Handling of Oxalate and Calcium Oxalate by Renal Tubule Epithelial Cells

De omgang met oxalaat en calcium oxalaat door epitheliale niertubuluscellen

Handling of Oxalate and Calcium Oxalate by Renal Tubule Epithelial Cells

De omgang met oxalaat en calcium oxalaat door epitheliale niertubuluscellen

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus Prof.dr. S.W.J. Lamberts en volgens besluit van het College voor Promoties. De openbare verdediging zal plaatsvinden op woensdag 20 april 2005 om 15.45 uur

door

Sjanette Jorina Marieke Schepers-Kok

geboren te Rotterdam

PROMOTIECOMMISSIE

Promotor:	Prof.dr. F.H. Schröder
Overige leden:	Prof. dr. P.M. ter Wee Prof. dr. P.E. de Jong Prof. dr. C.H. Bangma
Copromotor:	Dr. C.F. Verkoelen

ABBREVIATIONS

CaOx	calcium oxalate
CCD-A	apical fluid collecting duct buffer
CCD-B	basal fluid collecting duct buffer
CD	collecting duct
CLSM	confocal laser scanning microscopy
COD	calcium oxalate dihydrate
COM	calcium oxalate monohydrate
COX	cyclo-oxygenase
DMEM	Dulbecco's minimum essential medium
dpm	desintegrations per minute
DT	distal tubule
EDTA	ethylenediamine tri-acetic acid
EG	ethylene glycol
FCS	fetal calf serum
FITC	fluorescein-isothiocyanate
GAG	glycosaminoglycan
HA	hyaluronan
HABP	hyaluronan binding protein
HAS	hyaluronan synthase
H_2O_2	hydrogen peroxide
kDa	kiloDalton
LDH	lactate dehydrogenase
LLC-PK_1	porcine kidney cells
MDCK	Madin Darby canine kidney cells
OPN	osteopontin
Ox	oxalate
PGE_2	prostaglandin E_2
PBS	phosphate buffered saline
PCM	pericellular matrix
PEA	particle exclusion assay
PT	proximal tubule
$RCCD_1$	rat cortical collecting duct cells
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gelelectrophoresis
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TER	transepithelial electrical resistance

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 NEPHROLITHIASIS

Nephrolithiasis is a common disease in industrialized countries. In Europe and North America about 5-10% of the population forms at least one stone in the kidney during lifetime and the incidence is rising. It is often considered a "disease of affluence" since the prevalence of stone formation is related to the economical situation: during war and recession it decreases, to increase during times of abundance [1, 2]. The incidence in men is two to three times higher than in women. After development of an initial stone, recurrence rates vary from 31 to 75% depending on duration of follow-up. About 50% of the patients forms a new stone within ten years [2-4]. Most stones are passed spontaneously, but about 25% of stones requires active removal with methods like extracorporeal shock wave lithotripsy and percutaneous nephrolithotomy [1, 3, 5].

Risk factors

During the past decades, a range of risk factors for renal stone formation has been identified: positive family history of stones, nutrition, nutritional deficiencies, life style and associated diseases [4, 6-9]. Excessive intakes of salt, animal protein and oxalate, as well as insufficient intake of fluid, vegetable fibres and calcium increase the risk of stone formation [8-13]. However, nephrolithiasis is not primarily a nutritional disorder, but nutrition should be regarded as an additional factor to underlying abnormalities like primary hyperparathyroidism, distal renal tubular acidosis and primary or enteric hyperoxaluria. Specific dietary advice and/or pharmacological intervention can lower the risk of recurrence for some patients [3, 4, 8, 14].

Hyperoxaluria

High oxalate concentrations increase the risk of CaOx crystallization [11, 15]. Dietary intake, endogenous production, intestinal absorption, and renal handling determine the amount of oxalate that appears in the urine. Most of the urinary oxalate originates from endogenous production in the liver [13]. Recent studies have shown that the mean dietary contribution is higher (± 45%) than the 10-20% that was reported earlier, but interindividual variability is ranging from 10 to 72 % [10]. In the intestine, the amount of oxalate available for absorption and subsequent urinary excretion can be limited by complex formation with calcium [9,

12, 15, 16], as well as by oxalate-degrading bacteria (*Oxalobacter formigenes*) [17].

Dietary oxalate loads increase the oxalate levels in plasma and thereby the filtered oxalate load. The oxalate concentrations in all nephron segments will increase accordingly. Oxalate-rich foodstuff, like spinach and chocolate, can cause transient hyperoxaluria with peak excretion rates comparable to those found in primary hyperoxaluria patients. Within 2 to 4 hours after ingestion, urinary oxalate excretion can duplicate or even triplicate in comparison to fasting values [6, 11, 18]. Oxalate load can be reduced by dietary measures as well as by bacterial oxalate breakdown in the gut. Reduction of calcium in given segments of the nephron is more complicated. Urine normally contains about ten times less oxalate than calcium. After an oral calcium load, the body tries to keep plasma calcium homeostasis by manipulating the intestinal uptake, bone metabolism as well as renal absorption. In persons with normal intestinal calcium absorption, little changes will occur in the composition of the proximal tubular fluid and the major increase in calcium will occur from the distal tubules onwards [19]. Restriction of dietary calcium intake will have an adverse effect on crystallization risks. Severe reduction of calcium intake causes the urinary oxalate excretion to rise, probably because the pool of oxalate available from absorption in the gut is increased [19]. Great changes in dietary calcium load will have less effect on the risk of CaOx super saturation than small increases in urinary oxalate. Hess et al. have studied the effects of increasing dietary calcium after 20-fold normal oxalate loading on intestinal oxalate absorption, hyperoxaluria and urinary crystallization. Urines of healthy subjects were studied after a normal calcium and oxalate-rich diet, oxalate and calcium-rich diet, or after a free-choice diet. Dietary hyperoxaluria was prevented and CaOx crystallization reduced when calcium intake was increased while eating oxalate-rich food [20]. Low calcium intake will lower the amount of calcium available for complexation of CaOx in the gut, leading to increased gastrointestinal absorption of oxalate and subsequent hyperoxaluria [7-11, 20-22]. In calcium-stone forming patients, idiopathic hypercalciuria frequently occurs, which may be ascribed to increased intestinal calcium absorption, increased bone resorption and decreased renal tubular calcium reabsorption. Increasing dietary calcium in normocalciuric as well as in dietary-independent hypercalciuric patients leads to reduction in mean

urinary oxalate levels. In contrast, in dietary-dependent hypercalciuric patients urinary oxalate does not decrease with a higher calcium supply, probably due to a higher calcium absorption under these conditions, leaving less calcium for complexation with oxalate in the intestinal lumen. Nevertheless, a reduction in calcium intake is undesirable for all stone forming patients, since it can worsen the bone condition [12].In primary hyperoxaluria (PH), metabolic overproduction of oxalate occurs and the oxalate excretion is strongly increased in comparison to normal persons (3-3.5 mmol / 24 h vs. 0.2-0.5 mmol/24 h)[23]. Primary hyperoxalurias are rare autosomal recessive disorders resulting from deficiency of hepatic alanine:glyoxylate aminotransferase (PH type I) or D-glycerate dehydrogenase/ glyoxylate reductase (PH type II). In the Netherlands the prevalence and incidence of primary hyperoxaluria type I are 2.9 per million and 0.15 per million per year, respectively [24]. Disease expression is more severe in type I, where systemic oxalate deposition occurs, than in type II [13, 16, 25]. Excess oxalate is excreted via the urine, resulting in increased calcium oxalate supersaturation and calcium oxalate crystalluria. Together with decreased urine inhibition of calcium oxalate crystal formation as compared with normal persons, this leads to an augmented risk of renal stone formation.

1.2 RENAL FUNCTION AND STONE FORMATION

The physiological function of the kidneys is to excrete endogenous (e.g. creatinine, ureum and oxalate) as well as exogenous (like drugs) waste products and maintaining body homeostasis. Human kidneys are composed of 1-2 million nephrons, which are the functional units. The nephron is a specialized structure that is involved in concentration and dilution of primary urine. Blood flow in the kidneys is 1.2-1.3 l per minute. Each day, 180 litres of fluid are filtered and concentrated to form about 1.5 l urine. During this process, nutrients and salts are conserved and waste products are excreted.

Each nephron consists of different segments that perform specific functions. The blood enters the nephron at the glomerulus, where ultrafiltrate is formed. Ultrafiltrate has the same composition as blood, but without macromolecules larger than 30 kD. From there on, primary urine flows through the proximal tubules and Loop of Henle to the distal

tubules. In the proximal tubules, glucose, sodium chloride, and water are resorbed and return to the blood stream, together with essential nutrients such as amino acids, proteins, bicarbonate, calcium, phosphate, and potassium. In the Loop of Henle, the urine concentrating process proceeds and in the distal tubule the salt- and acid-base balance of blood is regulated. The final urine is formed in the collecting ducts, that drain the urine into the calyces from where it enters the ureter [26].

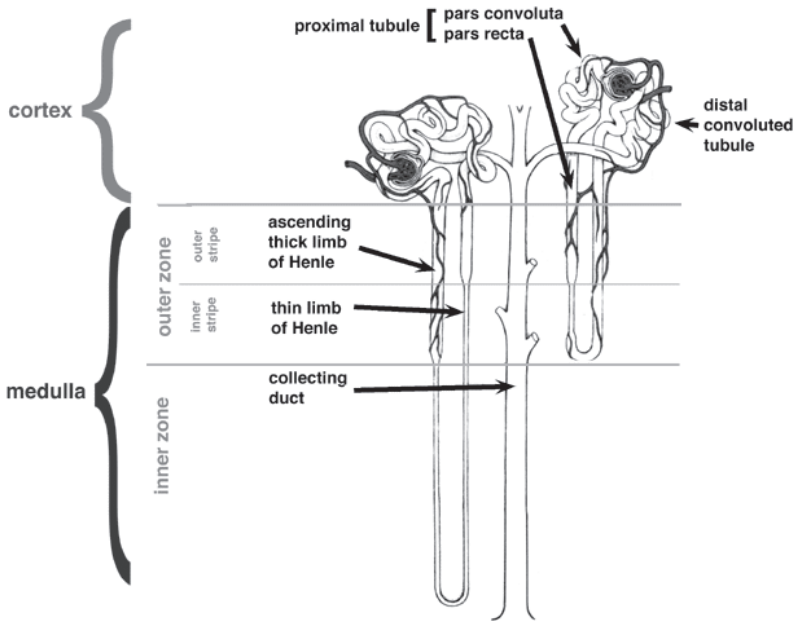


Figure 1. Schematic overview of the nephron

Crystallization

Kidney stones are composed of numerous small crystals that accumulate to ultimately form a stone. Crystals can precipitate in the urinary tract when the urine is supersaturated, i.e. when the concentration of salts is higher than what can be kept in solution. Calcium phosphate (CaP) can precipitate already in the loop of Henle or distal part of distal tubules, while calcium oxalate (CaOx) supersaturation usually occurs later in the nephron, in the collecting ducts. CaP that is precipitated in the earlier parts of the nephron dissolves as it travels through the tubules to the collecting ducts, where the pH is lower. At that site, CaP can act as

nucleation site for CaOx crystals [27-29]. On the basis of super-saturations, CaOx nucleation can first occur in the loops of juxtamedullar nephrons. High plasma calcium moves the chance of CaOx nucleation further upstream. Only in the extreme case of a serum oxalate of 50 μM, which is about five times higher than peak values reached in stone formers, CaOx can form in all loops and even in the proximal tubules [19].

Crystal retention

Before crystals can turn into an actual stone, they have to be retained in the kidney. Different opinions exist about this process. On one hand, it is believed to be a "free particle disease", while on the other hand, it could be a "fixed particle disease". In the first opinion, crystals grow and aggregate to the point at which they become too large to pass through the tubular lumen and become trapped. Alternatively, crystal retention in the kidney could be dependent on the interaction between crystals and the epithelium lining the renal tubules, even when crystals are small [28-32]. It is hypothesized that this occurs when the epithelium lining the renal tubules becomes susceptible to crystal binding. Under pathological conditions, crystal binding molecules that are normally absent from the cell surface might be expressed, enabling crystal-cell interaction. It remains to be determined what the triggers are that render the epithelium adherent to crystals. In addition, the nature of the crystal binding molecules has to be elucidated. Evidence has been provided for the existence of tubular damage in idiopathic CaOx stone formers. Urinary excretion of γ-glutamyl transpeptidase, beta-galactosidase, N-acetyl-beta-glucosaminidase and angiotensin I converting enzyme is higher than normal in these patients [33].

Renal stones are preferentially located in the calyces near the papillary tip. In kidneys of stone forming patients, crystalline material was found attached to the epithelium in collecting ducts near the papillary tip. These submucosal structures, consisting mainly of calcium phosphate (CaP or apatite), are termed "Randall's plaques" [34, 35]. It is believed that these sites could serve as nidus for the formation of renal stones. Evan et al. found that in intrarenal biopsies of idiopathic calcium-stone formers, obtained during percutaneous nephrolithotomy, scattered microscopic CaP deposits were located in the basement membrane of the thin loop of Henle. Larger plaques were extending from there to the papillary interstitium. In this study, no plaque was found in kidneys of non-stone

formers or among patients with stones from obesity-related bypass procedures [34, 35].

Inhibitors of stone formation

So-called "inhibitors of crystallization" can influence crystal-crystal interaction. These are compounds that can interfere with nucleation, growth, agglomeration, or retention of crystals, thereby diminishing the risk of actual stone formation. Small precipitated crystals can be harmlessly excreted with the urine. Urinary macromolecules, like proteins and glycosaminoglycans, can affect the growth and aggregation of these crystals, acting as either promoters or inhibitors [36-42]. Among those are prothrombin fragment 1, inter-α-trypsin inhibitor, Tamm Horsfall protein, osteopontin, albumin, and α_1-microglobulin. Small organic or inorganic compounds, like citrate, pyrophosphate, and magnesium, can also act as inhibitors of stone formation. These molecules all have affinity for calcium oxalate crystals and can be found inside the organic matrix that surrounds the crystals [36, 43-46]. Studies have been conducted to investigate possible differences in urine composition between stone formers and normal subjects, but indisputable distinctions are not yet obtained [40, 45, 47-49].

1.3 RENAL CELL CULTURE

Crystal-cell interaction is frequently studied using renal tubular epithelial cells in tissue culture. Although cell lines are not always a perfect representation of cell behaviour *in vivo,* they form reliable models to repeatedly perform experiments under reproducible conditions. In the studies that are described here, renal tubule epithelial cell lines were cultured on porous supports in a two-compartment system, to mimic the *in vivo* situation. The lower compartment represents the 'blood-side' of the epithelium, while the upper or apical compartment represents the tubular lumen or 'urine-side' of the epithelium. In contrast to cells cultured on impermeable plastic surfaces, cells cultured on inserts are able to take up nutrients from their basal side. Subsequently, they develop into functional polarized monolayers with a higher degree of differentiation than cells grown on impermeable surfaces. These confluent layers have preserved many of their *in vivo* characteristics [50].

In the experiments described here, MDCK-I and $RCCD_1$ cells were used as model systems for the distal part of the nephron. MDCK-I is a substrain of the original MDCK cell line, that was derived from the kidney of a normal Cocker Spaniel in 1958 [51, 52]. When cultured on porous supports, MDCK-I cells develop in to well-differentiated polarized monolayers, that have retained most characteristics of distal tubule/collecting duct cells [50, 53]. MDCK strain I cells develop a high transepithelial electrical resistance (>10.000 $\Omega.cm^2$), are covered with short and stubby microvilli and express low levels of alkaline phosphatase (AP) and γ-glutamyltranspeptidase (γ-GT) [50, 54]. These cells also exhibit significant carbonic anhydrase activity [55] and their inward current can be activated by vasoactive intestinal polypeptide [56]. $RCCD_1$ cells are isolated from rat collecting duct and are characterized by high transepithelial resistances (>3.000 $\Omega.cm^2$) and specific hormonal sensitivities. The cell line contains intercalated as well as principal cells. Their morphological appearance resembles that of intact CCD, with cuboid cells that are connected by tight junctions [57].

$LLC\text{-}PK_1$ and MDCK-II cells are used as model for the proximal tubule epithelium. Confluent monolayers formed by these cells are leaky (<200 $\Omega.cm^2$), are endowed with a luxurious brush border packed with tall microvilli and their membranes express high levels of AP and γ-GT activity [50]. $LLC\text{-}PK_1$ cells were isolated from porcine kidney [58]. These cells actively reabsorb essential nutrients and water across the epithelium [59]. MDCK strain II cells are derived from the parental MDCK cell line [54]. In contrast to strain I cells, these cells express characteristics of proximal as well as distal tubular cells.

It is important to confirm if findings obtained with cell lines represent the actual situation in the kidney. Therefore, primary cultures of renal tubular cells can be used. Since experiments with these cells are performed directly after their isolation from the kidney, they have retained most of their *in vivo* characteristics. Proximal and distal tubular cells are isolated from normal human kidney tissue, which became available thorough nephrectomies. Fluorescence-activated cell sorting (FACS) in combination with specific monoclonal antibodies is used to identify the segmental origin of the cells. When passage 1 cells reached confluence, hormonal stimulation and antigen expression were used to verify that these cells retained their *in vivo* behaviour as close as possible [60].

1.4 CRYSTAL-CELL INTERACTION IN CELL CULTURE

To unravel the mechanisms that might be involved in the development of renal stones, we studied crystal-cell interactions in cell culture. It was previously shown that the affinity of MDCK-I, MDCK-II, and LLC-PK_1 cells for COM crystals was high during subconfluence, when they were proliferating. Confluent proximal as well as distal tubule epithelial cells cultured on impermeable surfaces for the most part retained their crystal binding capacities. When cultured on permeable supports in a two-compartment model, differences in crystal retention between distal- and proximal tubule epithelial cells occurred. Cells derived from the proximal part of the nephron retained their high affinity for COM crystals during their growth to confluence [50, 61], while distal tubule/collecting duct cells on the other hand became non-adherent to crystals at confluence. An explanation for this might be that, i*n vivo*, the proximal tubular cells normally do not encounter crystals and therefore do not require defence mechanisms against their retention. In distal tubule/ collecting duct cells the transition from adherence to non-adherence occurred at the time when the tight junctions became functional [50, 61-63], indicating that intact monolayers that are likely to encounter crystals indeed possess a protective mechanism against crystal retention.

Renal tubular injury

It is hypothesized that, while normal intact epithelium in the distal part of the nephron is non-adherent or 'protected' against crystal binding, cell stress or injury can turn the tubular lining into a crystal-binding state [29, 62, 64, 65]. Indeed, cell culture studies have shown that cells derived from distal parts of the nephron, when grown into intact confluent mono-layers, are non-adherent to COM crystals. At times when the epithelial integrity is lost due to mechanical damage (scrape wounding) of the monolayer, crystal binding temporarily occurs until the cell layer is recovered. After closure of the wound and restoration of tight junctions, the epithelium becomes non-adherent again [62].

Oxalate toxicity

Recent investigations by our group focussed on the identification of potential stressors, capable of inducing injury to confluent tubular epithelial cells, which could in turn lead to enhanced affinity for crystals. About 80% of all renal stones are composed of calcium oxalate (CaOx)

and oxalate is considered one of the most harmful endogenous agents in this field of investigation. Cell culture studies have provided evidence that oxalate and/or calcium oxalate crystals are damaging to the renal tissue [66-68]. CaOx crystals are internalized by proximal tubular cells [69-72], which might lead to cell stress and activation [72-74]. In a calcium-containing environment, oxalate ions precipitate as CaOx crystals. This mechanism complicates the results from most cell culture studies, in which oxalate was added to the cells in calcium-containing cell culture medium. According to a study by Belliveau et al., addition of as little as 50 µM oxalate to DMEM (containing 1.8 mM $CaCl_2$) results in precipitation of about 45% of the oxalate with calcium from the medium. When 1 mM oxalate is added, 94% of the oxalate precipitates as CaOx [75]. To study the effects of oxalate ions alone, thus in absence of CaOx crystals, a calcium free buffer has to be used. Despite the fact that *in vivo* a calcium free environment does not occur in the kidney, these conditions can be created in cell culture to distinguish between effects caused by oxalate or crystals. In the two-compartment model, detachment of cells due to calcium deprivation can be prevented by addition of calcium-containing medium to the basal compartment (blood-side), while the apical (urine-) side of the cells receives calcium free medium.

Oxalate concentrations in the tubular fluid vary considerably per nephron segment. In ultrafiltrate, the concentration is similar to that in plasma (± 2 µmol/l). In the proximal tubules, this rises to approximately 17 µmol/l. During passage of the urine through the distal parts of the nephron, oxalate concentration can increase up to about 120 µmol/l. The maximal concentration of oxalate reported in final urine is 1 mmol/l [27, 28]. When the 24-hour excretion exceeds 450 µmol, this is usually considered as hyperoxaluria [23]. It has been reported that cells from proximal tubular origin (LLC-PK_1) and distal tubular origin (MDCK) respond differently to oxalate exposure, proximal tubular cells appearing to be the most sensitive to oxalate toxicity [74]. In studies presented in Chapters 5 and 6 of this thesis, we used the two-compartment cell culture model to investigate whether CaOx crystals and/or oxalate ions are indeed toxic to renal tubule epithelial cells.

1.5 IDENTIFICATION OF CRYSTAL BINDING MOLECULES

To obtain more insight into the mechanisms of crystal-cell interaction *in vivo*, identification of crystal binding molecules *in vitro* can provide important information. Certain criteria have to be met before a molecule can be identified as crystal binding molecule. First of all, the molecule in question must have affinity for crystals; second, it must be present at the cell surface at moments when crystal binding occurs and third, at moments when the epithelium is non-adherent, the potential crystal binding molecule has to be absent from the epithelium. Fourth, the removal of the molecule in question should result in lower levels of binding. It is however conceivable that other crystal binding molecules can compensate for the removal of a single type of crystal binding molecule. Molecules that meet these four criteria in cell culture studies can only serve as true crystal binding molecules when their expression *in vivo* is rationalized by existing physiological mechanisms.

Various potential crystal-binding molecules have been proposed during the past years. These molecules may play distinct roles in crystal-cell interaction, depending on the cell line used or the type of damage that is inflicted to the cells. It is conceivable that more than one crystal-binding molecule is involved in the pathogenesis of kidney stone disease. In a review by Asselman and Verkoelen, the candidate crystal binding molecules that have been proposed during the past decade and the way these might be connected to each other are discussed [76].

Hyaluronan (HA), a large polysaccharide was first identified as a major crystal binding molecule during proliferation and injury repair in studies with MDCK-I cells [77]. Furthermore, it was shown to function as crystal binding molecule in primary cultures of human renal epithelial cells [63]. HA at the cell surface attracts water and forms a thick viscous ‘coat’ or pericellular matrix (PCM) at the cell surface, that is stabilized by proteins and proteoglycans that form bridges between the HA chains. Due to the unique water-absorbing properties of the networks and the negative-charge repulsion between the various macromolecules, the whole construction straightens to extend several microns outward from the surface. PCMs play an important role in cell migration, proliferation and injury repair. The hydrated matrix facilitates movement of cells during tissue remodelling [78-83].

Recent data from a study with rats given ethylene glycol in drinking water to induce crystal formation and retention have provided more evidence for the concept that HA is involved in stone disease. Injured/regenerating tubule epithelial cells express HA on their apical surface, and at these sites calcium oxalate crystals are found attached to the tubule wall [84].

Other investigators studied the role of phospholipids, like phosphatidylserine (PS), in crystal attachment. PS is normally confined to the inner leaflet of the cell membrane and is expressed at the outer leaflet as a classic response to toxic stimuli. Cao et al. have shown that exposure of MDCK cells to oxalate (0.25-0.50 mM) induced re-distribution of phosphatidylserine to the cell-surface and crystal binding [85]. Crystal binding was also enhanced when PS expression in the outer leaflet of the membrane was stimulated by exogenous addition of PS or by membrane lipid scrambling agents [86].

Preincubation with positively charged compounds reduced crystal binding to BSC-1 cells, suggesting that crystals are attracted by negatively charged cell surface molecules [87]. Since terminal sialic acid residues account for most of the negative charge of the cell-surface, sialic acid was proposed as crystal binding molecule. Lieske et al. have shown that sialidase (neuraminidase) treatment of BSC-1 cells reduced crystal binding to these cells. These authors concluded that sialic acid-containing cell surface glycoconjugates are critical determinants of face-specific crystal nucleation upon the renal cell surface. The spatial three-dimensional organization of the sialic acid-containing glycoconjugates at the cell surface, rather than the amount of sialic acid, may enable their interaction with the crystal surface [87, 88]. However, investigations by our group showed that in MDCK-I cells sialic acid does not seem to act directly as crystal binding molecule, as it is more heavily expressed on non-adherent confluent cells than on subconfluent cells with affinity for crystals. Nevertheless, neuraminidase treatment significantly reduced COM crystal binding to subconfluent MDCK-I cells [89]. Sialic acid could be of importance in COM crystal binding, by stabilizing the HA-rich pericellular matrix or by regulating the CD44-mediated binding of HA to the cell surface [90, 91]. Other candidate crystal-binding molecules under investigation are annexin II [92] and nucleolin-like protein [93].

Extracellular matrix (ECM) molecules are also suggested to act as crystal binding molecules under certain conditions. After denudating injury, for example, molecules like collagen IV, fibronectin and laminin could be exposed. Urine sediments from stone-forming patients were shown to contain collagen IV-positive mucous threads with affinity for crystals [94]. Fibronectin was strongly expressed on crystal-containing tubules in a stone-forming rat model [95]. A study by Kramer et al. using CAKI-1 cells also suggested a role for collagen I and -IV and fibronectin as potential crystal binding molecules [96].

It has been demonstrated that the cell surface properties of the crystal binding phenotype differ considerably from that of cells that are non-adherent to crystals. The binding phenotype is characterized by the luminal expression of HA, OPN and CD44. We do not exclude the possibility that one or more of the aforementioned molecules also belong to the crystal binding phenotype. The relative contribution of these molecules to crystal retention remains to be resolved.

1.6 SCOPE OF THIS THESIS

The studies in the present thesis were performed to obtain more insight in the response of different renal tubular cell types to calcium oxalate crystals and oxalate ions. There are many conflicting data in the literature that for a large part may result from the use of renal tubular cells derived from different segments of the nephron and by the cell culture methods used. In this thesis attempts were made to clarify some of the existing indistinctness and contradiction. In *Chapter 2,* we investigated if urinary crystallization inhibitors were also capable of inhibiting the binding of calcium oxalate crystals to renal tubular epithelial cells. In *Chapter 3*, we studied the ability of renal tubular cells in culture to form hyaluronan-rich pericellular matrices with affinity for crystals. In *Chapter 4,* we addressed the potential significance of crystal binding and endocytosis in the pathophysiology of renal stone disease. In *Chapter 5,* we studied the possible damaging effect of the interaction of renal tubular cells with crystals and *Chapter 6* is devoted to the proposed nephrotoxic effect of free (soluble) oxalate. Finally, the results are discussed in *Chapter 7*.

1.7 REFERENCES

1. Ansari MS, Gupta NP: Impact of socioeconomic status in etiology and management of urinary stone disease. *Urol Int* 70:255-261, 2003
2. Stamatelou KK, Francis ME, Jones CA, Nyberg LM, Curhan GC: Time trends in reported prevalence of kidney stones in the United States: 1976-1994. *Kidney Int* 63:1817-1823, 2003
3. Tiselius HG: Epidemiology and medical management of stone disease. *BJU Int* 91:758-767, 2003
4. Anderson RA: A complementary approach to urolithiasis prevention. *World J Urol* 20:294-301, 2002
5. Cirillo M, Stellato D, Panarelli P, Laurenzi M, De Santo NG: Cross-sectional and prospective data on urinary calcium and urinary stone disease. *Kidney Int* 63:2200-2206, 2003
6. Balcke P, Zazgornik J, Sunder-Plassmann G, Kiss A, Hauser AC, Gremmel F, Derfler K, Stockenhuber F, Schmidt P: Transient hyperoxaluria after ingestion of chocolate as a high risk factor for calcium oxalate calculi. *Nephron* 51:32-34, 1989
7. Curhan GC: Epidemiologic evidence for the role of oxalate in idiopathic nephrolithiasis. *J Endourol* 13:629-631, 1999
8. Leonetti F, Dussol B, Berthezene P, Thirion X, Berland Y: Dietary and urinary risk factors for stones in idiopathic calcium stone formers compared with healthy subjects. *Nephrol Dial Transplant* 13:617-622, 1998
9. Siener R, Ebert D, Nicolay C, Hesse A: Dietary risk factors for hyperoxaluria in calcium oxalate stone formers. *Kidney Int* 63:1037-1043, 2003
10. Holmes RP, Goodman HO, Assimos DG: Contribution of dietary oxalate to urinary oxalate excretion. *Kidney Int* 59:270-276, 2001
11. Krishnamurthy MS, Hruska KA, Chandhoke PS: The urinary response to an oral oxalate load in recurrent calcium stone formers. *J Urol* 169:2030-2033, 2003
12. Nishiura JL, Martini LA, Mendonca CO, Schor N, Heilberg IP: Effect of calcium intake on urinary oxalate excretion in calcium stone-forming patients. *Braz J Med Biol Res* 35:669-675, 2002
13. Williams AW, Wilson DM: Dietary intake, absorption, metabolism, and excretion of oxalate. *Semin Nephrol* 10:2-8, 1990
14. Herrmann U, Schwille PO, Schmiedl A, Fan J, Manoharan M: Acute effects of calcium sodium citrate supplementation of a test meal on mineral homeostasis, oxalate, and calcium oxalate crystallization in the urine of healthy humans--preliminary results in patients with idiopathic calcium urolithiasis. *Biomed Pharmacother* 53:264-273, 1999
15. Hatch M, Freel RW: Renal and Intestinal Handling of Oxalate following Oxalate Loading in Rats. *Am J Nephrol* 23:18-26, 2003
16. Sutton RA, Walker VR: Enteric and mild hyperoxaluria. *Miner Electrolyte Metab* 20:352-360, 1994

17. Troxel SA, Sidhu H, Kaul P, Low RK: Intestinal Oxalobacter formigenes Colonization in Calcium Oxalate Stone Formers and Its Relation to Urinary Oxalate. *J Endourol* 17:173-176, 2003
18. Nguyen NU, Henriet MT, Dumoulin G, Widmer A, Regnard J: Increase in calciuria and oxaluria after a single chocolate bar load. *Horm Metab Res* 26:383-386, 1994
19. Kok DJ: Clinical implications of physicochemistry of stone formation. *Endocrinol Metab Clin North Am* 31:855-867, 2002
20. Hess B, Jost C, Zipperle L, Takkinen R, Jaeger P: High-calcium intake abolishes hyperoxaluria and reduces urinary crystallization during a 20-fold normal oxalate load in humans. *Nephrol Dial Transplant* 13:2241-2247, 1998
21. Holmes RP, Kennedy M: Estimation of the oxalate content of foods and daily oxalate intake. *Kidney Int* 57:1662-1667, 2000
22. von Unruh GE, Voss S, Sauerbruch T, Hesse A: Dependence of oxalate absorption on the daily calcium intake. *J Am Soc Nephrol* 15:1567-1573, 2004
23. Larsson L, Tiselius HG: Hyperoxaluria. *Miner Electrolyte Metab* 13:242-250, 1987
24. Van Woerden CS, Groothoff JW, Wanders RJ, Davin JC, Wijburg FA: Primary hyperoxaluria type 1 in The Netherlands: prevalence and outcome. *Nephrol Dial Transplant* 18:273-279, 2003
25. Milliner DS, Wilson DM, Smith LH: Phenotypic expression of primary hyperoxaluria: comparative features of types I and II. *Kidney Int* 59:31-36, 2001
26. Junqueira LC, Carneiro J, Kelley RO: Nier en urinewegen, in *Functionele Histologie*, edited by J. J, P. N, E. W, 6 ed, Utrecht, Wetenschappellijke Uitgeverij Bunge, pp 463-486, 1993
27. Hojgaard I, Tiselius HG: Crystallization in the nephron. *Urol Res* 27:397-403., 1999
28. Kok DJ: Intratubular crystallization events. *World J Urol* 15:219-228, 1997
29. Mandel N: Mechanism of stone formation. *Semin Nephrol* 16:364-374, 1996
30. Khan SR, Hackett RL: Retention of calcium oxalate crystals in renal tubules. *Scanning Microsc* 5:707-711; discussion 711-702, 1991
31. Finlayson B, Reid F: The expectation of free and fixed particles in urinary stone disease. *Invest Urol* 15:442-448, 1978
32. Kok DJ, Khan SR: Calcium oxalate nephrolithiasis, a free or fixed particle disease. *Kidney Int* 46:847-854, 1994
33. Baggio B, Gambaro G, Ossi E, Favaro S, Borsatti A: Increased urinary excretion of renal enzymes in idiopathic calcium oxalate nephrolithiasis. *J Urol* 129:1161-1162, 1983
34. Hering F, Briellmann T, Luond G, Guggenheim H, Seiler H, Rutishauser G: Stone formation in human kidney. *Urol Res* 15:67-73, 198735. Evan AP, Lingeman JE, Coe FL, Parks JH, Bledsoe SB, Shao Y, Sommer AJ, Paterson RF, Kuo RL, Grynpas M: Randall's plaque of patients with nephrolithiasis begins in basement membranes of thin loops of Henle. *J Clin Invest* 111:607-616, 2003
36. Worcester EM: Inhibitors of stone formation. *Semin Nephrol* 16:474-486, 1996

37. Atmani F, Khan SR: Role of urinary bikunin in the inhibition of calcium oxalate crystallization. *J Am Soc Nephrol* 10 Suppl 14:S385-388, 1999
38. Wesson JA, Worcester EM, Wiessner JH, Mandel NS, Kleinman JG: Control of calcium oxalate crystal structure and cell adherence by urinary macromolecules. *Kidney Int* 53:952-957., 1998
39. Wesson JA, Worcester EM, Kleinman JG: Role of anionic proteins in kidney stone formation: interaction between model anionic polypeptides and calcium oxalate crystals. *J Urol* 163:1343-1348, 2000
40. Tiselius HG, Hallin A, Lindback B: Crystallisation properties in stone forming and normal subjects' urine diluted using a standardised procedure to match the composition of urine in the distal part of the distal tubule and the middle part of the collecting duct. *Urol Res* 29:75-82, 2001
41. Asplin JR, Arsenault D, Parks JH, Coe FL, Hoyer JR: Contribution of human uropontin to inhibition of calcium oxalate crystallization. *Kidney Int* 53:194-199, 1998
42. Worcester EM, Beshensky AM: Osteopontin inhibits nucleation of calcium oxalate crystals. *Ann N Y Acad Sci* 760:375-377, 1995
43. Dawson CJ, Grover PK, Ryall RL: Inter-alpha-inhibitor in urine and calcium oxalate urinary crystals. *Br J Urol* 81:20-26, 1998
44. Verkoelen CF, Romijn JC, Cao LC, Boeve ER, De Bruijn WC, Schroder FH: Crystal-cell interaction inhibition by polysaccharides. *J Urol* 155:749-752, 1996
45. Hedgepeth RC, Yang L, Resnick MI, Marengo SR: Expression of proteins that inhibit calcium oxalate crystallization in vitro in the urine of normal and stone-forming individuals. *Am J Kidney Dis* 37:104-112., 2001
46. Verkoelen CF, Schepers MS: Changing concepts in the aetiology of renal stones. *Curr Opin Urol* 10:539-544, 2000
47. Marengo SR, Resnick MI, Yang L, Chung JY: Differential expression of urinary inter-alpha-trypsin inhibitor trimers and dimers in normal compared to active calcium oxalate stone forming men. *J Urol* 159:1444-1450, 1998
48. Atmani F, Glenton PA, Khan SR: Identification of proteins extracted from calcium oxalate and calcium phosphate crystals induced in the urine of healthy and stone forming subjects. *Urol Res* 26:201-207, 1998
49. Doyle IR, Ryall RL, Marshall VR: Inclusion of proteins into calcium oxalate crystals precipitated from human urine: a highly selective phenomenon. *Clin Chem* 37:1589-1594, 1991
50. Verkoelen CF, van der Boom BG, Kok DJ, Houtsmuller AB, Visser P, Schroder FH, Romijn JC: Cell type-specific acquired protection from crystal adherence by renal tubule cells in culture. *Kidney Int* 55:1426-1433, 1999
51. Gaush CR, Hard WL, Smith TF: Characterization of an established line of canine kidney cells (MDCK). *Proc Soc Exp Biol Med* 122:931-935, 1966
52. Richardson JC, Scalera V, Simmons NL: Identification of two strains of MDCK cells which resemble separate nephron tubule segments. *Biochim Biophys Acta* 673:26-36, 1981
53. Svennevig K, Prydz K, Kolset SO: Proteoglycans in polarized epithelial Madin-Darby canine kidney cells. *Biochem J* 311:881-888., 1995

54. Hansson GC, Simons K, van Meer G: Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. *Embo J* 5:483-489, 1986
55. Pfaller W, Gstraunthaler G, Kersting U, Oberleithner H: Carbonic anhydrase activity in Madin Darby canine kidney cells. Evidence for intercalated cell properties. *Ren Physiol Biochem* 12:328-337, 1989
56. Griffiths NM, Rugg EL, Simmons NL: Vasoactive intestinal peptide control of renal adenylate cyclase: in vitro studies of canine renal membranes and cultured canine renal epithelial (MDCK) cells. *Q J Exp Physiol* 74:339-353, 1989
57. Blot-Chabaud M, Laplace M, Cluzeaud F, Capurro C, Cassingena R, Vandewalle A, Farman N, Bonvalet JP: Characteristics of a rat cortical collecting duct cell line that maintains high transepithelial resistance. *Kidney Int* 50:367-376, 1996
58. Hull RN, Cherry WR, Weaver GW: The origin and characteristics of a pig kidney cell strain, LLC-PK. *In Vitro* 12:670-677, 1976
59. Verkoelen CF, Kok DJ, van der Boom BG, de Jonge HR, Schroder FH, Romijn JC: LLC-PK1 cells as a model system to study proximal tubule transport of water and other compounds relevant for renal stone disease. *Urol Res* 27:109-115, 1999
60. Helbert MJ, Dauwe S, De Broe ME: Flow cytometric immunodissection of the human nephron in vivo and in vitro. *Exp Nephrol* 7:360-376, 1999
61. Bigelow MW, Wiessner JH, Kleinman JG, Mandel NS: Calcium oxalate crystal attachment to cultured kidney epithelial cell lines. *J Urol* 160:1528-1532., 1998
62. Verkoelen CF, van der Boom BG, Houtsmuller AB, Schroder FH, Romijn JC: Increased calcium oxalate monohydrate crystal binding to injured renal tubular epithelial cells in culture. *Am J Physiol* 274:F958-965, 1998
63. 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
64. Wiessner JH, Hasegawa AT, Hung LY, Mandel GS, Mandel NS: Mechanisms of calcium oxalate crystal attachment to injured renal collecting duct cells. *Kidney Int* 59:637-644., 2001
65. Riese RJ, Mandel NS, Wiessner JH, Mandel GS, Becker CG, Kleinman JG: Cell polarity and calcium oxalate crystal adherence to cultured collecting duct cells. *Am J Physiol* 262:F177-184., 1992
66. Koul S, Fu S, Koul H: Oxalate exposure promotes reinitiation of the DNA synthesis and apoptosis of HK-2 cells, a line of human renal epithelial cells. *Ann N Y Acad Sci* 1010:292-295, 2003
67. Khan SR, Thamilselvan S: Nephrolithiasis: a consequence of renal epithelial cell exposure to oxalate and calcium oxalate crystals. *Mol Urol* 4:305-312., 2000

68. Scheid CR, Koul H, Hill WA, Lieske JC, Toback FG, Menon M: Oxalate ion and calcium oxalate crystal interactions with renal epithelial cells, in *Kidney Stones:Medical and Surgical Management*, edited by Coe FL, Favus M.J., Pak C.Y.C. , Parks J.H., Preminger G.M., Philadelphia, Lippincott-Raven Publishers, 1996, pp 129-143
69. Lieske JC, Norris R, Swift H, Toback FG: Adhesion, internalization and metabolism of calcium oxalate monohydrate crystals by renal epithelial cells. *Kidney Int* 52:1291-1301., 1997
70. Lieske JC, Deganello S: Nucleation, adhesion, and internalization of calcium-containing urinary crystals by renal cells. *J Am Soc Nephrol* 10 Suppl 14:S422-429, 1999
71. Schepers MS, Duim RA, Asselman M, Romijn JC, Schroder FH, Verkoelen CF: Internalization of calcium oxalate crystals by renal tubular cells: A nephron segment-specific process? *Kidney Int* 64:493-500, 2003
72. Khan SR, Byer KJ, Thamilselvan S, Hackett RL, McCormack WT, Benson NA, Vaughn KL, Erdos GW: Crystal-cell interaction and apoptosis in oxalate-associated injury of renal epithelial cells. *J Am Soc Nephrol* 10 Suppl 14:S457-463., 1999
73. Thamilselvan S, Khan SR, Menon M: Oxalate and calcium oxalate mediated free radical toxicity in renal epithelial cells: effect of antioxidants. *Urol Res* 31:3-9, 2003
74. Thamilselvan S, Hackett RL, Khan SR: Cells of proximal and distal tubular origin respond differently to challenges of oxalate and calcium oxalate crystals. *J Am Soc Nephrol* 10 Suppl 14:S452-456, 1999
75. Belliveau J, Griffin H: The solubility of calcium oxalate in tissue culture media. *Anal Biochem* 291:69-73, 2001
76. Asselman M, Verkoelen CF: Crystal-cell interaction in the pathogenesis of kidney stone disease. *Curr Opin Urol* 12:271-276, 2002
77. Verkoelen CF, Van der Boom BG, Romijn JC: Identification of hyaluronan as a crystal-binding molecule at the surface of migrating and proliferating MDCK cells. *Kidney Int* 58:1045-1054, 2000
78. Lee GM, Johnstone B, Jacobson K, Caterson B: The dynamic structure of the pericellular matrix on living cells. *J Cell Biol* 123:1899-1907, 1993
79. Knudson W, Aguiar DJ, Hua Q, Knudson CB: CD44-anchored hyaluronan-rich pericellular matrices: an ultrastructural and biochemical analysis. *Exp Cell Res* 228:216-228, 1996
80. Knudson W, Knudson CB: Assembly of a chondrocyte-like pericellular matrix on non-chondrogenic cells. Role of the cell surface hyaluronan receptors in the assembly of a pericellular matrix. *J Cell Sci* 99:227-235, 1991
81. Heldin P, Pertoft H: Synthesis and assembly of the hyaluronan-containing coats around normal human mesothelial cells. *Exp Cell Res* 208:422-429, 1993
82. Hedman K, Kurkinen M, Alitalo K, Vaheri A, Johansson S, Hook M: Isolation of the pericellular matrix of human fibroblast cultures. *J Cell Biol* 81:83-91, 1979

83. 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-E112, 2003
84. 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
85. Cao LC, Jonassen J, Honeyman TW, Scheid C: Oxalate-induced redistribution of phosphatidylserine in renal epithelial cells: implications for kidney stone disease. *Am J Nephrol* 21:69-77, 2001
86. Bigelow MW, Wiessner JH, Kleinman JG, Mandel NS: Surface exposure of phosphatidylserine increases calcium oxalate crystal attachment to IMCD cells. *Am J Physiol* 272:F55-62., 1997
87. Lieske JC, Leonard R, Swift H, Toback FG: Adhesion of calcium oxalate monohydrate crystals to anionic sites on the surface of renal epithelial cells. *Am J Physiol* 270:F192-199, 1996
88. Lieske JC, Toback FG, Deganello S: Sialic acid-containing glycoproteins on renal cells determine nucleation of calcium oxalate dihydrate crystals. *Kidney Int* 60:1784-1791, 2001
89. Verkoelen CF, van der Boom BG, Kok DJ, Romijn JC: Sialic acid and crystal binding. *Kidney Int* 57:1072-1082, 2000
90. Tammi R, MacCallum D, Hascall VC, Pienimaki JP, Hyttinen M, Tammi M: Hyaluronan bound to CD44 on keratinocytes is displaced by hyaluronan decasaccharides and not hexasaccharides. *J Biol Chem* 273:28878-28888, 1998
91. Katoh S, McCarthy JB, Kincade PW: Characterization of soluble CD44 in the circulation of mice. Levels are affected by immune activity and tumor growth. *J Immunol* 153:3440-3449, 1994
92. Kumar V, Farell G, Deganello S, Lieske JC: Annexin II is present on renal epithelial cells and binds calcium oxalate monohydrate crystals. *J Am Soc Nephrol* 14:289-297, 2003
93. Sorokina EA, Kleinman JG: Cloning and preliminary characterization of a calcium-binding protein closely related to nucleolin on the apical surface of inner medullary collecting duct cells. *J Biol Chem* 274:27491-27496, 1999
94. Kohri K, Kodama M, Ishikawa Y, Katayama Y, Matsuda H, Imanishi M, Takada M, Katoh Y, Kataoka K, Akiyama T, et al.: Immunofluorescent study on the interaction between collagen and calcium oxalate crystals in the renal tubules. *Eur Urol* 19:249-252, 1991
95. Tsujihata M, Miyake O, Yoshimura K, Kakimoto KI, Takahara S, Okuyama A: Fibronectin as a potent inhibitor of calcium oxalate urolithiasis. *J Urol* 164:1718-1723., 2000
96. Kramer G, Steiner GE, Prinz-Kashani M, Bursa B, Marberger M: Cell-surface matrix proteins and sialic acids in cell-crystal adhesion; the effect of crystal binding on the viability of human CAKI-1 renal epithelial cells. *BJU Int* 91:554-559, 2003

CHAPTER 2

URINARY CRYSTALLIZATION INHIBITORS DO NOT PREVENT CRYSTAL BINDING

M.S.J. Schepers, B.G. van der Boom,
J.C. Romijn, F.H. Schröder and C.F. Verkoelen

The Journal of Urology 2002; 167:1844-1847

ABSTRACT

Purpose. Renal stone formation requires the persistent retention of crystals in the kidney. Calcium oxalate monohydrate (COM) crystal binding to Madin Darby canine kidney strain I (MDCK-I), a cell line that resembles the epithelium in the renal distal tubule/collecting duct, is developmentally regulated, while LLC-PK_1 cells (American Type Tissue Collection), which are widely used as model for the renal proximal tubule, bind crystals irrespective of their stage of epithelial development. Whereas to our knowledge the binding molecules for COM at the surface of LLC-PK_1 cells are still unknown, crystals adhere to the hyaluronan (HA) rich pericellular matrix transiently expressed by mobile MDCK-I cells. In the current study we investigated whether crystal binding to either cell type is influenced by urinary substances, including glycoprotein inhibitors of crystallization.

Materials and Methods. We studied crystal binding to MDCK-I cells during wound repair, to confluent LLC-PK_1 cells and to HA immobilized on a solid surface using [^{14}C]COM pretreated or not pretreated with urine obtained from healthy male volunteers. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis were performed to assess whether the crystals became coated with urine derived proteins.

Results. Western blot analysis demonstrated that pretreated COM crystals were covered with protein inhibitors of crystallization. However, this protein coat had no significant effect on the level of crystal binding to either cell type. In contrast, the adherence of urine treated crystals to immobilized hyaluronan was significantly reduced.

Conclusions. The adherence of crystals to pericellular matrixes may encompass more than their simple fixation to the polysaccharide HA. Calcium oxalate crystal retention is not prevented by coating crystals with urinary constituents such as glycoproteins and, therefore, may predominantly depend on the surface properties of the renal tubular epithelium.

INTRODUCTION

The production of concentrated urine frequently initiates crystal formation in the kidney. Persistent inadequate elimination of crystal material with urine eventually can lead to the formation of a stone in the urinary tract. Retention of crystals may be caused by their attachment to epithelial cells lining the renal tubules. We [1] and others [2-4] developed cell culture models to investigate the mechanisms involved in crystal-cell interaction. Most of our studies are performed with Madin Darby canine kidney (MDCK) strain I cells[5], which exhibit morphological and functional characteristics of the epithelium found in the renal distal tubule/collecting duct [6]. At confluence MDCK-I cells form highly polarized functional monolayers with transepithelial resistances over 5000 Ω x cm^2. Calcium oxalate monohydrate (COM), the most common crystalline component of kidney stones, abundantly adheres to the surface of developing MDCK-I cultures but not to confluent monolayers. Since the renal epithelium in vivo occurs in continuous cell layers, it suggests that a healthy epithelium is non adherent. However, the resistance to crystal binding is temporarily lost at the time of intact monolayer wounding and during the subsequent healing process. During repair COM crystals bind to the hyaluronan (HA)rich pericellular matrix surrounding wounded areas. Soon after wounds are healed HA disappears from the surface and the epithelium regains its nonadherent properties. From these observations it was speculated that tissue injury may initiate the stone forming process[1, 7, 8]. Developmentally regulated COM binding is not the only form of crystal-cell interaction. Crystals avidly adhere to mobile LLC-PK_1 cells in subconfluent cultures as well as to growth-inhibited cells during confluence[6].

Crystallization in the tubular fluid is under the control of various high and low molecular weight urinary constituents. A considerable part of this inhibitory activity is provided by glycoproteins, such as members of the inter-α-trypsin inhibitor and prothrombin families of proteins, osteopontin, Tamm-Horsfall protein (THP) and α_1-microglobulin. The appreciation of these urinary inhibitors has led to the hypothesis that pathological calcification in the urinary tract is caused by alterations in the amount or the structure of these compounds[9]. It has been suggested that crystal-cell interaction can also be prevented by urine constituents[9]. Although this idea seems to be supported by results obtained with purified inhibitors of crystallization [10, 11], there is no solid evidence that crystal-cell

interaction indeed is modulated in the presence of physiological concentrations of naturally occurring urinary constituents. In the current study we investigated the effect of urine pretreatment on crystal-cell interaction.

MATERIALS AND METHODS

Cell culture.
Cells were seeded on 24 mm. polycarbonate porous filter inserts (Corning Costar, Badhoevedorp, The Netherlands) at a concentration of $1x10^6$ cells/insert [7]. As described previously [7], strips of cells were scraped off from confluent MDCK-I monolayers. Crystal binding was studied 2 days after injury to MDCK-I and 7 days after seeding to LLC-PK_1 (ATCC).

Pretreatment of COM with human urine.
Fresh morning midstream urine samples were obtained from 10 healthy male volunteers between 30 and 50 years old who were selected from the laboratory staff. Urine specimens did not receive any additives and were not centrifuged. They were maintained at room temperature and used for the various experiments the same morning. COM crystals were prepared as described previously [7]. Radiolabeled crystals (1.45 mg) were centrifuged for 5 minutes at 1000 x gravity. The supernatant was discarded and the crystal pellet was incubated in 1 ml. urine for 1 hour under end-over-end rotation at room temperature, centrifuged, washed and re-suspended in calcium oxalate saturated distilled water. To assess whether sufficient amounts of protein were available to cover the crystals an experiment was performed. Nonradiolabeled COM crystals (3.6, 7.25 or 14.5 mg.) were added to 5 ml. urine (0.72, 1.45 and 2.9 mg./ml., respectively). After 2-hour end-over-end incubation the crystal were centrifuged, washed and dissolved in 1 ml. 0.25 M. ethylene diamine tetraacetic acid. The amount of protein adsorped was determined by spectrophotometry using a protein assay (Roche Diagnostics GmbH, Mannheim, Germany). The method of studying crystal binding has been previously described in detail [7].

Sodium dodecyl sulfate (SDS- polyacrylamide gel elctrophoresis (PAGE) and Western blot analysis.

Urinary constituents were allowed to cover the crystal surface by the addition of 22 mg. non-radiolabeled COM to 50 ml. freshly voided urine. The crystals were incubated for 2 hours at room temperature under end-over-end rotation. After centrifugation at low speed the pellet was washed, demineralized in 0.25 M ethylene diamine tetraacetic acid, pH 8 for 2 days at 4^0 C, dialyzed and freeze-dried. SDS-PAGE was performed with a 1.0 mm 10% mini-slab gel using a mini-Protean II apparatus (BioRad Laboratories, Hercules, California). Samples of 20 µg. protein and molecular weight standards were electrophoresed under nonreducing conditions. Proteins were visualized using a silver staining kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) or blotted onto nitrocellulose. After electrophoresis gels and nitrocellulose membranes were equilibrated in the blotting buffer containing 16.5 mM. TRIS, 150 mM. glycine and 10% volume/volume methanol. Proteins were transferred for 1 hour at 4^0C. Immunochemical staining was performed using different polyclonal antibodies, including sheep-α-human inter-α-trypsin-inhibitor, sheep-α-human prothrombin, sheep-α-human α_1-microglobulin (The Binding Site,Birmingham, United Kingdom) and mouse-α-human THP (Cedarlane Laboratories Ltd., Hornby, Canada). Nonspecific protein binding sites were blocked by incubating for 60 minutes in phosphate buffered saline-0.1% Tween-5% nonfat dry milk. The membranes were incubated overnight with the primary antibody, diluted 1:1000, at 4^0C followed by 60 minutesd of incubation with the secondary antibody, diluted 1:2,500, using rabbit-α-sheep or mouse-α-sheep horseradish peroxidase (DAKO, Glostrup, Denmark). The membranes were then developed for 1 minute using a chemiluminescence substrate kit (Roche Diagnostics GmbH, Mannheim, Germany).

Enzyme treatment and HA staining.

After rinsing with a physiological saline solution cells were treated at 37°C, pH 5.3 for 1 hour with 5 units per ml Streptomyces hyaluronidase (EC 4.2.2.1, Sigma-Aldrich, Zwijndrecht, The Netherlands) in serum-free Dulbecco's modified Eagle's medium. The method of staining HA as well as the procedure for coating plastic wells with this polysaccharide have been previously described [1].

RESULTS

To study the effect of urine constituents on crystal-cell interaction we measured crystal adherence after pretreatment with urine derived from healthy males. Crystal adherence was studied to confluent LLC-PK_1 cultures 7 days after seeding and during the recovery of the MDCK-I cultures from scrape damage 2 days after injury. Although binding was slightly decreased in some experiments, this treatment did not prevent crystal adherence of urine treated crystals. Compared with the adherence of untreated crystals preincubation with 10 different urines reduced mean COM binding plus or minus standard deviation to LLC-PK_1 and MDCK-I cells to 92% ± 8% and 89% ± 12%, respectively, which was not statistically significant (table 1). To coat COM crystals with urinary constituents a standard amount of 1.45 mg. crystals was pre-incubated in 1 ml. undiluted urine. This ratio was chosen, such that urinary proteins were in excess compared with the amount of crystals. Figure 1 shows the linear relationship of protein adsorption and increasing amounts of crystals. An average of 1 ml. urine contained 0.1 mg. protein, of which approximately 10% became adsorbed to the the standard amount of COM crystals. [^{14}C]COM binding was not different using crystals generated in urine instead of pre-incubated in urine (data not shown).

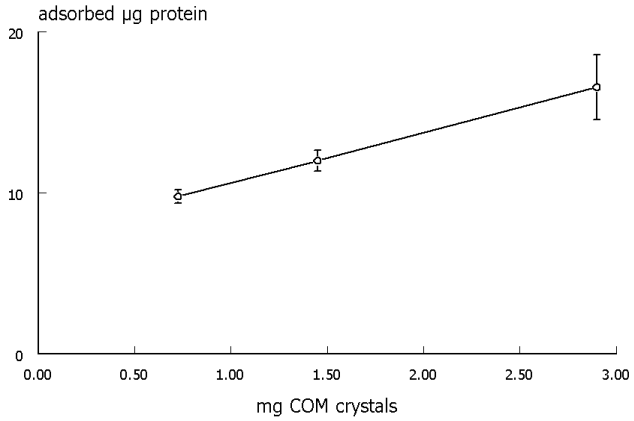


Figure 1. *Adsorption of urinary proteins to COM crystals. To coat COM crystals with urinary constituents 1.45 mg. crystals were pre-incubated in 1 ml. undiluted urine at room temperature for 2 hours. There was linear relationship op protein adsorption and increasing amounts of crystals.*

Table 1. Binding of [^{14}C] COM crystals pretreated with urine as percent of untreated crystals in 3 inserts.

Urine Sample No.	Mean % MDCK-I ± SD 2 Days After Injury	Mean % LLC-PK$_1$ ± SD 7 Days After Seeding
1	78 ± 10	111 ± 19
2	78 ± 9*	88 ± 3*
3	118 ± 17	75 ± 1*
4	80 ± 10*	51 ± 8*
5	93 ± 4	20 ± 1
6	71 ± 6*	111 ± 10
7	73 ± 22	74 ± 6 *
8	89 ± 4	97 ± 3
9	102 ± 16	94 ± 3
10	90 ± 9	96 ± 8
Av.	89 ± 12	92 ± 8

**Significantly decreased compared to the binding of untreated COM crystals (Student's t-test $p < 0.05$)*

To assess whether urinary inhibitors of crystallization became adsorbed onto the crystal surface COM crystals incubated with urine were demineralized and analyzed by silver staining. This technique revealed 4 major bands in the gel at approximately 80, 60, 36 and 20 kDa (Fig.2, A). Corresponding Western blots were stained with antibodies against a number of known urinary inhibitors of crystallization (Fig.2, B). Using anti-α_1-microglobulin we detected 2 major bands at a molecular weight of 80 and 35 kDa. in lane 1. ITI related proteins were present as 1 major band at 55 kDa. in lane 2. We found 2 main bands for the prothrombin family of proteins at 40 and at 30 kDa. in lane 3, while a large spot of THP was found at 98 kDa. in lane 4.

Previously we have identified HA as crystal binding molecule at the surface of migrating and proliferating MDCK-I cells. Since mobile as well as growth-arrested LLC-PK$_1$ cells are susceptible to crystal attachment, it is possible that these cells constitutively express HA. Therefore, we studied the presence or absence of HA at the cell surface using biotinylated HA binding protein coupled to avidin-fluorescein isothiocyanate and visualized by confocal microscopy. Scans done

perpendicular to the cell layer (xz scan) showed HA at the surface of mobile MDCK-I cells covering the whole apical cell surface (Fig. 3A).
Treating MDCK-I cells with Streptomyces hyaluronidase completely abolished the HA binding protein staining (not shown). Irrespective of their stage of epithelial development LLC-PK1 cells never expressed surface associated HA (Fig. 3B). Treatment of mobile cells with Streptomyces hyaluronidase caused a significant mean reduction versus controls in crystal binding to MDCK-I cells (100% ± 7% versus 45% ± 7+, p< 0.05), but not to LLC-PK1 cells (100% ± 8% and 95 ± 6%, respectively).
Previously we have demonstrated that crystals adhere to the surface of plastic wells pre-coated with HA [1]. This method was used in the current series to study selectively the effect of urinary constituents with affinity for COM on crystal binding to HA. Pre-coating wells with HA resulted in higher levels of binding, which was reduced to background levels by Streptomyces hyaluronidase. Although the magnitude of the effect varied, coating the crystals with urine significantly reduced their binding to HA coated wells ($p<0.05$, table 2).

Table 2. Binding of [^{14}C] COM crystals pretreated with urine as percentage of untreated crystals. Average ± SD of three wells, p-values according to Student's t-test.

Before treatment	Mean % COM binding
Control	100
Streptomyces Hyaluronidase	3 ± 1
Urine 11	4 ± 1
Urine 12	20 ± 1
Urine 13	18 ± 2
Urine 14	35 ± 7
Urine 15	22 ± 2

**Versus controls all pretreatment values were significantly decreased (Student's t-test $p < 0.05$)*

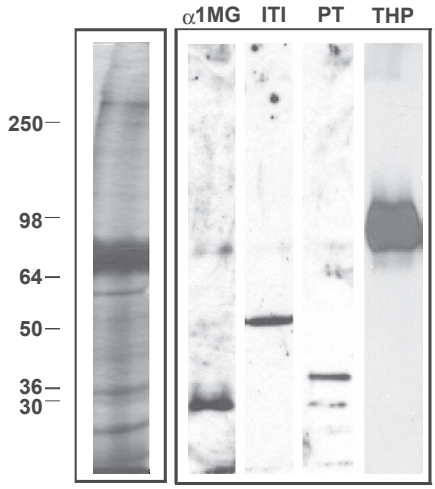


Figure 2. *Analysis of COM-associated urinary proteins. (A) Silver staining revealed 4 major bands in the gel at approximately 80, 60, 36 and 20 kDa as well as a number of minor bands. (B) Corresponding Western blots show 2 major bands for α_1-microglobulin (α_1MG) at 80 and 35 kDa, inter-α-trypsin inhibitor (ITI)-related proteins as one major band at 55 kDa, prothrombin (PT) proteins in 2 bands at 40 and 30 kDa. and broad band of THP at 98 kDa.*

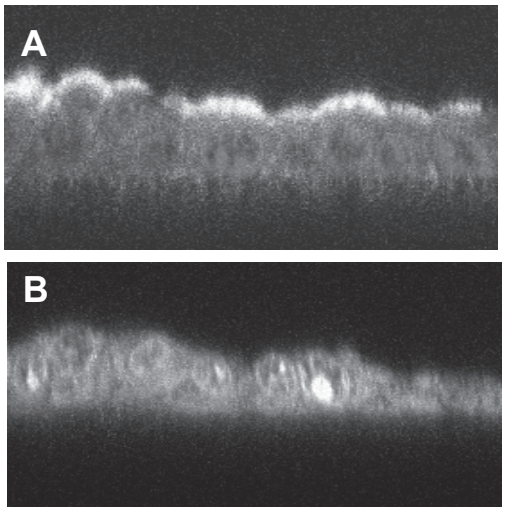


Figure 3*. Presence or absence of HA at the surface of subconfluent MDCK-I and LLC-PK$_1$ cells on confocal microscopy after staining with HA binding protein, coupled to fluorescein isothiocyanate. Cell nuclei were counterstained with propidium iodide. Scans were done perpendicular to cell surface as xz-scan. A. HA staining appears as a bright layer covering MDCK-I cells. B, no bright layer appears on LLC-PK$_1$ cells.*

DISCUSSION

This study shows that pretreatment of COM crystals with urine derived from healthy men does not prevent crystal binding to renal tubular cells in culture. Nevertheless, SDS-PAGE and Western blot analysis nevertheless revealed that many known urinary protein inhibitors of crystallization adsorbed onto the crystal surface, including α_1-microglobulin, THP, and inter-α-trypsin-inhibitor- and prothrombin-related proteins. To study the potential effect of urinary constituents on crystal-cell interaction we selected 2 cell types, namely LLC-PK_1 cells, which constitutively express crystal binding molecules, and MDCK-I cells, which are susceptible to crystal binding only during locomotion. MDCK-I and LLC-PK_1 cells are representative of the distal tubule/collecting duct and proximal convoluted tubule, respectively. When considering the levels of stone salt supersaturation, in contrast to that in the proximal convoluted tubule, the epithelium in the distal tubule/collecting duct requires protection against encountering crystals. In line with their suspected origin LLC-PK_1 cells are endowed with a well developed brush-border specialized for binding and endocytosing molecules that escaped the glomerular barrier, whereas MDCK-I cells are covered with a smaller amount of short and stubby microvilli[6]. The current study shows that urinary constituents cannot prevent the possible early as well as late nephron mode of crystal attachment**.** Since the effects of urinary inhibitors on crystal growth, aggregation and retention are often bracketed together [9, 11], our observations could make a distinction between the role of these substances in renal stone disease.

It has been reported that crystal attachment can be inhibited by pre-incubating COM with negatively charged low- and high molecular urinary constituents[10, 11]. In those studies glycoproteins, such as nephrocalcin and uropontin, were isolated and purified from biological fluids[11], whereas purified citrate and polysaccharides were commercially obtained[10, 11]. It is questionable whether these substances inhibited crystal adherence at concentrations within the physiological range. For example, citrate inhibited crystal binding at concentrations greater than 50 μM [11], whereas normocitraturia is approximately 2 μM. Heparan sulfate and chondroitin sulfate only marginally inhibited crystal attachment, whereas the level of crystal binding was increased in the presence of HA [10]. Most inhibitory capacity was obtained with anionic compounds that are not normally present in the urine, such as

synthetic polysaccharides[10], poly-aspartate[11] and heparin[10, 11]. In the current study we selected the urine of healthy males because the highest levels of inhibitory activity are most likely expected in the urine of men that do not form kidney stones. On the other hand, it is conceivable that the kidneys of stone formers are triggered to the overall or local excretion of protective compounds to prevent crystal-cell interaction. Ebusino et al. reported that the presence of 10% urine during the incubations inhibited the adherence of calcium oxalate crystals to MDCK cells by approximately 50% [12]. In that series the inhibitory effect of stone former derived urine was not significantly different from that of healthy controls, which seems to contradict the possibility that stone formers excrete higher levels of protective substances. The idea that stoneformers excrete higher levels of urinary crystallization inhibitory proteins was recently disproved [13]. To verify the results of Ebusino et. al [12], we also performed part of our experiments in the presence of 10% urine. When compared with pre-treated crystals, the inhibition levels inclined to be somewhat higher in the presence of urine (data not shown). Considering the amount of inhibitors available in 10% versus whole urine, it was most likely caused by other factors. Renal medullary cells are routinely under osmotic stress. MDCK cells are capable of regulating their cell volume under anisotonic conditions. When suddenly exposed to anisotonic extracellular fluid, they initially swell or shrink to return subsequently to the original cell volume [14]. Our crystal binding studies were performed under isosmotic conditions (310-320 mOsmol/ kg. water). It is possible that the inhibitory effect in the presence of urine is mediated by an acute adaptive response of the plasma membrane to osmotic stress. The effect of osmotic variations on crystal binding clearly merits investigations that are more detailed. Finally in contrast to the results of cell culture, urinary constituents significantly reduced the level of [^{14}C] COM binding to immobilized HA, suggesting that crystal binding to synthesized plasma membrane associated HA differs from binding to purified HA. It may have been caused by differences in the spatial structure, state of hydration, molecular mass of MDCK-I synthesized HA or by the presence of stabilizing proteins in the pericellular matrix. More detailed studies are required to investigate whether HA immobilized on a solid surface is representative for the structural organization of HA in organic cell coats. In conclusion, together the results of this study show that urinary protein inhibitors of crystallization have only a minor role in the attachment of

COM crystals to renal tubular cells, suggesting that crystal retention primarily depends on the composition of the cell surface.

REFERENCES

1. Verkoelen CF, Van der Boom BG, Romijn JC: Identification of hyaluronan as a crystal-binding molecule at the surface of migrating and proliferating MDCK cells. *Kidney Int* 58:1045-1054, 2000
2. Lieske JC, Huang E, Toback FG: Regulation of renal epithelial cell affinity for calcium oxalate monohydrate crystals. *Am J Physiol Renal Physiol* 278:F130-137, 2000
3. Wiessner JH, Hasegawa AT, Hung LY, Mandel GS, Mandel NS: Mechanisms of calcium oxalate crystal attachment to injured renal collecting duct cells. *Kidney Int* 59:637-644., 2001
4. Thamilselvan S, Byer KJ, Hackett RL, Khan SR: Free radical scavengers, catalase and superoxide dismutase provide protection from oxalate-associated injury to LLC-PK1 and MDCK cells. *J Urol* 164:224-229., 2000
5. Hansson GC, Simons K, van Meer G: Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. *Embo J* 5:483-489, 1986
6. Verkoelen CF, van der Boom BG, Kok DJ, Houtsmuller AB, Visser P, Schroder FH, Romijn JC: Cell type-specific acquired protection from crystal adherence by renal tubule cells in culture. *Kidney Int* 55:1426-1433, 1999
7. Verkoelen CF, van der Boom BG, Houtsmuller AB, Schroder FH, Romijn JC: Increased calcium oxalate monohydrate crystal binding to injured renal tubular epithelial cells in culture. *Am J Physiol* 274:F958-965, 1998
8. Verkoelen CF, Schepers MS: Changing concepts in the aetiology of renal stones. *Curr Opin Urol* 10:539-544, 2000
9. Worcester EM: Inhibitors of stone formation. *Semin Nephrol* 16:474-486, 1996
10. Verkoelen CF, Romijn JC, Cao LC, Boeve ER, De Bruijn WC, Schroder FH: Crystal-cell interaction inhibition by polysaccharides. *J Urol* 155:749-752, 1996
11. Lieske JC, Leonard R, Toback FG: Adhesion of calcium oxalate monohydrate crystals to renal epithelial cells is inhibited by specific anions. *Am J Physiol* 268:F604-612, 1995
12. Ebisuno S, Umehara M, Kohjimoto Y, Ohkawa T: The effects of human urine on the adhesion of calcium oxalate crystal to Madin-Darby canine kidney cells. *BJU Int* 84:118-122, 1999
13. Hedgepeth RC, Yang L, Resnick MI, Marengo SR: Expression of proteins that inhibit calcium oxalate crystallization in vitro in the urine of normal and stone-forming individuals. *Am J Kidney Dis* 37:104-112., 2001
14. Borgmann S, Dorge A: Adaptation of Madin-Darby canine kidney cells to hypertonic medium: an electron microprobe analysis. *Kidney Int Suppl* 67:S133-135., 1998

CHAPTER 3

PERICELLULAR MATRIX FORMATION BY RENAL TUBULE EPITHELIAL CELLS IN RELATION TO CRYSTAL BINDING

M.S.J. Schepers, M. Asselman, R.A.J. Duim,
J.C. Romijn, F.H. Schröder and C.F. Verkoelen

Nephron Experimental Nephrology 2003; 94:e103-e112

ABSTRACT

Background/Aim. Retention of crystals in the kidney ultimately leads to renal stone formation. Hyaluronan (HA) has been identified as binding molecule for calcium oxalate monohydrate crystals. The association of high molecular mass (M_r) HA with cell surface receptors such as CD44 gives rise to pericellular matrix (PCM) formation by many eukaryotic cells in culture. Here, we study the ability of several renal tubular cell lines to assemble PCMs and to synthesize high-M_r HA during proliferation in relation to crystal retention.
Methods. PCM assembly by MDCK-I, MDCK-II and LLC-PK_1 cells was visualized by particle exclusion assay. Metabolic labeling studies were performed to estimate the cellular production of HA. The expression of CD44 and HA was studied using fluorescent probes, and crystal binding was quantified with radiolabeled calcium oxalate monohydrate.
Results. PCMs were formed, and HA was expressed by most MDCK-I and some MDCK-II, but not by LLC-PK_1 cells. All cell types expressed CD44 at their apical surface. MDCK-I, MDCK-II secreted, respectively, 14.7 ± 1.6 and 0.5 ± 0.2 pmol [^{3}H]glucosamine incorporated in high-M_r HA, whereas LLC-PK_1 did not secrete HA. *Streptomyces* hyaluronidase treatment significantly decreased crystal binding ($\mu g/cm^2$) to MDCK-I cells (from 8.6 ± 0.4 to 3.9 ± 0.9), but hardly to MDCK-II cells (from 10.2 ± 0.2 to 9.6 ± 0.1) or LLC-PK_1 cells(from 10.2 ± 0.8 to 9.9 ± 0.3).
Conclusions. There are various forms of crystal binding to renal tubular cells in culture. Crystal attachment to MDCK-I cells and some MDCK-II cells involves PCM assembly that requires high-M_r HA synthesis. HA production and PCM formation do not play a role in crystal binding to LLC-PK_1 and the majority of MDCK-II cells. It remains to be determined which form of binding is involved in renal stone disease.

INTRODUCTION

Calcium oxalate kidney stones are composed of innumerable crystals formed by calcium and oxalate ions in the tubular fluid. Although the solubility product of calcium oxalate is exceeded already shortly after glomerular filtration, crystal nucleation is postponed to later nephron segments by tubular fluid constituents collectively indicated as "crystallization inhibitors". Despite the presence of these substances in

the primary urine, crystals are formed occasionally in the kidneys of stone formers as well as of subjects not forming stones. Crystal formation is harmless as long as the crystals are efficiently eliminated with the urine. The epithelial lining in the late nephron and also in more distal parts of the urinary tract is provided with a nonadherent surface. People are at risk of urinary tract stone formation when these antiadherent properties somehow are compromised [1-3].

Confluent monolayers formed in culture by renal distal tubule/collecting duct-like MDCK-I cells [4] and by primary cultures of human distal renal tubular cells [5] are nonadherent to calcium oxalate monohydrate (COM) crystals, the most common crystalline phase in kidney stones. In contrast, subconfluent cultures and monolayers recovering from mechanical injury are quite susceptible to crystal binding. From these observations, it was speculated that crystal retention in the kidney is caused by some form of tissue damage [6]. The polysaccharide hyaluronan (HA) was subsequently identified as major crystal-binding molecule (CBM) at the surface of renal tubular cells in locomotion [5, 7].

HA is a high molecular mass (M_r) polysaccharide (up to 10^4 kD) consisting of repeating units of disaccharides formed by glucuronic acid and N-acetylglucosamine (GlcNAc). HA is known for its ability to form "cell coats" or pericellular matrices (PCMs) surrounding eukaryotic cells, including fibroblasts [8], chondrocytes [9], keratinocytes [10], arterial smooth muscle cells [11] and tumor cells [12]. Linear polysaccharide chains synthesized by specific HA synthases (HAS-1, HAS-2 and HAS-3) at the inner surface of the plasma membrane are extruded into the extracellular space. Once outside the cell, the HA forms complexes with HA-binding proteins (HABPs) [13]. Due to the unique water-absorbing properties of these networks and the negative-charge repulsion between the various macromolecules, the whole construction straightens to extend up to several microns outward from the surface. These swollen coats remain associated with the cell surface by their HA synthases [13, 14] or via transmembrane HABPs, such as CD44 [15-17]. During embryonic development, tissue repair, and inflammation, these coats provide the microenvironment for many cellular activities such as proliferation, migration, and cell division [18-21].

In the kidney, large amounts of HA are found in the inner medullary portion and in the papilla, where it plays a role in renal water homeostasis [22]. Although the renal cortex usually contains only small amounts of HA, a pronounced accumulation is observed during

interstitial nephritis, ischemic injury and allograft rejection [23-26]. These inflammatory disease states in the kidney are frequently accompanied by an upregulated expression of CD44 by renal tubular cells [5, 27-29]. The identification of HA as CBM at the surface of activated renal tubule epithelial cells prompted us to study HA biology related processes, including PCM formation, HA synthesis, and CD44 expression by various renal tubular cell types in culture.

MATERIALS AND METHODS

Cell culture

MDCK-I and MDCK-II cells [30] were kindly provided by Prof. G. van Meer (Laboratory for Cell Biology and Histology, Academic Medical Centre, Amsterdam, The Netherlands). These strains are isolated from the wild-type American Type Culture Collection (ATCC) MDCK cell line. Although it is not possible to assign MDCK-I cells to a specific nephron segment, these cells exhibit many characteristics of the renal distal tubule/collecting ducts [30, 31], whereas MDCK strain II cells consists of cells with characteristics of the renal proximal and distal tubule [4]. LLC-PK_1 cells obtained from the Americam Type Culture Collection have widely been used as a model for the renal proximal tubule [32, 33]. Routinely, the cells are grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, N.Y., USA) supplemented with 10% fetal calf serum (PAA Laboratories, Linz, Austria) and weekly trypsinized and replated. To obtain subconfluent cultures, the cells were seeded at a plating density of 1 x 10^6 cells/24-mm polycarbonate porous filter insert (Transwell; Corning Costar, Badhoevedorp, The Netherlands). Two days after seeding, the inserts contain on average 1-2 x 10^6 cells. Although at this point the growth substrate is already entirely covered with cells, the maximal amount of cells per insert has not yet been reached, and tight junctions are not yet formed. Tests were routinely performed to assure that the cells used in this study were not contaminated with mycoplasma.

Particle exclusion assay

The presence of cell-associated coats was visualized by a particle exclusion assay, as first described by Clarris and Fraser [34]. This assay is based on the inability of relatively large particles, such as fixed red

blood cells, to penetrate HA-rich cell coats. Because conventional fixation/staining methods lead to the collapse of these hydrated coats, the particle exclusion assay is a useful technique to visualize their true size. Cells were seeded at $1x10^4$/well in DMEM + 10% fetal calf serum in 24-well plates (15 mm in diameter; Corning Costar). The next day, 50 µl of a suspension of formalin-fixed mouse red blood cells (10^9/ml) was added in serum-free DMEM. After settling for 15 min., the cells were inspected by phase-contrast microscopy for the presence of cell coats. The dependence of cell coats on HA was tested by treating coat-bearing cells with 5 U/ml *Streptomyces* hyaluronidase (E.C. 3.2.1.35; Sigma–Aldrich Chemie, Zwijndrecht, The Netherlands) which specifically digests HA. To determine the number of coat-bearing cells and the size of the coats, digital micrographs were taken from 100 randomly selected cells per cell line. These images were analyzed by KS-400 V2.0 image analysis software. Morphometric analysis was limited to single cells, and the size of the PCM was expressed as the ratio between the surface areas of the coat and the cell (coat/cell ratio) calculated from their diameters. Since the cell border is not clearly visualized by phase-contrast microscopy, we considered a coat to be present, if this ratio was equal to or larger than 1.50.

HA synthesis

Metabolic labeling studies were performed to explore the capability of the cells to synthesize HA. Proliferating cells seeded at a density of $1x10^6$/insert were starved overnight in low- glucose DMEM (1 g/l). One day after seeding, they were labeled in low-glucose medium with 74 kBq/ml [^{3}H]glucosamine – D-1-[^{3}H]GlcNAc hydrochloride (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). Two days after seeding, the apical fluid was collected. To distinguish between HA and other secreted glycoconjugates, we subjected portions of the apical fluid to differential enzyme digestion. One portion was left untreated (control) to measure the total glycoconjugate secretion. The second portion was incubated with papain overnight to digest proteins in the sample, but not the papain-resistant polysaccharide HA. Papain (E.C. 3.4.22.2; Sigma-Aldrich Chemie) was used at a final concentration of 5 U/ml at 60 °C and pH 6.0. To check whether the papain-resistant fraction indeed consisted of HA, the third portion was treated with papain as described above, followed by additional treatment with 5 U/ml *Streptomyces* hyaluronidase for 1 h at 37 °C. The macromolecules in the various

portions were assayed by Sephacryl S-1000 gel filtration, using 55 x 15-mm columns, that were equilibrated with the elution buffer (0.05 M Tris, 0.15 M NaCl, pH 8.0). Fifty-microliter samples were applied to each column and eluted in 250-µl fractions with the Tris-NaCl buffer. The elution pattern showed two major peaks, the first consisting of high-M_r [^{3}H]GlcNAc-labeled molecules (>10^3 kD) and the second with molecules <10^3 kD (including excess radiolabel). Synthesis was expressed as picomoles of [^{3}H]GlcNAc incorporated into glycoconjugates. This was calculated from the total amount of label added per insert (2.1 nmol) and the specific activity (107 GBq/mmol) of the precursor. The presence of cell surface-associated [^{3}H]GlcNAc-labeled HA was determined by measuring the release of radiolabel during 1 h incubation of the cells with serum-free DMEM with or without *Streptomyces* hyaluronidase. Additional release of [^{3}H]GlcNAc by the enzyme-treated cells, as compared with the spontaneous release by cells incubated without enzyme, was considered to represent cell surface-associated HA. [^{3}H]GlcNAc incorporation into the cells was determined by solubilizing the cells in 500 µl 4 M guanidine/2% Triton X-100/0.05 M sodium acetate buffer, pH 6.0. It was expressed as percentage of the total amount of precursor added per insert.

Hyaluronan and CD44 expression

Subconfluent cells grown on inserts were fixed for 10 min. in 3.7% formaldehyde/0.1% glutaraldehyde (v/v). Blocking of nonspecific binding as well as dilution of antibodies was done using 3% nonfat dry milk in phosphate-buffered saline/Tween 20 (w/v). HA was visualized using 5 µg/ml biotinylated hyaluronic acid binding protein (Seikagaku, Tokyo, Japan) coupled to 1:250 fluorescent avidin (avidin-FITC; Vector Laboratories, Burlingame, Calif., USA). CD44 was visualized using rat antimouse CD44 (IM7; PharMingen International, Woerden, The Netherlands), diluted 1:50, after coupling to FITC-conjugated rabbit antirat IgG (Southern Biotechnology Associates Inc, Birmingham, Ala., USA), diluted 1:100. The cells were incubated with primary antibodies overnight at 4°C, while incubation with secondary antibodies was performed for 1 h in the dark at room temperature. In some pictures, nuclei were counterstained with 1.0 µg/ml propidium iodide in phosphate-buffered saline. Confocal images were made using an LSM 410 laser-scanning microscope (Zeiss, Oberkochen, Germany). A 488-

nm argon laser was used to excite the FITC and a 543-nm laser to visualize propidium iodide.

Generation of COM crystals

A solution of sodium oxalate was prepared by adding 1 ml 0.37 MBq/ml [^{14}C]oxalic acid (Amersham International, Little Chalfont, UK) to 0.25 ml 200 mM sodium oxalate. A calcium chloride solution was prepared by adding 0.25 ml 200 mM calcium chloride to 9.5 ml distilled water. Mixing the two solutions at room temperature (final concentration of 5 mM for both oxalate and calcium) immediately resulted in the nucleation of radiolabeled COM. After settling for 3 days, crystals were washed three times with and resuspended in CaOx-saturated H_2O in a final volume of 5 ml (1.46 mg CaOx crystals/ml). The same procedure, but without [^{14}C]oxalic acid was used for the generation of nonradiolabeled COM. This procedure results in crystals with an average size of 1-2 μm [35]. Larger (5 μm) crystals for particle exclusion and confocal microscopy (CLSM) studies were prepared by using a final concentration of 1 mM for both calcium and oxalate [35].

Crystal binding

Subconfluent cells on inserts were pretreated or not with *Streptomyces* hyaluronidase in serum-free DMEM at a concentration of 5 U/ml, pH 5.3 at 37°C for 60 min, after which [^{14}C]COM crystal binding was performed as described earlier [7]. Briefly, radiolabeled crystals were added to the cultures and allowed to adhere for 1 h, after which the inserts were extensively rinsed to remove all nonassociated crystals, cut out, and counted in a liquid scintillation counter (Packard, Meriden, Conn., USA). The number of associated crystals was calculated from the disintegrations per minute per filter and usually expressed as micrograms per square centimeter.

Statistical analysis

Data were summarized as mean ± SD. The release of HA from the cell surface and the coat/cell ratios were analyzed by the unpaired Student t test. ANOVA was used to analyze the amount of [^{3}H]GlcNAc-labeled glycoconjugates secreted into the apical fluid. Differences were considered significant at $p < 0.05$.

RESULTS

Particle exclusion assay

To investigate whether renal tubular cells are able to form HA-rich pericellular coats, we applied a particle exclusion assay. Phase-contrast microscopy revealed that 24 h after seeding most MDCK-I cells (~91%) indeed were surrounded by prominent areas that excluded fixed red blood cells. Cell coats were also observed in a minority of the MDCK-II cells (~23 %), but LLC-PK_1 cells did not form PCMs (Fig. 1). The dependence of the cell coats on HA was shown by the disappearance of PCMs surrounding MDCK cells, shortly after the addition of hyaluronidase (Fig. 1, right corner).

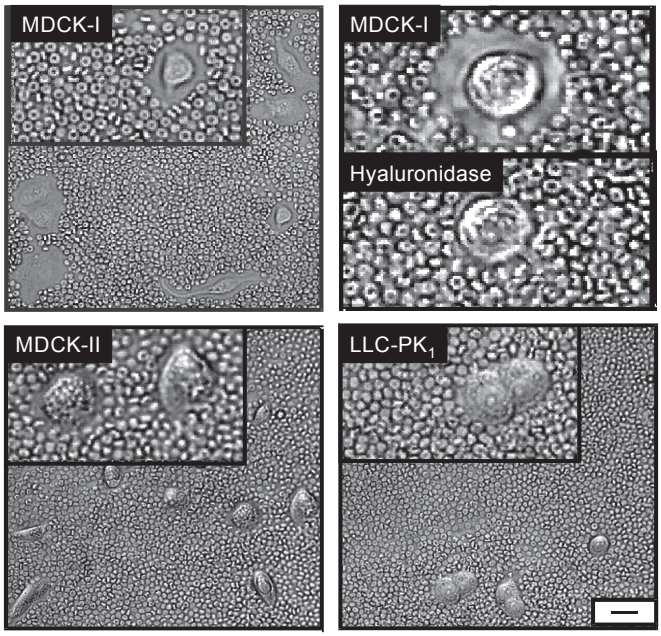


Figure 1. Particle exclusion assay. *Proliferating MDCK-I cells are surrounded by PCMs. The PCMs disappear shortly after the addition of Streptomyces hyaluronidase (top, right). PCMs are formed by a minority of the MDCK-II cells, but not by LLC-PK_1 cells. Bar = 10 μm.*

The size of the PCMs was expressed as coat/cell ratio. Coats surrounding MDCK-I and MDCK-II cells cultured in the presence of 10% fetal calf serum were similar in size (Table 1). To assess whether coat formation depended on the presence of serum components, we studied PCM assembly in serum-deprived cells. After a 4-hour plating period in serum-containing DMEM, the cells were washed and further cultured overnight under serum-free conditions. This serum deprivation did not alter the percentage of coat-bearing MDCK-I and MDCK-II cells, or the coat/cell ratios (Table 1).

Table 1. Coat/cell ratios.

	10% serum coat/cell ratio	% cells with coat	serumdeprived coat/cell ratio	% cells with coat
MDCK-I	2.07 ± 0.48	91	1.99 ± 0.39	94
MDCK-II	1.94 ± 0.34	23	1.60 ± 0.10	21
LLC-PK_1	< 1.50	not applicable	<1.50	not applicable

Using the particle exclusion assay, coat/cell ratios were measured for 100 cells per cell line. Due to the limitations of phase-contrast microscopy, a cell is considered coat-bearing when the coat/cell ratio is ≥ 1.50. Mean ± SD calculated for coat-bearing cells only. Statistical analysis by Student's t-test.

Metabolic labeling studies

Since most MDCK-I and some MDCK-II cells, but none of the LLC-PK_1 cells, assembled PCMs, we hypothesized that these differences could arise from variations in the synthesis of high-M_r HA between those cell types. For that reason, we studied the ability of the various cell types to synthesize HA by measuring the incorporation of [^{3}H]-N-acetyl-glucosamine ([^{3}H]GlcNAc) into high-M_r glycoconjugates. Two days after seeding, comparable amounts of [^{3}H]GlcNAc were incorporated in MDCK-I, MDCK-II and LLC-PK_1 cells (5.3-9.1 % of applied label). Representative elution patterns of apical culture medium collected from cells after 24-hour metabolic labeling are shown in figure 2. Untreated supernatants collected from MDCK-I cells contained two major peaks,

the first one between fractions 6 and 13, containing labeled macromolecules larger than 10^3 kD, and a second much larger peak between fractions 14 and 30, containing excess precursor molecules and radiolabeled glycoconjugates smaller than 10^3 kD (Fig. 2, top left). The high-M_r peak decreased about 50% in size after papain treatment, whereas the remaining macromolecular substances were completely digestible by bacterial hyaluronidase. Although MDCK-II cells also produced high-M_r glyconconjugates, the amounts were much smaller than those produced by MDCK-I, whereas LLC-PK_1 did not produce [^{3}H]-labeled molecules >10^3 kD (Fig. 2). The amount of [^{3}H]GlcNAc incorporated in high-M_r HA was estimated from the amount (pmol) of high-M_r molecules remaining after papain treatment (Table 2, Papain) minus the calculated amount (pmol) of high-M_r molecules remaining after the papain-treated sample was further digested with hyaluronidase (Table 2, Papain + hyaluronidase). The estimated amounts of label incorporated in high-M_r HA for MDCK-I, MDCK-II and LLC-PK_1 were, respectively, 14.7, 0.5 and 0 pmol.

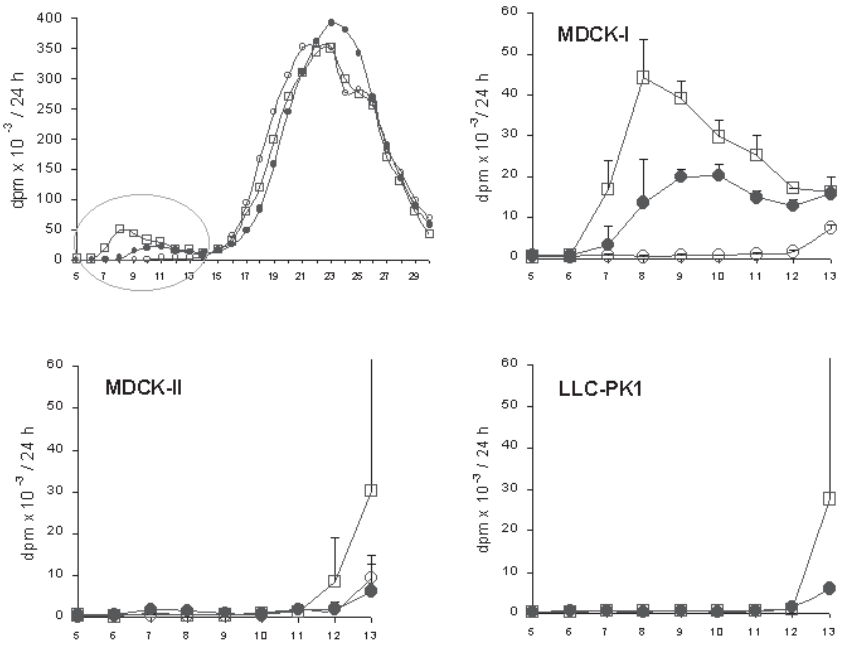


Figure 2

Table 2. Hyaluronan synthesis.

	[^{3}H]GlcNAc in glycoconjugates >10^3 kD, pmol				
	papain	-	papain + hyaluronidase	=	high-M_r HA
MDCK-I	15.5 ± 2.2	-	0.8 ± 0.2*	=	14.7 ± 1.6
MDCK-II	1.2 ± 0.3	-	0.7 ± 0.1*	=	0.5 ± 0.2
LLC-PK$_1$	0.6 ± 0.1	-	0.6 ± 0.1	=	0

*Aliquots of the apical fluid of [^{3}H]GlcNAc-labeled subconfluent cells were analyzed by Sephacryl S-1000 gel filtration for the presence of high-M_r glycoconjugates. Papain was added to the samples to digest proteins (papain) after which hyaluronidase was added to digest HA (papain + hyaluronidase). The difference between the papain- and hyaluronidase-digested fractions represents the amount high-M_r HA in the original sample; high-M_r HA = (papain) – (papain + hyaluronidase). Results expressed as picomoles [^{3}H]GlcNAc incorporated into high-M_r glycoconjugates in the total apical fluid volume. Mean ± SD of three independent samples of a representative experiment. Statistical analysis by ANOVA. *Significantly reduced as compared with samples treated with papain alone ($p < 0.05$).*

Figure 2. High-M_r hyaluronan synthesis. *Aliquots of the apical fluid of [^{3}H]GlcNAc-labeled MDCK-I, MDCK-II and LLC-PK$_1$ cells were analyzed by Sephacryl S-1000 gel filtration for papain- and hyaluronidase-sensitive high-M_r glycoconjugates. The whole elution pattern derived from MDCK-I cells is depicted in the top left image, whereas the other three images are enlargements of fraction 6-13 obtained from by the various cell types. The left top image shows a first peak between fractions 6-13 containing molecules >10^3 kD and a second peak between fractions 14 and 30 containing molecules <10^3 kD and excess [^{3}H]GlcNAc precursor. Untreated samples were run to assess total cellular production of high-Mr glycoconjugates (open squares). Aliquots were treated with papain to digest proteins (closed circles) and subsequently treated with hyaluronidase to digest HA (open circles). Mean ± SD of three independent samples of a representative experiment.*

Table 3. Cell surface-associated hyaluronan.

	[^{3}H]GlcNAc released from the cell surface, pmol				
	hyaluronidase	-	spontaneous	=	HA
MDCK-I	9.7 ± 0.3*	-	6.0 ± 0.6	=	3.7 ± 0.4
MDCK-II	2.9 ± 0.2*	-	2.3 ± 0.5	=	0.6 ± 0.3
LLC-PK$_1$	1.3 ± 0.3	-	1.1 ± 0.1	=	0.2 ± 0.1

*The amount of cell surface bound HA was estimated from the spontaneous and Streptomyces hyaluronidase-induced release of radioactivity from metabolically ([^{3}H]GlcNAc) labeled cells (hyaluronidase-induced release - spontaneous release = HA). Mean ± SD of three independent inserts derived from a representative experiment. Statistical analysis by Student's t test (one tailed). *Significantly higher than spontaneous release ($p < 0.05$).*

The amount of cell-surface-associated HA was estimated from the amounts of label released by *Streptomyces* hyaluronidase minus that released by vehicle alone. Hyaluronidase released significantly more label from MDCK-I than from MDCK-II, whereas this treatment did not release significant amounts of radioactivity from LLC-PK$_1$ cells (Table 3).

Cell surface HA and CD44 expression

Confocal microscopy staining using biotinylated HABPs revealed that MDCK-I cells were entirely covered with a thin layer of HA, whereas MDCK-II cells expressed a discontinuous layer of HA and LLC-PK$_1$ cells did not express HA (Fig. 3). Since the transmembrane protein CD44 plays an important role in the retention of HA-containing coats in many eukaryotic cell types, we also studied the expression of CD44 by the various proliferating renal tubular cells. Immunohistochemical studies revealed that CD44 was expressed by subconfluent cultures of all three cell types (Fig. 3).

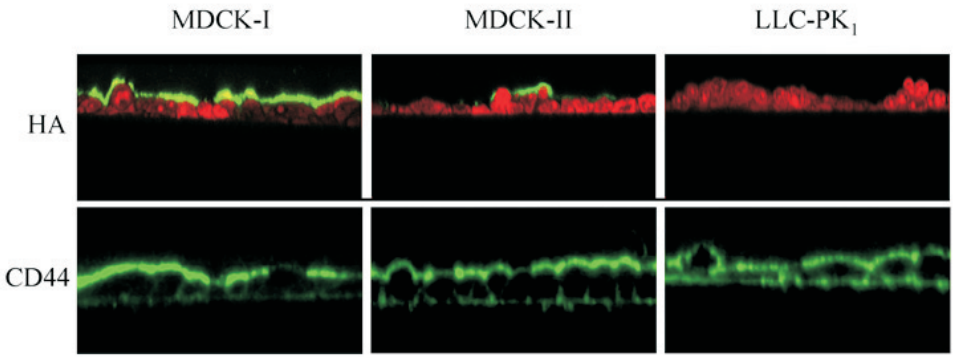


Figure 3. HA and CD44 expression. *Confocal images of proliferating cells stained with specific probes for HA and CD44 were made perpendicular to the growth substrate. MDCK-I cells uniformly expressed HA, whereas the HA staining pattern was dispersed at the surface of MDCK-II cells. LLC-PK$_1$ cells were HA negative (top row). All three cell types expressed CD44 at their apical surface (bottom row). x 63 .*

Table 4. Crystal binding.

	[^{14}C]COM crystal binding (μg/cm^2)	
	untreated	hyaluronidase
MDCK-I	8.60 ± 0.40	3.88 ± 0.90*
MDCK-II	10.23 ± 0.18	9.64 ± 0.12*
LLC-PK$_1$	10.15 ± 0.84	9.94 ± 0.27

Subconfluent cultures were incubated for 1 h with Streptomyces hyaluronidase or left untreated prior to incubating the cells for 1 h with [^{14}C]COM crystals.
*Mean ± SD of three independent inserts. Statistical analysis by Student's t test. *Significantly different as compared with untreated controls ($p < 0.05$).*

COM crystal binding

To determine whether HA-rich PCMs were involved in the binding of COM crystals to proliferating cells, subconfluent cultures of MDCK and LLC-PK_1 were treated with *Streptomyces* hyaluronidase prior to the incubation with [^{14}C]COM. In all cell lines, COM binding to control cells was high. Hyaluronidase treatment significantly reduced crystal binding to MDCK-I, hardly affected the affinity of MDCK-II for the crystals and had no effect on the adherence of crystals to LLC-PK_1 (Table 4).

DISCUSSION

This study shows that practically all MDCK strain I cells and part of the MDCK strain II cells assemble PCMs during proliferation, whereas LLC-PK_1 cells are unable to form these "cell coats". High-M_r HA is synthesized by proliferating MDCK cells, but not by LLC-PK_1 cells. The PCM formation plays an important role in COM crystal binding to MDCK-I cells, but is less important in the attachment of crystals to the other two cell lines.

Confocal microscopy studies showed that subconfluent cultures of MDCK-I are entirely covered with HA, while MDCK-II cultures are partially positive, and LLC-PK_1 cells do not express cell surface HA. The coats as well as the surface expression of HA disappear shortly after the addition of *Streptomyces* hyaluronidase, an enzyme that specifically cleaves β-1,4-glycosidic bonds in HA chains, indicating the crucial role of this polysaccharide in PCM formation.

The question that we subsequently addressed is why some of our cells form PCMs whereas others do not. Could it be that cells without PCMs do not express cell surface receptors with affinity for HA, or are these coat-free cells unable to produce high-M_r HA? Confocal microscopy studies showed that during subconfluence all three cell types expressed CD44 at their apical membrane (Fig. 3). Although we did not in detail study the HA-binding capacity of these membrane receptors, the presence of CD44 at the luminal surface suggests that the cells possess the required machinery for PCM retention. Furthermore, metabolic labeling studies demonstrated that proliferating MDCK-I cells produced relatively large amounts of high-Mr HA, whereas only very small amounts of HA were synthesized by MDCK-II cells and LLC-PK_1 did not produce high-M_r HA. These results indicate that only those cells that

are able to synthesize macromolecular HA assemble PCMs. Coat-forming MDCK-I cells not only produced significant amounts of high-M_r HA, but also relatively large amounts of high- M_r glycoproteins. Large proteoglycans such as aggrecan and versican serve as HABPs in the HA networks surrounding other cell types, such as chondrocytes [18]. It is, therefore, possible that MDCK-I cells not only produce the required amount high-M_r HA for successful PCM formation, but also the HA-binding glycoconjugates needed for coat stabilization [13].

Because in some cell types in culture PCM formation is supported by fetal calf serum components [36-38], we investigated coat formation by MDCK cells during serum deprivation. The number of coats as well as their sizes were not different under serum-deprived conditions, indicating that the PCMs surrounding renal tubular cells are predominantly composed of endogenous components. Coats formed by MDCK-II cells exhibited coat/cell ratios similar to those formed by MDCK-I cells. The two MDCK strains are both isolated from wild-type MDCK which was derived from the whole kidney of a dog. Because MDCK-II cells exhibit properties of more than one segment of the nephron, these observation suggests that MDCK strain II is not homogeneous and also seems to contain some MDCK-I cells.

The finding that LLC-PK_1 cells are unable to synthesize high-M_r HA and assemble PCMs seems to suggest that these coats are not formed in the renal proximal tubule. However, HA synthesis and PCM assembly are basic tools of most eukaryotic cells and there are reports about the synthesis of HA by various renal tubular cells, including proximal tubular cells. Mouse cortical tubular cells and primary cultures of human renal proximal tubular cells secrete HA, especially in response to cytokines [23, 39]. Recently, we observed that primary cultures of both human proximal and distal tubular cells were covered with HA [5]. It is possible that LLC-PK_1 and the majority of MDCK-II cells have lost the ability to synthesize high-M_r HA, emphasizing potential shortcomings in cell culture studies. Another possibility is that certain cell types are not supposed to synthesize and secrete HA or that there are species differences. More detailed studies are clearly warranted to answer these and other questions. For the time being, the subconfluent proximal-tubule-like LLC-PK_1 cells in Figure 4 are provided with an "imaginary PCM" (dotted line).

Subconfluent cultures formed by all three cell types are highly susceptible to COM crystal binding. Treatment with hyaluronidase

significantly reduced the affinity of the MDCK-I cell surface for crystals, whereas it hardly or not affected crystal binding to MDCK-II and LLC-PK_1 cells (Table 4). Thus, this study shows that coats formed by HA at the surface of MDCK-I cells serve as major CBMs, but that these coats do not play an important role in crystal binding to LLC-PK_1 or MDCK-II cells. From these observations it can be deduced that HA certainly is not the only constituent of the renal tubular cell surface with affinity for calcium oxalate crystals. The finding that the enzymatic removal of the PCMs from MDCK-I cells only leads to a 50% reduction in crystal binding suggests the presence of apical membrane CBMs beneath the coats. Previously, we have demonstrated that at confluence not only HA disappears from the MDCK-I cell surface, but since these cells practically become nonadherent, apparently also the CBMs that were located underneath the coat [7]. Most likely the latter group of CBMs represents epithelial-polarity-related plasma membrane components which are translocated to the basolateral membrane after the development of a functional epithelium. COM crystals avidly adhere to coat-free LLC-PK_1 cells, indicating that crystal binding to this cell line is mediated by cell surface constituents distinct from HA. Previously, we reported that this cell type does not lose its affinity for COM crystals after its development to intact polarized monolayers [4]. This suggests that, besides possible polarization-dependent CBMs, proximal tubular cells also express cell surface CBMs that do not disappear from the apical membrane at confluence or that disappearing CBMs are replaced by others. The identity of CBMs located beneath HA-rich PCMs expressed by late- nephron cells in locomotion or by early-nephron cells without PCMs is still unknown. It is possible that under these conditions sialic acid residues are exposed in a configuration that permits crystal attachment [2, 40] or that the cells express nucleonin-like calcium-binding proteins [41]. Another possibility is that these CBMs are “sticky” extracellular matrix molecules, such as laminin, collagen IV [42], fibronectin, or osteopontin [43], molecules that normally are restricted to the basolateral membrane. A schematic summary of CBM expression by MDCK-I and LLC-PK_1 is depicted in figure 4. This presentation does not include the results obtained with MDCK-II because this cell line seems to be a mixture of the proximal- and distal-tubule-like cell types.

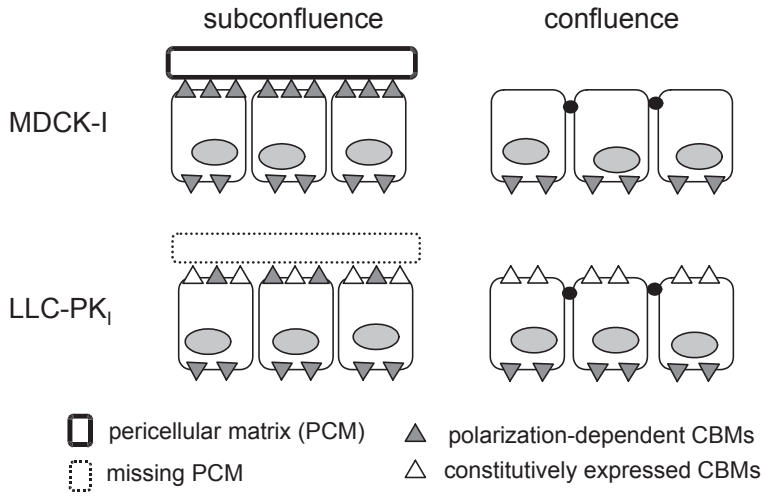


Figure 4. Crystal binding molecules (CBMs). *At subconfluence, MDCK-I cells express PCMs and polarization-dependent CBMs which all disappear from the luminal side of the cultures at confluence. Although LLC-PK$_l$ cells populating subconfluent cultures do not express PCMs, these coats presumably will be formed by activated proximal tubular cells in the kidney, which is why the missing PCM is depicted as a dotted line. Furthermore, it is hypothesized that LLC-PK$_l$ cells in locomotion express polarization-dependent and -independent CBMs, of which the latter type (constitutive CBM) is still present at the apical surface of confluent monolayers.*

Assuming that the surface properties of MDCK-I cells indeed are representative of cells lining the renal distal tubule/collecting duct and that those of LLC-PK$_l$ are largely in agreement with cells in the renal proximal tubule, then what is the significance of this and earlier reports in relation to crystal retention? In the first place, our studies suggest that proliferating renal tubular cells are always susceptible to crystal binding. However, such massive proliferation by renal tubular cells does not take place unless the renal tubular epithelium is significantly injured. In the second place, crystals adhere to early-nephron cells in their normal quiescent state, but not to late-nephron cells. Considering the stone salt concentrations in the tubular fluid, crystallization is a rare event in the early nephron and only to be expected when plasma oxalate is considerably raised, such as, for example in primary hyperoxalurias or after oxalate intoxications. Taken together, these observations suggest that proximal tubular cells are constitutively covered with molecules with

affinity for calcium oxalate crystals, whereas distal tubular/collecting duct cells as a rule are protected from crystal binding.
Collectively, the results from this investigation provide evidence for the concept that crystal retention in the renal distal tubule/collecting ducts requires PCM formation, whereas the formation of calcium oxalate crystals in the renal proximal tubule inevitably leads to crystal binding and nephrocalcinosis. The appreciation of these various forms of crystal retention may have its repercussion in the management of the various forms of nephrolithiasis, including those associated with inborn errors in oxalate metabolism and idiopathic recurrent calcium oxalate stone disease.

REFERENCES

1. Khan SR, Hackett RL: Retention of calcium oxalate crystals in renal tubules. *Scanning Microsc* 5:707-711; discussion 711-702, 1991
2. Lieske JC, Toback FG, Deganello S: Sialic acid-containing glycoproteins on renal cells determine nucleation of calcium oxalate dihydrate crystals. *Kidney Int* 60:1784-1791, 2001
3. Asselman M, Verkoelen CF: Crystal-cell interaction in the pathogenesis of kidney stone disease. *Curr Opin Urol* 12:271-276, 2002
4. Verkoelen CF, van der Boom BG, Kok DJ, Houtsmuller AB, Visser P, Schroder FH, Romijn JC: Cell type-specific acquired protection from crystal adherence by renal tubule cells in culture. *Kidney Int* 55:1426-1433, 1999
5. Verhulst A, Asselman M, Persy VP, Schepers MSJ, 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 non-adherent epithelium. *J Am Soc Nephrol*, in press
6. Verkoelen CF, van der Boom BG, Houtsmuller AB, Schroder FH, Romijn JC: Increased calcium oxalate monohydrate crystal binding to injured renal tubular epithelial cells in culture. *Am J Physiol* 274:F958-965, 1998
7. Verkoelen CF, Van der Boom BG, Romijn JC: Identification of hyaluronan as a crystal-binding molecule at the surface of migrating and proliferating MDCK cells. *Kidney Int* 58:1045-1054, 2000
8. Hedman K, Kurkinen M, Alitalo K, Vaheri A, Johansson S, Hook M: Isolation of the pericellular matrix of human fibroblast cultures. *J Cell Biol* 81:83-91, 1979
9. Knudson CB, Nofal GA, Pamintuan L, Aguiar DJ: The chondrocyte pericellular matrix: a model for hyaluronan-mediated cell-matrix interactions. *Biochem Soc Trans* 27:142-147, 1999
10. Pienimaki JP, Rilla K, Fulop C, Sironen RK, Karvinen S, Pasonen S, Lammi MJ, Tammi R, Hascall VC, Tammi MI: Epidermal growth factor activates

hyaluronan synthase 2 in epidermal keratinocytes and increases pericellular and intracellular hyaluronan. *J Biol Chem* 276:20428-20435, 2001

11. Evanko SP, Johnson PY, Braun KR, Underhill CB, Dudhia J, Wight TN: Platelet-derived growth factor stimulates the formation of versican-hyaluronan aggregates and pericellular matrix expansion in arterial smooth muscle cells. *Arch Biochem Biophys* 394:29-38, 2001
12. Simpson MA, Wilson CM, Furcht LT, Spicer AP, Oegema TR, Jr., McCarthy JB: Manipulation of hyaluronan synthase expression in prostate adenocarcinoma cells alters pericellular matrix retention and adhesion to bone marrow endothelial cells. *J Biol Chem* 277:10050-10057, 2002
13. Laurent TC, Fraser JR: Hyaluronan. *Faseb J* 6:2397-2404, 1992
14. Weigel PH, Hascall VC, Tammi M: Hyaluronan synthases. *J Biol Chem* 272:13997-14000, 1997
15. Lesley J, Hyman R: CD44 structure and function. *Front Biosci* 3:D616-630, 1998
16. Peach RJ, Hollenbaugh D, Stamenkovic I, Aruffo A: Identification of hyaluronic acid binding sites in the extracellular domain of CD44. *J Cell Biol* 122:257-264, 1993
17. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B: CD44 is the principal cell surface receptor for hyaluronate. *Cell* 61:1303-1313, 1990
18. Knudson CB, Knudson W: Hyaluronan-binding proteins in development, tissue homeostasis, and disease. *Faseb J* 7:1233-1241, 1993
19. Toole BP: Hyaluronan in morphogenesis. *J Intern Med* 242:35-40, 1997
20. Chen WY, Abatangelo G: Functions of hyaluronan in wound repair. *Wound Repair Regen* 7:79-89, 1999
21. Mohamadzadeh M, DeGrendele H, Arizpe H, Estess P, Siegelman M: Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. *J Clin Invest* 101:97-108, 1998
22. Hansell P, Goransson V, Odlind C, Gerdin B, Hallgren R: Hyaluronan content in the kidney in different states of body hydration. *Kidney Int* 58:2061-2068., 2000
23. Feusi E, Sun L, Sibalic A, Beck-Schimmer B, Oertli B, Wuthrich RP: Enhanced hyaluronan synthesis in the MRL-Fas(lpr) kidney: role of cytokines. *Nephron* 83:66-73, 1999
24. Jun Z, Hill PA, Lan HY, Foti R, Mu W, Atkins RC, Nikolic-Paterson DJ: CD44 and hyaluronan expression in the development of experimental crescentic glomerulonephritis. *Clin Exp Immunol* 108:69-77., 1997
25. Johnsson C, Tufveson G, Wahlberg J, Hallgren R: Experimentally-induced warm renal ischemia induces cortical accumulation of hyaluronan in the kidney. *Kidney Int* 50:1224-1229, 1996
26. Wells A, Larsson E, Hanas E, Laurent T, Hallgren R, Tufveson G: Increased hyaluronan in acutely rejecting human kidney grafts. *Transplantation* 55:1346-1349, 1993
27. Benz PS, Fan X, Wuthrich RP: Enhanced tubular epithelial CD44 expression in MRL-lpr lupus nephritis. *Kidney Int* 50:156-163, 1996
28. Lewington AJ, Padanilam BJ, Martin DR, Hammerman MR: Expression of CD44 in kidney after acute ischemic injury in rats. *Am J Physiol Regul Integr Comp Physiol* 278:R247-254., 2000

29. Sibalic V, Fan X, Loffing J, Wuthrich RP: Upregulated renal tubular CD44, hyaluronan, and osteopontin in kdkd mice with interstitial nephritis. *Nephrol Dial Transplant* 12:1344-1353, 1997
30. Hansson GC, Simons K, van Meer G: Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. *Embo J* 5:483-489, 1986
31. Richardson JC, Scalera V, Simmons NL: Identification of two strains of MDCK cells which resemble separate nephron tubule segments. *Biochim Biophys Acta* 673:26-36, 1981
32. Nielsen R, Birn H, Moestrup SK, Nielsen M, Verroust P, Christensen EI: Characterization of a kidney proximal tubule cell line, LLC-PK1, expressing endocytotic active megalin. *J Am Soc Nephrol* 9:1767-1776., 1998
33. Verkoelen CF, Kok DJ, van der Boom BG, de Jonge HR, Schroder FH, Romijn JC: LLC-PK1 cells as a model system to study proximal tubule transport of water and other compounds relevant for renal stone disease. *Urol Res* 27:109-115, 1999
34. Clarris BJ, Fraser JR: On the pericellular zone of some mammalian cells in vitro. *Exp Cell Res* 49:181-193, 1968
35. Verkoelen CF, Romijn JC, de Bruijn WC, Boeve ER, Cao LC, Schroder FH: Association of calcium oxalate monohydrate crystals with MDCK cells. *Kidney Int* 48:129-138, 1995
36. Blom A, Pertoft H, Fries E: Inter-alpha-inhibitor is required for the formation of the hyaluronan- containing coat on fibroblasts and mesothelial cells. *J Biol Chem* 270:9698-9701, 1995
37. Gallivan EK, Crombleholme TM, Moriarty KP: Effect of fetal serum on fibroblast pericellular matrix formation. *J Surg Res* 64:128-131, 1996
38. Huang L, Yoneda M, Kimata K: A serum-derived hyaluronan-associated protein (SHAP) is the heavy chain of the inter alpha-trypsin inhibitor. *J Biol Chem* 268:26725-26730, 1993
39. Jones S, Phillips AO: Regulation of renal proximal tubular epithelial cell hyaluronan generation: Implications for diabetic nephropathy. *Kidney Int* 59:1739-1749., 2001
40. Verkoelen CF, van der Boom BG, Kok DJ, Romijn JC: Sialic acid and crystal binding. *Kidney Int* 57:1072-1082, 2000
41. Sorokina EA, Kleinman JG: Cloning and preliminary characterization of a calcium-binding protein closely related to nucleolin on the apical surface of inner medullary collecting duct cells. *J Biol Chem* 274:27491-27496, 1999
42. Kohri K, Kodama M, Ishikawa Y, Katayama Y, Matsuda H, Imanishi M, Takada M, Katoh Y, Kataoka K, Akiyama T, et al.: Immunofluorescent study on the interaction between collagen and calcium oxalate crystals in the renal tubules. *Eur Urol* 19:249-252, 1991
43. Yamate T, Kohri K, Umekawa T, Konya E, Ishikawa Y, Iguchi M, Kurita T: Interaction between osteopontin on madin darby canine kidney cell membrane and calcium oxalate crystal. *Urol Int* 62:81-86, 1999

CHAPTER 4

INTERNALIZATION OF CALCIUM OXALATE CRYSTALS BY RENAL TUBULAR CELLS: A NEPHRON SEGMENT-SPECIFIC PROCESS?

M.S.J. Schepers, R.A.J. Duim, M. Asselman,
J.C. Romijn, F.H. Schröder and C.F. Verkoelen

Kidney International 2003; 64(2):493-500

ABSTRACT

Background. Crystal retention in the kidney is caused by the interaction between crystals and the cells lining the renal tubules. These interactions involve crystal attachment, followed by internalization or not. Here, we studied the ability of various renal tubular cell lines to internalize calcium oxalate monohydrate (COM) crystals.
Methods. Crystal-cell interactions are studied by light-, electron- and confocal microscopy with cells resembling the renal proximal tubule [porcine kidney (LLC-PK_1)], proximal/distal tubule [Madin-Darby Canine Kidney II (MDCK-II)] and distal tubule and/or collecting ducts [Madin Darby Canine Kidney I (MDCK-I), rat cortical collecting duct 1 ($RCCD_1$)]. Crystal-binding strength and internalization are characterized and quantified with radiolabeled COM.
Results. Microscopy studies showed that crystals were firmly embedded in the membranes of LLC-PK_1 and MDCK-II cells to be subsequently internalized. On the other hand, crystals bound only loosely to MDCK-I and $RCCD_1$ and were not taken up by these cells. Crystal uptake by LLC-PK_1 and MDCK-II, expressed in $\mu g/10^6$ cells, is temperature-dependent and gradually increased from 0.88 and 0.15 in 30 minutes, respectively, to 4.70 and 3.85, respectively, after five hours, whereas these values never exceeded background levels in MDCK-I and $RCCD_1$ cells.
Conclusions. The adherence of COM crystals to renal cells with properties of the proximal tubule is inevitable and actively followed by their uptake, whereas crystals attached to cells resembling the distal tubule and/or collecting duct are not internalized. Since crystal formation usually occurs in segments beyond the renal proximal tubule, crystal uptake may be of less importance in the etiology of idiopathic calcium oxalate stone disease.

INTRODUCTION

Kidney stones are composed of myriad microliths pasted with organic material. Although most stones are found in the renal pelvis, the stone-forming process actually starts already in the nephrons. Crystals are frequently precipitated in the renal tubular fluid of stone-forming as well as non-stone-forming individuals [1]. Following their formation in the nephron, the crystals are rapidly covered with inhibitors of crystal-

lization, which facilitates their excretion [2]. This elimination process is thwarted, however, when the crystals adhere to the tubular lining [3]. Several investigators in the past have proposed the concept that the onset of nephrolithiasis lies within the nephron. More than 30 years ago, Malek and Boyce [4] collected renal biopsies from idiopathic calcium oxalate stone formers who underwent nephrolithotomy, and from patients who were operated for other renal disorders. They found microliths lying in nephrons of all stone formers, whereas intranephronic calculosis, as this phenomenon was called, was observed in only a minority of the non-stone formers. Later, Hering et al. [5] found microliths inside the renal collecting ducts of stone formers who required nephrectomy. More recently, Lieske et al. [6] not only found intra-nephronic crystals in renal biopsies of a patient with type I primary hyperoxaluria, but strikingly they also found crystals inside renal tubular cells. Cell culture studies subsequently showed that calcium oxalate monohydrate (COM) crystals were taken up by renal tubular cells, and that this process stimulated cell proliferation [7, 8]. On the basis of these results, the authors proposed that this response of renal cells to crystals could play a role in the development of kidney stones. Although we extensively studied the adherence of COM crystals to renal tubule epithelial cells in culture, crystal uptake received little attention. The polysaccharide hyaluronan (HA) was identified as major crystal binding molecule at the surface of Madin-Darby canine kidney (MDCK) strain I cells in locomotion, whereas HA-negative quiescent cells in confluent cultures are non-adherent [9]. The level of crystal binding to rat cortical collecting duct ($RCCD_1$) cells also significantly decreased during their growth to confluence (unpublished observations). Crystal binding to porcine kidney ($LLC\text{-}PK_1$) and Madin-Darby canine kidney II (MDCK-II) cells is quite different because, irrespective of their stage of development, these cells constitutively express crystal binding molecules [10]. The present investigation was conducted to study the ability of various cell types to internalize calcium oxalate crystals.

METHODS

Cell culture

MDCK-I and MDCK-II cells [11] were kindly provided by Prof. G. van Meer (Laboratory for Cell Biology and Histology, Amsterdam Medical Center, The Netherlands). LLC-PK_1 cells were obtained from the American Type Culture Collection (ATCC). Routinely, the cells were grown in Dulbecco's modified Eagle's Medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum (PAA Labs, Linz, Austria) and replated weekly. $RCCD_1$ cells [12] were kindly provided by Dr. M. Blot-Chabaud (INSERM U246, Faculté de Médecine Xavier Bichat, Paris, France). Routinely, these cells were grown in DMEM/F12 supplemented with 14 mmol/L $NaHCO_3$, 2 mmol/L glutamine, 5 µg/mL transferrin, 5 µg/mL insulin, 1.3 ng/mL sodium selenite, 10 µg/mL EGF, 5 x 10^{-8} mmol/L T_3, 10 U/mL penicillin-streptomycin and 2% FCS in collagen-coated flasks (collagen-G; 100 µg/mL, Biochrom KG, Berlin, Germany) and replated weekly. Most studies described in the present investigation were obtained using subconfluent cultures. The rationale to use subconfluent cultures was that crystals do not adhere to collecting duct cells populating confluent cultures, which makes it impossible to study crystal binding and uptake at that stage of epithelial development. To obtain subconfluent cultures, cells were seeded at a plating density of 1 x 10^6 cells on 24 mm polycarbonate porous filter inserts (Corning Costar, Badhoevedorp, The Netherlands). $RCCD_1$ cells were grown on collagen-coated inserts. Two days post-seeding the inserts contain on average 1 to 2 x 10^6 cells. We considered these cultures subconfluent as it takes a few more days before the maximal amount of cells per insert (3 to 4 x 10^6 cells) and the maximal transepithelial electrical resistances (TER) are reached [11,12]. Routinely, polymerase chain reaction (PCR) analyses were performed on DNA isolated from cell culture- conditioned medium for the presence of mycoplasma. Cells used in this study were not contaminated with mycoplasma.

Crystal formation

A solution of sodium oxalate was prepared by adding 10 µL 37 MBq/mL [^{14}C] oxalic acid (Amersham Int.plc, Buckinghamshire, UK) to 0.25 mL 200 mmol/L sodium oxalate. A calcium chloride solution was prepared by adding 0.25 mL 200 mmol/L calcium chloride to 9.5 mL distilled water. Mixing the two solutions at room temperature (final concentration

of 5 mmol/L for both oxalate and calcium) immediately resulted in the nucleation of radiolabeled COM. After settling for three days, crystals were washed three times with, and resuspended in, CaOx-saturated H_2O in a final volume of 5 mL (1.46 mg COM crystals/mL). The same procedure but without [^{14}C] oxalic acid was used for the generation of nonradiolabeled COM. The crystals have an average size of 1 to 2 µm [13]. For the light (phase-contrast) microscopy studies we used a slightly different protocol in which the final concentration of both calcium and oxalate was 1 mmol/L, which resulted in larger crystals with an average size between 5 to 6 µm [11].

Phase-contrast microscopy

Twenty-four hours post-seeding on solid growth substrates, crystals were added to proliferating small islands of cells and allowed to adhere for 30 minutes, after which all nonadhered crystals were removed and the cell surface-associated crystals inspected (magnification, x200) under a phase-contrast microscope (Axiovert 25) coupled with an AxioCam camera scanner (Carl Zeiss Vision GmbH, Munchen-Hallbergmoos, Germany).

Scanning electron microscopy

Cells cultured on porous supports were fixed in 2.5% glutaraldehyde in 0.15 mol/L cacodylate buffer for 4 hours. After washing in 0.1 mol/L cacodylate buffer for 3 hours, the samples were postfixed in OsO_4 in 0.1 mol/L cacodylate buffer for 4 hours, washed again in cacodylatebuffer for 3 hours, dehydrated in a graded series of ethanol and finally critical point dried. After mounting on stubs, a conductive layer was sputtercoated on the samples and examined in a Philips SEM 525 (Philips, Eindhoven, The Netherlands) (magnification, x 20.000).

Transmission electron microscopy

Cells were fixed in 1.5% (vol/vol) glutaraldehyde in 0.1 mol/L cacodylate/HCl buffer for 1 hour at 37°C. After aldehyde fixation, cells were postfixed in 1% (weight/vol) OsO_4 in the same buffer system, to which $K_4Fe(CN_6).3H_2O$ was added to a final concentration of 0.05 mol/L for 1 hour at room temperature. The cells were routinely dehydrated and embedded in Epon. Ultrathin sections (30 to 50 nm) were sectioned with diamond knives, collected on unfilmed 400 mesh copper grids and

inspected in a Zeiss EM 902 transmission electron microscope (Zeiss, Oberkochen, Germany) operating at 80 kV (magnification, x 2500).

Crystal binding

Radiolabeled crystals were added to the cultures and allowed to adhere for one hour, after which the inserts were extensively rinsed to remove all nonassociated crystals, cut out, and counted in a liquid scintillation counter (Packard, Meriden, CT, USA). The number of associated crystals was calculated from the disintegrations per minute (dpm)/filter [9]. The effect of hyaluronan removal on crystal-cell association was studied by treating the monolayers with 5 U/mL *Streptomyces* hyaluronidase (E.C. 3.2.1.35; Sigma–Aldrich Chemie, Zwijndrecht, The Netherlands) in serum-free DMEM, pH 5.3 at 37°C for 1 hour before the addition of radiolabeled crystals. Then crystal-binding measurements were performed as described above. Experiments were performed to examine the firmness of crystal binding. Cultures were incubated with [^{14}C]COM crystals for 1 hour. After removal of non-adhered crystals by extensive washings, the cells were treated for 10 minutes with either 70% ethanol or with a mixture of 3.7% formaldehyde/0.1% glutaraldehyde. Released crystals were removed, the inserts cut out and the remaining cell-associated radioactivity counted. All [^{14}C]COM binding experiments were repeated at least three times, using three independent inserts per group.

Confocal laser scanning microscopy

A combination of light reflection (to visualize the crystals) and fluorescent-labeled phalloidin (to visualize the cells) was used to monitor the fate of cell-associated crystals by confocal microscopy. In time-lapse studies, nonradiolabeled COM crystals were incubated with subconfluent cultures for the indicated periods of time at 37°C and 4°C. All nonattached crystals were removed by extensive washings and the cells were fixed for 10 minutes with formaldehyde (3.7%), followed by membrane permeabilization with 70% ethanol for 10 minutes. The inserts were then washed, cut out, and incubated for 15 minutes with 2 μg/mL fluorescein isothiocyanate-conjugated (FITC) phalloidin (Sigma-Aldrich Chemie), washed two times with phosphate-buffered saline (PBS) and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). A 488 nm argon laser was used to excite the FITC-phalloidin. The extra- and/or intracellular localization of the crystals was detected by 633 nm (red) Kr-laser light reflection. A 560 nm beam splitter separated the FITC

emission signal and the 633 nm signal reflected by the crystals. The FITC signal was passed through a 510 to 540 nm band-pass filter to block the reflection from the 488 nm laser. No blocking filter was used for the light reflection signal. The mounted inserts were extensively inspected, images (magnification, x63) were made of at least 10 random fields per insert with a Zeiss LSM 410 laser scanning confocal microscope (Zeiss) from which representative images were selected. Experiments were repeated at least three times.

Quantification of crystal internalization

To assess if cell surface-associated crystals could be internalized, [^{14}C]COM crystals were added and incubated for the indicated periods of time, after which all nonadhered crystals were washed away. The cells were subsequently treated for 5 to 10 min. with 0.25% trypsin to digest cell surface glycoconjugates and thereby release most of the crystals attached to the cell exterior. TER measurements indicated that this mild enzyme treatment did not yet release the cells from each other or from the growth substrate. This method does not discriminate between crystals that are halfway through the membrane and those that are entirely inside the cell. With this restriction in mind, we considered the remaining cell-associated radiolabeled crystals as internalized.

RESULTS

Phase-contrast microscopy

The adherence of calcium oxalate monohydrate (COM) crystals to proliferating cells was viewed using light microscopy. These observations showed remarkable differences in the manner by which crystals were attached to LLC-PK$_1$ and MDCK-II compared to MDCK-I cells. The crystals appeared to be lying flat on the surface of the cell islands formed by LLC-PK$_1$ and MDCK-II. On the other hand, crystals were predominantly found at the periphery of the cell groups formed by MDCK-I (Fig.1). These observations prompted us to inspect the attachment of crystals to these cell types more closely.

Crystal binding

Hyaluronan (HA) is one of the major crystal binding molecules at the surface of MDCK-I cells [9]. To determine whether HA also plays a role in crystal binding to LLC-PK_1 and MDCK-II, these cells were treated with *Streptomyces* hyaluronidase prior to incubating subconfluent cultures with radiolabeled COM crystals. Hyaluronidase significantly reduced the level of binding to MDCK-I cells, whereas this enzyme had no or negligible effect on crystal binding to LLC-PK_1 and MDCK-II cells (Fig.1).

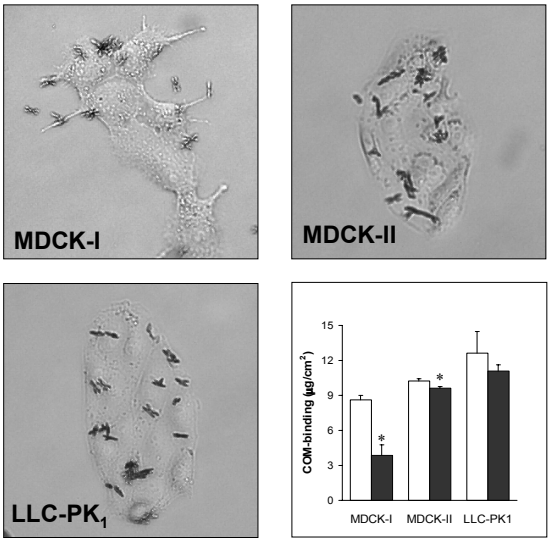


Figure 1. Phase-contrast microscopy. *Crystals associated with small groups of proliferating Madin-Darby canine kidney strain I (MDCK-I),Madin-Darby canine kidney strain II (MDCK-II), and porcine kidney (LLC-PK_1) cells. The crystals cover the entire surface of MDCK-II and LLC-PK_1, whereas they seem to be restricted to the periphery of MDCK-I (magnification, x200). The bar graph in the lower righthand corner depicts the effect of hyaluronidase treatment on [^{14}C] calcium oxalate monohydrate (COM) binding. Open bars represent control; filled bars represent hyaluronidase. *P < 0.05 is statistically significant compared to control.*

Figure 2. Scanning electron microscopy (SEM). *(A) SEM image of calcium oxalate monohydrate (COM) crystals generated by 5 mmol/L of both calcium and oxalate. (B) SEM image of the surface of Madin-Darby canine kidney strain II (MDCK-II) cells cultured on porous supports, showing numerous microvilli. (C) The same cells, but after incubation with COM crystals (magnification, x20,000). (D) Transmission electron microscopy (TEM) image of COM crystal ghosts inside MDCK-II (magnification, x2500*).

Electron microscopy

Electron microscopy (EM) images were made to study the interaction between crystals and the different cell types more in detail. Scanning electron microscopy (SEM) images showed the attachment of COM crystals to the surface of MDCK-II cells (Fig. 2) that is abundantly covered with microvilli (Fig. 2). Figure 2 suggests that the crystals became embedded in the plasma membrane and even entered the cells. Similar results were obtained with LLC-PK_1 (not shown). SEM images of crystal binding to MDCK-I cells were entirely different, however. Although our standard [^{14}C]COM crystal-binding studies indicated that relatively large amounts of crystals were able to bind to proliferating MDCK-I, these crystals were hardly recovered in the SEM images. This suggests that these crystals are lost somewhere during preparation, a process that requires extensive washing steps. To assess if crystals actually entered certain cell types, the cells were incubated for two hours with COM crystals, after which single cell suspensions were prepared with trypsin to be inspected by transmission electron microscopy (TEM). These images indeed provided evidence for the presence of crystals inside MDCK-II cells in the form of so-called ghosts (Fig. 2). Crystal ghosts were also seen in LLC-PK_1 cells, but never inside MDCK-I cells (not shown).

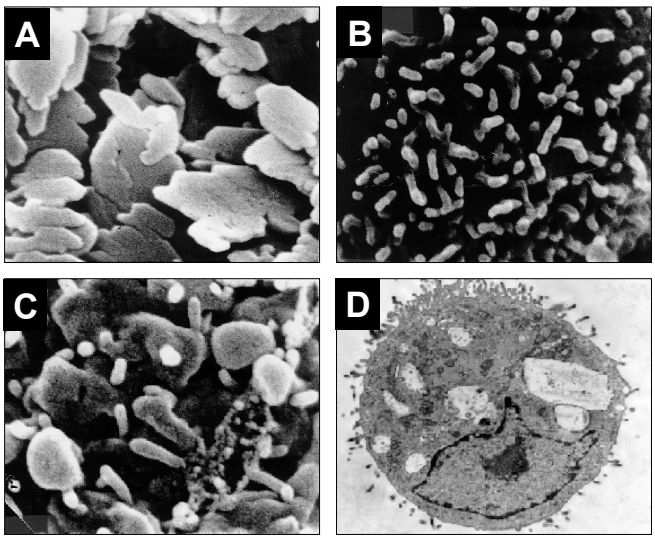


Figure 2

Effect of fixatives and enzymes on the release of cell surface-associated [^{14}C]COM

The presence in the SEM images of crystals at the surface of LLC-PK_1 and MDCK-II cells, and their absence from MDCK-I, as well as the adherence of crystals to these cells under phase-contrast microscopy, suggest that there are differences in the way by which crystals attach to these cell types. To test this concept, radiolabeled COM crystals were allowed to adhere to the various cell types, after which the cells were treated either with ethanol to dehydrate the cell surface or with formaldehyde/ glutaraldehyde to cross-link membrane proteins. These treatments released relatively large amounts of crystals from MDCK-I. Ethanol had the most profound effect and liberated approximately 60% of the cell surface-associated [^{14}C]COM, whereas formaldehyde/ glutaraldehyde released about 40% of adhered crystals. Neither fixation method, however, significantly liberated crystals from MDCK-II or LLC-PK_1 cells (Table 1).

Table 1. Effect of fixatives on the release of pre-bound COM crystals.

	Untreated	Ethanol	Formaldehyde/ glutaraldehyde
MDCK-I	100 ± 5	39 ± 6*	60 ± 6*
MDCK-II	100 ± 12	92 ± 4	88 ± 6
LLC-PK_1	100 ± 5	98 ± 7	87 ± 6

Abbreviations are: COM, calcium oxalate monohydrate; LLC-PK_1 , porcine kidney; MDCK-I, Madin-Darby canine kidney strain I; MDCK-II, Madin-Darby canine kidney strain II. Subconfluent cultures formed by LLC-PK_1, MDCK-II and MDCK-I cells preincubated with [^{14}C]COM crystals for one hour were treated for 10 minutes with ethanol or formaldehyde/glutaraldehyde to study the release of radiolabeled crystals. Results are expressed as percentage crystals remaining associated with the cells. Average ± SD of three independent inserts. Statistical analysis was performed using Mann-Whitney U-test. * *Significantly different compared to control (P<0.05).*

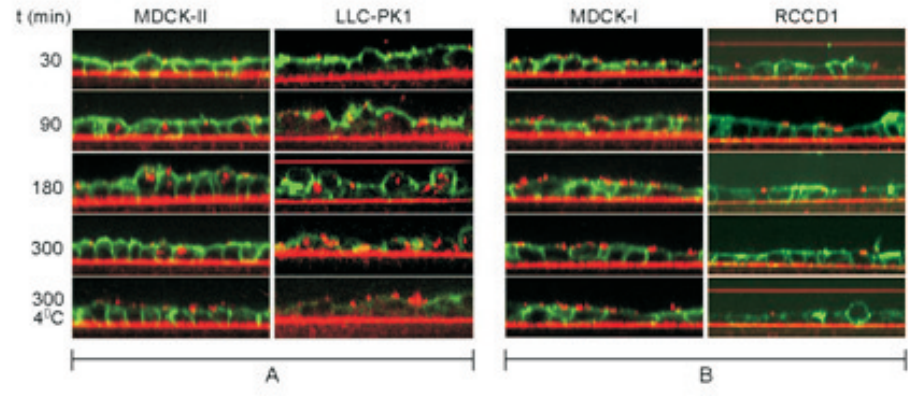


Figure 3. Confocal laser scanning microscopy (CLSM). *The cells are visualized using phalloidin-FITC (green), and COM by light reflection (red). A) Subconfluent cells with surface properties of the proximal tubule (MDCK-II and LLC-PK$_1$) internalize crystals, unless they are kept at 4^{0}C. B) Subconfluent cells with surface properties of the distal tubule and/or collecting ducts (MDCK-I and RCCD$_1$) do not internalize crystals. Note that although the cultures appear to be confluent we consider them subconfluent because the number of cells per insert is still increasing and the tight junctions have not yet been fully developed (magnification 63x).*

Confocal microscopy

To assess the fate of cell surface-associated crystals, we performed time-lapse studies using confocal microscopy. Nonradiolabeled COM crystals were added to subconfluent cultures formed by the various cell lines. Confocal images made perpendicular to the cell surface showed that after half an hour at 37°C, crystals were exclusively located at the surface of all cell types. After 90, 180 and 300 minutes, however, crystals were found inside LLC-PK$_1$ and MDCK-II cells, unless they were kept in the cold (300 minutes, 4^{0}C), indicating that crystal uptake is an active process. It should be noted that crystals were also taken up by confluent LLC-PK$_1$ and MDCK-II cells, indicating that the uptake of crystals is not crucially dependent on their stage of development (not shown). In contrast, crystals were never found inside MDCK-I cells. Since MDCK-I cells form hyaluronan-rich cell coats [3,24], it cannot be excluded that these coats make crystal internalization impossible by preventing the crystals from directly contacting the cell surface. Confocal images, however, revealed that crystals were also not taken up by cells that were made coat-free with hyaluronidase prior to the addition of crystals (not shown). To examine whether the inability to take up crystals could be an intrinsic property of late nephron cell types, crystal uptake was studied in

$RCCD_1$, another renal collecting duct cell line. The vast majority of the $RCCD_1$ cells also did not internalize COM crystals (Fig. 3).

Quantification of crystal internalization

Experiments were performed to quantify crystal uptake. In time-lapse studies, radiolabeled COM crystals were allowed to interact with the cells. The cultures were subsequently briefly treated with trypsin to trypsin gradually increased from background levels of 0.15 ± 0.03 $\mu g/10^6$ cells at 30 minutes to 3.85 ± 0.04 $\mu g/10^6$ cells after 300 minutes (Fig. 4), indicating crystal internalization. Crystals were also taken up by LLC-PK_1, but with a somewhat different time-course. Uptake showed a steep rise from 0.88 ± 0.11 to 3.23 ± 0.21 $\mu g/10^6$ cells between 30 and 90 minutes, followed by a slower increase to 4.70 ± 0.77 μg COM crystals per million LLC-PK_1 cells in the next hours. More than 90% of the [^{14}C]COM associated with MDCK-I was released by trypsin at all time points, and the amount of radiolabeled COM that remained associated with the cell surface after enzyme treatment never reached values above background (~0.4 $\mu g/10^6$ cells). We speculated that the polymer hyaluronan, the major binding substance at the surface of MDCK-I, might have prevented the crystals from contacting the membrane. To find out if the cell surface expression of hyaluronan is responsible for the inability of MDCK-I cells to take up crystals, uptake was measured by cells that were pre-treated with hyaluronidase. The uptake assay demonstrated that crystals that were able to bind to MDCK-I after hyaluronidase treatment also did not enter the cells (not shown). [^{14}C]COM uptake studies performed with $RCCD_1$ cells showed that even after an incubation period of five hours at 37°C, more than 90% of the crystals associated with the cell surface could be removed with trypsin. In the time lapse studies the amount of crystals internalized by $RCCD_1$ never exceeded background levels (Fig. 4).

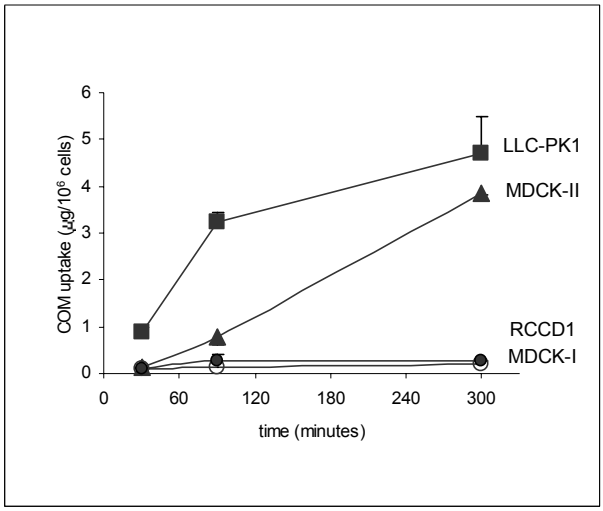


Figure 4. Quantitative crystal uptake assay. *Subconfluent cultures were incubated with [^{14}C]COM for increasing periods of time, after which all non-adhered crystals were removed by extensive washings. The cells were then briefly treated with trypsin to liberate crystals associated with the cell surface but not incorporated in the plasma membrane. Remaining cell-associated crystals are considered internalized. The uptake of crystals by MDCK-II is linear between 30 and 300 minutes (▲), whereas crystal internalization by LLC-PK$_1$ cells shows a steep rise in the first 90 minutes, followed by a more gradual increase thereafter (■). Crystals are not taken up by MDCK-I (●) or RCCD$_1$ (○). Average ± SD of three independent inserts of a representative experiment.*

DISCUSSION

This study shows that the adherence of calcium oxalate monohydrate (COM) crystals to renal tubular cells is not necessarily followed by crystal uptake. Although crystals avidly bind to subconfluent LLC-PK$_1$, MDCK-II, MDCK-I and RCCD$_1$ cells, they are eagerly internalized by LLC-PK$_1$ and MDCK-II, but not by MDCK-I and RCCD$_1$.

Light microscopy images showed that crystals entirely covered the surface of LLC-PK$_1$ and MDCK-II cells, whereas their adherence to MDCK-I cells seemed to be restricted to the cell periphery (Fig.1). Using radiolabeled COM it was demonstrated that, once attached to the surface of LLC-PK$_1$ or MDCK-II, only few crystals are liberated by fixation methods such as dehydration or protein crosslinking (Table 1). Moreover, brief treatment with trypsin did not liberate many of the crystals attached

to these two cell types. Hence, COM crystals became firmly anchored to the surface of LLC-PK_1 and MDCK-II cells. Electron microscopy images showed that crystals indeed became tightly embedded in the plasma membrane to ultimately end up inside these cells (Fig.2). These observations were confirmed by a [^{14}C]COM uptake assay, showing that the internalization of crystals by LLC-PK_1 and MDCK-II cells is temperature- and time-dependent (Fig.4). Previously, we identified the polysaccharide hyaluronan (HA) as an important crystal binding molecule at the surface of MDCK-I cells [9]. However, pretreatment of LLC-PK_1 and MDCK-II cells with hyaluronidase did not affect the affinity of these cell types for crystals indicating that this form of binding does not depend on HA (Fig.1). Crystals became only loosely attached to MDCK-I cells. Fixation or short trypsin treatment liberated most of the crystals bound to these cells. The affinity of MDCK-I cells for COM crystals is significantly reduced after pretreatment with hyaluronidase [9]. The quantitative uptake assay confirmed that MDCK-I cells are unable to internalize COM crystals. The possibility that uptake is impeded by HA coats was canceled out by the observation that crystals also did not enter MDCK-I cells that were treated with hyaluronidase prior to the addition of crystals (not shown). COM crystals also were not taken up by $RCCD_1$ cells. Although an occasional $RCCD_1$ cell contained a crystal, the quantitative uptake assay clearly demonstrated that the COM crystal uptake by $RCCD_1$ cells is negligible. Collectively, these data show that crystals are taken up by LLC-PK_1 and MDCK-II, but not by MDCK-I and $RCCD_1$.

LLC-PK_1 and MDCK-II cells, exhibit many characteristics of the renal proximal tubule. Confluent monolayers formed by these cells are leaky (<200 $\Omega.cm^2$), are endowed with a luxurious brush border packed with tall microvilli, and their membranes express high levels of alkaline phosphatase (AP) and γ-glutamyltranspeptidase (γ-GT) activity [10]. In addition, LLC-PK_1 cells actively transport essential nutrients and water across the epithelium [14]. MDCK-I and $RCCD_1$ cells, on the other hand, resemble the renal collecting ducts in many respects. MDCK-I cells, isolated from the original MDCK cell line, develop a high trans-epithelial electrical resistance (>10,000 $\Omega \cdot cm^2$), are covered with short and stubby microvilli and express low levels of AP and γ-GT [10]. MDCK-I exhibits significant carbonic anhydrase activity [15] and its inward current can be activated by vasoactive intestinal polypeptide [16]. $RCCD_1$ cells are isolated from rat collecting ducts; this cell line is also characterized by

high transepithelial resistances (>3,000 $\Omega \cdot cm^2$) and specific hormonal sensitivities. Their morphological appearance resembles that of intact cortical collecting duct (CCD) [12]. On the basis of these results it is speculated that the ability of renal tubular cells to internalize crystals depends on their segmental origin.

Up until now, crystal uptake studies have been predominantly performed with cells resembling the renal proximal tubule. Lieske et al. [17], for example, observed that African green monkey BSC-1 or parental MDCK (ATCC) cells rapidly took up calcium oxalate crystals. However, little is known about the segmental origin of BSC-1, whereas the original MDCK cell line contains various cell types including proximal tubular cells [12]. Crystal uptake has further been studied in the proximal tubular opossum kidney cell line, OK-1 [18], and again by several investigators in the original MDCK cell line [19-21]. Thus, these results are in agreement with those obtained with LLC-PK_1 and MDCK-II in the present study.

In vivo, the uptake of crystals followed by their translocation to the renal interstitium is typical for animals fed a stone-inducing diet, or for primary human forms of chronic hyperoxaluria [6, 22, 23]. In rats, hyperoxaluria is commonly generated by the addition of oxalate precursors such as ethylene glycol to the drinking water. Stone-inducing or, rather, crystal-inducing diets usually aim at increasing the amount of oxalate that is filtered at the glomerulus, thereby resembling primary forms of hyperoxaluria rather than idiopathic forms of calcium oxalate nephrolithiasis. High oxalate in ultrafiltrate may already lead to the formation of crystals in the proximal tubule and thus inevitably to crystal binding and uptake. Following this train of thoughts, it is reasonable to assume that evolution provided those areas in the urinary tract in which the urine can be very concentrated with a crystal-repulsive epithelial lining. Previously, we found that an intact monolayer formed by late nephron MDCK-I cells indeed is nonadherent to COM crystals. These anti-adherence properties were temporary lost, however, during the healing of wounds made in confluent monolayers [24]. Evidence was provided that during tissue repair and remodeling crystals adhered to the HA-rich zones surrounding the wounded areas [9]. From these observations it was speculated that crystal binding in late segments of the nephron and remaining part of the urinary tract requires some form of tissue damage [25, 26].

During the concentration process in the kidney, oxalate gradually increases from, on average, 2 µmol/L in ultrafiltrate to approximately

300 µmol/L in the final urine. Oxalate is poorly soluble in the tubular fluid and calcium oxalate crystals are occasionally formed in anyone's kidneys. Although it is difficult to exactly pinpoint the segment in the nephron where crystal formation takes place, it is assumed to occur somewhere beyond the thin limbs [27-29]. Hence, proximal tubular cells are not used to encounter crystals, which might explain why this epithelium is not provided with an anti-adherent surface. However, the concentration of oxalate can be 10 to 20 times higher in plasma of patients suffering from inborn errors of oxalate metabolism [30]. Because the upper limits of supersaturation in the primary urine depend on the amount of oxalate that is filtered at the glomerulus, it is plausible that crystals are formed earlier in the nephrons of these patients. This implies that in primary hyperoxalurias crystals are formed at sites in the nephron where the epithelium is not protected against their adherence.

The essence of the present study is not the observation that crystals adhere to HA or to other cell surface constituents, but that certain cell types constitutively bind and take up crystals, whereas others do not. The possibility that crystal binding and uptake cannot be avoided in proximal tubules and that epithelial injury-induced binding in the collecting ducts does not involve crystal uptake may have consequences for the treatment of the various forms of renal stone disease. In patients suffering from primary forms of hyperoxaluria measures should be taken to avoid crystal adherence in the proximal tubule, whereas in idiopathic recurrent calcium oxalate renal stone disease the best approach could be the prevention of tissue damage in the distal tubule and/or collecting ducts.

REFERENCES

1. Kok DJ, Khan SR: Calcium oxalate nephrolithiasis, a free or fixed particle disease. *Kidney Int* 46:847-854, 1994
2. Worcester EM: Inhibitors of stone formation. *Semin Nephrol* 16:474-486, 1996
3. Schepers MS, van der Boom BG, Romijn JC*, et al.*: Urinary crystallization inhibitors do not prevent crystal binding. *J Urol* 167:1844-1847, 2002
4. Malek RS, Boyce WH: Intranephronic calculosis: its significance and relationship to matrix in nephrolithiasis. *J Urol* 109:551-555, 1973
5. Hering F, Briellmann T, Luond G*, et al.*: Stone formation in human kidney. *Urol Res* 15:67-73, 1987
6. Lieske JC, Spargo BH, Toback FG: Endocytosis of calcium oxalate crystals and proliferation of renal tubular epithelial cells in a patient with type 1 primary hyperoxaluria. *J Urol* 148:1517-1519, 1992

7. Lieske JC, Walsh-Reitz MM, Toback FG: Calcium oxalate monohydrate crystals are endocytosed by renal epithelial cells and induce proliferation. *Am J Physiol* 262:F622-630, 1992
8. Lieske JC, Swift H, Martin T*, et al.*: Renal epithelial cells rapidly bind and internalize calcium oxalate monohydrate crystals. *Proc Natl Acad Sci U S A* 91:6987-6991, 1994
9. Verkoelen CF, Van der Boom BG, Romijn JC: Identification of hyaluronan as a crystal-binding molecule at the surface of migrating and proliferating MDCK cells. *Kidney Int* 58:1045-1054, 2000
10. Verkoelen CF, van der Boom BG, Kok DJ*, et al.*: Cell type-specific acquired protection from crystal adherence by renal tubule cells in culture. *Kidney Int* 55:1426-1433, 1999
11. Hansson GC, Simons K, van Meer G: Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. *Embo J* 5:483-489, 1986
12. Blot-Chabaud M, Laplace M, Cluzeaud F*, et al.*: Characteristics of a rat cortical collecting duct cell line that maintains high transepithelial resistance. *Kidney Int* 50:367-376, 1996
13. Verkoelen CF, Romijn JC, de Bruijn WC*, et al.*: Association of calcium oxalate monohydrate crystals with MDCK cells. *Kidney Int* 48:129-138, 1995
14. Verkoelen CF, Kok DJ, van der Boom BG*, et al.*: LLC-PK1 cells as a model system to study proximal tubule transport of water and other compounds relevant for renal stone disease. *Urol Res* 27:109-115, 1999
15. Pfaller W, Gstraunthaler G, Kersting U*, et al.*: Carbonic anhydrase activity in Madin Darby canine kidney cells. Evidence for intercalated cell properties. *Ren Physiol Biochem* 12:328-337, 1989
16. Griffiths NM, Rugg EL, Simmons NL: Vasoactive intestinal peptide control of renal adenylate cyclase: in vitro studies of canine renal membranes and cultured canine renal epithelial (MDCK) cells. *Q J Exp Physiol* 74:339-353, 1989
17. Lieske JC, Toback FG: Regulation of renal epithelial cell endocytosis of calcium oxalate monohydrate crystals. *Am J Physiol* 264:F800-807., 1993
18. Goswami A, Singhal PC, Wagner JD*, et al.*: Matrix modulates uptake of calcium oxalate crystals and cell growth of renal epithelial cells. *J Urol* 153:206-211, 1995
19. Kohjimoto Y, Ebisuno S, Tamura M*, et al.*: Adhesion and endocytosis of calcium oxalate crystals on renal tubular cells. *Scanning Microsc* 10:459-468, 1996
20. Ebisuno S, Kohjimoto Y, Tamura M*, et al.*: Histological observations of the adhesion and endocytosis of calcium oxalate crystals in MDCK cells and in rat and human kidney. *Urol Int* 58:227-231, 1997
21. Campos AH, Schor N: Mechanisms involved in calcium oxalate endocytosis by Madin-Darby canine kidney cells. *Braz J Med Biol Res* 33:111-118, 2000
22. de Water R, Boeve ER, van Miert PP*, et al.*: Pathological and immuno-cytochemical changes in chronic calcium oxalate nephrolithiasis in the rat. *Scanning Microsc* 10:577-587; discussion 587-590, 1996

23. Khan SR: Animal models of kidney stone formation: an analysis. *World J Urol* 15:236-243, 1997
24. Verkoelen CF, van der Boom BG, Houtsmuller AB*, et al.*: Increased calcium oxalate monohydrate crystal binding to injured renal tubular epithelial cells in culture. *Am J Physiol* 274:F958-965, 1998
25. Asselman M, Verkoelen CF: Crystal-cell interaction in the pathogenesis of kidney stone disease. *Curr Opin Urol* 12:271-276, 2002
26. Verkoelen CF, Schepers MS: Changing concepts in the aetiology of renal stones. *Curr Opin Urol* 10:539-544, 2000
27. Belliveau J, Griffin H: The solubility of calcium oxalate in tissue culture media. *Anal Biochem* 291:69-73, 2001
28. Kok DJ: Intratubular crystallization events. *World J Urol* 15:219-228, 1997
29. Hojgaard I, Tiselius HG: Crystallization in the nephron. *Urol Res* 27:397-403., 1999
30. Milliner DS, Wilson DM, Smith LH: Phenotypic expression of primary hyperoxaluria: comparative features of types I and II. *Kidney Int* 59:31-36, 2001

CHAPTER 5

CRYSTALS CAUSE ACUTE NECROTIC CELL DEATH IN RENAL PROXIMAL TUBULE CELLS, BUT NOT IN COLLECTING TUBULE CELLS

M.S.J. Schepers, E.S. van Ballegooijen,
C.H. Bangma and C.F. Verkoelen

Submitted

ABSTRACT

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 E_2 (PGE_2) secretion, DNA synthesis ([^{3}H]thymidine), total cell numbers, reactive oxygen species (H_20_2) 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 PGE_2, H_20_2 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 H_20_2 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.

INTRODUCTION

Kidney stones are composed of numerous microcrystals accumulated in the kidney. The human kidney produces concentrated urine commonly supersaturated with poorly soluble salts like calcium oxalate. The precipitation of these salts as crystals is common and harmless as long as these crystals are excreted with the urine. To ensure their efficient elimination, the renal epithelial lining should be nonadherent in nephron segments with high levels of supersaturation. Compared to persons not forming stones, the levels of stone salt supersaturation are usually higher in urine of recurrent stone formers, which is why these patients often excrete higher amounts of crystals. Crystal binding to renal tubular cells may be the underlying cause of nephrocalcinosis and nephrolithiasis. Perhaps there are pathological circumstances during which healthy cells are transformed in a crystal binding phenotype.

The impression that the crystals themselves could be actively involved in this process came from studies performed in animals and in cell culture[1-4]. These studies suggested that calcium oxalate crystals generate free radicals that are damaging to the renal tubular cells. There are some questions, however, about the model systems used. In animals, CaOx crystalluria cannot exist without hyperoxaluria which makes it difficult to discriminate between effects caused by crystals or by oxalate. Another point of concern is that most calcium oxalate toxicity studies are performed with renal proximal tubular cells while crystal formation is extremely unlikely in this nephron segment.

In the present investigation, we studied the possible toxic effect of calcium oxalate crystals to renal proximal and collecting tubule cells grown as confluent monolayers on permeable growth substrates in a two-compartment culture system. In this model system, the apical compartment is representative for the tubular fluid whereas the basal compartment is representative for the interstitial space and blood supply. Crystals are added at the luminal side of the monolayers in serumfree growth medium, while serum-containing growth medium is added to the basal compartment. Thus, during their apical exposure to CaOx crystals,

the cells have free access to serum components from the basolateral membrane. The results show that under these conditions CaOx crystals are toxic to renal proximal tubular cells, but not to collecting duct cells.

MATERIALS AND METHODS

Cell culture

MDCK-I and MDCK-II cells were kindly provided by Prof. G. van Meer (Laboratory for Cell Biology and Histology, Amsterdam Medical Center, The Netherlands)[5]. LLC-PK_1 cells were obtained from the American Type Culture Collection (ATCC). $RCCD_1$ (rat renal cortical collecting duct) cells [6] were kindly provided by Dr. M. Blot-Chabaud (INSERM U246, Faculté de Médecine Xavier Bichat, Paris, France). Routinely, cells were grown in Dulbecco's modified Eagle's Medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum (PAA Labs, Linz, Austria) and weekly replated. Cells are cultured at intermediate densities in polystyrene tissue culture treated 75 cm^2 flasks (Falcon, BD Biosciences, Bedford, USA) and the culture medium refreshed twice a week. For experiments cells are trypsinized and seeded at a concentration of $1x10^6$ cells/polycarbonate permeable support (Transwells, 24 mm diameter inserts, 4.7 cm^2 surface area, 0.4 μm pore size, Corning Costar, Badhoevedorp, The Netherlands). Within 6-7 days the cells proliferate and migrate into confluent monolayers with, depending on the cell type, a cell density of 4-6 x 10^6 cells.

To study crystal-cell interaction, confluent monolayers were washed after which serumfree DMEM (1.5 ml) was added to the apical compartment whereas the basal compartment received DMEM 10% FCS (2.6 ml) and 100 μl (146 μg) COM crystal suspension was added at the apical side of the monolayers.

Tight junction formation is assessed by transepithelial electrical resistance (TER) measurements. TER is measured in ohms (Ω) after placing the insert in an Endohm 24 connected to a Voltohm meter (World Precision Instruments, Sarasota, USA). Monolayers are considered confluent as soon as the highest TERs are reached. Routinely, PCR analyses were performed on DNA isolated from cell culture conditioned medium for the presence of mycoplasma. Cells used in this study were not contaminated with mycoplasma.

Crystal formation in cell culture medium.
To reveal at which concentrations oxalate induces crystal formation in culture medium, increasing amounts of oxalate were added to DMEM containing 1.8 mM calcium. Sodium oxalate was dissolved in Milli-Q distilled water and added at the appropriate concentration in a small volume on the bottom of a tube. Subsequently 50 ml DMEM was added at room temperature and the tube immediately vigorously shaken. After centrifugation at low speed (1000 rpm), the pellet was inspected by phase-contrast microscopy.

Morphological studies
Several staining procedures were used to visualize aspects of crystal-induced cell responses by confocal microscopy. To monitor cell-associated crystals, light reflection was applied to visualize the localization of the crystals and fluorescein isothiocyanate (FITC)-conjugated phalloidin to stain polymeric F actin in the cell cytoskeleton. At the various time points, filter inserts were washed three times with physiological saline (PBS) and fixed for 15 minutes in ethanol 70%. Previously, it was demonstrated that fixation with ethanol releases most of the crystals that are loosely associated with the cell surface[7]. Ethanol-fixed cells are subsequently incubated for 15 minutes with 5 µg/ml phalloidin-FITC (Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands), washed 3 times and mounted in Vectashield (Vector Laboratories, Burlingame, USA). To study the subcellular localization of crystals under the various conditions, XY-scans in a focal plane (horizontal) and cross-sectional XZ-scans (vertical) were made with a Zeiss LSM 510 Meta (Zeiss, Oberkochen, Germany)[8]. A 488-nm argon laser was used to excite the FITC-phalloidin. Crystals were detected by 633-nm (red) Kr-laser light reflection. A 560-nm beam splitter separated the FITC emission signal and the 633-nm signal reflected by the crystals. The FITC signal was passed through a 510- to 540-nm band-pass filter to block the reflection from the 488-nm laser. No blocking filter was used for the light reflection signal. The mounted inserts were extensively inspected, images (magnification 63x) were made of at least 10 random fields per insert from which representative images were selected. Experiments were repeated at least three times.

Calcium oxalate monohydrate (COM) crystal binding

A solution of radiolabeled sodium oxalate was prepared by adding 10 µl 37 MBq/ml [^{14}C]oxalic acid (Amersham Int.plc, Buckinghamshire, UK) to 0.25 ml 200 mM sodium oxalate. A calcium chloride solution was prepared by adding 0.25 ml 200 mM $CaCl_2$ to 9.5 ml distilled water. Mixing the two solutions at room temperature (final concentration of 5 mM for both oxalate and calcium) immediately resulted in the nucleation of radiolabeled COM. After settling for three days, crystals were washed three times with and resuspended in CaOx-saturated H_2O in a final volume of 5 ml (1.46 mg COM crystals/ml). In this study the cells were incubated with 100 µl crystal suspension (146 µg/insert or 32 µg/cm^2). The same procedure but without [^{14}C]oxalic acid was used for the generation of non-radiolabeled COM. The crystals prepared with this method consist entirely of calcium oxalate monohydrate (COM) with an average size of 1-2 µm[9].

To quantify the amount of COM crystals that remained associated with the monolayers in time, radiolabeled crystals were added at the luminal side of confluent monolayers in serumfree DMEM, while in the basal compartment the cells received DMEM 0.5% FCS. Cells were incubated for one hour, after which the inserts were rinsed to remove all non-associated crystals, cut out and placed in a scintillation vial. To extract radioactivity, 1 ml 1M perchloric acid was added and the amount of radioactivity was counted in a liquid scintillation counter (Packard, UK). The amount of associated crystals was calculated from the dpm/filter. The remaining inserts were extensively washed after which the apical compartment received serumfree DMEM whereas DMEM 10% FCS was added to the basal compartment. The procedure to assess the amount of cell-associated radioactivity was subsequently repeated 1, 2, 7 and 15 days post-incubation.

To study the ability of the cells to process internalized crystals the following study was conducted. Monolayers were incubated for one hour with [^{14}C]COM according the method described above. Twenty-four hours post-incubation, released or loosely attached crystals were removed through extensive washing of the monolayers. During the days thereafter and starting two-days post-incubation, the fluid in the apical and basal compartments was collected and centrifuged. The amount of radioactivity was counted in 100 µl portions in the supernatants and in the pellets after dissolution in 1M PCA. The amount of [^{14}C]COM that remained associated with the monolayers was counted in a scintillation counter.

Hydrogen peroxide (H_2O_2)

The reactive oxygen intermediate hydrogen peroxide (H_2O_2) was measured in the apical fluid of cells exposed to various concentrations of oxalate using the Amplex Red Hydrogen/Peroxide assay kit (A-22188; Molecular Probes, Leiden, The Netherlands). Since phenol red in the culture medium interferes with the absorbance measurements the studies are performed with a buffer containing (in mM): 124 NaCl, 25 $NaHCO_3$, 2 $NaH_2PO_4.2H_2O$, 5 KCl, 0.5 $MgCl_2.6H_2O$, 5 Na-acetate, pH 7.4, 310-320 mosmol/kg H_2O from now on called calcium-free buffer B, added to the apical fluid compartment, while the basal compartment receives DMEM 10% FCS without phenolred and sodium pyruvate (Gibco). A standard curve is prepared at 10 µmol/l to 1.25 µmol/l. Samples, standards and blank are aliquoted to a 96-well plate. A working solution of 100 µmol/l Amplex red reagent and 0.2 U/ml Horseradish peroxidase (HRP) is prepared and added to all the samples, standards and blank. The plate was protected from light and incubated for 30 minutes at room temperature. The absorbance was read at 560 nm using a Bio-Rad 550 microplate reader.

Prostaglandin E_2 (PGE_2).

PGE_2 secreted into the luminal compartment is measured in a switch enzyme-linked immunoassay (EIA) Kit (Cayman). This assay is based on the competition between PGE_2 and a PGE_2-acetylcholinestrase (AchE) conjugate (as tracer) for a limited amount of PGE_2 monoclonal antibody. Because the concentration of the PGE_2 tracer is held constant while the concentration of PGE_2 varies, the amount of PGE_2 tracer that is able to bind to the PGE_2 antibody is inversely proportional to the concentration of PGE_2 in the well. PGE_2 standard curves are prepared by diluting 10 mg/mL PGE_2 in EIA buffer to produce a concentration range of 0-1000 µg PGE_2/mL. Each plate or set of strips contains a blank-, a non-specific binding-, a maximum binding and a total activity control. Standard curve samples (50 µl), controls and experimental samples are added to the wells. Each well receives 50 µl tracer except the total activity and the blank wells. The wells subsequently receive 50 µl PGE_2 monoclonal antibody except the total activity well, the non-specific binding well, and the blank well. The plate is covered with plastic film and incubated for 18 hours at 4°C. The wells and rinsed extensively with wash buffer after which 200 µL Ellman's Reagent is added to each well and 5 µl tracer to the total activity well. The plate is then covered with plastic film and

developed in the dark on an orbital shaker. When the blank absorbance is between 0.3-0.8 units, the plate is read in a Bio-Rad 550 microplate reader at 405-420 nm wavelengths.

[^{3}H]Thymidine incorporation

To assess the effect of crystals on DNA synthesis, monolayers were incubated for 24h with 146 μg COM after which all non-associated crystals were removed by extensive washing and the monolayers received fresh DMEM 10% FCS containing 3.7 KBq/ml [methyl-^{3}H]thymidine (Amersham). After a pulse labeling period of 3 h, the cultures were washed 3 times with PBS, the filters cut out and counted in a liquid-scintillation counter. Results are expressed in dpm/filter insert.

Crystal-induced apoptotic or necrotic cell death

To assess the nature of crystal-induced cell death, annexin V (A.G. Scientific, Inc, USA) staining was applied to reveal the surface exposure of phosphatidylserine (apoptosis) and propidium iodide (Sigma Aldrich Chemie) to reveal the loss of plasma membrane integrity (necrosis). At the various time points, filter inserts were washed and unfixed cells were incubated for 15 minutes with FITC labeled annexin V (1:40), washed 3 times and subsequently incubated for 15 minutes with 1 μg/ml propidium iodide, washed and mounted in Vectashield (Vector Laboratories). Another widely applied method to study apoptosis is DNA fragmentation staining with Hoechst 33258 (Sigma Aldrich Chemie). At the various time points, filter inserts were washed and incubated with 0.1 mg/ml Hoechst, washed and mounted in Vectashield. In some experiments propidium iodide influx or Hoechst in combination with light reflection (crystals) was applied to assess if crystal-cell interaction is accompanied with apoptotic or necrotic cell death. Incubation of the cells for 4 hours with 1.0 μM antimycin A (Sigma Aldrich) was used as positive control for apoptosis, while cell fixation with ethanol to permeabilize the membrane served as positive control for necrosis. Phase-contrast microscopy was applied to monitor the release of crystal-containing cells in the apical fluid compartment.

Statistics.

ANOVA or Student's t-tests were performed using triplicate samples and repeated at least two times to assess the statistical significance between

data points. Differences were considered statistically significant at $p<0.05$.

RESULTS

Oxalate-induced crystal formation in culture medium

One of the motives to study the cellular response to calcium oxalate crystals was that oxalate is often added to calcium-containing culture media without sufficiently taking into account the risk for crystal formation. Effects attributed to oxalate in these studies can be partially or entirely be caused by crystals. In Fig.1 we show the oxalate concentrations inducing calcium oxalate crystal formation in DMEM (1,8 mM calcium). Calcium oxalate crystals were formed after the addition of ≥0.5 mM sodium oxalate (Fig.1).

Morphological studies.

Confluent monolayers were incubated for various periods of time with COM crystals and studied by confocal microscopy (CLSM) to monitor crystal localization and to reveal possible crystal-induced alterations in cell shape or behavior compared to cells of the same age that did not receive crystals. XY-images of phalloidin-FITC stained cells in combination with light reflection demonstrated no particles in cells that did not receive crystals (Fig.2A) and crystals inside MDCK-II, 1 day (Fig. 2B) and 7 days (Fig. 2C) after a one-hour incubation with 146 µg COM, which was also observed in LLC-PK_1 cells (not shown). As reported previously, crystals did not bind to and were not internalized by MDCK-I or $RCCD_1$ cells [7]. After 24 hours, the morphological appearance of crystal-containing cells was rather disorderly and images made perpendicular to the growth substrate (XZ-images) revealed the release of crystal-containing cells into the apical fluid (Fig. 2D). Exfoliation of cells with crystals was confirmed by phase-contrast microscopy showing birefringent crystals in and around released single cells (Fig. 2E). After this initial vigorous response, the cells in the monolayers seemed to accept internalized crystals. After 7 days the quiescent and functional monolayers still contained cells with internalized crystals (Fig. 2C).

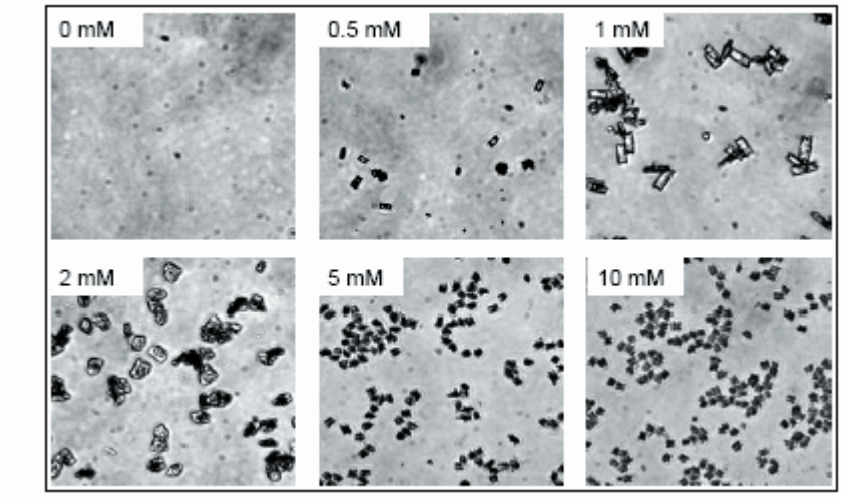


Figure 1. *Calcium oxalate crystal formation after adding sodium oxalate to DMEM. Crystals are detected by phase-contrast microscopy at oxalate concentrations ≥0.5 mM added to 1.8 mM calcium-containing growth medium.*

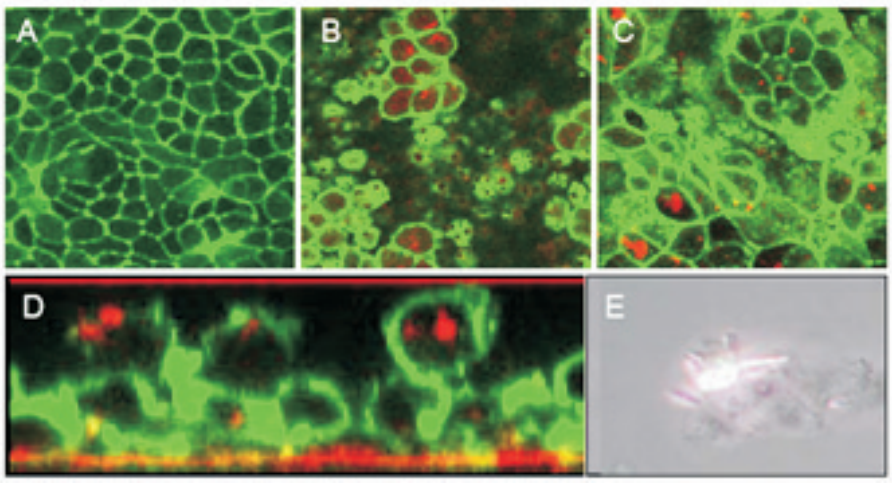


Figure 2. *CLSM images of MDCK-II cells cultured on permeable supports in a two-compartment culture system. Cells (green) are visualized with FITC-conjugated phalloidin abd crystals (red) using light reflection (A). Confluent MDCK-II cells incubated for one hour with 146 µg COM crystals. Internalized crystals are found inside cells after one (B) and seven days (C). An image made perpendicular to the growth substrate 24 hours post-incubation revealed that crystal-containing cells were exfoliated from the cultures (D), which was confirmed by the presence of crystal-containing cells in the supernatant by phase-contrast microscopy (E).*

Calcium oxalate monohydrate (COM) crystal binding

Since it is not possible from CLSM images to appreciate quantitative variations, this study was repeated with radiolabeled crystals. Confluent monolayers of MDCK-II cells were incubated for one hour with 146 µg [^{14}C]COM. After extensive washing to remove crystals that were not bound to the cells, the amount of monolayer-associated radioactivity was quantified immediately (t=1h) and 1, 2, 7 and 15 days later (Fig. 3). After one hour (t=1) a relatively large amount of crystals were associated with the monolayers. After the first 24 hours, the amount of monolayer-associated radioactivity was reduced by approximately 50% (T_d~21h), to further decline at a much slower rate in the next 14 days (T_d~312h). As mentioned above, crystals were eliminated during the initial response by the release of crystal-containing cells from the monolayers, but we do not exclude the possibility that part of the crystals were translocated back into the apical fluid or rapidly dissolved intracellularly.

To study the cellular handling of endocytosed crystals during the late response, MDCK-II cells were incubated for one hour with [^{14}C]COM crystals. After extensive washing at t=24 hour, the cells were monitored for an additional period of time. Two, 4 and 7 days later, the apical and basal fluid were collected, centrifuged, the filter inserts cut out and radioactivity counted in the pellets, supernatants and in the cells. Of the total amount of radioactivity eliminated by the monolayer in 5 days (~20 x10^3 dpm), approximately 20% (4.3 x10^3 dpm) was recovered in the apical fluid as solid material, ~30% (6.2 x10^3 dpm) as ion in the apical fluid and ~50% (9.8 x10^3 dpm) as ion in the basal fluid. Since the volume ratio in the apical (1500 µl) and basal (2600 µl) compartment also is approximately 3:5, this suggests that the ion concentration on both sides of the monolayer equilibrated through passive paracellular diffusion. Although about 80% of the eliminated crystals are recovered as ions in the apical and basal fluid compartment this does not necessarily imply that the cells were able to dissolve them. Because the fluid in both compartments was not saturated with calcium and oxalate it is also possible that eliminated crystals dissolved in the growth medium.

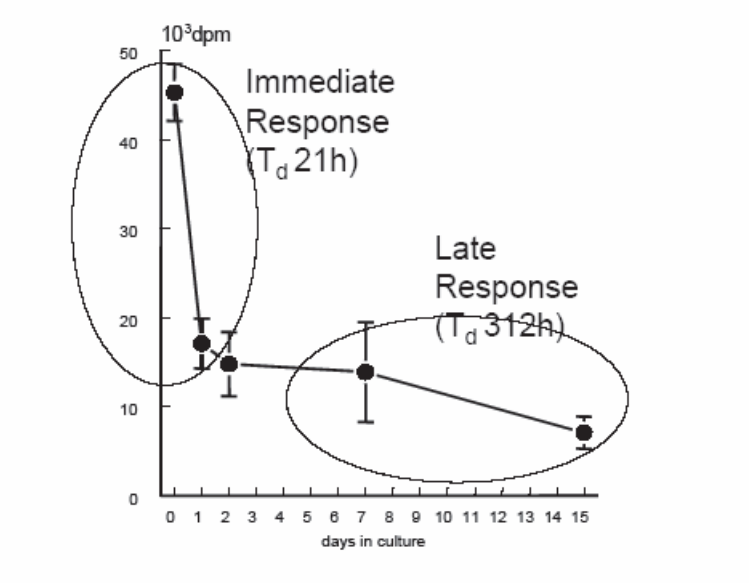
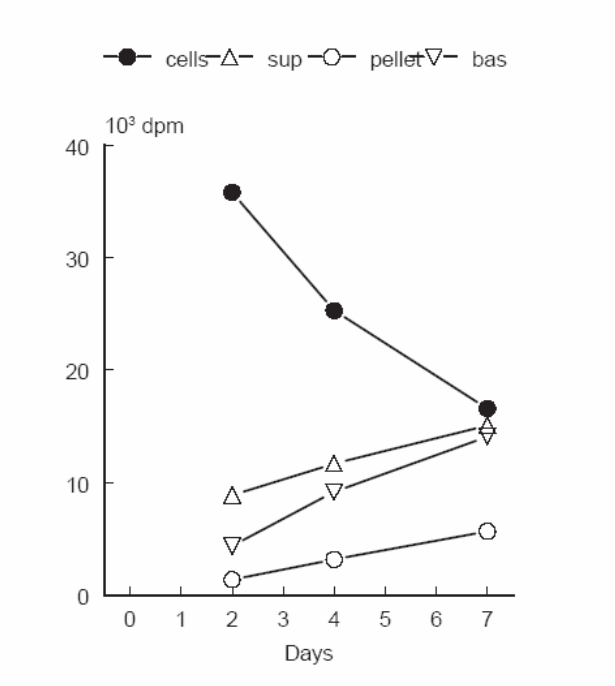


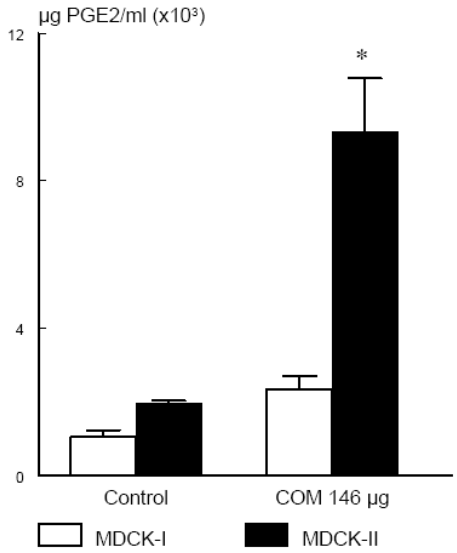
Figure 3. *The elimination of [14C]COM by MDCK-II cells showed a biphasic pattern. Within the first 24 hours approximately half of the amount of the crystals that initially became associated with the cells was removed (early response with T_d=21h). The cells required another two weeks to eliminate again 50% (late response with T_d=312 h). The early response may deal with crystals in the plasma membrane, while the late response may deal withinternalized crystals.*



Prostaglandin E_2 (PGE_2).
The secretion of prostaglandin E_2 (PGE_2) into the luminal compartment was measured as marker for crystal-induced inflammation. $RCCD_1$ and LLC-PK_1 appeared to be less suitable for these studies because they produced no or hardly detectable levels of PGE_2. Arachidonic acid is converted to eicosanoids like PGE_2 by cyclooxygenases (COX) and it is known that certain cell lines, including LLC-PK1, have defects in COX activity[10]. These studies were therefore primarily performed with the two MDCK strains. Earlier we found that MDCK cells indeed express COX-2 mRNA (unpublished results). After seeding cells at high densities, both cell types secreted relatively high levels of PGE_2, which steeply decreased after the cultures became confluent (not shown). The exposure of confluent MDCK-II monolayers for 24h to COM induced levels of PGE_2 secretion that were about 5 times higher than those secreted by control cultures. COM did not increase the secretion of PGE_2 by MDCK-I cells (Fig. 5).

Hydrogen peroxide (H_2O_2)
The production of reactive oxygen species has been proposed as one of the mechanisms by which oxalate and calcium oxalate crystals exert their damaging effect. The production of the reactive oxygen intermediate H_2O_2 was measured in the apical fluid of subconfluent cultures, confluent control cultures, confluent cultures exposed for 24 hours to 146 µg COM and mechanically damaged confluent monolayers. Compared to confluent cultures, H_2O_2 secretion was not higher in subconfluent cultures, indicating that the generation of reactive oxygen species is not a physiological event of growth-activated cells. Although the response was larger in MDCK-II, exposure to COM crystals resulted in increased levels of H_2O_2 production in both MDCK cell lines, as well as in scrape-injured monolayers (Fig. 6).

Figure 4. *The elimination of internalized [^{14}C]COM during the late response. The crystals are gradually removed from the cultures (●). Approximately 20% is was retrieved in the luminal compartment as insoluble (centrifugable) material (○). About 80% was recovered as ions that were equally distributed between both the apical (Δ) and basal (∇) fluid compartment. Because the medium in both fluid compartments was not saturated with calcium oxalate, it is not entirely clear if crystals are dissolved inside cells or in the growth medium.*



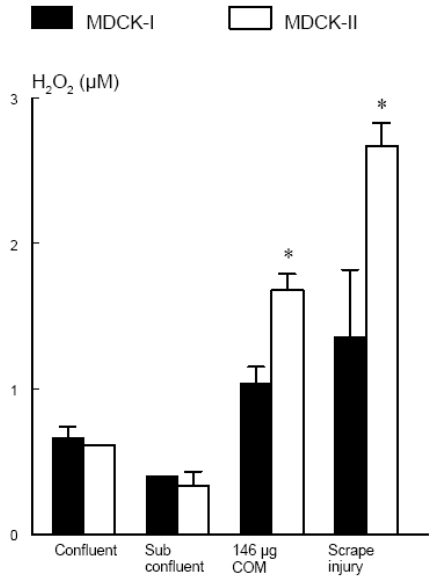


Figure 5 ***Figure 6***

Figure 5. *Prostaglandin E2 secretion in response to COM. The addition of COM crystals to confluent monolayers increased PGE2 secretion in MDCK-II, but not in MDCK-I.*

Figure 6. *Confluent monolayers are incubated with 146 µg COM crystals or scrape-damaged with the tip of a sterile culture pipette. One day after the addition of crystals or after mechanical damage, H2O2 is measured in the apical fluid compartment. The 24 hours conditioned medium of subconfluent and confluent cultures served as controls.*

[^{3}H]thymidine incorporation

To study the effect of crystals on DNA synthesis, cells with and without crystals were pulse-labeled (3 h) with [^{3}H]thymidine, directly or one day after a 24 hour incubation with 146 µg COM. [^{3}H]Thymidine incorporation was significantly higher 2 days after incubating MDCK-II with COM crystals, while COM did not affect MDCK-I proliferation (Table 1). Considering the release of MDCK-II cells from the monolayer (24h post-COM) and the time required for DNA synthesis to become significantly increased (48h post-COM), these results suggest that the

cells in the monolayer proliferated to replace released cells (regeneration).

Table 1. [^{3}H]thymidine incorporation in MDCK cells, 24 and 48 hours after incubation with 146 µg COM crystals. *Results expressed as 10^3 dpm/filter insert, (n=3). The incorporation of thymidine was increased in MDCK-II, but not in MDCK-I cells. One day post-incubation the p-value of the unpaired Student's t-test was not yet <0,05 (p=0.067), while 2 days post-incubation* the incorporation of [^{3}H]thymidine in COM treated MDCK-II cells was significantly higher compared to untreated controls (p=0.041).*

Cell line		24h	48h
MDCK-I	Control	16.6 ± 0.96	9.06 ± 0.88
	COM	15.7 ± 1.36	8.83 ± 2.62
MDCK-II	Control	5.13 ± 0.27	3.95 ± 0.23
	COM	5.63 ± 0.21	4.97* ± 0.55

Crystal-induced apoptotic or necrotic cell death

To assess if the released crystal-containing cells during the early response are viable or not, these cells were studied for various forms of cell death. Studies were performed in all cell lines. LLC-PK1 cells were not suitable because there were relatively many necrotic cells in untreated confluent monolayers. The results obtained with RCCD1 were comparable to those obtained with MDCK-I. Confluent monolayers of both MDCK strains were exposed for 24h to COM crystals and then incubated with propidium iodide (PI) to find out if crystals are capable to induce pathological cell death (necrosis). Earlier, we have demonstrated that crystals bind directly to the plasma membranes of MDCK-II and LLC-PK_1 cells, while crystals did not bind to confluent MDCK-I and RCCD1 cells and some force prevented crystals from directly contacting the plasma membrane of proliferating MDCK-I cells. This force appeared to be the invisible pericellular matrix surrounding mobile cells[11]. Our present study shows few necrotic cells in untreated confluent MDCK-II cultures (Fig.7A). The addition of COM crystals,

however, within 24 h induced massive cell necrosis as indicated by the ability of PI to enter non-fixed cells (Fig.7B). The crystal-containing exfoliated cells from figure 1 appeared to be necrotic cells (Fig.7C). Figure 8 shows that pathological cell death only occurs in MDCK-II cells that bind and internalize crystals (Fig.8 F), but not in non-adherent MDCK-I cells (Fig.8B, D). In addition, this figure shows that seven days after COM addition, crystals are still present in the MDCK-II cultures, but do no longer cause necrosis (Fig.8H).
To reveal if crystals can induce programmed cell death (apoptosis), cells were incubated for 24 hours with COM after which they were stained with Hoechst 33258 to visualize the DNA. Untreated controls had few apoptotic cells (Fig.9A). Whereas treatment with the apoptosis-inducer antimycin resulted in DNA fragmentation (Fig. 9C), COM crystals did not (Fig.9B). Apoptosis was also studied with annexin V that specifically binds to phophatidylserine at the surface of apoptotic cells. Annexin V bound only minimally to untreated confluent MDCK-I and MDCK-II monolayers (Fig. 10A and D). While treatment with antimycin A resulted in enhanced levels of annexin V binding (Fig.10C and G), 24 hours with COM did not increase annexin V binding in either cell type (Fig.10B and E). Crystals were clearly present in MDCK-II (reflection signals in red in Fig.10E) and absent in MDCK-I (no reflection signals in the laser light in Fig.10B), reinforcing the findings in figure 6 and in previously published results [7, 12] that, in contrast to confluent MDCK-II, confluent MDCK-I monolayers do not bind or take up crystals (Fig.10 E).

DISCUSSION

This study shows that calcium oxalate monohydrate (COM) crystals are toxic to renal proximal tubular cells, but not to renal collecting tubular cells. Crystals rapidly bind to and are taken up by renal proximal tubular cells, where they induce inflammation (PGE_2), oxidative cell stress (H_20_2), DNA synthesis ([^{3}H]thymidine) and pathological cell death (necrosis). The response to crystals was less marked and without necrotic cell death in renal collecting tubule cells. Two weeks after their initial contact, significant amounts of crystals were still found inside proximal tubular cells, indicating that these cells do not easily remove crystals. Proximal tubular cells showed a biphasic response to crystals. Within 24 hours, approximately half of the cell-associated crystals were released

back into the apical fluid compartment (early response), while another two weeks were required for the cells to remove again half of the internalized crystals (late response). In contrast to the late response, the early response was accompanied by inflammation-mediated cell death, suggesting that after surviving crystal internalization, the cells accepted the presence of crystals in their interior. Crystals induced increased levels of H_20_2 in proximal tubular cells as well as in scrape-damaged monolayers, indicating that any form of tissue damage may lead to H_20_2 generation. Although crystals also slightly increased the production of H_2O_2 in MDCK-I, it should be noted that these cells were non-physiologically covered for 24 hours with a relatively large amount of crystals that resulted in some cellular response. Finally, annexin V binding, DNA fragmentation and propidium iodide influx studies clearly demonstrated that crystals induce necrotic cell death without apoptosis.

The concept that crystals induce tissue damage was initially reported in non-renal tissues such as joints[13], soft tissues[14], cysts of the oral cavity[15], synovial fibroblasts [16] and endothelial cells[17]. Since kidney stones are largely composed of crystals this concept was also introduced in urolithiasis research.

Evidence came from rats fed a crystal-inducing diet and from studies performed in cell culture. In 1992 Lieske and Toback[18] reported that the internalization of calcium oxalate monohydrate crystals by BSC-1 and MDCK cells stimulated DNA synthesis. Hackett and coworkers [19]subsequently found that crystal-containing MDCK and LLC-PK1 cells were rapidly released from the cultures, which was confirmed in other studies [4, 18, 20-25]. Koul et al found that COM activates p38 mitogen-activated protein kinase (MAPK) in LLC-PK_1 [1, 26]. Crystals increased the mitochondrial production of reactive oxygen species in LLC-PK1 and MDCK cells with a subsequent depletion of the antioxidant status. The damaging effect of crystals to MDCK cells was inhibited by antioxidants[27]. The concept was born that not only oxalate, but also calcium oxalate crystals cause oxidative stress in renal tubular cells. Although these data collectively support the concept that crystal-cell interaction is damaging to renal tubular cells there is concern about the validity of this hypothesis. Let's look at the model systems used. Animals, usually rats, are made hyperoxaluric with ammonium oxalate, sodium oxalate, hydroxyproline or ethyleneglycol. Animals treated with these agents excrete higher levels of renal tubular enzymes and histological examination shows the deposition of crystals at sites

where the renal tubules are clearly damaged [28-33]. Recently, our group found that oxalate itself most likely is not nephrotoxic (paper submitted), suggesting that calcium oxalate crystals cause renal tissue damage in rats but the question is how? Based on the results in the present paper and on data in the literature it is conceivable that an oxalate overload immediately leads to the precipitation of crystals in ultrafiltrate, or that somewhere in the nephron tubules are obstructed by large crystal aggregates. It is less likely, however, that subtle crystal-cell interactions trigger inflammatory responses in late segments of the nephron.

In cell culture, damaging effects of crystals were found in MDCK [19, 27, 34-36], LLC-PK1 [20, 26, 27, 35, 37], OK-1 [24], BSC-1 [21, 38-40] and NRK52E cells [41-43]. MDCK was established in 1958 from the whole kidney of a dog. The heterogeneous nature of this cell line became clear by the isolation of various strains with completely different properties [5, 44-46]. The LLC-PK1 and OK-1 cells are widely used as model for the renal proximal tubule and BSC-1 and NRK52E cannot be assigned to a particular segment of the nephron. Thus, although crystal formation occurs in later parts of the nephron, such as the collecting ducts, crystal-cell interaction studies are performed with proximal tubular cells, mixed cell types, or undefined cell types, but not with collecting tubule cells. Data in the literature combined with the results presented in this paper suggest that crystals induce inflammation in cells that are not used to encounter crystals, such as fibroblast [16], endothelial cells [17] and renal proximal tubular cells (this study), but that they do not harm cell types that are frequently confronted with crystals in their environment, like renal collecting duct cells (this study) and most likely the urothelial cells in the remaining urinary tract. To avoid misunderstanding it should be emphasized that this does not include crystals-induced obstruction in the nephron or urinary tract. The limitations of this study are that we used cell lines derived from different species and it is questionable if they are representative for the human kidney. In addition, these cell types may have lost important functions in cell culture. We cannot exclude the possibility that crystals affect cells in nephron segments that are not included in our set of cell lines. Finally, it is possible that crystals trigger intracellular transduction pathways that were not monitored in our study.

Taken together, our results show that calcium oxalate crystals induce an inflammatory reaction in proximal tubular cells, but not in collecting tubular cells. Crystals bind and enter proximal tubular cells provoking an

inflammatory response leading to the exfoliation of necrotic crystal-containing cells that are replaced by new cells. This early reaction is followed by a much milder response during which non-necrotic crystal-containing cells are gradually eliminated from the monolayer. Crystals did not induce inflammation or necrosis in collecting tubule cells and it is unlikely that they are damaging to the epithelium in the late nephron.

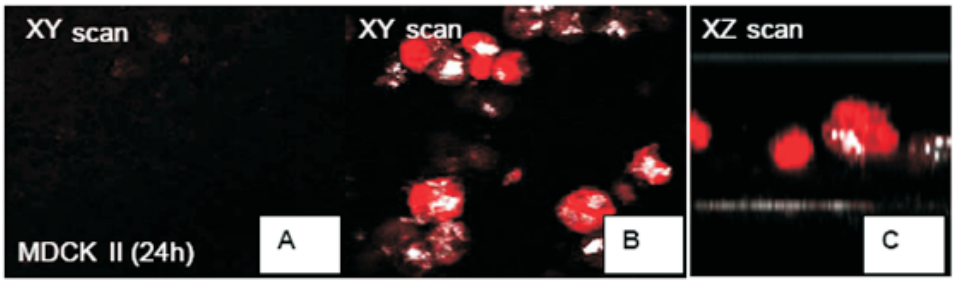


Figure 7. *Crystal-induced pathological cell death. Confluent monolayers of MDCK-II cells are incubated for 24 h with 146 µg COM after which the influx of propidium iodide (red) in living cells is studied by CLSM. Crystals are visualized by light reflection (white). There is no influx of propidium iodide in controls (A). One day after the addition of crystals, crystal-containing necrotic cells are seen all over the cultures (B). An image made perpendicular to the growth substrate revealed that most of the released crystal-containing cells are necrotic (C).*

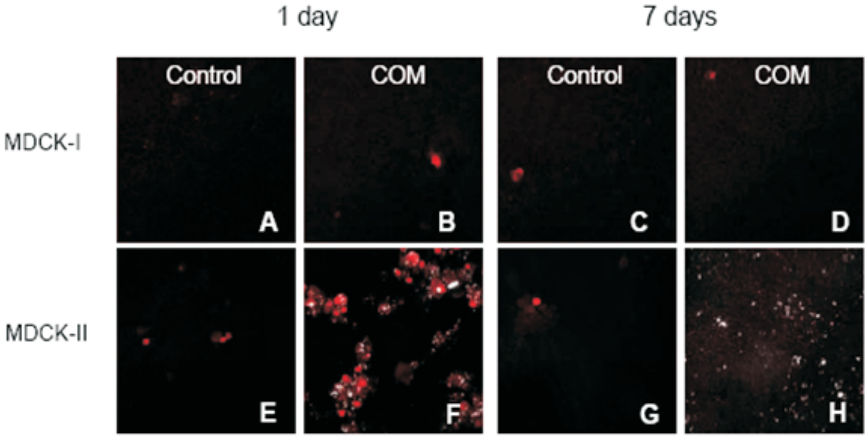


Figure 8*. Influx of propidium iodide (red) in MDCK cells in confluent monolayers, 1 and 7 days after COM (light reflection, white) addition. Influx only occasionally occurs in control MDCK-I cells (A, C) and is not increased by COM (B, D). Influx seldom occurs in control MDCK-II cells (E and G). One day after the addition of COM, crystals became associated with MDCK-II cells (F), causing necrosis (F). Seven days later COM was still present in MDCK-II, but did no longer cause cell necrosis (H).*

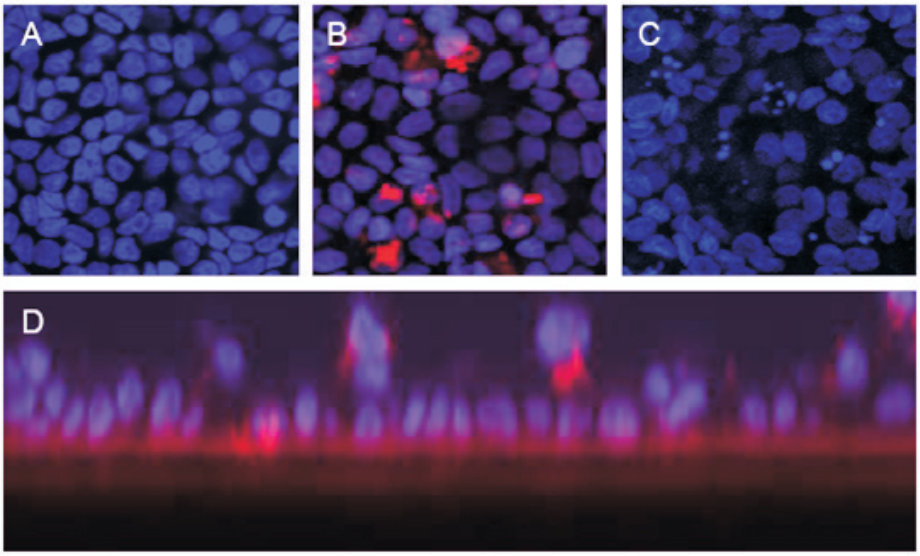


Figure 9. *Hoechst staining (blue) of confluent MDCK-II cell monolayers 24 hours after the addition of COM crystals (light reflection, red). DNA is not fragmented in untreated controls (A) or in cells that received COM crystals (B), while DNA fragmentation is clearly observed in MDCK-II cells treated with antimycin A (C). A CLSM XZ-image made perpendicular to the growth substrate showing that exfoliated crystal-containing cells are not apoptotic.*

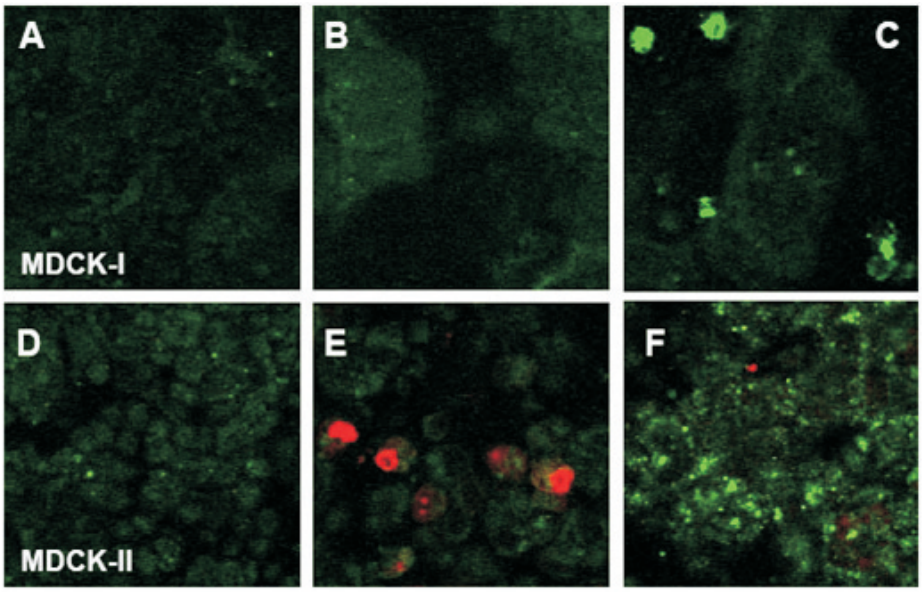


Figure 10

Figure 10. *Annexin V exhibits minimal binding to confluent monolayers of MDCK cells (A and D). The ability of annexin V to bind to MDCK-I and MDCK-II is increased after pretreatment with antimycin A C (and F). COM crystals do not increase annexin V binding to MDCK-I or MDCK-II cells (B and E). Propidium iodide influx occurs only in MDCK-II cells incubated with crystals (E).*

REFERENCES

1. Koul HK: Role of p38 MAP kinase signal transduction in apoptosis and survival of renal epithelial cells. *Ann N Y Acad Sci* 1010:62-65, 2003
2. Khan SR, Thamilselvan S: Nephrolithiasis: a consequence of renal epithelial cell exposure to oxalate and calcium oxalate crystals. *Mol Urol* 4:305-312., 2000
3. Scheid CR, Koul H, Hill WA, Lieske JC, Toback FG, Menon M: Oxalate ion and calcium oxalate crystal interactions with renal epithelial cells, in *Kidney Stones:Medical and Surgical Management*, edited by Coe FL, Favus M.J., Pak C.Y.C. , Parks J.H., Preminger G.M., Philadelphia, Lippincott-Raven Publishers, 1996, pp 129-143
4. Khan SR: Calcium oxalate crystal interaction with renal tubular epithelium, mechanism of crystal adhesion and its impact on stone development. *Urol Res* 23:71-79, 1995
5. Hansson GC, Simons K, van Meer G: Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. *Embo J* 5:483-489, 1986
6. Blot-Chabaud M, Laplace M, Cluzeaud F, Capurro C, Cassingena R, Vandewalle A, Farman N, Bonvalet JP: Characteristics of a rat cortical collecting duct cell line that maintains high transepithelial resistance. *Kidney Int* 50:367-376, 1996
7. Schepers MS, Duim RA, Asselman M, Romijn JC, Schroder FH, Verkoelen CF: Internalization of calcium oxalate crystals by renal tubular cells: A nephron segment-specific process? *Kidney Int* 64:493-500, 2003
8. Verkoelen CF, van der Boom BG, Houtsmuller AB, Schroder FH, Romijn JC: Increased calcium oxalate monohydrate crystal binding to injured renal tubular epithelial cells in culture. *Am J Physiol* 274:F958-965, 1998
9. Verkoelen CF, Romijn JC, de Bruijn WC, Boeve ER, Cao LC, Schroder FH: Association of calcium oxalate monohydrate crystals with MDCK cells. *Kidney Int* 48:129-138, 1995
10. Lifschitz MD: LLC-PK1 Cells derived from pig kidneys have a defect in cyclooxygenase. *J Biol Chem* 257:12611-12615, 1982
11. 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-E112, 2003
12. Verkoelen CF, van der Boom BG, Kok DJ, Houtsmuller AB, Visser P, Schroder FH, Romijn JC: Cell type-specific acquired protection from crystal adherence by renal tubule cells in culture. *Kidney Int* 55:1426-1433, 1999

13. Denko CW, Whitehouse MW: Experimental inflammation induced by naturally occurring microcrystalline calcium salts. *J Rheumatol* 3:54-62, 1976
14. Denko CW, Petricevic M: Sympathetic or reflex footpad swelling due to crystal-induced inflammation in the opposite foot. *Inflammation* 3:81-86, 1978
15. Aldred MJ, Sugar AW: Calcium oxalate crystals in a nasolabial cyst. *J Oral Maxillofac Surg* 44:149-152, 1986
16. Hasselbacher P: Stimulation of synovial fibroblasts by calcium oxalate and monosodium urate monohydrate. A mechanism of connective tissue degradation in oxalosis and gout. *J Lab Clin Med* 100:977-985, 1982
17. Falasca GF, Ramachandrula A, Kelley KA, O'Connor CR, Reginato AJ: Superoxide anion production and phagocytosis of crystals by cultured endothelial cells. *Arthritis Rheum* 36:105-116, 1993
18. Lieske JC, Walsh-Reitz MM, Toback FG: Calcium oxalate monohydrate crystals are endocytosed by renal epithelial cells and induce proliferation. *Am J Physiol* 262:F622-630, 1992
19. Hackett RL, Shevock PN, Khan SR: Madin-Darby canine kidney cells are injured by exposure to oxalate and to calcium oxalate crystals. *Urol Res* 22:197-203, 1994
20. Hackett RL, Shevock PN, Khan SR: Alterations in MDCK and LLC-PK1 cells exposed to oxalate and calcium oxalate monohydrate crystals. *Scanning Microsc* 9:587-596, 1995
21. Lieske JC, Toback FG: Interaction of urinary crystals with renal epithelial cells in the pathogenesis of nephrolithiasis. *Semin Nephrol* 16:458-473, 1996
22. Kohjimoto Y, Ebisuno S, Tamura M, Ohkawa T: Interactions between calcium oxalate monohydrate crystals and Madin- Darby canine kidney cells: endocytosis and cell proliferation. *Urol Res* 24:193-199, 1996
23. Kohjimoto Y, Ebisuno S, Tamura M, Ohkawa T: Adhesion and endocytosis of calcium oxalate crystals on renal tubular cells. *Scanning Microsc* 10:459-468, 1996
24. Goswami A, Singhal PC, Wagner JD, Urivetzky M, Valderrama E, Smith AD: Matrix modulates uptake of calcium oxalate crystals and cell growth of renal epithelial cells. *J Urol* 153:206-211, 1995
25. Ebisuno S, Kohjimoto Y, Tamura M, Inagaki T, Ohkawa T: Histological observations of the adhesion and endocytosis of calcium oxalate crystals in MDCK cells and in rat and human kidney. *Urol Int* 58:227-231, 1997
26. Koul HK, Menon M, Chaturvedi LS, Koul S, Sekhon A, Bhandari A, Huang M: COM crystals activate the p38 mitogen-activated protein kinase signal transduction pathway in renal epithelial cells. *J Biol Chem* 277:36845-36852, 2002
27. Khand FD, Gordge MP, Robertson WG, Noronha-Dutra AA, Hothersall JS: Mitochondrial superoxide production during oxalate-mediated oxidative stress in renal epithelial cells. *Free Radic Biol Med* 32:1339-1350, 2002
28. Selvam R, Bijikurien T: Effect of citrate feeding on free radical induced changes in experimental urolithiasis. *Indian J Exp Biol* 30:705-710, 1992

29. Khan SR: Animal model of calcium oxalate nephrolithiasis, in *Calcium oxalate in biological systems*, edited by Khan SR, Boca Raton, FL, CRC Press, 1995, pp 343-359
30. de Bruijn WC, Boeve ER, van Run PR, van Miert PP, Romijn JC, Verkoelen CF, Cao LC, Schroder FH: Etiology of experimental calcium oxalate monohydrate nephrolithiasis in rats. *Scanning Microsc* 8:541-549, 1994
31. 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
32. Khan SR, Shevock PN, Hackett RL: Acute hyperoxaluria, renal injury and calcium oxalate urolithiasis. *J Urol* 147:226-230, 1992
33. Khan SR, Shevock PN, Hackett RL: Urinary enzymes and calcium oxalate urolithiasis. *J Urol* 142:846-849, 1989
34. Thamilselvan S, Khan SR: Oxalate and calcium oxalate crystals are injurious to renal epithelial cells: results of in vivo and in vitro studies. *J Nephrol* 11 Suppl 1:66-69, 1998
35. Khan SR, Byer KJ, Thamilselvan S, Hackett RL, McCormack WT, Benson NA, Vaughn KL, Erdos GW: Crystal-cell interaction and apoptosis in oxalate-associated injury of renal epithelial cells. *J Am Soc Nephrol* 10 Suppl 14:S457-463., 1999
36. Kohjimoto Y, Kennington L, Scheid CR, Honeyman TW: Role of phospholipase A2 in the cytotoxic effects of oxalate in cultured renal epithelial cells. *Kidney Int* 56:1432-1441, 1999
37. Thamilselvan S, Khan SR, Menon M: Oxalate and calcium oxalate mediated free radical toxicity in renal epithelial cells: effect of antioxidants. *Urol Res* 31:3-9, 2003
38. Lieske JC, Spargo BH, Toback FG: Endocytosis of calcium oxalate crystals and proliferation of renal tubular epithelial cells in a patient with type 1 primary hyperoxaluria. *J Urol* 148:1517-1519, 1992
39. Lieske JC, Swift H, Martin T, Patterson B, Toback FG: Renal epithelial cells rapidly bind and internalize calcium oxalate monohydrate crystals. *Proc Natl Acad Sci U S A* 91:6987-6991, 1994
40. Lieske JC, Toback FG: Regulation of renal epithelial cell endocytosis of calcium oxalate monohydrate crystals. *Am J Physiol* 264:F800-807., 1993
41. Umekawa T, Chegini N, Khan SR: Oxalate ions and calcium oxalate crystals stimulate MCP-1 expression by renal epithelial cells. *Kidney Int* 61:105-112, 2002
42. Umekawa T, Chegini N, Khan SR: Increased expression of monocyte chemoattractant protein-1 (MCP-1) by renal epithelial cells in culture on exposure to calcium oxalate, phosphate and uric acid crystals. *Nephrol Dial Transplant* 18:664-669, 2003
43. Miller C, Kennington L, Cooney R, Kohjimoto Y, Cao LC, Honeyman T, Pullman J, Jonassen J, Scheid C: Oxalate toxicity in renal epithelial cells: characteristics of apoptosis and necrosis. *Toxicol Appl Pharmacol* 162:132-141, 2000

44. Balcarova-Stander J, Pfeiffer SE, Fuller SD, Simons K: Development of cell surface polarity in the epithelial Madin-Darby canine kidney (MDCK) cell line. *Embo J* 3:2687-2694, 1984
45. Valentich JD: Morphological similarities between the dog kidney cell line MDCK and the mammalian cortical collecting tubule. *Ann N Y Acad Sci* 372:384-405, 1981
46. Gekle M, Wunsch S, Oberleithner H, Silbernagl S: Characterization of two MDCK-cell subtypes as a model system to study principal cell and intercalated cell properties. *Pflugers Arch* 428:157-162, 1994

CHAPTER 6

OXALATE IS TOXIC TO RENAL TUBULAR CELLS ONLY AT SUPRAPHYSIOLOGICAL CONCENTRATIONS

M.S.J. Schepers, E.S. van Ballegooijen,
C.H. Bangma and C.F. Verkoelen

Submitted

ABSTRACT

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), trans-epithelial electrical resistances (TER), prostaglandin E_2 (PGE_2) secretion, lactate dehydrogenase (LDH) release, DNA synthesis ([^{3}H]thymidin incorporation), total cell numbers, reactive oxygen species (H_20_2) 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 PGE_2, LDH and H_20_2 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.

INTRODUCTION

Most kidney stones are predominantly composed of calcium oxalate (CaOx). Oxalate is a metabolic end product that is freely filtered at the glomerulus and excreted with the urine. Oxalate is a major risk factor in CaOx renal stone disease because of its poor solubility in the presence of calcium. In addition, it has been proposed that an increased oxalate

delivery to the kidney is nephrotoxic. Urinary oxalate excretion is low in healthy subjects (0.1-0.45 mmoles/24 h), somewhat higher in idiopathic CaOx renal stone disease (0.45-0.65 mmoles/24h) and considerably higher in primary hyperoxaluria (1-3.5 mmoles/24h) [1, 2]. The concept that oxalate is toxic to the kidney came from studies performed in animals [3-6] and cell culture [7-9]. These studies suggested that oxalate generates free radicals that are damaging to the renal tubular cells. There are some questions, however, about the model systems used to reach this conclusion. In animals, hyperoxaluria cannot exist without crystalluria that makes it difficult to discriminate between effects caused by either oxalate or calcium oxalate. Since oxalate rapidly forms crystals in calcium-containing growth media, the same problem may also apply to studies performed in cell culture. Another point of concern is that renal tubular cells are grown on plastic dishes receiving culture media containing 1-2 mM calcium and oxalate in the same compartment. Especially in the high oxalate range (≥1 mM oxalate) this leads to calcium depletion. Finally, most oxalate toxicity studies in cell culture are performed with renal proximal tubular cells while high oxalate occurs at the end of the nephron.

In the present investigation we studied the possible toxic effect of oxalate to renal proximal and collecting tubule cells grown as confluent monolayers on permeable growth substrates in a two-compartment culture system. Oxalate is added to the apical compartment in a simple buffer without calcium or serum, while growth medium containing calcium and serum is added to the basal compartment. Thus, during their apical exposure to oxalate, the cells have free access to nutrients and calcium from the basolateral membrane. The results show that under these conditions oxalate is toxic only at supraphysiological concentrations.

MATERIALS AND METHODS

Cell culture

MDCK-I and MDCK-II cells were kindly provided by Prof. G. van Meer (Laboratory for Cell Biology and Histology, Amsterdam Medical Center, The Netherlands). LLC-PK_1 cells were obtained from the American Type Culture Collection (ATCC). $RCCD_1$ (rat renal cortical collecting duct) cells [10] were kindly provided by Dr. M. Blot-Chabaud (INSERM U246, Faculté de Médecine Xavier Bichat, Paris, France). Routinely, cells were grown in Dulbecco's modified Eagle's Medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum (PAA Labs, Linz, Austria) and weekly replated. For experiments, cells are grown on permeable supports in a two-compartment culture system (Transwells, 24 mm diameter inserts, 4.7 cm^2 surface area, 0.4 μm pore size, Corning Costar, Badhoevedorp, The Netherlands). In this culture system, growth medium containing 10% serum is added to the basal fluid compartment, while the apical compartment receives either growth medium or calcium and serum-free buffer B containing (in mM): 124 NaCl, 25 $NaHCO_3$, 2 $NaH_2PO_4.2H_2O$, 5 KCl, 0.5 $MgCl_2.6H_2O$, 5 Na-acetate, pH 7.4, 310-320 mosmol/kg H_2O. Cells are trypsinized and seeded at a concentration of $1x10^6$ cells/polycarbonate permeable support (Corning Costar). Within 6-7 days the cells proliferate and migrate into confluent monolayers with a cell density of on average 4-6 x 10^6 cells. Routinely, PCR analyses were performed on DNA isolated from cell culture conditioned medium for the presence of mycoplasma. Cells used in this study were not contaminated with mycoplasma.

Coulter counter

To reveal at which concentrations oxalate induces crystal formation in culture medium, increasing amounts of oxalate were added to DMEM containing 1.8 mM calcium. Sodium oxalate was dissolved in Milli-Q distilled water and pipetted in the appropriate concentration in a small volume at the bottom of a tube. Subsequently 50 ml DMEM was added at room temperature and the tube vigorously shaken. After centrifugation at low speed (1000 rpm), the pellet was taken up in a smaller volume and measured in a Coulter Multisizer (Coulter Electronic Ltd, Luton, UK). The number of particles was determined in 500 μl sample volumes using a 70 μm orifice tube.

Transepithelial electrical resistances (TERs)
Tight junction formation is assessed by transepithelial electrical resistance (TER) measurements. TER is measured in ohms (Ω) after placing the insert in an Endohm 24 connected to a Voltohm meter (World Precision Instruments, Sarasota, USA). Monolayers are considered confluent as soon as the highest TERs are reached. Cells were incubated for 6 hours with increasing oxalate concentrations after which TERs were measured. The monolayers were subsequently washed and fresh serum and calcium-containing culture medium was added to both compartments to monitor if monolayers with decreased TERS are capable to re-establish the electrical resistance.

Morphological studies
Fluorescein isothiocyanate (FITC)-conjugated phalloidin was applied to stain polymeric F actin in the cell cytoskeleton. At the various time points, filter inserts were washed three times with physiological saline (PBS), fixed for 15 minutes in ethanol 70% and incubated for 15 minutes with 5 μg/ml phalloidin-FITC (Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands), washed 3 times and mounted in Vectashield (Vector Laboratories, Burlingame, USA). A 488-nm Ar laser was used to excite the FITC-phalloidin. Mounted inserts were extensively inspected, images (magnification 63x) were made of at least 10 random fields per insert with a Zeiss LSM 410 laser scanning confocal microscope (Zeiss, Oberkochen, Germany) from which representative images were selected. Experiments were repeated at least three times.

Lactate dehydrogenase (LDH) release
LDH (EC 1.1.1.27) was measured in the luminal compartment with pyruvate as substrate using a standard autoanalyzer.

Prostaglandin E_2 (PGE_2).
PGE_2 secretion into the luminal compartment was measured in a switch enzyme-linked immunoassay (EIA) Kit (Cayman). This assay is based on the competition between PGE_2 and a PGE_2-acetylcholinestrase (AchE) conjugate (as tracer) for a limited amount of PGE_2 monoclonal antibody. Because the concentration of the PGE_2 tracer is held constant while the concentration of PGE_2 varies, the amount of PGE_2 tracer that is able to bind to the PGE_2 antibody is inversely proportional to the concentration of PGE_2 in the well. PGE_2 standard curves are prepared by diluting 10

mg/mL PGE_2 in EIA buffer to produce a concentration range of 0-1000 µg PGE_2/mL. Each plate or set of strips contains a blank-, a non-specific binding-, a maximum binding and a total activity control. Standard curve samples (50 µl), controls and experimental samples are added to the wells. Each well receives 50 µl tracer except the total activity and the blank wells. The wells subsequently receive 50 µl PGE_2 monoclonal antibody except the total activity well, the non-specific binding well, and the blank well. The plate is covered with plastic film and incubated for 18 hours at 4°C. The wells and rinsed extensively with wash buffer after which 200 µL Ellman's Reagent is added to each well and 5 µl tracer to the total activity well. The plate is then covered with plastic film and developed in the dark on an orbital shaker. When the blank absorbance is between 0.3-0.8 units, the plate is read at 405-420 nm wavelengths in a Bio-Rad 550 microplate reader.

H_2O_2 determination

The reactive oxygen intermediate hydrogen peroxide (H_2O_2) was measured in the apical fluid of cells exposed to various concentrations of oxalate using the Amplex Red Hydrogen/Peroxide assay kit (A-22188; Molecular Probes, Leiden, The Netherlands). Since phenol red in the culture medium interferes with the absorbance measurements the studies are performed with buffer B, added to the apical fluid compartment, while the basal compartment receives DMEM 10% FCS without phenolred and sodium pyruvate (Gibco). A standard curve is prepared at 10 µmol/l to 1.25 µmol/l. Samples, standards and blank are added to a 96-well plate. A working solution of 100 µmol/l Amplex red reagent and 0.2 U/ml Horseradish peroxidase (HRP) is prepared and added to all the samples, standards and blank. The plate was protected from light and incubated for 30 minutes at room temperature. The absorbance was read at 560 nm using a Bio-Rad 550 microplate reader.

[^{3}H]Thymidine incorporation

To assess the effect of oxalate on DNA synthesis, confluent monolayers are incubated for 24h with various oxalate concentrations added to the apical compartment in calcium-free buffer B, while DMEM 10% FCS was added to the basal compartment. The next day (24 h) or one day later (48 h) the inserts are extensively washed and the fluid in both compartments replaced with fresh DMEM 10% FCS containing 3.7 KBq/ml [methyl-^{3}H]thymidine (Amersham). The cells are pulse-labeled for 3h, washed 3

times with PBS; the filters are cut out and counted in a liquid-scintillation counter. Results expressed in dpm/filter insert.

Apoptosis and necrosis

To assess the nature of crystal-induced cell death, annexin V (A.G. Scientific, Inc, USA) staining was applied to reveal the surface exposure of phosphatidylserine (apoptosis) and propidium iodide (Sigma Aldrich Chemie) to reveal the loss of plasma membrane integrity (necrosis). At the various time points, filter inserts were washed and unfixed cells incubated for 15 minutes with FITC labeled annexin V (1:40), washed 3 times and subsequently incubated for 15 minutes with 1 µg/ml propidium iodide, washed and mounted in Vectashield (Vector Laboratories, Burlingame, USA). Another widely applied method to study apoptosis is DNA fragmentation staining with Hoechst 33258 (Sigma Aldrich Chemie). At the various time points, filter inserts were washed and incubated with 0.1 mg/ml Hoechst, washed and mounted in Vectashield. In some experiments Hoechst dye (DNA fragmentation) and light reflection (crystals) were simultaneously applied to assess if crystal-cell interaction was accompanied with physiological cell death (apoptosis), while propidium iodide (membrane permeabilization) in combination with light reflection (crystals) were simultaneously applied to reveal if crystal-cell interaction led to pathological cell death (necrosis). Incubation of the cells for 4 hours with 1.0 µM antimycin A was used as positive control for apoptosis, while cell fixation with ethanol to permeabilize the membrane served as positive control for necrosis. Phase-contrast microscopy was applied to monitor the release of crystal-containing cells in the apical fluid compartment.

Statistical analysis

All experiments were performed at least three times. The results are presented as means ± SD 0f three independent inserts. Statistical analysis was performed with 0ne-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

RESULTS

Coulter counter.

The pH of DMEM was approximately 7.3 and was not influenced by oxalate. The measurements showed that in order to study the effect of oxalate alone the experiments should be performed in a calcium-free buffer because crystals are rapidly formed in culture medium (Fig.1).

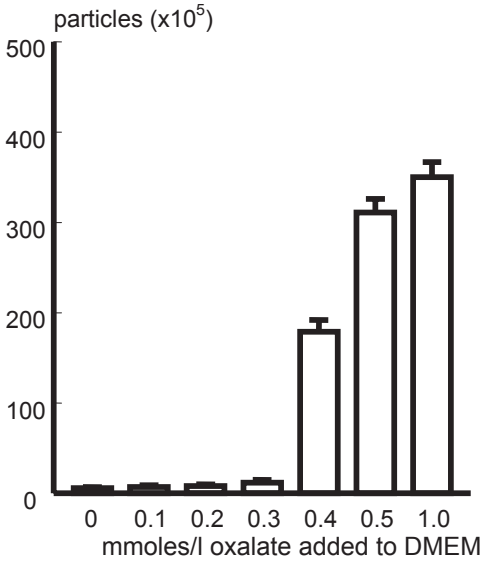


Figure 1. *Crystals are formed in culture medium (DMEM) at oxalate concentrations above 400 μM.*

Morphological studies.

Because other investigators found that the exposure of renal tubular cells in culture to oxalate leads to the spontaneous release of cells from the growth substrate [7], we started our studies by monitoring the morphological behavior of the cells in response to oxalate. Confluent monolayers were apically exposed for 24h with 0, 0.2, 0.5, 1, 5 and 10 mM oxalate after which the cell cytoskeleton was stained with phalloidin-FITC and inspected by confocal microscopy. The scans made perpendicular to the growth substrate (xz-scans) clearly show that cells are not released from the monolayer at any of the concentrations used (Fig.2). In MDCK-I cells, the first morphological alterations are observed

not sooner than at 10 mM oxalate and in MDCK-II cells at 5 mM oxalate (Fig.2). At these concentrations the cells lose their normal cubical shape, they begin to round up. Although cellular stress responses do not necessarily lead to alterations in cell shape, soluble oxalate certainly does not easily release renal tubular cells from their growth substrates. Comparable results were obtained with LLC-PK_1 and RCCD1 cells (not shown).

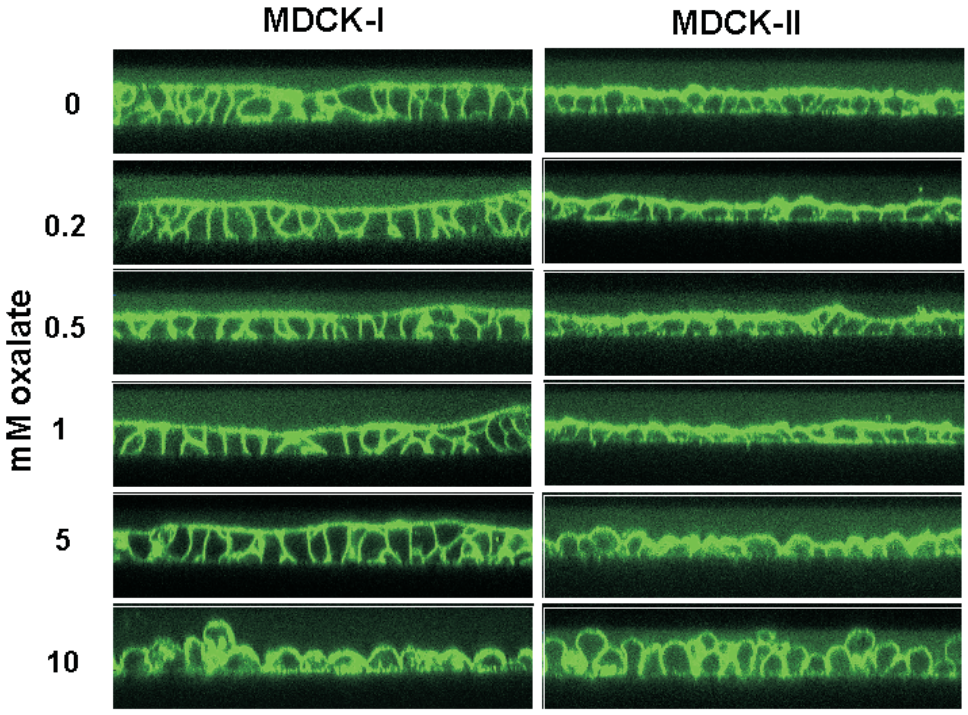


Figure 2. *Morphological alterations in renal tubular cells exposed for 24 hours to increasing oxalate concentrations. The cells are cultured as confluent monolayers on porous supports in a two-compartment culture system in which oxalate is added to the apical fluid compartment in a calcium and serum-free buffer, while DMEM with calcium and serum is added to the basal fluid compartment. The first visible effect of oxalate is observed in MDCK-I cells at 10 mM oxalate and in MDCK-II cells at 5 mM oxalate. Note that even at these extremely high oxalate concentrations the cells are not released from the growth substrate.*

Transepithelial electrical resistances (TERs)
To study oxalate-induced effects on functional properties such as monolayer epithelial barrier integrity, TER was measured across confluent monolayers exposed for 6h to increasing concentrations of oxalate (0, 1, 5 and 10 mM) in a calcium-free buffer. Collecting duct cell monolayers are much more tight (~5000 $\Omega.cm^2$) than proximal tubular cell monolayers (~ 100-200 $\Omega.cm^2$). In MDCK-II and LLC-PK1, TER was not influenced by 1 and 5 mM oxalate, but a significant reduction was seen at 10 mM. Surprisingly, both cell lines were capable to re-establish their TERs within one to two days (Fig.3). Although TER dropped across MDCK-I and RCCD1 monolayers directly after oxalate exposure, this was also observed in untreated controls, indicating that manipulation of the cells or the change of the apical fluid was responsible for the decrease in TER at this point in time. The effect of oxalate on TER across MDCK-I seemed to be concentration-dependent. Re-establishment of the electrical resistance was seen with 0, 1 and 5 mM oxalate, but not with 10 mM. TER across RCCD1 had difficulty to regain its starting value but there were only small differences between untreated controls and monolayers treated with 1 and 5 mM oxalate. TER hardly re-established from 10 mM oxalate.

Lactate dehydrogenase (LDH) release.
The release of LDH was measured as marker for plasma membrane damage. It should be noted that the absolute amounts of LDH secreted or released by these cells varied depending on the developmental stage of the monolayers. Comparisons could be made, however, when matching untreated controls of the same age were included. Confluent monolayers of MDCK-I and MDCK-II cells were exposed for 24h to increasing oxalate concentrations ranging from 0.2 to 10 mM. Up to 1 mM, the amount of LDH in the apical compartment was not different from untreated controls. Increased levels of LDH were measured in response to 5 and 10 mM oxalate in MDCK-II and MDCK-I (Fig.4).

Prostaglandin E_2 (PGE_2).
To reveal a possible inflammatory response to oxalate we measured the secretion of PGE_2 in the apical fluid compartment after 24h exposure to oxalate concentrations ranging from 0.2-10 mM. The secretion of PGE_2 by untreated controls of MDCK-I and MDCK-II was not higher after 24

hours ≤1mM oxalate. However, increased levels of PGE_2 were measured in response to 5 and 10 mM oxalate (Fig. 5).

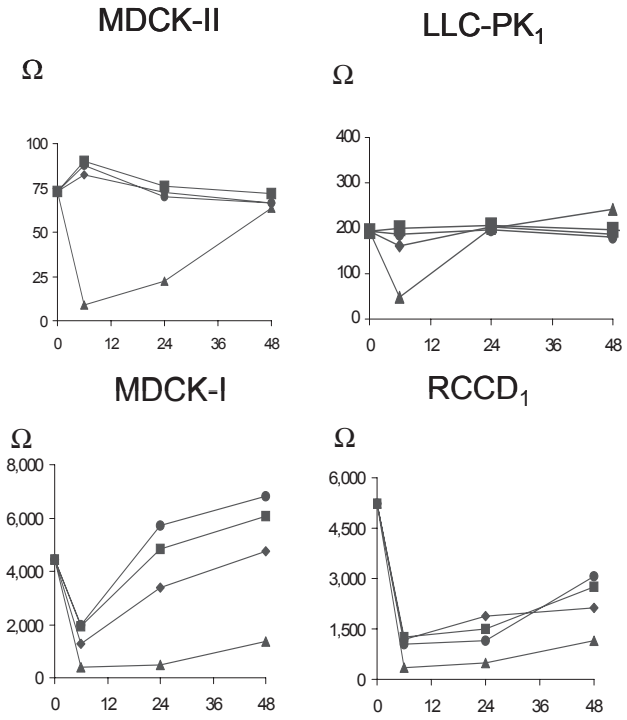


Figure 3. *Transepithelial electrical resistance measurements across confluent monolayers of various renal tubular cell types in response to a six hour exposure to increasing concentrations of oxalate (•Control, ■1 mM, ◆5 mM, ▲10 mM). Oxalate is added in a calcium and serum-free buffer to the apical fluid compartment while DMEM containing calcium and serum is added at the basolateral side. After oxalate treatment the cells received DMEM with calcium 10% serum in both fluid compartments to monitor their ability to recover from potential oxalate-induced injury. Note that TERs across the two proximal tubular cell types LLC-PK1 and MDCK-II are much lower than those across the two collecting duct cell types, MDCK-I and RCCD1.*

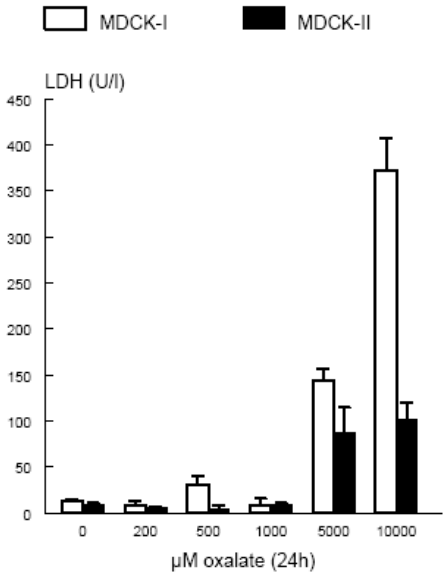


Figure 4

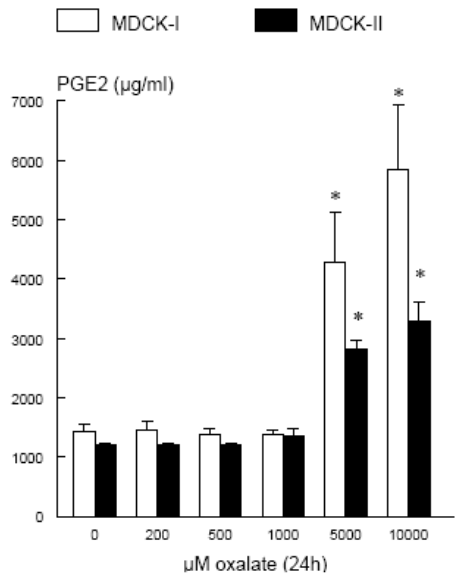


Figure 5

Figure 4. *Release of LDH in the apical fluid of confluent renal tubular cell monolayers cultured on permeable supports in a two-compartment culture system and treated for 24 hours with increasing concentrations of oxalate. Oxalate is added in a calcium and serum-free buffer to the apical fluid compartment while DMEM containing calcium and serum is added at the basolateral side. Means ± SD of a representative experiment are shown. Statistical analysis was performed using ANOVA ($p < 0.05$, $n=3$). * Significantly different compared to 0 mM oxalate.*

Figure 5. *PGE_2 secretion in the apical fluid of confluent renal tubular cell monolayers cultured on permeable supports in a two-compartment culture system and treated for 24 hours with increasing concentrations of oxalate. Oxalate is added in a calcium and serum-free buffer to the apical fluid compartment while DMEM containing calcium and serum is added at the basolateral side. Oxalate-induced inflammation occurs in both cell types at oxalate concentrations ≥5 mM. Means ± SD of a representative experiment are shown. Statistical analysis was performed using ANOVA ($p < 0.05$, $n=3$).*
** Significantly different compared to 0 mM oxalate.*

H_2O_2 determination

To study the ability of oxalate to generate reactive oxygen species the concentration of H_2O_2 was measured in the luminal fluid of confluent monolayers formed by MDCK-I and MDCK-II cells in response to a 24h exposure to increasing concentrations of oxalate (0, 0.2, 0.5, 1, 5 and 10 mM). Although in MDCK-II cells there seemed to be a relatively small increase in untreated controls and in monolayers exposed to the lowest dose of oxalate 0.2 mM, this probably reflects the vulnerability of this assay rather than a true effect on the cells. Compared with untreated controls, a significant increase in H_2O_2 production by MDCK-II was seen only at the highest oxalate concentration used (10 mM). Also the production of H_2O_2 by MDCK-I cells was increased with 10 mM oxalate (Fig.6). To study if there are also other conditions during which the production of H_2O_2 is increased we measured H_2O_2 in the apical fluid of scrape-damaged monolayers and during subconfluency. Whereas the production of H_2O_2 was relatively low in subconfluent and confluent cultures (not shown), relatively high levels of H_2O_2 were measured in the luminal fluid of scrape-damaged confluent monolayers, indicating that H_2O_2 production is not an exclusive cellular response to oxalate (Fig.6).

[^{3}H]Thymidine incorporation and total cell numbers.

Previously, oxalate was found to induce DNA synthesis [8, 11], which prompted us to study the incorporation of [^{3}H]thymidine in response to oxalate. Whereas a 24h exposure to 1.0 mM oxalate did not affect DNA synthesis in both MDCK strains, thymidine incorporation was increased in MDCK-II cells treated for 24h with 5 and 10 mM oxalate (Fig. 7). Although the incorporation of thymidine was not yet increased in MDCK-I cells at this point in time, these levels were increased one day later (not shown). Thus, although at much higher oxalate concentrations, we hereby confirm the ability of oxalate to stimulate DNA synthesis.

Now there are two possibilities: 1) oxalate has a mitogenic effect on renal tubular cells, or 2) the increase in DNA synthesis reflects damage-induced regeneration. To answer this question we decided to count the cells in a hemocytometer following oxalate exposure. The results show that relatively low oxalate concentration (≤ 1mM) had no effect on the total cell numbers and that high oxalate (≥ 5mM) decreased total cell numbers (Fig.8).

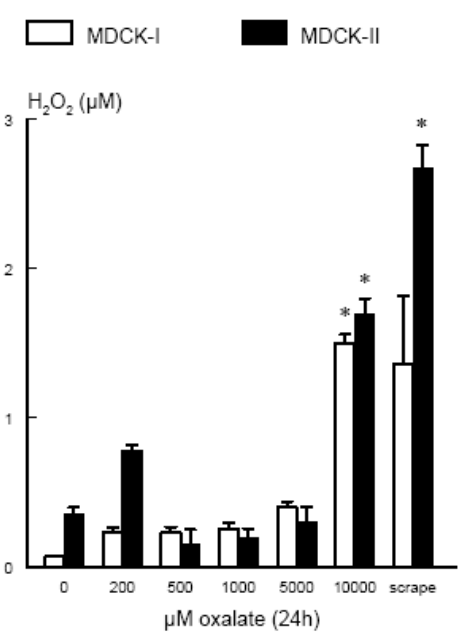


Figure 6

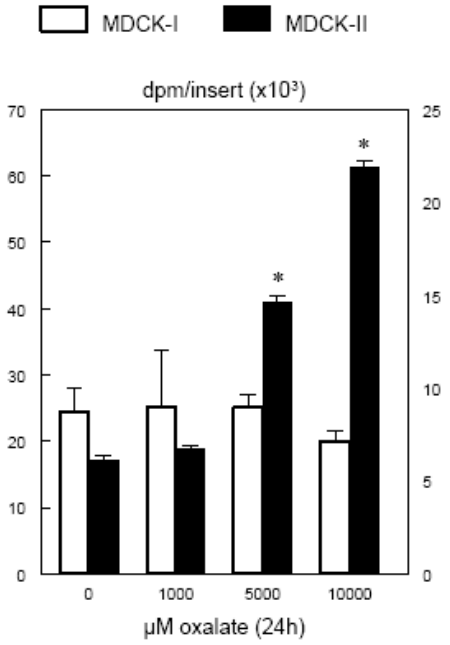


Figure 7

Figure 6. *H_2O_2 in the apical fluid of confluent renal tubular cell monolayers cultured on permeable supports in a two-compartment culture system and treated for 24 hours with increasing concentrations of oxalate or scrape-damaged with the tip of a 10 ml culture pipette. Oxalate is added in a calcium and serum-free buffer to the apical fluid compartment, while DMEM containing calcium and serum is added at the basolateral side. Oxalate-induced H_2O_2 generation occurs in both cell types at oxalate concentrations ≥ 5 mM. Means ± SD of a representative experiment are shown. Statistical analysis was performed using ANOVA ($p< 0.05$, n=3). * Significantly different compared to 0 mM oxalate.*

Figure 7. *Incorporation of [^{3}H]thymidine measured in 3 hours pulse labeling studies in renal tubular cells pre-exposed for 24 hours to increasing oxalate concentrations. Cells are cultured as confluent monolayers on permeable supports in a two-compartment culture system. Oxalate is added in a calcium and serum-free buffer to the apical fluid compartment while DMEM containing calcium and serum is added at the basolateral side. Exposure for 24 hours to ≥5 mM oxalate leads to elevated levels of thymidine incorporation in MDCK-II. Increased levels of thymidin incorporation were also seen in MDCK-I exposed to 5 and 10 mM oxalate but this occurred 2 days (48h) post-injury (not shown). Means ± SD of a representative experiment are shown. Statistical analysis was performed using ANOVA ($p< 0.05$, n=3). * Significantly different compared to 0 mM oxalate.*

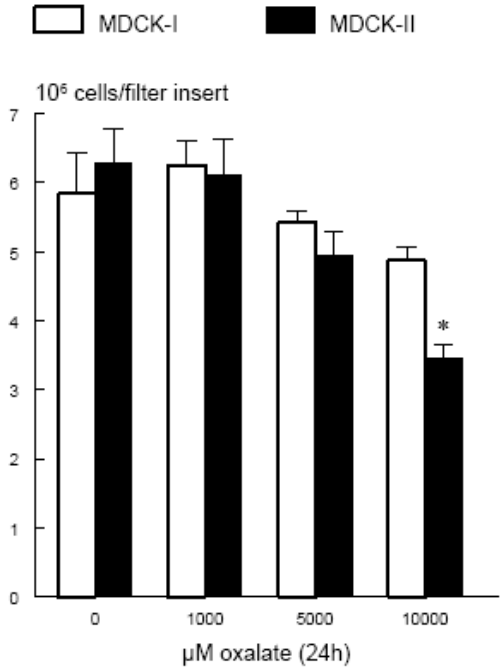


Figure 8. *Total cell numbers counted with a hemocytometer in confluent renal tubular cell monolayers cultured on permeable supports in a two-compartment culture system and treated for 24 hours with increasing oxalate concentrations. Oxalate is added in a calcium and serum-free buffer to the apical fluid compartment while DMEM containing calcium and serum is added at the basolateral side. This figure shows that the oxalate-induced increase in DNA synthesis with ≥5 mM oxalate (Fig.7) is accompanied by decreased total cell numbers. Means ± SD of a representative experiment are shown. Statistical analysis was performed using ANOVA ($p < 0.05$, $n=3$). * Significantly different compared to 0 mM oxalate*

Apoptosis and necrosis

The observation that the total cell numbers decreased after incubation with high oxalate concentrations indicated that oxalate can be lethal to renal tubular cells. Since the existing literature is contradictory on the mode of oxalate-induced cell death [12-15] we decided to study physiological (apoptosis) and pathological (necrosis) cell death in response to oxalate. Confluent monolayers were exposed to oxalate concentrations ranging from 0.2-10mM. Hoechst staining studies showed that although oxalate concentration-dependently reduced the total amount of cells in MDCK-I as

well as in MDCK-II, there were no signs of DNA fragmentation and therefore no indication for the involvement of apoptosis in this process. Treatment with the apoptosis inducer antimycin A, on the other hand, clearly resulted in DNA fragmentation in both cell types (Figure 9).
We selected a second method to assess oxalate-induced apoptotic or necrotic cell death namely a combination of the binding of annexin V to surface exposed phosphatidylserine (PS), which occurs during programmed cell death or apoptosis and the ability of propidiumiodide (PI) to enter the cell interior that occurs during unprogrammed cell death or necrosis. Again antimycin A was used as marker for apoptotic cell death. These studies showed that in contrast to antimycin, oxalate exposure to both cell types did not lead to increased annexin V binding, thereby confirming the observation that high oxalate does not induce apoptosis. On the other hand, PI entered MDCK-II cells after a 24h exposure to 5mM oxalate, while 10mM was required to cause the same effect in MDCK-I (Fig.10). The oxalate-induced reduction in total cell numbers therefore was caused by necrotic cell death.

DISCUSSION

This study shows that oxalate is not very toxic to renal tubular cells in culture. Inflammation (PGE_2), membrane damage (LDH), oxidative cell stress (H_20_2), DNA synthesis ([^{3}H]thymidine) and pathological cell death (necrosis) are observed only at extremely high oxalate concentrations ($\geq$5 mM). There is not much difference between renal proximal and collecting tubule cells in their response to oxalate. In time, high oxalate increased DNA synthesis without higher total cell numbers indicating that this reflects repair from oxalate-induced tissue damage rather than growth stimulation. Although high oxalate induced increased levels of H_20_2 this was also achieved by scrape injury, indicating that any form of tissue damage may lead to H_20_2 generation. The production of limited amounts of free radicals most likely has a functional role in the cellular response to tissue injury, rather than that oxalate-induced free radical production is responsible for the observed tissue damage. Finally, annexin V binding, DNA fragmentation and propidium iodide influx studies clearly demonstrated that high oxalate leads to necrotic cell death but not to apoptosis.

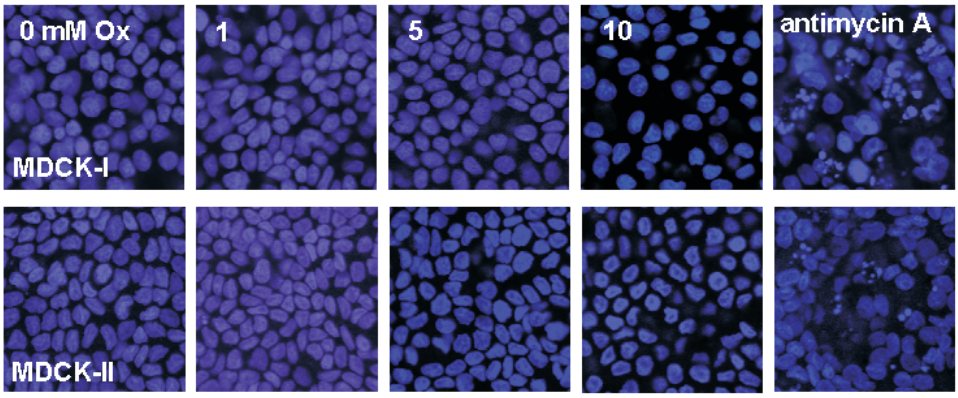


Figure 9. *Confluent monolayers cultured on a porous support in a two-compartment culture system exposed for 24 hours to increasing oxalate concentrations are stained with Hoechst to detect apoptosis. Oxalate is added in a calcium and serum-free buffer to the apical fluid compartment while DMEM containing calcium and serum is added at the basolateral side. This image shows that oxalate does not induce DNA fragmentation at any of the concentrations used. As positive control the cells are treated with antimycin A. Note that the cell density decreases at 10 mM oxalate.*

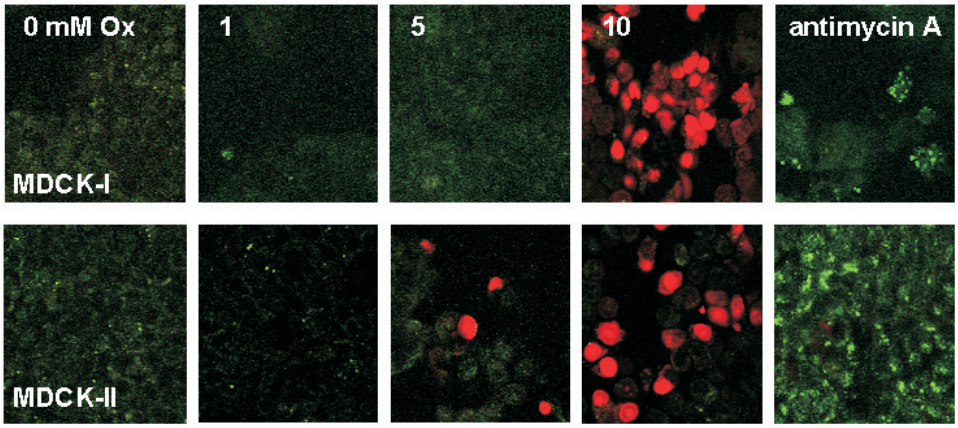


Figure 10. *Confluent monolayers cultured on a porous support in a two-compartment culture system exposed for 24 hours to increasing oxalate concentrations are stained with annexin V and propidium iodide to detect apoptosis and necrosis. Oxalate is added in a calcium and serum-free buffer to the apical fluid compartment while DMEM containing calcium and serum is added at the basolateral side. This image shows that oxalate does not lead to increased annexin V binding at any of the concentrations used. As positive control the cells are treated with antimycin A. Oxalate leads to the influx of propidiumiodide at 5 mM in MDCK-II and 10 mM in MDCK-I.*

The concept that oxalate toxicity may play an important role in the pathophysiology of renal stone disease initially was derived from studies in hyperoxaluric rats [3, 16]. The accumulation of hydroxyl radicals and decreased free radical scavengers were found in organs of hyperoxaluric rats [4]. The idea that hyperoxaluria causes free radical-mediated inflammation was supported by the beneficial effects of free radical scavengers like vitamin E, glutathione, mannitol and allopurinol [17, 18] and anti-inflammatory drugs such as calcium agonists, ACE inhibitors and fish oil [19-21]. The results obtained in animal studies inspired several investigators to study the effect of oxalate on renal tubular cells in culture [7, 8, 12, 22]. Studies performed with various cell lines demonstrated that oxalate provoked several cellular responses varying from activation of DNA synthesis to apoptotic and necrotic cell death. Oxalate-induced cell injury appeared to result from free radical-induced stress-activated protein kinase-mediated inflammation [8, 11, 13-15, 22-29]. Despite the overwhelming number of publications in this area, there is concern about the validity of this hypothesis.

Let's first look at the animal studies. Animals are treated with oxalate or oxalate precursors [5, 30, 31]. These methods successfully increase oxalate in blood and ultrafiltrate. In the primary urine, oxalate rapidly precipitates as calcium oxalate crystals [32]. Renal tissue damage in hyperoxaluric rats cannot be exclusively attributed to oxalate because crystals could also cause it. The choice to study the damaging effect of oxalate in cell culture therefore seems logical. Unfortunately, also this approach has it pitfalls. In most oxalate toxicity studies, renal tubular cells are cultured on tissue culture plastic [7-9, 22, 23, 25]. The disadvantage of this method is that confluent monolayers can be maintained only for a relatively short period of time. At confluence the cells assemble tight junctions and begin to transport salt and water from the apical to the basolateral side of the monolayer leading to the formation of fluid-filled domes between cells and growth substrate. In addition, these cells rapidly suffer from the lack of nutrients since they normally feed from the basal membrane [33, 34]. As a result of these conditions confluent monolayers rapidly begin to detach from their growth support. Another problem is the addition of oxalate to calcium-containing growth medium. Belliveau et al. [35] recently investigated the equilibrium parameters for calcium oxalate solubility in culture media. It was found that the addition of 1 mM oxalate to culture medium leads to >50% precipitation resulting in equilibrium oxalate concentrations of ≤60

μM. Microscopy and Coulter Counter measurements confirmed the formation of particles at oxalate concentrations ≥0.4 mM added to Dulbecco's essential modified medium (Fig.1). Another problem is oxalate-induced depletion of calcium from the growth medium. Calcium is an essential ingredient of growth media. Without sufficient amounts of calcium, the tight junctions will disassemble leading to cell rounding and release. Thus, also in cell culture it is difficult to differentiate between damaging effects caused by oxalate, calcium oxalate crystals or the lack of calcium.

In the present study we tried to circumvent many of these problems by culturing the cells on permeable growth substrates in a two-compartment culture system, a method that enables us to add oxalate to the apical compartment, while calcium and serum are added to the basal compartment. The results show that oxalate is toxic to renal tubular cells, but only at very high concentration (≥5 mM). The literature is controversial on the mode of oxalate-induced cell death. Some investigators found that oxalate induces apoptosis as well as necrosis [13, 14], while others found that oxalate leads to necrotic cell death [12]. Our study clearly shows that high oxalate causes necrosis but no apoptosis.

The experiments in this investigation were designed to study the damaging effect of luminal oxalate on renal tubular cells in culture. Limitations of this study are: 1) In contrast to most previous oxalate toxicity studies [7, 25, 29], our cells were cultured on permeable growth substrates and did not receive calcium and serum in the apical compartment. From the present study it is not entirely clear which of these variables is responsible for the relative insensitivity of the cells to oxalate. 2) The addition of oxalate to the apical compartment leads to apical-to-basal oxalate leakage resulting in gradual decreased luminal oxalate concentrations. The addition of oxalate to both fluid compartments is unusable because of the presence of calcium in the basal compartment. [^{14}C]oxalate flux studies demonstrated that the apical-to-basal paracellular flux of oxalate is much higher across proximal tubular cells than collecting duct cells [36]. In the proximal tubular cell types, estimated oxalate equilibrium in both fluid compartments is reached in approximately 12 hours, while numerous days are required to reach this point in collecting tubular cells. During a 24 hours incubation period the apical-to basal leakage across intact collecting tubular monolayers is neglectible, while in proximal tubular cells the luminal concentration of oxalate gradually decreases in time. Starting, for example, with 10 mM in

the apical compartment, oxalate leaks into the basal compartment until its concentration in both fluid compartments is ~3.7 mM. Despite the gradual reduction in luminal oxalate we do not believe that this alters the conclusion that oxalate is not very toxic to renal tubular cells in culture. 3) We used cell lines derived from different species and it is questionable if they are representative for the human kidney. 4) Cell lines may have lost important functions in cell culture. 5) Perhaps oxalate affects renal tubular cell types that were no part of our set of cell lines. 6) Oxalate may trigger intracellular transduction pathways that were not measured in our study.

Collectively the data of this study show that oxalate causes inflammation-mediated necrotic cell death in renal tubular cells at supra-physiological concentrations (≥5 mM), but not at oxalate concentrations that are within the physiological range (≤1 mM).

REFERENCES

1. Milliner DS, Wilson DM, Smith LH: Phenotypic expression of primary hyperoxaluria: comparative features of types I and II, *Kidney Int* 59: 31-36, 2001
2. Larsson L, Tiselius HG: Hyperoxaluria, *Miner Electrolyte Metab* 13: 242-250, 1987
3. Khan SR, Finlayson B, Hackett RL: Histologic study of the early events in oxalate induced intranephronic calculosis, *Invest Urol* 17: 199-202, 1979
4. Kurien TB, Selvam R: Induction of lipid peroxidation in calcium oxalate stone formation, *Indian J Exp Biol* 27: 450-453, 1989
5. Selvam R, Bijikurien T: Effect of citrate feeding on free radical induced changes in experimental urolithiasis, *Indian J Exp Biol* 30, 705-710, 1992
6. Baggio B, Gambaro G, Ossi E, Favaro S, Borsatti A: Increased urinary excretion of renal enzymes in idiopathic calcium oxalate nephrolithiasis, *J Urol* 129: 1161-1162, 1983
7. Hackett RL, Shevock PN, Khan SR: Madin-Darby canine kidney cells are injured by exposure to oxalate and to calcium oxalate crystals, *Urol Res* 22: 197-203, 1994
8. Koul H, Kennington L, Nair G, Honeyman T, Menon M, Scheid C: Oxalate-induced initiation of DNA synthesis in LLC-PK1 cells, a line of renal epithelial cells, *Biochem Biophys Res Commun* 205: 1632-1637, 1994
9. Scheid CR, Koul HK, Kennington L, Hill WA, Luber-Narod J, Jonassen J, Honeyman T, Menon M: Oxalate-induced damage to renal tubular cells, *Scanning Microsc* 9: 1097-1105; discussion 1105-1097, 1995
10. Blot-Chabaud M, Laplace M, Cluzeaud F, Capurro C, Cassingena R, Vandewalle A, Farman N, Bonvalet JP: Characteristics of a rat cortical

collecting duct cell line that maintains high transepithelial resistance, *Kidney Int* 50: 367-376, 1996

11. Koul H, Kennington L, Honeyman T, Jonassen J, Menon M, Scheid C: Activation of c-myc gene mediates the mitogenic effects of oxalate in LLC-PK1 cells, a line of renal epithelial cells, in *Kidney Int* 50: 1525-1530, 1996
12. Knoll T, Steidler A, Trojan L, Sagi S, Schaaf A, Yard B, Michel MS, Alken P: The influence of oxalate on renal epithelial and interstitial cells, *Urol Res* 32: 304-309, 2004
13. Miller C, Kennington L, Cooney R, Kohjimoto Y, Cao LC, Honeyman T, Pullman J, Jonassen J, Scheid C: Oxalate toxicity in renal epithelial cells: characteristics of apoptosis and necrosis, *Toxicol Appl Pharmacol* 162: 132-141, 2000
14. Khan SR, Byer KJ, Thamilselvan S, Hackett RL, McCormack WT, Benson NA, Vaughn KL, Erdos GW: Crystal-cell interaction and apoptosis in oxalate-associated injury of renal epithelial cells, *J Am Soc Nephrol* 10Suppl 14: S457-463, 1999.
15. Wiessner JH, Hasegawa AT, Hung LY, Mandel NS: Oxalate-induced exposure of phosphatidylserine on the surface of renal epithelial cells in culture, *J Am Soc Nephrol* 10 Suppl 14: S441-445, 1999
16. Dykstra MJ, Hackett RL: Ultrastructural events in early calcium oxalate crystal formation in rats, *Kidney Int* 15: 640-650, 1979
17. Lenin M, Thiagarajan A, Nagaraj M, Varalakshmi P: Attenuation of oxalate-induced nephrotoxicity by eicosapentaenoate-lipoate (EPA-LA) derivative in experimental rat model, *Prostaglandins Leukot Essent Fatty Acids* 65: 265-270, 2001
18. Lenin M, Latha LM, Nagaraj M, Varalakshmi P: Mitigation of free radical toxicity in hyperoxaluric condition by a novel derivative eicosapentaenoate-lipoate, *Hum Exp Toxicol* 21: 153-158, 2002
19. Toblli JE, Ferder L, Angerosa M, Inserra F: Effects of amlodipine on tubulointerstitial lesions in normotensive hyperoxaluric rats, *Hypertension* 34: 854-858, 1999
20. Toblli JE, Stella I, de Cavanagh E, Angerosa M, Inserra F, Ferder L: Enalapril prevents tubulointerstitial lesions by hyperoxaluria, *Hypertension* 33: 225-231, 1999
21. Toblli JE, Ferder L, Stella I, De Cavanaugh EM, Angerosa M, Inserra F: Effects of angiotensin II subtype 1 receptor blockade by losartan on tubulointerstitial lesions caused by hyperoxaluria, *J Urol* 168: 1550-1555, 2002
22. Scheid C, Koul H, Hill WA, Luber-Narod J, Kennington L, Honeyman T, Jonassen J, Menon M: Oxalate toxicity in LLC-PK1 cells: role of free radicals, *Kidney Int* 49: 413-419, 1996
23. Hackett RL, Shevock PN, Khan SR: Alterations in MDCK and LLC-PK1 cells exposed to oxalate and calcium oxalate monohydrate crystals, *Scanning Microsc* 9: 587-596, 1995
24. Bhandari A, Koul S, Sekhon A, Pramanik SK, Chaturvedi LS, Huang M, Menon M, Koul HK: Effects of oxalate on HK-2 cells, a line of proximal tubular epithelial cells from normal human kidney, *J Urol* 168: 253-259, 2002

25. Scheid C, Koul H, Hill WA, Luber-Narod J, Jonassen J, Honeyman T, Kennington L, Kohli R, Hodapp J, Ayvazian P, Menon M: Oxalate toxicity in LLC-PK1 cells, a line of renal epithelial cells, *J Urol* 155: 1112-1116, 1996
26. Thamilselvan S, Hackett RL, Khan SR: Cells of proximal and distal tubular origin respond differently to challenges of oxalate and calcium oxalate crystals, *J Am Soc Nephrol* 10 Suppl 14: S452-456, 1999
27. Kohjimoto Y, Kennington L, Scheid CR, Honeyman TW: Role of phospholipase A2 in the cytotoxic effects of oxalate in cultured renal epithelial cells, *Kidney Int* 56: 1432-1441, 1999
28. Kohjimoto Y, Honeyman TW, Jonassen J, Gravel K, Kennington L, Scheid CR: Phospholipase A2 mediates immediate early genes in cultured renal epithelial cells: possible role of lysophospholipid, *Kidney Int* 58: 638-646, 2000
29. Chaturvedi LS, Koul S, Sekhon A, Bhandari A, Menon M, Koul HK: Oxalate selectively activates p38 mitogen-activated protein kinase and c-Jun N-terminal kinase signal transduction pathways in renal epithelial cells, *J Biol Chem* 277: 13321-13330, 2002
30. Khan SR: Animal model of calcium oxalate nephrolithiasis, *Calcium oxalate in biological systems*, edited by Khan SR, Boca Raton, FL, CRC Press, pp 343-359, 1994
31. de Bruijn WC, Boeve ER, van Run PR, van Miert PP, Romijn JC, Verkoelen CF, Cao LC, Schroder FH: Etiology of experimental calcium oxalate monohydrate nephrolithiasis in rats, *Scanning Microsc* 8: 541-549, 1994
32. 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
33. Handler JS, Green N, Steele RE: Cultures as epithelial models: porous-bottom culture dishes for studying transport and differentiation, *Methods Enzymol* 171: 736-744, 1989
34. Rodriguez-Boulan E, Nelson WJ: Morphogenesis of the polarized epithelial cell phenotype, *Science* 245: 718-725, 1989
35. Belliveau J, Griffin H: The solubility of calcium oxalate in tissue culture media, *Anal Biochem* 29: 69-73, 2001
36. Verkoelen CF, Romijn JC , de Bruijn WC, Boeve ER, Cao LC: Absence of a transcellular oxalate transport mechanism in LLC-PK1 and MDCK cells cultured on porous supports, *Scanning Microsc* 7: 131-138, discussion 1038-1040, 1993

CHAPTER 7

GENERAL DISCUSSION

7 .1 INFLUENCE OF URINE ON CRYSTAL-CELL INTERACTION

The development of a renal stone largely depends upon the structural composition of the surface epithelium lining the renal tubules. CaOx crystals occur in the distal tubules and in the collecting ducts. It is crucial that the tubular epithelium in this region does not retain crystals, so that they can be excreted with the urine. Cell surface protection against crystal adherence is of less importance to the proximal tubular epithelium, as crystallization is not to be expected there. Crystallization in the early parts of the nephron is exceptional and can only occur in patients with late stages of primary hyperoxaluria or after ethylene glycol poisoning. As discussed in Chapter 1, individual parts of the nephron contain specialized epithelial cells. Although the prime function of these cells is not to bind or repel crystals, they have to be able to cope with them if needed.

Previous studies by our group and others have shown that cell lines derived from renal collecting tubular epithelium lose their affinity for calcium oxalate monohydrate (COM) crystals at confluence [1, 2]. Injury inflicted to the epithelium by scraping cells from the monolayer, triggers cells at the edge of the wound to migrate and proliferate. During this process, they regain their crystal binding capacity. It is possible that *in vivo*, urinary proteins can influence the binding of crystals to the epithelium by interacting with the surface of the crystals and/or the cells. Theoretically, while in non-stone forming individuals the quality and quantity of these urinary inhibitors is sufficient to prevent the stone-forming process, in stone-formers the urine might be less able to prevent crystallization, aggregation and crystal retention.

Several studies have been undertaken to identify markers for stone formation in urine of stone forming patients. Considering the amount of proteins present in urine, detecting small differences between healthy subjects and stone-formers is a difficult process. Not only are there variations in urine composition within groups, even in one person there can be day-to-day variations. Comparing whole urines, e.g. using 2D-gel electrophoresis, looking for differences in the complete protein pattern is like looking for a needle in a hay stack. Given that there are indeed different expression patterns found, one has to be sure that those are derived from stone formation in the kidney. The final urine contains a

mixture of components secreted along the entire urinary tract and filtered blood proteins, making it virtually impossible to backtrack them to a specific part of the kidney, or even one specific nephron segment. Even when specific markers are produced along the renal tubules, the possibility exists that these are transported to the basal side of the epithelium instead of into the tubular fluid. If this happens, one cannot detect those in the urine but in the blood. Therefore, it might be a better strategy to initially narrow down the search to known crystallization inhibitors like osteopontin (OPN), prothrombin (PT) and inter-α-inhibitor (IαI). These proteins are known to be present in the organic crystal matrix [3] and are also involved in injury repair or inflammation [4, 5]. The presence of these substances in the urine might provide a clue for the presence of an activated, crystal-binding epithelium lining the renal tubules.

A comparison between urines of clinically active stone formers (SFs) and healthy individuals using sodium dodecyl sulphate- polyacrylamide electrophoresis (SDS-PAGE) did not bring to light differences in relative densities of CaOx crystallization inhibitors osteopontin and prothrombin, but inter-α-inhibitor trimer was about twofold increased. The hypothesis that the incidence of CaOx nephrolithiasis is higher in men because of a generalized decrease in CaOx crystallization inhibitors compared to women was not supported by the results of this and other studies [6, 7].

Another approach is to study substances that can be found in the stone matrix. In this manner, only molecules with affinity for crystals or for other substances in the crystal matrix are collected, concentrating the pool of potential markers. Crystallization can be induced in urine samples through the addition of oxalate, after which the crystal matrix is investigated [8-12]. After dissolving the crystals with EDTA, the remaining molecules can be separated by gel electrophoresis to be subsequently identified. The group of Khan induced CaOx as well as CaP crystals in urine of normal subjects and stone formers and subsequently identified protein components that became entrapped in the crystal matrices. There were no differences between stone formers and normal subjects observed in either the amount of crystals formed or the organic crystal matrix. Albumin, inter-α-inhibitor related proteins, α-1-micro-globulin, osteopontin (OPN), Tamm-Horsfall protein (THP) and pro-thrombin (PT)-related proteins were detected. The main constituents

were albumin, PT-related proteins and OPN. CaP crystals, which contained more proteins than CaOx crystals, also contained a large amount of THP [12].

Incubation of CaOx crystals in physiological urine (pH 5.5) led to less aggregation of CaOx crystal seeds compared to controls. Alkaline urine (pH 8.0) on the other hand did not affect the crystal morphology [13]. Urinary macromolecules can influence the CaOx crystal structure, as shown by Wesson et al. In an *in vitro* system at pH 7.5, in the presence of urinary inhibitors of crystallization like OPN and PT, the favoured form of crystals shifted from CaOx monohydrate (COM) to CaOx dihydrate (COD). In cell culture, COM have greater affinity for the cell surface than COD [14]. Instead of using animal models or cell culture for mimicking the development of renal stones, some investigators have developed in *vitro* model systems to grow stones out of small calcium oxalate fragments by incubating them in flowing (artificial) urine. In these "stone-farms" the impact of specific urinary components on stone growth composition can be investigated [15-17].

In Chapter 2 of this thesis, we studied the effects of urine from normal healthy males on COM crystal binding to LLC-PK_1 cells and to MDCK-I cells during wound healing. During incubation of CaOx crystals in urine, crystallization inhibitors with affinity for the crystals form a coat around them, altering their surface characteristics. This might affect the binding of CaOx to the renal tubular epithelial cells. If urine should indeed prevent crystal retention, then COM crystal binding to injured cells would be similar to that of undamaged MDCK-I monolayers that lost their affinity for crystals. The results of our study showed that COM crystals that were coated with urinary proteins bound to the epithelium almost to the same extent as uncoated crystals. In a parallel series of experiments, we compared the binding of treated and untreated crystals to LLC-PK_1 cells that constitutively bind crystals during all stages of development. The results were comparable to those obtained with MDCK-I. Thus, the cell surface properties of the epithelium appeared to be of more importance for crystal retention than the presence of urinary inhibitors. In other words, if the epithelium has become susceptible to CaOx crystal binding, the crystals will bind regardless of the presence of inhibitors in the urine.

In another study, Kumar et al. found that coating of CaOx crystal with whole urine decreased crystal binding to MDCK-I cells cultured on impermeable substrates, while urine from which molecules bigger than 10 kDa were filtered out did not. Apparently, these macromolecules were responsible for the inhibiting effects. Urine exerted its effect on the crystals, and not on the cell surface, as pre-treatment of the cells did not affect crystal binding [18]. These authors incubated their crystals in a larger volume of urine than we did, which might in part explain the differences between the results of the studies. It is indeed possible that alterations in the urinary protein-to-crystal ratio result in differences in the type or amount of proteins in the coat of the crystals, affecting their affinity for the epithelium. The effect of urine pH on CaOx crystal binding to both healthy (polarized) and injured (nonpolarized) primary IMCD cells was investigated by the group of Mandel. Below pH 6 the negative charge on the crystal surface decreases and the attachment to injured cells enhanced. At lower pH, more proteins adhered to the crystal surface and the tertiary structure of the adsorbed protein was altered. Coating of crystals with urine macromolecules at pH 5, 6 or 7 greatly reduced their binding to normal as well as injured epithelia. At all pH values, THP was the most abundant protein in the crystal coating. Removal of one crystallization inhibitor from the total pool of urinary proteins is unlikely to enhance crystal binding to the epithelium, since other inhibitors will fill in the gap. Therefore it seems more useful to study the effect of whole urine instead of that of isolated urinary proteins. As shown in this study, alterations in the urine pH have more effect on the type and structure of proteins attached to the crystals. [19].

From these studies, it can be concluded that urinary components can indeed influence the interaction between CaOx crystals and the renal tubular epithelium. However, effects are depending on the cell surface composition of the epithelium as well as on the urine composition, especially the pH. Although I do not dispute the finding that urinary macromolecules can inhibit the binding of crystals to a certain extent, most likely the cell surface properties of the epithelium are far more important. At times when the tubular lining becomes adherent to crystals, the protective effects of urinary inhibitors are limited, since they cannot completely block the binding of crystals

7.2 HYALURONAN AND PERICELLULAR MATRIX FORMATION

A few years ago, the polysaccharide hyaluronan (HA) was identified as a key crystal binding molecule at the cell surface of proliferating and migrating MDCK-I cells. More detailed investigations have shown that HA is not just a simple cell surface molecule.

Hyaluronan is produced by hyaluronan synthases (HAS), that are located inside the cell membrane. HA synthesis takes place on the cytoplasmic side of the plasma membrane, after which the product is extruded across the plasma membrane into the extracellular space. There are three types of synthases (HAS-1, -2 and -3) that have specific enzymatic properties, synthesizing hyaluronan of varying molecular weights. HAS-1 is the least active of the three HAS proteins and drives the synthesis of high molecular weight HA ($> 10^6$ Da). HAS-2 is more active than HAS-1, is widely expressed throughout embryonic development and also produces high molecular weight HA. HAS-3, the most active of the three HAS-proteins, drives the synthesis of short HA chains ($< 2 \times 10^5$ Da) [20-22]. Hyaluronan sugar chains are connected to the cell surface via hyaluronan synthases (HAS-1, -2 or -3) and/or via receptors like CD44 [23, 24]. The hyaluronan molecules can get as large as 10 million Da and are intertwined and stabilized via large proteoglycans and hyaluronan-binding proteins (HABP), forming a relatively thick water-attracting layer. This cell coat is often called a pericellular matrix (PCM) [25-27]. PCMs are formed by many cell types, like fibroblasts, chondrocytes and vascular smooth muscle cells. During proliferation and wound repair, they are creating a path for migrating cells. Cell coats are also protecting the cell surface against invasions of e.g. bacteria [28-31]. In cell culture, metastatic prostate adenocarcinoma cells assembled PCMs to enable adhesion to bone marrow endothelial cells [32].

In the kidney, HA is normally present only in the medullary interstitium, where it is involved in water homeostasis. It is not expressed at the luminal surface of the tubular epithelium in healthy kidneys [33-35]. Hyaluronan can be upregulated in the kidney during renal inflammatory diseases like interstitial nephritis[36], acute ischemic injury [37, 38], autoimmune renal injury [39] and acutely rejecting human kidney grafts [40].

The production of hyaluronan can be induced by pro-inflammatory stimuli [41]. Eicosanoids are important local mediators of inflammation. Arachidonic acid (AA) is metabolized by cyclooxygenase (COX) into eicosanoids including leukotriens, thromboxane, prostacyclin and prostaglandins [42, 43]. During inflammation activated cytosolic phospholipase A_2 ($cPLA_2$) cleaves AA from membrane phospholipids as rate limiting step in the production of eicosanoids. There are two different COX isoenzymes, COX-1 and COX-2. COX-1 is constitutive in many organs and cells and catalyzes the prostaglandin (PG) synthesis believed to support physiologic functions. The COX-2 isoform is also constitutive in some tissues, but unlike COX-1, this isoenzyme is markedly induced by cytokines and growth factors and catalyzes the synthesis of pro-inflammatory prostaglandins [44]. There is a relationship between the production of eicosanoids and hyaluronan. Prostaglandin E_2 (PGE_2) stimulates the production of HA in cultured rabbit pericardial mesothelial cells and in fibroblasts[45, 46]. Hyaluronan increases glomerular COX-2 protein expression in a p38 MAP-kinase-dependent process [47] and in renal tubular cells hyaluronan-induced COX-2 promoted the production of eicosanoids[48]. The production of HA in the glomerulus depends on prostaglandins [49, 50]. Interaction of HA with cell surface receptors triggers the production of pro-inflammatory cytokines, chemokines and adhesion molecules [51]. In renal tubular cells, low molecular weight HA stimulates COX-2, but not COX-1. The expression of monocyte chemoatractant protein (MCP-1) is induced by low molecular weight, but not high molecular weight HA [47, 48, 52].

Using our cell culture models, we found that the majority of MDCK-I cells form hyaluronan-rich PCMs during migration, while only few MDCK-II cells are able to do so (*Chapter 3*). In order to form cell coats, sufficient high MW HA has to be produced. Furthermore, the presence of cell surface receptors like CD44 is required to connect the HA chains to the cell surface and hyaluronan binding proteins (HABPs), e.g. large proteoglycans, are necessary to stabilize the cell coat [25, 26]. During proliferation, MDCK-I cells produced about six fold more cell-surface associated HA than MDCK-II cells, while LLC-PK_1 cells did not synthesize cell-associated HA. We determined the amount of high molecular weight HA ($> 10^6$ Da) that was secreted into the apical fluid of subconfluent cells and found that MDCK-I cells produced significant amounts of high MW HA, while MDCK-II cells synthesized only small

amounts of high MW HA. In addition, MDCK-I cells assembled large amounts of high MW proteoglycans, necessary for stabilizing the peri-cellular matrix. Results from this study provide evidence for the concept that PCM formation is necessary for crystal retention in the renal distal tubule-collecting duct. As a rule, these cells are normally protected form crystal binding. Proximal tubular cells appear to be constitutively covered with molecules with affinity for CaOx crystals. [53].

Primary cultures of tubular epithelial cells derived from human kidneys behaved similar to the MDCK cell lines. At subconfluence, proximal as well as distal tubular cells were susceptible to COM crystal binding. Distal tubular cultures lost this affinity for crystals when they achieved confluence. As with the cell lines, HA was expressed at the cell surface of proliferating subconfluent cells where it formed a PCM, but was absent from the confluent cultures. To strengthen those observations, it was found that in damaged rat kidneys hyaluronan was expressed at the luminal side of the tubules, while HA was not found in the lumen of intact tubules [5].

Recent data from a study with rats given ethylene glycol in drinking water to induce crystal formation and -retention have provided more evidence for the concept that HA might be involved in stone disease. In the renal cortex as well as outer stripe of outer medulla (OSOM, HA is virtually undetectable in control rats. EG-treated rats display HA in the cortex and the OSOM at days 4 and 8 after beginning of treatment. Injured/regenerating tubule epithelial cells express HA on their apical surface, and at these sites calcium oxalate crystals are found attached to the tubule wall. Interestingly, the presence of HA at the apical surface was accompanied by that of osteopontin (OPN) and CD44. In healthy kidneys, CD44 and OPN are not expressed at the luminal side of the renal tubules [54]. OPN and HA are both ligands for the cell surface receptor CD44. OPN is known as urinary inhibitor of crystallization [55]. At the apical cell surface, it can act as crystal binding molecule [5, 54].

Hyaluronan has been shown to be an important crystal binding molecule in cell culture as well as in animal models for nephrolithiasis. Production of HA is stimulated during proliferation and injury repair. Hyaluronan was found inside the organic renal stone matrix [56, 57]. Detection of HA in human stone forming kidneys would provide evidence for its role

in crystal-cell interaction *in vivo*. It is however virtually impossible to pinpoint the exact sites where stone formation is initiated. Nevertheless, studies will be undertaken to investigate the presence of HA in biopsy material obtained at the site of Randall's plaques, where stones have been attached. In theory, crystalline material that has been in contact with activated hyaluronan-covered tubular epithelial cells before continuing its way through the nephron might have affinity for the surface of Randall's plaques. Crystals that are not retained by the tubular epithelium and therefore not coated with HA, on the other hand, are less likely to bind to Randall's plaques.

7.3 FATE OF CELL-BOUND CRYSTALS

Cells derived from different parts of the nephron interact with crystals in specific ways that appear to vary from segment to segment. Normal confluent distal tubule-like cells do not have affinity for CaOx crystals, while proximal tubule-like cells bind crystals at all stages of development [1]. To study the fate of crystals that have been bound to the cell surface in distal as well as proximal tubule-like cells, we therefore used proliferating cells. As discussed in Chapter 4, renal tubular cell lines exposed to CaOx crystals during subconfluence initially retain them at the apical surface. When the time of exposure is extended, proximal- and distal tubule-like cells respond differently. While the first ones internalize the crystals, the latter ones keep the crystals on the outside of the cells. This is probably due to the fact that proximal tubular cells i*n vivo* normally do not encounter CaOx crystals and therefore do not display defence mechanisms against their retention. Crystallization in the early parts of the nephron is exceptional and can only occur in patients with late stages of primary hyperoxaluria or after ethylene glycol poisoning. Otherwise, plasma oxalate values are not high enough to cause nucleation calcium oxalate nucleation [58].

Lieske et al. have discussed a patient with type 1 primary hyperoxaluria, requiring a combined liver-kidney transplantation. A biopsy two weeks after the operation displayed calcium oxalate crystals inside the renal tubular cells. These crystals appeared to stimulate cell proliferation. Crystals seemed to be moved from the tubular lumen through the

basement membrane into the interstitium. One month after transplantation, calcium oxalate deposits were no longer observed [59].

The presence of intracellular crystals was also shown in rats kept on a so-called crystal inducing diet of ethylene glycol (EG) alone or in combination with ammonium chloride (AC). Most crystals were observed within the tubular lumen in the renal medulla, where they were firmly attached to the cell surface and covered by neighbouring epithelial cells. A smaller number of crystals was present in the intercellular spaces and inside the epithelial cells and the interstitium. Intracellular oedema and blebbing of the microvilli occurred in the crystal-containing tubules[60]. Some investigators believe that these intracellular crystals can travel through the basement membrane into the interstitium[61]. When crystals are anchored to the basement membrane of the peripheral collecting duct, they can aggregate and move towards the papillary surface to form a stone nidus[60].

Ebisuno et al. studied kidney tissues from rats and humans, looking for adhesion and/or endocytosis of CaOx crystals. Sprague-Dawley rats that were peritoneally injected with sodium oxalate had formed CaOx crystals inside their kidneys. These crystals were located inside the tubular lumen or internalized into the membrane in the cortical region, whereas in the papilla crystal aggregates were located freely inside the degenerated tubular lumen, together with cell debris. In human kidney tissue obtained from renal stone formers without underlying disorders like primary hyperoxaluria, before the era of ESWL, crystals were observed inside the tubular lumen or attached to the tubular epithelium in about half of the specimens. Some crystals were seen inside the cells [62].

It is believed that CaOx crystals that are taken up by renal tubular epithelial cells act as stressor, leading to cell activation or injury. The internalization of CaOx crystals has been observed in different types of renal tubular epithelial cells in culture, like, LLC-PK_1 , BSC-1 , OK-1, MDCK and NRK-52E [60, 62-72]. The crystals come in contact with microvilli on the apical cell surface, after which they protrude through the membrane into the cell. Lieske et al. reported already in 1992 that CaOx crystals are avidly internalized by monkey kidney BSC-1 and canine kidney MDCK cells, where they initiate DNA synthesis and induce cell proliferation [67]. Large amounts of CaOx crystals might be

toxic to MDCK cells, as shown by Hackett et al. Addition of 500 µg/ml CaOx crystals led to some degree of cell detachment and release of enzymes (LDH, NAG, LAP, γ-GT). Similar effects were observed of 200 µg/ml oxalate ions [66, 73]. By contrast, our group did not observe toxic effects of CaOx crystals on MDCK cells at 100 µg/ml (or 32 µg/cm^2) [74].

In Chapter 5 of this thesis, we investigated whether CaOx crystals can actually induce damage to renal tubule epithelial cells. The responses of confluent proximal and distal tubular cells to increasing amounts of CaOx were described. While collecting tubule cells (MDCK-I) do not bind, take up or respond to CaOx crystals, renal proximal tubular epithelial cells have to cope with CaOx crystals that are bound and internalized. In MDCK-II cells an early and a late response could be distinguished. After their association with the plasma membrane COM crystals were rapidly taken up by both proximal tubule cells to remain lodged inside the cells for weeks. Within the first 24 hours, about 50% of the cell-associated COM were released back into the apical fluid. The other half of the crystals were gradually eliminated by the release of crystals and/or crystal-containing cells during the following fortnight. The early proximal tubular response was characterized by an increased secretion of PGE_2 and incorporation of [^{3}H]thymidine accompanied by the release of crystal-associated necrotic cells without signs of apoptosis. PGE_2 secretion declined to control levels after the immediate response, while crystal-containing cells remained incorporated in the otherwise functional monolayers. This study shows that COM triggers acute inflammation and necrosis in proximal tubular cells. It is however unlikely for these cells to encounter crystals *in vivo*. Collecting duct cells did not bind and internalize crystals. We did not observe crystal-induced activation, injury or cell death. 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.

7.4 OXALATE TOXICITY

During the past decade, many research groups have focussed on studying the role of oxalate toxicity in the development of nephrolithiasis. Oxalate toxicity was extensively studied using animal models, but also in cell culture.

Animal models

Studies performed with laboratory animals have indicated that injury to the epithelium is required for crystal retention. A commonly used model for renal stone formation in rats is the addition of ethylene glycol to the drinking water, which leads to chronic hyperoxaluria and CaOx crystal formation Administration of EG not only leads to increased excretion of urinary oxalate, but also decreases urinary pH and citrate excretion. Persistent crystalluria can be observed within the first week of this diet and nephrolithiasis is induced within 2-3 weeks [75-77]. Ethylene glycol (EG) is metabolized into a range of intermediates, such as glyco-aldehyde, glycolate, glyoxylate and oxalate. Less than one percent of EG is ultimately turned into oxalate. In a study by Huang et al., increased urine lipid peroxides and enzymuria (GAL, NAG) were found in rats that were on a diet supplemented with 0.75% EG in drinking water for 7 days. In addition to this, renal tubular dilatation and infiltration of macrophages/monocytes in the interstitium were found. In the early phase of chronic hyperoxaluria, increased production of free radicals occurred. Acute oxalate infusion increases the production of free radicals in the kidney in a dose-dependent manner [78-80]. In rabbits, addition of 0.75% EG to drinking water led to crystal deposition in tissues. Apoptotic changes occurred prominently in distal tubular and collecting duct cells, but less in proximal tubular cells. [81] Recent results from our group have demonstrated that in rats fed a diet supplemented with 0.5% or 0.75 % ethylene glycol (EG) in drinking water tubular injury was induced without loss of renal function. In these rats, crystals were formed from day 1 but at that moment those were not retained inside the tubules. Injury to the tubules could not be detected, and CD44, HA and OPN were absent form the luminal surface of the epithelium. At day 4, tubular injury could be detected in EG-treated rats, but not in control rats. CaOx crystals were especially detected at sites were injured/regenerating cells were present. At these sites, HA and OPN were present at the cell surface, where they could serve as crystal binding molecules [54].

It is generally believed that high oxalate concentrations and/or crystals caused by an EG-supplemented diet provide the noxious stimulus for tubular injury. However, in studies with experimental animals, effects are seen of ethylene glycol and oxalate together with all their intermediate metabolites. To rule out the possibility that observed effects are not caused by CaOx crystals or by oxalate itself, but by other products of metabolism, cell culture studies are required.

Cell culture models

For over a decade, investigators have been studying the effects of oxalate and CaOx crystals on renal tubular epithelial cells in culture. Most of these studies were performed using cells from proximal tubular origin, like LLC-PK_1. From early studies the idea arose that oxalate was harmful to the cells, since time- and concentration dependent changes in cell morphology were observed, together with increased membrane permeability, DNA fragmentation and even cell death. These effects were observed upon exposure to 1 to 4 mM oxalate, added in calcium-containing DMEM or buffer [82]. Although the authors denied the formation of CaOx crystals under these circumstances, based on estimations made using the EQUIL program, this appears to be inaccurate. In a more recent study, Belliveau et al. performed measurements on the solubility of oxalate in calcium-containing cell culture media and found that addition of 1.0 mM oxalate to normal DMEM medium, containing 1.8 mM $CaCl_2$, causes precipitation of 94.3 % of the available oxalate. Instead of the calculated 0.35 mM free oxalate, there is actually only 60 μM free oxalate left under these circumstances [83]. When cells grown on plastic wells are exposed to high oxalate concentrations, precipitation of calcium from the tissue culture medium with oxalate leads to calcium depletion and subsequent cell detachment

When we started to investigate oxalate toxicity in the two-compartment cell culture model, we expected to find damaging effects to renal tubular epithelial cells. Surprisingly, even LLC-PK_1 and MDCK-II cells, with properties of the proximal tubules, could withstand oxalate concentrations up to 1 mM without undergoing massive injury. These findings contradicted those obtained by Scheid et al., who published a large number of studies on oxalate toxicity in LLC-PK_1 cells [82, 84-92]. This discrepancy might be contributed to cell culture techniques, since in most studies cells were cultured on impermeable tissue culture plastic rather

than on permeable supports in a two-compartment model. Cells cultured on plastic surfaces are less well-developed than those cultured on permeable supports. Since cells normally feed from their basal side, plastic-cultured cells are more likely to suffer from lack of nutrients upon reaching confluence. This might make them more susceptible to damaging stimuli. Indeed, we found that incubating the cells with high oxalate in calcium-containing medium, such that all calcium was complexed with oxalate, caused rounding and detachment of the cells from the substrate, an effect that was similar to that of incubation in calcium free medium. LLC-PK_1 cells that were cultured to confluence in plastic wells spontaneously detached from the substrate when the maximal amount of cells per well was reached. (unpublished results). As described in Chapter 6, in our cell culture model only extremely high oxalate concentrations (5 and 10 mM) triggered an inflammatory response and necrotic cell death. Apparently, the availability of nutrients and calcium in the basal compartment provides the proximal tubular cells with a protective mechanism against oxalate-induced injury.

In contrast to proximal tubular cells, distal tubule/collecting duct cells *in vivo* are used to encounter higher oxalate concentrations (up to 0.3-1 mM). Therefore, it seems more physiologically relevant to study the effects of oxalate on cells derived from the late part of the nephron. We examined MDCK-I and $RCCD_1$ cells after exposure to increasing concentrations of oxalate and found little or no effects of up to 5 mM oxalate. Only at the extreme supraphysiological concentration of 10 mM oxalate, morphological changes, inflammatory responses and cell death occurred. The results from our studies suggest that oxalate in the kidney is not as damaging to the tubular epithelium as has been believed during the past years. Oxalate appears not to be the stressor leading to activation of renal tubular epithelium, turning it into a crystal-binding instead of a crystal-repelling phenotype.

7.5 CONCLUSIONS

Crystallization in the renal tubules is a common occurrence that is normally harmless. Intact epithelium in the distal parts of the nephron is not susceptible to crystal binding. When renal tubular epithelial cells are exposed to a stressor and are activated or injured, this might turn the epithelium into a crystal-binding state. This thesis was conducted to obtain more insight in the response of renal tubular cells to oxalate and calcium oxalate and to clarify some of the contradictions in nephrolithiasis research literature.

Our results show that particular cellular responses such as crystal uptake and crystal-induced inflammation and necrosis are characteristic for cells derived from nephron segments such as the proximal tubule where crystallization does not take place. In the distal tubules and collecting ducts, where crystallization is common, the epithelium is not susceptible to crystal binding, unless it is activated/injured. In other words, the reported crystal-induced cell death most likely is not physiologically relevant.
We also found that the adherence of crystals to a crystal binding collecting tubule phenotype is not prevented by pre-coating crystals with urinary proteins. This undermines the theory that crystal binding requires structurally abnormal or insufficient proteins. On the other hand, this finding reinforces the concept that crystal binding depends on the structural composition of the cell surface in certain areas of the nephron. Finally, our studies disproved the idea that high urinary excretion of oxalate could injure the renal tubular epithelium thereby setting the stage for crystal attachment and stone development. Studies performed in our model in which the cells are cultured on permeable supports in a two compartment culture system revealed that oxalate is not very toxic at all and that most of the damaging effects reported by other most likely were caused by crystals that were formed in calcium-rich culture medium.

urinary proteins. This undermines the theory that crystal binding requires structurally abnormal or insufficient proteins. On the other hand, this finding reinforces the concept that crystal binding depends on the structural composition of the cell surface in certain areas of the nephron. Finally, our studies disproved the idea that high urinary excretion of oxalate could injure the renal tubular epithelium thereby setting the stage for crystal attachment and stone development. Studies performed in our model in which the cells are cultured on permeable supports in a two compartment culture system revealed that oxalate is not very toxic at all and that most of the damaging effects reported by other most likely were caused by crystals that were formed in calcium-rich culture medium.

7.6 REFERENCES

1. Verkoelen CF, van der Boom BG, Kok DJ, Houtsmuller AB, Visser P, Schroder FH, Romijn JC: Cell type-specific acquired protection from crystal adherence by renal tubule cells in culture. *Kidney Int* 55:1426-1433, 1999
2. Wiessner JH, Hasegawa AT, Hung LY, Mandel GS, Mandel NS: Mechanisms of calcium oxalate crystal attachment to injured renal collecting duct cells. *Kidney Int* 59:637-644., 2001
3. Worcester EM: Inhibitors of stone formation. *Semin Nephrol* 16:474-486, 1996
4. Khan SR, Johnson JM, Peck AB, Cornelius JG, Glenton PA: Expression of osteopontin in rat kidneys: induction during ethylene glycol induced calcium oxalate nephrolithiasis. *J Urol* 168:1173-1181, 2002
5. 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
6. Marengo SR, Resnick MI, Yang L, Chung JY: Differential expression of urinary inter-alpha-trypsin inhibitor trimers and dimers in normal compared to active calcium oxalate stone forming men. *J Urol* 159:1444-1450, 1998
7. Hedgepeth RC, Yang L, Resnick MI, Marengo SR: Expression of proteins that inhibit calcium oxalate crystallization in vitro in the urine of normal and stone-forming individuals. *Am J Kidney Dis* 37:104-112., 2001
8. Morse RM, Resnick MI: A new approach to the study of urinary macromolecules as a participant in calcium oxalate crystallization. *J Urol* 139:869-873, 1988
9. Doyle IR, Ryall RL, Marshall VR: Inclusion of proteins into calcium oxalate crystals precipitated from human urine: a highly selective phenomenon. *Clin Chem* 37:1589-1594, 1991
10. Suzuki K, Mayne K, Doyle IR, Ryall RL: Urinary glycosaminoglycans are selectively included into calcium oxalate crystals precipitated from whole human urine. *Scanning Microsc* 8:523-530, 1994
11. Dawson CJ, Grover PK, Ryall RL: Inter-alpha-inhibitor in urine and calcium oxalate urinary crystals. *Br J Urol* 81:20-26, 1998
12. Atmani F, Glenton PA, Khan SR: Identification of proteins extracted from calcium oxalate and calcium phosphate crystals induced in the urine of healthy and stone forming subjects. *Urol Res* 26:201-207, 1998
13. Santin M, Motta A, Cannas M: Changes in the surface conditioning of calcium-salt crystals treated with physiological and alkaline urine. *Br J Urol* 82:97-103, 1998
14. Wesson JA, Worcester EM, Wiessner JH, Mandel NS, Kleinman JG: Control of calcium oxalate crystal structure and cell adherence by urinary macromolecules. *Kidney Int* 53:952-957., 1998
15. Ananth K, Kavanagh JP, Walton RC, Rao PN: Enlargement of calcium oxalate stones to clinically significant size in an in-vitro stone generator. *BJU Int* 90:939-944, 2002

16. Kavanagh JP: Methods for the study of calcium oxalate crystallisation and their application to urolithiasis research. *Scanning Microsc* 6:685-704; discussion 704-685, 1992
17. Chow K, Dixon J, Gilpin S, Kavanagh JP, Rao PN: A stone farm: development of a method for simultaneous production of multiple calcium oxalate stones in vitro. *Urol Res* 32:55-60, 2004
18. Kumar V, Farell G, Lieske JC: Whole urinary proteins coat calcium oxalate monohydrate crystals to greatly decrease their adhesion to renal cells. *J Urol* 170:221-225, 2003
19. Wiessner JH, Hung LY, Mandel NS: Crystal attachment to injured renal collecting duct cels: Influence of urine proteins and pH. *Kidney Int* 63:1313-1320, 2003
20. Weigel PH, Hascall VC, Tammi M: Hyaluronan synthases. *J Biol Chem* 272:13997-14000, 1997
21. Itano N, Sawai T, Yoshida M, Lenas P, Yamada Y, Imagawa M, Shinomura T, Hamaguchi M, Yoshida Y, Ohnuki Y, Miyauchi S, Spicer AP, McDonald JA, Kimata K: Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J Biol Chem* 274:25085-25092, 1999
22. Spicer AP, Olson JS, McDonald JA: Molecular cloning and characterization of a cDNA encoding the third putative mammalian hyaluronan synthase. *J Biol Chem* 272:8957-8961., 1997
23. Underhill C: CD44: the hyaluronan receptor. *J Cell Sci* 103:293-298, 1992
24. Knudson W: The hyaluronan receptor, CD44, in (vol 1999), 1999
25. Lee GM, Johnstone B, Jacobson K, Caterson B: The dynamic structure of the pericellular matrix on living cells. *J Cell Biol* 123:1899-1907, 1993
26. Yu Q, Banerjee SD, Toole BP: The role of hyaluronan-binding protein in assembly of pericellular matrices. *Dev Dyn* 193:145-151, 1992
27. Knudson W, Aguiar DJ, Hua Q, Knudson CB: CD44-anchored hyaluronan-rich pericellular matrices: an ultrastructural and biochemical analysis. *Exp Cell Res* 228:216-228, 1996
28. Evanko SP, Angello JC, Wight TN: Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 19:1004-1013, 1999
29. Yamagata M, Saga S, Kato M, Bernfield M, Kimata K: Selective distributions of proteoglycans and their ligands in pericellular matrix of cultured fibroblasts. Implications for their roles in cell-substratum adhesion. *J Cell Sci* 106:55-65, 1993
30. Knudson CB, Nofal GA, Pamintuan L, Aguiar DJ: The chondrocyte pericellular matrix: a model for hyaluronan-mediated cell-matrix interactions. *Biochem Soc Trans* 27:142-147, 1999
31. Chen WY, Abatangelo G: Functions of hyaluronan in wound repair. *Wound Repair Regen* 7:79-89, 1999
32. Simpson MA, Wilson CM, Furcht LT, Spicer AP, Oegema TR, Jr., McCarthy JB: Manipulation of hyaluronan synthase expression in prostate adenocarcinoma cells alters pericellular matrix retention and adhesion to bone marrow endothelial cells. *J Biol Chem* 277:10050-10057, 2002

33. Hansell P, Goransson V, Odlind C, Gerdin B, Hallgren R: Hyaluronan content in the kidney in different states of body hydration. *Kidney Int* 58:2061-2068., 2000
34. Wells AF, Larsson E, Tengblad A, Fellstrom B, Tufveson G, Klareskog L, Laurent TC: The localization of hyaluronan in normal and rejected human kidneys. *Transplantation* 50:240-243, 1990
35. Knepper MA, Saidel GM, Hascall VC, Dwyer T: Concentration of solutes in the renal inner medulla: interstitial hyaluronan as a mechano-osmotic transducer. *Am J Physiol Renal Physiol* 284:F433-446, 2003
36. Sibalic V, Fan X, Loffing J, Wuthrich RP: Upregulated renal tubular CD44, hyaluronan, and osteopontin in kdkd mice with interstitial nephritis. *Nephrol Dial Transplant* 12:1344-1353, 1997
37. Johnsson C, Tufveson G, Wahlberg J, Hallgren R: Experimentally-induced warm renal ischemia induces cortical accumulation of hyaluronan in the kidney. *Kidney Int* 50:1224-1229, 1996
38. Lewington AJ, Padanilam BJ, Martin DR, Hammerman MR: Expression of CD44 in kidney after acute ischemic injury in rats. *Am J Physiol Regul Integr Comp Physiol* 278:R247-254., 2000
39. Feusi E, Sun L, Sibalic A, Beck-Schimmer B, Oertli B, Wuthrich RP: Enhanced hyaluronan synthesis in the MRL-Fas(lpr) kidney: role of cytokines. *Nephron* 83:66-73, 1999
40. Wells A, Larsson E, Hanas E, Laurent T, Hallgren R, Tufveson G: Increased hyaluronan in acutely rejecting human kidney grafts. *Transplantation* 55:1346-1349, 1993
41. Mohamadzadeh M, DeGrendele H, Arizpe H, Estess P, Siegelman M: Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. *J Clin Invest* 101:97-108, 1998
42. Moreno JJ: Regulation of arachidonic acid release and prostaglandin formation by cell-cell adhesive interactions in wound repair. *Pflugers Arch* 433:351-356, 1997
43. Koralewska-Makar A, Johnsson C, Bruun A, Stenevi U, Ehinger B: COX-2 inhibitors prolong trauma-induced elevations of iris hyaluronan. *J Ocul Pharmacol Ther* 19:385-395, 2003
44. Harris RC: Cyclooxygenase-2 in the kidney. *J Am Soc Nephrol* 11:2387-2394, 2000
45. Honda A, Sekiguchi Y, Mori Y: Prostaglandin E2 stimulates cyclic AMP-mediated hyaluronan synthesis in rabbit pericardial mesothelial cells. *Biochem J* 292 (Pt 2):497-502, 1993
46. Karlinsky JB, Goldstein RH: Regulation of sulfated glycosaminoglycan production by prostaglandin E2 in cultured lung fibroblasts. *J Lab Clin Med* 114:176-184, 1989
47. Dunlop ME, Muggli EE: Hyaluronan increases glomerular cyclooxygenase-2 protein expression in a p38 MAP-kinase-dependent process. *Kidney Int* 61:1729-1738, 2002
48. Sun LK, Beck-Schimmer B, Oertli B, Wuthrich RP: Hyaluronan-induced cyclooxygenase-2 expression promotes thromboxane A2 production by renal cells. *Kidney Int* 59:190-196, 2001

49. Mahadevan P, Larkins RG, Fraser JR, Dunlop ME: Effect of prostaglandin E2 and hyaluronan on mesangial cell proliferation. A potential contribution to glomerular hypercellularity in diabetes. *Diabetes* 45:44-50, 1996
50. Mahadevan P, Larkins RG, Fraser JR, Fosang AJ, Dunlop ME: Increased hyaluronan production in the glomeruli from diabetic rats: a link between glucose-induced prostaglandin production and reduced sulphated proteoglycan. *Diabetologia* 38:298-305, 1995
51. McKee CM, Penno MB, Cowman M, Burdick MD, Strieter RM, Bao C, Noble PW: Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *J Clin Invest* 98:2403-2413, 1996
52. Beck-Schimmer B, Oertli B, Pasch T, Wuthrich RP: Hyaluronan induces monocyte chemoattractant protein-1 expression in renal tubular epithelial cells. *J Am Soc Nephrol* 9:2283-2290, 1998
53. 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-E112, 2003
54. 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
55. Worcester EM, Beshensky AM: Osteopontin inhibits nucleation of calcium oxalate crystals. *Ann N Y Acad Sci* 760:375-377, 1995
56. Roberts SD, Resnick MI: Glycosaminoglycans content of stone matrix. *J Urol* 135:1078-1083, 1986
57. Wakatsuki A, Nishio S, Iwata H, Ochi K, Takeuchi M, Matsumoto A: Possible role of hyaluronate in experimental renal stone formation in rabbits. *J Urol* 133:319-323, 1985
58. Kok DJ: Clinical implications of physicochemistry of stone formation. *Endocrinol Metab Clin North Am* 31:855-867, 2002
59. Lieske JC, Spargo BH, Toback FG: Endocytosis of calcium oxalate crystals and proliferation of renal tubular epithelial cells in a patient with type 1 primary hyperoxaluria. *J Urol* 148:1517-1519, 1992
60. Khan SR: Calcium oxalate crystal interaction with renal tubular epithelium, mechanism of crystal adhesion and its impact on stone development. *Urol Res* 23:71-79, 1995
61. de Bruijn WC, Boeve ER, van Run PR, van Miert PP, Romijn JC, Verkoelen CF, Cao LC, Schroder FH: Etiology of experimental calcium oxalate monohydrate nephrolithiasis in rats. *Scanning Microsc* 8:541-549, 1994
62. Ebisuno S, Kohjimoto Y, Tamura M, Inagaki T, Ohkawa T: Histological observations of the adhesion and endocytosis of calcium oxalate crystals in MDCK cells and in rat and human kidney. *Urol Int* 58:227-231, 1997
63. Lieske JC, Hammes MS, Toback FG: Role of calcium oxalate monohydrate crystal interactions with renal epithelial cells in the pathogenesis of nephrolithiasis: a review. *Scanning Microsc* 10:519-533, 1996
64. Kohjimoto Y, Ebisuno S, Tamura M, Ohkawa T: Adhesion and endocytosis of calcium oxalate crystals on renal tubular cells. *Scanning Microsc* 10:459-468, 1996

65. Kohjimoto Y, Ebisuno S, Tamura M, Ohkawa T: Interactions between calcium oxalate monohydrate crystals and Madin- Darby canine kidney cells: endocytosis and cell proliferation. *Urol Res* 24:193-199, 1996
66. Hackett RL, Shevock PN, Khan SR: Madin-Darby canine kidney cells are injured by exposure to oxalate and to calcium oxalate crystals. *Urol Res* 22:197-203, 1994
67. Lieske JC, Walsh-Reitz MM, Toback FG: Calcium oxalate monohydrate crystals are endocytosed by renal epithelial cells and induce proliferation. *Am J Physiol* 262:F622-630, 1992
68. Lieske JC, Swift H, Martin T, Patterson B, Toback FG: Renal epithelial cells rapidly bind and internalize calcium oxalate monohydrate crystals. *Proc Natl Acad Sci U S A* 91:6987-6991, 1994
69. Lieske JC, Toback FG: Regulation of renal epithelial cell endocytosis of calcium oxalate monohydrate crystals. *Am J Physiol* 264:F800-807., 1993
70. Lieske JC, Norris R, Swift H, Toback FG: Adhesion, internalization and metabolism of calcium oxalate monohydrate crystals by renal epithelial cells. *Kidney Int* 52:1291-1301., 1997
71. Lieske JC, Deganello S: Nucleation, adhesion, and internalization of calcium-containing urinary crystals by renal cells. *J Am Soc Nephrol* 10 Suppl 14:S422-429, 1999
72. Khan SR, Byer KJ, Thamilselvan S, Hackett RL, McCormack WT, Benson NA, Vaughn KL, Erdos GW: Crystal-cell interaction and apoptosis in oxalate-associated injury of renal epithelial cells. *J Am Soc Nephrol* 10 Suppl 14:S457-463., 1999
73. Hackett RL, Shevock PN, Khan SR: Alterations in MDCK and LLC-PK1 cells exposed to oxalate and calcium oxalate monohydrate crystals. *Scanning Microsc* 9:587-596, 1995
74. Verkoelen CF, Romijn JC, de Bruijn WC, Boeve ER, Cao LC, Schroder FH: Association of calcium oxalate monohydrate crystals with MDCK cells. *Kidney Int* 48:129-138, 1995
75. Khan SR: Animal models of kidney stone formation: an analysis. *World J Urol* 15:236-243, 1997
76. de Water R, Boeve ER, van Miert PP, Vermaire CP, van Run PR, Cao LC, de Bruijn WC, Schroder FH: Pathological and immunocytochemical changes in chronic calcium oxalate nephrolithiasis in the rat. *Scanning Microsc* 10:577-587; discussion 587-590, 1996
77. Khan SR: Role of renal epithelial cells in the initiation of calcium oxalate stones. *Nephron Exp Nephrol* 98:e55-60, 2004
78. Huang HS, Ma MC, Chen J, Chen CF: Changes in renal hemodynamics and urodynamics in rats with chronic hyperoxaluria and after acute oxalate infusion: Role of free radicals. *Neurourol Urodyn* 22:176-182, 2003
79. Huang HS, Ma MC, Chen J, Chen CF: Changes in the oxidant-antioxidant balance in the kidney of rats with nephrolithiasis induced by ethylene glycol. *J Urol* 167:2584-2593, 2002
80. Huang HS, Chen CF, Chien CT, Chen J: Possible biphasic changes of free radicals in ethylene glycol-induced nephrolithiasis in rats. *BJU Int* 85:1143-1149, 2000

81. Sarica K, Yagci F, Bakir K, Erbagci A, Erturhan S, Ucak R: Renal tubular injury induced by hyperoxaluria: evaluation of apoptotic changes. *Urol Res* 29:34-37, 2001
82. Scheid C, Koul H, Hill WA, Luber-Narod J, Kennington L, Honeyman T, Jonassen J, Menon M: Oxalate toxicity in LLC-PK1 cells: role of free radicals. *Kidney Int* 49:413-419, 1996
83. Belliveau J, Griffin H: The solubility of calcium oxalate in tissue culture media. *Anal Biochem* 291:69-73, 2001
84. Scheid CR, Koul H, Hill WA, Lieske JC, Toback FG, Menon M: Oxalate ion and calcium oxalate crystal interactions with renal epithelial cells, in *Kidney Stones:Medical and Surgical Management*, edited by Coe FL, Favus M.J., Pak C.Y.C. , Parks J.H., Preminger G.M., Philadelphia, Lippincott-Raven Publishers, 1996, pp 129-143
85. Cao LC, Honeyman T, Jonassen J, Scheid C: Oxalate-induced ceramide accumulation in Madin-Darby canine kidney and LLC-PK1 cells. *Kidney Int* 57:2403-2411., 2000
86. Cao LC, Jonassen J, Honeyman TW, Scheid C: Oxalate-induced redistribution of phosphatidylserine in renal epithelial cells: implications for kidney stone disease. *Am J Nephrol* 21:69-77, 2001
87. Kohjimoto Y, Kennington L, Scheid CR, Honeyman TW: Role of phospholipase A2 in the cytotoxic effects of oxalate in cultured renal epithelial cells. *Kidney Int* 56:1432-1441, 1999
88. Kohjimoto Y, Honeyman TW, Jonassen J, Gravel K, Kennington L, Scheid CR: Phospholipase A2 mediates immediate early genes in cultured renal epithelial cells: possible role of lysophospholipid. *Kidney Int* 58:638-646, 2000
89. Koul H, Kennington L, Nair G, Honeyman T, Menon M, Scheid C: Oxalate-induced initiation of DNA synthesis in LLC-PK1 cells, a line of renal epithelial cells. *Biochem Biophys Res Commun* 205:1632-1637, 1994
90. Koul H, Kennington L, Honeyman T, Jonassen J, Menon M, Scheid C: Activation of c-myc gene mediates the mitogenic effects of oxalate in LLC-PK1 cells, a line of renal epithelial cells. *Kidney Int* 50:1525-1530, 1996
91. Scheid CR, Koul HK, Kennington L, Hill WA, Luber-Narod J, Jonassen J, Honeyman T, Menon M: Oxalate-induced damage to renal tubular cells. *Scanning Microsc* 9:1097-1105; discussion 1105-1097, 1995
92. Scheid C, Koul H, Hill WA, Luber-Narod J, Jonassen J, Honeyman T, Kennington L, Kohli R, Hodapp J, Ayvazian P, Menon M: Oxalate toxicity in LLC-PK1 cells, a line of renal epithelial cells. *J Urol* 155:1112-1116, 1996

SUMMARY

Nephrolithiasis (renal stone disease) is a common disorder in industrialized countries. About 10% of the population will develop kidney stones during lifetime. Approximately 50% of the patients that formed a renal stone for the first time will do that again within a period of 10 years. Although renal stone disease generally is not life threatening, stones can be very painful and invasive clinical interventions are required for their removal. Since nephrolithiasis is such a widespread disease, clinical procedures and the allied absence from work have a substantial economical impact. For that reason, it is important to disclose the abnormalities underlying stone disease. With this knowledge we might be able to develop strategies to prevent stone recurrence.

Risk factors for stone formation are a positive family history of stones, nutrition, nutritional deficiencies, life style and associated diseases. Excessive intakes of salt, animal protein and oxalate, as well as insufficient intake of fluid, vegetable fibres and calcium increase the risk of stone formation. Specific dietary advice and/or pharmacological interventions can lower the risk of recurrence for some patients.

In the kidneys, concentrated urine is produced. Each day, 180 litres of fluid are filtered at the glomerulus. During this process, essential nutrients and salts are reabsorbed together with most of the water and waste products are excreted. The concentration of components that are not or less extensively reabsorbed rises towards the end of the nephron. When super saturation occurs, i.e. when the concentration of salts gets too high to be kept in solution, crystals can spontaneously be formed (nucleation). Urine normally contains compounds that interfere with the nucleation, growth, agglomeration or retention of crystals. Renal stones can be formed when crystals are not eliminated with the urine, but are retained in the kidney. Different theories exist about the retention of crystals in the kidney. According to the “fixed particle” theory, crystals grow and aggregate until they become too large to pass through the tubular lumen and become e trapped. In contrast, according to the “free particle” theory, crystal retention could be dependent on the interaction between the renal tubular epithelium and the crystals, even when those are small. The epithelium has to become susceptible to crystal binding, which might occur under pathological conditions. Urinary constituents can influence the interaction between crystals and between crystals and the epithelium. In Chapter 2, we investigated the inhibitory effect of

urinary components on CaOx crystal binding to a susceptible epithelium. Crystal binding was studied using renal tubular epithelial cell lines with characteristics from different parts of the nephron. Since hyaluronan (HA) was earlier identified as major crystal binding molecules in MDCK-I cells during migration and proliferation, the role of HA in crystal binding was more extensively investigated in different cell lines (Chapter 3). The fate of crystals that have been bound to the renal tubular epithelium varies depending on the origin of the epithelium. Proximal tubular epithelium usually does not encounter crystals and do not display defence mechanisms against their retention. Crystals that are retained by this cell type are subsequently internalized. In contrast, distal tubule/collecting duct cells often come in contact with crystals and are normally protected against crystal binding. The epithelium from this part of the nephron can be turned into a crystal-binding phenotype during pathological conditions. Even then, crystals are not internalized by this cell type (Chapter 4).

It is believed that CaOx crystals that are taken up by renal tubular epithelial cells act as stressor, leading to cell activation or injury. For this reason, we investigated the effects of long-term exposure of confluent renal tubular epithelial cells grown on permeable supports to a high dose of CaOx crystals (32 $\mu g/cm^2$). Our studies showed that collecting tubule cells (MDCK-I), that were protected against crystal binding, did not bind take up or respond to crystals. Proximal tubular cells, by contract, bound and internalized CaOx crystals, after which an early and a late response could be distinguished. The early response was characterized by an increased secretion of prostaglandin E_2 (PGE_2) and a proliferative response. At the same time, crystal-containing necrotic cells were released. After this immediate response, PGE_2 levels declined to control levels and crystal-containing cells remained incorporated in the normal functioning monolayer. COM triggers acute inflammation and necrosis in proximal tubular cells, but do not induce tissue injury in collecting duct cells (Chapter 5).

Calcium oxalate (CaOx) is the most common component of renal stones. The soluble amount of CaOx is about fourfold higher in normal urine than in water. Oxalate has a bigger influence on the soluble amount than calcium. Urinary oxalate is increased in many idiopathic stone formers. This might be ascribed to increased intestinal calcium absorption, increased bone resorption and decreased renal tubular calcium reabsorption. Oxalate is believed to be toxic to renal tubular epithelial

cells. We investigated oxalate toxicity using our two-compartment cell culture model, comparing proximal tubular cells and collecting tubule cells. Contrary to our expectations, physiological oxalate concentrations did not exert damage to either cell type. At extreme supraphysiological concentrations, oxalate did induce morphological alterations, inflammatory responses and cell death (Chapter 6).

SAMENVATTING

Nephrolithiasis (niersteenvorming) is een algemeen voorkomende ziekte in geïndustrialiseerde landen. Ongeveer één op de tien mensen zal tijdens zijn leven nierstenen ontwikkelen. Van de patiënten die voor de eerste keer een steen gevormd hebben zal ongeveer de helft dat binnen tien jaar nog een keer doen. Hoewel niersteenziekte niet levensbedreigend is kunnen stenen veel pijn veroorzaken en zijn de klinische procedures om ze te verwijderen erg ingrijpend. Niersteenvorming is een wijdverbreide ziekte, daarom hebben de klinische procedures en het daarmee samenhangende werkverzuim een grote economische impact. Om die reden is het belangrijk de onderliggende afwijkingen van steenziekte bloot te leggen. Met deze kennis kunnen we mogelijk strategieën ontwikkelen om het terugkeren van een steen te voorkomen.
Risicofactoren voor steenvorming zijn het voorkomen van stenen in de familie, voeding, voedingstekorten, levensstijl en onderliggende ziekten. Overmatige inname van zout, dierlijk eiwit en oxalaat verhogen de kans op steenvorming, net zoals onvoldoende inname van vocht, vezels en calcium. Specifieke dieetadviezen en/of farmacologisch ingrijpen kan voor sommige patiënten de kans op recidive voorkomen.
In de nieren wordt geconcentreerde urine geproduceerd. Iedere dag wordt 180 liter vloeistof gefiltreerd aan de glomerulus. Tijdens dit proces worden essentiële voedingsstoffen en zouten teruggeresorbeerd met het grootste deel van het water en worden afvalstoffen uitgescheiden. De concentratie van stoffen die niet of slecht geresorbeerd worden neemt toe naar het einde van het nephron. Wanneer supersaturatie optreedt, d.w.z. wanneer de concentratie van zouten te hoog wordt om in oplossing gehouden te kunnen worden, kunnen zich spontaan kristallen vormen (nucleatie). Urine bevat normaliter bestanddelen die de nucleatie, groei, agglomeratie of retentie van kristallen beïnvloeden. Nierstenen kunnen gevormd worden wanneer kristallen niet met de urine uitgescheiden worden, maar achterblijven in de nier. Er bestaan verschillende theorieën over de retentie van kristallen in de nier. Volgens de “fixed particle” theorie blijven kristallen groeien en samenklonteren tot ze te groot worden om door het lumen van de tubulus te passeren en blijven steken. Volgens de “free particle”theorie, daarentegen, zou het achterblijven van kristallen afhankelijk kunnen zijn van de interactie tussen het epitheel van het niertubuli en de kristallen, zelfs als deze klein zijn. Het epitheel is ontvankelijk geworden voor kristalbinding, wat onder pathologische

omstandigheden kan gebeuren. Urine bestanddelen kunnen invloed uitoefenen op de interactie tussen kristallen onderling en tussen kristallen en het epitheel. In hoofdstuk 2 hebben wij het remmende effect van urinebestanddelen op de binding van CaOx kristallen aan een ontvankelijk epitheel onderzocht. Hyaluronzuur (HA) was al eerder geïdentificeerd als belangrijk kristalbindend molecuul in MDCK-I cellen tijdens migratie en proliferatie. Om die reden hebben we de rol van HA uitgebreider bestudeerd in meerdere cellijnen (hoofdstuk 3). Het lot van kristallen die aan het epitheel van de niertubuli gebonden zijn is afhankelijk van de oorsprong van dat epitheel. In de proximale tubuli komt het epitheel normaalgesproken niet in contact met kristallen en heeft daarom geen mechanisme ontwikkeld om achterblijven van kristallen te voorkomen. Kristallen die plakken aan dit celtype worden vervolgens in de cel opgenomen. Cellen uit de distale tubuli en verzamelbuizen komen daarentegen veelvuldig in contact met kristallen en zijn onder normale omstandigheden beschermd tegen kristalbinding. Het epitheel uit dit gedeelte van het nephron kan onder pathologische omstandigheden een kristalbindend fenotype tentoonspreiden. Zelfs onder die condities worden kristallen door deze cellen niet opgenomen (hoofdstuk 4).
Men gelooft dat CaOx kristallen die door cellen opgenomen worden fungeren als stressor en leiden tot activering of beschadiging van cellen. Wij hebben daarom de effecten onderzocht van blootstelling van confluente, op permeabele filters gekweekte, cellen aan een hoge dosis calcium oxalaat kristallen. De resultaten van onze studie wezen uit dat verzamelbuiscellen (MDCK-I), die beschermd zijn tegen kristalbinding, kristallen binden noch opnemen, noch een reactie op de kristallen vertonen. Cellen afkomstig uit de proximale tubulus (MDCK-II), binden CaOx kristallen en nemen deze op in de cellen, waarna een vroege en een late reactie onderscheiden kunnen worden. De vroege respons kenmerkt zich door een toegenomen uitscheiding van prostaglandine E2 (PGE_2) en een groei reactie. Tegelijkertijd worden necrotische, kristallen bevattende, cellen losgelaten. Na deze onmiddellijke respons keert de PGE_2 uitscheiding terug naar het oorspronkelijke niveau en blijven de cellen die kristallen bevatten in de normaal functionerende monolaag. De COM kristallen brengen acute ontsteking en necrose in proximale tubulus cellen teweeg, maar veroorzaken geen weefselschade in verzamelbuiscellen (hoofdstuk 5).

Calcium oxalaat (CaOx) is het meest voorkomende bestanddeel van nierstenen. De oplosbare hoeveelheid CaOx in urine is ongeveer viermaal zo hoog als in water. De invloed van oxaalzuur op de oplosbare hoeveelheid is groter dan die van calcium. Oxaalzuur in de urine is verhoogd bij veel idiopathische steenvormers. Dit kan toegeschreven worden aan toegenomen intestinale calcium absorptie, verhoogde botresorptie en verlaagde calcium reabsorptie in de niertubuli. Oxaalzuur wordt verondersteld toxisch te zijn voor het tubulaire nierepitheel. Wij hebben de toxiciteit van oxaalzuur onderzocht in ons tweecompartimenten celkweekmodel, warbij we proximale tubuluscellen en verzamelbuiscellen vergeleken hebben. Tegen onze verwachting in brachten fysiologische oxaalzuurconcentraties geen schade toe aan elk van deze celtypen. Slechts wanneer extreem hoge concentraties oxaalzuur toegediend werden, veroorzaakten deze morfologische veranderingen, ontstekingsreacties en celdood (hoofdstuk 6).

LIST OF PUBLICATIONS

1. Verkoelen CF and Schepers MSJ (2000). Changing concepts in the aetiology of renal stones. Current Opinions in Urology, 10:539-544.

2. Schepers MSJ, Boom van der BG, Romijn JC, Schröder FH and Verkoelen CF (2002). Urinary crystallization inhibitors do not prevent crystal binding. Journal of Urology, 167:1844-1847

3. Verhulst A, Asselman M, Persy VP, Schepers MSJ, Helbert MF, Verkoelen CF and De Broe ME (2003). Crystal retention capacity of cells in the human nephron: Involvement of CD44 and its ligans hyaluronic acid and osteopontin in the transition of a crystal binding- into a non-adherent epithelium. Journal of the American Society of Nephrology, 14(1): 107-115.

4. Schepers MSJ, Asselman M, Duim RAJ, Romijn JC, Schröder FH and Verkoelen CF (2003). Pericellular matrix formation by renal tubule epithelial cells in relation to crystal binding. Nephron Experimental Nephrology, 94(3): e103- e112.

5. Schepers MSJ, , Duim RAJ, Asselman M, Romijn JC, Schröder FH and Verkoelen CF (2003). Internalization of calcium oxalate crystal by renal tubule epithelial cells: a nephron segment-specific process?. Kidney International, 64(2): 493-500.

6. Schepers MSJ, Van Ballegooijen ES, Bangma CH and Verkoelen CF. Crystals cause acute necrotic cell death in renal proximal tubule cells, but not in collecting tubule cells.
   Submitted

7. Schepers MSJ, Van Ballegooijen ES, Bangma CH and Verkoelen CF. Oxalate is toxic to renal tubular cells only at supraphysiological concentrations.
   Submitted

CURRICULUM VITAE

Marieke Schepers-Kok werd op 23 maart 1976 te Rotterdam geboren. Zij is getrouwd met Paul Schepers en moeder van Jeroen. In juni 1994 behaalde zij haar diploma aan het Gymnasium Juvenaat H. Hart te Bergen op Zoom. Vervolgens studeerde zij Biomedische Gezondheids-wetenschappen aan de Katholieke Universiteit Nijmegen, met als afstudeerrichting Toxicologie. Tijdens deze studie liep ze stage bij de afdeling Celfysiologie (prof. dr. R.J.M. Bindels) en Reumatologie (dr. F.A. van der Loo) van deze universiteit. Na haar afstuderen werkte zij korte tijd bij de afdeling Endocrinologie en Voortplanting (dr. A. Themmen) van de Erasmus Universiteit, waarna zij in september 1999 AIO werd bij de afdeling Experimentele Urologie. Hier voerde zij onder leiding van dr. C.F. Verkoelen het in dit proefschrift beschreven onderzoek uit.

DANKWOORD

Tot slot wil ik iedereen bedanken die betrokken is geweest bij de totstandkoming van dit proefschrift. Zonder jullie was het me niet gelukt. Ik wil beginnen met mijn steengroep-collega's van het eerste uur te bedanken, allereerst mijn begeleider en co-promotor Carl Verkoelen. Het was niet altijd gemakkelijk en we hebben elkaar af en toe flink dwars gezeten, maar het is ons toch gelukt. Mijn promotor, professor Schröder wil ik bedanken voor zijn vertrouwen en steun in de afgelopen jaren en vooral deze laatste maanden. Burt: jij hebt me de eerste jaren geweldig geholpen en me de technieken geleerd om dit proefschrift tot een goed einde te brengen. Het was vreemd toen jij weg ging. Verder wil ik Dik, Hans, Cindy, Ruud, Niels, Saima en Paul bedanken voor hun hulp en adviezen. Rejiv, jij hield de discussies binnen de groep levendig. Ronald, hopelijk bevalt de informatica je beter dan het labwerk. Eddy, we hebben maar kort samengewerkt, maar zonder jou waren de laatste twee papers niet gelukt. Mijn dank is zeer groot. Tenslotte Marino, steun en toeverlaat in moeilijke tijden. We hebben heel wat besproken bij een bakkie koffie: van het opzetten van experimenten, tot kapotte computers en het schrijven van papers, en alles daar tussenin. Ik ben benieuwd naar jouw boekje!

In de loop der jaren heb ik heel wat verschillende kamergenoten gehad op Be 331: Johan en Nuria, het was gezellig dat eerste jaar. Mazen, met jou een kamer delen was een bijzondere ervaring. Richard, Marino, Rute, Leonie, Dennis en Karin: ik mis onze appelflappen-meetings. Wij hadden de drukste, maar zeker ook de gezelligste AIO-kamer van het JNI. Uiteraard wil ik ook alle collega's van het lab bedanken voor hun hulp en steun in de jaren van mijn promotie-onderzoek. Ik heb van jullie allemaal wel iets geleerd en het was fijn om bij jullie te mogen uitrazen als dingen niet naar wens verliepen (jullie weten wel wat ik bedoel). Naast de collega's van Urologie en Pathologie wil ik ook Adriaan Houtsmuller en Pim van Schalkwijk bijzonder bedanken voor hun hulp. Tenslotte wil de Belgische collega's, in het bijzonder Anja Verhulst en professor de Broe bedanken voor de samenwerking en de gastvrije ontvangst in het labo in Antwerpen.

Last but not least wil ik ook mijn familie bedanken. Paul, ook al snap je nog steeds niets van mijn werk, dankzij jou ben ik toch steeds doorgegaan als ik er even geen heil meer in zag. Mama, jij wist al voor ik mijn vakkenpakket moest kiezen dat dit mijn toekomstige werk zou worden. Papa en mama, bedankt voor jullie steun en vertrouwen en voor al het oppassen tijdens het schrijven van dit proefschrift. Marjolein: toch weer een dame Kok bij Cluster 12. Het laboratorium is wel heel iets anders dan het Sophia, maar we kunnen maar beter niet ruilen. Jeroen, je bent nog te klein om er iets van te beseffen, maar jij weet mij altijd weer op te vrolijken na een lange dag schrijven. Tegen de rest van mijn familie en schoonfamilie zou ik willen zeggen: dit is het dan, hier heb ik al die tijd aan gewerkt.

De Nierstichting wil ik bedanken voor het financieren van dit onderzoek en SUWO voor de bijdrage in de drukkosten van dit proefschrift.