Crystal Retention Capacity of Cells in the Human Nephron: Involvement of CD44 and Its Ligands Hyaluronic Acid and Osteopontin in the Transition of a Crystal Binding- into a Nonadherent Epithelium

ANJA VERHULST,* MARINO ASSELMAN,† VEERLE P. PERSY,* MARIEKE S.J. SCHEPERS,† MARK F. HELBERT,* CARL F. VERKOELEN,† and MARC E. DE BROE*†

*Department of Nephrology-Hypertension, University of Antwerp, Antwerp, Belgium; and †Department of Experimental Urology, Erasmus Medical Center Rotterdam, Rotterdam, The Netherlands.

Abstract. Nephrolithiasis requires formation of crystals followed by their retention and accumulation in the kidney. Crystal retention can be caused by the association of crystals with the epithelial cells lining the renal tubules. The present study investigated the interaction between calcium oxalate monohydrate (COM) crystals and primary cultures of human proximal tubular (PTC) and distal tubular/collection duct cells (DTC). Both PTC and DTC were susceptible to crystal binding during the first days post-seeding (4.9 ± 0.8 µg COM/cm²), but DTC lost this affinity when the cultures developed into confluent monolayers with functional tight junctions (0.05 ± 0.02 µg COM/cm²). Confocal microscopy demonstrated the expression of the transmembrane receptor protein CD44 and its ligands osteopontin (OPN) and hyaluronic acid (HA) at the apical membrane of proliferating tubular cells; at confluence, CD44 was expressed at the basolateral membrane and OPN and HA were no longer detectable. In addition, a particle exclusion technique revealed that proliferating cells were surrounded by HA-rich pericellular matrices or “cell coats” extending several microns from the cell surface. Disintegration of these coats with hyaluronidase significantly decreased the cell surface affinity for crystals. Furthermore, CD44, OPN, and HA were also expressed in vivo at the luminal side of tubular cells in damaged kidneys. These results suggest that the intact distal tubular epithelium of the human kidney does not bind crystals, and that crystal retention in the human kidney may depend on the expression of CD44-, OPN-, and HA rich cell coats by damaged distal tubular epithelium.

debroe@uia.ua.ac.be

Kidney stone development requires the formation of crystals in the tubular fluid followed by their retention and accumulation in the kidney. Whereas crystal formation predominantly depends on the composition of the tubular fluid, crystal retention might depend on the composition of the renal tubular epithelial cell surface (1–4). Per day, the human kidney forms about 1.5 L of urine from 150 to 180 L of ultrafiltrate. As a result of this concentration process, tubular fluid often becomes supersaturated with calcium salts, leading to the spontaneous nucleation of crystals. Providing distal tubules, collecting ducts, ureters, bladder, and the urethra with a nonadherent surface might be a natural defense mechanism against crystal retention, which is hampered when the anti-adherence properties are compromised.

Damage to epithelial cells lining the renal tubules may play a crucial role in the disturbance of this defense mechanism. In rats, the deposition of crystals in the kidneys is higher when their crystal-inducing diet is combined with nephrotoxic agents (5,6). Likewise, crystals adhere to damaged bladder urothelium, but not to the healthy tissue (7). The urinary of recurrent stone-formers contains enhanced levels of renal tubular cell-derived enzymes (8) and cytokines (9), indicating that the renal tissue is injured in these patients. In cell culture, it became evident that proliferation (1), scrape damage (2), or modification of cell membrane properties (10) showed increased crystal binding when compared with intact monolayers. The glycosaminoglycan hyaluronic acid (hyaluronan; HA) was identified as one of the major cell surface crystal binding molecules in these cultures (3,11).

Hyaluronic acid is a high–molecular mass polysaccharide (ranging from 1 to 10 million D) consisting of multiple repeating disaccharides of glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc). HA performs several important biologic functions in vertebrates. In connective tissues, it mainly serves as a structural component; during embryonic development and repair processes, it provides hydrated matrices through which cells can move (12,13). HA also plays a role in the communication of the cell interior with the environment through its major cell surface receptor, CD44 (14,15). The transmembrane

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protein CD44 has also been identified as a receptor for the phosphoprotein osteopontin (OPN) (16,17). OPN is an extracellular matrix molecule like HA but also a key cytokine during tissue repair and inflammation (18).

CD44, HA, and OPN are upregulated in damaged kidneys during renal disease states (19–21). The interaction between these molecules may play an important regulatory role in renal diseases by their influence on cell adhesion, migration, and chemotaxis (13,18,22,23). Besides their role in inflammation, HA and OPN are also known for their affinity for calcium crystals (3,24). It is therefore possible that crystal retention is caused by the expression of these CD44 ligands in the renal tubules.

Although crystal-cell interaction studies have been performed with cells derived from various species (1,10,25), as far as we know, this process has not yet been investigated with human kidney cells. Here, we studied the involvement of CD44, HA, and OPN in calcium oxalate monohydrate (COM) crystal binding to primary cultures of human renal cells.

Materials and Methods

Isolation, Purification, and Culture of Proximal and Distal Human Tubular Kidney Cells

Proximal and distal tubular cells were isolated as described previously (26–28). Briefly, normal human kidney tissue, which became available through nephrectomies performed for an oncological indication was collected and processed in a sterile manner. Macroscopically normal tissue was decapsulated. Cortex and outer stripe of outer medulla were dissected, cut into pieces of ≤1 mm², and digested in collagenase D solution (Roche, Ottweiler, Germany), supplemented with DNAse (Sigma, St Louis, MO). The suspension was shaken vigorously for 2 h at 37°C and sieved through a 120-μm sieve. The resulting cell suspension was loaded on top of a discontinuous Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient with densities 1.04 and 1.07 g/ml. After centrifugation, cells from the intersection were carefully aspirated, washed, and brought into culture. These cultures of human proximal (PTC) and distal, in a lesser amount also collecting duct cells (DTC), were grown for the indicated periods of time on polycarbonate Transwell filters (pore size, 0.4 μm; Costar, Cambridge, UK) in α-MEM (Life Technologies, Rockville, MD) modified according to Gibson d’Ambrosio (29) supplemented with 10% fetal calf serum.

Development of Functional Monolayers

Isolated cells were seeded at a concentration of 2 × 10⁵ cells/porous growth substrate. During a time period of 15 d, cell density was determined every 2 d by counting the cells in a hemocytometer. To assess the assembly of tight junctions during this process, trans-epithelial electrical resistances (TER) were measured by an Endohm-24 tissue resistance measurement chamber (World Precision Instruments, Sarasota, FL). Although both subconfluent and confluent cultures appear as a serried row of cells, only monolayers with measurable TER are considered as confluent.

Preparation of CaOx Crystals

Calcium oxalate monohydrate (COM) crystals were prepared as described earlier (3). Briefly, a solution of sodium oxalate (labeled or not with [14C]) was mixed with a calcium chloride solution at room temperature (final concentration of 5 mmol/L for both calcium and oxalate). COM crystals (radiolabeled or not) were formed immediately. The crystal suspension was allowed to equilibrate for 3 d and then washed three times with (sodium and chloride-free) Ca oxalate–saturated water and was resuspended in 5 ml of this solution (1.46 mg COM crystals/ml).

[14C] Calcium Oxalate Crystal Association

The cells were washed with PBS to be replaced by buffer A (140 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 50 mM urea, pH 6.6, 310 to 320 mM osm/kg H₂O) in the apical compartment and buffer B (124 mM NaCl, 25 mM NaHCO₃, 2 mM Na₂HPO₄, 5 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 8.3 mM D-glucose, 4 mM L-alanine, 5 mM Na acetate, 6 mM urea, and 10 mg/ml BSA, pH 7.4, 310 to 320 mM osm/kg H₂O) in the basolateral compartment. Buffer A was representative for the tubular fluid and buffer B for renal peritubular capillary plasma. Subsequently, 50 μl of the radiolabeled crystal suspension (16 μg/cm²) was added to the apical compartment and incubated for 60 min at 37°C. Filters were rinsed extensively to remove the non-adhered crystals and transferred to a scintillation vial, 0.5 ml of 1 M perchloric acid was added, and radioactivity was quantified. The amount of associated crystals was expressed as μg/cm².

Imaging Crystal-Cell Association by Confocal Laser Scanning Microscopy

After 60 min of incubation with non-radioactive COM crystals and extensive PBS washings to remove all non-adhered crystals, the cells were fixed in 4% formaldehyde/0.1% glutaraldehyde for 10 min. The filters were labeled for epithelial membrane antigen (EMA), a specific marker for distal tubular and collecting duct cells (26), to identify these cells. Filters were blocked with normal donkey serum in 1% BSA, incubated overnight with rat anti-human EMA antibody (Seralab, Leicestershire, UK), and subsequently with FITC-conjugated donkey anti-rat secondary antibody (Jackson, West Grove, PA). Crystals were detected by their light reflection. Confocal microscopy (Zeiss LSM 410, Oberkochen, Germany) was applied to visualize the fluorescence label of EMA and the crystals by their light reflection. Images reconstructed perpendicular to the porous growth substrate were used to discern apical from basolateral cell labeling.

Hyaluronidase Treatment

Cells were treated with hyaluronidase (hyaluronoglucosaminidase, EC 3.2.1.35; Sigma) dissolved in α-MEM, pH 5.5, at a concentration of 25 U/ml for 1 h at 37°C. Controls were incubated with identical medium without hyaluronidase.

HA, CD44, and OPN Staining

Human tubular kidney cells were fixed in 4% formaldehyde/0.1% glutaraldehyde for 10 min, washed, and blocked with 3% milk powder for HA staining and with normal donkey serum in 1% BSA for OPN and CD44 staining. Cells were subsequently incubated with biotinylated HA-binding protein (Seikagaku, Falmouth, MD) or mouse antihuman CD44 antibody (Bendermedystems, Vienna, Austria) or goat anti-human OPN antibody (OP189; C.M. Giachelli, University of Washington), followed by the appropriate secondary labels: FITC-labeled streptavidin (Vector, Burlingame, CA), FITC-labeled donkey anti-mouse IgG (Dako, Glostrup, Denmark), or FITC/Cy3-labeled donkey anti-goat IgG (Jackson).

HA, CD44, and OPN staining were combined with a propidium iodide cell staining to localize these molecules in the cell layer or with an EMA staining (as described above, but a secondary antibody
labeled with Cy3 was used). Filters were mounted in Vectashield and analyzed by confocal microscopy as described above. Negative controls for staining procedures (substitution of the primary label by a pre-immune serum or where this was not possible omitting of the primary label) showed no signal.

**Particle Exclusion Assay**

The particle exclusion technique, based on the inability of particles such as fixed red blood cells, to penetrate gelatinous cell coats, was performed as described earlier (30) to detect cell coats or pericellular matrices surrounding the cells. Culture medium was removed and replaced by a high-density (10^6/ml) suspension of formaldehyde-fixed mouse red blood cells in PBS. After settling for 10 min, the cultures were analyzed with an Axiosvert 25 phase-contrast microscope coupled to an AxioCam camera scanner (Zeiss, Munich-Hallbergmoos, Germany).

**OPN, HA, and CD44 Staining on Human and Rat Kidneys**

Tissue from one postrenal obstructed human kidney and from ischemic and normal rat kidneys was fixed in methacarn for 4 h and paraffin embedded. Different tissue sections were stained for HA, OPN, and CD44. Therefore, the sections were blocked with 1% BSA for HA staining and with normal horse serum for OPN and CD44 staining and incubated with the primary labels (the same as for cell culture staining).

For OPN and CD44 staining, sections were then incubated with secondary labels, biotinylated horse anti-goat and horse anti-mouse antibodies (Vector), respectively. Finally, avidin-biotin peroxidase complex (Vector) and diaminobenzidine were used to detect HA, OPN, and CD44 signals. Sections were counterstained with methylgreen, and the ischemic rat kidney also with periodic acid-Shiff reagent. Negative control sections showed no signal.

**Statistical Analyses**

All experiments were performed in duplicate on independent filters and on cultures of at least two different kidney specimens. The results are presented as mean ± SD. [14C] COM binding with or without hyaluronidase treatment was statistically analyzed with a Mann-Whitney U test.

**Results**

**Culture Characteristics**

The isolation procedure collects renal cortical tubular cells and the cultures therefore contain PTC and DTC. To differentiate between PTC and DTC, the cells were stained for EMA, a cell surface antigen expressed by DTC, but not by PTC. This staining procedure showed that DTC (EMA-positive) and PTC (EMA-negative) were approximately equally represented 5 d post-seeding. However, in time this ratio changed in favor of the EMA-positive cells, indicating a time-dependent enrichment in DTC. When the cultures were terminated 15 d post-seeding, the majority (more than 90%) of all cells in the culture originated from the distal tubule.

**Development of a Functional Monolayer**

To monitor the formation of tight junctions during the growth of the cells into confluent monolayers, the TER was measured starting 4 d post-seeding. The density of the cells on the growth substrates was determined by counting the cells. It was found that the TER was still very low 4 d after seeding. Thereafter the resistance gradually increased. After the cells reached their maximal density, TER attained maximal values as well (Figure 1). Thus, TER is low at subconfluence and much higher at confluence.

**Crystal Association**

To establish the crystal association capacity of subconfluent (proliferating) versus confluent (functional) human tubular epithelium, it was investigated in tubular cell cultures, starting at day 5 and ending at day 15. The association of crystals was quantified by adding [14C]-labeled COM crystals to these cell cultures. These time-series studies showed a high level of crystal association with proliferating cells in subconfluent cultures, which gradually decreased in time to very low levels on day 15 (Figure 2).

**Identification of Cells Associating with Crystals**

Human urine does not become supersaturated until the distal nephron. As a consequence, the crystal association capacity of DTC is of primary importance compared with that of PTC. Because the human tubular cell cultures contained both PTC and DTC, it was important to be able to distinguish between these cells. We therefore investigated the ability of the crystals...
to become associated with the surface of PTC (EMA-negative) and DTC (EMA-positive). These studies showed that, whereas COM crystals associated with PTC as well as DTC at subconfluence, this was entirely different at confluence (Figure 3). On day 9, crystal association was severely reduced and appeared to be limited to PTC. Fifteen days post-seeding, PTC represented less than 10% of the cells, which could explain the low levels of crystal binding at this time.

**Hyaluronic Acid**

To determine the role of HA in the adherence of crystals to human tubular cells, HA localization was investigated by confocal microscopy and related to the crystal adherence capacity of these cells. HA was present on the apical cell surface (Figure 4A) of proliferating, subconfluent PTC and DTC, but could no longer be detected on cell cultures of day 9 (Figure 4B). As shown above, crystals did not associate with DTC at this time. During the further course of the experiment, HA was absent from the tubular cells. The role of HA in the crystal binding process was further investigated by hyaluronidase treatment of days 5 and 15 cultures (Figure 4C). Crystal binding was significantly reduced after enzymatic digestion of HA on day 5 ($P = 0.02$). In contrast, hyaluronidase treatment did not alter crystal binding on day 15.

**Osteopontin**

To assess a possible role of OPN in crystal interaction with tubular cells, the same cultures were used for OPN staining. We showed previously that OPN was present on the apical cell membrane of proliferating DTC (31). In parallel with the HA expression, OPN disappeared almost completely when cell cultures became more confluent (Figure 5) and remained absent in the further course of the experiments.

**CD44**

The expression of HA and OPN at the cell surface suggests that these cells also express the major receptor for these ligands, namely the transmembrane protein CD44. Confocal microscopy studies showed that CD44 indeed is expressed at the apical surface of subconfluent cultures (Figure 6). However, on day 9 CD44 had disappeared from the cell surface (at the same time as HA and OPN) and was translocated to the basolateral cell membranes (Figure 6).

**Pericellular Matrix**

To assess whether HA-expressing cells were also capable to assemble pericellular matrices, the particle exclusion assay was applied. Proliferating human tubular kidney cells were able to produce a translucent, red blood cell–impermeable, pericellular matrix as illustrated in Figure 7 A and B. The addition of *Streptomyces* hyaluronidase results in the disappearance of the coat within minutes, indicating that this pericellular matrix structurally depends on HA (Figure 7C).

**Tubular Localization of HA in Damaged Kidneys**

The whole concept of crystals that are retained in the tubules of an injured kidney by their adherence to specific crystal binding molecules is based on the expression of these mole-

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*Figure 2.* Crystal binding to human tubular cell cultures was quantified using $^{14}$C-labeled calcium oxalate monohydrate (COM) crystals. The results are presented as mean ± SD. Crystal binding is high on subconfluent cultures and gradually decreases in time to very low levels on day 15.

*Figure 3.* Crystal-cell interactions analyzed by confocal microscopy. Crystals are visible by their light reflection (red). Epithelial membrane antigen (EMA) is immunofluorescently stained (green). Upper panels demonstrate overviews of the cultures at days 5, 9, and 15; lower panels demonstrate side views. Crystals interact to both distal tubular/collecting duct cells (DTC; EMA-positive) and proximal tubular cells (PTC; EMA-negative) on day 5, and selectively to PTC cells on day 9. No crystals are observed on day 15 in cultures, which almost entirely consisted of DTC. Magnification, ×630.
cules on damaged tubular epithelium. This is true for OPN and CD44; HA however is until now reported only in the renal interstitium. Therefore, we investigated tubular HA localization in an obstructed human kidney and in ischemic and normal rat kidneys. Although interstitial staining dominated, several tubules showed clear HA staining on their apical/luminal side (Figure 8, B through D). From periodic acid-Schiff counterstaining, it became clear that HA-positive tubules were distal tubules. This luminal HA expression was not present in normal kidneys (Figure 8A). To confirm OPN and CD44 presence in damaged tubules, we included sections from the human kidney stained for these molecules. Both OPN and CD44 were, like HA, present at the luminal side of the tubules (Figure 8, E and F).

Discussion
In the present study, we investigated the affinity of primary cultures of human renal PTC and DTC for COM crystals.
Association studies using radiolabeled COM crystals showed that subconfluent cultures were susceptible to crystal adherence, but that this affinity for crystals was lost at confluence. The renal tubular cells in this study were derived from the cortex and therefore contained several cell types. To reveal whether crystals became associated with the surface of PTC or DTC, crystal-cell association was visualized by light reflection and combined with an immunostaining for a DTC marker (EMA). The cultures initially contained approximately equal amounts PTC and DTC, both having affinity for crystals. During growth to confluence, DTC became more numerous compared with the PTC. Confluent cultures almost entirely consisted of DTC without affinity for COM crystals. These results are in agreement with earlier observations that COM crystals are unable to adhere to confluent monolayers formed by MDCK-I cells but that they do bind to proliferating cells (1). It appeared that PTC did associate with crystals, also in confluent monolayers when DTC lost this capacity. Again, these findings are consistent with previous observations describing that LLCPK-1 cells associate with crystals both in proliferating and confluent conditions (32). Probably, PTC do not require protection against crystal adherence because of crystal absence in the proximal nephron.

The adhesion molecule CD44 was randomly expressed at the plasma membranes of proliferating PTC and DTC, whereas CD44 was directed to the basolateral membrane at confluence. Proliferating cells also expressed OPN and HA at their surface, whereas these well-known CD44 ligands were no longer detectable in confluent monolayers. Considering their affinity for calcium oxalate crystals, OPN and HA should therefore be considered serious candidate crystal-binding molecules. The particle exclusion assay showed that human renal tubular cells were capable to assemble cell coats or pericellular matrices. The coats disappeared shortly after the addition of Streptomyces hyaluronidase, indicating their structural dependency on HA. The observation that hyaluronidase also reduced the affinity of the cell surface for COM crystals favors HA rather than OPN as the effective crystal-binding molecule under these conditions. However, Yamate et al. (33) described OPN as crystal-binding molecule on MDCK cells. On the other hand, OPN is described as a urinary inhibitor of crystal growth (34–36); therefore, the exact role of this protein in the process of nephrolithiasis remains uncertain. In addition, we cannot rule out the importance of other molecules previously identified as crystal-binding molecules, including sialic acid–containing glycoproteins (25), phosphatidyl serine (37), collagen (38), and nucleolin (39). Hyaluronidase treatment on day 5 inhibited crystal association by only 50%. This can be partially explained by the presence of PTC at that time. Crystals still associated with PTC when HA had disappeared from the cultures. Therefore molecules other than HA must be involved in crystal association with PTC. We did not investigate the identity of these molecules as PTC normally do not encounter crystals. Furthermore, we cannot exclude that molecules other

Figure 8. (A through D) Staining of HA on normal and ischemic rat kidneys and on an obstructed human kidney. In the normal rat kidney, HA staining is limited to the interstitium of the medulla (left) and absent from the cortex (right) (A). In a postischemic rat kidney (B and C: periodic acid-Schiff counterstained) and an obstructed human kidney (D), HA is present at the luminal side of cortical tubules (arrows). Periodic acid-Schiff staining of the ischemic kidney makes clear that the HA-positive tubules are distal tubules. In the highly fibrotic, obstructed kidney, the tubular staining is overwhelmed by interstitial staining. (E and F) OPN and CD44 staining on the human obstructed kidney. Like HA, OPN (E) and CD44 (F) are present on the luminal side of cortical tubules (arrows). Magnifications: ×400; inserts, ×600s.
than HA are responsible for crystal associations with DTC. It seems reasonable that these molecules become more available to crystals when HA is absent.

HA visualization by confocal microscopy resulted in a narrow signal covering the cell surface. The narrowness of this signal is an artifact of cell fixation and dehydration. Once outside the cell, HA forms networks by complexing with HA-binding proteins (40). Due to the water-binding properties of HA, the network swells to materialize into a pericellular matrix. This cell coat remains at the cell surface by the interaction of HA with specific membrane receptors such as CD44 (40). One of the functions of this coat is to create an environment in which cells can proliferate and migrate. A pericellular matrix is particularly observed surrounding mobile cells during embryonic development, tissue repair, inflammation, and tumorigenesis (12). Occurring in vivo, such a coat could cause loss of tubular lumen diameter and thereby retain crystals in the kidney. Luminal coat formation is reported to play a role in the development of vascular disease and atherosclerosis as well (13).

Consequently, it seems that upon cell activation, the expression of crystal binding molecules like OPN and HA at the apical cell surface of DTC, is accompanied by the development of a strong crystal-binding, gelatinous cell coat. Elucidating a role for OPN in the process of coat formation could shed a new light on the exact role of this protein in the complex occurrence of renal stones. As soluble OPN competes with HA for binding of CD44 (17), it is possible that OPN plays a role in regulating coat assembly.

Whereas there were many reports concerning CD44 and OPN expression in the renal tubules (41–44), it was much less clear whether HA also can be present in the tubules. So far, HA was found only in the renal interstitium (20,42,45–50). We studied the expression of CD44 and its ligands especially in the tubules of damaged kidneys. These studies clearly demonstrated the expression of not only OPN and CD44, but also that of HA in the renal tubules, suggesting that renal tissue damage is accompanied by the formation of HA rich-cell coats.

The results described in this article were obtained using cells of the cortex and the outer stripe of the outer medulla. Although these parts of the kidney contain, besides the proximal nephron, the straight and convoluted distal nephron, and important parts of the collecting duct system, it would be relevant to extend these observations to the renal medulla, the likely site of stone formation. During this study, the crystal retention capacity of human tubular epithelium was investigated, to our knowledge, for the first time. To this end, primary tubular cell cultures, isolated from normal human kidney tissue were an excellent model. Cultures evolved from proliferating, subconfluent cells into confluent and functional monolayers. In the same time, the DTC lost their capacity to associate with COM crystals. These results imply that human distal tubular epithelium is not susceptible to crystal binding under normal physiologic conditions but that retention is promoted by epithelial disturbances. Furthermore, in this cell culture model, evidence was provided that upregulated cell surface expression of HA, OPN, and their receptor CD44, as well as the formation of a HA-dependent cell coat, may play a crucial role in the process of crystal retention. Finally, it was observed in vivo that besides OPN and CD44, HA was also present in the tubules of damaged kidneys, a prerequisite for their being involved in the development of renal stones.

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References


