

Crystal Retention in Renal Stone Disease: A Crucial Role for the Glycosaminoglycan Hyaluronan?

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The mechanisms that are involved in renal stone disease are not entirely clear. In this article, the various concepts that have been proposed during the past century are reviewed briefly and integrated into current insights. Much attention is dedicated to hyaluronan (HA), an extremely large glycosaminoglycan that may play a central role in renal stone disease. The precipitation of poorly soluble calcium salts (crystal formation) in the kidney is the inevitable consequence of producing concentrated urine. HA is a major constituent of the extracellular matrix in the renal medullary interstitium and the pericellular matrix of mitogen/stress-activated renal tubular cells. HA is an excellent crystal-binding molecule because of its size, negative ionic charge, and ability to form hydrated gel-like matrices. Crystal binding to HA leads to crystal retention in the renal tubules (nephrocalcinosis) and to the formation of calcified plaques in the renal interstitium (Randall's plaques). It remains to be determined whether one or both forms of renal crystal retention are involved in the development of kidney stones (nephrolithiasis).

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Renal stone disease has tormented people throughout the ages. Despite enormous developments in nephrology and urology, we still do not know exactly how kidney stones are formed and how to prevent them. Kidney stones are built from numerous tiny crystals that commonly are pasted together with organic material. The formation of crystals in the kidney is normal and harmless provided that they are excreted with the urine. The difference between stone formers and non-stone formers is that crystals stay behind in kidneys of stone formers. An acute renal colic that is caused by a tiny stone's passing down the ureter can be extremely painful. Small stones (≤ 5 mm) often pass without medical intervention, but larger stones (≥ 10 mm) usually must be removed. With the advent of extracorporeal shock wave lithotripsy and improved surgical procedures, the removal of kidney stones no longer is as primitive as a few hundred years ago. Nevertheless, recurrent stone disease causes significant morbidity in the Western world, and in patients with inborn errors in endogenous oxalate synthesis, pathologic renal calcifications ultimately may destroy the kidney. The human kidney concentrates the urine to preserve water and essential nutrients and to eliminate waste products. Approximately 180 L/d blood is filtered, only 1 to 1.5 L of which is excreted as urine. During this process, the primary urine becomes supersaturated with poorly soluble waste salts such as calcium phosphate (CaP) and calcium oxalate (CaOx), leading to crystal formation. To ensure their elimination, the urinary tract should be nonadherent to crystals. The ability to

excrete adequately crystalline material is perturbed in stone formers. Current treatment strategies are based on reducing the levels of supersaturation through dietary recommendations and drugs. As long as we do not know exactly how crystals are retained in the kidney, it will remain difficult to develop successful treatment strategies. There must be crystal-binding sites in the kidneys of stone formers that allow crystals to settle and accumulate into a stone. The concept that stone formation depends on a preexisting renal lesion was proposed in 1937 by Randall (1) and in 1978 by Finlayson and Reid (2). The essential difference between these two historic papers is that Randall expected the preexisting lesions on the renal papillae, whereas Finlayson expected them in the renal tubules.

Renal Stone Disease

Renal stone disease can be classified into two major groups: Nephrocalcinosis and nephrolithiasis. Whereas the definition of nephrolithiasis (kidney stones) usually does not lead to misunderstanding, the description of nephrocalcinosis is less clear. We define nephrocalcinosis as the retention of crystals in the renal tubules. Nephrocalcinosis occurs when the epithelial cells that line the renal tubules become susceptible to crystal attachment (3). Although the clinical consequence of nephrocalcinosis is not entirely clear, it may lead to abnormal renal tubular cell function, inflammation, and renal tissue damage. The start of nephrocalcinosis (microscopic nephrocalcinosis) seldom is demonstrated in a clinical setting because the initial lesions cannot be detected with current imaging techniques and renal biopsies are not performed in early stages of metabolic disease. In chronic renal disorders, crystal retention in the renal tubules often develops into macroscopic nephrocalcinosis. Ultimately, renal tubular cells may overgrow the initial lesions, leading to relatively large diffuse areas of tubular calcification in the renal

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cortex or medulla that are visible by conventional radiography and ultrasonography. Nephrocalcinosis and nephrolithiasis should not be confused with the deposition of calcium salts in the renal interstitium. Interstitial CaP precipitates are common in individuals who are older than 35 yr and should be considered a relatively benign form of renal calcification (4). Although the papillary interstitium apparently has a large storage capacity, this form of renal calcification eventually may become pathologic as is discussed later in the review.

Crystallization

Urinary supersaturation refers to a state in which stone salts are soluble at much higher concentrations in urine than in water. The explanation for this phenomenon is that urinary glycoproteins, glycosaminoglycans (GAG), citrate, and magnesium form complexes with these salts so that they can be kept in solution at much higher concentrations. Already at the beginning of the 20th century, it was known that “crystalloids” are relatively insoluble in water, but at much higher concentrations, they are soluble in urine or blood as a result of “protective colloids,” such as mucins, albumin, chondroitin sulfate, and nucleic acids (5). It took several decades until it was demonstrated that urine indeed was capable of inhibiting cartilage calcification in tibiae of ricketic rats (6). The attractive concept was born that stone formers may produce less or abnormal urinary inhibitors of crystallization (6). During the subsequent years, a large number of papers were published on urinary macromolecular inhibitors, including GAG such as chondroitin sulfate, heparan sulfate, dermatan sulfate, and hyaluronan (HA) and glycoproteins such as osteopontin (OPN), nephrocalcin, Tamm-Horsfall protein (THP), uropontin, crystal matrix protein (F1 prothrombin fragment), and uronic acid-rich protein (bikunin) (7,8). Recently, it was proposed that proteins that are incorporated into the organic stone matrix may support their dissolution inside cells (9). Despite these extensive studies, we still do not know whether one of these substances is crucial in stone disease prevention or whether normal individuals excrete more or superior macromolecules (10). Because crystal formation in the kidney is common, crystals may be a problem only for individuals who are predisposed to retain them in the kidney.

Randall's Plaque

Early in the 20th century, Randall published a series of papers in which he described the early development of stones on the renal papillae. Between 1935 and 1938, he examined a large series of seemingly normal kidneys at the autopsy table. In approximately 20% of the kidneys, he found cream-colored areas near the tips of the papilla that seemed to be located partially beneath the epithelium. These areas were plaques of calcium deposited in the papillary interstitium. In 6% of the kidneys, renal calculi were observed growing on and adhered to a renal papilla. An important finding was that the plaque on the papilla consisted of calcium carbonate and phosphate, whereas the attached stone usually was composed of a different salt, predominantly CaOx. These observations convinced Randall that kidney stones grow from calcium plaques on the renal

papilla. In the second part of the 20th century, several researchers opposed this hypothesis after it became clear that plaques occur predominantly in people who are older than 50 yr, whereas the clinical peak stone age is between 20 and 50 yr. In addition, it was found that the plaque incidence in the general population was much higher than the stone incidence, and according to Prien (11), stones actually rarely were found attached to the papilla. Despite the declined interest in Randall's theory during the latter part of the 20th century, the theory never completely lost its attraction (12–17), and there even is a renewed interest in Randall's plaques. Evan and colleagues (18–20) took intraoperative biopsies in kidneys of idiopathic calcium stone formers, patients with stones as a result of obesity-related bypass procedures, and non-stone formers after nephrectomy. This work is innovative because it is extremely difficult to obtain renal tissue from living stone formers. In agreement with earlier observations by Vermooten (21) and Stout (22), the authors found that calcium plaque seemed to originate in the basement membranes of the thin loops of Henle and spread from there into the interstitium to beneath the urothelium. Patients who had undergone bypass surgery ($n = 4$) and non-stone formers ($n = 4$) did not produce plaques. In idiopathic stone formers ($n = 15$), a correlation was found between papillary surface coverage by Randall's plaque and the number of stones formed and urinary calcium, whereas urine volume and pH were inversely related (23,24). It remains to be determined whether the new data provided by Evan and colleagues sufficiently refute the earlier opposition against Randall's concept. However, it is worthwhile to re-evaluate the value of this “old” hypothesis with modern research techniques.

Free- and Fixed-Particle Theory

In 1978, Finlayson and Reid (2) advocated the fixed-particle theory as opposed to the free-particle theory that was advanced 10 yr earlier by Vermeulen and Lyon (25). The free-particle theory was based on experiments that were performed in rats that received large amounts of hyperoxaluric agents and showed that particles grow fast enough to obstruct the ducts of Bellini. Finlayson and Reid used physicochemical, physiologic, and anatomic parameters to analyze the plausibility of this concept in the human kidney. They came to the conclusion that free particles cannot grow fast enough to cause stone disease in the upper urinary tract. Although Finlayson and Reid rejected Randall's theory, they did not show how crystals are retained in the renal tubules but expected an initiation of “an entire new area of stone matrix-related studies upon the mechanism(s) of particle fixation.” This prediction was correct because their work indeed was followed by an extensive search for crystal-binding molecules (CBM) in the renal tubules. Later, Kok and Khan (26) proposed that crystal agglomeration could lead to the development of particles that were large enough to obstruct the renal tubules. Khan (27–31) was one of the first investigators to demonstrate that crystal retention in the rat kidney or bladder requires some form of epithelial damage.

Crystal Retention in the Renal Tubules

In the earlier mentioned study by Evan *et al.* that used biopsies that were taken from the kidney during a surgical procedure (18), the investigators did not observe crystals in the renal tubules of idiopathic stone formers or non–stone-forming control subjects, whereas crystals were found in renal tubules of patients who underwent obesity-related bypass procedures. Remarkably, these were CaP crystals while the urine was also supersaturated with CaOx (18). From these observations, it was concluded that renal stone formation does not involve the retention of CaOx crystals in the nephrons (32). This is not the first time that investigators searched for crystals in the kidney. Previously, this type of study commonly was performed with kidneys that were obtained at autopsy. Most of these studies are relatively old and have to be interpreted with caution because the underlying disorders frequently are not well defined and different definitions are used for calcium deposits in the kidney, including calcium infarcts (33), lime deposits (4), “kalkmetastasen” (34), nephrocalcinosis (22), microscopic calculi (35), renal medullary calcification (36), and simply crystals in the kidney (37). To avoid confusion, these terms are not used in the next brief summary, in which we merely point out where, according to the authors, the crystals were found in the kidney. Already in 1862, Henle (33) found calcifications completely filling the renal tubules. In routine autopsies, Beer (4) in 1904 found calcium deposits in the interstitium and renal tubules in practically all kidneys of individuals who were older than 35 yr, whereas Stout *et al.* (22) in 1955 found calcium deposits in the interstitium and renal tubules in 80 (54%) of 147 cases. In 1964, Bennington *et al.* (37) found CaOx crystals in the renal tubules in 32 (6.4%) of 500 cases, and, more recently, Ebisuno *et al.* (38) found CaOx crystals in the renal tubules in 19 (50%) of 38 CaOx stone formers. In contrast, Anderson and McDonald (35) in 1946 observed calcium deposits everywhere in 168 kidneys except in the renal tubules; also in 1971, Haggitt and Pitcock (36) did not see crystals in renal tubules of 100 successive autopsy kidneys. How is it possible that some investigators find CaOx crystals in renal tubules whereas others do not? Tissue preparation for routine histology requires tissue fixation, dehydration, clearing, and paraffin embedding. The paraffin-embedded material is cut into thin sections that are deparaffinized by running through xylenes to alcohols to water, stained with hematoxylin and eosin, dehydrated through alcohols to xylenes, and covered with a coverslip. There are two critical moments during which crystals can be lost, namely during sectioning, as a result of differences in hardness between crystals and tissue, and during the graded alcohol and washing steps in processing sections with open tubules. From our own studies, we know that the percentage of remaining crystals is low and often detectable only when the initial amount of crystals was high. In electron microscopy studies, the recovery of calcium crystals is even more difficult. Besides the aforementioned effects of tissue processing, the remaining crystals often are dissolved by postfixation with aggressive chemicals such as osmium tetroxide, which is why the localization of crystals in the renal tissue can be deduced only from their “ghosts” (39,40). There is another complicating factor in

clinical studies: The so-called “stone clinic effect,” which refers to the effect on crystal formation of encouraging a high intake of fluid and avoiding dietary excesses (41). Perhaps most crystals already were eliminated from kidneys of fasting and hydrated stone formers by the time the biopsies were taken in the study by Evan *et al.* (18). Crystals could be associated more firmly with the collecting ducts in patients who underwent an intestinal bypass procedure for obesity because they frequently develop nephrocalcinosis (42). The approach to take biopsies from kidneys of patients during percutaneous nephrolithotomy undoubtedly is valuable, but there may be pitfalls. To call to mind the words of Finlayson and Reid, “It is usually difficult to prove something to be true, whereas it is frequently possible to show something to be not true” (2). The retention of crystals in the renal tubules unquestionably plays a role in preterm infants (43), transplanted kidneys (44), sarcoidosis (45), primary hyperoxaluria (46), Dent’s disease (47), carbonic anhydrase deficiency (48), renal tubular acidosis (49–51), Bartter’s syndrome (35), cystic fibrosis (52), Sjögren’s syndrome (53), patients who undergo intestinal bypass surgery (19,42), and brushite stone formers (54).

Crystal–Cell Interactions

Mandel, Lieske, and Toback were pioneers in crystal–cell interaction studies. The group of Mandel initially studied CaOx crystal–induced membranolysis of red blood cells as a model for effects of crystals on cell membranes (55,56). Later, they found that crystal binding to renal tubular cells in culture depends on membrane lipid asymmetry (57–59), epithelial polarity (59,60), membrane fluidity (61), cell differentiation (62), the cell types used (62–64), the presence of urinary macromolecules (65), and exposure to oxalate (66). Lieske and Toback and colleagues (67–72) observed that CaOx monohydrate (COM) crystals are endocytosed, thereby stimulating the expression of immediate early genes, OPN, and cell division. COM internalization was inhibited by THP, fibronectin, TGF- β 2, and heparin (69). Once inside the cell, crystals slowly dissolved in lysosomes (73). A role for crystal uptake in renal stone disease is questionable, however, because intracellular crystals seldom are observed in the kidney (3,74), and in cell culture, crystals are taken up by proximal tubular cells but not by cells that are derived from segments where crystals are expected to be formed (*e.g.*, the distal tubule, collecting ducts) (75,76). COM and hydroxyapatite crystals adhered to negatively charged cell surface molecules by a process that could be inhibited by GAG, polyglutamic acid, polyaspartic acid, nephrocalcin, uropontin, and citrate but not by THP (77,78). Uric acid crystal binding did not depend on anionic cell surface binding molecules (79). CaOx dihydrate (COD) nucleated directly on the cell surface *via* their [101] face (80), to become associated with the membrane through their [100] face (81,82). Crystal binding stimulated additional crystal attachment and was inhibited by arachidonic acid or other compounds that raise intracellular cAMP (83). Urinary macromolecular inhibitors may inhibit not only crystal growth and agglomeration but also the interaction between crystals and renal tubular cells (crystal retention). Studies that were performed with COM and renal tubular cells in culture showed that urinary glycoconjugates that were derived from healthy men seemed to have some inhibitory potential (84,85), and

this inhibitory power was somewhat lower in idiopathic male CaOx stone formers (86). Although perhaps a matter of interpretation, it seems that when the epithelium becomes susceptible to crystal attachment, the demonstrated inhibitory capacity of urinary macromolecules is not sufficient to prevent crystal retention.

Collectively, these results significantly increased our insights into the processes and mechanisms that are involved in the interaction between precipitated stone salts and renal tubular cells. Another school of investigators searched for stressors that may stimulate crystal attachment. Most attention has been devoted to oxalate, an anionic dicarboxylate that frequently is increased in the urine of kidney stone formers (hyperoxaluria). Oxalate is considered a major risk factor in renal stone disease because of its poor solubility and possible toxicity. The idea that ionized oxalate is harmful to kidney cells was derived from studies that were performed in animals (30,87–91) and cell culture (90–95). The general idea is that oxalate causes oxidative stress, thereby generating toxic free radicals (96–98). The oxalate toxicity hypothesis currently is under debate, however. A recent animal study demonstrated that the production of free radicals probably is the consequence of oxalate-mediated renal tubular injury rather than the initiating cause (99). In cell culture studies, it was found that oxalate cannot become very high in the presence of physiologic amounts of calcium (100,101) and that oxalate seemed to be relatively harmless in the absence of calcium (101). Accumulating evidence suggests that the assumed oxalate-induced cell injury in fact often is caused by crystals (75,102,103). This has consequences for the clinical relevance of these studies, because the levels of crystalluria usually are much higher in animal studies than in patients, and cell culture studies often are performed with renal tubular cells that are not supposed to encounter crystals (*i.e.*, proximal tubular cells) (104). It must be emphasized that studies with renal tubule epithelial cells in culture should be interpreted with caution because there are many pitfalls. Conditions in culture may deviate substantially from local conditions in the kidney. Primary cultures or cell lines may have lost important signaling pathways and functions (LLC-PK1 and MDCK strain II cells, *e.g.*, almost entirely lost the eicosanoid pathway and the ability to synthesize and express HA [105]). As a result of adaptation to the artificial environment, cells in culture also may gain certain functions. Other variables are segmental origin (*e.g.*, proximal, distal, collecting duct), growth substrates (*e.g.*, solid, permeable), growth medium (supplemented or not with serum and/or other additives), growth conditions (*e.g.*, subconfluent, confluent), and the correct polarized distribution of membrane components at confluence. It also is important that cells have the ability to bring proliferation to a standstill at confluence (as though they are normal cells). In our hands, confluent monolayers remain functional on permeable supports for 1 to 2 d, after which they have the tendency to form multilayers. Taken together, cell culture models are useful to elucidate pathways and mechanisms that are difficult to study *in vivo*. Nevertheless, progress can be made only after verification of the (patho)physiologic relevance of the results in animal and human kidneys.

CBM

Our group entered this field in the early 1990s. In one of our first papers, we described the model system that we apply for these studies, with renal tubular cells grown on permeable supports in a two-compartment culture system (Transwells) (106). This model was chosen because the group of Mandel found a relationship between crystal binding and the polarized distribution of membrane components in collecting duct cells (60), and it was known that renal tubular cells obtain higher levels of polarity on permeable supports (107). The MDCK cell line was selected because it was reported to originate from the distal nephron, a segment where, on the basis of the levels of supersaturation, crystals are to be expected. Later, it became clear that wild-type MDCK cultures contain more than one cell type, after which we switched to MDCK strain I, a cell type that more closely resembles the renal collecting tubule (76). Our studies usually are performed with COM because this is the most common crystalline phase of kidney stones. MDCK-I cells are not susceptible continuously to crystal binding. Crystals adhere to proliferating cells in subconfluent cultures but hardly to growth-inhibited cells in confluent cultures, suggesting that an intact epithelium is nonadherent. After scrape injury, crystals indeed selectively bind to regenerating cells in the wound. The epithelium re-obtains its nonadherent properties soon after the wounds are closed (108). Apparently, proliferating and migrating cells express cell surface molecules with affinity for crystals, whereas growth-inhibited cells do not. The identification of a cell-surface CBM should be based on several criteria (109). Crystals should bind in the presence of the CBM, whereas crystal binding must be much lower in its absence. Evidence must be provided that crystals actually physically interact with the CBM in question and that the CBM also plays an important role in the binding of crystals in kidneys of animals and finally in kidneys of patients. The group of Mandel proposed that crystals bind to negatively charged phosphatidylserine (PS), a phospholipid that appears at the outer leaflet of the lipid bilayer during physiologic cell death (apoptosis) (58). The group of Lieske, however, suggested that crystals adhere to the terminal sialic acid residues of membrane glycoconjugates that provide the cell surface most of its negative charge (110). In our model system, these molecules do not seem to be decisive in crystal binding. Annexin V binding studies revealed that MDCK-I cells with affinity for crystals are not necessarily apoptotic. Although PS might be a CBM, this phospholipid does not play a role in crystal binding to nonapoptotic proliferating and migrating cells (109). Lectin binding studies using *Maackia Amurensis II* and *Sambucus Nigra* that bind sialic acid residues that are, respectively, α 2,3 and α 2,6-linked to penultimate galactose of cell-surface glycoconjugates demonstrated that crystal-binding as well as non-crystal-binding MDCK-I cells express α 2,3 and α 2,6-linked sialic acid residues. Metabolic labeling studies using [3 H]glucosamine, a precursor of N-acetylneuraminic acid (sialic acid), demonstrated that neuraminidase (sialidase) cleaves more radioactively labeled molecules from confluent than from subconfluent cultures. Therefore, there is an inverse relationship between the amount of sialic acid residues and crystal binding, which does not support

the concept that sialic acid is a CBM (111). Nevertheless, crystal binding to subconfluent cultures was greatly reduced after neuraminidase treatment, suggesting that in subconfluent cultures, sialic acid residues may support the expression of a true CBM. Most likely, polysialic acid also does not play a role in crystal binding because endo-N-acetylneuraminidase, an enzyme that hydrolyzes poly- α -2,8-sialosylcarbohydrate units, did not release radiolabel and did not reduce crystal binding. Finally, free sialic acid molecules in solution did not bind to COM (111). Other proposed CBM are OPN (112,113), nucleolin-like protein (114,115), collagen IV (116), and annexin II (117). The potential value of these molecules in crystal retention requires further verification according to the formulated criteria for the identification of a CBM. Support for the role of annexin II in crystal binding recently came from an experimental cell culture model for Dent's disease (118). Dent's disease is a genetic disorder that is associated with low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, kidney stones, and renal failure caused by mutations in a renal chloride channel gene, *CLCN5*. Crystal binding was studied to renal collecting duct (mIMCD-3) cells in which *clc-5* was disrupted by antisense *clc-5* or by overexpression of truncated *clc-5*. Disruption of *clc-5* resulted in the translocation of annexin II from the cytoplasm to the luminal cell surface, leading to increased levels of crystal binding that could be blocked with annexin II antibodies (118). It will be of interest to find out whether *clc-5* disrupted cells also are triggered to express CD44, HA, and OPN. Membrane-associated annexin II has been reported to regulate extracellular matrix (ECM) metalloproteinase inducer (Emmprin) activity (119), which stimulates HA biosynthesis in mammary carcinoma cells (120). However, annexin II-expressing cells also may represent an entirely different class of crystal-

binding cells. The aforementioned criteria for the identification of a CBM were applied to reveal that the GAG HA is a CBM (121). As mentioned above, proliferating and migrating MDCK-I cells are highly susceptible to COM crystal binding, whereas growth-arrested cells in confluent cultures are not. Crystal binding to mobile cells is much lower after the cells are treated with *Streptomyces* hyaluronidase (Hyal), an enzyme that digests HA but no other GAG or proteins (121). Biotinylated HA-binding protein binds to the luminal surface of cells in subconfluent or healing cultures but not to cells in confluent cultures. Crystals bind to plastic wells that are precoated with high molecular weight HA but not to uncoated wells, and COM binding to HA-precoated wells is abolished after Hyal treatment. Glucosamine is not only a precursor of N-acetylneuraminic acid (sialic acid) but also of N-acetylglucosamine, one of the monosaccharide building blocks of HA. Metabolic labeling studies with [3 H]glucosamine in combination with Sephadex G-50 size gel exclusion chromatography demonstrated that Hyal cleaves high molecular weight HA from subconfluent but not from confluent cultures. In collaboration with the group of De Broe (122), these studies were repeated and confirmed in primary cultures of human renal tubular cells. Human crystal-binding cells seemed to express not only HA but also OPN and the transmembrane protein CD44, a receptor for both HA and OPN (123–125). After wound healing or at confluence, HA and OPN no longer are detectable at the cell surface, whereas the expression of CD44 is restricted to basolateral domains of the plasma membrane (Figure 1) (126). Particle exclusion studies revealed that an organic matrix that is invisible with routine microscopy surrounded MDCK-I cells and primary cultures of human renal tubular cells. This pericellular matrix (PCM) disappeared after the addition of Hyal, indicating that the PCM depends on HA (105,122).

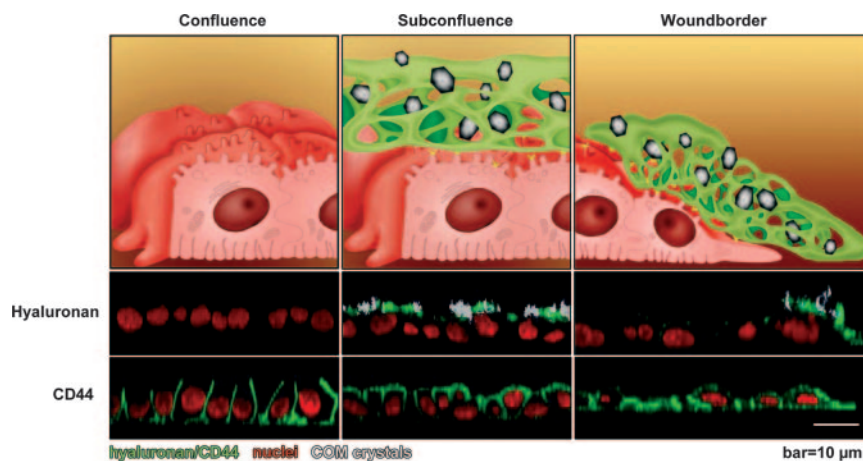


Figure 1. (Top) Confocal laser scanning microscopy (CLSM) scans made perpendicular to the growth substrate (xz scans) of MDCK strain I cells in confluent (left) and subconfluent (middle) cultures and during repair of scrape injury (right). The cells are incubated for 1 h with calcium oxalate monohydrate (COM) crystals and then stained for hyaluronan (HA) and the HA receptor CD44 (there are no suitable antibodies for the staining of osteopontin in canine MDCK cells). All nonadherent crystals are removed by washing; therefore, all remaining crystals are attached firmly to the cell surface. Confluent monolayers that developed functional tight junctions (transepithelial electrical resistance $>5000 \Omega/\text{cm}^2$) do not express HA, express CD44 exclusively at the basolateral plasma membrane, and are nonadherent to crystals. Crystals avidly bind to subconfluent cultures that express luminal HA and CD44. Crystals also avidly bind to the migrating cells at a wound edge, and these cells express HA and CD44 at their luminal surface.

After the identification of CD44-, HA-, and OPN-expressing cells as crystal-binding phenotype in culture, our next assignment was to verify these findings *in vivo*. Rats were treated for 1, 4, and 8 d with 0, 0.5, and 0.75% ethylene glycol (EG), after which we studied crystal retention and the expression of our crystal-binding phenotype in the kidney. Control animals did not express HA and CD44 in the renal tubules, whereas OPN expression was restricted to specific nephron segments. This staining pattern was not altered after 1 d of treatment, and there were no visible alterations in tissue morphology. After 4 and 8 d of EG, however, the epithelial lining in the renal tubules clearly was damaged, and HA, OPN, and CD44 were expressed at the luminal surface of proliferating cell nuclear antigen-positive and flattened (regenerating) cells in various segments of the nephron. Although EG almost immediately induced crystalluria, crystals initially were not retained in the kidney. After 4 and 8 d of EG, however, there was a dose- and time-dependent retention of crystals in the renal tubules. Crystals were found to be associated exclusively with renal tubular cells that expressed luminal HA, OPN, and CD44.

Our next objective was to reveal whether our crystal-binding phenotype also is observed in kidneys of patients with renal stone disease. For this purpose, two patient populations that frequently develop nephrocalcinosis—preterm infants and renal transplant patients—were selected (44,127,128). Renal tissue from 52 human fetuses with gestational age of 15 to 40 wk was obtained from the archive of the Antwerp University Hospital, Belgium. These preterm neonates died soon after birth (<1 d), and the expression of our marker molecules could be studied in the absence of diet and fluid intake influences. Another 18 kidneys were obtained from preterm neonates who lived for at least 4 d from the Erasmus Medical Center, The Netherlands. This group received a diet that is known to promote urinary supersaturation for at least 4 d. Two protocol kidney biopsies at 12 and 24 wk after transplantation were obtained from 10 renal transplant patients from the Medical School Hannover, Germany. Sections were stained for HA, OPN, and von Kossa (calcium). Fetal and transplanted kidneys invariably expressed HA and OPN at the luminal surface of renal distal tubular cells. Whereas there were no crystals in the renal tubules of the 52 preterm neonates who died at birth, nephrocalcinosis developed in seven of 18 surviving preterm neonates, indicating that crystal retention in the renal tubules requires diet- or drug-related crystal formation. Crystals also were found in the renal tubules of transplanted kidneys. It is interesting that between 12 and 24 wk after transplantation, tubular crystal retention increased from 20 to 60% in this patient group, suggesting that crystal retention in the renal tubules requires the production of concentrated urine. In both patient groups, crystals were selectively found in distal tubules where the epithelial cells expressed HA and OPN. The expression of the crystal-binding phenotype therefore seems to be an essential prerequisite for crystal retention in both patient groups (3). As is discussed next, HA is expressed by renal tubular cells that are activated to become mobile. The fetal kidney contains mobile cells because these kidneys are not developed completely, and transplanted kidneys probably contain remodeling cells as a result of repair

from ischemic- and/or immunosuppressive drug-induced renal tissue damage. The observation that crystals become attached to the same phenotype in two entirely different patient populations suggests that crystal binding to HA-, OPN-, and CD44-expressing cells represents a general mechanism to retain crystals in nephron.

HA

HA is a linear GAG that is composed of multiple units of glucuronic acid and N-acetylglucosamine (1,4-GlcUA-1,3-GlcNAc)_n. Apart from the PCM, high molecular weight HA (>10⁶ Da) also is a prominent constituent of the ECM in connective tissues. Because one disaccharide is approximately 400 Da, high molecular weight HA is a chain of >2500 disaccharide repeats. As a result of its expanded random coil structure, HA chains occupy huge tissue domains with the ability to entrap large amounts of solvent. Hydrated PCM provide the microenvironment that is conducive to adjustments in cell shape and epithelial architecture during dynamic morphogenetic processes such as inflammation, wound healing, embryonic development, and cancer (129). HA also directly influences cell behavior through its ability to communicate with the cell interior *via* cell-surface receptors, such as CD44 and CD168 (receptor for HA-mediated motility) (129–134). HA differs from other GAG in that it is not sulfated, not produced in the Golgi, and not incorporated in proteoglycans. HA is produced by HA synthase (HAS) proteins that are located at the inner face of the plasma membrane, where it is polymerized and simultaneously extruded across the membrane into the extracellular space. Three mammalian HAS genes have been identified. HAS1 is considered a house-keeping HAS, whereas HAS2 and HAS3 are regulatory enzymes (135). Eukaryotic cells produce HA that varies from extremely high molecular weight (>10⁶ Da) to very low molecular weight (approximately 2 × 10⁴ Da). Depending on its size, HA performs a wide range of activities in the body. High molecular weight HA provides structural functions, is involved in immune cell adhesion and receptor-mediated signal transduction, and is antiangiogenic and immunosuppressive, whereas low molecular weight HA is angiogenic, immunostimulatory, and inflammatory (130,136,137). HA catabolism involves the cell-surface receptor CD44, Hyal, and two lysosomal enzymes, β-glucuronidase and β-N-acetylglucosaminidase. Three human genes, HYAL1, 2, and 3, are found tightly clustered on chromosome 3p21.3, coding for Hyal-1, -2, and -3. Hyal-1 and -2 constitute the major Hyal of somatic tissues. Hyal-2 is anchored to the plasma membrane by a glycosylphosphatidylinositol link and cleaves high molecular weight HA to a limit product of approximately 2 × 10⁴ Da (approximately 50 disaccharides). Hyal-1 digests HA to small oligosaccharides at permissive pH in the lysosomes. Urine contains relatively high Hyal-1 activity and HA fragments of approximately 15 disaccharide units (6 × 10³ Da) (136,138–141). HA plays an important role in renal development. The Has2-null is an embryonic lethal mouse, with ECM that are less hydrated and more compact than normal (142). In the developing chick embryo kidney, HA accumulates at early stages of tubular epithelium formation. Differentiation of the epithelium to mature tubules and

renal corpuscles is accompanied by decreasing HA and increasing Hyal (143). Studies in our laboratory demonstrated that HAS2 mRNA and the production of high molecular weight HA are upregulated in subconfluent (developing) cultures and downregulated in confluent (mature) cultures (126). As mentioned previously, HA is expressed by renal tubular cells in human fetal kidneys but not in adult kidneys (3).

HA in the Healthy Kidney

HA is abundant in the renal medullary interstitium but almost undetectable in the cortex, and renal tubular cells normally do not express HA. In the renal medulla, HA provides structural support for nephron segments and blood vessels and plays an important role in renal water handling. Histochemical and physiologic studies demonstrated that antidiuretic hormone-mediated movement of water out of the renal collecting duct is facilitated by the release of Hyal with subsequent breakdown of HA in the renal medullary interstitium. The general picture is that at low urine volumes, Hyal is high and HA is low in the papillary tissue, whereas Hyal is low and HA is high during water diuresis (144–149). Rat renomedullary interstitial cells produce three-fold more HA under hypo-osmotic conditions than under hyperosmotic conditions, suggesting that these fibroblasts regulate the production of HA in the renal interstitium (147). Schmidt-Nielsen (150), for the first time, underscored the sponge-like properties of the papillary interstitium. The wall of the renal pelvis is equipped with a pacemaker that produces rhythmic contractions to push fluid into the calyces (150,151). As a result of the hydrostatic pressure on the epithelial barrier, water enters the collecting duct cells through selective water channels to leave the cells *via* basolateral water channels as a result of the vacuum that is caused by the elastic forces that expand the papilla during recoil. In the interstitium, water is taken up by HA, to be squeezed out again during the next pelvic contraction to flow into the ascending vasa recta (152). How does this lead to facultative water reabsorption from a hyperosmotic environment? According to Knepper *et al.* (152), purified water is leaving the HA sponge because cationic solutes are trapped by the carboxyl (COO^-) groups in the compressed matrix. Therefore, the selective reabsorption of water from the collecting ducts seems to be achieved by a two-step process in which pure water is translocated from the tubular lumen to the interstitium through water-selective cell membrane channels to be absorbed subsequently by the HA matrix, the run-through fraction of which consists of purified water that subsequently drains into the blood stream (Vincent C. Hascall, Department of Biomedical Engineering, The Cleveland Clinic Foundation, Cleveland, OH, personal communication, January 20, 2006). Measurements in slices of the rat kidney showed that the osmotic gradient increases steeply within the medulla along the corticopapillary axis (153). While the cortex is still isosmotic (approximately 300 mOsmol/kg H_2O), the osmolality gradually rises to >2000 mOsmol/kg H_2O in the papillary tip (urine is between 1000 and 2000 mOsmol/kg H_2O) (152,154). Although this osmotic gradient depends predominantly on NaCl and urea, concentration gradients also are found for less abundant solutes. Calcium

and oxalate, for example, are several-fold higher in the renal papilla than in blood or urine (155). It is not clear how these compounds (and phosphate) can become so high in the papillary interstitium. Perhaps they are actively pumped into the interstitium by membrane transport proteins (156,157). Despite their extremely high concentrations, poorly soluble calcium salts do not precipitate continuously in the papillary interstitium. Most likely, Ca^{2+} also becomes associated with the COO^- groups in the HA matrix, thereby preventing CaP precipitation (Figure 2). In fact, HA should be considered an enormous inhibitor of crystallization that effectively prevents papillary calcification. Because interstitial HA is low during antidiuresis, the highest risk for crystal formation most likely occurs during periods of water deprivation. Conversely, high fluid intake leads to high interstitial HA, which protects against crystallization. The pH in the renal tissue must be permissive for CaP nucleation (approximately pH 7) because interstitial CaOx deposits seldom are observed. Precipitated calcium crystals (CaP) may bind to the gel-like HA matrix, which could play an important role in interstitial plaque formation (Randall's plaques). It is conceivable that HA-rich plaque eroded through the pelvic wall serves as anchor for calcium crystals that are present in the primary urine. Therefore, although entirely speculative, HA could be an inhibitor of crystallization as long as calcium salts are in solution (the carboxyl groups of the HA chains compete with anions such as phosphate and oxalate for binding to calcium), whereas HA may serve as binding substance for precipitated calcium salts (because of the affinity of these crystals for high molecular weight HA) (Figure 3).

HA in the Diseased Kidney

HA is upregulated in the kidney during inflammatory renal disease states such as interstitial nephritis (158), acute ischemic injury (3,159,160), autoimmune renal injury (161), acutely rejecting human kidney grafts (162), acute tubular necrosis (122), and obstructed kidneys and EG poisoning (74). During these disease states, HA becomes expressed in areas of the kidney where it normally is absent, such as in the corticointerstitium and on the luminal surface of renal tubular cells (3,74,158). The source of HA in the cortical interstitium is not entirely clear. HA could be produced by interstitial fibroblasts; cells of the immune system, such as dendritic cells, macrophages, and lymphocytes; or renal tubular cells (158,163–167). Proximal tubular cells, such as HK-2, and human primary cultures synthesize more HA in response to scrape-injury, IL-1 β , high D-glucose, and bone morphogenic protein-7 through intracellular signaling pathways, including mitogen-activated protein kinase-dependent, NF- κ B-activated HAS2. In proximal tubular cells, increased HAS mRNA is accompanied by decreased Hyal mRNA (168). HA also has been proposed to serve as a binding molecule in proximal tubular cells, but this time not for crystals but as binding molecules for infiltrating monocytes (168). MDCK-I cells and primary cultures of mixed human proximal and distal renal tubular cells synthesize more HA during proliferation and in response to mechanical damage (121,122,126). The production of HA by scrape-damaged MDCK-I cultures is highly polarized and directed toward the

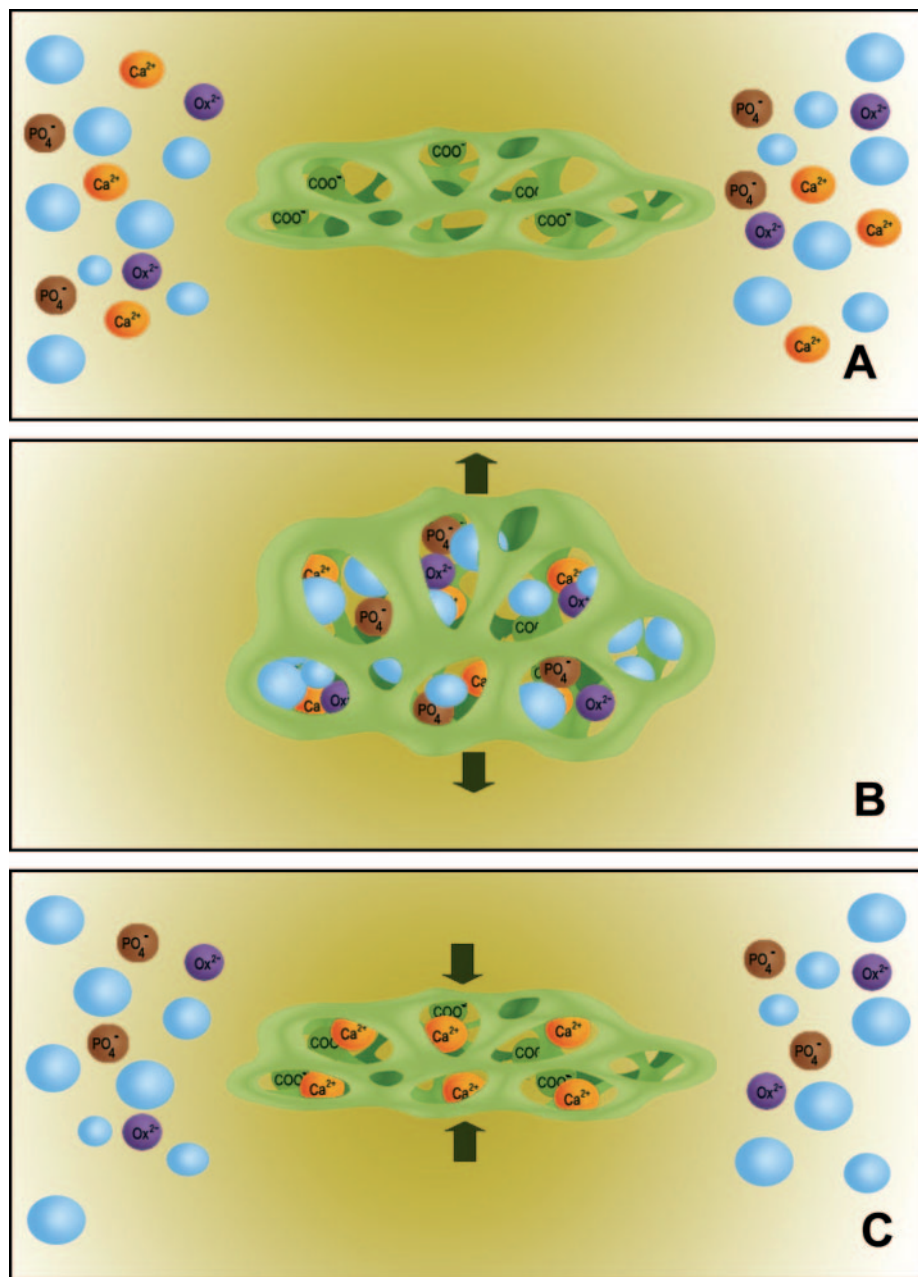


Figure 2. The appreciation of the sponge-like properties of HA in the renal interstitium by Schmidt-Nielsen (150) resulted in new insights into the concentration of solutes in the renal inner medulla (152). According to this concept, water is translocated from the tubular lumen to the interstitium during the relaxation phase of the pelvocaliceal contraction–relaxation cycle *via* water-permeable structures in the descending limbs of Henle and collecting ducts (A). In the papillary interstitium, newly arrived water is absorbed by the HA-rich matrix together with local solutes (B). Positively charged molecules are trapped by negatively charged carboxyl (COO^-) groups in the matrix. During the next peristaltic contraction, purified water is squeezed out of the matrix (C) into the ascending vasa recta. The interaction between Ca^{2+} and COO^- , most likely, also inhibits the precipitation of stone salts (CaP), thereby preventing the renal papillae from becoming mineralized. Green, COO^- ; blue, H_2O ; orange, Ca^{2+} ; purple, Ox^{2-} ; brown, PO_4^- . Illustration courtesy of Nihal Yildirim (PhD student, Department of Urology, Erasmus Medical Center, Rotterdam, The Netherlands).

luminal side of the epithelium (Figure 2) (126), suggesting that the major function of newly produced HA is in the luminal microenvironment rather than in the surrounding tissue. HA-expressing renal tubular cells invariably also express the HA receptor CD44 (74,122,158,160,169,170). It is interesting that HA

is one of the major constituents of the organic matrix of kidney stones. Several researchers found that HA is present in kidney stones in fractions that are disproportionate to its urinary concentrations (171–174). Many processes and mechanisms in HA biology in the diseased kidney are unexplored, including the

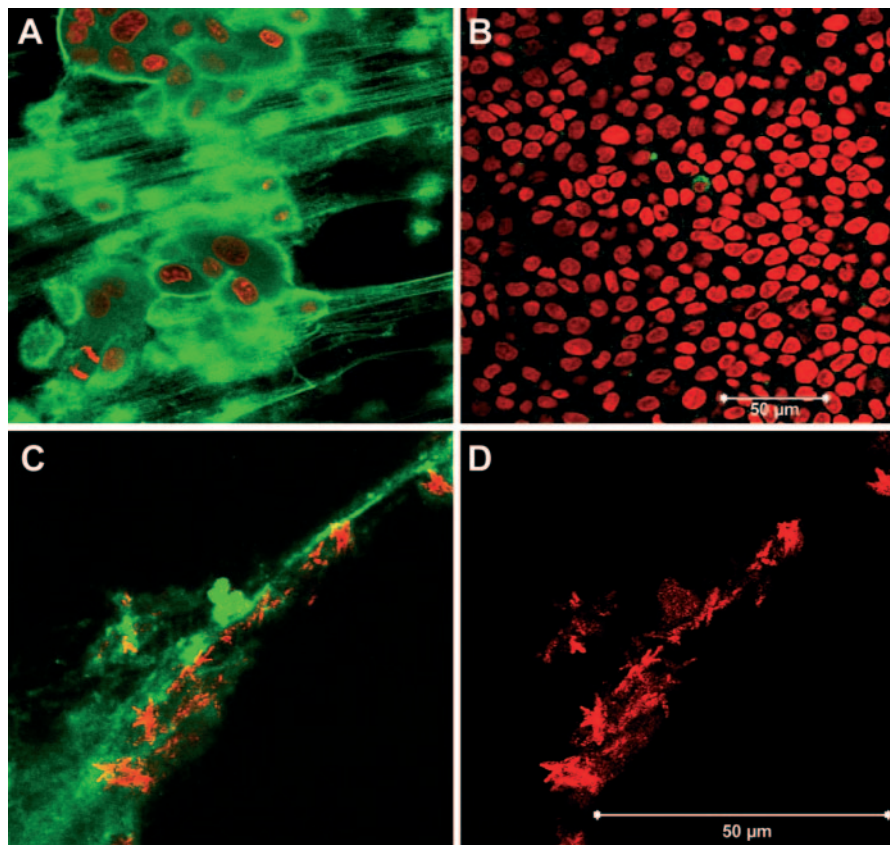


Figure 3. (A) Pericellular matrix (PCM), or glycocalyx rich in the glycosaminoglycan HA associated with the surface of mobile MDCK-I cells visualized by CLSM. (B) Confluent monolayers do not stain for HA. (C) COM crystals attached to HA in the PCM. (D) Light reflection only. Green in A and C, HA stained with biotinylated HA binding protein (bHABP) coupled to avidin-FITC; red in A and B, nuclei stained with propidium iodide after membrane permeabilization with ethanol; red in C and D, COM crystals reflecting in the laser light.

functional significance of HA in the cortical interstitium, the cross-talk between various cell types (*e.g.*, renal tubular cells, interstitial cells, inflammatory cells), CD44-HA signaling pathways, the functional significance of HA in the renal tubules, the regulation of HA synthesis in the various segments of the nephron, the role of interstitial HA on renal water homeostasis, and the role of HA in Randall's plaque formation.

Theoretical Concepts

The inadequate elimination of crystals with the urine is caused by their retention in the renal tubules (nephrocalcinosis), their accumulation into stones (nephrolithiasis), or both. The question is whether there is a link between these two forms of renal stone disease or they should be considered two entirely different phenomena. To be more specific, does the accumulation of crystals in the renal calyces require previous crystal attachment in the renal tubules or not? Another intriguing question to be answered is whether nephrolithiasis depends on the presence of Randall's plaques. When reduced to these key questions, the number of possible scenarios is limited (Figure 4).

Nephrocalcinosis

This form of renal calcification is caused by the continuous expression of crystal-binding cells in the renal tubules. The

expression of the crystal-binding phenotype is activated by specific mitogen/stress conditions, such as epithelial development in fetal kidneys and regeneration in transplanted kidneys (3).

Nephrocalcinosis and Nephrolithiasis

Crystals that are attached to the PCM of activated renal tubular cells occasionally are released back into the tubular fluid. Covered with sticky organic cell material, these crystals are predestined to bind and accumulate at allocated sites in the renal calyces, such as anatomic death corners and/or Randall's plaques. In the absence of these fixation points, nephrocalcinosis can be found without nephrolithiasis.

Idiopathic Nephrolithiasis-1

Specific stress conditions intermittently transform renal collecting tubular cells into their crystal-binding phenotype. Although the putative stress conditions are unknown, they could be diet related. Cells with inborn errors in membrane transport proteins, for example, may become stress activated by a dietary overload. After recuperation, the cells re-obtain their non-crystal-binding phenotype. During this activation/inactivation cycle, crystals transiently become attached to renal tubular cells. In analogy to the former scenario, once released back into

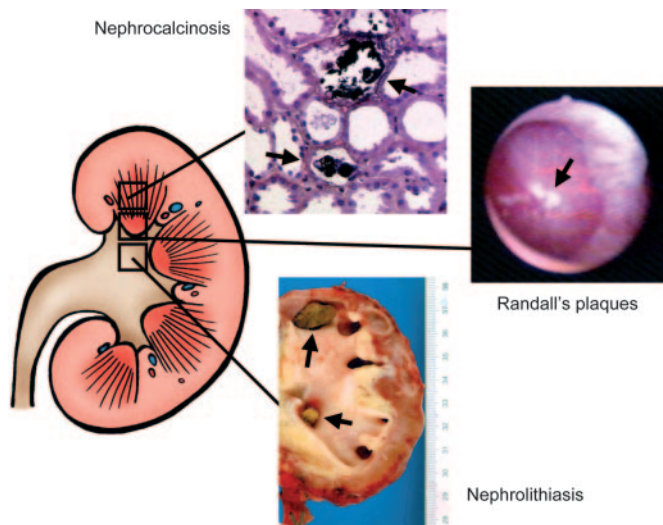


Figure 4. Could there be a relationship among nephrocalcinosis, Randall's plaques, and nephrolithiasis? (Top) Nephrocalcinosis in a patient with primary hyperoxaluria, stained with hematoxylin and von Kossa (calcium stain). (Right) Randall's plaques at the surface of the papillae; image of a film made during percutaneous nephrolithotomy. (Bottom) Stones in the kidney. Top photo courtesy of Benjamin Vervae (Department of Nephrology, University of Antwerp, Belgium) in cooperation with Jaap Groothof and Christiaan van Woerden (Department of Nephrology, Amsterdam Medical Center, The Netherlands). Right photo courtesy of Paul Verhagen (Department of Urology, Erasmus Medical Center, Rotterdam, The Netherlands). Bottom photo courtesy of Frank van de Panne (Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands). Left illustration courtesy of Nihal Yildirim (Department of Urology, Erasmus Medical Center, Rotterdam, The Netherlands).

the tubular fluid, these crystals are predestined to produce stones in the renal calyces.

Idiopathic Nephrolithiasis-2

Crystals do not require previous attachment in the renal tubules to develop into mature stones in the renal calyces, for example, by binding to HA-rich Randall's plaques (18,32).

Summary and Future Directions

The principle that crystal retention in the kidney leads to renal calcification is indisputable. Randall's plaques are caused by the deposition of crystals in the interstitium. Although crystals could be translocated from the tubular lumen to the interstitium, they probably are precipitated in the interstitial space itself. The HA-rich interstitial matrix retains precipitated CaP, leading to plaque formation. Nephrocalcinosis is caused by crystal binding to the epithelial cells in the distal tubule. Each form of nephrocalcinosis will have its specific mitogen/stress conditions that lead to the expression of crystal-binding cells in the renal tubules. The initial site of crystal retention in idiopathic stone formation remains an open question. To explore these concepts, future studies should include (1) crystal reten-

tion in snap-frozen tissue sections; (2) the development of drugs that are capable of coating crystals to facilitate their urinary elimination; (3) the composition of Randall's plaque; (4) the role of HA in Randall's plaque formation; (5) the content, localization, and molecular weight of HA in kidney stones; (6) the expression of renal tubular crystal-binding cells in nephrocalcinosis-associated disorders (*e.g.*, Dent's disease, primary hyperoxaluria); (7) the potential link between annexin II and HA biology; (8) HA biosynthesis regulation in the papillary interstitium; (9) HA biosynthesis regulation in the renal tubules; (10) the interaction between Randall's plaques and crystals; (11) definitive proof of where and when crystals are damaging in the kidney; and (12) the identification of urine, blood, and renal tissue markers to predict the risk for renal crystal retention (*e.g.*, HA, Hyal, HA-binding proteins). When these studies yield a coherent explanation for the retention and accumulation of crystals in the kidney, new treatment strategies can be designed for persistent stone patients.

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