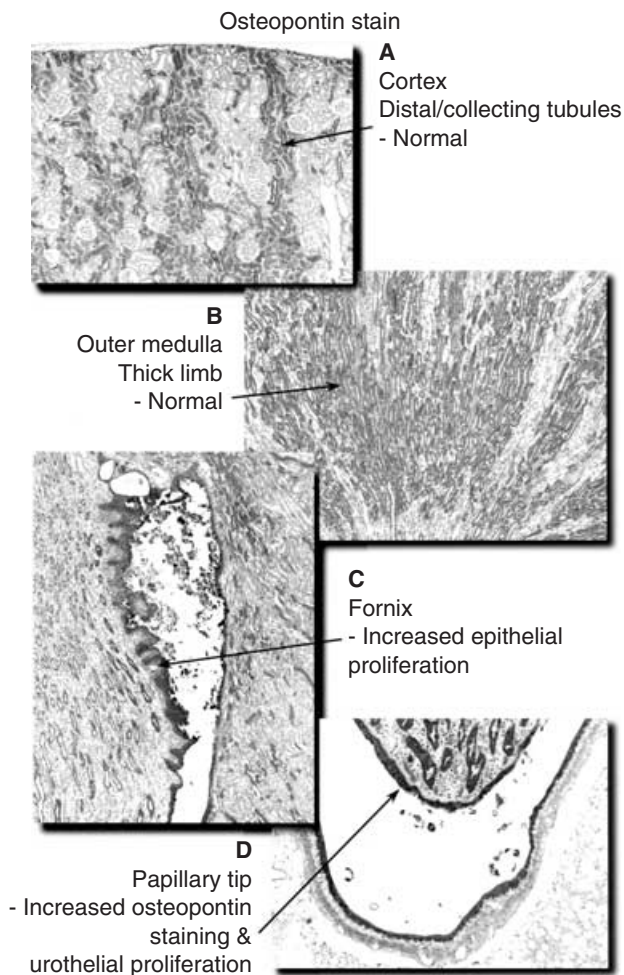


Fig. 4. High magnification views of selected areas of the osteopontin stained section seen in Figure 3. (A) Osteopontin staining of the cortical distal and collecting tubules. The distal tubules stained more intensely than the collecting tubules. (B) Osteopontin staining of the thick ascending limbs. (C) A site of proliferation of the urothelium at the fornix. Intense osteopontin staining is observed in the proliferated urothelial cells and nearby stone material. (D) A similar region of urothelial proliferation and osteopontin staining at the side of the papillary tip of nearby stone material [magnification $\times 25$ (A); $\times 25$ (B); $\times 25$ (C); and $\times 20$ (D)].

Our observation of calcium oxalate crystal entrapment at sites of urothelial cell proliferation near or at the fornix, which leads to the positioning of these crystals beneath the urothelium, has been previously documented. In humans, Carr [48] described a process by which calcium oxalate crystals would be moved from the urinary space to lymphatic channels at the surface of renal papilla and form forniceal “geodes.” In rats, Lilien et al [49] reported a similar observation in animals fed a pyridoxine (vitamin B₆)-deficient diet. These authors also proposed a pathway by which calcium oxalate crystals in the urinary space would be entrapped by the urothelium and then moved to the interstitium to be accessible to renal tubules or lymphatics. The morphologic data from our present GHS rat study strongly suggests that the calcium oxalate, and not

calcium phosphate, crystals in the urinary space initiate a proliferative response in the cells of the urothelium. After this mitogenic process progresses, nearby calcium oxalate crystals become entrapped and are moved to the interstitial side of these cells as the urothelium continues to thicken. Because we never see calcium oxalate crystals in tubular lumens or deposition beneath the normal urothelial cells, we have concluded that the calcium oxalate crystals found beneath the urothelium at the fornix originated from the urinary space.

The calcium oxalate, but not the calcium phosphate, solid phases induced proliferation of the urothelium. Previous studies in several renal cell lines, including porcine normal kidney tubular epithelial cells (LLC-PK1), African green monkey cells (BSC-1), Madin-Darby canine kidney cells (MDCK) and the primary human renal proximal tubular epithelial cells (RPTEC) have shown that oxalate induces immediate early response genes, leading to increased DNA synthesis and cellular proliferation [50–53]. The findings of the current study, that calcium oxalate crystals induce cellular proliferation, are in accord with these prior *in vitro* studies.

In vitro, calcium oxalate crystals have been shown to bind in a stereo-specific manner to renal tubular cells [54]. Following binding, the cells react to the solid phase by internalizing the crystals, which promote changes in gene expression and cytoskeletal organization and induce cellular proliferation [55–58]. In the current study, while we did not observe internalization of the crystals, we did observe marked proliferation of the urothelium in direct contact with calcium oxalate crystals. This proliferation indicates almost certain alteration in gene expression and cytoskeletal organization. There was no apparent cellular proliferation in response to the calcium phosphate crystals. Further studies will be necessary to determine the mechanism(s) by which the interaction of the calcium oxalate, but not calcium phosphate, crystals with the urothelial cells induces proliferation.

Finlayson and Reid [59] reasoned that since the renal ultrafiltrate traverses the tubule in a matter of minutes there is insufficient time for ions to nucleate and then aggregate into a clinically significant stone. Some have postulated that the adherence of the crystals to the urothelium would allow sufficient time for crystal aggregation and stone formation. In the current study, crystals were not found adherent to the tubular lumen but only found in the urinary space. The crystals did not appear to be adherent to cells, rather the calcium oxalate crystals located in the fornix appeared to be trapped by the proliferating urothelium. Perhaps these calcium oxalate crystals cannot be rapidly cleared due to the absence of directed urinary flow in the fornix resulting in crystal trapping and subsequent cellular proliferation.

Ethylene glycol added to the drinking water of rats induces the rapid formation of calcium oxalate kidney

stones. Histologic analysis of the kidneys from these rats reveal marked cellular damage, including disruption of the proximal and distal tubules with apoptosis and necrosis [26, 27, 51, 53]. These cellular degradation products are thought to induce heterogeneous nucleation of calcium oxalate crystals at physiologic tubular concentrations of oxalate. Markers of cell injury are expressed. In the current study, using only light microscopy, we did not observe evidence of tubular cell disruption or injury. Perhaps the difference between the cellular response of the rats in this study and that observed after ethylene glycol administration is due to the cellular toxicity observed after ethylene glycol administration [32–34]. Poldelski et al [34] demonstrated that the metabolites of ethylene glycol induce multisystem organ injury. In isolated mouse tubular segments and in renal tubular cell lines, they found that principle metabolites of ethylene glycol, glycoaldehyde and glyoxylate, were both highly toxic, causing profound adenosine triphosphate (ATP) depletion, lactate dehydrogenase (LDH) release, and Human Renal Proximal Tubular cell (HK2) death. Neither oxalate nor ethylene glycol itself induced this cellular toxicity. Additionally ethylene glycol administration results in severe, life-threatening metabolic acidosis [28–31]. Thus, the renal tubular cellular injury induced by ethylene glycol may be a function of the metabolites of ethylene glycol and/or the metabolic acidosis induced by metabolism of ethylene glycol and not oxalate or calcium oxalate crystals.

Evan et al [60] have recently demonstrated that in hypercalciuric calcium oxalate stone formers the initial site of crystal deposition is the interstitium surrounding the thin limb of Henle's loop. There is crystal extension to the collecting tubule and then to the papilla, which then erodes into the urinary space. The initial crystal phase is apatite, a calcium phosphate complex, which can be a heterogeneous nucleation site for calcium oxalate crystal formation in the urinary space. In patients who form stones after intestinal bypass procedures the crystals, again composed of calcium phosphate, initially form in the collecting duct where they act as a nucleating site for calcium oxalate crystals. Thus, the initial site of crystal deposition appears dependent upon the principle mechanism of stone formation [61]. Similar to humans, the GHS rats, not given hydroxyproline, form a calcium phosphate solid phase [10–13, 18, 23]. Why the initial site of stone formation in the GHS rats given hydroxyproline differs from the initial site of stone formation in these human studies is unclear. Perhaps the difference is due to the relative rapidity of stone formation in the GHS rat compared to humans. It is possible that with future study, other mechanisms of stone formation in humans, will more closely resemble that in the GHS rat.

As in the GHS rat, the crystal phase initially deposited in humans is composed of calcium and phosphate [60]. In the GHS rats, only with the addition of hydroxypro-

line to increase urine oxalate excretion, did the rats form calcium oxalate stones [23]. The urine of the GHS rats, without the addition of hydroxyproline, is markedly supersaturated not only with respect to calcium phosphate but with respect to calcium oxalate as well. The initial calcium phosphate crystal formation in the GHS rat appears due to an increase in the upper limit of metastability with increasing urinary supersaturation with respect to calcium oxalate but not to calcium phosphate [15]. The increase in the upper limit of metastability with respect to calcium oxalate would decrease the likelihood of forming this solid phase. A similar increase in the upper limit of metastability with respect to calcium oxalate, but not calcium phosphate, to that observed in the GHS rat has been confirmed in humans [62].

Thus we have demonstrated localization and tissue reaction to the stones formed by the GHS rats. We have shown that all stones appear to be localized to the urinary space and that the calcium oxalate, but not the calcium phosphate, stones induce marked proliferation of the urothelium resulting in sequestration of the stones. Osteopontin is induced and may help to retard further stone formation. The mechanisms by which the calcium oxalate, but not the calcium phosphate stones induce the cellular proliferation and by which the sequestration occurs only in the fornix, remain to be determined.

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