Hyaluronan Biology and Regulation in Renal Tubular Epithelial Cells and its Role in Kidney Stone Disease

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"No matter how counterintuitive it may seem, basic research has proven over and over to be the lifeline of practical advances in medicine. Without advances, medicine regresses and reverts to witchcraft."

Dr. Arthur Kornberg, winner of the 1959 Nobel Prize in Physiology or Medicine.

Contents

Abbreviations

AA	arachidonic acid
ATL	ascending thin limb
CaOx	calcium oxalate
CCD	cortical collecting duct
CD	collecting duct
CLSM	confocal laser scanning microscopy
CNT	connecting tubule
COM	calcium oxalate monohydrate
COX	cyclo-oxygenase
Da	Dalton
DCT	distal convoluted tubule
DMEM	Dulbecco's modified minimal essential medium
dpm	disintegrations per minute
DT	distal tubule
DTL	descending thin limb
EDTA	ethylenediaminetetraacetic acid
EG	ethylene glycol
EMA	epithelial membrane antigen
ESWL	extracorporal shock-wave lithotripsy
FCS	fetal calf serum
G	glomerulus
γ-GT	γ-glutamyltranspeptidase
GAGs	glycosaminoglycans
GLcUA	glucuronic acid
GLcNAc	N-acetylglucosamine
HA	hyaluronan
HAS	hyaluronan synthase
[^{3}H] GlcNAc	[^{3}H] glucosamine
IM	inner medulla
IMCD	inner medullary collecting duct
ISOM	inner stripe of the outer medulla
LAP	leucine amino peptidase
MD	macula densa
MDCK	Madin Darby canine kidney
MEM	α-minimal essential medium
M_r	molecular weight

NSAIDs	non-steroidal anti-inflammatory drugs
OMCD	outer medulla collecting duct
OPN	osteopontin
OSOM	outer stripe of the outer medulla
PBS	phosphate-buffered saline
PCMs	pericellular matrices
PCNA	proliferating cell nuclear antigen
PCNL	percutaneous nephrolitholapaxy
PCT	proximal convoluted tubule
PAS	periodic acid-Schiff
PGE_2	prostaglandin E_2
PST	proximal straight tubule
PT	proximal tubule
RT-PCR	reverse transcriptase-polymerase chain reaction
TAL	thick ascending limb
TER	transepithelial electrical resistance
TLH	thin limb of Henle
URS	ureterorenoscopy

Chapter 1

General introduction

In part derived from Curr Opin Urol. 2002;12:271-276. Crystal-cell interaction in the pathogenesis of kidney stone disease. M. Asselman and C.F. Verkoelen

1.1 KIDNEY STONES

Renal stone disease is a widespread problem afflicting more and more people throughout the world. Epidemiological studies show an increase in incidence and prevalence rates. In North America and Europe the yearly incidence is estimated to be about 0.5% [1, 2]. The prevalence of kidney stones in the USA has risen in two decades from 3.2% to 5.2% [3]. The lifetime risk is about 10-15% in the developed world, but can be as high as 20-25% in the middle east [1, 2]. Kidney stone disease often presents as an episode of acute renal colic with characteristic severe intermittent pain in the flank or lower abdomen, vomiting and haematuria caused by a calculus obstructing the ureter [4]. Renal stone disease has a substantial impact on the health care system. For example, the total annual cost for urolithiasis in the United States in 1995 was estimated to be $1.83 billion [5]. Nephrolithiasis is likely to recur and recurrence rates are reported to be 50% in 10 years and 75% in 20 years [1]. Apparently the development of minimal invasive techniques for stone removal, such as extracorporal shock-wave lithotripsy (ESWL), ureterorenoscopy (URS) and percutaneous nephrolitholapaxy (PCNL) together with current standards to avoid stone recurrence such as general measures and metabolic therapy have *not* resolved the increasing problem of (recurrent) stone formation [4, 6, 7]. Therefore scientific research is warranted to better understand the etiology of stone disease aimed at preventing kidney stone formation in healthy people or stone recurrence in patients.

Kidney stones are composed of innumerable small crystals cemented together with organic material (stone matrix). Kidney stone formers can be categorized on the basis of the composition of their stones in two groups, namely calcium stone formers and non-calcium stone formers [4]. The most common are the first group of calcium stone formers accounting for 75-80% [1, 8, 9]. Calcium stones are composed of mixtures of calcium oxalate (calcium oxalate monohydrate and/or calium oxalate dihydrate) and calcium phosphate (including apatite or brushite), only calcium oxalate or rarely calcium phosphate [4]. Non-calcium stones include uric acid / urate stones (uric acid / ammonium urate and sodium urate), infection stones (struvite, i.e. magnesium ammonium phosphate and carbonate apatite), cystine stones [1, 4] and miscellaneous types of highly uncommon stones associated with xanthine, 2,8-dihydroxyadenine, protein matrix, and drugs (for example indinavir and triamterene) [1]. Data on the pathophysiology of kidney stone formation have been acquired via population-based epidemiology, human metabolic studies, and basic science experiments [1].

1.2 EPIDEMIOLOGICAL STUDIES

Epidemiological investigations show that the disease is 2-3 times more common in males than in females. The modern western lifestyle and diet increases the risk of stone formation. Global

epidemiological studies show that the incidence of upper urinary tract calculi increases with prosperity [10]. Recent studies show a correlation between nephrolithiasis and cardiovascular risk factors such as being overweight/obesity [11, 12], hypercholesterolemia and hypertension [13]. The association of nephrolithiasis and the socioeconomic status is also apparent from the fact that in Europe an almost explosive increase in stone prevalence occurred in the two so-called stone waves which followed World Wars I and II in the affected countries [10]. Stone disease predominantly affects middle-aged white males, but from metabolic studies it was shown that ethnicity seems to be of less importance than dietary and other environmental factors [14] and also worldwide the diversity is likely to be due to nutritional-environmental and socio-political-economic factors [15]. About 2-5% of the population in Asia, 8-15% in Europe and North America, and 20-25% in the middle east develop renal stones in their lifetime [2]. There are only a few geographical areas in which stone disease is rare, e.g. in Greenland and in the coastal areas of Japan [7].

1.3 HUMAN METABOLIC STUDIES

Several metabolic risk factors have been identified such as low urine volume, hypercalciuria, hypocitraturia, hyperoxaluria, low urine pH or hyperuricosuria, cystinuria and urinary tract infection. These risk factors encompass several pathophysiologic conditions in diseases (including genetic disorders) that are associated with an increased risk of stone formation, such as absorptive hypercalciuria, mutation in the renal chloride transporter gene CLC-5 in Dent's disease and primary and secondary hyperparathyroidism (hypercalciuria), acidosis or acid retention in distal renal tubular acidosis, metabolic acidosis and a consumption of a diet rich in meat (hypocitraturia), excess of oxalate in diet, bowel pathology or primary and secondary hyperoxaluria (hyperoxaluria), high purine intake, myeoloproliferative diseases, enzymatic defects, uricosuric drugs, genetic primary renal leak, i.e. renal transport defects and the metabolic syndrome of gouty diathesis (hyperuricosuria), congenital mutations of dibasic aminoacid transporter SLC3A1 gene (cystinuria) and urea-splitting organisms (urinary tract infections) [1, 2]. Amongst these and other diseases associated with stone formation (hyperparathyroidism, renal tubular acidosis, cystinuria, primary hyperoxaluria, jejunoileal bypass, Crohn's disease, intestinal resection, malabsorptive conditions, sarcoidosis and hyperthyroidism) other risk factors for recurrent stone formation include strong family history of stone formation, medication associated with stone formation (calcium supplements, vitamin D supplements, acetazolamide, ascorbic acid in megadoses (>4 g/day), sulphonamides, triamterene and indinavir), anatomical abnormalities associated with stone formation (tubular ectasia in medullary sponge kidney, pelvo-ureteral junction obstruction, caliceal diverticulum, caliceal cyst, ureteral stricture, vesico-ureteral reflux, horseshoe kidney and ureterocele) [4] and dietary factors (reduced fluid intake and dehydration, high animal protein intake, high oxalate intake,

reduced intake of potassium-rich citrus fruits, high sodium intake) [16]. To discuss all of these individual conditions is beyond the scope of this thesis. It should be noted however that these conditions do not apply to the vast majority of stone forming patients which are categorized as idiopathic stone formers.

1.4 BASIC SCIENCE EXPERIMENTS

There are two topics that are involved in the earliest events of kidney stone formation and have been studied extensively; (A) crystal formation (supersaturation, crystal nucleation, crystal growth and aggregation), and (B) crystal retention in the kidney.
Before discussing crystal formation a description of the functional anatomy of the nephron, the basic structural and functional unit of the kidney, and collecting duct system is given in figure 1.1. The human kidney consists of more than 1.2 million of nephrons. The nephron consists of segments with different cell populations each with their own specific functions. These segments are the glomerulus, proximal tubule, loop of Henle and distal tubule. The collecting ducts are formed through the joining of on average eleven nephrons and fuse with each other (on average eight) to form a papillary duct that drains into the calyces via the ducts of Bellini. The initial step in urine formation is the filtration of plasma. Each minute a liter of blood flows into the glomerular capillaries. About 150 to 250 liters of ultrafiltrate is formed per day providing an efficient mechanism for excretion of the continuously produced nitrogenous waste metabolites. The kidney has also a homeostatic role in regulating the volume and composition of body fluids that is the net result of discrete transport processes that occur in series along the length of individual nephrons and results in either diluted or concentrated urine. The composition of final urine encompasses three processes; glomerular filtration, tubular reabsorption and tubular secretion [17].

(A) Crystal formation (supersaturation, crystal nucleation, crystal growth and aggregation) [18-20].
As a direct result of the renal function of water preservation, urine becomes supersaturated with slightly soluble salts like calcium oxalate and –phosphates [21]. Supersaturation is a thermodynamically unstable state and occurs when the amount in solution of these salts exceeds the solubility [19]. Supersaturation for calcium phosphate is first achieved in the loop of Henle, while that for calcium oxalate occurs in the distal tubules. Calcium phosphate dissolves again in later nephron segments as the fluid is acidified there and these dissolving calcium phosphate crystals act as heterogenous nucleator for calcium oxalate crystals formed in the distal tubule [19]. Crystal nucleation is a thermodynamically more stable state and depends on the supersaturation level and the stability of the first nuclei [19]. Thus, when supersaturation is high enough and lasts long enough or promoters are present, crystals nucleate in the urine and are excreted as crystalluria. Both types of nuclei may increase in size by growth and/or aggregation [20, 21].

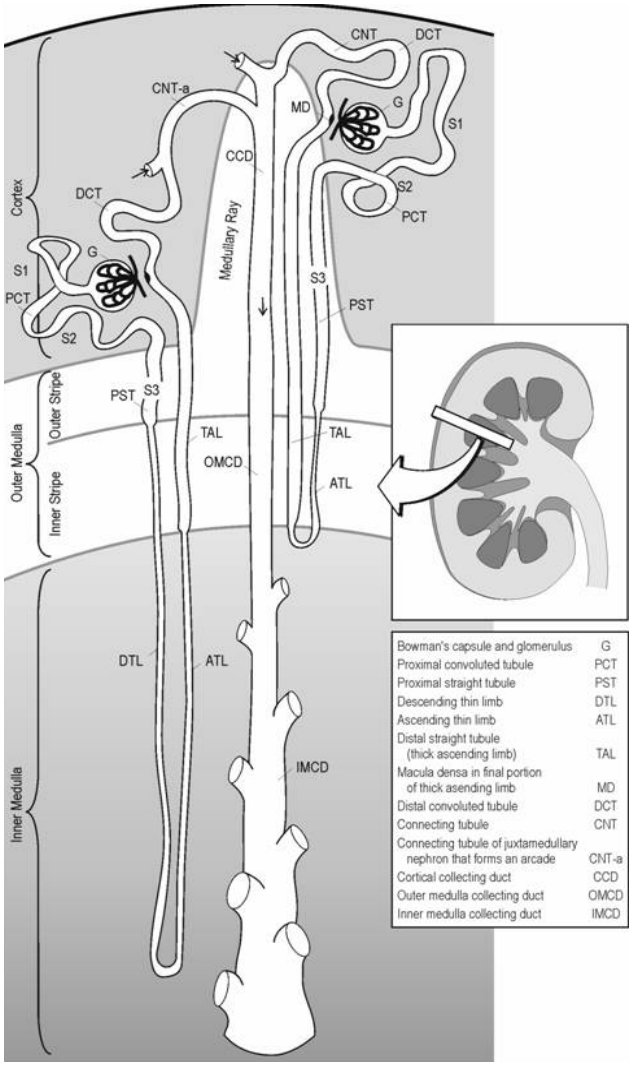

Figure 1.1

Functional anatomy of the nephron and the collecting duct system. The kidney comprises a large number of functional units called nephrons. They form two major anatomical regions: the outer region or cortex and the inner region or medulla. In the medulla an outer and inner zone can be distinguished of which the former can be subdivided in an outer stripe and an inner stripe, based on the different segments of the nephron present in each zone and subzone. The components of the nephron are depicted in the figure. Starting at the glomerulus (G), the proximal tubule consists of an initial convoluted part (PCT) (S1 and S2 segment) followed by a straight part (PST) (S3 segment). The transition from the proximal tubule to the thin descending limb is abrupt. After a hairpin loop, the thin descending limb continues in a thin ascending limb and, with an abrupt transition also, in a thick ascending limb (TAL). The latter, which is the straight part of the distal tubule, extends upwards into outer medulla and cortex towards the glomerulus of the same nephron. The following segment of the nephron, the distal convoluted tubule (DCT) extends into the connecting tubule (CNT), which is the transition region to the collecting duct (CD). The straight part of the proximal tubule, the thin limb and the straight part of the distal tubule form the "loop of Henle". The figure shows one superficial, short-looped, nephron and one deep (or juxtamedullary), long-looped, nephron.

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Urine contains several compounds that have been investigated on their role on each of these processes of supersaturation, nucleation, crystal growth and aggregation, acting as either promoters or inhibitors of crystallization. For example, stone salts are at much higher concentrations soluble in urine than in water due to chelation (complexation) of calcium (eg, citrate) or oxalate (eg, magnesium) [19].Three groups of modulators of crystal formation in urine have been recognized; low molecular weight compounds like citrate, magnesium and pyrophosphate, high molecular weight compounds like glycosaminoglycans and proteins (Tamm-Horsfall protein, nephrocalcin, Inter-α-Inhibitor, osteopontin, urinary prothrombin fragment-1, etc) and lipids or cellular membranes [21]. It is postulated that an imbalance between promoter and inhibitor activity could act as a causative mechanism in stone formation, but despite extensive studies none of the known urinary inhibitors, except for citrate [22], have been definitively proven to play a key role in calcium stone formation, or to have any clinical and therapeutic application [23].

(B) Crystal retention ('free particle' versus 'fixed particle' theory)

Kidney stones cannot be formed as long as crystals are washed away with the urine downstream the urinary tract. However, when crystals are retained in the kidney it becomes possible that crystals aggregate and become large enough to form a stone. Crystalluria is a physiological phenomenon in mammals whereas kidney stone formation is not [18, 19, 21, 24-26]. Despite the fact that healthy people continuously form crystals in their kidneys, they do not develop stones. Robertson measured that up to 7,200 crystals/ml of urine, or approximately 1.1 x 10^7 crystals/day, are being excreted [18, 26, 27]. Apparently, additional mechanisms are required to initiate the stone-forming process. Crystal retention could result from crystal aggregates that became too large in size to freely pass the renal tubules ('free particle' theory), or from crystal binding to renal tubular epithelial cells ('fixed particle' theory) [28, 29]. The likelihood of individual crystals growing large enough to be trapped within the measured urine transit time through the nephron of 3-4 minutes is very small [30]. By 1978, Finlayson and Reid [28] had already proposed that some form of fixation is required for the accumulation of crystals in the kidney. This concept is in agreement with an earlier histological observation in renal tissue of a hyperoxaluric patient, which showed the attachment of relatively small calcium oxalate crystals to the renal tubular epithelium [31]. Cell-culture studies have been performed to obtain insights into the susceptibility of the cell surface to crystal attachment, and to uncover cell-surface crystal-binding molecules [32].

1.5 CRYSTAL-CELL INTERACTION IN THE PATHOGENESIS OF KIDNEY STONE DISEASE

Khan *et al*. [33] were among the first to study crystal-cell interaction in animals. In the early 1990s, both Wiessner *et al*. [34] and Lieske *et al*. [35] had started to study this topic in cell culture. Their studies, and those of others, have contributed considerably to the concept that the interaction between crystals and renal tubular cells plays a role in the pathophysiology of nephrolithiasis and that, under pathological conditions, renal tubular cells express cell-surface molecules that have an affinity for crystals ("crystal-binding molecules") [32]. These observations inspired several investigators to unmask the nature of these molecules. In the next section, the experiments that have led to the proposal of substances as crystal-binding molecule are described.

1.6 CRYSTAL-BINDING MOLECULES

Mandel's group [34, 36] investigated the possible role of plasma-membrane phospholipids in the attachment of calcium oxalate monohydrate crystals to primary cultures of rat inner medullary collecting duct (IMCD) cells and continuous cultures of IMCD (cIMCD) cells. Bigelow *et al*. [37] induced alteration of the membrane-lipid composition of inner medullary collecting duct cells, by both exogenous addition of phospholipid liposome suspensions and by exposure to a calcium ionophore (A23187). Both methods revealed that the expression of phosphatidylserine at the cell surface greatly enhanced crystal binding. The anionic phospholipid phosphatidylserine normally occurs exclusively in the inner leaflet of the lipid bilayer of the plasma membrane as a result of its inwardly directed transport, catalyzed by an energy-dependent aminophospholipid translocase. The levels of crystal binding were again low after treatment of phosphatidylserine-expressing cells with a phosphatidylserine-specific ligand (annexin V). On the basis of these observations, the authors proposed that crystal retention might be caused by the appearance, under pathological conditions, of phosphatidylserine at the surfaces of epithelial cells lining the collecting ducts. Recently, the group of Scheid [38] demonstrated that the same effects on phosphatidylserine exposure and crystal binding were observed after exposing wild-type Madin-Darby canine kidney (MDCK) cells to toxic oxalate concentrations.

In 1996, Lieske *et al*. [39] described the adherence of calcium oxalate monohydrate crystals to African green monkey renal epithelial (BSC-1) cells and wild-type MDCK cells. Crystal binding was reduced after preincubation of the cells with positively charged compounds, suggesting that crystals adhere to negatively charged cell surface components [39]. Pretreatment of the crystals with these cationic compounds did not influence crystal binding, indicating that they affected the cell surface rather than the crystal surface. Since most of the cell-surface negative

charge is attributable to terminal sialic acid residues, these investigators subsequently studied the effect of sialidase (neuraminidase) on crystal binding. This treatment significantly reduced the binding of crystals. Pretreatment of the cells with sialic acid-specific lectins could also reduce crystal binding. Therefore, these authors proposed that crystals can bind to sialic acid. Next, the authors launched the concept that two populations of (poly)anions, one anchored to the apical plasma membrane and the other free in tubular fluid (e.g. proteins, glycosaminoglycans, citrate), can be viewed as competitors for the crystal surface. Alterations in the quality and quantity of either population of anions could alter this competitive balance and thereby determine whether or not crystals bind to the renal tubular cell surface. Recently, Lieske demonstrated that neuraminidase and protease treatment of African green monkey renal epithelial (BSC-1) cells and cIMCD cells induced freshly nucleated calcium oxalate dihydrate crystals to adhere to the cell surface via the (100) face rather than the (001) face [40]. From these observations, it was speculated that sialic-acid-containing cell surface glycoconjugates are critical determinants of face-specific crystal nucleation upon the renal cell surface. According to these authors, the spatial three-dimensional organization of these anionic sugar residues, rather than the amount of sialic acid, may enable their interaction with complementary atomic arrays on the crystal surface.

A number of investigators have focused on extracellular matrix molecules as potential crystal-binding substances. Kohri *et al*. [41] observed that calcium oxalate crystals adhered to collagen type IV-positive cell clumps in primary cultures of rat IMCD cells, and that collagen IV-positive mucous threads with an affinity for crystals were present in urine sediments from patients with stones. These observations prompted Kohri *et al*. [41] to speculate that renal tubular basement membrane collagen IV is a potential crystal-binding molecule. Yamate *et al*. [42] explored the role of osteopontin in calcium oxalate monohydrate crystal binding to wild-type MDCK cells. The addition of osteopontin to MDCK cells resulted in increased crystal deposition on the cell surfaces. The fluorescence intensity of osteopontin at the cell surface could be reduced by osteopontin antibodies, thrombin, cyclic Arg-Gly-Asp (RGD) peptides and tunicamycin, and these compounds also reduced crystal binding. From these observations, it was proposed that osteopontin might serve as a crystal-binding molecule [42].

Besides sialic acid, glycosaminoglycans make a substantial contribution to the overall negative charge of the cell surface. These molecules have therefore frequently been postulated as potential crystal-binding molecules. Glycosaminoglycans such as chondroitin sulfate and heparan sulfate are the polysaccharide side-chains of cell-surface-associated glycoconjugates (e.g. proteoglycans and glycolipids). Crystal-binding studies by our group with MDCK strain I cells, a clone of wild-type MDCK cells, showed that calcium oxalate monohydrate crystal binding is much higher to proliferating cells in subconfluent cultures than to growth-arrested cells populating confluent monolayers [43]. During the process of cell proliferation, crystal binding can

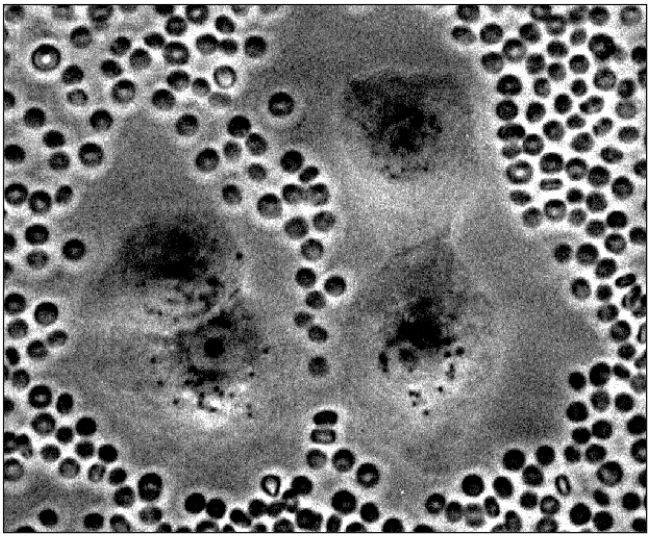

Figure 1.2

Pericellular matrices surrounding MDCK-I cells. In our confocal laser scanning microscopy images, hyaluronan is usually represented as a relatively thin line covering the cell surface. It should be noted, however, that the staining method requires cell fixation, and that during this process the pericellular matrix is dehydrated and collapses onto the cell surface. To visualize the pericellular matrix, we adopted the 'particle exclusion assay', described by Knudson *et al.*, in which fixed red blood cells are added to living coat-bearing cells. This method is based on the inability of these particles to penetrate the viscous cell coats. This method gives a true impression of the size of these (otherwise invisible) cell coats viewed under a phase-contrast microscope.

From: Asselman & Verkoelen: Curr Opin Urol, Volume 12(4).July 2002.271-276.

be reduced by chondroitinase ABC or testicular hyaluronidase, but not by heparinase III. Therefore, chondroitin sulfate and/or hyaluronan, in contrast to heparan sulfate, could be involved in crystal binding. Since chondroitinase ABC and testicular hyaluronidase degrade chondroitin sulfate as well as hyaluronan, these studies were repeated with *Streptomyces* hyaluronidase, an enzyme that specifically digests hyaluronan. The results obtained were identical, which favored the view that hyaluronan is a crystal-binding molecule [43]. Studies with purified hyaluronan provided evidence that calcium oxalate monohydrate crystals can actually bind to this polymer. Next, metabolic labeling studies revealed that the surface of subconfluent MDCK-I cultures contained much higher amounts of *Streptomyces* hyaluronidase-cleavable material than did confluent monolayers. In addition, hyaluronidase treatment significantly reduced crystal binding to subconfluent cultures, whereas it did not further affect the already low levels of binding to intact monolayers. Finally, confocal laser scanning microscopy revealed that hyaluronan is indeed expressed by crystal-attracting cells in subconfluent cultures but not by non-adherent confluent cells. After scrape-damage of intact monolayers, calcium oxalate monohydrate crystals selectively adhered to hyaluronan-expressing migrating and proliferating cells in the wound [44]. These studies indisputably identified hyaluronan as a binding molecule for crystals at the surfaces of MDCK-I cells in locomotion (figure 1.2 and 1.3).

A recent study by Sorokina *et al*. [45] confirmed that intact, quiescent monolayers of well-differentiated cultures of IMCD cells have little affinity for calcium oxalate monohydrate cultures, whereas proliferating cells populating subconfluent cultures or migrating cells in scraping areas of mature monolayers have a high affinity for crystal binding. Previously these authors isolated and identified a membrane glycoprotein related to nucleolin, designated nucleolin-related protein (NRP), from rat IMCD cells as crystal binding molecule [46]. The polarized distribution and expression of NRP and cell differentiation was associated with crystal binding [45].

In MDCK-I cells another apical membrane glycoprotein with affinity for calcium oxalate monohydrate crystals was recently isolated and identified, namely annexin-II [47]. Crystal binding could be decreased significantly by incubation with antibodies against annexin-II as well

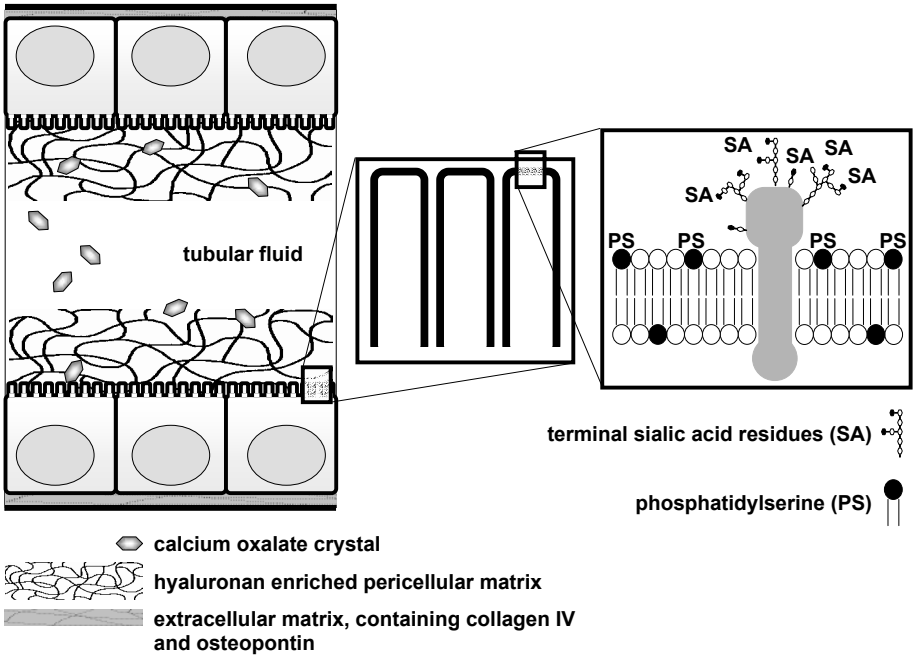

Figure 1.3

Schematic representation of the relative size and localization of candidate crystal-binding molecules in relation to the sizes of the crystals and the diameter of the lumen of the distal tubule/collecting duct. The distal tubules/collecting ducts are the sites in the nephron where, considering the levels of calcium oxalate supersaturation, crystals are to be expected. According to the calculations made by Kok and Khan [29], the diameter of the lumen of the distal tubule/collecting duct is 20-60 µm. Spontaneous nucleated calcium oxalate crystals are depicted with an average size of 1-2 µm. This size could be underestimated, since crystals frequently form aggregated clumps interspersed with organic material. Although the sizes of the cells lining the renal tubules vary, an average height of approximately 10 µm is certainly not unrealistic. This illustration clearly shows the microscopic scale of sialic acid residues (SA) and phospholipids, such as phosphatidylserine (PS), in comparison to the crystal size and especially with respect to pericellular and extracellular matrix molecules.
From: Asselman & Verkoelen: Curr Opin Urol, Volume 12(4).July 2002.271-276.

as nucleolin. The authors concluded that annexin-II expressed at the apical membrane of MDCK-I cells could serve as crystal-binding molecule, but they also suggested that multiple cell surface molecules mediate crystal adhesion since neither of these antibodies completely abolished crystal adhesion [47]. Also in mouse IMCD-3 cells an increase in annexin A2 expression at the plasma membrane resulted in an increased crystal-binding capacity, which could be reduced by pretreatment with anti-annexin A2 antibodies [48].

1.7 SCOPE OF THE THESIS

In summary, two major conclusions could be drawn from cell crystal-cell interaction experiments that were performed by the group of Verkoelen et al.

1) It was found that MDCK-I cells in intact monolayers with high levels of transepithelial resistance have no affinity for calcium oxalate crystals and crystals do not bind to their cell surface. In contrast, proliferating cells in subconfluent cultures and migrating/regenerating cells at the border of the wound of scrape-damaged cultures with low levels of transepithelial resistance have a high affinity for calciumoxalate crystals and crystals bind to the cell surface of these cells [44].
Hence, a different phenotype of mature, fully differentiated distal tubular cells as opposed to proliferating/regenerating dedifferentiating cells is accompanied by a different affinity of the cell surface for calcium oxalate crystals.

2) Next, it was found in MDCK-I cells in culture that the high molecular mass polysaccharide hyaluronan serves as crystal binding substance based on the following results: (a) crystals bind to hyaluronan-expressing cells at subconfluence but not to cells in confluent cultures that do no longer express hyaluronan; (b) metabolic labeling studies showed that the surface of proliferating cells contains substantially higher levels of radiolabeled hyaluronan; (c) crystal binding could be decreased by *Streptomyces* hyaluronidase, an enzyme that specifically digests hyaluronan; and (d) during wound healing, hyaluronan-binding protein binds to migrating and proliferating flattened cells in damaged areas but not to cells in intact monolayers [43].

Subsequently the validity of these results was confirmed in primary cultures of human renal cells [49]. Studies were repeated in primary cultures of human proximal (PTC) and distal tubular/collecting duct cells (DTC) [49]. Cells were susceptible to crystal binding during the first days post-seeding but lost this affinity when cultures developed into confluent monolayers with functional tight junctions. The transmembrane receptor protein CD44 and its ligands osteopontin and hyaluronan were expressed at the apical membrane of proliferating tubular cells, whereas at confluence, CD44 was expressed at the basolateral membrane and OPN and

hyaluronan were no longer detectable. In addition, a particle exclusion technique revealed that proliferating cells were surrounded by hyaluronan-rich pericellular matrices or "cell coats" extending several microns from the cell surface. Disintegration of these coats with hyaluronidase significantly decreased the cell surface affinity for crystals. These results suggested (1) that the intact distal tubular epithelium of the human kidney does not bind crystals, and (2) that crystal retention in the human kidney may depend on the expression of CD44-, osteopontin-, and hyaluronan rich cell coats by damaged distal tubular epithelium [49].

General aims of the present study were;

1. To further explore hyaluronan biology in renal distal tubular epithelial cells *in vitro*.
2. To study whether the conclusions drawn from these *in vitro* studies also apply to the *in vivo* situation (rat and human).
3. To evaluate whether crystal binding to hyaluronan-expressing cells could be modulated.

Since studies with renal tubular cells in culture indicate that hyaluronan and osteopontin and their mutual cell surface receptor CD44 play an important role in calcium oxalate crystal binding during wound healing, this concept was investigated *in vivo* by treating rats for 1, 4, and 8 days with ethylene glycol (0.5 and 0.75%) in their drinking water to induce renal tubular cell damage and calcium oxalate crystalluria (**Chapter 2**).

In **Chapter 3** hyaluronan biology in renal epithelial cells was further studied to determine the polarized distribution of hyaluronan and its cell-surface receptor CD44 by MDCK-I cells.

All of these cell culture and rat studies indicate that crystal retention in the distal nephron is limited to proliferating/regenerating tubular cells expressing hyaluronan and osteopontin at their luminal surface. Fetal and transplant kidneys contain proliferating and/or regenerating cells since nephrogenesis is not completed until 36 weeks of gestation, while ischemia and nephrotoxic immunosuppressants may lead to injury and repair in renal transplants. In **Chapter 4** the expression of hyaluronan and osteopontin and the appearance of tubular calcifications in both human fetal/preterm and transplanted kidneys are described.

In **Chapter 5** the effect of nonsteroidal anti-inflammatory drugs on calcium oxalate crystal binding by hyaluronan-expressing proliferating/regenerating renal tubular epithelial cells in culture was studied based on the hypothesis that hyaluronan synthesis is regulated by cyclooxygenase-induced prostaglandin E_2 (PGE_2) production.

In the general discussion (**Chapter 6**) the clinical relevance of these results will be discussed as well as treatment strategies and future studies and perspectives.

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Chapter 2

Calcium oxalate adherence to hyaluronan-, osteopontin-, and CD44-expressing injured/ regenerating tubular epithelial cells in rat kidneys

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ABSTRACT

Background

Retention of crystals in the kidney is an essential early step in renal stone formation. Studies with renal tubular cells in culture indicate that hyaluronan (HA) and osteopontin (OPN) and their mutual cell surface receptor CD44 play an important role in calcium oxalate (CaOx) crystal binding during wound healing.

Methods

This concept was investigated *in vivo* by treating rats for 1, 4, and 8 d with ethylene glycol (0.5 and 0.75%) in their drinking water to induce renal tubular cell damage and CaOx crystalluria. Tubular injury was morphologically scored on periodic acid-Schiff–stained renal tissue sections and tissue repair assessed by immunohistochemical staining for proliferating cell nuclear antigen. CaOx crystals were visualized in periodic acid-Schiff–stained sections by polarized light microscopy, and renal calcium deposits were quantified with von Kossa staining. HA was visualized with HA-binding protein and OPN and CD44 immunohistochemically with specific antibodies and quantified with an image analyzer system.

Results

Already after 1 d of treatment, both concentrations of ethylene glycol induced hyperoxaluria and CaOx crystalluria. At this point, there was neither tubular injury nor crystal retention in the kidney, and expression of HA, OPN, and CD44 was comparable to untreated controls. After 4 and 8 d of ethylene glycol, however, intratubular crystals were found adhered to injured/regenerating (proliferating cell nuclear antigen positive) tubular epithelial cells, expressing HA, OPN, and CD44 at their luminal membrane.

Conclusion

In conclusion, the expression of HA, OPN, and CD44 by injured/regenerating tubular cells seems to play a role in retention of crystals in the rat kidney.

INTRODUCTION

The development of kidney stones requires formation of crystals followed by their retention in the kidney [1]. Crystal retention could be caused by adherence of crystals to the epithelial cells lining the renal tubules [2]. Although many investigators recognized a role for renal tubular injury in the pathophysiology of nephrolithiasis, definite proof for this concept and the mechanisms involved are not yet available [3-7].

Most kidney stones are predominantly composed of precipitated calcium salts, the most common of which is calcium oxalate monohydrate [5]. Studies with renal tubular epithelial cells in culture showed that confluent monolayers of distal tubule/collecting duct–like MDCK-I cells are nonadherent to calcium oxalate monohydrate crystals [8]. In contrast, crystals bind to cells in subconfluent cultures and in confluent monolayers recovering from mechanical injury [9]. The glycosaminoglycan hyaluronan (HA) was identified as a major crystal-binding molecule at the surface of MDCK-I cells [10] and of human renal tubular cells in primary culture [11]. In addition, it was found that crystal-binding cells not only expressed HA at their apical surface but also osteopontin (OPN) and CD44 [11].

HA is a high molecular mass polysaccharide (>10^6 Da), composed of linear polymers of a repeating disaccharide structure of alternating glucuronic acid and N-acetylglucosamine. In the kidney, HA is hardly detectable in the cortex but is abundantly present as the main component of the renal inner medullary interstitium [12]. HA in the kidney is upregulated during various inflammatory disease states [13-17]. OPN is a glycoprotein and is widely spread throughout organs and tissues, including the kidney. In the healthy kidney, OPN is confined to the distal parts of a subset of nephrons. During various types of inflammation, however, renal OPN is severely upregulated in most segments of the nephron [13, 14, 18-20]. The transmembrane protein CD44 is a cell surface receptor for both HA and OPN [21, 22] and also is upregulated during inflammation in the kidney [13, 14]. The present study was conducted to investigate whether *in vivo* renal tubular injury and HA, OPN, and CD44 expression are involved in crystal retention.

MATERIALS AND METHODS

Experimental Design

Male Wistar rats (300 to 350 g) were obtained from the Central Animal Breeding Center (Harlan, Zeist, the Netherlands) and divided into three groups ($n = 9$ each) receiving drinking water supplemented with 0, 0.5, or 0.75% (vol/vol) ethylene glycol (EG) for 1, 4, and 8 d. All animals had free access to standard chow. Twenty-four hours before the indicated times, rats were housed individually in metabolic cages to collect 24-h urine samples and to monitor fluid

intake. Urine samples were divided into portions of 5 ml, one portion of which was acidified with 100 µl of 1 M hydrochloric acid and stored at -20°C until analysis. Animals were sedated and killed; kidneys were extracted and decapsulated; and sagittal slices were immediately fixed in either methacarn (60% methanol, 30% chloroform, 10% acetic acid) or Dubosq-Brasil fixative (47% ethanol, 11.7% H2O, 23.5% formaldehyde, 17.6% acetic acid, and 4 mM picric acid) for 4 h, rinsed with 70% ethanol, and embedded in low-melting-point paraffin (52°C; BDH Laboratory Supplies, Poole, UK). Serum specimens were collected and frozen at -20°C until biochemical analysis. The experiments were approved by the local University Animal Committee and carried out in accordance with the Netherlands Experiments on Animals Act (1977) and the European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, March 18, 1986).

Urine and Serum Biochemistry

Urinary oxalate was determined in acidified urine portions with a quantitative enzymatic colorimetric assay (Sigma Diagnostics, Dei- senhofen, Germany). For determination of urinary citrate, the ultraviolet method with the test combination of Boehringer Mannheim (Darmstadt, Germany) was used. The concentrations of calcium in urine and bicarbonate, calcium, and creatinine in serum were determined on a routine autoanalyzer system (Vitros 750 XRC). Urine samples were centrifuged at 5000 x *g*, and sediments were inspected by optical and polarized light microscopy (Zeiss Axioplan microscope, Oberkochen, Germany).

Tubular Morphology

Methacarn-fixed, paraffin-embedded renal tissue sections (4 µm) were stained with periodic acid-Schiff (PAS), and nuclei were counterstained with methyl green. Histologic damage was evaluated with a morphologic scoring system (table 2.1) in proximal tubules (PT), thin limbs of Henle (TLH), distal tubules (DT; including thick ascending limbs [TAL]) and collecting ducts (CD). Tubules were morphologically inspected by a reproducible procedure, which comprised a random selection of the first tubular cross-section, followed by shifting the microscopic field over fixed distances according to a standardized pattern (x300 magnification). The cortex, outer stripe of the outer medulla (OSOM), inner stripe of the outer medulla (ISOM), and inner

Table 2.1

Scoring system for the evaluation of tubular morphology of PT, TLH, DT (including TAL), and CD*.

Score	PT	TLH	DT (TAL)/CD
0	Intact tubule with normal appearance	Intact tubule with normal appearance	Intact tubule with normal appearance
1	Tubule with luminal cell debris	Tubule with luminal cell debris	Tubule with luminal cell debris
2	Tubule with loss of brush border	–	Dilated tubule
3	Tubule with flattened cells	–	Tubule with flattened cells

*PT, proximal tubules; TLH, thin limbs of Henle; DT, distal tubules; TAL, thick ascending limbs; CD, collecting ducts.

medulla of each kidney section were evaluated. In the cortex, PT (S1–S2) and DT were evaluated (n = 50 and 25, respectively); in the OSOM, PT (S3) and DT (TAL) were evaluated (n = 50 and 25, respectively); in the ISOM, TLH and DT (TAL) were evaluated (n = 25 each); and in the inner medulla, TLH and CD were evaluated (n = 25 each). PT and DT could be distinguished according to at least one of the following morphologic criteria: topographical localization, tubular size and form, cytoplasmic density and position of the nuclei, and presence or absence of brush border and basolateral cell aspect. Tubules in the cortex and OSOM were scored as PT only when a brush border could be identified; if not, then they were scored as DT. In the ISOM and inner medulla, TLH could be distinguished from DT (TAL) and CD by tubular size and position of the nuclei.

Proliferation was determined by immunohistochemical staining for proliferating cell nuclear antigen (PCNA) using the PC10 mAb (DAKO, Glostrup, Denmark) as described previously [23]. Routinely, sections were counterstained with PAS and nuclei were stained with methyl green. In a number of sections, PAS staining was omitted, allowing optimal visualization of crystals. Expression of PCNA was quantified morphometrically with an image analyzer system (KS-400 V2.0 image analysis software) in the cortex + OSOM and ISOM + inner medulla, by measuring positive signals in 25 and 15 randomly chosen microscopic fields, respectively (x250 magnification). Measurements were expressed as fractional positive area of the tissue section.

Crystal Retention

During the evaluation of tubular morphology, each tubule was additionally inspected by polarized light microscopy for the presence of crystals. In this way, crystal retention was assessed and sites of crystal location were correlated with tubular morphology.

Calcium deposits were also visualized by von Kossa staining. Deparaffinized Dubosq-Brasil–fixed 4-µm tissue sections were incubated in 5% silver nitrate for 45 min. Slides were rinsed in water, incubated in 1% pyrogallic acid for 3 min, rinsed in water, fixed in 5% sodium thiosulfate for 1 min, and counterstained with hematoxylin and eosin. In each sagittal kidney section, calcium deposits were quantified by counting the total number of positive stained crystals in the cortex + OSOM and ISOM + inner medulla.

HA, OPN, and CD44 Expression

Renal tissue sections were stained for HA, OPN, and CD44 as described previously [11]. Briefly, methacarn-fixed tissue sections were blocked with 1% BSA for HA and with normal horse serum for OPN and CD44 staining and incubated with primary labels (biotinylated HA-binding protein, Seikagaku, Falmouth, MD; goat anti-human OPN antibody, OP189, C.M. Giachelli, University of Washington; or mouse anti-human CD44 antibody, Bender MedSystems, Vienna, Austria). For OPN and CD44, sections were subsequently incubated with secondary labels,

biotinylated horse anti-goat and horse anti-mouse antibodies (Vector Laboratories, Burlingame, CA), respectively. Finally, avidin-biotin peroxidase complex (Vector) and diaminobenzidine were used to detect HA, OPN, and CD44. Sections were counterstained with methyl green. No staining was observed when primary labels were omitted.

Expression of HA, OPN, and CD44 was quantified morphometrically with KS-400 V2.0 image analysis software in the cortex and OSOM, by measuring positive signals in 10 and six randomly chosen microscopic fields, respectively (x200 magnification). Measurements were expressed as fractional positive area of the tissue section. OPN and CD44 were also quantified in the remaining part of the medulla, by analyzing six randomly chosen microscopic fields of the ISOM and inner medulla. HA was not quantified in this region, because the well known abundant amount of HA in the interstitium of the inner medulla stains nearly the entire tissue [12].

Statistical Analyses

Data are expressed as mean ± SEM. $P < 0.05$ was considered to be significant, using *t* test. Correlation analysis between total tubular PCNA expression per sagittal kidney section and the total amount of positive von Kossa renal calcium deposits per sagittal kidney section in individual rats was performed by the nonparametric Spearman's rank order test. $P < 0.01$ was considered significant (two-tailed). Computations were performed using SPSS version 10.

RESULTS

Urine and Serum Biochemistry

In the control group, the average urinary oxalate and calcium excretion was 0.95 ± 0.05 and 3.24 ± 0.79 mg/24 h, respectively (figure 2.1), and the urinary sediment did not contain crystals (figure 2.2 A). Administration of 0.5 and 0.75% EG to the drinking water induced within 24 h a significant concentration-dependent hyperoxaluria (4.37 ± 1.71 and 7.78 ± 4.46 mg/24 h, respectively). In the 0.5% EG group, oxalate gradually increased further to reach its maximum level at day 8, whereas in the 0.75% EG group, urinary oxalate reached its maximum level already at day 4. Urinary calcium was decreased at day 1 and was undetectable at day 4 and day 8 (figure 2.1). At day 1, calcium oxalate crystals were observed in urinary sediments of both EG groups (figure 2.2 B). Oxalate was determined in acidified urine portions, which dissolves crystals and therefore represents the total amount of oxalate, including oxalate precipitated with calcium. Because calcium was determined in urine that was not acidified, it represents the amount of free calcium ions. Thus, the decreased amounts of urinary calcium apparently resulted from the formation of CaOx crystals.

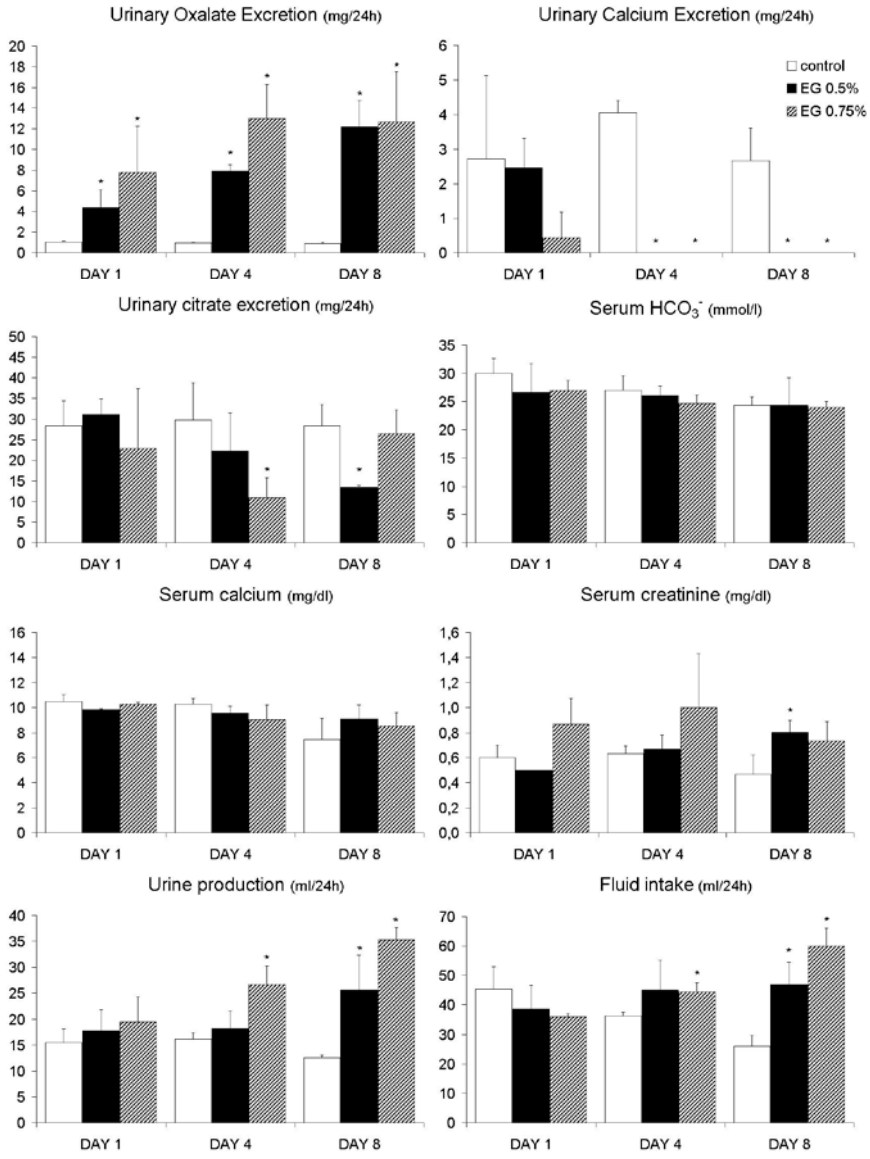

Figure 2.1

Urine- and serum biochemical analysis, urine production, and fluid intake. Statistical analysis by *t* test. *Significantly different compared with controls at the same point in time ($P < 0.05$).

In the 0.5% EG group at day 8 and in the 0.75% EG group at days 4 and 8, increased diuresis and fluid intake were observed compared with controls. Thus, the addition of EG led to polyuria, which may be secondary to the osmotic effect of EG excreted in the urine unchanged [24] or simply because these rats drank more water (figure 2.1). There was no increase in serum

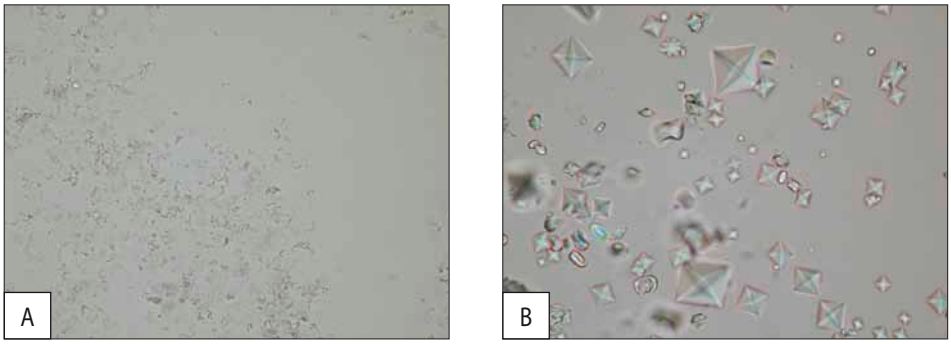

Figure 2.2

Urinary sediment inspected by polarized light microscopy of a control rat (A) and a 0.5% ethylene glycol (EG)-treated rat (B) after 1 d. (A) Some debris was observed, but no urinary crystalline material. (B) Numerous urinary calcium oxalate crystals in the EG-treated rat are clearly visible. Magnification, x400.

creatinine, except for a slight increase in the low-dose EG at day 8, indicating that renal function was preserved in these rats. These biochemistry data are in accordance with earlier observations in rats treated with EG [25].

EG caused a metabolic acidosis, as can be derived from the concentration-dependent decrease in urinary citrate after 4 d. This metabolic acidosis was relatively mild, however, as serum bicarbonate and calcium were not affected and a compensatory homeostatic response seemed to normalize urinary citrate after continued EG challenge (figure 2.1).

Tubular Morphology

The relatively low concentrations of EG in the present study did not result in frank necrosis but in mild changes in tubular morphology. At day 1, tubular morphology was comparable to controls, but at days 4 and 8, different degrees of injury/regeneration were found in tubules (figure 2.3). PT of the OSOM (S3) and, to a lesser extent, PT in the cortex (S1–S2) suffered the most morphologic damage, for the most part with loss of brush border (score 2). Tubules with flattened cells (score 3) were also observed, predominantly in DT (TAL) of the OSOM. Because a tubule with flattened cells was scored only as PT if there was still brush border recognizable, the number of PT with score 3 could actually have been higher. In TLH and CD at days 4 and 8, increased amounts of luminal cell debris (score 1) were observed, but the majority of these tubules had a normal morphology (score 0). Importantly, cell debris in the tubular lumen in the distal nephron could also have been derived from an "upstream" section of the nephron, and the tubule concerned could actually have been completely healthy.

At day 1, PCNA staining was comparable to control kidneys, in which tubular expression of this protein was sparse (figure 2.4 A), whereas at days 4 and 8, PCNA staining was clearly upregulated in tubules in rats that received EG (figure 2.4 B).

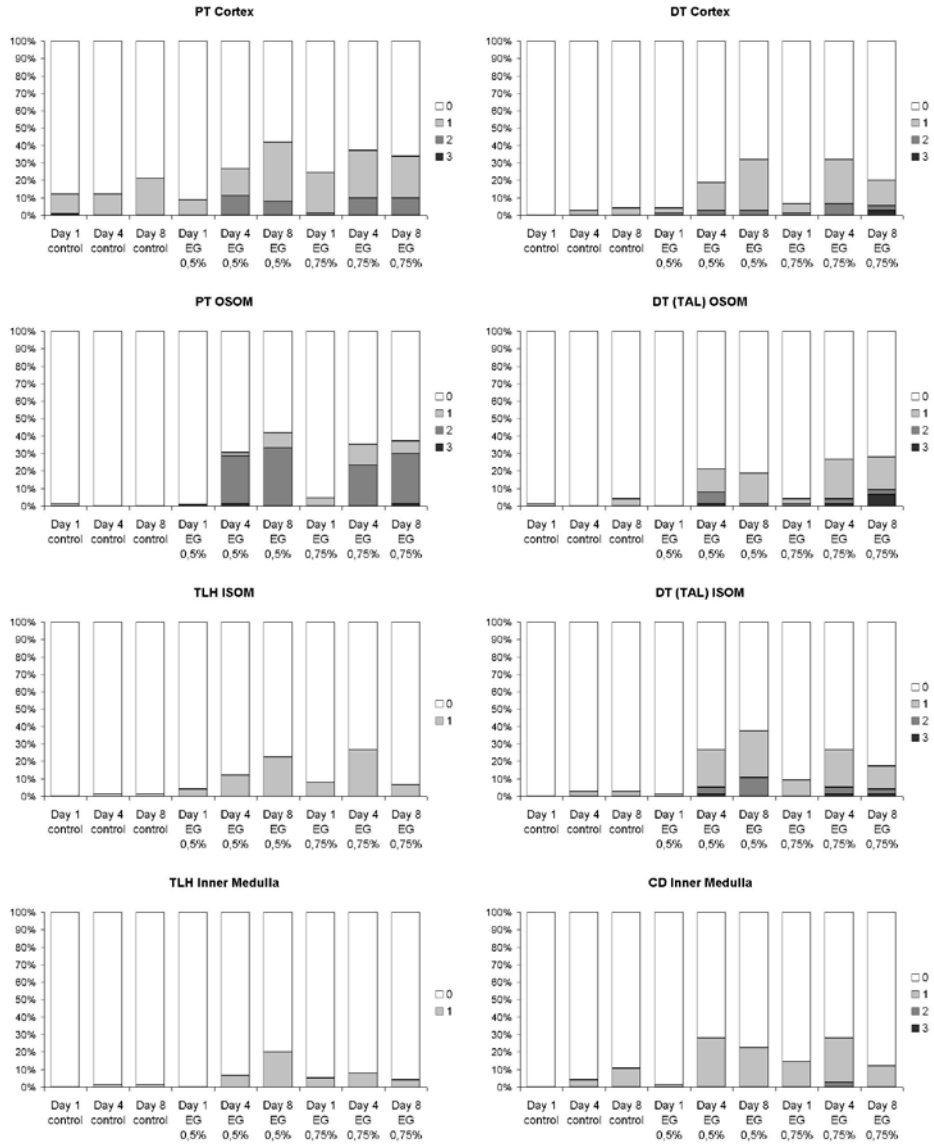

Figure 2.3

Evaluation of tubular morphology with the scoring system of table 2.1 in proximal tubules (PT), thin limbs of Henle (TLH), distal tubules (DT; including thick ascending limbs [TAL]), and collecting ducts (CD 0 = intact tubule with normal appearance, 1 = tubule with luminal cell debris, 2 = PT: tubule with loss of brush border/DT (TAL) and CD: dilated tubule, 3 = tubule with flattened cells).

Crystal Retention

No crystals were found in control rat kidneys (figure 2.5). After 1 d of 0.5 and 0.75% EG, no crystals were observed by polarized light microscopy, consistent with a marginal amount of

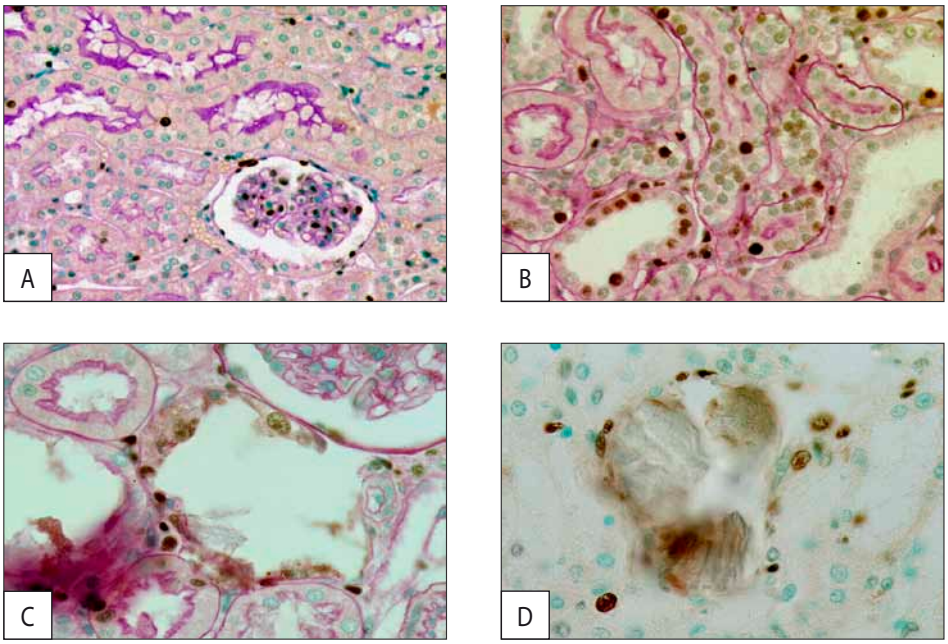

Figure 2.4

Proliferating cell nuclear antigen (PCNA) staining in a kidney of a control rat (A) and of a rat that received 0.75% EG for 8 d (B through D). In D, periodic acid-Schiff (PAS) counterstaining was omitted. (A) Sparse PCNA staining, mainly present in the interstitium and glomeruli in a control rat. (B) Strongly upregulated PCNA in the tubules of an EG-treated rat. (C and D) Crystals associated with PCNA-positive, flattened cells. Magnifications: x400 in A, x200 in B, x630 in C and D. Color image: see appendix page 147.

positive von Kossa signals. At days 4 and 8, however, an increasing number of crystals were found attached to the luminal membrane of tubular epithelial cells, corresponding with a markedly increased number of positive von Kossa signals (figure 2.5). Crystals were smaller in size than the tubular lumen and not seen intracellularly or in the interstitium.

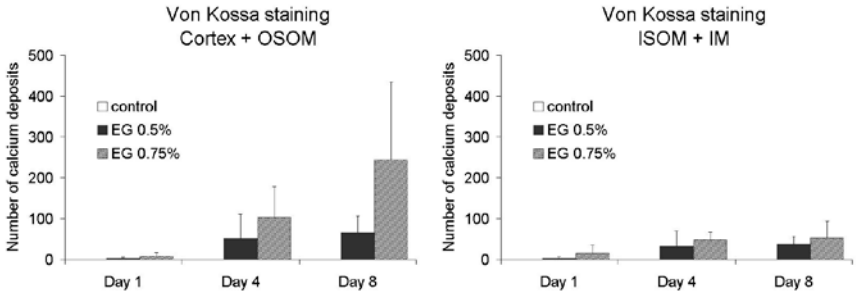

Figure 2.5

Quantification of renal calcium deposits on von Kossa–stained tissue sections.

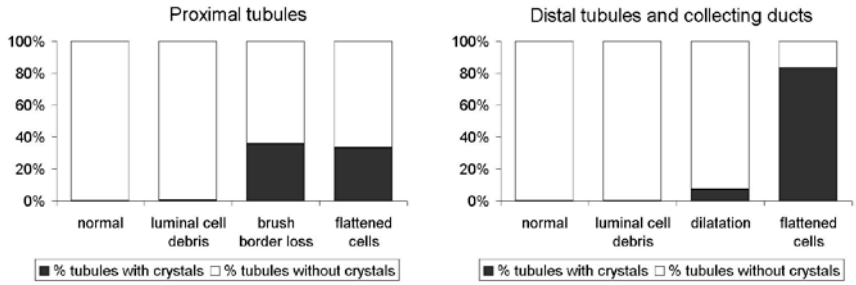

Figure 2.6

Retention of crystals in relation to tubular morphology. In PT, DT (including TAL), and CD, crystals were not observed in tubules with score 0 (normal appearance) or score 1 (luminal cell debris). Crystals were exclusively retained at the luminal surface of tubular cells in PT, DT, and CD with score 2 (loss of brush border in PT and dilated tubules in DT and CD) and score 3 (tubules with flattened cells).

In figure 2.6, retention of crystals and tubular morphology is plotted in a graph. In PT, DT (TAL), and CD, crystals were not observed in histologic normal tubules (score 0) or tubules with luminal cell debris (score 1). Crystals were found adhered to tubular cells in PT with loss of brush border and DT (TAL) and CD with dilation of the tubular lumen (score 2), and in tubules with flattened cells (score 3). Crystals were not observed in TLH (data not shown). Figure 2.7 shows a DT with small crystals at the luminal membrane of flattened tubular epithelial cells (score 3). Furthermore, these flattened cells were PCNA positive, proliferating cells (figure 2.4, C and D). Approximately 93% of the observed crystals in PCNA-stained renal tissue sections were found to be adhered to PCNA-positive cells.

Correlation analysis was performed between the total amount of tubular PCNA expression and the amount of positive von Kossa calcium deposits in sagittal kidney sections of individual rats

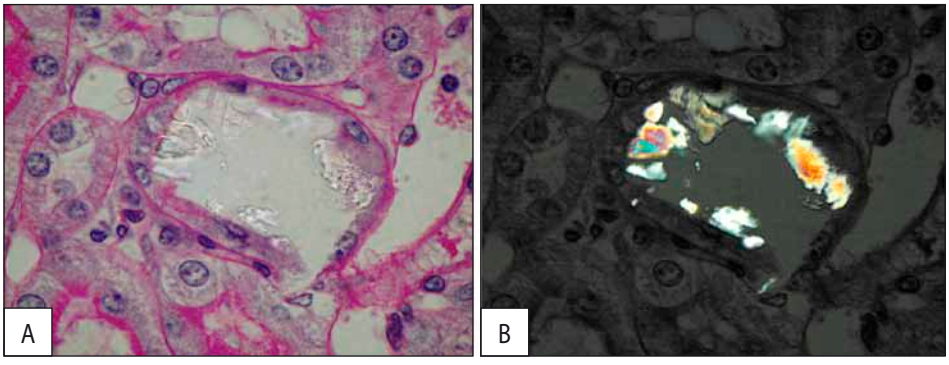

Figure 2.7

PAS and methyl green–stained renal tissue section of a rat treated with 0.75% EG for 4 d by optical (A) or polarized (B) light microscopy. (A) A dilated DT is shown with flattened epithelial cells (score 3). (B) In the lumen of this DT, crystals that are smaller than the tubular lumen are clearly visible and located at the luminal side of the injured/regenerating flattened epithelium. Magnification, x1000. Color image: see appendix page 147.

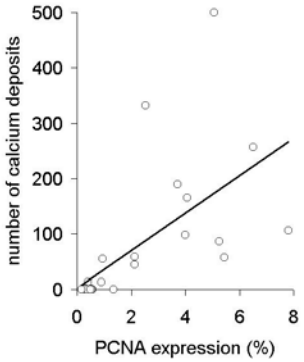

Figure 2.8

Correlation analysis was performed between the total amount of tubular PCNA expression and the amount of positive von Kossa calcium deposits in sagittal kidney sections of individual rats (Spearman's Rho = 0.819, $P < 0.0001$).

(figure 2.8). Tubular cell regeneration was associated with crystal adhesion/retention (Spearman's Rho = 0.819; $P < 0.0001$).

HA, OPN, and CD44 Expression

HA in the cortex and OSOM of control rats was scarcely observed in the interstitium and around a few glomerular capsules. After 1 d, HA expression in EG-treated rats was comparable to controls (figure 2.9). After 4 and 8 d, however, HA was upregulated in a focal pattern throughout the cortex and OSOM of EG-treated rats (figure 2.9), primarily in the interstitium but also at the luminal membrane of tubular cells (figure 2.10 A). Strikingly, in the majority of cases, crystals were found at the luminal surface of HA-expressing cells (figure 2.10 A).

OPN was expressed in a limited number of distal tubular epithelial cells of control rats. After 1 d of EG, OPN expression was not different from controls, except for a small increase in the OSOM of the moderate-dose EG group (figure 2.9). At days 4 and 8, however, OPN was significantly upregulated in the kidneys of EG-treated rats (figure 2.9). Both PT and DT showed a rise in OPN immunostaining (figure 2.10, B and C). The classical OPN expression pattern was observed, with Golgi apparatus immunostaining in PT and at the apical membrane in DT, allowing differentiation between PT and DT (previously described after renal ischemia/reperfusion) [19, 26]. Crystals were retained in apical OPN-expressing DT. Crystals were positive for OPN, which was evident by comparison between optical and polarized light microscopy (figure 2.10, B and C).

CD44 was hardly expressed in the rat kidney, except for some interstitial cells and glomeruli. After 1 d of EG, CD44 expression was not significantly different from controls (figure 2.9). After 4 and 8 d of EG, however, CD44 in the cortex, OSOM, and medulla was markedly upregulated (figure 2.9). CD44 expression was prominent in a focal pattern along basolateral and apical

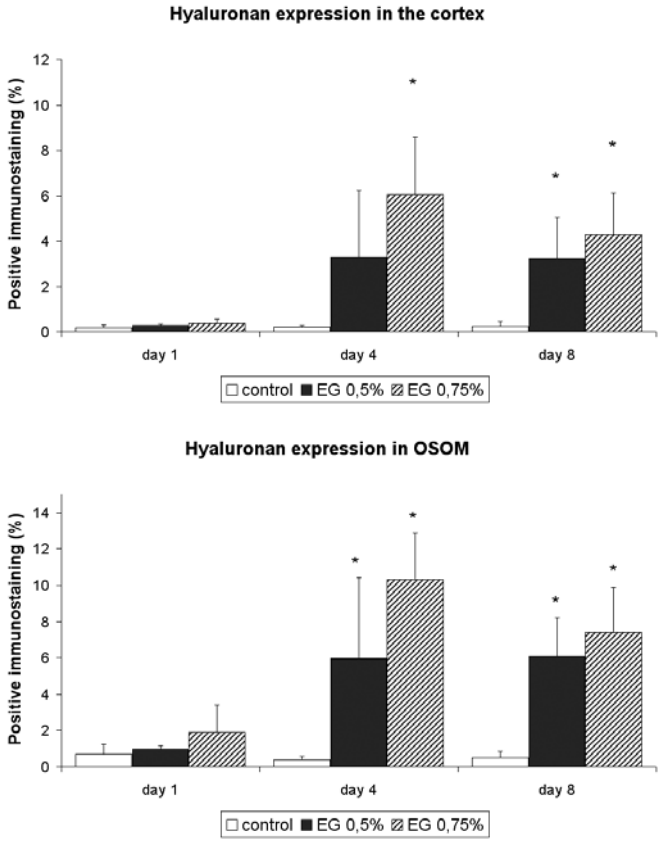

Figure 2.9

Quantification of renal hyaluronan (HA) expression using computerized image analysis software (KS400). Data are expressed as fractional positive area (%). Statistical analysis by *t* test. *Significantly increased compared with controls at the same point in time ($P < 0.05$).

tubular cell membranes (figure 2.10 D). Crystals were observed frequently closely at sites where cells expressed CD44 at their luminal membrane (figure 2.10 D).

DISCUSSION

In the present study, we investigated the role of HA, OPN, and CD44 in retention of crystals in renal tubules damaged by EG. The EG model is extremely suitable for this purpose since this agent not only is toxic to the nephron but also generates urinary CaOx crystals [25, 27-30]. For investigating the earliest events involved in crystal retention, these studies were performed shortly (1, 4, and 8 d) after the addition of EG to the drinking water and at relatively low concentrations to avoid massive tissue damage. One of the major findings was that there is no

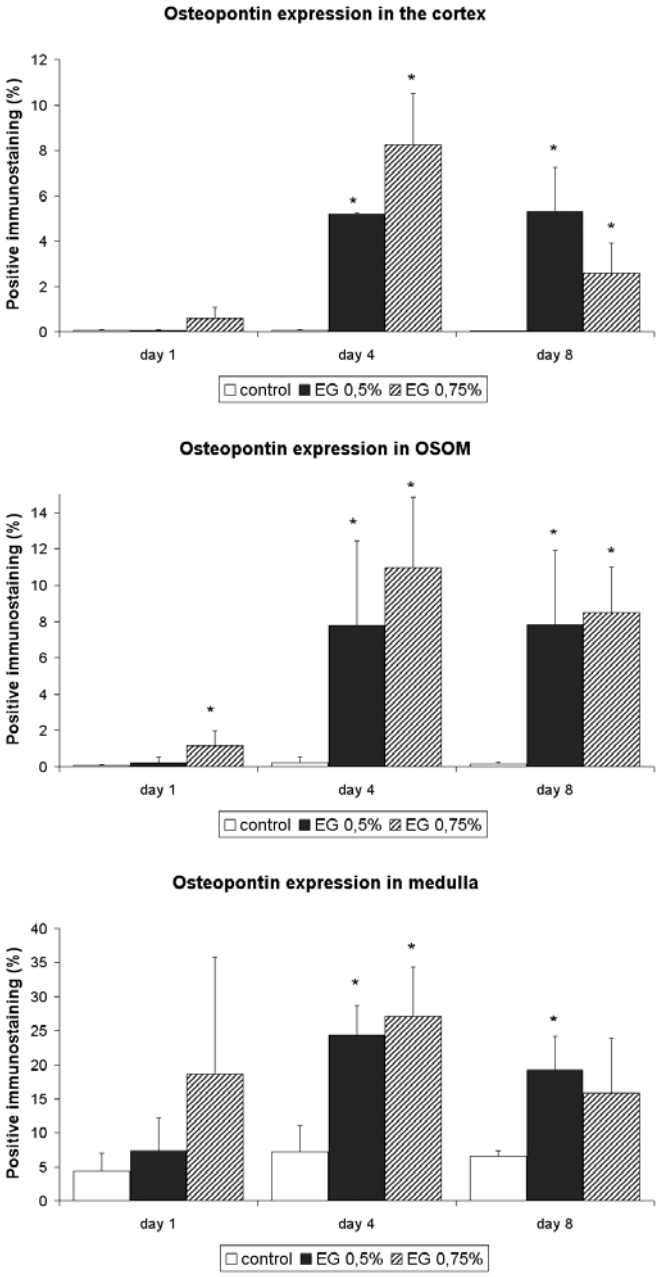

Figure 2.9

Quantification of renal osteopontin (OPN) expression using computerized image analysis software (KS400). Data are expressed as fractional positive area (%). Statistical analysis by *t* test. *Significantly increased compared with controls at the same point in time ($P < 0.05$).

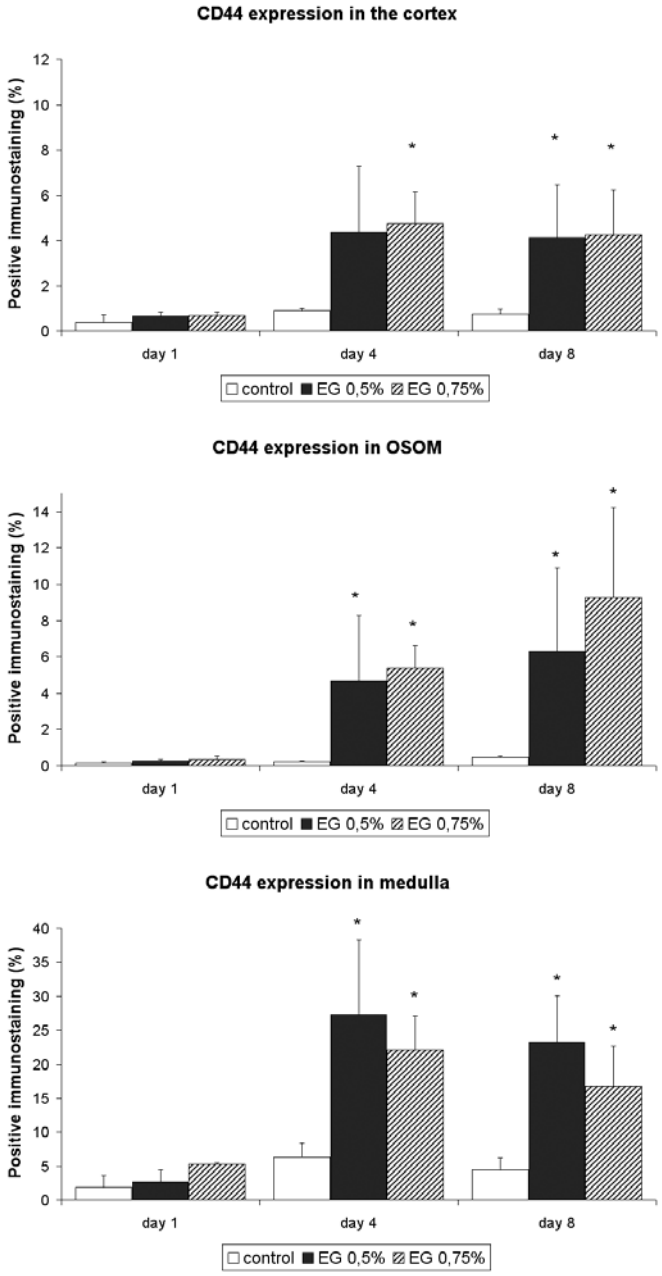

Figure 2.9

Quantification of renal CD44 expression using computerized image analysis software (KS400). Data are expressed as fractional positive area (%). Statistical analysis by *t* test. *Significantly increased compared with controls at the same point in time ($P < 0.05$).

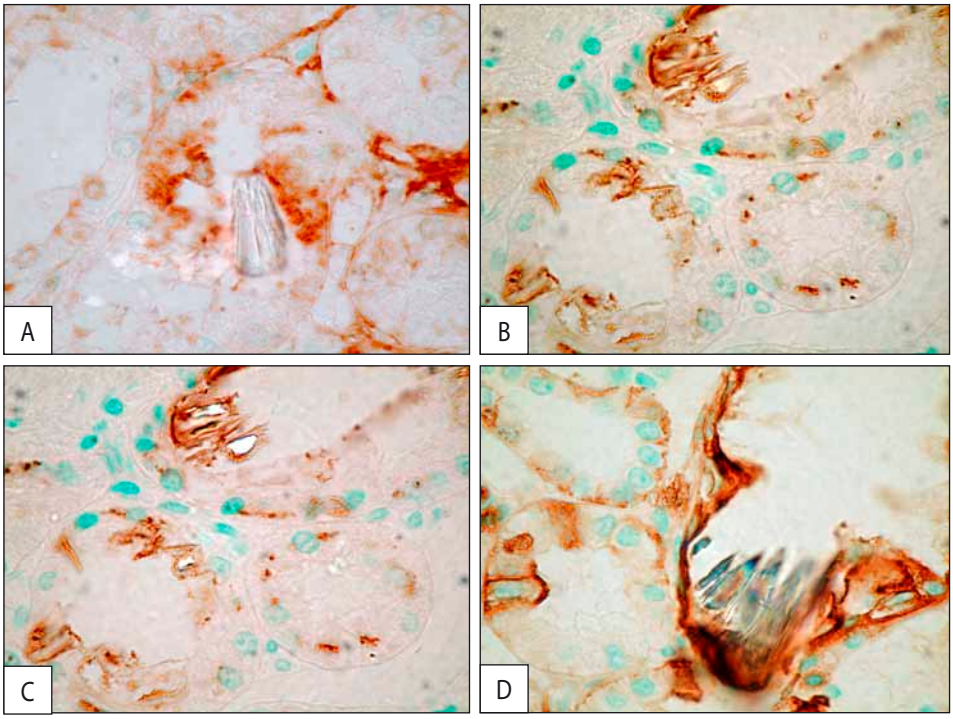

Figure 2.10

(A) The outer stripe of the outer medulla (OSOM) stained for HA of a rat with 0.75% EG in the drinking water for 8 d. HA is upregulated in the interstitium as well as at the apical membrane of tubular epithelial cells. A crystal is shown close to cells that express HA at their luminal membrane. **(B and C) The OSOM stained for OPN after 8 d of 0.75% EG**, showing increased typical Golgi apparatus immunostaining in PT and at the apical cell membrane in DT. (C) Crystals visualized with polarized light microscopy. Retained crystals stained for OPN are smaller than the tubular lumen and closely associated with apical OPN-expressing epithelium. **(D) The cortex stained for CD44 after 8 d of 0.75% EG.** CD44 is upregulated at basolateral and apical tubular cell membranes. Polarized light microscopy shows birefringent crystals in the lumen of a tubule closely associated with the surface of flattened cells positive for CD44. Magnification, x1000. Color image: see appendix page 148.

crystal retention in the absence of tubular injury/regeneration but that crystals are retained as soon as renal tubules are injured/regenerating. Crystals were found adhered to the luminal surface of HA-, OPN-, and CD44-expressing injured/regenerating cells. The results of this study therefore strongly suggest that crystal retention in the kidney requires tubular epithelial injury accompanied by luminal expression of HA, OPN, and CD44.

EG, which itself is not nephrotoxic, is metabolized in the liver to several intermediates, including glycoaldehyde, glycolate, glyoxylate, and oxalate [27]. It is still a matter of debate which of these metabolites are responsible for the damage to renal tubular cells [28, 29]. Oxalate precipitates as CaOx in the primary urine as a result of its poor solubility. The nephrotoxic effect of EG has also been attributed to these crystals [25, 31]. However, autopsy and renal biopsy studies in humans

did not support the concept that crystals are the primary cause of EG toxicity [27]. Considering this controversy, it is impossible in the present study to distinguish between crystals binding to injured/regenerating cells and the alternative explanation that crystal deposition causes cell injury (and consequently cells bind crystals). Crystals did not seem to be retained because of their size, because no crystal aggregates occluding the tubular lumen were observed, but crystals were generally smaller (figures 2.4, C and D, 2.7, A and B, and 2.10, A, B, C, and D).

In urolithiasis research, animals are usually treated with relatively high concentrations of EG for several weeks [25, 30, 31]. Also in these studies, crystals are retained in tubules that are clearly injured [25, 31, 32]. In the present study, the short period of treatment (1 to 8 d) with relatively low concentrations of EG did not result in frank necrosis or cast formation but in a mild form of injury/regeneration. Renal tubular injury was morphologically scored (see table 2.1), and PCNA was used to assess cell proliferation. PCNA is an auxiliary protein for DNA polymerase δ and required for both DNA replication and DNA repair. In proliferating cells, it is upregulated in cell nuclei mainly during the S-phase (DNA synthesis phase) of the cell cycle [33]. Consequently, it is widely used as a marker for proliferating cells. Immunohistochemical PCNA staining is a validated method for evaluating epithelial regeneration of the kidney after renal damage [23]. It is well known that in the kidney, tubular cell injury and regeneration occur concurrently [23]. Hence, the flattened, PCNA-positive tubular cells after 4 and 8 d of EG treatment (figure 2.4 B) are dedifferentiated proliferating cells.

HA is a high molecular mass polysaccharide found in many tissues, where it performs a great range of biologic functions [34, 35]. In the kidney, HA is normally not expressed at the luminal surface of tubular cells and is present only in the medullary interstitium, where it provides structural stability to the tubules and contributes to concentrating the urine [12]. HA expression in the renal cortex is upregulated in renal inflammatory diseases such as interstitial nephritis [13], acute ischemic injury [14, 17], autoimmune renal injury [15], and acutely rejecting human kidney grafts [16]. HA accumulates in wounded tissue shortly after injury to form loose hydrated matrices that allow cell division and migration [36, 37]. During recent years, we searched extensively for crystal-binding molecules at the surface of renal tubular cells in culture [10, 11, 38, 39]. HA was identified as major crystal-binding substance, based on the following results: (*1*) crystals bind to HA-expressing cells at subconfluence but not to cells in confluent cultures that do no longer express HA; (*2*) metabolic labeling studies showed that the surface of proliferating cells contains substantially higher levels of radiolabeled HA; (*3*) crystal binding could be decreased by *Streptomyces* hyaluronidase, an enzyme that specifically digests HA; and (*4*) during wound healing, HA-binding protein binds to migrating and proliferating flattened cells in damaged areas but not to cells in intact monolayers [10]. The co-localization of intraluminal CaOx crystals with HA-expressing tubular cells in the present study suggests for the first time, to our knowledge, a role for HA in crystal retention *in vivo*.

The glycoprotein OPN is widely distributed in the body and has been implicated in several physiologic and pathologic processes, including cell adhesion, migration, signaling, inflammation, and biomineralization [18, 40, 41]. In the kidney, the expression of OPN is upregulated during renal injury [18, 19, 42], including induced by EG [31]. The role of OPN under these circumstances is unclear, but OPN seems to be involved in mediating macrophage accumulation and interstitial fibrosis [18, 43]. The role of OPN in urolithiasis is controversial. OPN has been proposed as an inhibitor of crystal formation and retention but also as a promoter of crystal retention [44-46]. Recently, crystal retention was studied in OPN knockout and wild-type mice treated with EG [44]. Noticeably, after 4 wk of treatment with 1% EG, no crystals were retained in wild-type mice, whereas some crystal retention was observed in the kidneys of OPN knockouts (14.3 ± 3 von Kossa signals per sagittal kidney section). From these observations, the authors concluded that OPN protects the kidney from crystal formation and retention. However, the crystal retention inhibitor function of OPN seems to be ineffective in the present study, because crystals covered with OPN but smaller in size than the tubular lumen became firmly associated with the cell surface (figure 2.10, B and C). This is in agreement with a previous study, in which it was demonstrated that urinary inhibitors of crystallization were unable to prevent the attachment of crystals to regenerating renal tubular cells [47]. Consequently, possibly because of the differences in species and study designs between the present study and the one performed by Wesson *et al.* [44], the role of OPN remains controversial.

CD44 is a ubiquitous transmembrane glycoprotein that is involved in many processes including inflammation [48]. CD44 serves as cell surface receptor for both HA and OPN [21, 22]. In fact, the biologic activity of HA and OPN predominantly depends on their interaction with CD44 [49-51]. In the kidney, the expression of CD44 is highly upregulated during various renal disease states [13, 14]. Hence, it is not surprising that an upregulated expression of CD44 in the renal tubules is accompanied by an increased expression of its ligands HA and OPN. Reports of the role of CD44 in renal stone formation are scarce. In cell culture, it was found that CD44 is expressed at the luminal surface of crystal-binding renal tubular cells but not on that of cells without affinity for crystals [11]. In the present study, crystals were also retained at sites where cells expressed CD44. In our opinion, crystals are not likely to become associated with CD44 but rather with HA and/or OPN.

The present study supports *in vivo* for the first time the concept that crystal retention is associated with HA expressed at the luminal surface of injured/regenerating cells. However, we cannot rule out the importance of other crystal-binding molecules, including sialic acid–containing glycoproteins [52], phosphatidyl serine [53], collagen [54], and nucleolin-related protein [55]. Furthermore, several other macromolecules have been implicated in CaOx crystallization and retention [56], including inter-α inhibitor–related proteins [32] such as bikunin [57] and prothrombin fragment 1 [58]. Although in the present study we focused on HA, OPN, and CD44,

the interrelationship between these and other molecules as part of complex cell biologic pathways in the pathophysiology of kidney stone disease remains to be determined [2].

Although the expression of HA, OPN, and CD44 by injured/regenerating tubular epithelial cells most likely is aimed to reestablishment of the epithelial barrier integrity and restoration of renal

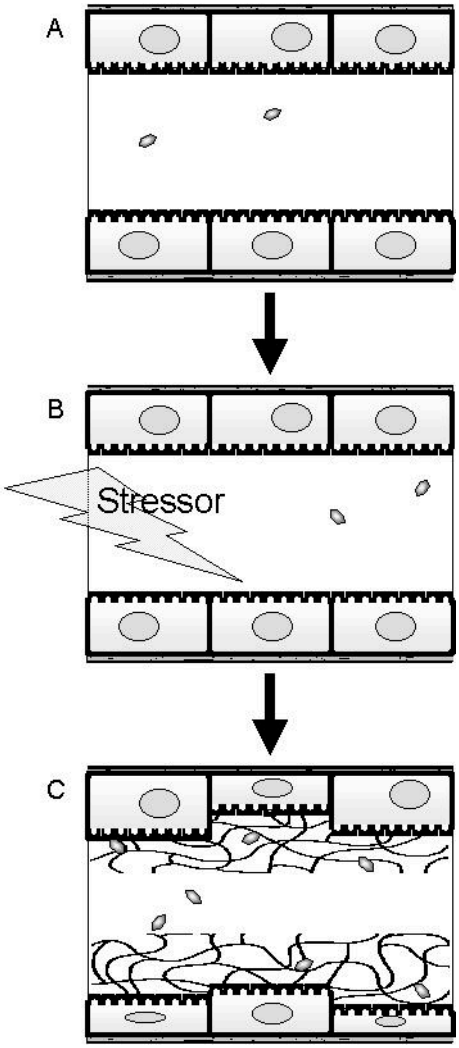

Figure 2.11

Paradigm of crystal retention. A schematic representation of a cross-section of a distal tubule is shown. (A) Under normal conditions, crystals do not bind to renal tubular epithelial cells and are harmlessly excreted in the urine. (B) Crystal retention is preceded by a stressor injuring the epithelium. (C) During the process of tubular epithelial regeneration and repair, flattened epithelial cells express HA, OPN, and CD44 at their apical membrane. HA is a cell surface crystal-binding molecule. Because these regenerating dedifferentiated tubular epithelial cells are susceptible to crystal binding, crystal retention may ensue.

function, a negative side effect could be that it turns a non–crystal-binding epithelium into a crystal-binding one, thereby setting the stage for crystal retention (figure 2.11). The clinical relevance of this concept was recently reinforced by observations in kidneys of preterm neonates, in which the development of nephrocalcinosis is common, showing that HA and OPN are abundantly expressed at the luminal membrane of proliferating tubular cells (unpublished results).

CONCLUSION

In conclusion, the results obtained in this study support the concept that the expression of HA, OPN, and CD44 by injured/regenerating tubular cells is a prerequisite for retention of crystals in the kidney.

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Chapter 3

Hyaluronan is Apically Secreted and Expressed by Proliferating or Regenerating Renal Tubular Cells

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ABSTRACT

Background

Hyaluronan has diverse biologic functions in the body, varying from structural tasks to cell stress-induced CD44-mediated activation of intracellular signaling pathways. Hyaluronan biology is relatively unexplored in the kidney. Previously, we identified hyaluronan as binding molecule for crystals in the renal tubules. Crystal retention is a crucial early event in the etiology of kidney stones. The present study was performed to determine the polarized distribution of hyaluronan and CD44 by renal tubular cells.

Methods

Madin-Darby canine kidney (MDCK) strain I and primary cultures of hu man renal tubular cells were grown on permeable supports in a two-compartment culture system. Studies were performed during growth and after scrape-injury. Metabolic labeling studies and an enzyme-linked hyaluronan -binding assay were used to measure the molecular mass and the amount of secreted hyaluronan in apical and basal medium. Confocal microscopy was applied to detect membrane hyaluronan and CD44. Hyaluronan synthase (HAS) mRNA expression was studied with reverse transcriptase-polymerase chain reaction (RT-PCR). The in vitro expression profile of hyaluronan was compared with that in biopsies of transplanted human kidneys with acute tubular necrosis.

Results

Proliferating cells produced more hyaluronan ($M_r > 10^6$ Da) than growth-inhibited cells in intact monolayers and up to 85% was targeted to the apical compartment, which was accompanied by increase d HAS2 mRNA expression and slightly decreased HAS3 mRNA, while HAS1 mRNA remained undetectable. Hyaluronan and CD44 were exclusively expressed at the apical surface of proliferating/regenerating cells. After (re)establishment of tight junctions, hyaluronan was no longer detectable while CD44 was targeted to basolateral membrane domains. In vivo in inflamed human kidneys hyaluronan was abundantly expressed in the cortical tubulointerstitial space as well as at the luminal surface of regenerating renal tubular cells.

Conclusion

These results demonstrate that the production of hyaluronan by renal tubular cells is activated during proliferation and in response to mechanical injury and that hyaluronan and CD44 expression is highly polarized. The targeted delivery of hyaluronan to the apical compartment suggests that hyaluronan produced by renal tubular cells supports proliferation/rege neration in the renal tubules, but that it does not contribute to hyaluronan accumulation in the renal interstitium. These data further support the concept that mitogen/stress-induced hyaluronan deposition in the renal tubules increases the risk for crystal retention and stone formation.

INTRODUCTION

A kidney stone is composed of many small crystals interspersed with organic material. Crystallization is the unavoidable physicochemical consequence of the urinary concentration process in the kidney and can not lead to stone formation as long as precipitated stone salts are efficiently excreted with the urine. Renal stone formation requires crystal retention. Previous studies indicate that hyaluronan plays a key role in this process [1, 2]. Studies with renal tubular epithelial cells in culture showed that confluent monolayers of distal tubule/collecting duct-like Madin-Darby canine kidney strain I (MDCK-I) cells are nonadherent to calcium oxalate monohydrate crystals [3]. In contrast, crystals bind to cells in subconfluent cultures and in confluent monolayers recovering from mechanical injury [4]. The glycosam inoglycan hyaluronan was identified as a major crystal-binding molecule at the surface of MDCK-I cells [1] and of human renal tubular cells in primary culture [5]. In addition, it was found that in vivo in rats that there is no crystal retention in the absence of tubular injury/regeneration, but that crystals are retained as soon as renal tubules are injured/regenerating. Crystals were found adhered to the luminal surface of hyaluronan-expressing injured/regenerating cells, therefore strongly suggesting that crystal retention in the kidney requires tubular epithelial injury accompanied by luminal expression of hyaluronan [2].

Hyaluronan (hyaluronic acid) is a glycosaminoglycan (GAG) composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine [6]. The biosynthesis of hyaluronan is unique, since most GAGs are synthesized in the Golgi network and linked to a core protein to form proteoglycans, while hyaluronan is synthesized at the plasma membrane as a free GAG. Hyaluronan synthases (HAS) are localized at the inner face of the plasma membrane and assemble a growing polymer of hyaluronan which is extruded through the membrane to the outside of the cell [7]. In mammalian cells three different HAS genes have been identified, encoding for three different HAS proteins, HAS1, HAS2, and HAS3. HAS1 drives the synthesis of high M_r hyaluronan and has been proposed as a "housekeeping" HAS. HAS2 also produces high M_r hyaluronan and is activated during embryonic development and inflammation. HAS3 drives the synthesis of short proinflammatory hyaluronan chains. Hyaluronan plays important roles in morphogenesis, tissue remodeling, inflammation, and cancer [6]. In many cell types hyaluronan binds to the extracellular domain of specifi c cell surface receptors such as CD44 to form cell coats or pericellular matrices (PCMs). CD44-hyaluronan interaction also directly activates intracellular signaling pathways [8-10].

In healthy kidneys, hyaluronan is hardly expressed in the cortex, but abundantly present in the medullary interstitium where it is involved in diluting/concentrating the urine [11, 12]. The expression of hyaluronan is strongly up-regulated in the cortex during various inflammatory renal disease states, such as immune-mediated renal tissue injury [13] allograft rejection [14], and ethylene glycol intoxication [2]. Increased hyaluronan expression is predominantly reported in

the cortical interstitium, but in ethylene glycol–treated rats hyaluronan not only accumulated in the renal interstitium, but also at the luminal membrane of tubular epithelial cells [2].
MDCK-I and primary cultures of human renal tubular cells express hyaluronan at subconfluence, but not at confluence [1, 5, 15]. Since crystal retention depends on the expression of hyaluronan at the apical surface and not on the basolateral plasma membrane, we here studied the polarized distribution of hyaluronan and CD44 in MDCK-I and primary cultures of human renal tubular cells during various growth stages and in response to mechanical damage. Cells were cultured on permeable supports in a two-compartment culture system to have free access to both apical and basal culture medium. The results show that mitogen/stress-activated cells are triggered to synthesize increased levels of high M_r hyaluronan which is predominantly secreted into the apical medium compartment. Activated cells express hyaluronan and CD44 at the apical but not at the basolateral membrane, while at confluence or after wound healing hyaluronan is not detectable and CD44 directed to the basal membrane. Increased hyaluronan biosynthesis is accompanied by increased levels of HAS2 mRNA expression, while the expression of HAS3 mRNA is slightly down-regulated. Our cells did not express HAS1 mRNA. During acute renal failure in humans, hyaluronan accumulated in the renal corticointerstitium but was also observed at the luminal surface of renal tubular cells. These results indicate that the increased production of high M_r hyaluronan by mitogen/stress-activated renal tubular cells is destined for the apical surface and that the expression of hyaluronan and CD44 is highly polarized under these conditions. This suggests that the increased synthesis of hyaluronan by activated renal tubular cells during inflammatory disease states does not contribute to the up-regulated expression of hyaluronan in the renal interstitium. These data further support the concept that the elimination of precipitated stone salts for the kidney is impeded by mitogen/stress-induced hyaluronan expression in the renal tubules.

METHODS

Cell culture

MDCK-I cells were kindly provided by Prof. Dr. G. van Meer, Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, The Netherlands. This strain is isolated from the original American Type Culture Collection MDCK cell line and resembles cortical collecting ducts segments [16]. The cells were routinely grown in plastic tissue culture flasks (Corning Costar Corporation, Badhoevedorp, The Netherlands) at 37°C in a humidified atmosphere of 5% CO_2 in air. Subculturing was performed weekly by releasing the cells from the culture flasks by incubation with 0.05% (wt/vol) trypsin and 0.02% (wt/vol) ethylenediaminetetraacetic acid (EDTA) (Bio Whittaker, Verviers, Belgium). The cells were cultured in Dulbecco's modified minimal essential medium (DMEM) (Gibco, Grand Island, NY, USA)

supplemented with 10% fetal calf serum (FCS) (PAA Labs, Linz, Austria), 2 mmol/L L-glutamine, 100 µg/mL streptomycin, and 100 IU/mL penicillin.

Primary cultures of human renal cortical tubular cells were used as previously described [17, 18]. Briefly, cells were obtained from tumor nephrectomy specimens, which was permitted by the Ethical Committee of the University of Antwerp. Macroscopically normal tissue was decapsulated. Cortex and outer stripe of outer medulla were dissected, cut into pieces of ±1 mm^3 and digested in collagenase D solution (Roche, Ottweiler, Germany), supplemented with DNAse (Sigma Chemical Co., St Louis, MO, USA). The suspension was shaken vigorously for 2 hours at 37°C and sieved through a 120 µm sieve. The resulting cell suspension was loaded on top of a discontinuous Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient with densities 1.04 and 1.07 g/mL. After centrifugation, cells from the intersection were carefully aspirated, washed and brought into culture in α-minimal essential medium (MEM) (Life Technologies, Rockville, MD, USA) modified according to Gibson d'Ambrosio et al [19] supplemented with 10% FCS. In mixed cultures proximal and distal tubular cells are recognized with segment-specific membrane markers. Leucine amino peptidase (LAP) was used for proximal tubular cells and epithelial membrane antigen (EMA) for distal tubular cells. Initially, subconfluent cultures of primary human tubular cells contain equally as much proximal as distal tubular cells. Interestingly, confluent mixed cultures appeared to consist almost entirely of EMA-positive cells that are no longer susceptible to crystal binding [5]. Thus primary cultures of human renal cortical tubular cells become enriched with distal tubular during their development into intact monolayers.

The cells used in these experiments were checked to be free from mycoplasma contamination.

Growth studies

Cells were cultured on permeable polycarbonate Transwell filter inserts (insert growth a rea 4.7 cm^2, 0.4 µm pore size) (Costar®) (Corning Costar Corporation). MDCK-I and primary cultures of renal tubular cells were seeded at a plating density of 1.0 x 10^6 cells/insert and cultured on these permeable supports in a two-chamber system. The total amount of cells/insert was determined at time intervals of 2 days by counting the cells in a hemocytometer. To monitor the assembly of tight junctions, transepithelial electrical resistances (TERs) were measured with an Epithelial Tissue Volt-ohm-meter connected to an Endohm-24 tissue resistance measurement chamber (World Precision Instruments; Sarasota, FL, USA). Since cells are seeded at a high density, both subconfluent and confluent cultures morphologically appear as a continuous row of cells. Cell cultures were considered confluent when the total number of cells/insert did not further increase and maximal TER values had developed.

Wound healing studies

Cells were grown to confluence and subsequently injured by scraping off 100 to 150 mm^2 (approximately one third of the total filter area) with the tip of a sterile 10 mL tissue culture pipette. Subsequently, the filters were washed with phosphate-buffered saline (PBS), fresh medium was added to the cells, and the process of wound healing monitored morphologically by light microscopy and functionally by measuring TERs.

Metabolic labeling studies

The production of high M_r hyaluronan was determined in subconfluent (1 day postseeding) and in confluent (7 days postseeding) MDCK-I cultures by a metabolic labeling protocol using r adiolabeled [^{3}H] glucosamine (D-1-[^{3}H] GlcNAc hydrochloride) (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) in combination with enzymatic digestion and Sephacryl S-1000 size exclusion chromatography. Cells in culture flasks were cultured overnight in modified DMEM with a reduced glucose content (1 mg/mL), after which they were trypsinized, seeded at a concentration of 1 x 10^6/insert, cultured for 1 day, and metabolically labeled for 24 hours with 2 µCi/mL [^{3}H] GlcNAc (subconfluence). Cells were also seeded at a concentration of 1 x 10^6/insert, cultured for 6 days in DMEM 10% FCS, incubated overnight in modified DMEM with a reduced glucose content (1 mg/mL), and than metabolically labeled for 24 hours with 2 µCi/mL [^{3}H] GlcNAc (confluence). One and 7 days postseeding, the average cell density subsequently was 0.9 x 10^6 and 4 x 10^6 cells/insert. After metabolic labeling, the apical and basolateral medium fractions (1500 and 2600 µL) were divided in three portions of 500 µL. To distinguish between hyaluronan and other secreted glycoconjugates, we subjected these portions of the apical and basolateral medium to differential enzyme digestion. One portion was left untreated (control) to assess total glycoconjugate production. The second portion was incubated with papain overnight to digest proteins in the sample and not the papain-resistant polysaccharide hyaluronan. Papain (E.C. 3.4.22.2) (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) was used at a final concentration of 5 U/mL at 60°C, pH 6.0. To check if the papain-resistant fraction indeed consisted of hyaluronan, the third portion was treated with papain as described above, followed by additional treatment with 5 U/mL *Streptomyces* hyaluronidase for 1 hour at 37°C. Of the various fractions, 150 µL was subsequently applied to a 13 x 60 mm Sephacryl S-1000 column (Amersham Biosciences) and eluted with 0.05 mol/L Tris, 0.15 mol/L NaCl (pH 10), with 250 µL fraction volumes. The elution pattern showed two major peaks, the first consisting of high M_r [^{3}H] GlcNAc–labeled molecules (>10^6 Da), and the second with smaller molecules (<10^6 Da). High M_r molecules were run-through in the early fractions 6 to 12, whereas smaller molecules were retarded by the column to be collected later in fractions 13 to 30. Accordingly, free precursor [^{3}H] GlcNAc was entirely recovered in these late fractions.

Cell surface-associated [^{3}H] GlcNAc-labeled hyaluronan was determined by measuring the release of radiolabel during 5 minutes of incubation of the cells wit h fresh serum-free DMEM

with or without *Streptomyces* hyaluronidase. Additional release of [^{3}H] GlcNAc by the enzyme-treated cells, compared to the spontaneous release by cells incubated without enzyme, was considered to represent cell surface-associated hyaluronan.

Reverse transcriptase-polymerase chain reaction (RT-PCR) studies

Primer pairs were designed to selectively amplify either HAS1, HAS2, or HAS3 based on published species-conserved sequences (Amersham Pharmacia Biotech Inc.). Primer sequences are listed below (figure 3.1). Total RNA and genomic DNA were isolated from subconfluent and confluent MDCK-I cells using, respectively, RNA-Bee RNA Isolation Solvent (Tel-Test Inc., Friendswood, TX, USA) and PurGene genomic isolation kit (BioPlastics, Landgraaf, The Netherlands) following the manufacturer's protocol. RNA was reverse transcribed using Maloney-murine leukemia virus (M-MLV) Reverse Transcriptase (Invitrogen, Breda, The Netherlands) and poly-T priming. Thirty cycles of PCR were performed using isoform-specific primers for HAS2 and POLR2A (RNA Polymerase II, large subunit) using either cDNA, genomic DNA as a positive control, or nonreverse transcribed mRNA as a negative control. HAS1 and HAS3 primers needed 35 cycles of PCR to detect an appropriate amount of DNA. In the case of HAS1 RT-PCR, mouse cDNA served as a second positive control. PCR products were visualized under ultraviolet light after gel electrophoresis in 2% agarose containing ethidium bromide.

Confocal laser scanning microscopy (CLSM)

Cells were fixed in formaldehyde/glutaraldehyde (3.7%/0.1%, vol/vol) for 10 minutes. Subsequently, cells were incubated for 60 minutes with 3% (wt/vol) low-fat dry milk in PBS/0.1% Tween-20 to block nonspecific binding. Cells were washed and incubated overnight at 4°C with 2% biotinylated hyaluronan-binding protein (bHABP) (Seikagaku Inc., Tokyo, Japan), rat anticanine CD44 (Serotec, Oxford, UK) or purified antimouse CD44 (IM7) (Becton Dickinson Biosciences Pharmingen, Woerden, The Netherlands) fo llowed by the appropriate secondary labels, respectively, fluorescein isothiocyanate (FITC)-conjugated avidin (fluorescein avidin D) (Vector Laboratories Inc., Burlingame, VT, USA), FITC- or TRITC-conjugated rabbit antirat IgG (H + L) (Southern Biotechnology Associates Inc., Birmingham, AL, USA), or FITC- or TRITC-conjugated rabbit antimouse IgG (Dako, Glostrup, Denmark). Occasionally, the stainings

Primer	Sequence (5' to 3')	Position
HAS-1 F	CAC TGT GTA TCC TGC ATC AG	936-956
HAS-1 R	CTT GGT AGC ATA ACC CAT GC	1080-1100
HAS-2 F	CTC AGT GTT ATA CAT GTC GAG TTT ACT TC	1852-1880
HAS-2 R	ACT GAT ACT GGA ATG AGT CCT ATG AA	1958-1983
HAS-3 F	ACT CTG CAT CGC TGC CTA CC	324-344
HAS-3 R	TAC ATG ACC TCA CGC TTG CC	634-692
POLR2A F	CGG ATG AAC TGA AGC GAA TG	469-488
POLR2A R	TTG TTA GAG TCC ACA AGC AG	735-754

Figure 3.1

The position of the oligonucleotide primers are based on human mRNA sequences.

were combined with a propidium iodide counterstaining to localize cell nuclei. Filters were mounted in vectashield and analyzed with a Zeiss LSM 410 CLSM (Zeiss, Oberkochen, Germany). A 488 nm Ar-laser was used to excitate FITC and a 543 nm laser to excitate TRITC or propidium iodide.

5-bromo-2'-deoxyuridine (BrdU) staining

To incorporate BrdU during wound healing, the cells were incubated for 2 hours with 30 µg/mL BrdU (Sigma Chemical Co.) in culture medium 10% FCS at 37°C in a humidified incubator 5% CO_2 in air. The inserts were washed with physiologic saline and fixed in 70% ethanol. The cells were subsequently incubated overnight with primary mouse monoclonal BrdU antibody IIB5 (1:20) (Euro-Diagnostica B.V., Arnhem, The Netherlands) at 4°C after which they were incubated with biotinylated secondary antibody (1:400) (Dako) at room temparature for 1 hour. Immunoreactivity was visualized by staining with streptavidin-peroxidase complex (1:50) (BioGenex, San Ramon, CA, USA) and 3,3-diaminobenzidine (DAB) (Dako) and cells were counterstained with hematoxylin.

Quantitation of hyaluronan production

The concentration of hyaluronan in culture medium was determined with a commercial quantitative kit, which is based on the specific binding of HABP with hyaluronan (Corgenix HA Test Kit, Denver, CO, USA). Medium of both the apical and basal compartment were collected after the indicated periods of time during growth and wound healing studies. This assay method measures all hyaluronan in the sample and does not discriminate between long or short hyaluronan chains.

Hyaluronan staining of human renal tissue

Renal biopsy tissue specimens of patients suffering from posttransplantation acute tubular necrosis were stained for hyaluronan as described previously [2]. Briefly, tissue was fixed in formaldehyde 4% and paraffin embedded. Sections were blocked with 1% bovine serum albumin (BSA) for hyaluronan staining and incubated with bHABP. Avidin-biotin peroxidase complex (Vector Laboratories) and DAB were used to detect hyaluronan. Nuclei were counterstained with methyl green. No staining was observed when bHABP was omitted.

RESULTS

Hyaluronan biosynthesis analyzed in metabolic labeling studies

Subconfluent and confluent MDCK-I cultures were metabolically labeled overnight with [^{3}H] GlcNAc after which culture medium in the apical and basal compartment was collected to assess the relative amount of [^{3}H] GlcNAc incorporated in macromolecular glycoconjugates.

Subconfluent cultures secreted relatively large amounts of radioactively labeled high M_r molecules into the apical compartment, whereas much smaller amounts were secreted into the basal compartment (figure 3.2). The production of high M_r glycoconjugates by confluent monolayers was ~20-fold lower and delivered into the apical medium. Only a relatively small amount of high M_r glycoconjugates produced by subconfluent cultures could be degraded with papain, whereas practically the remaining high M_r molecules could be digested by

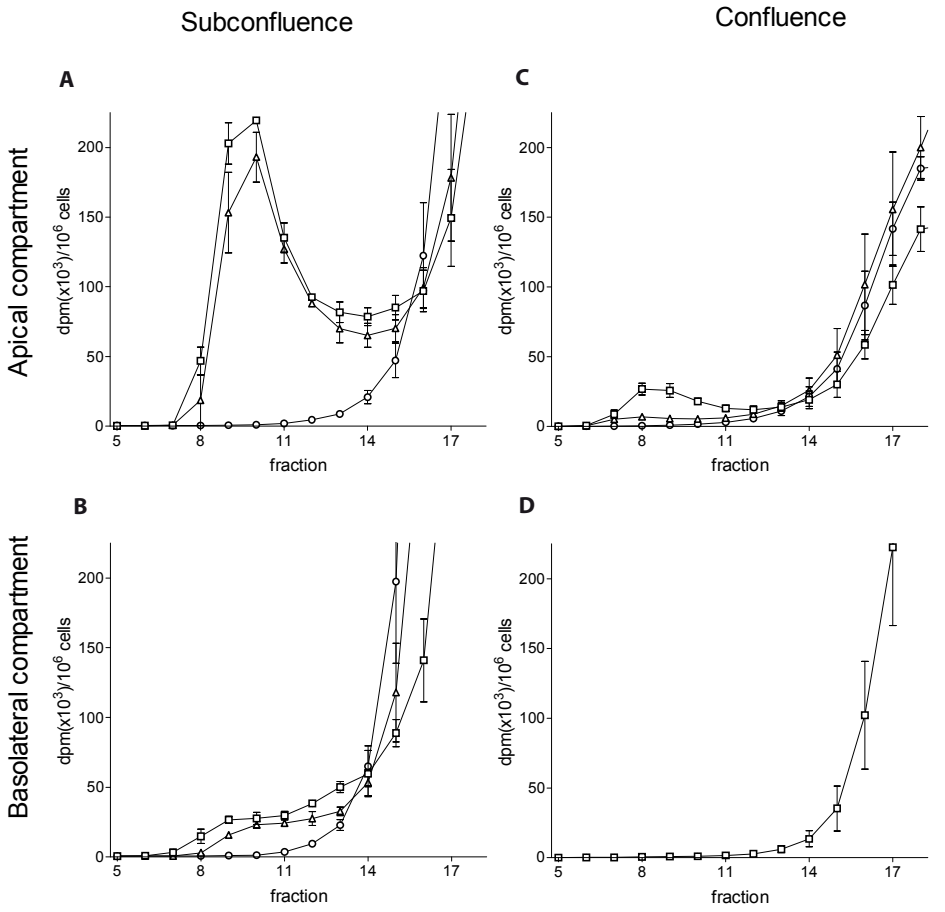

Figure 3.2

Production of high Mr glycoconjugates by Madin-Darby canine kidney strain I (MDCK-I) cells cultured on permeable supports in a two-compartment culture system, and analyzed by metabolic labeling studies using [3H] glucosamine in combination with specific enzyme digestion and Sephacryl S-1000 size exclusion chromatography. Proliferating cells in subconfluent cultures deliver relatively large amounts of high M_r [^{3}H] glucosamine-labeled glycoconjugates into the apical compartment of which the far majority is hyaluronidase-digestible (A), whereas at this point in epithelial development much smaller amounts of hyaluronidase-digestible macromolecules are delivered into the basal medium (B). Growth-inhibited cells in confluent cultures deliver only samll amounts of hyaluronidase- and papain-digestible macromolecules into the apical compartment (C), while confluent monolayers no longer secrete molecules $>10^6$ Da into the basal compartment (D). Symbols are: (□), untreated; (△), papain-treated; (○), papain pretreated + hyaluronidase treated.

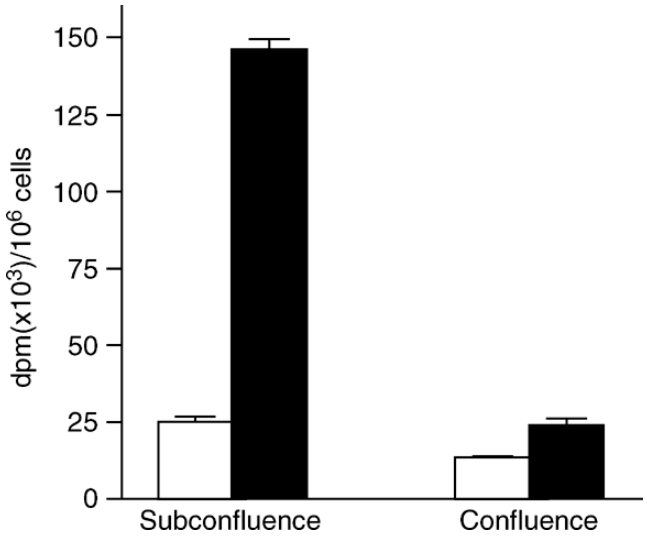

Figure 3.3

Streptomyces hyaluronidase (■)-cleavable [3H] glucosamine incorporated in cell surface gylcoconjugates of subconfluent and confluent Madin-Darby canine kidney strain I (MDCK-I) cells grown on culture system compared to spontaneous release of [3H]-glucosamine in DMEM alone.

Streptomyces hyaluronidase. Thus, proliferating MDCK-I cells produced high M_r glycoconjugates of which the majority consisted of hyaluronan. Considering their size, most papain-sensitive glycoconjugates consist of proteoglycans. Although relatively small differences were observed in the secretion of papain-sensitive molecules (ratio apical basal medium 2:1), most hyaluronidase-digestable glycoconjugates were delivered almost exclusively into the apical compartment (ratio apical basal medium 7:1) (figure 3.2).

To assess the amount of cell surface-associated [^{3}H] GlcNAc-labeled glycoconjugates metabolically labeled MDCK-I cells in subconfluent and in confluent cultures were treated with hyaluronidase. Hyaluronidase was more effective in releasing radiolabel from subconfluent cultures (figure 3.3).

Quantitation of hyaluronan production

An enzyme-linked HABP assay was applied to measure the amount of hyaluronan in the apical and basal medium bathing MDCK-I cells during the healing of wounds made in confluent monolayers. Confluent monolayers of MDCK-I cells synthesized relatively low amounts of hyaluronan (figure 3.4). After scrape-damaging intact monolayers the production of hyaluronan was strongly up-regulated. During regeneration, most of the de novo produced hyaluronan, was targeted to the apica l compartment (figure 3.4). The apical versus basal ratios however were lower than those in the metabolic labeling studies suggesting that a considerable part of hyaluronan measured with the quantitative assay method consisted of shorter hyaluronan

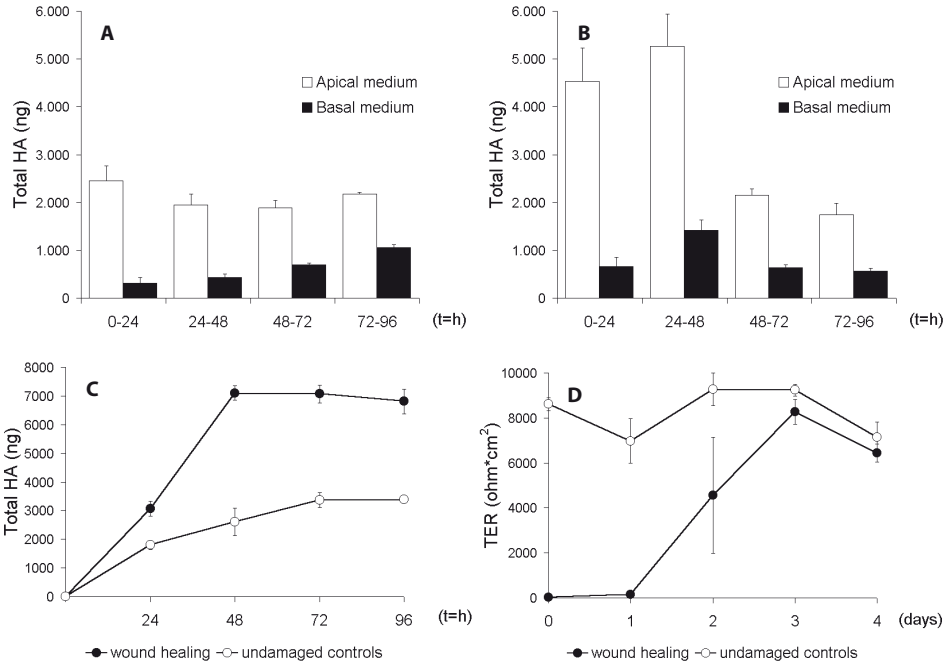

Figure 3.4

Quantitative determination of hyaluronan by an enzyme-linked binding protein assay in the apical and basal compartment of intact Madin-Darby canine kidney strain I (MDCK-I) monolayers (A) and during the healing of wounds made in confluent monolayers (B), grown on permeable supports in a two-compartment culture system. Hyaluronan was measured in daily replaced Dulbecco's modified Eage's medium (DMEM) supplemented with 10% fetal calf serum (FCS) on 1, 2, 3, and 4 days postconfluence or postinjury (A and B) and without replacing the culture medium (C). Wound closure was monitored by transepithelial electrical resistance (TER) measurements (D).

chains. The synthesis of hyaluronan normalized to the levels in undamaged controls as soon as the wound were healed and monolayers reestablished high TERs.

Four days after the initiation of primary cultures of human renal tubular cells, culture medium of the apical and basal compartment was collected every 48 hours and replaced with fresh medium. The highest amount of hyaluronan was measured at subconfluence (i.e., the second time that medium was replaced) (figure 3.5). Also in primary cultures most synthesized hyaluronan was secreted into the apical compartment (figure 3.5).

RT-PCR studies

Expression of HAS1, HAS2, and HAS3 mRNA in subconfluent and confluent MDCK-I cultures was assessed with RT-PCR using specific primers designed to amplify species-conserved HAS1, HAS2, and HAS3 sequences. Primers were designed for POLR2A for the normalization of RNA levels. Using HAS2 primers, a prominent product of the expected size from both cDNA and genomic DNA was evident after 30 cycles of PCR (and not noted in the negative control).

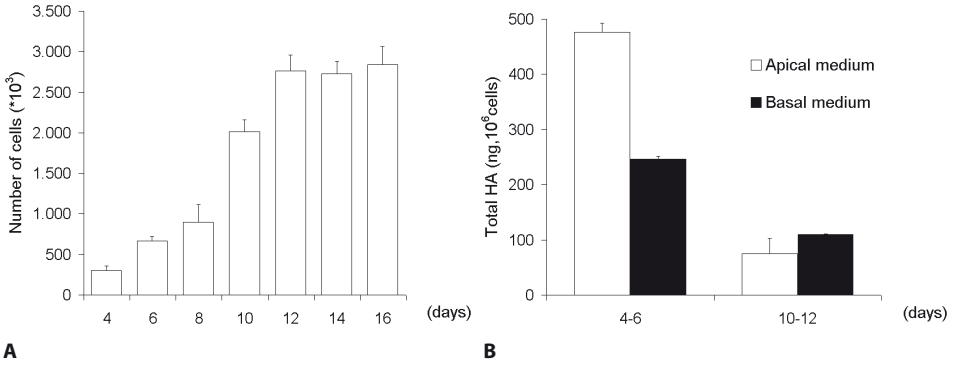

Figure 3.5

Determination of hyaluronan by enzyme-linked binding protein assay (B) in the apical and basal compartment of primary cultures of human renal tubular cells during their growth to confluence (A) on permeable supports in a two-compartment culture system.

Sequence analysis of the product confirmed the expression of HAS2 mRNA (figure 3.6). According to these results, HAS2 is clearly up-regulated in subconfluent MDCK-I cells. The designed HAS3 primers were also able to ampl ify the expected DNA fragment sizes from cDNA and genomic DNA, but this time 35 cycles of PCR were required to detect an appropriate amount of DNA. HAS3 sequences were detected in these DNA fragments by sequence analysis. According to these data there is a slight downregulation of HAS3 in subconfluent MDCK-I cells (figure 3.6). Both sequences of the HAS2 and HAS3 PCR products are shown aligned with the published human sequence in figure 3.7. The homology of the canine HAS2 and HAS3 sequences to published sequences from other species (including human, rat, mouse, and bull) is >92%. HAS1-specific primers were unable to amplify any HAS1 product from MDCK-I cDNA but did amplify HAS1 from genomic DNA, although this fragment contains an intron sequence.

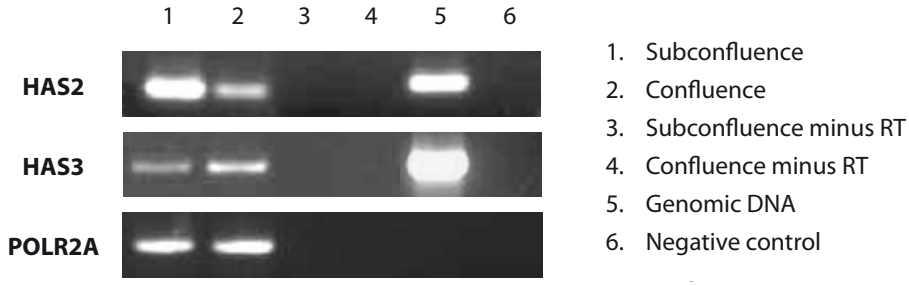

Figure 3.6

Hyaluronan synthase (HAS) mRNA expression in subconfluent and confluent cultures of Madin-Darby canine kidney strain I (MDCK-I) cells. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed using HAS2 and HAS3 gene-specific primers as described in the **Methods** section. Gene-specific primers for the large subunit of RNA polymerase II (POLR2A) were used for the normalization of RNA levels.

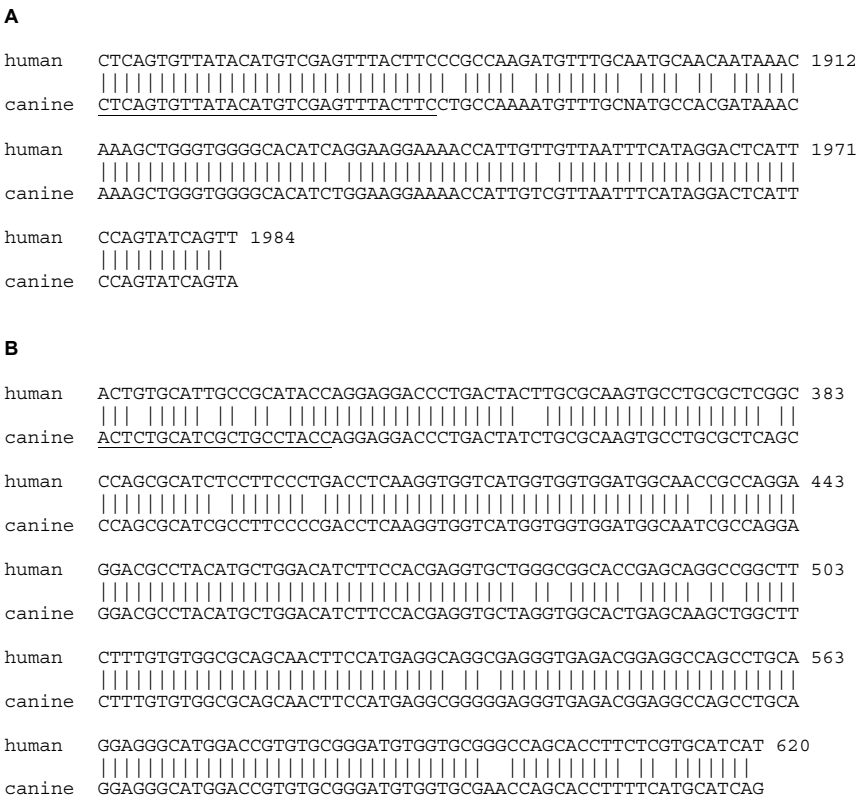

Figure 3.7

Sequences of polymerase chain reaction (PCR) products from hyaluronan synthase 2 (HAS2) and HAS3 reverse transcriptase (RT)-PCR (canine) are shown aligned with the human sequences. The canine HAS1 PCR product was not detectable and could not be sequenced. Underlined segments indicate the sites of primer annealing. (A) Sequence of PCR product for HAS2. (B) Sequence of PCR product from HAS3.

Also an amplification of HAS1 product was obtained with mouse and human cDNA. All of the DNA fragments had the expected sizes. Sequence analysis of the DNA fragments of MDCK-I genomic DNA and mo use cDNA confirmed the presence of HAS1 DNA sequences. From these findings it was concluded that HAS1 is hardly expressed in MDCK-I cells (not shown).

CLSM

Two days postseeding, when monolayers still have low TERs (~200 Ω cm^2), bHABP bound to the entire apical cell surface, but not at basolateral domains. This apical staining was less prominent after 4 days when cultures approached confluence and were actively assembling tight junctions (TER ~3000 Ω cm^2). After 6 days the TERs were maximal high (>5000 Ω cm^2) and bHABP did not bind to the cell surface (figure 3.8).

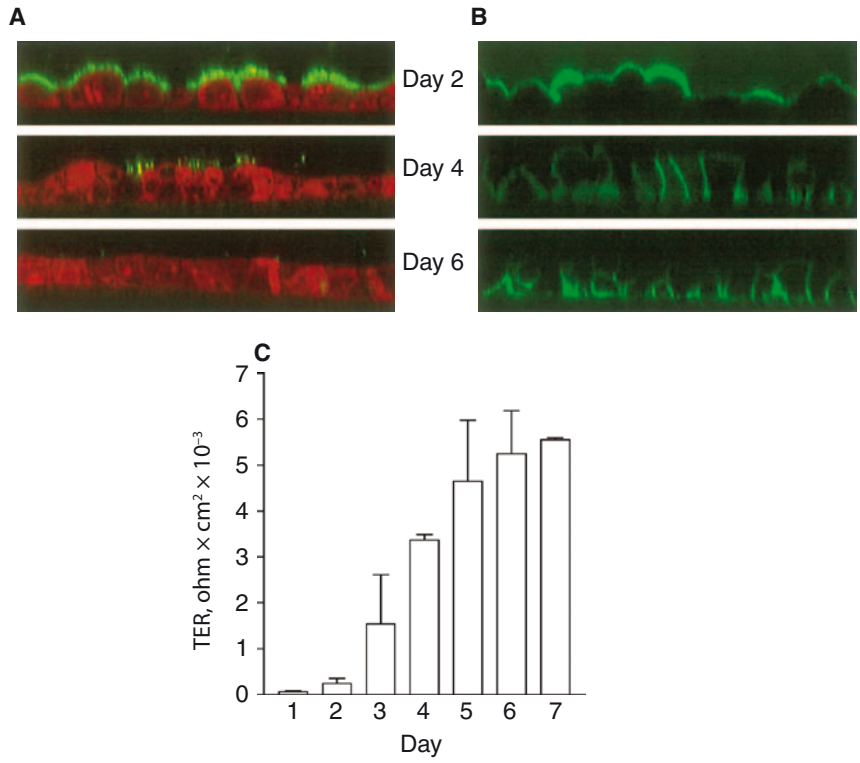

Figure 3.8

Confocal microscopy images of the membrane localization of hyaluronan [biotinylated hyaluronan binding protein (bHABP)-fluorescein isothiocyanate (FITC) + propidium iodide] (A) and C44 (IM7-FITC) (B) during the development of Madin-Darby canine kidney strain I (MDCK-I) cultures into confluent monolayers with functional tight junctions (C). TER is transepithelial electrical resistance. Color image: see appendix page 148.

Confluent MDCK-I monolayers did not express hyaluronan. After scrape injury, however, migrating cells at the border of the wound became flattened and again expressed hyaluronan at their membrane (figure 3.9, A and B), while cells in undamaged areas did not express hyaluronan. After the wounds were healed and tight junctions were reestablished hyaluronan again disappeared from the cell surface.

Two days postseeding CD44 was expressed exclusively at the apical surface of cells (TERs ~200 Ω cm^2). Four days postseeding (TERs ~3000 Ω cm^2) CD44 was predominantly found in the lateral space between the cells. Six days postseeding (maximal high TERs >5000 Ω cm^2), the membrane expression of CD44 was restricted to the basal side of the monolayers. Thus, in MDCK-I cells during their growth to confluence CD44 expression is polarized, at first at the apical surface, subsequently at lateral sites, and finally at the basal plasma membrane when tight junction formation is fully established (figure 3.8). Double-staining of CD44 (red) and

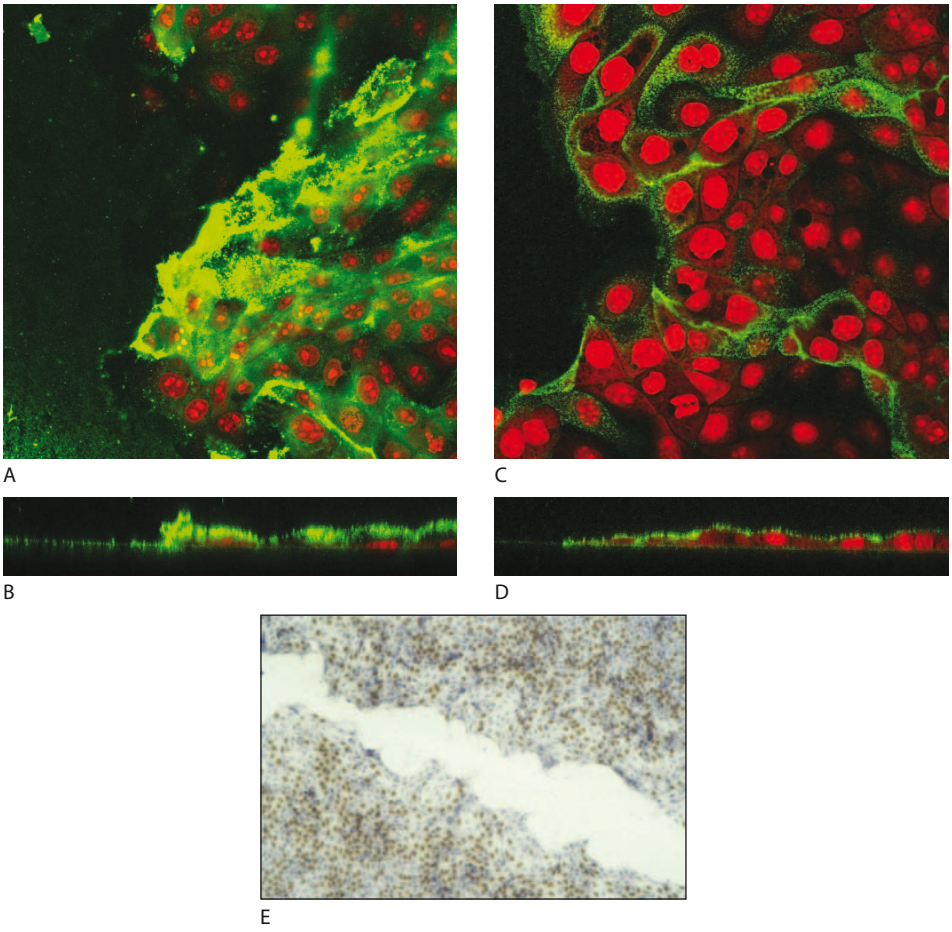

Figure 3.9

Confocal microscopic images of hyaluron [biotinylated hyaluron binding protein (bHABP)-fluorescein isothiocyanate (FITC)] (A and B) and CD44 (IM7-FITC) (C and D) expressed by flattened cells at the border of the wound. B and D are scans made perpendicular to the cultures (xz scans) showing flattened cells infiltrating the denuded areas. **(E) Light microscopic image of 5-bromo-2′-deoxyuridine (BrdU)-stained cells during wound healing showing few BrdU-positive cells at the leading edge of the wound and abundant staining in areas somewhat distant from the wound.** This BrdU staining indicates that the falttened cells at the border of the wound are migrating cells rather than proliferating cells. Color image: see appendix page 149.

hyaluronan (green) showed a clear colocalization at the apical surface of proliferating MDCK-I cells populating subconfluent cultures (figure 3.10).

In confluent monolayers, the expression of CD44 was restricted to basal membranes. After scrape injury, migrating cells at the border of the wounds began to express CD44 at their apical membrane (figure 3.9, C and D). After the wounds were healed and TERs were reestablished, CD44 expression was again selectively targeted to the basal membrane.

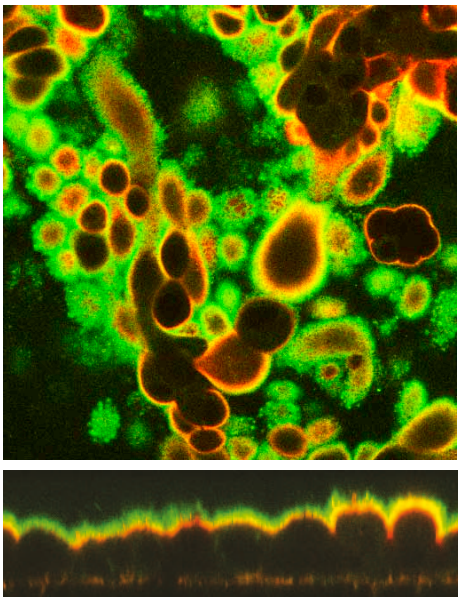

Figure 3.10

Confocal microscopy xy and xz images showing the colocalization of hyaluronan [biotinylated hyaluron binding protein (bHABP)-fluorescein isothiocyanate (FITC) (green) and its major cell surface receptor CD44 [IM7-TRITC] (red) at the apical plasma membrane of subconfluent cultures of Madin-Darby canine kidney strain I (MDCK-I) cells grown on a permeable support in a two-compartment culture system. Color image: see appendix page 150.

BrdU staining

To reveal if the cells at the border of a scrape wound are proliferating, 1 day postinjury mechanically damaged cultures were incubated with the proliferation marker BrdU. This study showed that the cells stained positively for BrdU somewhat distal from the wound, but that BrdU staining was hardly observed in the infiltrating flattened cells at the border of the wound, indicating that these are migrating rather than proliferating cells (figure 3.9 E).

Hyaluronan staining in human renal tissue

Acute tubular necrosis is a pathologic condition that is characterized by massive damage and regeneration of tubular epithelial cells, processes which are known to take place concurrently in the kidney [20]. Hyaluronan staining in renal tissue biopsy specimens not only showed an up-regulated expression in the cortex but also that regenerating tubular cells in vivo express hyaluronan at their apical membrane (figure 3.11).

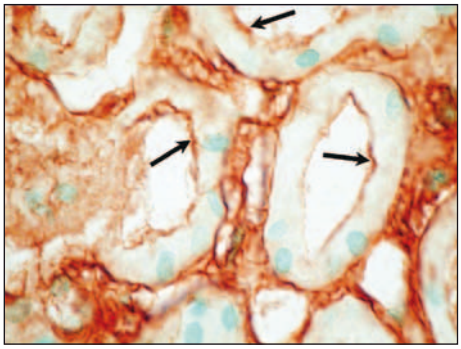

Figure 3.11

Hyaluronan staining in the cortex of a renal tissue biopsy specimen of a patient suffering from acute tubular necrosis, showing expression of hyaluronan at the luminal membrane of tubular cells and also in the cortical interstitium. Arrows indicate hyaluronan expressed at the luminal cell membrane (×1000 magnification). Color image: see appendix page 150.

DISCUSSION

This study shows that subconfluent cultures formed by MDCK-I cells and primary cultures of human renal tubu lar cells synthesize higher amounts of hyaluronan than confluent cultures. The increased production of hyaluronan by proliferating cells is accompanied by increased expression of HAS2 mRNA, while HAS3 is slightly down-regulated and HAS1 remains undetectable. The increased hyaluronan production is polarized since in both cell types, hyaluronan is secreted predominantly into the apical medium compartment. During wound healing, regenerating renal tubular cells also secreted increased amounts of hyaluronan into the apical compartment. Hyaluronan is expressed at the apical, but not the basolateral membrane of proliferating or regenerating cells, indicating that the membrane expression of hyaluronan is polarized as well. The apical expression of hyaluronan was invariably accompanied with the apical expression of CD44. At confluence or after wound healing, hyaluronan as well as CD44 is no longer detectable at the apical surface. Hyaluronan entirely disappeared, while CD44 was targe ted to basal domains of the plasma membrane.

To our knowledge there is only one other report dealing with the polarized secretion of hyaluronan by epithelial cells [21]. The secretion of hyaluronan by confluent retinal epithelial cells in primary culture grown on permeable supports is also highly polarized, with 82% to 95% of the hyaluronan delivered into the apical medium. Apparently, retinal pigment cells constitutively express and deliver hyaluronan from the apical surface to support the interphotoreceptor matrix of the eye [21]. The polarized expression and secretion of hyaluronan by renal tubular cells is *not* constitutive, but restricted to proliferating and regenerating cells, indicating that remodeling/regenerating renal tubular cells require hyaluronan at the luminal side of the epithelium, while terminally differentiated cells do not. From our study it is unclear whether the relatively small amount of hyaluronan in the basal compartment was secreted

from the basal membrane or derived from transepithelial leakage. The fact that hyaluronan did not accumulate under the monolayers and that hyaluronan staining was found at the apical cell surface and not between cells indicates that the porous growth substrate was no barrier for hyaluronan and that hyaluronan indeed is predominantly secreted from the apical membrane. Soon antibodies directed against HASs will be available which will elucidate the epithelial development-dependent subcellular localization of the various HAS proteins. Remarkably, the secretion/expression of hyaluronan and the expression of CD44 is polarized at times when the tight junctions are not yet fully assembled. Although many membrane proteins and lipids require functional tight junctions as a diffusion barrier in the plane of the lipid bilayer, for other membrane components cell-substratum and/or cell-cell contact is sufficient to induce and maintain epithelial cell surface segregation [22]. Apparently, tight junction formation is not an absolute requirement for HAS and CD44 to be targeted to the apical plasma membrane. The polarized distribution of CD44 by MDCK cells was demonstrated earlier, but in these studies CD44 did not entirely reach the basal membrane [23, 24]. Most likely, our cells obtained higher levels of terminal differentiation because the confluent monolayers were kept in culture for a longer period of time.

The interaction of hyaluronan with cell surface receptors such as CD44 and receptor for hyaluronan-mediated motility (RHAMM) activates intracellular signaling pathways during cell migration/proliferation [25]. Hyaluronan receptor binding also plays a role in anchoring hyaluronan coats to the cell surface [26]. Also in our study apical CD44 invariably colocalizes with hyaluronan, suggesting receptor-ligand interaction [27]. The interaction between CD44 and hyaluronan was studied earlier in proliferating SV40-transformed mouse cortical tubular (MCT) cells [28]. CD44 was randomly expressed over the membrane and the cells constitutively bound exogenously added hyaluronan. CD44-hyaluronan binding could be modulated with CD44 antibodies, suggesting that CD44 indeed served as a hyaluronan receptor. The polarized membrane distribution of CD44 was not further addressed and it was not reported if MCT cells were capable to synthesize and express endogenous hyaluronan.

In an impressive series of recent publications Philips et al [29] revealed several aspects of renal hyaluronan biology using immortalized human proximal tubule human kidney 2 (HK-2) cells and human primary cultures of proximal tubular cells. In agreement with our studies, scrape-damaging HK-2 monolayers led to an increased hyaluronan production, which in HK-2 cells appeared to be mediated by the extracellular signal-regulated kinase (ERK) pathway. HK-2 cell migration during wound healing was increased by exogenous hyaluronan. In human proximal tubulular cells, HAS2 mRNA expression and hyaluronan secretion could be stimulated with interleukin (IL)-1β and high levels of glucose, but not by transforming growth factor-β (TGF-β) or other growth factors. In this study, HAS2 mRNA expression was inducible, whereas HAS3 was consitutively expressed and HAS1 was undetectable. This HAS expression pattern is identical to that found by us in activated MDCK-I cells. The activated transcription of HAS2 in activated proximal tubular cells was coupled to the transcription factor nuclear factor-κB

(NF-κB) [30]. IL-1β and high glucose also influenced the functional behavior of cell surface CD44 leading to increased hyaluronan binding and uptake [31]. The hyaluronan production and expression by confluent HK-2 cells was also stimulated by bone morphogenic protein-7 (BMP-7). Among other things, this type of stimulation increased the amount of HAS2-mediated cell surface hyaluronan, decreased the expression of hyaluronidases and increased the binding of monocytes to the cell surface [32]. In HK-2 cells, the synthesis of extracellular matrix molecules collagen III and IV were activated with TGF-β, which could be blocked with CD44 antibodies, mitogen-activated protein (MAP) kinase inhibitors or hyaluronan [33]. CD44- hyaluronan also decreased TGF- β1–dependent proximal tubular cell functions [34]. Collectively, these results suggest that hyaluronan performs various functions during inflammatory renal disease states.

There are some important differences between the studies performed by Philips et al and our studies. HK-2 cells constitutively express hyaluronan, while our cells express hyaluronan only after some form of activation. Since hyaluronan is not expressed by renal tubular cells in healthy kidneys, this suggests that HK-2 cells are continuously activated, a phenomenon typical for immortalized cells or cancer cells. While we culture our cells on porous supports in a two compartment culture system, the HK-2 cells are cultured on conventional solid growth substrates. This makes it difficult to appreciate if the various agonists and extracellular and pericellular matrix molecules exerted their effects at the apical or basolateral plasma membrane. In agreement with activated HK-2 cells, stimulation of MDCK-I cells leads to an increased production of (high M_r) hyaluronan and increased levels of HAS2 mRNA.

The expression of hyaluronan is up-regulated in the cortical interstitium during various inflammatory renal disease states [2, 13, 14]. It was only recently reported that the expression of hyaluronan in kidneys of ethylene glycol treated rats was not only increased in the renal interstitium, but that hyaluronan also appeared at the luminal surface of the epithelial cells lining the renal tubules [2]. This expression of hyaluronan by renal tubular cells could be overlooked in the past due to the overwhelming hyaluronan expression in the interstitium, or by the removal of cell surface hyaluronan by fixation and washing procedures [35]. Here, it is shown that in *mildly fixed* and *washed* renal biopsy specimens of patients with acute tubular necrosis, the expression of hyaluronan is not only up-regulated in the cortical interstitium, but also at the luminal surface of renal tubular cells (figure 3.10). Since acute tubular necrosis is accompanied by tubular damage and regeneration [20], this suggests that also in the human kidney, hyaluronan is expressed by regenerating renal tubular cells. The source of hyaluronan in the cortical interstitium remains unclear. Although we cannot entirely exclude some apical-to-basal hyaluronan leakage our results suggest that renal tubular cells are not the main source for hyaluronan in the renal interstitium. Perhaps interstitial fibroblasts account for most of the newly formed hyaluronan during acute renal inflammation. The engagement of CD44 and hyaluronan in the renal tubules may communicate with and thereby activate interstitial cells through signal transduction pathways, or interstitial fibroblasts are triggered by low M_r

hyaluronan secreted from the basolateral membrane of stress-activated renal tubular cells. It has been demonstrated that normal rat cortical and medullary fibroblasts in culture are capable to synthesize hyaluronan [36]. Another possibility is that during inflammation tubular epithelial cells become fibroblasts via epithelial to mesenchymal transdifferentiation [37]. This is entirely speculative, however, and additional studies are required using appropriate model systems to investigate this issue more in detail.

Our interest in hyaluronan production and expression by renal tubular cells came from investigations into the possible involvement of renal tubular cells in the pathophysiology of nephrolithiasis. Renal stones can only form when crystals somehow are retained in the kidney. It was found that (calcium oxalate) crystals bind to renal tubular cells in subconfluent, but not in confluent cultures [1, 4]. Hyaluronan was identified as crystal binding molecule [1]. Recently, we studied this concept in rats treated with ethylene glycol, a nephrotoxic and (calcium oxalate) crystal-inducing agent [2]. Although ethylene glycol almost immediately resulted in crystalluria, several days were required for the renal tubular epithelium to become damaged/regenerate and crystals to be retained. In agreement with our in vitro data, crystals deposited in the tubules were closely associated with regenerating cells that expressed hyaluronan and CD44 at their luminal surface. Studies are underway in which we will explore possibilities to inhibit the proliferation/stress-induced expression of hyaluronan by renal tubular cells in culture and in rats, aimed at limiting or preventing accumulation of crystals in the kidney.

CONCLUSION

The results of the present study show that hyaluronan biosynthesis is activated during proliferation and in response to mechanical injury and that the membrane distribution of hyaluronan and CD44 is highly polarized despite the absence of functional tight junctions. The targeted delivery of hyaluronan to the apical medium compartment suggests that hyaluronan supports cell growth and remodeling in the renal tubules. These data further support the concept that mitogen/stress-induced hyaluronan deposition in the renal tubules increases the risk for crystal retention.

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Chapter 4

Preconditioning of the Distal Tubular Epithelium of the Human Kidney Precedes Nephrocalcinosis

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ABSTRACT

Background

Preterm neonates and renal transplant patients frequently develop nephrocalcinosis. Experimental studies revealed that crystal retention in the distal nephron, a process that may lead to nephrocalcinosis, is limited to proliferating/regenerating tubular cells expressing hyaluronan and osteopontin at their luminal surface. Fetal and transplant kidneys contain proliferating and/or regenerating cells since nephrogenesis is not completed until 36 weeks of gestation, while ischemia and nephrotoxic immunosuppressants may lead to injury and repair in renal transplants. This prompted us to investigate the expression of hyaluronan and osteopontin and to correlate this to the appearance of tubular calcifications both in fetal/preterm and transplanted kidneys.

Methods

Sections of fetal/preterm kidneys and protocol biopsies of transplanted kidneys (12 and 24 weeks posttransplantation from the same patients) were stained for osteopontin, hyaluronan, and calcifications (von Kossa).

Results

Hyaluronan and osteopontin were expressed at the luminal surface of the epithelial cells lining the distal tubules of all fetal kidneys at birth and in all kidney graft protocol biopsies 12 and 24 weeks posttransplantation. In 7 out of 18 surviving (at least 4 days) preterm neonates crystal retention developed. In renal allografts a striking increase (from 2/10 to 6/10) in tubular crystal retention between 12 and 24 weeks posttransplantation was observed. In addition, crystals were selectively retained in distal renal tubules containing cells with hyaluronan and osteopontin at their luminal surface.

Conclusion

The results of this study show that luminal expression of hyaluronan and osteopontin preceded renal distal tubular retention of crystals in preterm neonates and renal transplant patients. We propose that the presence of this crystal binding phenotype may play a general role in renal calcification processes.

INTRODUCTION

Nephrocalcinosis, recently defined [1] as increased calcium content of the kidney, is observed in specific patient populations, including renal transplant patients and preterm neonates. Renal calcifications in preterm neonates, detected by roentgenograms or ultrasonography, were first described in 1982 [2]. Varying incidences (17% to 64%) have been reported since then [3]. Nephrocalcinosis in preterm neonates is the cause of later complications such as urinary tract obstruction and infection resulting in decreased renal function on the longer term [4, 5]. Increases in urinary stone salts saturation because of the calcium and vitaminD–rich diet, low urinary citrate concentration, and long-term furosemide treatment are all factors that have been previously considered to explain the enhanced crystal formation in tubules of preterms [3, 6, 7]. Recently, intratubular microcalcifications have been described in 13% of transplanted kidneys 26 weeks posttransplantation [8] and in 42% 1 year posttransplantation [9]. Importantly, these intratubular calcifications have been associated with poor long-term graft survival [10]. As in other individuals, a protein-rich diet may result in high urinary calcium and oxalate concentrations and hence intratubular crystallization [6, 11].

The exact mechanisms leading to nephrocalcinosis in these two patient groups, however, are unclear. The localization of renal calcifications in preterm neonates is unknown, while in transplanted kidneys nephrocalcinosis clearly presents as intratubular microcalcifications [9, 10]. Recent investigation has shown involvement of hyaluronan, osteopontin, CD44, nucleolin-related-protein, annexin II, and Tamm-Horsfal-protein in the processes of tubular crystal formation and/or retention [12-16]. An important step in the development of nephrocalcinosis is the retention/trapping of passing crystals to the luminal surface of cells in the distal nephron. Our own studies, both in vitro and in vivo, have demonstrated that calcium crystals do not adhere to an intact epithelium but solely to a proliferating/regenerating epithelium with dedifferentiated cells expressing hyaluronan, osteopontin, and their receptor CD44 at the luminal surface [12, 16]. Luminal hyaluronan and osteopontin expression is absent/sparse in normal renal epithelium but extensively up-regulated following renal damage and the subsequent proliferation/regeneration [12, 17-22]. During nephrogenesis, hyaluronan may play a role during branching morphogenesis [23] and osteopontin has been proposed to be involved in tubulogenesis [24]. In proliferating renal tubular cell cultures, hyaluronan is identified as a major crystal-binding molecule since hyaluronidase treatment diminished crystal adhesion [16, 25]. Although controversial, osteopontin is also considered to be a crystal-binding molecule, it is found associated with hyaluronan and their mutual receptor CD44 on a crystal-binding epithelium, and therefore may play a role in crystal retention [12, 16, 26, 27].

In an attempt to extend our experimental results showing the important role of the epithelial phenotype in crystal retention, in a human clinical setting, we investigated two patient populations presenting with a high incidence of nephrocalcinosis (i.e., preterm born infants and renal transplant patients) [3, 9]. Kidneys of both populations contain proliferating/regenerating

nephrons since nephrogenesis is not completed until 36 weeks of gestation and posttransplant kidneys are damaged and regenerate because of ischemia, nephrotoxic drugs (e.g., cyclosporine) and chronic graft rejection.

METHODS

Patients

Renal tissue from 52 human fetuses with gestational age 15 to 40 weeks was available (arch ive Antwerp University Hospital, Professor Dr. Van Marck). These preterm neonates died soon after birth (<1 day) and expression of hyaluronan and osteopontin during nephrogenesis could be studied, in the absence of any influence from diet and fluid intake. Consequently, these kidneys did not contain crystals. Therefore, tubular crystal localization and colocalization with luminal osteopontin and hyaluronan was studied in 18 preterm neonates who lived for at least 4 days (Dr. R. De Krijger, Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands) and hence received a diet known to promote urinary crystallization for at least 4 days. Two kidney biopsies, 12 and 24 weeks posttransplantation, were available from 10 transplant-patients (Transplant Center, Medical School Hannover, Hannover, Germany). The protocol biopsy program at the Hannover Medical School is part of the routine medical care following transplantation and had been approved by the local ethi cal committee. Patients were informed well about the program before transplantation. Written consent was obtained and participation in the program was not a requirement for transplantation. The use of archive material for research is permitted by local ethical committees on the understanding of anonymity.

Hyaluronan and osteopontin staining

For hyaluronan staining, sections were incubated 20 minutes with 0.1% bovine serum albumin (BSA), 4 hours with biotinylated hyaluronan-binding-protein (Seikagaku, Tokyo, Japan) (1/10000), 1 hour with avidin-biotinylated-peroxidase-complex (Vector Laboratories, Burlingame, CA, USA) and finally with peroxidase substrate [diaminobenzidine (DAB)]. Nuclei were methyl green counterstained.

Osteopontin was stained with LF123, a polyclonal rabbit-antihuman osteopontin antibody (provided by Dr. Fisher, National Institutes of Health, Bethesda, MD, USA). Sections were incubated 20 minutes with normal goat serum, overnight with LF123, and subsequently 30 minutes with biotinylated goat-antirabbit antibody (Vector Laboratories). Avidin-biotinylated-peroxidase-complex (Vector Laboratories) and peroxidase substrate [DAB or aminoethyl carbazole (AEC)] were used for detection. Nuclei were methyl green counterstained.

von Kossa staining

Calcium deposits were visualized by von Kossa staining. Sections were incubated 45 minutes in 5% silver nitrate, 3 minutes with 1% pyrogallic acid, and 1 minute fixed in 5% sodium thiosulfate. Sections were hematoxylin and eosin counterstained.

Tubular crystals

Renal tissue from preterm neonates that lived at least 4 days and from von Kossa–positive transplant patients was used. Three sequential sections, stained for von Kossa, hyaluronan and osteopontin were used to evaluate the expression pattern of hyaluronan and osteopontin in tubules with von Kossa–positive deposits.

RESULTS

Hyaluronan and osteopontin

Developing kidneys

Hyaluronan expression was present in the interstitium of fetal kidneys during the whole period of nephrogenesis studied (week 15 to 40). However, the amount of interstitial hyaluronon diminished along with the decrease in interstitial volume resulting from tubular growth. Besides the interstitium, hyaluronan was found at the luminal surface of the tubules. This tubular localization was observed, both in cortex and medulla, from week 15 to 40. During later nephrogenesis (from around week 27), luminal hyaluronan was especially evident in newly formed, immature tubules of the medulla and outer layer of the cortex. Tubular osteopontin expression was present during nephrogenesis (week 15 to 40) both in cortex and medulla. Between week 15 and 21, however, expression was minimal and only intracellular. Luminal osteopontin expression was clearly present from week 24 on and persisted until week 40. Since it is not possible to allow proximal and distal nephron identification in immature kidneys, hyaluronan and osteopontin expression could not be attributed to specific parts of the nephron (figure 4.1, A to F).

Transplanted kidneys

Twelve and 24 weeks posttransplantation all biopsies showed the presence of hyaluronan and osteopontin at the luminal surface of distal tubules (figure 4.1, G and H).

Tubular crystals

Developing kidneys

Seven out of 18 von Kossa–stained sections from preterm neonates that lived for at least 4 days showed intratubular calcium-containing inclusions in distal tubules. This was in contrast

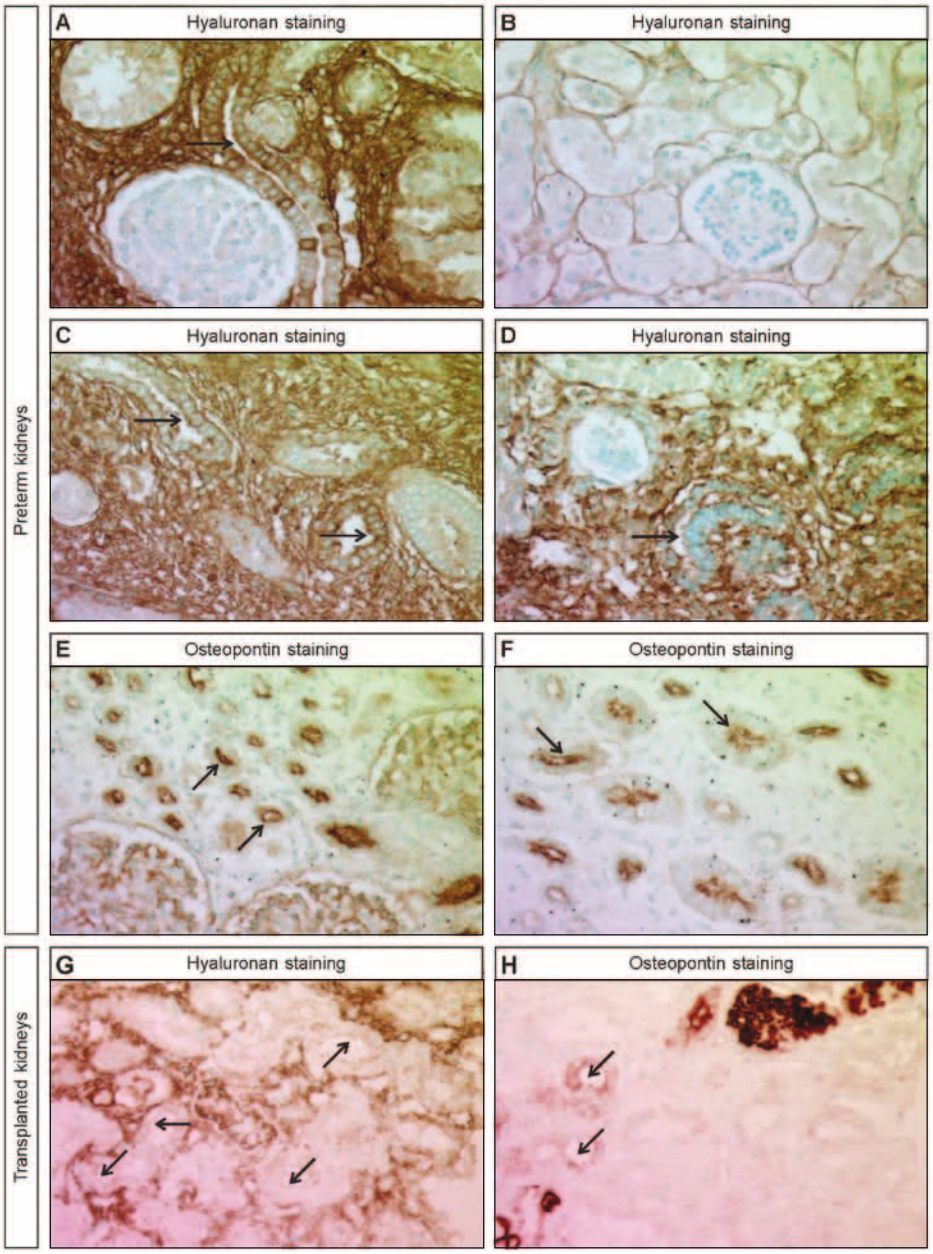

Figure 4.1

Hyaluronan and osteopontin staining on fetal (A to F) and transplanted (G and H) kidneys. Fetal kidneys of 18 weeks show widespread interstitial hyaluron staining in both cortex (A) and medulla (C), while at 27 weeks this staining becomes much less extensive together with the cortical interstial volume (B). Luminal staining in the tubules is present in cortex and medulla (A and C, arrows). At 28 weeks hyaluronon is mainly present in the immature, developing tubules of the outer cortex (D). Osteopontin staining is clearly present in many tubules of both cortex (E) and medulla (F) of a 27-week-old fetus (arrows). Both hyaluronon (G) and osteopontin (H) are expressed at the luminal surface (arrows) of the tubules in a kidney 24 weeks after transplantation (200×). Color image: see appendix page 151.

to sections from preterm neonates who lived less than 1 day since none of those showed any von Kossa–positive staining (figure 4.2). By means of von Kossa, hyaluronan-stained and osteopontin-stained serial sections, it was shown that crystal containing tubules also expressed luminal hyaluronan or osteopontin. Interstitial calcifications were not present.

Transplanted kidneys

Analyzing biopsies, obtained in the same patients (N = 10), 12 and 24 weeks posttransplantation, luminal expression of hyaluronan and osteopontin was observed to precede the retention of crystals in the tubules (figure 4.2 and 4.3). Indeed, 12 weeks posttransplantation there was extensive hyaluronan and osteopontin staining at the luminal membrane of distal tubules in all biopsies, whereas only two out of ten patients showed tubular crystals (von Kossa–positive tubules). At 24 weeks after transplantation, however, in six biopsies out of ten, calcium containing crystals were found attached to distal tubular cells expressing hyaluronan and osteopontin. As in preterm neonates, in the transplanted kidneys interstitial calcifications were never observed, in the transplanted kidneys.

DISCUSSION

The extensive expression of hyaluronan and osteopontin during nephrogenesis is in agreement with the important role of these molecules described during kidney development [23, 24], while in transplanted kidneys the up-regulated expression probably occurs in response to ischemic renal damage and cyclosporine toxicity [17, 21, 28-30].

It can be concluded from this study that nephrocalcinosis in preterm neonates, like in transplanted kidneys, arises from intratubular calcifications. Furthermore, this study corroborates our observation in ethylene glycol (induces both crystalluria and tubular damage)–treated rats that crystal retention is associated with tubular regeneration and luminal expression of hyaluronan and osteopontin [12]. From these rat experiments, however, it could not be concluded whether the retained crystals themselves induced epithelial damage/regeneration and luminal expression of hyaluronan and osteopontin, or whether crystals were retained because of an already existing (ethylene glycol-metabolites induced [31-33]) tubular damage/regeneration and hence hyaluronan and osteopontin up-regulation. The current study shows that luminal hyaluronan and osteopontin expression in distal tubules precedes crystal retention. Hyaluronan and osteopontin are expressed in all fetal kidneys in view of their immaturity, which may result in nephrocalcinosis after preterm birth because of subsequent crystalluria resulting from diet and/or medication. In transplanted patients, ischemia/reperfusion damage during transplantations followed by the exposure to nephrotoxic drugs and on the longer-term chronic allograft rejection may be responsible for acute and sustained expression of hyaluronan and osteopontin, respectively. Exposing these kidneys to a protein-rich/

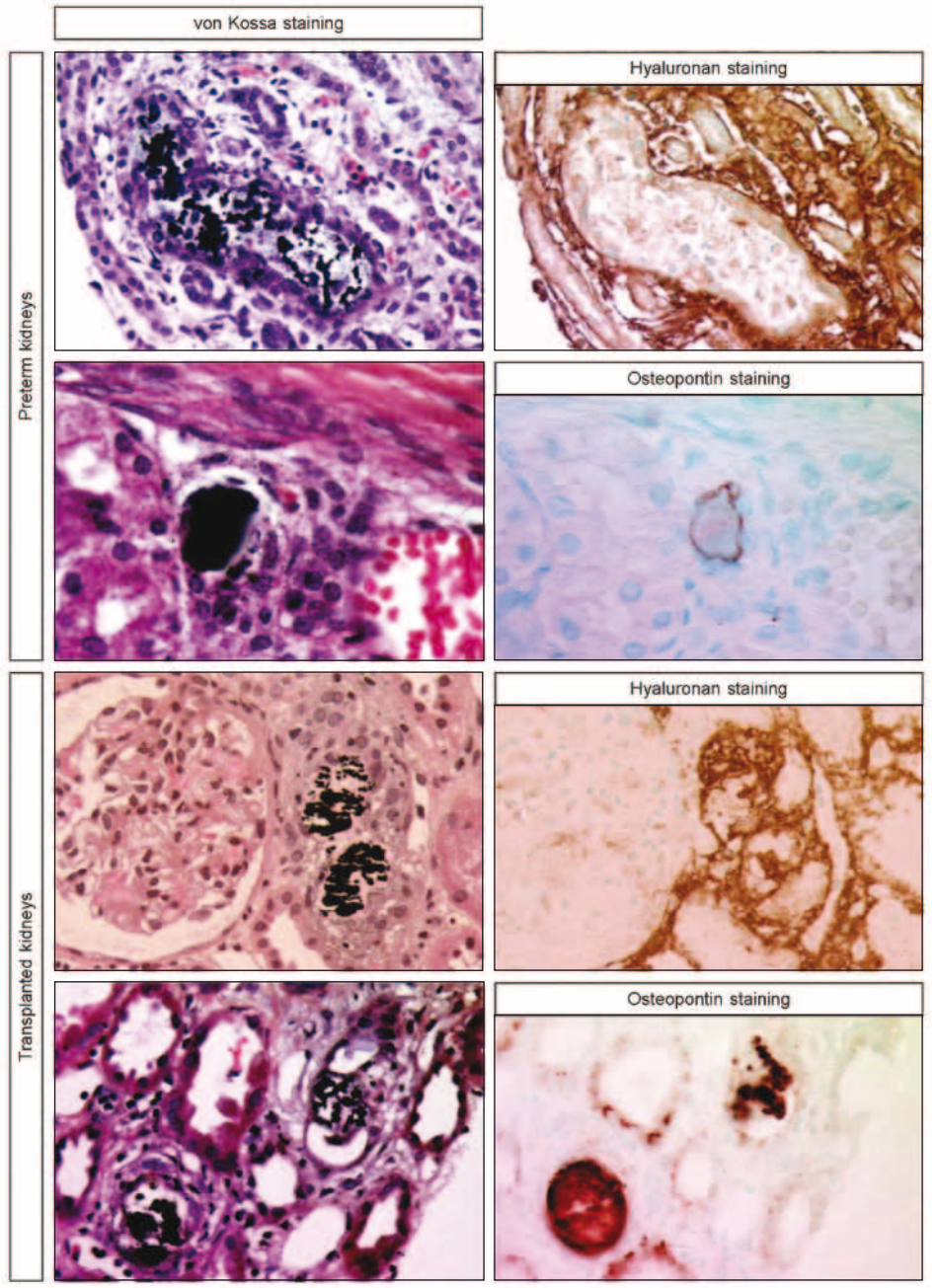

Figure 4.2

Serial sections from preterm and transplanted kidneys stained for von Kossa and hyaluronan or osteopontin. The von Kossa–positive tubules are clearly positive for hyaluronan or osteopontin (320×, except second row 640×). Color image: see appendix page 152.

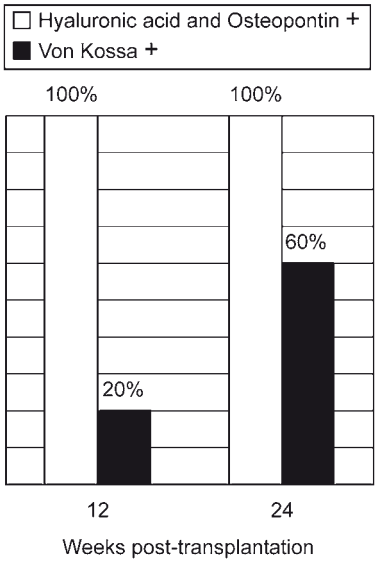

Figure 4.3

Twelve weeks posttransplantation hyaluronan and osteopontin were extensively present at the luminal membrane of distal tubules in all biopsies, whereas only two patients showed tubular crystals (von Kossa–positive tubules). At 24 weeks after transplantation, however, in six biopsies calcium crystals were found attached to distal tubular cells expressing hyaluronan and osteopontin.

crystalluria-inducing diet can result in nephrocalcinosis. This is in agreement with the recent study of Pinheiro et al [10] who associated acute tubular necrosis after transplantation with a predisposition to the deposition of tubular crystals.

CONCLUSION

Expression of crystal-binding molecules (hyaluronan and osteopontin) at the luminal surface of the proliferating/regenerating distal tubular cells precedes crystal retention to the distal nephron epithelium, the first step of nephrocalcinosis.

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Chapter 5

Cyclooxygenase-2 inhibitors decrease hyaluronan-dependent calcium oxalate crystal binding to regenerating renal tubular cells

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ABSTRACT

Background

Previously, we reported *in vitro* and *in vivo* studies indicating that expression of the glycosaminoglycan hyaluronan at the luminal cell membrane of regenerating tubular cells leads to nephrocalcinosis (retention of calcium oxalate crystals inside the tubular lumen of the nephron). Here, we investigated in cell culture if this process can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs).

Methods

[^{14}C] Calcium oxalate monohydrate (COM) crystal binding was studied in scrape-damaged monolayers of MDCK-I cells, a cell line that resembles the renal collecting duct. During the repair process cells were treated with the non-selective cyclooxygenase (COX) inhibitor indomethacin (1-100 μM) or COX-2 selective inhibitors nimesulide and meloxicam (50-100 μM). An enzyme-linked immunoassay and an enzyme-linked hyaluronan-binding assay were used to measure in culture medium the amount of prostaglandin E_2 and hyaluronan production, respectively.

Results

Transepithelial electrical resistance (TER) measurements demonstrated that wound healing required approximately four days to restore high TERs, which was slightly inhibited by the highest concentration of indomethacin and nimesulide, and not by meloxicam. Regenerating cells at the borders of the wound expressed hyaluronan at their apical cell membrane, secreted increased amounts of PGE_2 and high molecular weight hyaluronan, and bound more COM crystals. The highest peak level of PGE_2 secretion occurred one day after scrape injury, and of hyaluronan two days after scrape injury. The highest level of COM crystal binding to hyaluronan-expressing regenerating tubular cells occurred two days post-injury. NSAIDs concentration-dependently inhibited the secretion of PGE_2 and hyaluronan, the cell surface expression of hyaluronan and hyaluronan-dependent COM crystal binding.

Conclusion

Collectively, these results indicate that the suppression of PGE_2 by cyclooxygenase inhibitors leads to a decrease in the production and expression of hyaluronan by regenerating MDCK-I cells. Hyaluronan-dependent crystal binding to regenerating MDCK-I cells is COX-2 mediated and can be inhibited by NSAIDs.

INTRODUCTION

Nephrocalcinosis and nephrolithiasis results from the retention of crystals in the kidney [1-3]. Previously, we identified hyaluronan (HA) as major binding molecule for calcium oxalate monohydrate (COM) crystals at the apical surface of mitogen/stress activated renal tubular cells in culture [4, 5]. Also *in vivo* in the kidneys of ethylene glycol-treated rats [6], preterm neonates and kidney transplantation patients [7], crystals were selectively found attached to the luminal surface of HA-expressing tubular cells.

Mitogen/stress conditions activate various cellular signal transduction systems including the eicosanoid pathway. After the release of arachidonic acid (AA) from membrane phospholipids by cytosolic phospholipase A_2, AA is converted by cyclooxygenase (COX) isoenzymes (COX-1 and COX-2) to eicosanoids such as prostaglandin E_2 (PGE_2). PGE_2 is the most prevalent AA metabolite in the kidney particularly in the collecting ducts [8]. Whereas COX-1 is constitutively expressed in most regions of the kidney, COX-2 is considered the inducible isoform upregulated for instance during inflammation [9-12]. The effects of COX inhibitors, commercially known as non-steroidal anti-inflammatory drugs (NSAIDs), in the kidney reflects different points of the drug action along the nephron where COX isoforms are expressed [13].

In many cell systems HA synthesis is linked to the eicosanoid pathway. Little is known about HA biosynthesis by renal tubular cells. PGE_2 stimulates hyaluronan synthase (HAS) enzyme activity in rabbit pericardial cells [14], lung fibroblasts [15] and rat mesangial cells [16]. Low molecular weight HA activates COX in glomeruli [17] and renal tubular cells [18], suggesting that HA plays an active role in its own production. NSAIDs can have opposing effects on HA biosynthesis. Indomethacin and mefenamic acid inhibited the synthesis of HA in murine Swiss 3T3 fibroblasts [19], whereas celecoxib stimulated HA synthesis in cartilage [20] and SC-236 and rofecoxib in the iris [21]. In renal proximal tubular cells, indomethacin did not affect the production of HA that was elevated by high glucose or cytokines (IL-β) [22]. In the renal inner medulla, HA plays an important role in concentrating and diluting the urine [23]. During acute water loading the content of HA increases in the papillary interstitium, while the opposite occurs during water deprivation. This increase in HA during excess water intake antagonizes medullary interstitial water reabsorption by changing the matrix properties of the interstitium resulting in resistance to water flow [24, 25]. NSAIDs prevented the accumulation of HA in the medullary interstitium of water-loaded rats, indicating that the synthesis of HA in the renal medulla is mediated by eicosanoids [26].

In the present study, we investigated in MDCK-I cells in culture in a scrape-wound model the effect of the COX-1/COX-2 inhibitor indomethacin and the highly selective COX-2 inhibitors nimesulide and meloxicam on COM crystal binding, PGE_2 secretion, HA secretion,

HA expression, LDH release and recovery of transepithelial resistance (TER). The results indicate that crystal binding depends on COX-2 mediated cell surface HA expression which is significantly inhibited by non-steroidal anti-inflammatory drugs at doses that do not affect cell viability or the regeneration capacity of the cells to restore epithelial barrier integrity. The clinical implication of this finding should be evaluated in an experimental *in vivo* model of calcium oxalate crystal retention in the kidney.

METHODS

Cell culture

MDCK-I cells were kindly provided by Prof. G. van Meer (Laboratory for Cell Biology and Histology, Amsterdam Medical Center, The Netherlands [27]. Routinely, cells were grown in Dulbecco's modified Eagle's Medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Hyclone, Perbio Science, Etten-Leur, The Netherlands) and weekly replated. To maintain the cell line, 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 three times a week. For experiments cells are trypsinized and seeded at high density (1 x 10^6 cells) on polycarbonate permeable supports (Transwells), 24 mm diameter inserts with 4.7 cm^2 surface area provided with pores of 0.4 µm pore size (Corning Costar, Badhoevedorp, The Netherlands). Within 6-7 days the cells develop into confluent monolayers with a cell density of on average 4-6 x 10^6 cells. One day post-seeding there is already cell-cell contact and the cultures seem confluent under light microscopy. In time, however, the cells become much more closely packed and taller and begin to assemble tight junctions after 4-5 days. Tight junction formation is assessed by transepithelial electrical resistance (TER) measurements. Monolayers are considered confluent as soon as the highest TERs are reached. Routinely, PCR analyses are performed on DNA isolated from cell culture conditioned medium for the presence of mycoplasma. Cells used in this study were not contaminated with mycoplasma.

Wound healing studies

For the wound healing studies, cells were grown to confluence and subsequently injured by scraping off 100-150 mm^2 (approximately one third of the total insert area) with the tip of a sterile 10 ml tissue culture pipette as previously described [4, 28, 29]. The inserts were washed with PBS, fresh medium without fetal calf serum was added and the process of wound healing monitored morphologically by light microscopy and functionally by measuring TERs.

Non-steroidal anti-inflammatory drugs

The non-selective COX-1/COX-2 inhibitor indomethacin and the highly selective COX-2 inhibitors nimesulide (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and meloxicam

(Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) were used. Indomethacin and nimesulide were dissolved in ethanol (EtOH) and meloxicam in DMSO and added to healing cultures at concentrations ranging from 1-100 µM. Cultures that received EtOH or DMSO alone served as controls.

[^{14}C] calcium oxalate monohydrate crystal binding

Radiolabeled calcium oxalate monohydrate (COM) crystals were prepared as previously described [28]. Briefly, a solution of sodium oxalate was prepared by adding 1 ml 0.37 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 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 precipitation of radiolabeled COM crystals. 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 CaOx crystals/ml). At the indicated periods of time, cells were washed with PBS to be replaced by buffer A (140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 50 mM urea, pH 6.6, 310 to 320 mOsm/kg H_20) in the apical compartment and buffer B (124 mM NaCl, 25 mM $NaHCO_3$, 2 mM Na_2HPO_4, 5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 8.3 mM D-glucose, 4 mM L-alanine, 5 mM Na acetate, 6 mM urea and 10 mg/ml bovine serum albumin, pH 7.4, 310 to 320 mOsm/kg H_2O) in the basal compartment. Subsequently 50 µl of the radiolabeled crystal suspension (16 µg/cm^2) was added to the apical compartment and incubated for 60 minutes at 37°C. Filters were rinsed extensively to remove all non-adhered crystals, cut-out and transferred to a scintillation vial. After the addition of 0.5 ml 1 M perchloric acid radioactivity was quantified in a scintillation counter. The amount of cell-associated crystals is expressed in µg/cm^2.

Prostaglandin E_2 secretion

During wound healing, culture medium was collected from the apical fluid compartment at the indicated time points. PGE_2 was measured by a switch enzyme-linked immunoassay (EIA) Kit (Cayman Biochemicals, USA). 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 contained a blank-, a non-specific binding-, a maximum binding and a total activity control. Standard curve samples (50 µl), controls and experimental samples were added to the wells. Each well received 50 µl tracer except the total activity and the blank wells. The wells subsequently received 50 µl PGE_2 monoclonal antibody except the total activity well, the non-specific binding well, and the blank well. The plate was covered

with plastic film and incubated for 18 hours at 4°C. The wells are rinsed extensively with wash buffer after which 200 µL Ellman's Reagent was added to each well and 5 µl tracer to the total activity well. The plate was then covered with plastic film and developed in the dark on an orbital shaker. When the blank absorbance was between 0.3-0.8 units, the plate was read at 405-420 nm wavelengths.

Hyaluronan production

During wound healing, culture medium was collected from the apical fluid compartment at the indicated time points. The total amount of HA was determined in a sandwich protein-binding assay in microplates according to the manufactures instructions (Corgenix). The assay uses microwells coated with a highly specific HA binding protein (HABP) from bovine cartilage to capture HA and an enzyme-conjugated version of HABP to detect and measure HA in the samples. Reference HA solutions were used to calculate test results in ng/ml. In short, samples and HA reference solutions were incubated for 1 hour in HABP-coated wells, subsequently the wells were washed and HABP conjugated with horseradish peroxidase (HRP) was added to form complexes with bound HA. Following a second washing step, a chromogenic substrate (TMB/H_2O_2) was added to develop a colored reaction. Stopping solution was added and the intensity of the color measured in a spectrophotometer at 450 nm. HA concentrations were calculated by comparing the absorbance of the sample against a reference curve prepared from the reagent blank and five HA reference solutions (50, 100, 200, 500, and 800 ng/ml). Linear regression was used to calculate the results. This assay measures HA molecules larger than 9 kDa (i.e. more than 20 repeating dimers of β-(1-4)-glucoronic acid and β-(1-3)-N-acetylglucosamine).

Hyaluronan expression

Cells were fixed in formaldehyde/glutaraldehyde (3.7%/0.1%, v/v) for 10 minutes. Subsequently, cells were incubated for 60 minutes with 3% (w/v) low-fat dry milk in PBS/0,1% Tween 20 to block non-specific binding. Cells were washed and incubated overnight at 4°C with 2% biotinylated HA-binding protein (bHABP; Seikagaku Inc., Tokyo, Japan) followed by the FITC-conjugated avidin (fluorescein avidin D; Vector Laboratories Inc., Burlingame, USA). Stainings were combined with a propidiumiodide counterstaining to localize cell nuclei. Filters were mounted in vectashield and analyzed with a Zeiss LSM 410 confocal laser scanning miscroscopy (CLSM; Zeiss, Oberkochen, Germany). A 488 nm Ar-laser was used to excitate FITC and a 543 nm laser to excitate propidiumiodide.

Lactate dehydrogenase release

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

Transepithelial resistance

TER is measured in ohms (Ω) after placing the insert in an Endohm 24 connected to a Voltohm meter (World Precision Instruments, Sarasota, USA) and expressed as $\Omega*cm^2$.

Statistical analysis

The results are presented as means ±SD of three independent inserts. Statistical analysis on NSAID concentration-dependent effects was performed with one-way analysis of variance (ANOVA) and the effect of 25 μM indomethacin on COM crystal binding, PGE_2- and HA-production with Student *t* test. Differences were considered significant at $P < 0.05$.

RESULTS

Indomethacin during wound healing

[^{14}C] calcium oxalate monohydrate crystal binding

We investigated whether the non-selective COX-inhibitor indomethacin affected the susceptibility of the cell surface for COM crystal binding during wound healing. MDCK-I cells do no longer express the crystal-binding molecule HA and practically loose their affinity for COM crystals as soon as cells are fully differentiated and have formed confluent monolayers with tight junctions (high levels of TER). Scrape-damage results in increased crystal binding to HA-expressing regenerating cells at the border of the wound. Crystal binding to healing cultures compared to undamaged cultures reaches its maximum level 2 days post-injury to gradually decline to control levels as the epithelial barrier is restored 4 days post-injury [4]. [^{14}C] COM crystal binding was measured on day 1, 2, 3 and 4 after scrape-damaging confluent monolayers and addition of 25 μM indomethacin or vehicle alone (EtOH) to the culture medium. Crystal binding to undamaged confluent control cultures was relatively low (0.80 ± 0.14 μg/cm^2). Two days post-injury crystal binding reached its maximum level (4.05 ± 1.17 μg/cm^2) and at this point in time 25 μM indomethacin resulted in a significant decrease of crystal binding (figure 5.1 A).

Prostaglandin E_2 secretion

The secretion of PGE_2 in the apical medium compartment was measured on day 1, 2, 3 and 4 post-injury in daily-replaced Dulbecco's modified Eagle's medium. PGE_2 secretion reaches its highest level already after 1 day post-injury to gradually decline thereafter (figure 5.1 B). PGE_2 production was significantly reduced at all time points by 25 μM indomethacin. From measurements in culture medium that was not replaced it is also apparent that the highest production of PGE_2 is reached at the first day post-injury and indomethacin significantly reduced these high levels at all indicated points in time (figure 5.1 C).

Hyaluronan production

The delivery of HA in the apical compartment was also measured after scrape-damaging confluent monolayers. Previously we have shown that scrape damage results in increased levels of HAS2 mRNA expression and high molecular weight (M_r) HA production which is predominantly targeted to the apical medium compartment [29]. Scrape-injury induced increased levels of HA production, with peak levels 2 days post-injury (figure 5.1 D). Thus, while peak levels PGE_2 were secreted 1 day post-injury (figure 5.1 B), peak HA levels were secreted 2 days post-injury (figure 5.1 D). The increased production of HA in response to tissue damage was significantly reduced by 25 μM indomethacin (figure 5.1 D). From measurements in culture medium that was not replaced it is also clear that the highest production of HA is reached two days post-injury and indomethacin significantly reduced these high levels at all indicated points in time (figure 5.1 E).

Indomethacin during wound healing, two days post-injury

[^{14}C] calcium oxalate monohydrate crystal binding

Since [^{14}C] COM crystal binding reached its maximum level 2 days post-injury, we investigated at this point in time crystal binding at increasing doses of the non-selective COX-inhibitor indomethacin (0, 1, 10, 25 and 100 μM). It was found that COM crystal binding was significantly decreased already at the lowest dose of 1 μM indomethacin compared to scrape-damaged cultures treated with vehicle alone (= 0 μM indomethacin). Crystal binding decreased even more to the lowest levels at concentrations of 25 and 100 μM indomethacin, which were equivalent to low crystal binding levels in undamaged control confluent cultures (figure 5.2 A).

Prostaglandin E_2 secretion.

Two days post-injury culture medium was collected and the amount of PGE_2 measured. Intact monolayers secreted relatively low amounts of PGE_2 (623 ± 112 pg/24h) in culture medium that was aspirated after 2 days. Scrape-injury resulted in an increased production of PGE_2. The increased secretion of PGE_2 was concentration-dependently inhibited by indomethacin (1-100 μM). Already at 1 μM indomethacin, PGE_2 concentrations were significantly reduced and were even much lower than in undamaged control confluent cultures (figure 5.2 B).

Hyaluronan production

Previously we have shown that during proliferation or in response to mechanical injury proliferating or regenerating MDCK-I cells express HA at their apical membrane and high M_r HA production is increased which is targeted to the apical medium compartment [29]. The increased HA production in healing cultures was concentration-dependently inhibited by indomethacin (1-100 μM) (figure 5.2 C). The amount of in hyaluronan in apical medium that was collected

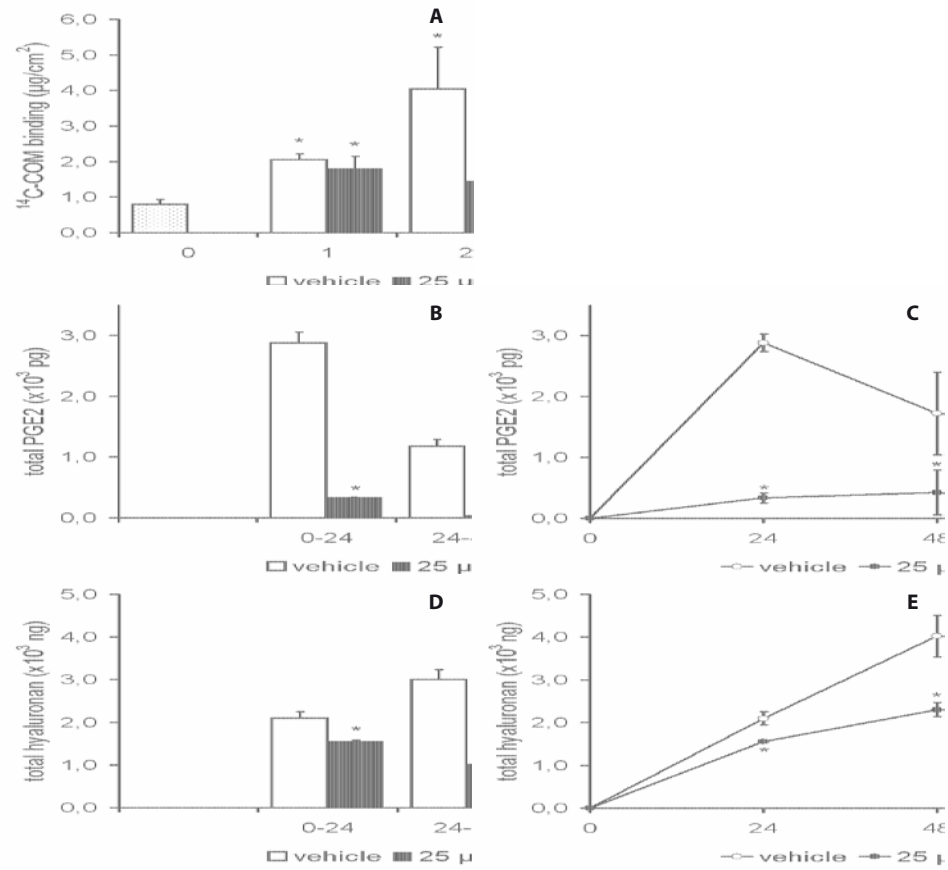

Figure 5.1

The effect of the non-selective COX-inhibitor indomethacin on [^{14}C] COM crystal binding (A), PGE_2 production (B-C) and HA production (D-E) during the process of wound healing in scrape-injured cultures of MDCK-I cells.

*Significantly lower compared to scrape-injured cultures with vehicle alone; **Significantly higher compared to undamaged control confluent cultures.

after two days showed a gradual decline and was significantly reduced compared to vehicle alone at concentrations of 25 µM and 100 µM indomethacin (figure 5.2 C).

Hyaluronan expression

HA was previously identified as major binding molecule for crystals at the apical membrane of flattened, migrating tubular epithelial cells closing wounds made in confluent monolayers [4]. Since indomethacin reduced COM crystal binding it was investigated whether indomethacin treatment also resulted in decreased HA expression. HA was stained with biotinylated hyaluronan binding protein (bHABP) in healing cultures treated or not treated with indomethacin. These studies demonstrated that HA is abundantly expressed at the border of the wound in

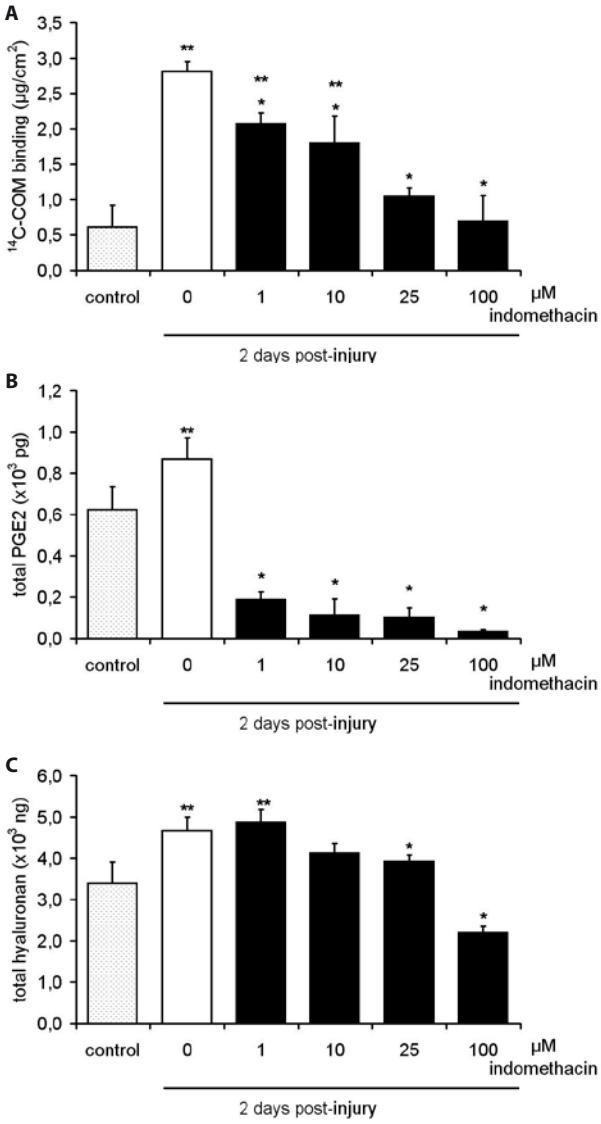

Figure 5.2

Dose-dependent effects of the non-selective COX-inhibitor indomethacin on [^{14}C] COM crystal binding (A), PGE_2 (B) and HA production (C), 2 days post-injury in a scrape-wound model of MDCK-I cells in culture.

*Significantly lower compared to scrape-injured cultures with vehicle alone; **Significantly higher compared to undamaged control confluent cultures.

cultures treated with vehicle alone one day post-injury, but 10 µM indomethacin reduced this expression and at concentrations of 25 µM indomethacin (figure 5.5 B) or higher, hyaluronan was nearly not detectable.

Nimesulide and meloxicam during wound healing, two days post-injury

[^{14}C] calcium oxalate monohydrate crystal binding

The effect of the highly selective COX-2 inhibitors nimesulide and meloxicam (0, 50 and 100 µM) on crystal binding at 2 days post-injury was investigated. Crystal binding was significantly decreased by 50 µM nimesulide, and decreased even more to low levels comparable to undamaged controls by 100 µM nimesulide (figure 5.3 A). The same pattern of decreasing levels of COM crystal binding was observed in meloxicam-treated cultures (figure 5.4 A).

Prostaglandin E_2 secretion

Both nimesulide and meloxicam (50 and 100 µM) effectively inhibited the increased secretion of PGE2 during wound healing (figure 5.3 B and 5.4 B).

Hyaluronan production

The increased HA production in healing cultures was concentration-dependently inhibited by nimesulide and meloxicam. HA production was significantly reduced by both 50 and 100 µM Nimesulide compared to increased HA production in scrape-damaged cultures treated with vehicle alone (figure 5.3 C). Meloxicam showed a similar pattern with increasing reduced levels of hyaluronan in the apical medium compartment (figure 5.4 C).

Hyaluronan expression

HA expression at the surface of flattened, migrating cells at the border of the wounds was strongly reduced by 50 µM nimesulide and was merely absent at 100 µM (figure 5.5 C). The observations in healing cultures treated with increasing doses of meloxicam were similar. HA was scarcely observed at the highest dose of 100 µM meloxicam (figure 5.5 D).

Lactate dehydrogenase release

LDH release was measured in healing cultures treated with increasing doses of indomethacin, nimesulide or meloxicam. Treatment with either NSAID did not increase LDH release compared to scrape-injured cultures treated with vehicle alone (table 5.1). Hence, the concentrations of

Table 5.1

The effects of the non-selective COX-inhibitor indomethacin (A) and the selective COX-2 inhibitors nimesulide (B) and meloxicam (C) on LDH release in the apical medium compartment 2 days post-injury in a scrape-wound model of MDCK-I cells in culture.

	LDH (U/l)				
Indomethacin (µM)	0	1	10	25	100
	2.7±1.8	4.0±0.5	2.9±1.8	2.9±2.5	3.6±1.6
Nimesulide (µM)	0	50	100		
	5.9±2.3	6.4±0.6	3.3±0.6		
Meloxicam (µM)	0	50	100		
	8.2±0.4	8.4±2.0	3.1±1.6		

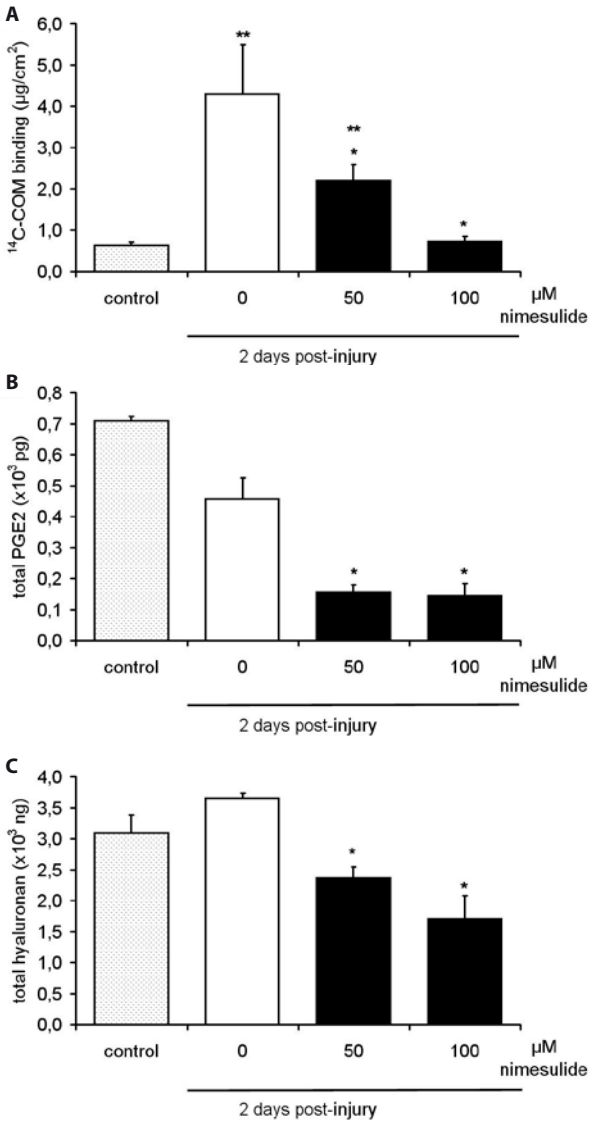

Figure 5.3

Dose-dependent effects of the selective COX-2 inhibitor nimesulide on [^{14}C] COM crystal binding (A), PGE_2 (B) and HA production (C), 2 days post-injury in a scrape-wound model of MDCK-I cells in culture.

*Significantly lower compared to scrape-injured cultures with vehicle alone; **Significantly higher compared to undamaged control confluent cultures.

NSAIDs that were used were not cytotoxic. The trypan blue exclusion test was also used to assess toxicity of NSAIDs and also showed that the concentrations of NSAIDs that were used in these studies were not injurious to cells (not shown).

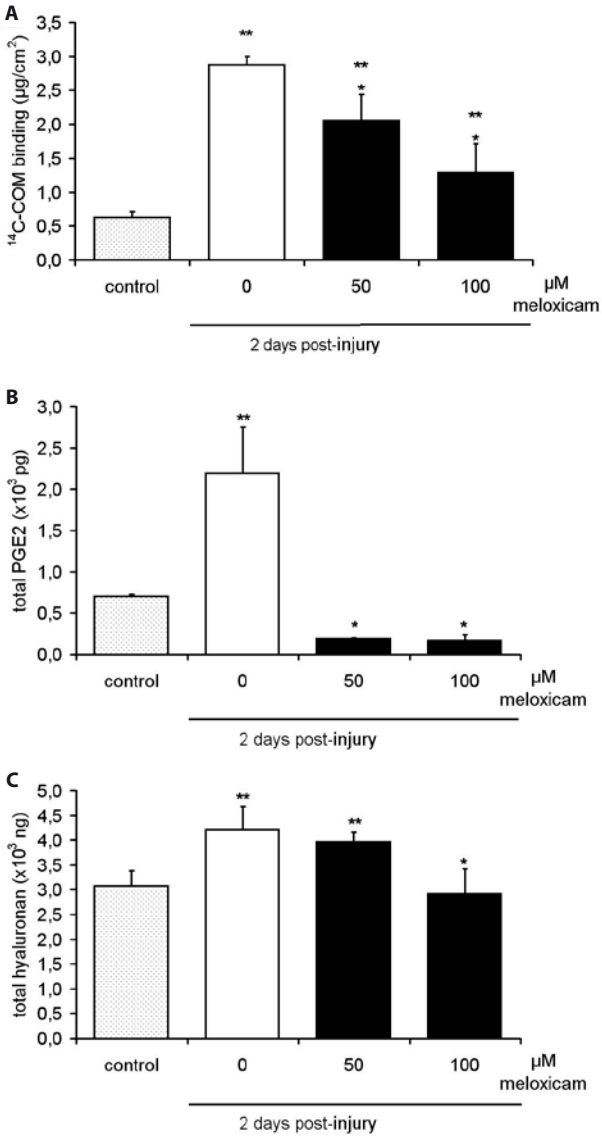

Figure 5.4

Dose-dependent effects of the selective COX-2 inhibitor meloxicam on [^{14}C] COM crystal binding (A), PGE_2 (B) and HA production (C), 2 days post-injury in a scrape-wound model of MDCK-I cells in culture.

*Significantly lower compared to scrape-injured cultures with vehicle alone; **Significantly higher compared to undamaged control confluent cultures.

Transepithelial electrical resistance

Previously we have shown with TER measurements, phase-contrast and confocal microscopy that after scraping-off approximately 30% of a confluent monolayer, approximately 4 days

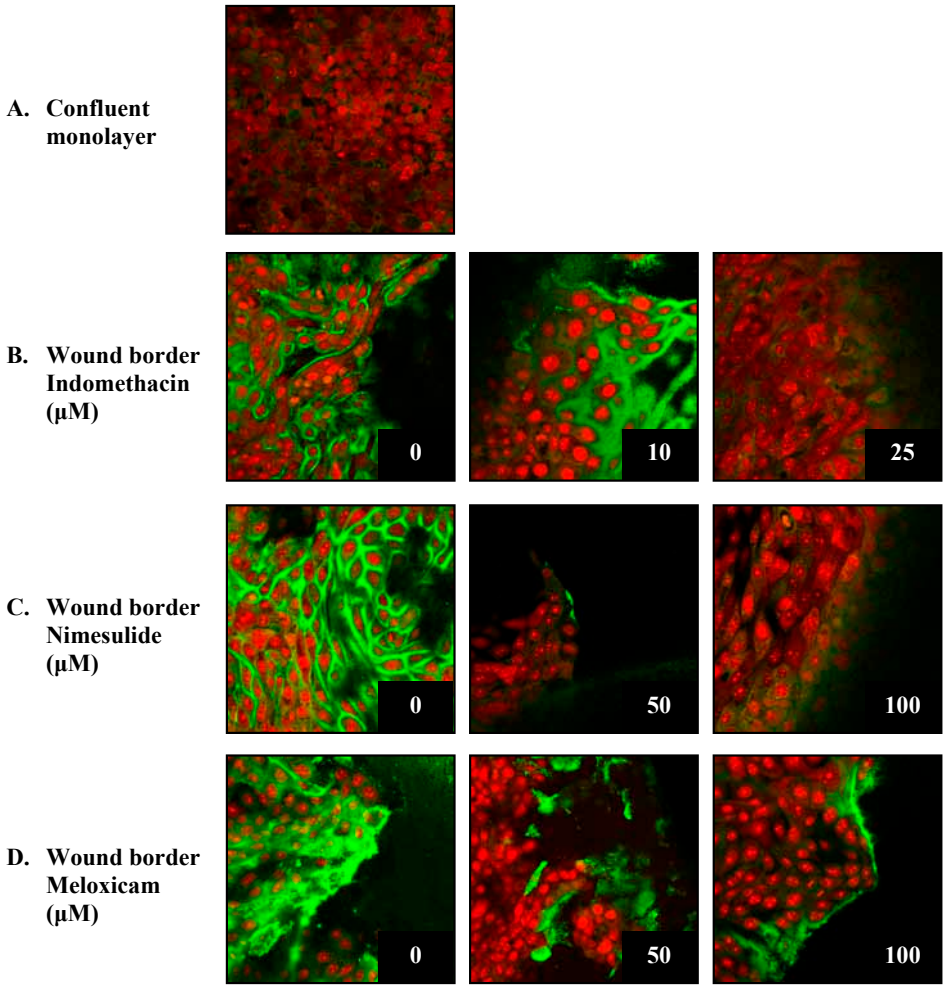

Figure 5.5

Confocal laser scanning microscopy studies. Color image: see appendix page 153.

are required for the cultures to fully re-establish the epithelial barrier integrity [28]. Whereas TERs are around 5000 Ω*cm^2 in confluent cultures grown in 10% FCS, they drop to values of 1000 - 2000 Ω*cm^2 in the absence of serum. TER is reduced to zero after scraping 30% of the monolayers. NSAIDs did not abolish tight junction formation since re-establishment of TER (with increasing levels of TER) were measured in all healing cultures treated with indomethacin, nimesulide or meloxicam at day 1, 2 3 and 4 post-injury (figure 5.6).

TER measurements in indomethacin treated cultures of 1 and 10 µM were comparable to controls. TER was lower compared to healing cultures treated with vehicle alone at the highest dose of 100 µM indomethacin. TER was also less high with 25 µM indomethacin at day 1, 2 and 3 post-injury but was not significantly different from control cultures 4 days post-injury (figure

5.6 A). The highest dose of 100 µM nimesulide resulted in less high levels of TER compared to controls, whereas TER was not significantly different from controls with 50 µM nimesulide (figure 5.6 B). Both concentrations of meloxicam that were used showed increasing levels of TER similar to control scrape-injured cultures (figure 5.6 C).

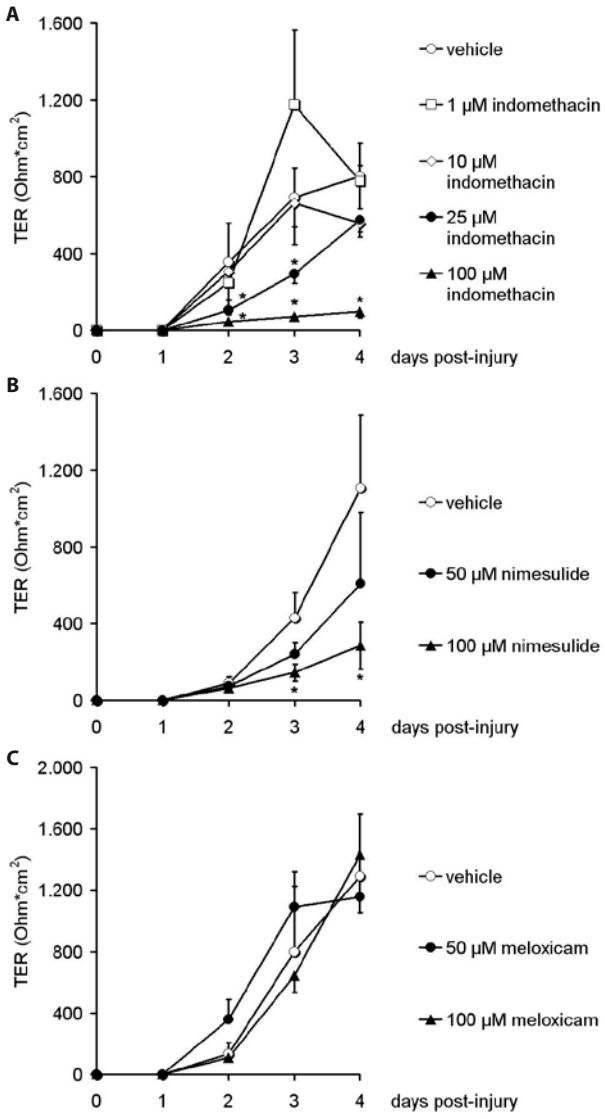

Figure 5.6

Dose-dependent effects of the non-selective COX-inhibitor indomethacin (A) and the selective COX-2 inhibitors nimesulide (B) and meloxicam (C) on recovery of transepithelial electrical resistance (TER) in a scrape-wound model of MDCK-I cells in culture.

*Significantly lower compared to scrape-injured cultures with vehicle alone.

DISCUSSION

Crystal retention is indispensable for the development of nephrocalcinosis and nephrolithiasis. There is mounting evidence that stone formers are predisposed to retain crystals in their kidneys. Crystallization is the physiological result from the urinary concentration process along the nephron. Cells lining the urinary tract are non-adherent to crystals in nephron segments where levels of supersaturation are high and crystals are being formed. Thus, calcium oxalate crystals do not bind to renal distal tubule/collecting duct cells, whereas they unrestrictedly adhere to proximal tubular cells [30]. It is not entirely clear how in the kidneys of stone forming patients the non-adherent crystal binding capacity of the cell surface is transformed into a crystal binding phenotype. Earlier studies have demonstrated that the crystal binding phenotype is characterized by the luminal expression of HA, OPN and CD44 by dedifferentiating/regenerating activated tubular epithelial cells [5, 6]. Although the relative contribution in crystal retention of these and perhaps other membrane molecules, including sialic acid [31], OPN [32], nucleolin-like protein [33], phosphatidylserine [34], extracellular matrix (ECM) proteins [35] and annexin II [36] remains to be determined, the results of the present study reinforce a major role for HA in crystal retention.

HA is a very large polysaccharide (>10^6 Da) composed of multiple units of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) dissacharides. Besides structural functions, HA also directly influences cell behaviour. HA mediates immune cell adhesion at sites of inflammation and HA-receptor binding triggers signal transduction events that, in concert with other ECM and cytoskeletal components, can direct cell trafficking under physiological and pathological conditions. HA also is the main component of pericellular matrices (PCMs) providing a microenvironment required for the movement of the cells during dynamic morphogenetic events such as embryonic tissue development, tissue regeneration and tumorigenesis [37-40]. HA is not expressed in normal, healthy nephrons. Its expression by renal tubular cells requires some form of stress activation [5, 6]. Inflammation also triggered colonic and aortic smooth muscle cells to assemble HA-rich PCMs which resulted in the retention of leukocytes at the sites of inflammation [41]. Once expressed, HA forms entangled molecular networks through steric interactions between individual HA molecules and aggregated HA binding proteins [42]. Previously, we have demonstrated that in cell culture this glycocalyx serves as binding substance for calcium oxalate crystals [4, 43].

Arachidonic acid (AA) and its eicosanoid metabolites play critical roles in modulating inflammatory responses in the kidney. The synthesis of PGE_2 is catalyzed by cyclooxygenase (COX) enzyme activity and COX inhibitors (NSAIDs) are powerful drugs in controlling the inflammatory process. The present study was undertaken to reveal if regeneration-induced crystal binding is inhibited by NSAIDs. The effect of the non-specific COX inhibitor indomethacin and

the COX-2 selective inhibitors nimesulide and meloxicam were studied on COM crystal binding to MDCK-I cells during the repair from scrape-wounds made in confluent monolayers. Scrape injury leads to a burst of cell proliferation distal from the wound and cell migration at the wound border, with a critical role for HA in the subsequent repair process [4, 44]. Our results show that the secretion of PGE_2 (figure 5.1, B and C) as well as that of HA (Figure 5.1, D and E) increased in response to injury, to gradually decline during the subsequent healing process. Peak levels of PGE_2 were measured one day post-injury, while those for HA were measured two days post-injury suggesting that -if interrelated- HA biosynthesis is activated down-stream the eicosanoid pathway. Mechanical stress-induced PGE_2 and HA secretion were dose-dependently decreased by indomethacin (1-100 μM), nimesulide or meloxicam (50-100 μM). Since nimesulide and meloxicam are COX-2 selective, the results suggest that activation of PGE_2 and HA depends on COX-2 rather than COX-1 enzyme activity[45].

Previously we have shown that proliferating MDCK-I cells produced more high M_r hyaluronan ($M_r > 10^6$ Da) than growth-inhibited cells in intact monolayers and up to 85% was targeted to the apical compartment, which was accompanied by increased HAS2 mRNA expression and slightly decreased HAS3 mRNA, while HAS1 mRNA remained undetectable [29]. These proliferating cells express HA at their apical membrane and avidly bind COM crystals. Also regenerating MDCK-I cells at the border of the wound of scrape-damaged monolayers express the crystal-binding molecule HA and secrete increased amounts of HA in the apical medium compartment. In the present study it is shown that NSAID treatment resulted in decreased levels of COM crystal binding, decreased HA secretion in the apical medium compartment and decreased HA expression at the apical cell membrane. These results strongly suggest that the crystal-binding capacity of these cells was decreased by reduced expression of the crystal-binding molecule HA. Figure 5.7 supports this conclusion. Indomethacin treated cultures were subsequently treated with *Streptomyces* hyaluronidase, an enzyme that specifically digests hyaluronan from the cell surface. COM crystal binding in cultures that were treated with 0 or 1 μM indomethacin could be further decreased with *Streptomyces* hyaluronidase to low levels comparable to undamaged control cultures, whereas at higher concentrations of indomethacin hyaluronidase treatment did not further decrease the already low levels of crystal binding. Together with the results of TER recovery during wound healing (figure 5.6 A), LDH release and trypan blue exclusion, this suggests that indomethacin reduced the hyaluronan-dependent crystal binding capacity of regenerating cells at concentrations that did not affect cell viability or tight junction formation.

The remarkable new finding in the present study is that NSAIDs effectively inhibit crystal binding. In contrast, Lieske et al. found that crystal binding to MDCK cells is increased by COX inhibitors [46]. However, this study was performed with the heterogeneous parental MDCK cells that may respond differently to blocking the eicosanoid pathway. In addition, it is not

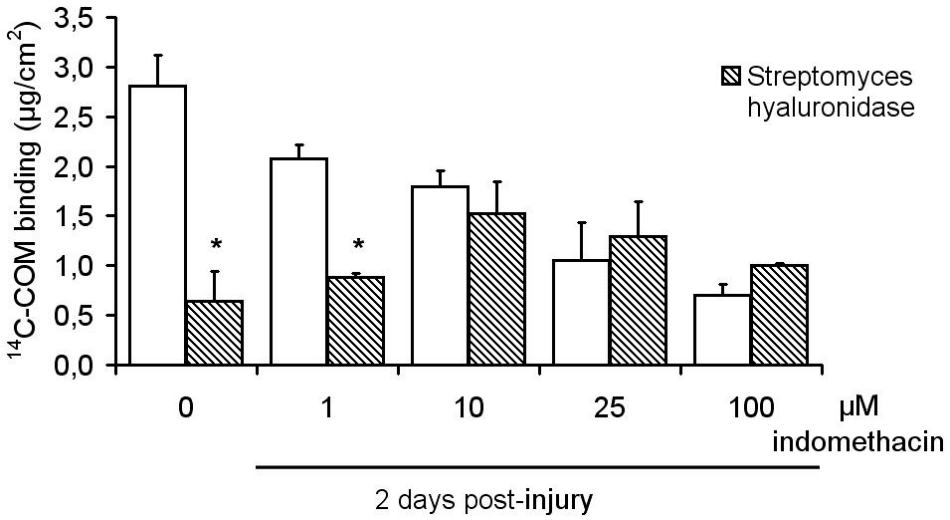

Figure 5.7

The effect of *Streptomyces* hyaluronidase treatment on [^{14}C] COM crystal binding by MDCK-I cells 2 days post-injury treated with increasing doses of indomethacin.

*Significantly lower compared to injured cultures that were not treated with *Streptomyces* hyaluronidase.

clear if these cells –which were cultured on plastic wells instead of permeable supports- retained HA biosynthesis in culture. Earlier we have shown that not all renal tubular cell lines are capable to produce and express HA [43]. HA biosynthesis depends on multiple mediators, effectors, enzymes, receptors and cellular signal transduction pathways and failure of only one of them could be sufficient to abrogate its expression at the cell surface. Our recent rat study showed that *in vivo* practically all activated renal tubular cells expressed HA at their luminal membrane [6]. Cells in culture have the tendency to lose apparently vital properties. It is known, for example, that LLC-PK1 cells have defective COX activity [47] and it is thus no surprise that these cells are unable to synthesize HA [43]. There are probably more stress conditions to activate HA biosynthesis in renal tubular cells. For example, HAS2 mRNA and HA biosynthesis were activated in renal proximal tubular cells by cytokines (IL-1β), high glucose [22] and bone morphogenic protein-7 (BMP-7) [48]. It should be emphasized, however, that HA biosynthesis regulation may differ in renal tubular cells derived from different parts of the nephron. Interestingly, Farell and Lieske recently studied COM crystal binding also in MDCK-I cells and found that PGE_2 addition to proliferating or healing cultures decreased crystal binding whereas NSAIDs increased crystal binding. The authors therefore hypothesized that intrarenal PGE_2 could serve a protective function by preventing adhesion of crystals to regenerating cells [49]. The authors also recommend future studies to elucidate the relative role of crystal-binding molecules that are present on the surface of injured and/or regenerating cells and the effect that PGE_2 has on their expression. The present study suggest that increased PGE_2 expression by regenerating MDCK-I cells stimulates the expression of the crystal-binding molecule HA,

since NSAIDs decreased PGE_2 and HA-depedent COM crystal binding. Addition of PGE_2 neither abolished the effect of NSAIDs on COM crystal binding, nor increased COM crystal binding in confluent monolayers (data not shown). The conclusions from the present study and the study performed by Farell and Lieske therefore seem to be contradictionary to each other. It would be a logic step to study the effect of NSAIDs in an *in vivo* rat model of crystal retention to elucidate this issue and study the effects on crystal retention, HA expression and PGE_2 production. In 1983 Buck et al. induced nephrocalcinosis in rats by intraperitoneal injections of calcium gluconate, and studied the effect of prostaglandin inhibition by NSAIDs [50]. It was found that calcium gluconate injections caused calcium deposits in the tubular lumen, which were absent in rats treated with indomethacin or flurbiprofen [50]. These results are in coherence with the present *in vitro* study.

Previously, we have studied calcium oxalate crystal retention in the kidneys of ethylene glycol treated rats. It was shown that ethylene glycol almost immediately induced calcium oxalate crystalluria, whereas mild tubular injury/regeneration was observed at a later point in time. Crystals were not retained in normal kidneys, but they were found at the luminal membrane of tubular epithelial cells as soon as cells were injured/regenerating and expressed HA at their luminal membrane. The association of retained crystals with HA-expressing cells strongly suggested that HA served as crystal-binding molecule *in vivo* [6]. These results showed that the induction of the crystal binding phenotype in the rat kidney is accompanied by crystal deposition. In coherence with our cell culture data this suggests a causal link. Since HA is expressed only by stress activated renal tubular cells, crystal retention in the kidney could be an inflammation-mediated process [4-6, 51].

In the meantime, we disclosed several clinical conditions during which crystals are found in human nephrons (nephrocalcinosis) associated with cell surface expression of HA (and OPN). In these clinical settings the expression of HA seems to be induced by the long term use of nephrotoxic agents such as cyclosporin in renal transplant patients and developmentally regulated in the renal tubules of preterm infants [7]. It is much more difficult, however, to obtain renal tissue from idiopathic recurrent stone formers or primary hyperoxaluria patients. Although we expect that HA also plays a role in crystal retention in the kidneys of these patients, direct evidence for this assumption has not yet been provided and the offending stimuli activating HA biosynthesis in these patients are unknown. It is unlikely that recurrent renal stone disease is initiated by mechanical injury. Our tissue damages studies should therefore be regarded as experimental models illustrating that non-adherent cells without luminal expression of HA could be transformed into HA expressing crystal-binding cells.

In general, eukaryotic cells respond in a reasonable standardized fashion to stressful conditions [52], such as activation of the mitogen-activated protein kinase (MAP kinase) pathway [52, 53] and

an enhanced production of HA seems to be a typical cell stress response [41, 54]. There are three major MAP kinase cascades: ERK, JNK and p38. ERK1/2 has been implicated in mitogenesis and cell differentiation, while JNK and p38 are activated by cell stress [52, 53]. Scrape-damaging HK-2 monolayers leads to MAPK kinase (MEK)-mediated ERK activation and increased HA secretion. Both p38 MAPK and HAS are activated in synoviocytes from rheumatic arthritis patients [55]. Interestingly, oxalate and calcium oxalate crystals stimulated p38 MAPK in renal proximal tubular cells [56] and it is conceivable that MAPK also activates HA biosynthesis under these conditions. Future studies will shed more light on stimuli that can induce HA-mediated crystal retention in recurrent renal stone disease.

The present study suggests that NSAIDs could be applied to prevent crystal retention in renal stone formers. However, the therapeutic use of non-selective COX inhibitors is limited due to gastrointestinal and renal side-effects [13, 57]. Although the gastrointestinal complications are reduced with COX-2 selective inhibitors, it is not entirely clear if they also improve renal safety [12]. The potential relationship between MAPK and HA opens the possibility to inhibit stress-induced HA-mediated crystal binding with MAPK inhibitors such as cytokine-suppressive anti-inflammatory drugs (CSAIDs), drugs that could spare normal prostaglandin-dependent renal physiology.

CONCLUSION

Taken together, our results show that NSAIDs inhibit crystal binding to HA at the surface of regenerating MDCK-I cells by a process that depends on COX-2 mediated HAS2 activation.

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Chapter 6

General discussion

6.1 INTRODUCTION

Calcium salts (and other poorly soluble waste salts) often precipitate as crystals in the urine when the concentration of urine increases along the kidney tubules. These crystals are normally washed away and excreted with the urine during micturition, which is a physiological phenomenon called crystalluria. Patients that suffer from kidney stones (so-called stone formers) differ from non-stone formers by the fact that in stone formers crystals are not being washed away but are retained and accumulate in their kidneys (crystal retention) to ultimately become cemented together with organic material into a kidney stone [1]. Almost 30 years ago leading investigators emphasized that crystal retention should be considered a crucial requisite for kidney stone formation and postulated that this process could take place inside the tubular lumen when crystals are retained at the luminal membrane of epithelial cells lining the renal tubules [2]. This concept inspired several researchers throughout the world to search for so-called "crystal binding molecules", i.e. molecules that are expressed by tubular epithelial cells that have affinity for crystals and could thereby play a role in the retention of crystals in the kidney [3, 4]. The hypothesis was formulated that these molecules are exclusively expressed by tubular cells of stone formers.

6.2 PREVIOUS WORK

Dr Carl Verkoelen and co-workers from the department of Urology of the Erasmus Medical Center Rotterdam started to work on the subject of crystal-cell interaction in the mid-nineties [5]. *In vitro* studies were performed with MDCK-I cells [6-8]. These cells are derived from a kidney of a normal Cocker Spaniel in 1958 [9, 10]. Their location of origin is assumed to be the distal renal tubules/collecting ducts [10-13]. The surface of these cells exists of two plasma membrane domains separated by junctional complexes. The apical membrane facing the tubular lumen has a protein and lipid composition different from the basolateral membrane which is in contact with the internal milieu, facing the mesenchymal space and the blood supply [14]. Cells were cultured on porous supports in a two-compartment system mimicking the *in vivo* situation and the use of these permeable supports has been shown to allow cells to develop into higher levels of morphological and functional differentiation compared to cells cultured on solid substrates [7]. Well-differentiated polarized monolayers have retained most characteristics of distal tubule/collecting duct cells with high levels of transepithelial electrical resistance and low levels of brush border enzyme activity (γ-GT and alkaline phosphatase). Phenotypically these cells form a tight layer of cells columnar in shape with short, stubby microvilli at their apical membrane [7, 14, 15].

Crystal-cell interaction studies were performed with these MDCK-I cells in culture, since the formation of crystals along the nephron is to be expected not earlier than the distal tubules and collecting ducts unless oxalate plasma concentrations are raised to unphysiologically high levels in rare cases such as ethylene glycol poisoning or severe primary hyperoxaluria [16]. Calcium oxalate monohydrate (COM) crystals were prepared by adding calcium chloride with sodium oxalate. To obtain radiolabeled crystals a small amount of [^{14}C] oxalate was added during the precipitation step. It was found that COM crystals do not adhere to fully differentiated cells populating confluent monolayers with high levels of transepithelial electrical resistance. In contrast, crystals avidly adhere to proliferating cells populating developing subconfluent cultures and to migrating cells at the border of scrape-damaged cultures with low levels of transepithelial electrical resistance [6]. Subsequently it was shown that the difference between the crystal binding phenotype of confluent cultures and subconfluent or scrape-damaged cultures could be explained by the fact that hyaluronan is expressed at the apical membrane of proliferating and regenerating cells and serves as crystal binding molecule whereas fully differentiated cells do not express hyaluronan [8].

Hyaluronan is an extremely large (M_r >10^6 Da) linear glycosaminoglycan composed of thousands repeating units of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) disaccharides. Hyaluronan chains form entangled networks providing a microenvironment conducive to proliferation and migration during processes like growth, development, regeneration and cancer [17]. We found that the pericellular matrices (PCMs) formed by hyaluronan extended several microns from the cell surface and that these coats disappeared at the cell surface when cells had developed into confluent monolayers [18]. Hyaluronan is an excellent binding molecule for crystals because it is negatively charged due to the carboxyl (COO^-) group of GlcUA, while the crystals are positively charged due to the calcium ions (Ca^{2+}) protruding from the crystal lattice.

The validity of these results was confirmed in primary cultures of human renal cells. As in MDCK-I cells, crystal adhesion declined and hyaluronan was no longer detectable as cells reached confluency and had established tight junctions [19]. It can therefore be concluded that the MDCK-I cell line provides a relevant and functional tool to study hyaluronan biology in epithelial cells lining the distal tubule/collecting duct.

We hypothesized from the results of these cell culture studies that the risk for crystal retention in the human kidney is increased when cells in the renal tubules express hyaluronan at their luminal cell membrane [4, 8, 20].

6.3 THE PRESENT THESIS

One of the main aims of the present thesis was to test this hypothesis, and to assess its clinical relevance. Therefore studies were performed in an *in vivo* experimental model of nephrolithiasis with rats, and in human renal tissue. Other aims of our studies were to further explore hyaluronan biology in relation to crystal binding in renal distal tubular epithelial cells *in vitro*, and to assess if crystal binding to hyaluronan-expressing regenerating cells could be modulated by cyclo-oxygenase inhibitors (NSAIDs).

In **Chapter 2** rats were treated for 1, 4 and 8 days with ethylene glycol (0.5% and 0.75%) in their drinking water to induce renal tubular cell damage and calcium oxalate crystalluria. There was no crystal retention in the absence of tubular injury/regeneration but crystals were retained as soon as renal tubules were injured/regenerating. Crystals were found adhered to the luminal surface of hyaluronan-, osteopontin- and CD44-expressing injured/regenerating cells. These results supported for the first time *in vivo* the concept that crystal retention is associated with hyaluronan expressed at the luminal surface of injured/regenerating cells [21].

In **Chapter 3** we showed that hyaluronan production by distal tubular epithelial cells is increased during proliferation and in response to mechanical injury during regeneration, which depends on increased hyluronan synthase 2 (HAS2) mRNA expression. The expression and secretion of hyaluronan and the membrane expression of its cell-surface receptor CD44 are highly polarized during proliferation and regeneration [22].

In **Chapter 4** we selected two patient populations (preterm neonates and kidney transplant patients) which commonly retain crystals in their renal tubules (nephrocalcinosis). Time-lapse studies of human renal tissue in these patients provided evidence that the expression of hyaluronan and osteopontin at the luminal cell membrane of the epithelium preceded the retention of crystals in both patient groups. From these results the clinical relevance of our work became more apparent and we proposed that this crystal binding mechanism may play a general role in the development of nephrocalcinosis.

In **Chapter 5** it was demonstrated in regenerating MDCK-I cells that the suppression in prostaglandin E_2 (PGE_2) production by non-selective cyclo-oxygenase inhibitiors (COX-1 and COX-2) as well as by COX-2 specific NSAIDs leads to reduced production and expression of hyaluronan and more importantly also in decreased levels of crystal binding.

In summary, our studies suggest that healthy tubular epithelium in the distal nephron – where crystals are to be expected- has no affinity for crystals, and that under normal conditions the kidney seems protected from crystal retention inside the renal tubules. However, the luminal

membranes of proliferating cells during development as well as regenerating/(re)differentiating cells during injury and repair have a high affinity for calcium oxalate crystals. This different crystal binding phenotype is explained by differences in expression of molecules at the apical cell membrane (CD44, hyaluronan and osteopontin) of which hyaluronan seems to be the most important crystal binding molecule through the formation of pericellular matrices. Hyaluronan is involved during nephrogenesis in kidney development and during tubular regeneration in epithelial repair. It plays a key role in (re)establishment of the epithelial barrier integrity and restoration of renal function, but as a negative side-effect it turns a non-crystal binding epithelium into a crystal-binding one due to its unique crystal binding properties [3, 6, 8, 18-23].

6.4 CRYSTAL RETENTION IN THE RENAL TUBULES

From this conclusion the question arises whether crystals and such hyaluronan-expressing tubular cells are found in the kidneys of (recurrent) stone formers. Since the hypothesis was launched 30 years ago that crystal retention could start in the renal tubules, extremely little evidence has been provided that crystals are present in the renal tubules of idiopathic kidney stone formers. The theory is that the risk of kidney stone formation increases if crystals are (transiently) hindered as they pass through the nephron as a result of crystal-cell interaction, but this has never been proven. On the other hand this is not surprising since a study design to evaluate this process in humans is practically impossible, because it is extremely difficult to obtain renal tissue specimens from stone forming patients to inspect renal tubules during the first initial steps of kidney stone formation. In 2003 Evan et al. forced a breakthrough by performing intraoperative renal biopsies obtained during percutaneous nephrolithotomy procedures to remove kidney stones in idiopathic calcium stone formers [24]. Strikingly, no intratubular crystals or tubular injury were noted. These patients had interstitial apatite crystal deposits beginning in the basement membranes of the thin limbs of the loops of Henle extending into the interstitium surrounding the collecting ducts and downwards towards the papillae just beneath the urothelium [25]. These suburothelial deposits called Randall's plaque are thought to serve as sites for stone attachment when they erode into the urinary space of the calyces [26]. Since no intratubular crystals or tubular injury were noted, does this mean that our work has been meaningless or irrelevant? We do not think so. The number of patients studied was relatively small (n=15) and it is unclear whether the selected patients represent the whole group of idiopathic stone formers. Furthermore it cannot be excluded that crystals were dissolved and/or washed by tissue processing steps [27]. However, we have to admit that their results at least undermine the concept that in idiopathic stone formers crystal retention already starts inside the tubular lumen of the nephron.

Our studies demonstrated that the expression of hyaluronan, CD44 and osteopontin at the luminal membrane of renal tubular cells in the distal nephron leads to a form of renal calcification better known as nephrocalcinosis. It has been postulated that nephrocalcinosis and nephrolithiasis (kidney stones) are interrelated, but the finding that crystals were absent in the tubular lumen of idiopathic stone formers suggest that these are two independent entities. Interestingly, there are diseases in which nephrocalcinosis occurs together with nephrolithiasis such as cystic fibrosis, Dent's disease, distal renal tubular acidosis and primary hyperoxaluria type 1 and 2. Studies in these groups of patients might reveal the possible interrelationship –if any- between nephrocalcinosis and nephrolithiasis [28]. Further studies are clearly warranted to definitely answer these questions.

6.5 NEPHROLITHIASIS AND NEPHROCALCINOSIS

What exactly do we mean with renal stone disease? Based on the arguments listed above, renal stone disease can be classified into two major categories: nephrolithiasis and nephrocalcinosis. While there is no discussion on the definition of nephrolithiasis (kidney stones), the description of nephrocalcinosis is less clear. The term nephrocalcinosis often refers to *diffuse renal parenchymal calcification demonstrable by radiology imaging techniques* [29], but there are also other definitions varying from *increased calcium content of the kidney* (eMedicine), to *a kidney disorder involving deposition of calcium and oxalate or phosphate in the renal tubules and interstitium* (Medical Encyclopedia), to *a condition characterized by precipitation of calcium phosphate in the tubules of the kidney, with resultant renal insuffiency* (WrongDiagnosis) and *renal lithiasis in which calcium deposits form in the renal parenchyma and result in reduced kidney function and blood in the urine* (Thesaurus). The definition seems to depend on the detection method used and it usually refers to a typical image during ultrasonography of the kidney in which diffuse scattering of hyperechogenic miniscule lesions are observed in the cortex and/or medulla [30]. The routine use of ultrasonography has revealed many conditions reported to be associated with nephrocalcinosis [31]. Ultrasonography and other radiological imaging techniques have their limitations, however, because they are unable to depict the exact location of the deposits in the kidney and are not sensitive enough to detect limited forms of early stages of nephrocalcinosis. Nephrocalcinosis should be subdivided on the basis of the location where calcium deposits are retained at a microscopic level into interstitial nephrocalcinosis (deposition of crystals in the renal medullary interstitium) and tubular nephrocalcinosis (retention of crystals in the renal tubules) (figure 6.1) [27].

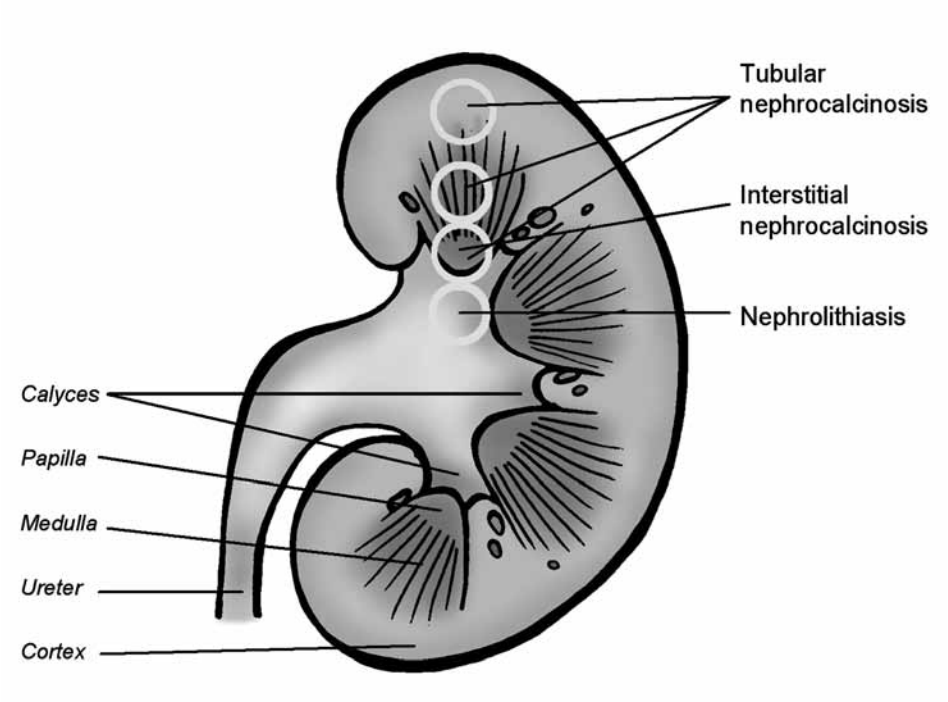

Figure 6.1
Kidney anatomy and the localization of the different manifestations of renal stone disease.

6.6 INTERSTITAL NEPHROCALCINOSIS

Interstitial nephrocalcinosis is defined as calcium phosphate deposits in the renal medullary interstitium. Its incidence rises with age [32]. This form of nephrocalcinosis is probably relatively benign until it erodes into the wall of the renal pelvis to become a Randall's plaque. These plaques could be the nidus for stone attachment in at least a subset of kidney stones, since calcium oxalate stones have been found attached these plaques [33]. Although calcium, phosphate and oxalate concentrations are extremely high in the renal medullary interstitium, only a minority of individuals form Randall's plaques. Randall's plaques are common in idiopathic calcium oxalate stone patients and suggests an alteration in the solubility of calcium phosphate in the renal interstitium [33]. In theory, the concentration of calcium phosphate in the interstitium could be increased in stone formers compared to normal individuals, or the inhibitors of crystallization could be less effective.

6.7 TUBULAR NEPHROCALCINOSIS

Tubular nephrocalcinosis is defined as calcium deposits inside the renal tubules and occurs if crystals are retained inside the nephron [20]. Our studies have contributed significantly to the understanding that crystals are retained when renal tubular cells are triggered to express hyaluronan at their luminal membrane [23]. In preterm infants proliferating/differentiating tubular cells express hyaluronan, because these kidneys have not yet fully developed and nephrogenesis is uncomplete. In renal transplants injured/regenerating tubular cells during injury/repair express hyaluronan, because these kidneys suffer from ischemia/reperfusion injury. In addition, these patients are treated with nephrotoxic immunosuppressive agents again leading to cell damage, regeneration and up-regulated hyaluronan biosynthesis and expression. Besides in preterm neonates [34] and kidney transplants [35], tubular nephrocalcinosis also occurs in sarcoidosis [36], primary hyperoxaluria [37], Dent's disease [38, 39], distal renal tubular acidosis [40-42], carbonic anhydrase deficiency [43], Bartter's syndrome [44, 45], cystic fibrosis [46, 47], Sjögren's syndrome [48], intestinal bypass surgery patients [49], Crohn's disease [33, 50], acute phosphate nephropathy [51] and brushite stone formers [4, 20, 28, 52].

At this point in time we do not know if or why the kidney contains regenerating cells under these other various conditions. One of the first steps for further research could be that renal biopsies in these aforementioned other groups of patients are also stained for hyaluronan and examined for the presence of intratubular crystals. In the meantime this has been performed in part by Vervaet et al. At the 5th eULIS Symposium 2007 (12th European Symposium on Urolithiasis) data was presented and awarded with the best poster prize that indeed in Dent's disease, sarcoidosis, primary hyperoxaluria and acute phosphate nephropathy the same association of crystals with hyaluronan expressing tubular epithelial cells was observed [53]. Recently, Evan *et al.* have shown both intratubular crystals and hyaluronan staining in biopsies from the kidneys of patients who had hyperoxaluria as the result of intestinal bypass surgery for obesity [82].

6.8 CLINICAL RELEVANCE

The most important reason to continue further research and expand our findings is the clinical relevance of crystal retention in patients. Tubular nephrocalcinosis usually does not lead to acute renal failure, except for example in ethylene glycol poisoning [54]. However, the long term effects of calcium deposits in the tubules are unknown because data on long term follow-up of patients with nephrocalcinosis are limited in the literature.

Only 26 years ago nephrocalcinosis was reported for the first time in preterm neonates by Hufnagle et al. in 1982 [55]. The intratubular deposits in these patients are predominantly calcium oxalate but also calcium phosphate crystals (observations in kidneys of neonatal autopsies) [56]. The incidence of tubular nephrocalcinosis has been reported to be as high as 83% in extremely preterm infants (gestation between 24 and 28 weeks) [57]. Nephrocalcinosis commonly disappears later in life, but Schell-Feith et al. showed in their large group of 83 patients with nephrocalcinosis that it persisted in 15% of patients after 30 months follow-up [34, 58]. These authors prospectively studied a large group of 201 preterm infants who developed nephrocalcinosis in 41% and recently published a paper of the largest follow-up up to date at a mean age of 7.5 ± 1 years old of 42 patients with nephrocalcinosis at term and 32 patients without nephrocalcinosis [59]. Significantly more ex-preterm infants with nephrocalcinosis developed (mild) chronic renal insufficiency (15%) than expected in healthy children, in contrast to ex-preterm infants without nephrocalcinosis (6%) [59]. Prematurity per se is associated with high blood pressure and distal tubular dysfunction (low plasma bicarbonate and early-morning urine osmolality) but nephrocalcinosis seems to have an additional risk on compromising renal function later in life [59]. In our opinion this could very likely be attributed to crystal retention which could eventually lead to irreversible damage of nephrons as a result of large crystal aggregates obstructing the tubular lumen. We have shown in preterm neonates that hyaluronan and osteopontin is expressed at the luminal membrane of proliferating distal tubular cells during nephrogenesis, which is in agreement with the important role that has been attributed to these molecules during kidney development [60, 61]. In preterm neonates supersaturation of calcium salts is increased because of a calcium and vitamin D rich diet, low urinary citrate and long-term furosemide treatment resulting in crystalluria [34, 62, 63]. We have shown that hyaluronan and osteopontin expressed at the luminal membrane of proliferating distal tubular cells precedes nephrocalcinosis in these infants, which strongly suggests that this increased crystal binding phenotype played a role in crystal retention [23]. The subsequent decline of kidney function in 15% of preterm infants with persistent nephrocalcinosis after 30 months follow-up described by Schell-Feith et al. therefore rather seems to be the result than the cause of crystal retention [58, 59].

Another condition in which nephrocalcinosis unmistakably plays a role is chronic allograft nephropathy in kidney transplants. The incidence of nephrocalcinosis after kidney transplantation and its relation with renal function and graft survival are not entirely clear. It has only recently been shown that in these patients nephrocalcinosis occurs and is associated with long-term graft survival [4, 23, 35, 64, 65]. Merneo et al. examined 40 allograft nephrectomies in patients with transplant kidney failure between 2 days and 11 years after transplantation who had neither primary or secondary hyperoxaluria as the cause of initial end-stage renal disease, nor a history of kidney stones. These authors showed that calcium oxalate crystal retention in the renal tubules is frequently observed in failing renal grafts, because in 88% of

allograft nephrectomies birefringent calcium oxalate crystals were observed which appeared to involve mainly the lumina of distal tubules and were located on the tubular lining epithelium [64]. Serum oxalate concentrations increase during chronic renal failure and accumulates in the body during dialysis. Within the first few days after transplantation oxalate is mobilized and excreted with the urine, and therefore it was proposed that the recipient's chronic renal failure was related to early calcium oxalate deposition in the renal tubules [64]. In another study, nephrocalcinosis in allograft biopsies was found in 76% of patients with post-transplantation acute tubular necrosis (ATN), whereas nephrocalcinosis was not observed in patients with normal renal function after transplantation [66].

In 213 patients with 586 protocol biopsies obtained 6 weeks, 3 and 6 months after transplantation, nephrocalcinosis increased from 6 to 18%. Although three months after transplantation some crystals were found in the interstitium, the majority was located inside the renal tubules [67]. In our own experience we noticed that crystals can be displaced during tissue sectioning . Our study of protocol renal allograft biopsies taken 3 and 6 months post-transplantation showed an increase in nephrocalcinosis from 20% to 60% [23]. In general, biopsies obtained from dysfunctional renal allografts more often contain crystals in the renal tubules than those from functional allografts. It should be noticed, however, that this does not establish the cause and effect relationship between poor renal graft function and nephrocalcinosis. Pinheiro et al. performed percutaneous renal transplant biopsies in 97 patients within three months post-transplantation and reanalyzed them after ten years. An overall incidence of calcium oxalate deposits of 53% was observed from which 86% of the patients the calcium oxalate deposits were already found in the first biopsy, which –as the authors themselves state- was higher than expected. The calcium oxalate deposits were only observed in the tubular lumen [65]. Chronic allograft nephropathy is the result of cumulative and incremental damage to nephrons as a result of ischemic injury and chronic stress by nephrotoxic immunosuppressive agents in which impaired tubular repair/regeneration eventually leads to fibrosis and loss of function [35]. Pinheiro et al. not only found a higher incidence of calcium oxalate deposits in patients with tubular dysfunction and acute tubular necrosis (ATN) in early post-transplantation biopsies but there was also an independent association between calcium oxalate deposition and a poorer allograft survival, because ATN-free patients with nephrocalcinosis had a worse graft survival than ATN-free patients without nephrocalcinosis [65]. The observation that patients with early nephrocalcinosis had a four-fold higher risk of losing the graft than those without nephrocalcinosis again indicates that renal dysfunction is the consequence rather than the cause of nephrocalcinosis. The authors themselves concluded that a high incidence of post-transplantation nephrocalcinosis may have a negative impact on graft survival [65].

It should be emphasized that all of the above mentioned studies were performed with renal tissue specimens prepared for routine histology. We [27] and others [68] have demonstrated,

however, that a substantial part of crystals in renal tubules are lost during routine tissue preparation. It is therefore not unlikely that the incidence and severity of nephrocalcinosis could even be underestimated.

We have shown in transplant kidneys that expression of hyaluronan and osteopontin is upregulated, which probably occurs in response to ischemic/reperfusion renal damage, cyclosporine toxicity and chronic allograft rejection. Subsequent biopsies of transplant kidneys showed an increase in crystal retention in time, which again suggests that this increased crystal binding phenotype played a role in crystal retention [23]. These results also indicate that crystals themselves were not injurious to tubular cells because tubular injury preceded crystal retention. These *in vivo* results are in correspondence with cell culture studies, which showed that calcium oxalate crystals are not injurious to distal tubular epithelial / collecting duct cells [69]. We propose that calcium oxalate crystals are not the primary cause of decline of renal function in kidney transplants of patients with nephrocalcinosis but crystal retention could eventually lead to irreversible damage to nephrons as a result of large crystal aggregates obstructing the tubular lumen.

Nephrocalcinosis also occurs in primary hyperoxaluria type 1, a genetic metabolic disorder of oxalate metabolism. This rare autosomal recessive inborn error of glyoxylate metabolism is characterized by a functional defect of the liver specific enzyme alanine glyoxylate aminotransferase (AGT) leading to hyperoxaluria and hyperglycolic aciduria [37]. In 57 primary hyperoxaluria type 1 patients in the Netherlands progression to renal insufficiency was statistically associated with the presence of nephrocalcinosis, detected by ultrasound and response to pyridoxine therapy also predicted outcome of renal function but progression to renal insufficiency was not associated with the level of urinary oxalate or with AGT activity [70]. In another paper it was shown that all patients with cortical nephrocalcinosis in primary hyperoxaluria developed end-stage renal disease [71].

6.9 TREATMENT STRATEGIES

Tubular nephrocalcinosis is the result of the combination of crystal formation and crystal retention. The risk of crystal formation is significantly higher when concentrations of citrate in the urine is low and concentrations of calcium and/or oxalate are high [72]. Efforts should therefore be made to keep urinary calcium and oxalate levels low and citrate concentrations high. Hyperoxaluria was found in 35% of kidney transplant recipients and hypocitraturia in 69% [73]. High urinary oxalate probably results from the release of oxalate that was accumulated during the dialysis period in body stores like bone and tissue [74]. Renal tubular acidosis (RTA) is common in preterm infants [63] and renal transplant patients [75]. RTA leads to hypercalciuria and

hypocitraturia [76]. To avoid crystal formation, these patients should be treated with potassium citrate. Potassium citrate treatment has several beneficial effects. It increases urinary pH to the physiological range which prevents further release of calcium carbonate from the bone and it leads to increased urinary citrate that binds calcium and thereby prevents calcium oxalate and calcium phosphate crystal formation. Crystal formation can also be influenced by adjustments in the diet. To avoid crystal formation as much as possible, it is recommended to avoid obesity and maintain an ideal body weight, drink at least 2 L water/day, limit sodium chloride (salt) intake, limit protein intake, consume normal amounts of dairy products (30 mmol calcium per day) and consume fruits and vegetables, except spinach and rhubarb [62, 77].

It is more difficult to prevent crystal retention by influencing the crystal binding phenotype of the distal tubular epithelium. Previously, it was found that prostaglandin E_2 (PGE_2) increases hyaluronan synthesis in renal glomerular cells and that this could be inhibited by the cyclooxygenase (COX) inhibitor (NSAID) indomethacin [78]. This prompted us to study the effect of NSAIDs on hyaluronan-mediated crystal binding to regenerating MDCK-I cells (Chapter 5). These *in vitro* studies showed that hyaluronan-dependent calcium oxalate crystal binding to regenerating dedifferentiating tubular cells is COX-2 mediated and can be modulated by NSAIDs which suggests that COX inhibitors could be used to reduce the risk for nephrocalcinosis in patients. This does not mean, however, that we propagate the use of NSAIDs for the prevention of nephrocalcinosis, because NSAIDs have important adverse effects on the function of the kidney due to the role of COX-2 in renin release, regulation of sodium excretion, and maintenance of renal blood flow [79]. The most important conclusion is that it is not impossible to lower the expression of hyaluronan at the surface of renal tubular cells temporarily, and thereby reducing the risk for nephrocalcinosis, but strategies should be developed that are safe to the kidney.

6.10 FUTURE STUDIES AND PERSPECTIVES

It is interesting to look again at earlier reports and interpret data following the line of thoughts that tubular crystal retention is the result from increased expression of hyaluronan at the luminal membrane of distal tubular epithelial cells during injury/regeneration. In the Lancet in 1994, for example, extracorporal shock-wave lithotripsy (ESWL) treatment of a calyceal stone in a reported case of a twenty-six years old patient with primary hyperoxaluria resulted in fatal renal failure within one month [80]. Since the age of three this patient had recurrent nephrolithiasis. One month after treatment haemodialysis was necessary and within three months symptoms of oxalosis became apparent: peripheral neuritis and cardiac rhythm abnormalities as a result of systemic deposition of calcium oxalate. Within two years a kidney transplantation failed due to oxalosis of the transplant and rejection, and she died of sepsis

after a cerebrovascular accident. The authors emphasized the fact that ESWL treatment causes renal damage in 63-85% and an immediate decline in total effective renal plasma flow [80,81]. Apparently the combination of hyperoxaluria and renal damage led to this catastrophic series of events resulting in renal failure, but the authors did not recognize the fact that renal damage might have caused an increased risk of crystal retention. In this patient subsequent kidney transplantation again failed as a result of widespread deposition of calcium oxalate crystals (oxalosis). It is plausible that injury in the transplant kidney due to ischemia/reperfusion and nephrotoxic drugs in combination with calcium oxalate crystalluria resulted in obstructive tubulopathy and graft failure in this patient.

The concept that crystal retention in the kidney leads to renal calcification disorders is indisputable. Interstitial nephrocalcinosis (Randall's plaques) result from the deposition of crystals in the renomedullary interstitium and there are strong indications that hyaluronan in the inner medullary interstitium plays a role in this process [20]. Tubular nephrocalcinosis is caused by the attachment of crystals to renal tubular cells. Each form of tubular nephrocalcinosis probably has its own specific mitogen/stress conditions leading to the expression of regenerating crystal binding cells in the renal tubules. The initial site of crystal retention in idiopathic stone formers remains an open question. Studying the mechanisms of retention of crystals inside the lumen of the renal tubules is needed because it is obvious that prevention of kidney stone disease is better than only treating symptomatic kidney stone disease. Another particularly relevant reason to extend these studies is the fact that tubular nephrocalcinosis could eventually lead to irreversible damage of nephrons since nephrocalcinosis has been found to be associated with decline of renal function in preterm neonates and patients with kidney transplants.

To explore these concepts future studies should include histological studies of snap frozen human renal tissue specimens of different kidney stone formers, and of other specific patient groups associated with nephrocalcinosis like Dent's disease and primary hyperoxaluria. Human renal tissue specimens are difficult to obtain whereas urine samples are not. Therefore, urine samples of well-defined patient populations should be collected and determined whether molecules can be detected in the urine which could have a relation with hyaluronan biosynthesis by renal tubular epithelial cells. The concentration of hyaluronan itself and its molecular size, for example, can be detected in the urine and quantified with an ELISA (Enzyme-Linked Immuno Sorbant Assay). The aim of such studies is to reveal whether there are urinary markers that can identify patients with tubular injury/regeneration and an increased risk for crystal retention. Urine samples of patients before and after ESWL-treatment for example could be used, since ESWL is known to temporarily cause renal damage. More interesting are urine samples during follow-up of post-transplantion patients, preterm neonates and primary hyperoxaluria patients. *In vitro* studies of renal distal tubular epithelial cells in culture should be

extended in order to determine which hyaluronan-binding proteins are involved in pericellular matrix formation and -degradation, because identification of such proteins could eventually give reason to test their presence in urine of patients and evaluate whether or not they could also be used as urinary markers of tubular injury/regeneration. The aim of such studies is to extend our knowledge of the mechanisms of retention and accumulation of crystals in the kidney, in order to ultimately design new strategies for the prevention and treatment of both nephrocalcinosis and nephrolithiasis in patients.

6.11 REFERENCES

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Summary

The incidence of renal stone disease has increased during the last decades. In North America and Europe the yearly incidence is estimated to be about 0.5%. The lifetime risk in white males and females to develop a kidney stone is 15% and 6%, respectively [1-3]. Patients who develop kidney stones (or so-called stone formers) can be categorized on the basis of the composition of their stone. The most common are calcium stones, which contain calcium oxalate and/or calcium phosphate. A kidney stone can cause flank pain and hematuria. When a stone migrates downwards from the kidney through the ureter towards the bladder it can cause excruciating flank pain, which is a typical phenomenon called renal colic. Small stone fragments usually can pass spontaneously whereas treatment is often required for bigger stones.

The treatment of kidney stones has changed dramatically after the introduction of ESWL ("extracorporeal shock wave lithotripsy") in the eighties and at this moment ESWL is frequently the first choice of treatment for kidney stone disease. The development of minimal invasive techniques with endoscopic equipment also has had an important role in the treatment of stones of the urinary tract (percutaneous nephrolitholapaxy, ureterorenoscopy, laser lithotripsy etc.). Open stone surgery therefore (practically) belongs to the past.

The techniques to remove kidney stones definitively have improved during the last decades. However, this did not cause a decline in the incidence of stone disease [4-6]. In fact, treatment is aimed at resolving symptoms by removing the stone and not at preventing kidney stone formation. The recurrence risk in a person to develop a second stone is 50% in 10 years. This thesis deals with the question how to prevent stone formation in the urinary tract. Therefore we need to extend our knowledge on the etiology of kidney stone disease, which at present is rather limited. Recurrent stone formers should be analyzed to evaluate whether a metabolic disorder causes an increased risk to develop stones, like for example hypercalciuria or hypocitraturia. In these patients specific dietary advice and medication can help to prevent recurrent stone disease [1, 2]. In a subset of patients however no metabolic disorder is found and these patients are therefore called idiopathic recurrent stone formers. Especially these patients indicate that we need to further investigate the etiology of renal stone disease [5].

Kidney stones are composed of innumerable small crystals and organic material. Historically kidney stone research focused on compounds in the urine which increase the risk of crystal formation and agglomeration. Different compounds in the urine act as promotors or inhibitors of crystal formation and agglomeration. From all of the known urinary promotors or inhibitors none of them (except for citrate) have been definitively proven to play a key role in calcium stone formation, or to have any clinical and therapeutic application [7, 8].

The presence of a kidney stone indicates that crystals must have been retained in the kidney. The formation of crystals along the nephron is to be expected not earlier than the distal

nephron. Crystals are being transported with the urine downwards the urinary tract and are excreted with the urine during micturition. The presence of crystals in the urine is a physiological phenomenon and occurs in both stone formers and non-stone formers. It has therefore been postulated that crystal retention has a crucial role in the development of kidney stones [9]. In the eighties this hypothesis led to studies of the tubular epithelium lining the lumen of the distal nephron. The aim was to identify molecules at the surface of tubular epithelial cells that bind crystals and could therefore play a role in the retention of crystals in the kidney [9].

The research group from Dr. Carl Verkoelen in Rotterdam has been performing cell culture studies to answer this question. It was shown that crystals do not bind to MDCK-I cells in culture that have developed into confluent monolayers in which tight junctions have been formed. In contrast, crystals avidly bind to proliferating cells during growth and regenerating cells during wound healing. Apparently this different crystal binding phenotype is explained by differences in expression of molecules at the apical cell membrane [10]. In 2000 Verkoelen *et al*. showed that hyaluronan is expressed at the apical membrane of proliferating and regenerating cells and serves as calcium oxalate crystal binding molecule [11]. Hyaluronan is a high molecular weight (M_r) polysaccharide and the main component of pericellular matrices (PCMs), and has excellent crystal binding properties because of its negative charge due to the carboxyl (COO^-) group of glucuronic acid (GlcUA) [12, 13]. PCMs are expressed by proliferating and migrating cells during growth, regenerating cells during wound healing and tumor cells [14]. These experiments have been repeated in primary cultures of human tubular cells by Verhulst *et al*. in which these results were confirmed [15]. Human tubular crystal-binding cells did not only express hyaluronan at the apical cell membrane, but also the receptor for hyaluronan, the transmembrane protein CD44 and another ligand of CD44, osteopontin (OPN) [16, 17]. Verhulst *et al*. also investigated expression of hyaluronan in renal tissue. Hyaluronan is normally hardly expressed in the renal cortex. During various inflammatory renal disease states however an increased expression of hyaluronan in the renal cortex has been reported, but this has been described in the renal cortical interstitium and not inside the renal tubules [18-22]. Verhulst *et al*. reported for the first time that hyaluronan is also expressed in the kidney at the luminal cell membrane of tubuli in a damaged human kidney by post-renal obstruction and in rat kidneys which were damaged by ischemia/reperfusion [15].

From these results we hypothesized that the risk for crystal retention in the human kidney is increased when tubular epithelial cells of the distal nephron express hyaluronan at their luminal membrane.

To test this hypothesis *in vivo* we performed an experiment in rats (**Chapter 2**). We needed a model in which both calcium oxalate crystalluria could be induced as well as damage to the tubular epithelium. We performed an experiment in which rats received ethylene glycol

added to their drinking water and compared these to rats which received normal drinking water. Ethylene glycol is a precursor of oxalate and therefore causes hyperoxaluria and subsequent calcium oxalate crystalluria. Ethylene glycol is also nephrotoxic which probably should be attributed to the ethylene glycol metabolites glycoaldehyde and glyoxylate [23]. Ethylene glycol almost immediately caused calcium oxalate crystalluria. Calcium oxalate crystals were not retained as long as there were no signs of tubular injury. Crystals were retained in the kidney as soon as ethylene glycol had caused mild damage and injured/regenerating tubular cells were observed. Calcium oxalate crystals were found at the luminal cell membrane of injured/regenerating tubular cells that expressed hyaluronan, CD44 and OPN at the luminal cell membrane [24]. It was shown for the first time *in vivo* that the retention of crystals in the kidney is associated with the expression of hyaluronan at the luminal cell membrane of injured/regenerating tubular epithelium [24].

Hyaluronan expression in the cortex of the kidney is strongly up-regulated during various inflammatory disease states, but mainly has been described in the cortical interstitium [21, 22, 24]. Because in ethylene glycol treated rats hyaluronan not only accumulated in the renal cortical renal interstitium but was also expressed at the luminal cell membrane of tubular epithelial cells, we decided to investigate in cell culture the possible role of tubular cells in this process. We therefore performed experiments in MDCK-I cells and primary cultures of human tubular epithelial cells during growth and wound healing. Cells were cultured on semipermeable filter inserts in a two-chamber system (**Chapter 3**) [25]. In this culture system the apical medium compartment can be analyzed separately from the basolateral medium compartment. It was shown that hyaluronan and CD44 are expressed at the apical cell membrane of proliferating cells together with increased HAS2 mRNA expression and slightly down-regulated HAS3 mRNA expression. As soon as confluent monolayers had developed with formation of tight junctions, hyaluronan was no longer expressed, and CD44 at the basolateral membrane. During wound healing CD44 and hyaluronan were also expressed at the apical cell membrane of regenerating cells at the border of the wounds. The secretion of high M_r hyaluronan is also increased during proliferation and regeneration and highly polarized and targeted to the apical medium compartment [25]. Since the increased production of high M_r hyaluronan by mitogen/stress-activated renal tubular cells is destined for the apical surface and the expression of hyaluronan and CD44 is highly polarized under these conditions, it was concluded that the increased synthesis of hyaluronan by activated renal tubular cells during inflammatory disease states most likely does not contribute to the up-regulated expression of hyaluronan in the renal interstitium but merely supports cell growth and remodeling in the tubules [25].

From these *in vitro* and *in vivo* experiments it was concluded that crystals in the lumen of the distal nephron only bind to proliferating or regenerating cells that express hyaluronan, OPN and CD44 at their luminal cell membrane. To evaluate the clinical relevance of this finding we

studied two different patient groups in which retention of crystals inside the tubular lumen has been described (**Chapter 4**) [26]. This condition is called tubular nephrocalcinosis and occurs in kidneys of preterm neonates [27] and renal transplant patients [28]. Kidneys of both patient groups contain proliferating or regenerating cells because nephrogenesis is not completed until 36 weeks of gestation and posttransplant kidneys are damaged and regenerate because of ischemia, nephrotoxic drugs and chronic graft rejection. In renal tissue of preterm neonates from gestational ages 15 to 40 weeks hyaluronan and OPN expression was shown at the luminal cell membrane of developing tubules [26]. In renal transplants in consecutive biopsies 12 and 24 weeks posttransplantation hyaluronan and OPN expression was also shown at the luminal cell membrane of distal tubuli [26]. In kidney tissue from both preterm neonates that lived for at least 4 days as well as posttransplantation patients calcium containing crystals were observed inside the tubular lumen by von Kossa staining and these crystals were associated with tubules in which hyaluronan and OPN were expressed at the luminal cell membrane. The number of patients posttransplantation in which crystals were identified increased from 20% after 12 weeks to 60% after 24 weeks. These findings in both patient groups suggest that the retention of crystals inside the tubular lumen is preceded by the expression of the crystal binding molecules hyaluronan and OPN [26].

Finally we asked ourselves whether it would be possible to inhibit the binding of crystals to hyaluronan at the surface of tubular epithelial cells. In different cell types hyaluronan synthesis during inflammation is stimulated by prostaglandin E_2 (PGE_2) [29-31]. Cyclooxygenase (COX) isoenzymes stimulate the production of PGE_2. COX-1 is constitutively expressed in the kidney, whereas COX-2 is the inducible isoform upregulated for instance during inflammation [32-35]. We therefore studied the effect of COX-inhibitors, or so-called NSAIDs (non-steroidal anti-inflammatory drugs), on calcium oxalate monohydrate (COM) crystal binding, PGE2 secretion, hyaluronan secretion, hyaluronan expression, lactate dehydrogenase (LDH) secretion and recovery of transepithelial resistance (**Chapter 5**). It was found that both the non-selective COX-1/COX-2 inhibitor indomethacin as well as the selective COX-2 inhibitors nimesulide and meloxicam decreased the binding of COM crystals to the surface of regenerating MDCK-I cells at the border of wounds in scrape-damaged monolayers, already at doses in which recovery of transepithelial resistance was unaffected. Inhibition of PGE_2 production by COX-inhibitors causes a decrease in hyaluronan production and expression and a decrease in hyaluronan-dependent COM crystal binding to the apical cell membrane of regenerating MDCK-I cells. Subsequently this finding should be tested in an experimental *in vivo* model of crystal retention in the kidney.

In the general discussion of this thesis (**Chapter 6**) the possible role of crystal retention in the tubular lumen in kidney stone disease is discussed. The main reasons to expand our findings and continue further research are described by emphasizing on the clinical relevance of

retention of crystals in tubular nephrocalcinosis, because there are indications that tubular nephrocalcinosis could cause irreversible damage to nephrons. A number of suggestions for future research are being given.

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Samenvatting

Vorming van nierstenen is de afgelopen decennia toegenomen. In Noord-Amerika en Europa wordt het aantal nieuwe gevallen per jaar geschat op ongeveer 0.5%. Het risico voor blanke mannen en vrouwen om in hun leven een steen te krijgen is respectievelijk 15 en 6% [1-3]. Patiënten die nierstenen vormen (zogenaamde niersteenvormers) kunnen worden onderverdeeld op basis van de samenstelling van hun steen. De meest voorkomende stenen zijn calciumhoudende stenen die bestaan uit calciumoxalaat en/of calciumfosfaat. Een niersteen kan flankpijn en bloed in de urine (hematurie) veroorzaken. Wanneer een steen vanuit de nier door een urineleider naar de blaas migreert kan dit gepaard gaan met helse pijn in de flank uitstralend naar de onderbuik, een karakteristiek ziektebeeld dat een niersteenkoliek wordt genoemd. Kleine steentjes worden doorgaans spontaan geloosd maar voor grotere fragmenten zijn vaak interventies nodig.

In de behandeling van nierstenen is de introductie van ESWL ("extracorporeal shock wave lithotripsy") begin jaren '80 een doorbraak geweest en op dit moment is ESWL doorgaans de eerste keus van behandeling voor nierstenen. Daarnaast heeft de ontwikkeling van minimaal invasieve technieken met behulp van endoscopisch instrumentarium een grote rol gespeeld in de behandeling van stenen in de urinewegen (percutane nefrolitholapaxie, ureterorenoscopie, laserlithotripsie etc.). Hierdoor behoort open steenchirurgie (vrijwel) tot het verleden.

De technieken om stenen te behandelen zijn in de afgelopen decennia dus duidelijk verbeterd. Dit heeft echter niet geleid tot een afname van het ontstaan van nierstenen [4-6]. Feitelijk is behandeling ook gericht op bestrijding van symptomen doordat stenen worden verwijderd en niet gericht op preventie van steenvorming. Het risico dat een persoon in zijn of haar leven opnieuw een steen vormt is ca. 50% in 10 jaar. Dit proefschrift handelt over de vraag hoe steenvorming in de urinewegen voorkomen zou kunnen worden. Hiervoor is kennis over het ontstaan van stenen nodig en die is nog vrij beperkt. Recidiverende steenvormers behoren geanalyseerd te worden of er bij hen een metabole oorzaak ten grondslag ligt aan een verhoogd risico op de vorming van stenen zoals hypercalciurie (een verhoogde calciumuitscheiding in de urine) of hypocitraturie (een verlaagde concentratie citraat in de urine). Dit kan leiden tot specifieke dieetadviezen en medicatie om de recidiefkans te verkleinen [1, 2]. In een deel van de patiënten wordt er echter geen metabole oorzaak gevonden en deze patiënten worden daarom zogenaamde idiopathische recidiverende steenvormers genoemd. Het feit dat er patiënten zijn die herhaaldelijk stenen maken waarvoor geen oorzaak wordt gevonden onderschrijft dus het gebrek aan kennis hierover en het nut van verder onderzoek hiernaar [5].

Nierstenen zijn samengesteld uit ontelbaar veel kristallen en organisch materiaal. Het onderzoek naar de vorming van nierstenen was historisch vooral gericht op factoren die de samenstelling van de urine beïnvloeden, waardoor er een verhoogde kans is op de vorming en agglomeratie van kristallen in de urine. Er zijn factoren die de vorming en agglomeratie

van kristallen in de urine bevorderen ("promoters") of remmen ("inhibitors"). Van alle urinebestanddelen die zijn onderzocht is er (behalve van citraat) van geen enkele duidelijk bewijs geleverd dat zij een overtuigende of klinisch toepasbare rol te spelen in de vorming van nierstenen [7, 8].

Wanneer er een niersteen is ontstaan zijn er blijkbaar kristallen achtergebleven in de nier om deze steen te vormen. Kristallen worden in het distale nephron (figuur 1.1) gevormd en worden meegevoerd naar het verzamelsysteem en met de urine tijdens het plassen geloosd. Vanwege het feit dat de aanwezigheid van kristallen in de urine een normaal fysiologisch fenomeen is en voorkomt bij zowel steenvormers als niet-steenvormers, is geopperd dat het achterblijven van kristallen in de nier ("crystal retention") een cruciale rol zou moeten spelen bij het ontstaan van nierstenen [9]. Deze hypothese heeft sinds de jaren '80 geleid tot onderzoek van het tubulusepitheel dat de luminale zijde van het distale nephron bekleedt. Het doel was om moleculen aan het oppervlak van tubuluscepitheelcellen te identificeren waaraan kristallen binden en hierdoor een rol zouden kunnen spelen in het achterblijven van kristallen in de nier [9].

De Rotterdamse onderzoeksgroep onder leiding van Dr. Carl Verkoelen heeft sinds de jaren '90 celkweekexperimenten uitgevoerd om deze vraag te beantwoorden. Het bleek dat er geen kristallen binden aan het oppervlak van MDCK-I cellen in celkweek als zij zijn uitgegroeid tot confluente monolagen waarin tight junctions zijn gevormd. Aan prolifererende cellen in kweek tijdens groei en aan regenererende cellen tijdens wondheling daarentegen binden kristallen zeer sterk. Het celoppervlak van deze geactiveerde cellen was blijkbaar anders, waardoor de verhoogde affiniteit om kristallen te binden ten opzichte van cellen in confluente monolagen werd verklaard [10]. In 2000 werd door Verkoelen *et al*. aangetoond dat calciumoxalaatkristallen binden aan deze cellen omdat zij hyaluronzuur tot expressie brengen [11]. Hyaluronzuur is een hoog moleculair gewicht polysacharide die het hoofdbestanddeel is van pericellulaire matrices (PCMs) en heeft uitstekende kristalbindende eigenschappen door zijn negatieve lading door de carboxyl(COO^-)groep van glucuronzuur (GlcUA) [12, 13]. PCMs komen onder andere tot expressie bij prolifererende en migrerende cellen tijdens groei en embryogenese, regenererende cellen tijdens wondheling en bij kankercellen [14]. Vervolgens zijn deze experimenten herhaald in primaire celkweken van humane tubulusepitheelcellen door Verhulst *et al*. en deze bevestigden deze resultaten [15]. Humane tubulusepitheelcellen die calciumoxalaatkristallen binden brengen niet alleen hyaluronanzuur tot expressie aan de apicale celmembraan, maar ook de receptor voor hyaluronzuur, het transmembraan eiwit CD44 en een andere ligand van CD44, namelijk osteopontin (OPN) [16, 17]. In het artikel door Verhulst *et al*. is tevens expressie van hyaluronzuur in nierweefsel onderzocht. Hyaluronzuur komt onder normale omstandigheden nauwelijks tot expressie in de cortex. Tijdens verschillende inflammatoire nierziektes daarentegen is er in de cortex een verhoogde expressie van hyaluronzuur aangetoond, maar

dit wordt beschreven in het interstitium en niet in de niertubuli [18-22]. Verhulst *et al*. hebben voor het eerst aangetoond dat hyaluronzuur in de nier ook tot expressie komt aan de luminale celmembraan in renale tubuli in een door postrenale obstructie beschadigde humane nier en in een rattennier die beschadigd was als gevolg van ischemie [15].

Naar aanleiding van deze bevindingen werd de hypothese geformuleerd dat het risico dat kristallen achterblijven in de nier verhoogd is wanneer tubulusepitheelcellen in het distale nephron hyaluronzuur tot expressie brengen.

Om deze hypothese *in vivo* te toetsen werd een rattenexperiment uitgevoerd (**Hoofdstuk 2**). Hiervoor was er een model nodig waarin zowel de vorming van calciumoxalaatkristallen in de urine werd geïnduceerd als schade aan de niertubuli. Er werd een experiment uitgevoerd waarin ratten ethyleenglycol kregen toegevoegd aan het drinkwater en deze werden vergeleken met ratten die gewoon drinkwater kregen. Ethyleenglycol is een precursor van oxaalzuur en veroorzaakt daardoor een verhoogde concentratie oxaalzuur in de urine (hyperoxalurie) waardoor calciumoxalaatkristallen in de urine worden gevormd. Daarnaast is ethyleenglycol schadelijk voor de nier, wat waarschijnlijk toegeschreven moet worden aan de ethyleenglycol metabolieten glycoaldehyde and glyoxylate [23]. Het bleek dat ethyleenglycol vrijwel direct calciumoxalaatkristalvorming in de urine veroorzaakt. Calciumoxalaatkristallen bleven echter niet in de nier achter op het moment dat er (nog) geen tekenen waren van tubulaire schade/regeneratie. Vanaf het moment dat ethyleenglycol milde schade had veroorzaakt en er beschadigde, regenererende tubulusepitheelcellen werden vastgesteld, bleven kristallen achter in de nier. Calciumoxalaatkristallen in de nier werden aangetroffen aan de luminale celmembraan van beschadigde/regenererende tubulusepitheelcellen die hyaluronzuur, OPN en CD44 aan de luminale celmembraan tot expressie brengen [24]. Dit experiment toonde voor de eerste keer *in vivo* aan dat het achterblijven van kristallen in de nier geassocieerd is met de expressie van hyaluronzuur aan de luminale celmembraan van beschadigd/regenererend tubulusepitheel [24].

Hyaluronzuurexpressie in de cortex van de nier is sterk verhoogd tijdens verschillende inflammatoire nierziekten, maar wordt voornamelijk geobserveerd in het corticale interstitium [21, 22, 24]. Omdat in het ethyleenglycol rattenexperiment hyaluronzuur niet alleen verhoogd tot expressie komt in het interstitium van de cortex maar ook aan de luminale celmembraan van het tubulusepitheel, werd in celkweek onderzocht wat de rol van tubulusepitheelcellen hierin zou kunnen zijn. Hiervoor werden experimenten uitgevoerd met MDCK-I cellen en primaire celkweken van humane tubulusepitheelcellen tijdens proliferatie en wondheling. Cellen werden gekweekt op semipermeabele filters in een 2-compartiment systeem (**Hoofdstuk 3**) [25]. In dit model kan het bovenste kweekmedium aan de apicale zijde van de cellen (het apicale mediumcompartiment) en het kweekmedium onder de cellen (het basolaterale medium

compartiment) afzonderlijk worden geanalyseerd. Het bleek dat tijdens groei hyaluronzuur en CD44 selectief tot expressie komen aan de apicale celmembraan van prolifererende cellen, wat gepaard ging met een verhoogde expressie van HAS2 mRNA en een licht verlaagde expressie van HAS3 mRNA. Zodra cellen uitgegroeid waren tot confluente monolagen waarin tight junctions waren gevormd kwam hyaluronzuur niet meer tot expressie, en CD44 aan de basolaterale celmembraan. Tijdens wondheling komen CD44 en hyaluronzuur ook tot expressie aan de apicale celmembraan van regenerende cellen aan wondranden. De secretie van hoog moleculair gewicht hyaluronzuur is eveneens verhoogd tijdens proliferatie en regeneratie en sterk gepolariseerd en gericht naar het apicale mediumcompartiment [25]. Op basis van de bevindingen dat tijdens groei en wondheling hoog moleculair gewicht hyaluronzuur selectief naar het apicale mediumcompartiment wordt gesecreteerd en aan de apicale celmembraan tot expressie komt, werd uit deze resultaten geconcludeerd dat het waarschijnlijk is dat tijdens inflammatoire aandoeningen van de nier de verhoogde synthese van hyaluronzuur door geactiveerde tubulusepitheelcellen niet bijdraagt aan de verhoogde expressie van hyaluronzuur in het interstitium van de cortex, maar een rol speelt tijdens celgroei en herstel in de niertubuli [25].

Uit deze *in vitro* en *in vivo* experimenten werd geconcludeerd dat kristallen in het lumen van het distale nephron alleen binden aan prolifererende of regenererende tubulusepitheelcellen die hyaluronzuur, CD44 en OPN aan de luminale celmembraan tot expressie brengen. Om de klinische relevantie van deze bevinding te evalueren werden twee patiëntenpopulaties onderzocht waarvan bekend is dat er kristallen worden gevormd en achterblijven in de tubuli (**Hoofdstuk 4**) [26]. Deze klinische conditie heet tubulaire nephrocalcinosis en wordt geobserveerd in nieren van tevroeggeborenen [27] en van niertransplantatiepatiënten [28]. Nieren van beide patiëntenpopulaties bevatten prolifererende of regenererende tubulusepitheelcellen, omdat de nier in ontwikkeling is tot week 36 van de zwangerschap en in transplantaatnieren schade/regeneratie optreedt als gevolg van ischemie, nefrotoxische medicatie en chronische rejectie. In nierweefsel van tevroeggeborenen werd tijdens de ontwikkeling van de nier van week 15 tot en met week 40 hyaluronzuur en OPN expressie aangetoond aan de luminale celmembraan in tubuli van zich ontwikkelende nephronen [26]. In transplantatienieren werd in opeenvolgende biopsieën 12 en 24 weken na transplantatie eveneens expressie van hyaluronzuur en OPN aan de luminale celmembraan van distale tubuli waargenomen [26]. In zowel nierweefsel van tevroeggeborenen die langer dan vier dagen leefden als van niertransplantatiepatiënten werden met behulp van von Kossa-kleuring calciumhoudende kristallen aangetoond in de tubuli en deze waren geassocieerd met tubuli waarin hyaluronzuur en OPN aan de luminale celmembraan tot expressie kwam. Het aantal patiënten na transplantatie waarin kristallen in het lumen van tubuli werden aangetoond steeg van 20% na 12 weken naar 60% na 24 weken. De waarnemingen in beide patiëntengroepen suggereren dat het achterblij-

ven van kristallen in de tubuli wordt voorafgegaan aan de expressie van de kristalbindende moleculen hyaluronzuur en OPN [26].

Ten slotte stelden we ons de vraag of er mogelijkheden zouden kunnen zijn om de binding van kristallen aan hyaluronzuur aan het oppervlak van tubulusepitheelcellen te remmen. Er zijn aanwijzingen in verschillende celtypes dat hyaluronzuur synthese tijdens inflammatie gestimuleerd wordt door prostaglandine E_2 (PGE_2) [29-31]. Cyclooxygenase (COX) isoenzymen stimuleren de vorming van PGE_2. COX-1 komt constitutief tot expressie in de nier, COX-2 daarentegen is de induceerbare isovorm die opgereguleerd is tijdens onder andere inflammatie [32-35]. Daarom hebben we in MDCK-I cellen in kweek het effect onderzocht van COX inhibitors, of zogenaamde NSAIDs (non-steroidal anti-inflammatory drugs), op calciumoxalaat monohydraat (COM) kristal binding, PGE2 secretie, hyaluronzuur secretie, hyaluronzuur expressie, lactaat dehydrogenase (LDH) secretie en herstel van transpitheliale weerstand (**Hoofdstuk 5**). Het bleek dat zowel de niet-selectieve COX-1/COX-2 inhibitor indomethacine als de selectieve COX-2 inhibitors nimesulide en meloxicam de binding van COM kristallen aan het oppervlak van regenererende MDCK-I cellen aan de wondranden van een door een kras beschadigde monolaag remmen, al bij lage doses die geen invloed hadden op het herstel van de transepitheliale weerstand tijdens wondheling van de cellagen. Remming van PGE_2 productie door COX-inhibitors leidde tot verminderde expressie en productie van hyaluronzuur en verminderde hyaluronzuurafhankelijke binding van COM kristallen aan de apicale celmembraan van regenererende MDCK-I cellen. Deze bevinding zou vervolgens getest moeten worden in een experimenteel diermodel.

In de algemene discussie van dit proefschrift (**Hoofdstuk 6**) wordt nader ingegaan op welke rol het achterblijven van kristallen in de niertubuli zou kunnen spelen in niersteenziekte. De belangrijkste redenen om het onderhavige onderzoek te continueren worden benadrukt door in te gaan op de klinische relevantie van het achterblijven van kristallen in tubulaire nephrocalcinosis, omdat er aanwijzingen zijn dat tubulaire nephrocalcinosis schadelijk is voor nephronen. Er worden tevens suggesties gedaan voor vervolgstudies.

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The OHF is a voluntary, not-for-profit health organization founded in 1989 to promote research to find a cure for oxalosis, primary hyperoxaluria and related stone disease. I greatly thank the OHF for their financial support.
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Prof.dr. A.J. van der Heijden, bedankt voor het zitting nemen in de kleine promotiecommissie en voor de beoordeling van het manuscript, evenals de overige leden Dr. J.W. Groothoff, Prof. dr. J.N.M. IJzermans en Prof.dr. A.A.B. Lycklama à Nijeholt.

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List of publications

Asselman M, Van Ballegooijen ES, Bangma CH, Verkoelen CF
Cyclooxygenase-2 inhibitors decrease hyaluronan-dependent calcium oxalate crystal binding to regenerating renal tubular cells.
Submitted 2008

Asselman M and Verkoelen CF
Fructose intake as a risk factor for kidney stone disease.
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Verhulst A, **Asselman M**, De Naeyer S, Vervaet BA, Mengel M, Gwinner W, D'Haese PC, Verkoelen CF, De Broe ME
Preconditioning of the distal tubular epithelium of the human kidney precedes nephrocalcinosis.
Kidney Int 2005 Oct;68(4):1643-7

Asselman M, Verhulst A, Van Ballegooijen ES, Bangma CH, Verkoelen CF, De Broe ME
Hyaluronan is apically secreted and expressed by proliferating or regenerating renal tubular cells.
Kidney Int 2005 Jul;68(1):71-83

Klijn AJ, **Asselman M**, Vijverberg MA, Dik P, De Jong TP
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Schepers MS, **Asselman M**, Duim RA, Romijn JC, Schröder FH, Verkoelen CF
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Verhulst A, **Asselman M**, Persy VP, Schepers MS, Helbert MF, Verkoelen CF, De Broe ME
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Asselman M and Verkoelen CF
Crystal-cell interaction in the pathogenesis of kidney stone disease.
Curr Opin Urol 2002 Jul;12(4):271-6

Appendix: Color images

CHAPTER 2

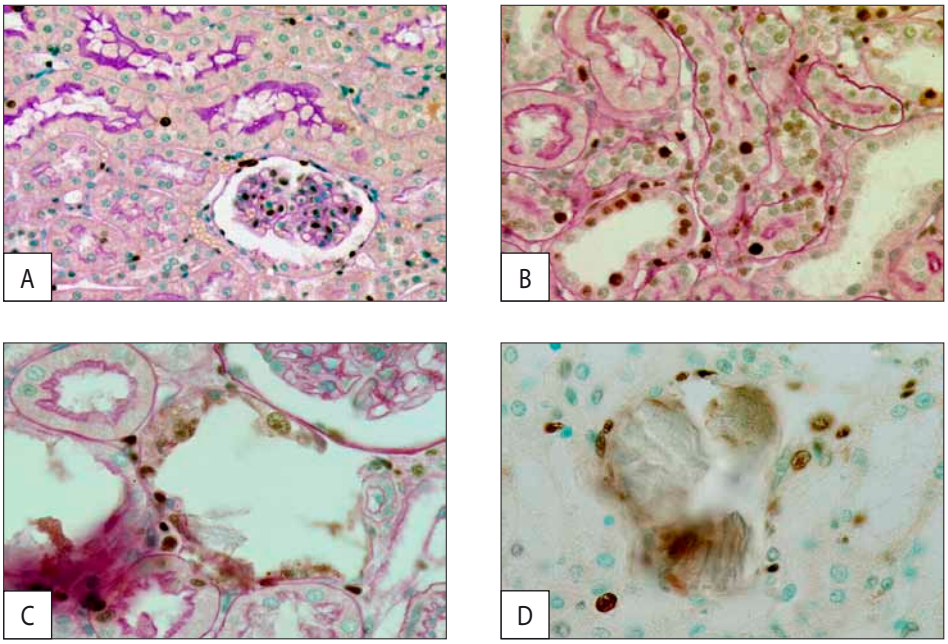

Figure 2.4
Proliferating cell nuclear antigen (PCNA) staining in a kidney of a control rat (A) and of a rat that received 0.75% EG for 8 d (B through D).

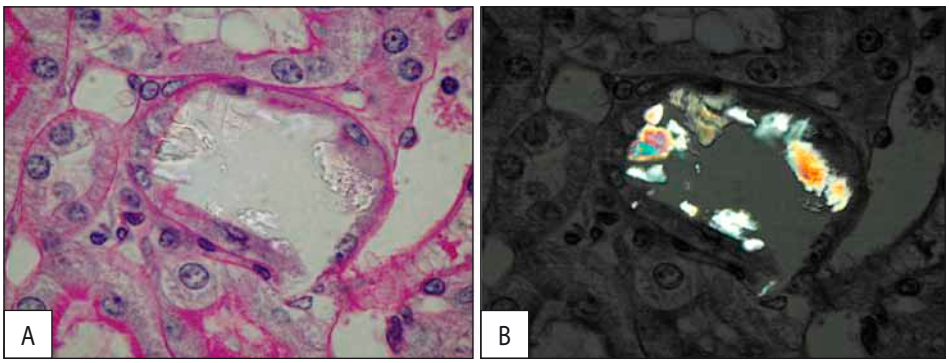

Figure 2.7
PAS and methyl green–stained renal tissue section of a rat treated with 0.75% EG for 4 d by optical (A) or polarized (B) light microscopy.

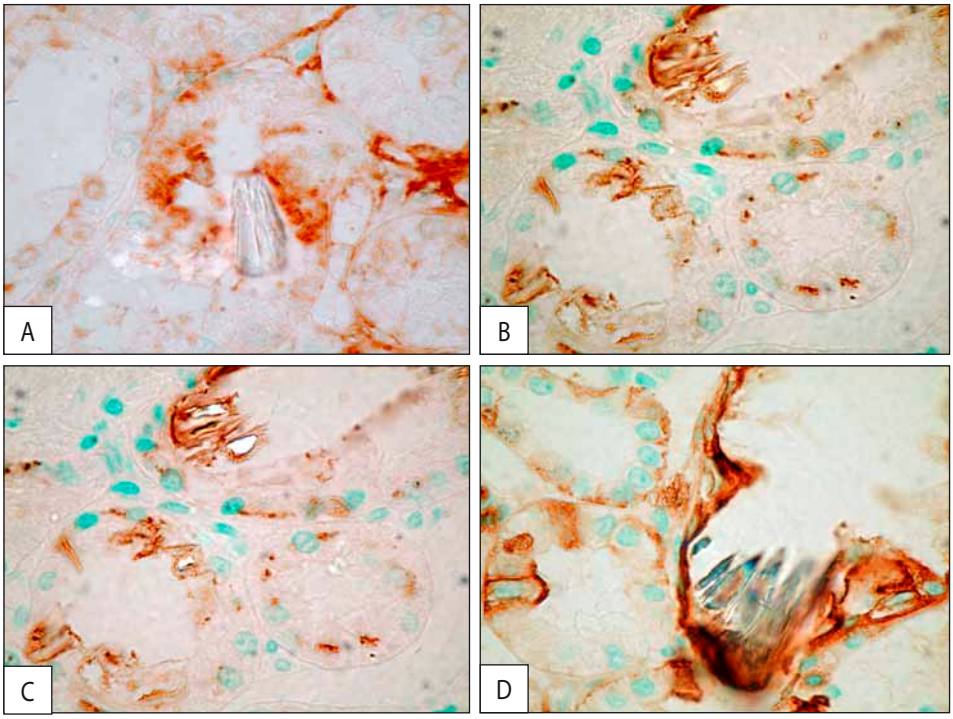

Figure 2.10

(A) The outer stripe of the outer medulla (OSOM) stained for HA of a rat with 0.75% EG in the drinking water for 8 d. (B and C) The OSOM stained for OPN after 8 d of 0.75% EG. (D) The cortex stained for CD44 after 8 d of 0.75% EG.

CHAPTER 3

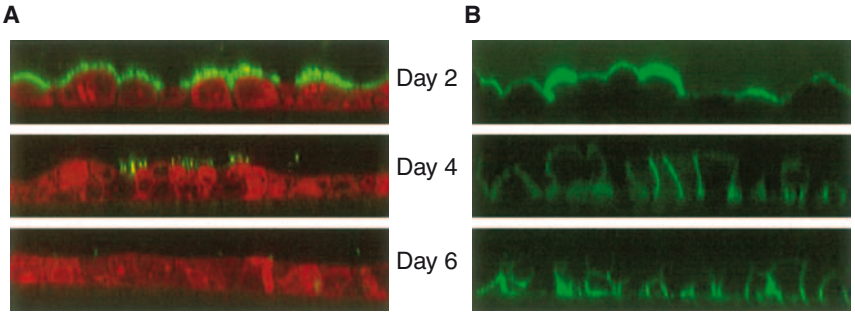

Figure 3.8

Confocal microscopy images of the membrane localization of hyaluronan [biotinylated hyaluronan binding protein (bHABP)-fluorescein isothiocyanate (FITC) + propidium iodide] (A) and C44 (IM7-FITC) (B) during the development of Madin-Darby canine kidney strain I (MDCK-I) cultures into confluent monolayers with functional tight junctions.

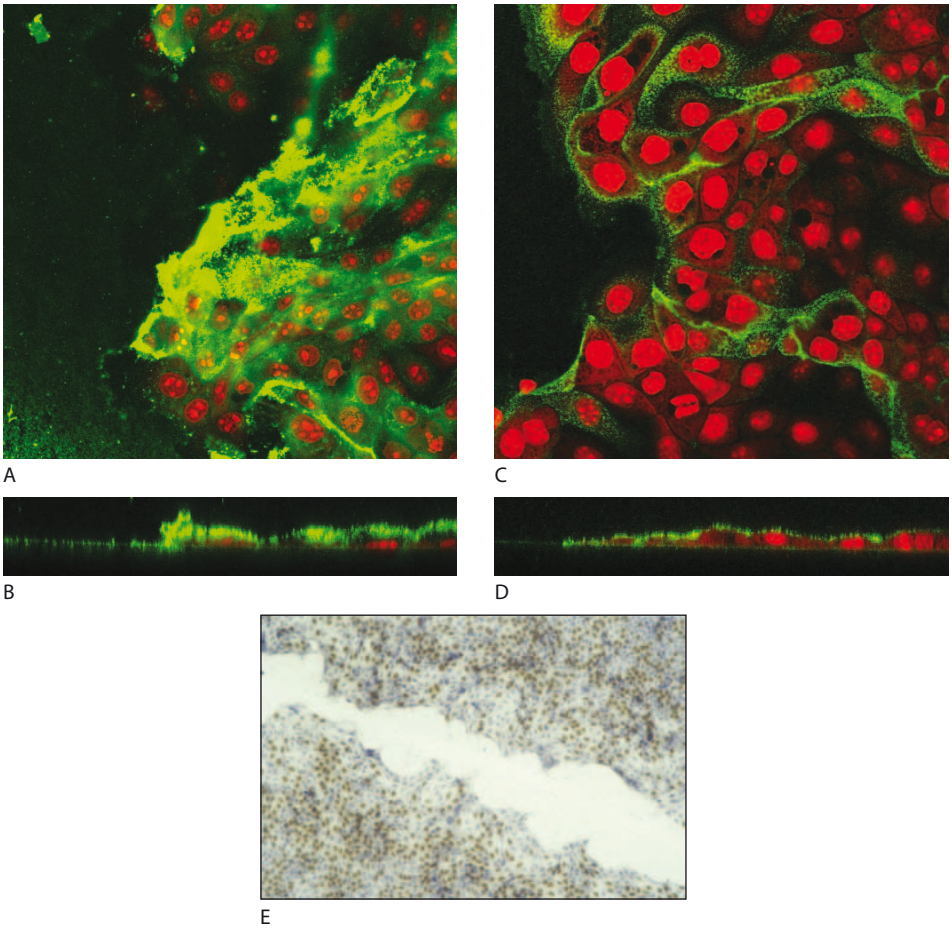

Figure 3.9

Confocal microscopic images of hyaluron [biotinylated hyaluron binding protein (bHABP)-fluorescein isothiocyanate (FITC)] (A and B) and CD44 (IM7-FITC) (C and D) expressed by flattened cells at the border of the wound. (E) Light microscopic image of 5-bromo-2′-deoxyuridine (BrdU)-stained cells during wound healing showing few BrdU-positive cells at the leading edge of the wound and abundant staining in areas somewhat distant from the wound.

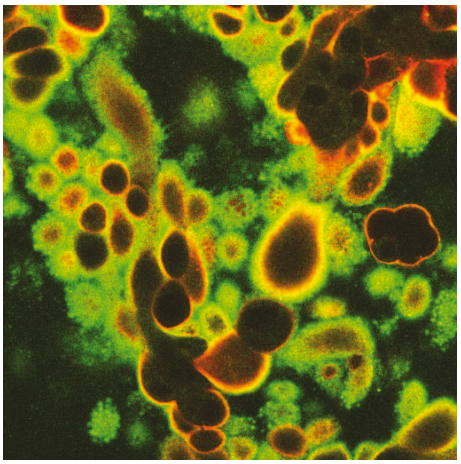

Figure 3.10

Confocal microscopy xy and xz images showing the colocalization of hyaluronan [biotinylated hyaluron binding protein (bHABP)-fluorescein isothiocyanate (FITC) (green) and its major cell surface receptor CD44 [IM7-TRITC] (red) at the apical plasma membrane of subconfluent cultures of Madin-Darby canine kidney strain I (MDCK-I) cells grown on a permeable support in a two-compartment culture system. .

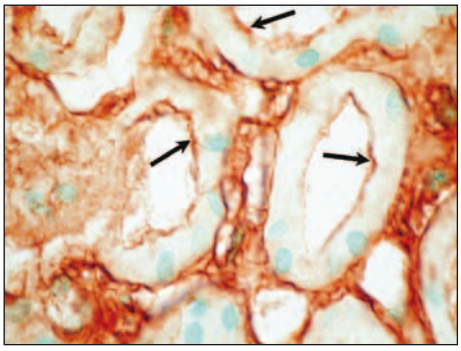

Figure 3.11

Hyaluronan staining in the cortex of a renal tissue biopsy specimen of a patient suffering from acute tubular necrosis, showing expression of hyaluronan at the luminal membrane of tubular cells and also in the cortical interstitium.

CHAPTER 4

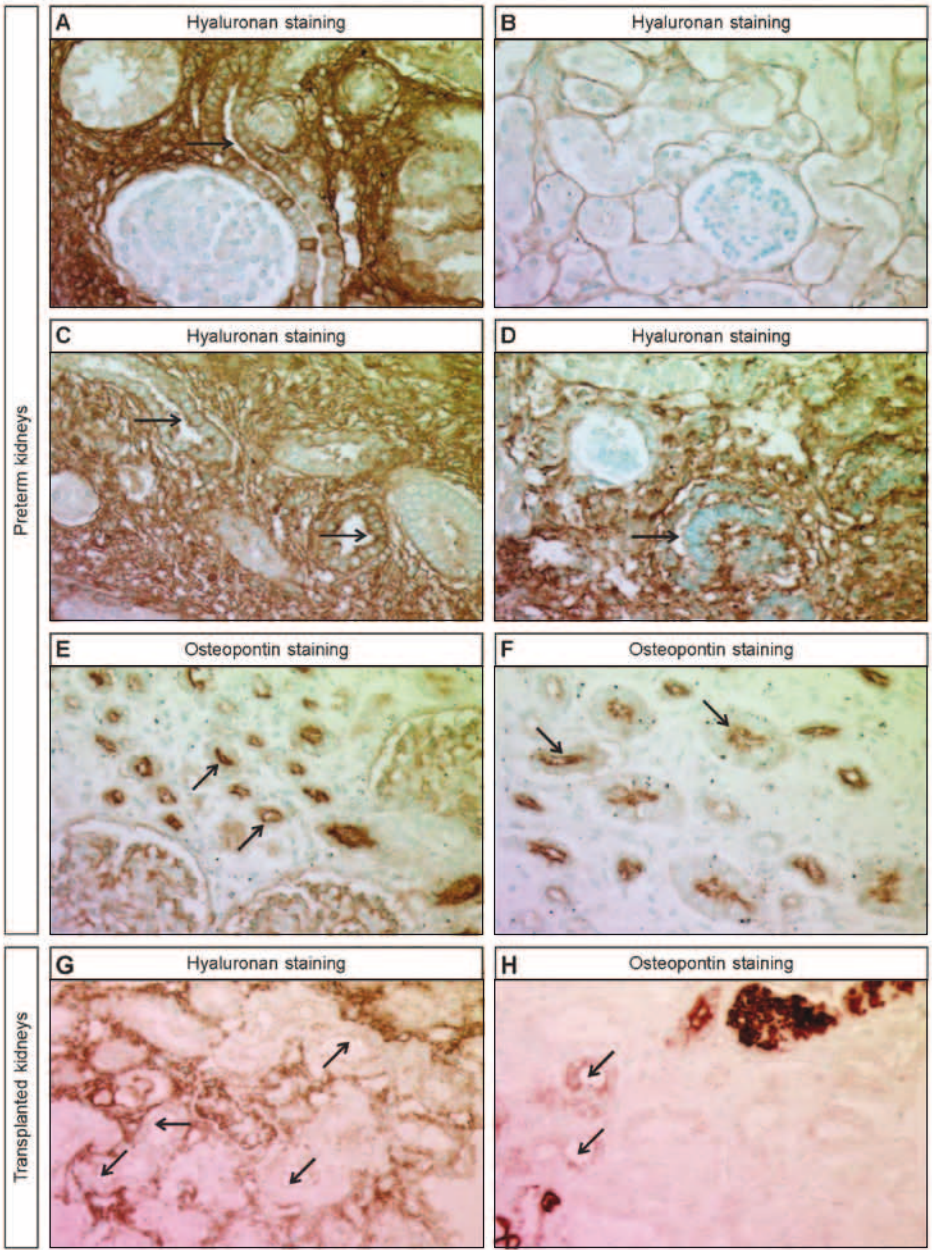

Figure 4.1

Hyaluronan and osteopontin staining on fetal (A to F) and transplanted (G and H) kidneys.

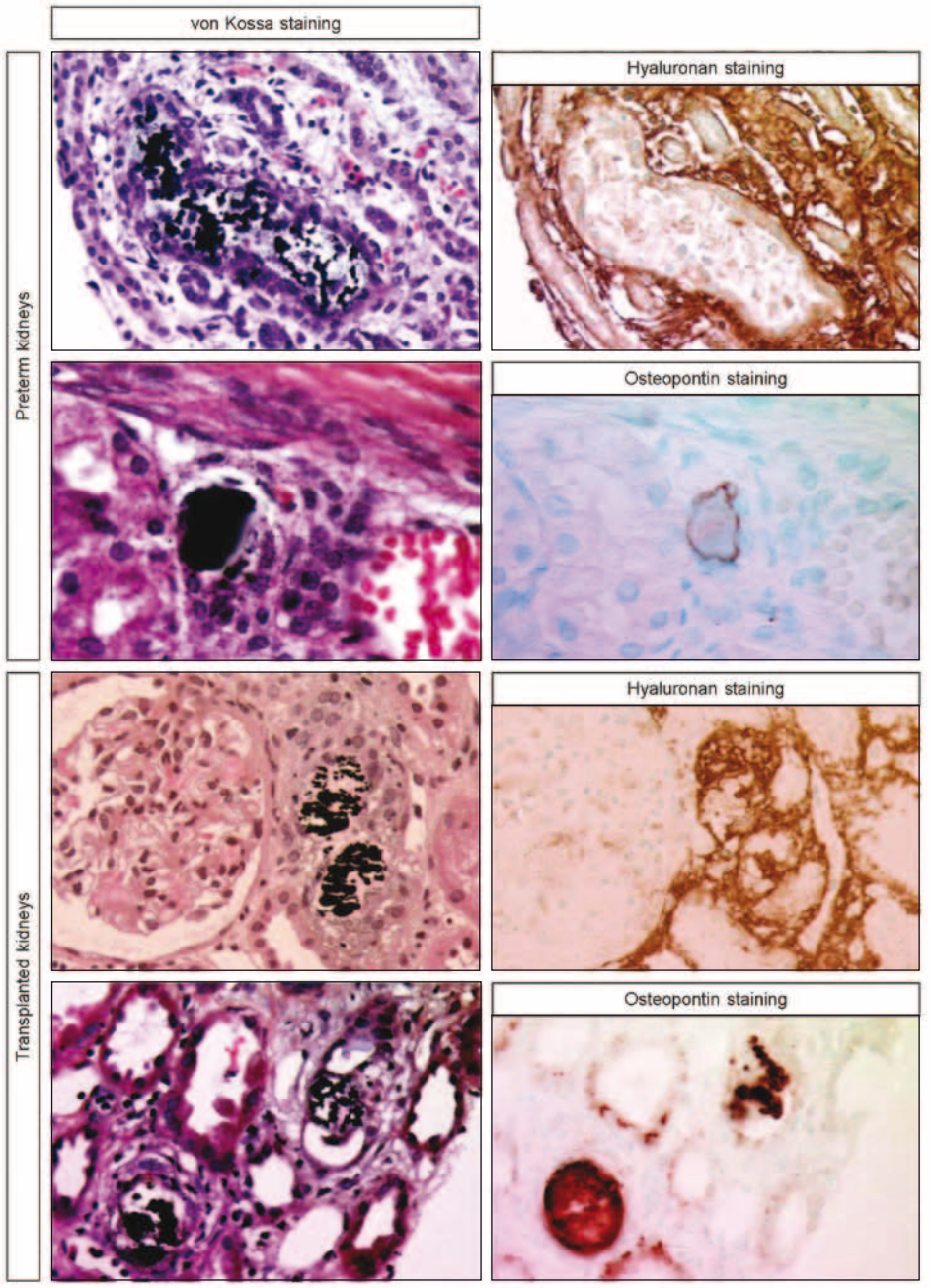

Figure 4.2

Serial sections from preterm and transplanted kidneys stained for von Kossa and hyaluronan or osteopontin.

CHAPTER 5

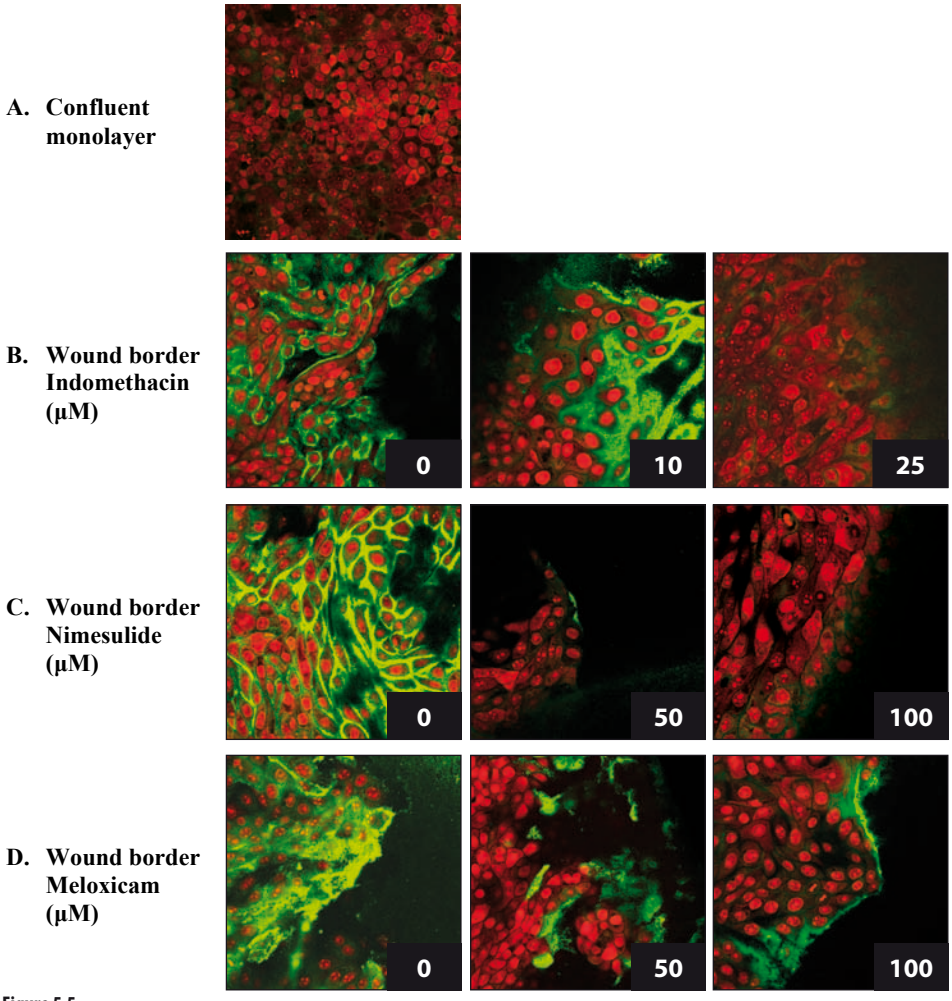

Figure 5.5

Confocal laser scanning microscopy studies.

Curriculum Vitae

Marino Asselman was born on May 22nd, 1973 in Goes, the Netherlands. After graduation from the St. Willibrordcollege in Goes in 1991 (VWO), he started medical school at the University of Utrecht. He graduated from medical school in January 1999.

In 1999 he worked as a M.D. at the department of pediatric urology at the Wilhelmina Children's Hospital, University Medical Center Utrecht (head: Dr. T.P.V.M. de Jong) and in 2000 he worked at the department of urology at the same institution (head: Prof.dr. T.A. Boon).

In 2001, after working three months as a M.D. at the department of urology of the Erasmus Medical Center Rotterdam (former head: Prof.dr. F.H. Schröder), he started working at the Josephine Nefkens Institute of the Erasmus University Rotterdam as a PhD student on the subject which has led to this thesis (supervisor: Dr. C.F. Verkoelen). The research on the earliest events in kidney stone formation at the department of urology has been done in close collaboration with co-workers at the department of Nephrology-Hypertension, University of Antwerp, Belgium (former head: Prof.dr. M.E. de Broe)

In 2004 he started his residency in general surgery at the St. Franciscus Gasthuis, Rotterdam (mentor: Dr. C.H. Wittens). From 2006 he is currently working as a resident in urology at the department of urology of the Erasmus Medical Center Rotterdam (head: Prof.dr. C.H. Bangma / mentor: Dr. G.R. Dohle). Next year he will continue his training at the department of urology of the St. Franciscus Gasthuis, Rotterdam (mentor: Dr. J.H. Blom) to finish his training in urology and become an urologist.

Marino Asselman received the "Van Stockum prize 2005" from the Dutch Urological Association for the best publication in the period 2003 – 2004 (J Am Soc Nephrol 2003 Dec, 14:3155-66), and was recently invited to serve a three-year term as an editorial board member of Kidney International.

He shares his life with Ineke C.M. Noyons and their three children Sarah (2003), Michiel (2005) and Alexander (2007).