

The Role Of Tubule Epithelial Cell Pericellular Matrix Formation In The Retention Of Calcium Oxalate Crystals

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Abstract Research Proposal

Crystal retention. Kidney stones are composed of numerous small crystals interspersed with organic matrix. Although crystals are occasionally formed in anyone's kidneys, considerable more crystals are formed during hyperoxaluria. The formation of crystals is harmless as long as they are efficiently eliminated with the urine. Their retention in the kidney, however, sooner or later leads to stone formation (nephrolithiasis). There is accumulating evidence that the retention of crystals is engendered by their adherence to the epithelial cells lining the renal tubules.

Crystal adherence. We used cell lines to study crystal-cell interaction in vitro. Cultured on permeable supports these cells grow into functional epithelial sheets. Calcium oxalate crystals, the most common type of crystal found in kidney stones, avidly adhere to the surface of growing cells but not to an intact epithelium, suggesting that the epithelium normally is non-adherent to crystals. After scrape-damaging an intact epithelium, crystals recommenced to adhere to migrating cells at the border of the wound. Soon after the wound was healed the epithelium regained its anti-adherent properties. From these results it was speculated that tissue damage followed by repair might play a crucial role in the development of stones in the kidney. It was demonstrated that a translucent pericellular coat surrounds developing and migrating cells with affinity for crystals. The sugar compound, hyaluronan appeared to be the main constituent of this organic matrix. Evidence was provided that calcium oxalate crystals predominantly adhered to this hyaluronan enriched cell coat covering wounds. Besides hyaluronan the pericellular matrix consists of hyaluronan binding proteins which are required to stabilize the three-dimensional structure of the coat, while hyaluronan itself is retained at the cell surface by specific membrane receptors. During the healing of wounds, hyaluronan not only forms a barrier to protect the interior from the invasion of potential harmful substances and microorganisms, but also actively mediates the repair process by directly stimulating cell migration. After wound healing, hyaluronan is taken up by specific cell surface receptors to be degraded inside the cells.

Clinical significance. There are numerous indications for renal tissue damage in the kidneys of stone patients. The presence of hyaluronan binding proteins and receptors in their urine and the accumulation of hyaluronan in kidney stones not only suggests that repair processes are in progress in the kidneys of stone patients, but also that these processes contribute to crystal retention.

Hypothesis. Pericellular matrix formation in the kidney leads to or aggravates the retention of crystals and stone formation.

Aims. In the present proposal we intend to study the regulation of pericellular matrix assembly by tubular kidney cells. More understanding in the mechanisms responsible for the appearance of this “sticky” substance at the surface of renal tubule epithelial cells could lead to novel therapeutic approaches to prevent recurrent stone formation.

Introduction

Previously, we found in cell culture that the renal tubular epithelium becomes susceptible to crystal binding during its recovery from injury. During repair proliferating and migrating cells begin to express hyaluronan, a polysaccharide with a high affinity for calcium oxalate crystals. Hyaluronan is not expressed at the luminal surface of undamaged or re-established. In the present research proposal these observations are investigated more in detail and extended.

Personnel

Marino Asselman, M.D., started on this OHF project. On the first of January 2004 Asselman started his urology training and was replaced by the technician Eddy van Ballegooijen.

Results and publications

Marino Asselman is first author of a review article entitled: **“Crystal-cell interaction in the pathogenesis of kidney stone disease”** [1].

Marino Asselman is second author of an article by Anja Verhulst, entitled **“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 non-adherent epithelium”** [2]. Verhulst is member of the collaborating group of Prof. Dr. De Broe, Department of Nephrology, University of Antwerp, Belgium, In this paper evidence was provided that the concept that crystal binding requires transition of a non-crystal binding cell into a crystal binding cell is not restricted to the adherence of crystals to dog-derived MDCK-I cells, but also applies to primary cultures of human renal tubular cells. In this study we found that, in contrast to non-crystal binding cells, crystal-binding cells also expressed osteopontin and CD44 at their surface. Interestingly, others found that CD44 is a cell surface receptor for hyaluronan as well as for osteopontin. This opens the possibility that the susceptibility of the cell surface to crystal attachment depends on the interplay between hyaluronan, osteopontin and CD44.

Marino Asselman is co-author of a paper by Marieke Schepers entitled: **“Internalization of calcium oxalate crystals by renal tubular cells: A nephron segment-specific process?”** [3]. In this study, performed with two late nephron (collecting duct) cell lines and two early nephron (proximal tubulus) cell lines, it is demonstrated that crystals do not directly bind to the membrane of late nephron cell types, but to (hyaluronan-rich) pericellular matrices surrounding these cells. On the other hand, crystals directly associate with apical plasma membranes of early nephron cells. Furthermore, it is demonstrated that crystal uptake is restricted to early

nephron cell types. These observations could be important for stone formation in kidneys of people with different underlying abnormalities. Crystal retention could be an extracellular event in the average calcium stone patient (where crystal precipitation occurs in the late nephron), whereas it could have an intracellular component under conditions where crystal formation occurs earlier in the nephron, such as in primary hyperoxalurias.

Marieke Schepers is another member of our group who obtained her PhD on 20 April 2005 on a thesis entitled: “**Handling of oxalate and calcium oxalate by renal tubule epithelial cells**”. (This thesis has been mailed to the OHF on 25 April 2005).

Marino Asselman is second author of an article entitled: “**Pericellular matrix formation by renal tubule epithelial cells in relation to crystal binding**”[4]. by Marieke Schepers. In this paper we investigated the ability of renal tubular cells (LLC-PK1, MDCK-I and MDCK-II) to synthesize hyaluronan and assemble pericellular matrices. It was found that MDCK strain I cells synthesize and secrete significant amounts of high molecular weight hyaluronan and almost all cells (>90%) assemble hyaluronan-dependent pericellular matrices. MDCK strain II cells produce much lower amounts of hyaluronan and only a minority of the cells (25%) form cell coats. LLC-PK1 cells are unable to synthesize hyaluronan and do not form pericellular matrices. Thus, pericellular matrix formation requires hyaluronan synthesis. Interestingly, LLC-PK1 is derived from the renal proximal tubule, MDCK-I from the collecting duct, whereas MDCK-II is heterogeneous and resembles both proximal and distal tubules. This opens the possibility that hyaluronan synthesis is cell type-specific. On the other hand it is also possible that the machinery to synthesize hyaluronan is lost in cells that are kept in culture for a long period of time (these cells were brought into culture about half a century ago!). Since hyaluronan is a “receptor” for calcium oxalate crystals it is not surprising that treatment with an enzyme that specifically degrades hyaluronan does not influence crystal binding to LLC-PK1, has little effect on crystal binding to MDCK-II and significantly reduced crystal binding to MDCK-I.

In collaboration with the group of Prof. Dr. de Broe we have performed a study in rats to test our hypothesis that pericellular matrix formation in the kidney leads to crystal retention. One of the major findings was that there is no crystal retention in the absence of tubular injury, but that crystals are retained as soon as renal tubules are injured. Crystals were found adhered to the luminal surface of hyaluronan-, osteopontin- and CD44-expressing injured/regenerating cells. The results of this study therefore strongly suggest that crystal retention in the kidney indeed requires tubular epithelial injury accompanied by luminal expression of HA, OPN and CD44. Asselman was first author of this paper entitled: “**Calcium oxalate crystal adherence to hyaluronan-, osteopontin-, and CD44-expressing injured/regenerating tubular epithelial cells in rat kidneys**” [5].

Asselman studied the distribution of hyaluronan and CD44 over the plasma membrane of proliferating and regenerating renal tubular cells (MDCK-I and primary

cultures of human renal tubular cells). This study showed that proliferating and/or regenerating renal tubular cells produce increased amounts of high molecular (M_r) mass HA, which is preferentially extruded from the apical membrane. Renal tubular cells triggered to produce more HA not only express the principal receptor CD44 at their luminal surface but also HA. After the cells become confluent or after the wounds are healed, HA completely disappears from the cultures whereas CD44 is restricted to basolateral domains. From these results it is proposed that CD44 and HA play a role in the repair from injury and that this role is located at the luminal side of the monolayer facing the tubular fluid. The attachment of crystals should be considered an unfortunate coincidence of circumstances. Asselman is first author of this article entitled: **“Polarized distribution of HA and CD44 during renal tubule epithelial development and wound healing”** [6].

Eddy van Ballegooijen, who replaced Asselman in 2004 provided essential technical assistance in collecting the data on two articles presented at the 7th international workshop on Primary Hyperoxaluria at Mayo Clinics, October 8-10, 2004.

Paper 1) **“Crystals cause acute necrotic cell death in renal proximal tubule cells, but not in collecting tubule cells”** (accepted for publication in *Kidney International*).

***Background** The interaction between renal tubular cells and crystals generated in the tubular fluid could play an initiating role in the pathophysiology of calcium oxalate (CaOx) nephrolithiasis. Crystals are expected to form in the renal collecting ducts, but not in the proximal tubule. In the present investigation, we studied the damaging effect of CaOx crystals on renal proximal- and collecting tubule cells in culture.*

Methods Studies were performed with the renal proximal tubular cell lines, LLC-PK1 and MDCK-II and the renal collecting duct cell lines, RCCD1 and MDCK-I. Confluent monolayers cultured on permeable growth substrates in a two-compartment culture system were apically exposed to calcium oxalate monohydrate (COM) crystals, after which several cellular responses were studied, including monolayer morphology (confocal microscopy), transepithelial electrical resistances (TER), prostaglandin E₂ (PGE₂) secretion, DNA synthesis ([³H]thymidine), total cell numbers, reactive oxygen species (H₂O₂) generation, apoptotic (annexin V, DNA fragmentation) and necrotic (propidiumiodide influx) cell death.

Results Crystals were rapidly taken up by proximal tubular cells and induced a biphasic response. Within 24 hours approximately half of the cell-associated crystals were released back into the apical fluid (early response). Over the next two weeks half of the remaining internalized crystals were eliminated (late response). The early response was characterized by morphological disorder, increased synthesis of PGE₂, H₂O₂ and DNA and the release of crystal-containing cells from the monolayers. These released cells appeared to be necrotic, but not apoptotic cells. Scrape-injured monolayers generated even higher levels of H₂O₂ than those generated in response to crystals. During the late response, crystals were gradually removed from the monolayers without inflammation-mediated cell death. Crystals did not bind to, were not taken up by and did not cause marked responses in collecting tubule cells.

Conclusions This study shows that CaOx crystals cause acute inflammation-mediated necrotic cell death in renal proximal tubular cells, but not in collecting tubule cells. The crystal-induced generation of reactive oxygen species by renal tubular cells is a general response to tissue damage and the increased levels of DNA synthesis seem to reflect regeneration rather than growth stimulation. As long as the renal collecting ducts are not obstructed with crystals, these results do not support an important role for crystal-induced tissue injury in the pathophysiology of calcium oxalate nephrolithiasis.

Paper 2) “Oxalate is toxic to renal tubular cells only at supraphysiological concentrations” (under review, *Kidney International*).

Background Oxalate-induced tissue damage may play an initiating role in the pathophysiology of calcium oxalate (CaOx) nephrolithiasis. The concentration of oxalate is higher in the renal collecting ducts (~0.1-0.5 mM) than in the proximal tubule (~0.002-0.1 mM). In the present investigation, we studied the damaging effect of oxalate to renal proximal-and collecting tubule cells in culture.

Methods Studies were performed with the renal proximal tubular cell lines, LLC-PK1 and MDCK-II and the renal collecting duct cell lines, RCCD1 and MDCK-I. Confluent monolayers cultured on permeable growth substrates in a two-compartment culture system were apically exposed to relatively low (0.2, 0.5 and 1.0 mM) and high (5 and 10 mM) oxalate concentrations, after which several cellular responses were studied, including monolayer morphology (confocal microscopy), transepithelial electrical resistances (TER), prostaglandin E₂ (PGE₂) secretion, lactate dehydrogenase (LDH) release, DNA synthesis ([³H]thymidin incorporation), total cell numbers, reactive oxygen species (H₂O₂) generation, apoptotic (annexin V, DNA fragmentation) and necrotic (propidium iodide influx) cell death.

Results Visible morphological alterations were observed only at high oxalate concentrations. Most oxalate concentrations had little effect on TER; only 10 mM oxalate decreased TER irrevocably in collecting tubule monolayers. Elevated levels of PGE₂, LDH and H₂O₂ were measured in both cell types after exposure to high, but not to low oxalate. Exposure to high oxalate resulted in elevated levels of DNA synthesis with decreasing total cell numbers. High, but not low oxalate, induced necrotic cell death without signs of programmed cell death.

Conclusion This study shows that oxalate is toxic to renal tubular cells, but only at supraphysiological concentrations.

Publications

1. Asselman M, Verkoelen CF: Crystal-cell interaction in the pathogenesis of kidney stone disease. *Curr Opin Urol* 12:271-276, 2002
2. Verhulst A, Asselman M, Persy VP, Schepers MS, Helbert MF, Verkoelen CF, De Broe ME: 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. *J Am Soc Nephrol* 14:107-115, 2003

3. Schepers MS, van der Boom BG, Romijn JC, Schroder FH, Verkoelen CF: Internalization of calcium oxalate crystals by renal tubular cells: A nephron segment-specific process? *Kidney Int*, 2003
4. Schepers MS, Asselman M, Duim RA, Romijn JC, Schroder FH, Verkoelen CF: Pericellular matrix formation by renal tubule epithelial cells in relation to crystal binding. *Nephron Exp Nephrol* 94:e103-112, 2003
5. Asselman M, Verhulst A, De Broe ME, Verkoelen CF: Calcium oxalate crystal adherence to hyaluronan-, osteopontin-, and CD44-expressing injured/regenerating tubular epithelial cells in rat kidneys. *J Am Soc Nephrol* 14:3155-3166, 2003
6. Asselman M VA, Verkoelen CF, ME De Broe.: Polarized distribution of HA and CD44 during renal tubule epithelial development and wound healing. *Kidney Int* 68:1-13, 2005
7. Crystals cause acute necrotic cell death in renal proximal tubule cells, but not in collecting tubule cells MSJ Schepers, ES van Ballegooijen, CH Bangma, CF Verkoelen, *Kidney Int.*, in press.

Summary

The results that we obtained in the dog-derived MDCK cells were repeated and confirmed in primary cultures of human renal tubular cells [2]. Next, it was found that crystals are endocytosed by proximal tubular cells but not by collecting tubular cells. Since crystals usually are not formed in the proximal tubule, there does not seem to be an important role for crystal internalization [3]. Certain cells in culture produce hyaluronan (HA) and HA-rich cell coats (or pericellular matrices;PCM) whereas others do not. We have the impression that PCM formation is normal but that some cell types have lost this ability in culture [4]. Calcium oxalate crystals are selectively retained in rat renal tubules containing HA, OPN and CD44 expressing epithelial cells. This is for the first time that the role of crystal binding molecules in vitro are confirmed in an animal model [5]. We have found that HA and CD44 are expressed at the luminal surface of crystal binding activated renal tubular cells and therefore highly polarized. As soon as the cells re-obtain their differentiated phenotype, HA entirely disappears from the plasma membrane, while CD44 is translocated to the basal aspect of the cell membrane [6]. Crystals cause inflammation-mediated necrosis in proximal tubular cells but not in collecting tubules cells [7]. Oxalate ions are not very toxic to either cell type. Necrosis is caused by oxalate concentration >5 mM (under review).