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PATHOLOGICAL AND IMMUNOCYTOCHEMICAL CHANGES IN CHRONIC CALCIUM OXALATE NEPHROLITHIASIS IN THE RAT

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Abstract

In the present study, we exposed rats to a crystal-inducing diet (CID) consisting of vitamin D_3 and 0.5% ethylene glycol (EG), and we investigated histologically the kidney damage induced by the deposition of calcium oxalate (CaOx) crystals. After 28 days, 50% of the animals had renal CaOx crystals, of which 60% also had small papillary stones. Most crystals were present in the cortex. The occurrence of these crystals coincided with morphological and cytochemical changes: glomerular damage, tubular dilatation and necrosis, and an enlargement of the interstitium. The number of epithelial and interstitial cells positive for the proliferating cell nuclear antigen (PCNA) was increased. Tamm-Horsfall protein (THP) was not only demonstrable in the thick ascending limb of the loop of Henle (TAL), but also frequently in glomeruli, in the proximal tubular epithelium, and in the papilla. In the lumen of the tubular system, it was associated with urinary casts. Reflection contrast microscopy (RCM) showed that the crystals were coated with a thin layer of THP. In spite of the high urinary oxalate concentrations, the above described cellular changes were not observed in CID-fed rats without renal crystals. We conclude, therefore, that in the kidney, the retained CaOx crystals rather than the urinary oxalate ions are responsible for the observed morphological and immunocytochemical changes.

Key Words: Calcium oxalate, urolithiasis, rat, vitamin D, ethylene glycol, Tamm-Horsfall protein, immunocytochemistry, renal pathology.

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Introduction

To understand the pathobiology of calcium oxalate (CaOx), nephrolithiasis rats have frequently been used. Their oxalate metabolism is comparable to man, although a fairly severe hyperoxaluria is required to induce intratubular crystal formation [23].

In man, hyperoxaluria and hypercalciuria are recognized risk factors for CaOx nephrolithiasis [13]. An increase in calcium and oxalate excretion will result in urinary supersaturation and enhance the potential for crystallization. Crystal formation, however, also depends on the presence of promoters and inhibitors. Low molecular weight compounds, such as citrate, magnesium, and pyrophosphate, and high molecular weight compounds, such as glycosaminoglycans (GAG's), nephrocalcin, osteopontin and Tamm-Horsfall protein (THP), are both involved in the inhibition of crystal nucleation, growth and agglomeration [42].

The amount of oxalate in urine is determined by dietary intake, gut absorption, endogenous production, and renal metabolism [23, 32, 41]. Increased serum levels of vitamin D have been found in 10-30% of all CaOx stone formers [19, 32, 38]. In rats, administration of vitamin D_3 and ethylene glycol (EG) induces chronic hyperoxaluria, crystalluria and renal deposition of CaOx crystals [11]. The underlying mechanism of this diet is thought to be as follows: EG is an oxalate precursor and vitamin D_3 induces an enhanced calcium absorption in the intestine [2, 32]. The induced hyperoxalemia leads to an increased entry of oxalate into the urine through both glomerular filtration and tubular secretion and to an increased deposition of CaOx in the kidney.

In rats, acute hyperoxaluria is associated with crystalluria and a release of renal enzymes [20, 21]. In these studies [20, 21], an increase in urinary oxalate coincided with a proportional elevation of the amount of CaOx crystals. These crystals become coated with cellular and urinary proteins, which promote their aggregation [4, 22, 23]. Free floating CaOx crystals are harmless. But, when crystals or crystal aggregates attach to the tubular epithelium, they may grow in size and dis-

turb the integrity of the renal tubular epithelium and the interstitium [4, 9, 28]. The appearance of CaOx crystals in the renal tubule has been associated with necrosis and enhanced cell proliferation of the tubular cells [14, 25, 27, 28]. This results in shedding of cellular components into the tubular lumina, which can initiate a further nucleation of CaOx salts. Experiments with cultured cells have shown that oxalate ions can be injurious and that uptake of CaOx crystals is associated with cell proliferation. This interaction with the crystals leads to detachment of the cells from the substrate and release of membranes and various enzymes into the culture medium [15, 31].

In a recent study, we paid attention to the EG-vitamin D_3 rat model and investigated the effect of the EG concentration in the drinking water on the deposition of CaOx crystals in the kidney [11]. In that study, it was found that an EG concentration of 0.8% or higher led to a consistent deposition of crystals in the kidney. The present study describes, by histology and immunocytochemistry, the cellular changes in the kidney, induced by a diet of vitamin D_3 and 0.5% EG. The fact that, at this low EG concentration, renal crystals were encountered in only a part of the animals, enabled us, by measuring various urinary parameters, to focus our attention to the question whether the deposition of the CaOx crystals or the hyperoxaluria itself is responsible for the observed renal morphological changes.

Material and Methods

Male Wistar rats, weighing 250-300 g, obtained from the Central Animal Breeding Center (Harlan, Zeist, The Netherlands), were acclimatized for seven days. Hyperoxaluria was induced by feeding EG and vitamin D_3 for 28 days (crystal-inducing diet; CID). EG (Sigma, St. Louis, USA) was supplemented to the drinking water to a final concentration of 0.5 vol.%. The vitamin D_3 (Sigma) was dissolved in cottonseed oil at a concentration of 1 $\mu g/ml$, of which 0.5 ml was orally administrated by stomach intubation every other day. Control animals received neither EG nor vitamin D_3. All rats received a standard rat chow (Diet AM II, Hope Farms, Woerden, The Netherlands) containing rough protein {23.3 weight % (wt.%)}, fat (6.4 wt.%), rough cell compound (3.2 wt.%), and a salt mixture, consisting of phosphorus (0.48 wt.%), magnesium (0.12 wt.%), sodium (0.24 wt.%), and potassium (0.8 wt.%). The calcium content varied per batch between 0.56-0.83% (mean: 0.70%).

After 28 days of feeding, the urines of CID-fed and control rats were collected during 24 hours, separately for each animal. The rats were individually kept in metabolic cages and the urines were collected on ice without any preservative during 24 hours. For each urine, the volume and the pH were determined. The amount of urinary calcium and oxalate were assessed with the appropriate kits {obtained from, respectively, Merck (Merck, Diagnostics, Darmstadt, Germany) and Sigma} adapted to the protocol of the autoanalyser (Eppendorf-Merck ELAN autoanalyser) [11]. Urinary GAG's were measured using 1,9-dimethylmethylene blue as substrate [10]. Urinary inhibitory activity was assessed by measuring the potential of the collected urines to inhibit crystal growth and crystal agglomeration of calcium oxalate seed crystals in a metastable solution [37]. Significance of the differences was statistically analyzed, using a two-tailed Mann-Whitney U Test. The animals were anaesthetized with ether, followed by an intramuscular injection with 2.5 ml of an aqueous solution of 12 wt.% urethane. For the light microscopical (LM) morphological studies, the kidneys were fixed by perfusion with 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) as described before [4, 9]. The kidneys were excised and scanned by radiography for the presence of CaOx crystals in the cortex and medulla. Of each kidney, five small tissue blocks were excised from the cortex and medulla, dehydrated in a graded series of ethanol, and embedded in Epon as described [4, 9]. The 1 μm thick sections were stained with toluidine blue and LM examined with a Zeiss Axiophote microscope (Zeiss, Oberkochen, Germany). CaOx crystals were visualized by LM under polarized light.

For the LM and the reflection contrast microscopical (RCM) immunocytochemical studies, the tissue was fixed by perfusion as described above, using either 1.5% glutaraldehyde (THP studies) or 4% paraformaldehyde (PCNA studies) in 0.1 M cacodylate buffer (pH 7.4) as fixative. For the LM immunocytochemical studies, the whole kidneys were embedded in paraffin. Four μm thick transversal sections, containing cortical and medullary tissue (including the papilla), were deparaffinized, blocked for endogenous peroxidase activity with 3% H_2O_2 in methanol, and finally incubated with a goat polyclonal antiserum against human THP (diluted 1/4000; Cappel/Organon Teknika, Boxtel, The Netherlands) for one hour at room temperature. In control experiments, sections were incubated with non-immune goat serum (diluted 1/4000) or with diluting buffer alone {phosphate-buffered saline, containing 1% bovine serum albumin (Sigma) and 0.1% Tween 20 (PBS-BSA)}. Proliferating cells were stained for the proliferating cell nuclear antigen (PCNA) using a mouse polyclonal antibody (clone PC10, Dakopatts, Glostrup, Denmark), as described [40]. Briefly, after blocking of endogenous peroxidase activity, slides were placed in 10 mmol citric acid buffer (pH 6.0) and warmed in a 700-W microwave oven at 50% power for 15 minutes. The slides were

Table 1. Urinary parameters and renal morphological changes of control rats and rats fed a CID for 28 days. Data are expressed as mean $\pm$ SD.

| | Control rats | CID rats | |
		Without renal crystals	With renal crystals
Oxalate excretion (μmol/24 h)	10.4 $\pm$ 1.8 (n = 4)	42.3 $\pm$ 28.9 (n = 6)	75.3 $\pm$ 44.8 (n = 8)
Calcium excretion (μmol/24 h)	16.1 $\pm$ 2.6 (n = 4)	8.7 $\pm$ 2.6 (n = 7)	8.5 $\pm$ 6.2 (n = 8)
GAG concentration (μg/ml)	6.8 $\pm$ 1.5 (n = 4)	13.2 $\pm$ 3.9 (n = 7)	8.1 $\pm$ 2.9 (n = 8)
Inhibition agglomeration (%)	65.2 $\pm$ 18.1 (n = 4)	44.2 $\pm$ 15.8 (n = 7)	59.1 $\pm$ 14.9 (n = 8)
Inhibition crystal growth (%)	28.8 $\pm$ 15.3 (n = 4)	28.1 $\pm$ 9.2 (n = 7)	41.3 $\pm$ 19.6 (n = 5)
Morphological changes*	absent	absent	+ to + + + +

*+ to + + + + indicates the degree of morphological damage.

Table 2. Parameters of individual rats with renal CaOx crystals. The rats were fed a CID for 28 days.

Rat number	1	2	3	4	5	6	7	8
Presence of crystals:								
- **radiography**	papilla	papilla	papilla	nephr.	nephr.	papilla	papilla	-
- **histology***	+	N.D.	+	+ + +	+ + +	+ + +	+	+ +
Oxalate excretion (μmol/24 h)	14.3	49.9	30.4	97.3	89.6	114.2	147.4	59.6
Calcium excretion (μmol/24 h)	20.8	9.6	11.8	7.2	2.2	1.7	9.4	4.9
GAG concentration (μg/ml)	10.6	7.5	10.9	6.5	5.8	9.7	3.1	10.9
Inhibition agglomeration (%)	58.7	70.6	30.7	74.5	64.3	49.9	65.2	N.D.
Inhibition crystal growth (%)	41.8	65.9	5.3	57.1	49.1	34.5	35.2	N.D.
Morphological changes*:								
1. **Damaged glomeruli (%)[1]**	9.3	N.D.	16.0	7.1	9.5	1.6	9.3	3.4
2. **Tubular changes**								
epithelial cell damage	+/-	N.D.	+/-	+ +	+ +	+	+	+
mitotic activity	+	N.D.	+ + +	+	+	+	+	+
luminal dilatation	-	N.D.	-	+ + +	+	+ +	+ +	+ +
3. **Interstitial changes**	+ +	N.D.	+ +	+ + + +	+ +	+	+ +	+

*-, +/-, and + to + + + + indicates the degree of expression of the indicated parameter.   [1]n = 70-100

N.D. = not determined; **papilla** = crystals in the papilla; **nephr.** = nephrocalcinosis (crystals outside the calyx and the tubular system, i.e., in the cortical and/or in the medullary parenchyma).

allowed to cool for 20 minutes at room temperature and incubated with the anti-PCNA antibodies (diluted 1/150) in PBS-BSA. In control experiments, the cells were incubated with non-immune mouse serum (diluted 1/150). The slides were then washed and the bound primary antibody was detected in a two-step procedure (biotin-streptavidin amplified system; Biogenex, San Ramon, CA, USA), using biotinylated anti-IgG and streptavidin labelled peroxidase (diluted 1/50 in PBS-BSA). Subsequently, the slides were incubated with a substrate mix-

ture of 3,3'-diaminobezidine (DAB; Fluka, Buchs, Switzerland) and 0.02% H_2O_2 in PBS for 10 minutes at room temperature. All sections were counterstained with methylgreen or Mayer's hematoxylin.

For the RCM immunocytochemical studies, small tissue blocks were embedded in Lowicryl K4M (Polysciences, Eppelheim, Germany), as described by de Water et al. [12]. The immunostaining for the demonstration of THP was performed using a two-step labelling procedure [34]. Briefly, 70-90 nm thick ultrathin sections
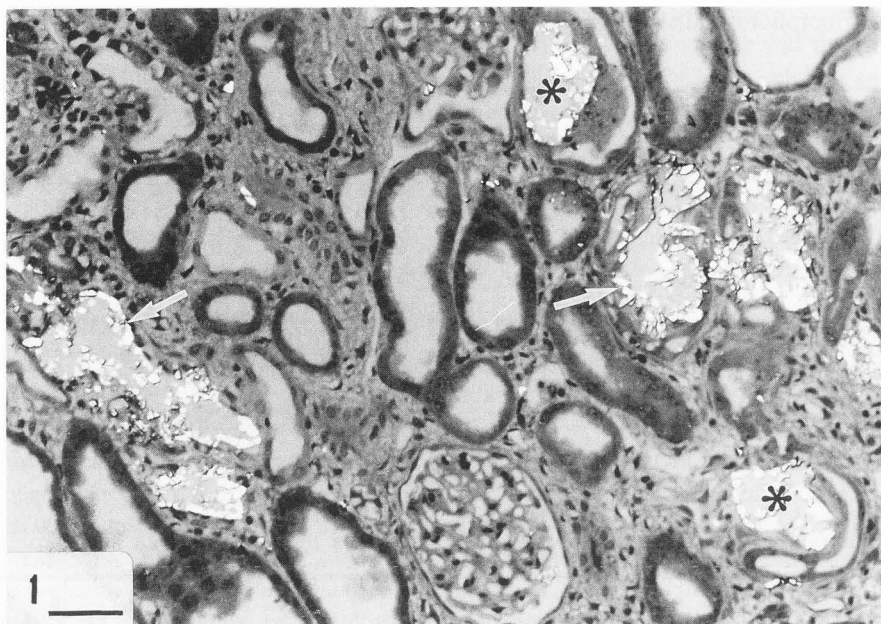
Figure 1. Micrograph showing a detail of the renal cortex of from a CID-fed rat with nephrocalcinosis. Crystals, visualized by polarized light, are present in the interstitium. A number of crystals are overgrown by the tubular epithelium. Note the enlargement of the interstitium. The tissue has been embedded in Epon and stained with toluidine blue. Bar = 70 μm.

were placed on aminosilane-coated glass slides. These sections were preincubated with PBS-BSA and then incubated with the THP antibodies (1/4000) for two hours at 37°C, followed by an indirect immunoperoxidase staining using diaminobezidine and 0.02% H_2O_2 in PBS buffer at pH 7.6. The sections were briefly counterstained with 1 wt.% aqueous light green and examined with Zeiss Axiophote microscope, equipped for epi-illumination and adapted for RCM as described by Cornelese-ten Velde et al. [7]. The ultrathin sections were observed with immersion oil, without coverslip.

Results

Urinary parameters

After 28 days of CID of vitamin D_3 and 0.5% EG, two groups of rats could be recognized: (1) rats with renal crystals (47%), and (2) rats without renal crystals (53%). Both groups were compared with rats fed only the standard chow (control rats; Table 1). All CID-fed rats showed a higher oxalate (p = 0.007) and GAG excretion (p = 0.048), but a lower calcium excretion than control rats (p = 0.0062). No obvious differences were observed in the urinary inhibitory activity of crystal agglomeration (p = 0.23) and crystal growth (p = 0.5). The group CID-fed rats with renal crystals and the group without renal crystals showed no significant differences in any of these urinary parameters (0.07 < p < 0.7). Histologically, renal morphological changes were only observed in the group of CID-fed rats with renal crystals (Table 1).

Crystal- and stone formation

The radiographs showed that within the group of CID-fed rats with renal crystals, there were two rats with CaOx crystals in the cortical- and/or in the medullary parenchyma (nephrocalcinosis) and five rats with small papillary stones (Table 2). In the rats with nephrocalcinosis, most crystals were light microscopically found in the interstitium of the cortex. Crystals were also observed in the lumina of proximal and distal tubules. These crystals regularly completely filled the tubular lumen, obstructing the tubular system of the corresponding nephron. There were also crystals that partly adhered to the tubular epithelium, and these crystals were mostly covered by a basement membrane and overgrown by epithelial cells (Fig. 1). Crystals were sometimes also observed in collecting tubules. These crystals were generally larger. In the medullary interstitium, hardly any crystals could be encountered.

Morphological changes

The presence of crystals in the cortex or medulla were associated with changes in individual nephrons and in the surrounding interstitium. This damage varied per rat, but paralleled the number of renal crystals. If by histology and by radiography no CaOx could be detected, no deviations in the renal histology were found and the tubular and interstitial cells were morphologically undamaged.

In the kidneys with crystals, roughly 2-15% of the glomeruli was morphologically changed. These changes consisted of an increase of the mesangium and a thickening of the capsule of Bowman, frequently coupled with an adhesion between the parietal and visceral layer (Fig. 2). The lumina of a number of proximal and distal tubules had collapsed. In these tubules, the intercellular
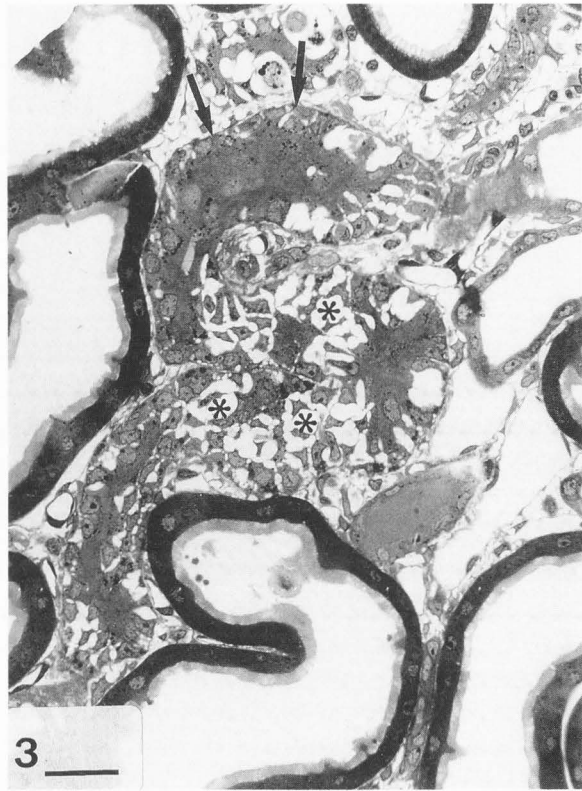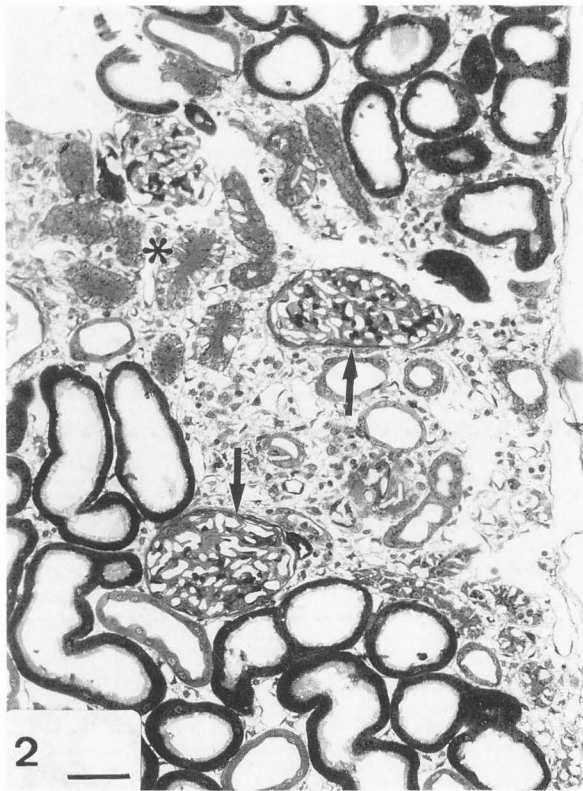
Figure 2 (at left). Micrograph showing two damaged glomeruli (arrows), both coalesced with the thickened capsules of Bowman. A number of adjacent tubules have been collapsed (*). Note the enlargement of the interstitium. Epon embedding and toluidine blue staining. Bar = 50 μm.

Figure 3 (at right). Micrograph of a collapsed tubule. Note the large intercellular spaces (*) between the epithelial cells and the intact underlying basement membrane (arrows). Bar = 24 μm.

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spaces between the epithelial cells were often grossly enlarged, such that it was difficult to distinguish these tubules from the surrounding interstitium (Fig. 3). The individual epithelial cells looked viable and, by light microscopy, the underlying basement membranes were mostly intact. The surrounding interstitium was enlarged. It contained an increased number of mononuclear cells and it showed signs of edema. The lumina of other tubules were dilated. Their epithelial cells had a flattened appearance. Since these cells had lost their brush border, it was practically impossible to determine whether these tubules were of proximal or distal origin.

In the lumen of the entire tubular system, Periodic acid-Schiff-positive, protein-like material (casts) and cell debris were frequently encountered.

Immunocytochemical changes

THP staining: In kidneys of control rats and the CID-fed rats without crystals, THP could only be localized in the epithelial of the thick ascending limb of the loop of Henle (TAL). Extracellularly, reaction product was not only found on the surface of the epithelial cells, but also sometimes in the tubular lumen. In the CID-fed rats with renal crystals, THP was regularly also demonstrable in glomeruli, i.e., in the urinary space and on the luminal surface of the parietal and visceral layer of the capsule of Bowman (Fig. 4). The affected glomeruli were morphologically more or less abnormal (Fig. 4A). THP was regularly found on the luminal surface of adjacent proximal tubules. In the lumen of the proximal and distal tubules, THP was often associated with cell debris and protein casts (Fig. 4B). It could occasionally be found in the neighbouring interstitium, mostly dispersed over a small area (Fig. 4C). CaOx crystals were less frequently found in the THP-positive tubules, but if these crystals were seen, they were positive for THP (Fig. 4D). In the papilla, THP was regularly found in the epithelial cells of the collecting tubules. If crystals or small stones were found on the papilla, they were positive for THP at the calyceal side (Fig. 4E). All interstitial crystals lacked reaction product. The RCM studies showed that all crystals present in the tubular
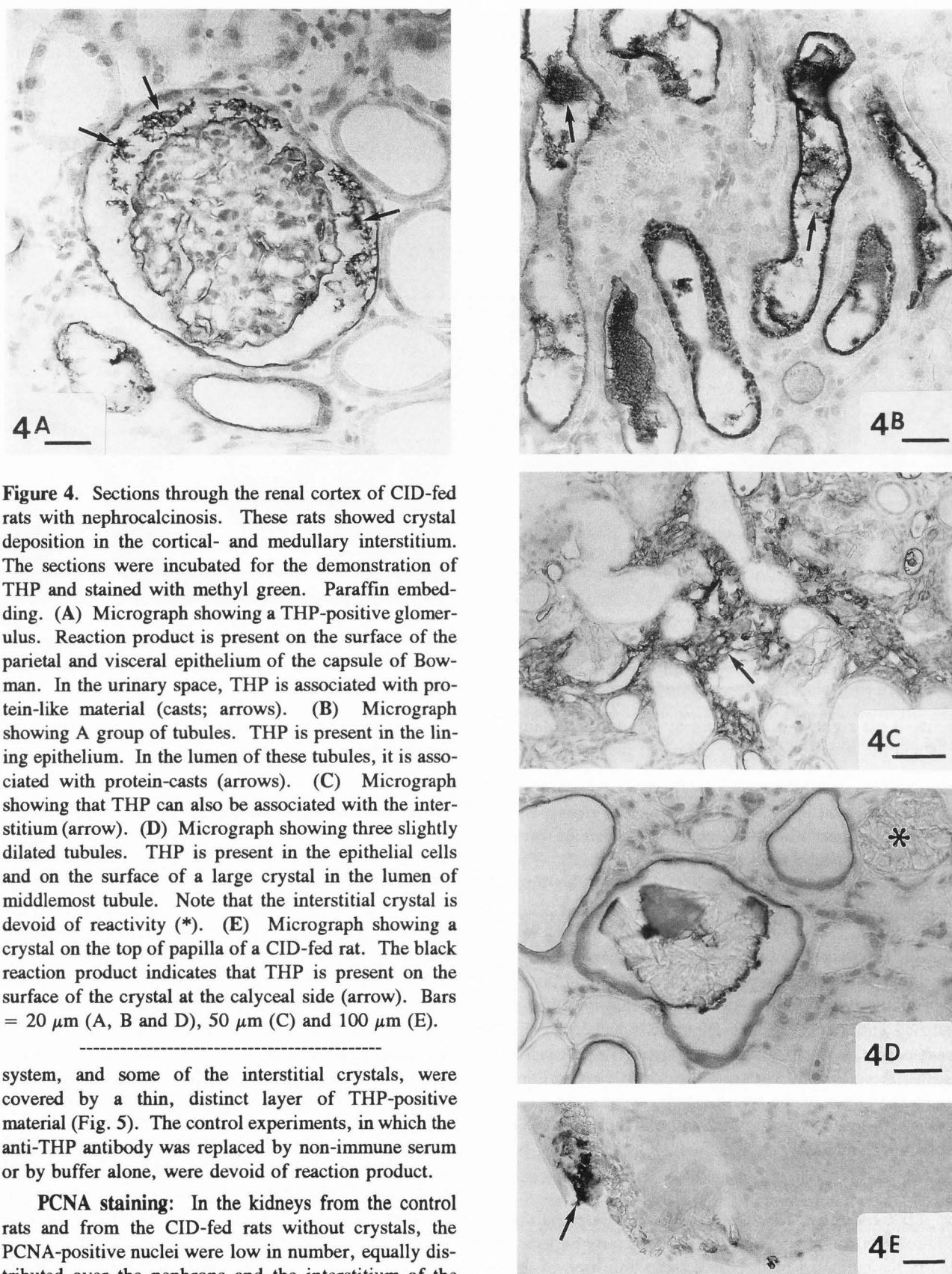
Figure 4. Sections through the renal cortex of CID-fed rats with nephrocalcinosis. These rats showed crystal deposition in the cortical- and medullary interstitium. The sections were incubated for the demonstration of THP and stained with methyl green. Paraffin embedding. **(A)** Micrograph showing a THP-positive glomerulus. Reaction product is present on the surface of the parietal and visceral epithelium of the capsule of Bowman. In the urinary space, THP is associated with protein-like material (casts; arrows). **(B)** Micrograph showing A group of tubules. THP is present in the lining epithelium. In the lumen of these tubules, it is associated with protein-casts (arrows). **(C)** Micrograph showing that THP can also be associated with the interstitium (arrow). **(D)** Micrograph showing three slightly dilated tubules. THP is present in the epithelial cells and on the surface of a large crystal in the lumen of middlemost tubule. Note that the interstitial crystal is devoid of reactivity (*). **(E)** Micrograph showing a crystal on the top of papilla of a CID-fed rat. The black reaction product indicates that THP is present on the surface of the crystal at the calyceal side (arrow). Bars = 20 μm (A, B and D), 50 μm (C) and 100 μm (E).

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system, and some of the interstitial crystals, were covered by a thin, distinct layer of THP-positive material (Fig. 5). The control experiments, in which the anti-THP antibody was replaced by non-immune serum or by buffer alone, were devoid of reaction product.

PCNA staining: In the kidneys from the control rats and from the CID-fed rats without crystals, the PCNA-positive nuclei were low in number, equally distributed over the nephrons and the interstitium of the cortex and the medulla.
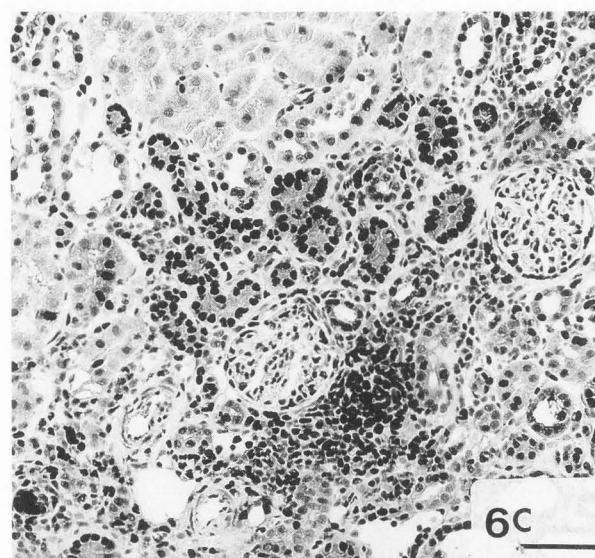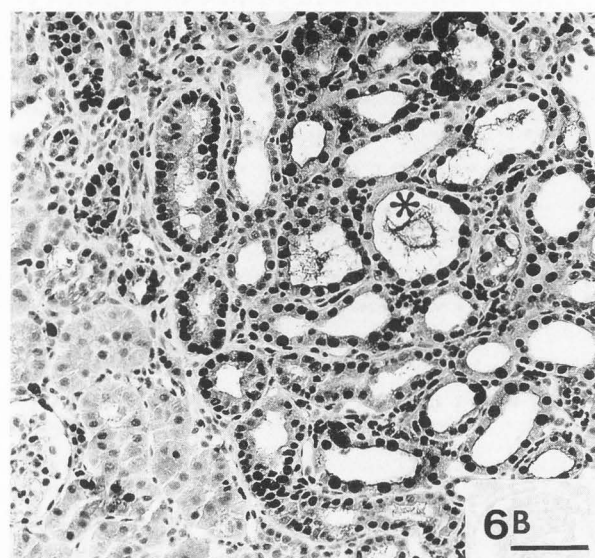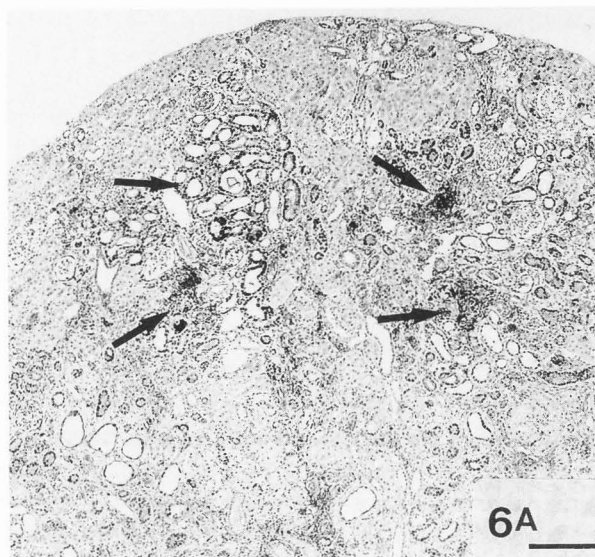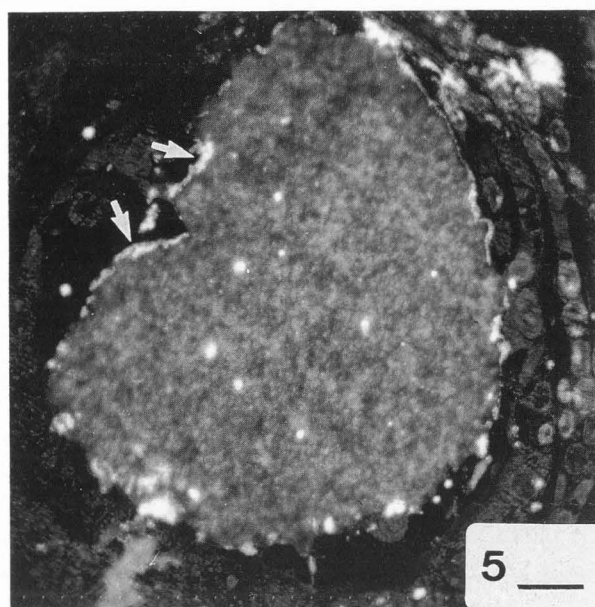
Figure 5. Reflection contrast micrograph of a crystal in the lumen of a proximal tubule. The crystal is coated with a layer of THP (arrows). The tissue was embedded in Lowicryl. Bar = 15 μm.

Figure 6. Kidney sections from a CID-fed rat incubated for the presence of PCNA. The kidney had crystals in the cortex and medulla. The tissue was embedded in paraffin and stained with hematoxylin. (A) At low magnification, numerous PCNA-positive nuclei are present in restricted areas (arrows). (B) Detail showing a cluster of PCNA-positive tubules. These tubules have a relatively high number of nuclei, which are all positive for PCNA. Note the crystal ghost in a tubular lumen (*). (C) Detail showing a cluster PCNA-positive nuclei in the interstitium. Note the presence of PCNA-positive tubules and glomeruli. Bars = 200 μm (A), and 50 μm (B, C).

In the kidneys from the CID-fed rats with crystals, the number of PCNA-positive nuclei had strongly increased in restricted areas (Fig. 6). In glomeruli, the tubular system, and in the surrounding interstitium, most PCNA-positive nuclei occurred in clusters. It was frequently observed that of one tubule, all epithelial cells were PCNA-positive, whereas a neighbouring tubule was without reactivity (Figs. 6A and 6B). The spatial relation between the crystals and the PCNA-positive cells is unclear, since all CaOx crystals were dissolved by the

cytochemical procedure.

Discussion

A CID of vitamin D_3 and EG results in a prominent increase of oxalate excretion. A concentration of 0.5% EG generated renal CaOx crystals, but only in eight of the fifteen animals (53%). When the EG concentration in the drinking water is raised, the number of animals with renal crystals is increased [11]. In the present study, we focused our attention on the CID of vitamin D_3 and 0.5% EG and correlated the presence of calcium crystals with changes in morphology, cytochemistry and a number of urinary parameters in individual rats.

Our results extend earlier observations that in the rat, renal deposition of CaOx crystals is associated with damage to the tubular system and the interstitium [1, 4, 8, 9]. This damage is comparable to that found in hyperoxaluric patients, in which the deposition of CaOx crystals within the nephron and interstitium causes tubular obstruction and tubular atrophy and enlargement of the interstitium by infiltration of mononuclear cells, and fibrosis [6, 28]. The glomeruli of these patients appear either unaltered or focally sclerotic. Also, in non-human primates, renal deposition of calcium oxalate is accompanied by proximal tubular necrosis, distal tubular dilatation and damage of some of the renal corpuscles, consisting of focal adhesions between the glomerulus and the capsular epithelium [36]. In the capsular spaces and in the lumen of tubular system protein, precipitates were regularly observed. The affected distal tubules were either dilated and had a flattened epithelium or had a reactive proliferation of the lining epithelial cells. These data agree with the results of the present study.

Whereas the present study strongly suggests that a high urinary oxalate concentration on itself does not induce morphological changes, it is conceivable that a high oxalate excretion leads to changes in cell physiology. Nouwen et al. [30] found that after a subcutaneous injection of gentamicin, injury of proximal epithelial cells leads to a temporary loss in the cytochemical expression of epidermal growth factor, THP, transferrin receptor and in binding of various lectins. Khan and Hackett [21] showed that after an intraperitoneal injection of sodium oxalate, the early phases of hyperoxaluria, with or without renal deposition of CaOx crystals, is associated with increased release of renal enzymes. In that study, it was also found that at four days of chronic hyperoxaluria without crystal deposition, all rat kidneys were morphologically normal. Although in vitro studies [15, 31] have shown that oxalate ions can be toxic for Madin-Darby canine kidney (MDCK) cells, it must be realized that the situation in vitro might be different from that observed in vivo. In the human and rat kidney, immunocytochemical light- and electron microscopical studies

have localized THP in the epithelium of the TAL [3, 17, 18, 43]. The present experiments showed that there is a change in the localization of THP after CID of EG and vitamin D_3, but only in those kidneys where CaOx crystals were encountered. It is not clear whether this change in THP localization is due to an induced THP synthesis by the lining tubular epithelium.

The rate of crystal nucleation, growth and aggregation are regulated by promoters and inhibitors [42]. Whether the endogenous production and urinary excretion of THP and other inhibitors is increased after CID is still unclear. Using an ELISA technique, it has been found that in cats, the urinary concentration of THP is increased after urolithiasis [35]. Our study showed that a CID of vitamin D_3 and 0.5% EG does not lead to changes in urinary inhibitor activity of crystal growth and crystal agglomeration. This suggests that in our rat model, the formation of renal CaOx crystals is related to urinary supersaturation of calcium and oxalate. Although in stone-forming patients the concentration of inhibitors has been reported to be reduced, a number of studies revealed on the average equal excretion rates in healthy subjects and in stone forming patients [16, 29, 32, 39]. It has also been suggested that in stone-forming patients, the inhibitors formed are functionally and structurally defective [2, 42]. Whether this is also the case in our rat model deserves further attention.

The cellular and molecular events that lead to renal stone formation are not known. There is evidence that proteins and cell membranes induce crystal formation. These cell components are nucleators of calcium salts, and the tubular epithelium can bind crystals and/or crystal aggregates [22, 23, 25]. The partial endocytosed material may serve as a nidus on which the crystals would grow to occlude, finally, the tubular lumen. Tubular obstruction induced by aggregated crystals leads unequivocally to tubular dilatation and an inverse current of urine towards the glomerulus. A similar phenomenon could be responsible for the THP-staining pattern observed in glomeruli and proximal tubules. Locally, it could be found in the urinary space and on the surface coat of the proximal epithelium, and on the visceral and parietal layer of the glomerulus. The obstructed ascending limbs continue to produce THP and the stasis of tubular fluid promotes its aggregation. In combination with other urinary substances, it forms the matrix of the luminal casts. It is conceivable that these casts induce additional tubular obstruction.

In the present study, it has been shown that CaOx crystals in the lumen of the tubular system are coated with THP. This finding is confirmed by the observation that crystals have affinity for (glyco)proteins [4, 22, 24, 26]. Why, especially in the paraffin sections, most interstitial crystals were negative for THP deserves further

investigation. Although it is known that THP can inhibit endocytosis of renal crystals [26], it is speculative to suppose that exclusively the uncoated crystals are endocytosed, and that these crystals pass the basement membrane and accumulate in the interstitium. It is also conceivable that the coat on the interstitial crystals is degraded, since the interstitial cells produce specific matrix components and enzymes, which may interfere with the crystal surface [14]. Alternatively, the interstitial crystals are formed *in situ*.

PCNA is an auxiliary protein of DNA polymerase delta [5, 33]. In the kidneys under study, this enzyme is most likely involved in DNA replication. Although not determined quantitatively, in our morphological studies, we regularly found mitotic interstitial and epithelial cells in the kidney with renal crystals. These mitotic figures were not observed in the kidneys without crystals and the kidneys from the animals fed the standard chow. Since we found that renal crystal deposition and hyperoxaluria is accompanied by damage and necrosis of the tubular epithelial cells, this enhanced mitotic activity is in all likelihood related to tissue repair as a consequence of tissue damage. The fact that in the tubular system these PCNA-positive cells occurred in clusters reflects the divergent regeneration states of these tubules. The occurrence of PCNA positive nuclei may be also be the result of DNA repair, but, so far, there are no reasons to assume that CaOx induces DNA damage. Our studies showed that the deposition of renal crystals is also accompanied by an enlargement of the interstitium. This was due to an increase of the intracellular matrix and a rise in the number of interstitial cells. The fact that also in the interstitium the PCNA positive cells increased in number suggests that the presence of crystals in the interstitium induces cell proliferation of interstitial cells. *In vitro* studies with BSC-1 and MDCK cells showed that the uptake of CaOx crystals is associated with increased cell proliferation and an increased expression of genes that regulate the synthesis of the extracellular matrix [25, 28]. These findings *in vitro* could correlate to the interstitial fibrosis observed in kidneys of patients with primary or secondary hyperoxaluria. Other *in vitro* investigations with O.K. CRL-1840 renal epithelial cells showed that crystal interaction stimulates the proliferation of both the epithelial and the interstitial cells [14]. In that study, there were indications that this proliferation is modulated by the extracellular matrix.

In summary, in the present study, we related in individual rats the presence of renal CaOx crystals with morphological and cytochemical changes of kidney cells. In the tubular lumen, the CaOx crystals are coated with a thin layer of THP. If these crystals are small enough, they can be endocytosed by the tubular epithelial cells [4]. If these crystals have grown into large complexes, covered by neighbouring epithelial cells, they can obstruct the tubular lumen, leading to glomerular and tubular damage. Both the small and large crystals are incorporated into the interstitium and give rise to local cell proliferation and enlargement of the interstitium. The fact that histologically no changes were observed in the CID-fed rats without renal CaOx crystals, but with high urinary oxalate concentrations, strongly suggests that the retained CaOx crystals and not the oxalate ions are responsible for the observed morphological changes.

Acknowledgements

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Discussion with Reviewers

R.L. Hackett: Several urinary parameters are altered with EG treatment; you chose to emphasize GAG's. Please comment on alterations in urinary citrate.
P.S. Chandhoke: Were other urinary parameters, such as urinary citrate, measured to explain the mechanism crystal formation in your animal model?
Authors: In urine, formation of CaOx crystals can be considered as an unbalance between supersaturation of calcium and oxalate and promoter activity on the one hand, and inhibitory activity on the other. In an accompanying study [11], in which we characterized our CID model more extensively, we decided to measure a fixed number of parameters: the urinary capacity of crystal growth- and agglomeration inhibition and the excretion of GAG's. Although vitamin D is supposed to raise the urinary concentration of certain inhibitors (e.g., osteopontin), we did not measure an increase in inhibitor activity of crystal growth and agglomeration. Therefore, the question of which inhibitors contribute to the urinary inhibitory activity after CID is the next point that needs to be clarified. This means that not only the citrate concentration has to be followed, but also the urinary concentration of osteopontin, THP, individual GAG's, and nephrocalcin.

R.L. Hackett: You describe interstitial edema as well as increased numbers of interstitial cells. Did you do actual cell counts taking into account alterations in interstitial tissue volume?
Authors: Morphologically, we noticed an obvious increase in the number of interstitial cells and an enlargement of the interstitial matrix. Cell counts or morphometric analyses were not done.

R.L. Hackett: Please comment in more detail as to the mechanism involved in the "clustering" of PCNA-positive nuclei. Are these clusters associated only with the presence of crystals?
Authors: PCNA has been identified as an auxiliary protein of DNA polymerase-delta, an enzyme most likely involved in the catalysis of DNA leading strand synthesis (S-phase). Although it is also involved in DNA repair, there are, so far, no indications that CaOx induces DNA damage. Actually, in the cell nucleus, two populations exist: one that is tightly associated with replication clusters, and another that is nucleoplasmic. The latter form is not detected after methanol treatment. S-phase nuclei show a prominent granular staining pattern, and as such is a cytochemical marker of proliferation activity. Cell proliferation plays an important role in tissue repair. The fact that the number of positive epithelial cells was considerably higher in certain tubules than in others, in all likelihood, reflects the divergent regeneration states of these tubules. In our rat model, tubular damage is caused by obstruction of the lumen by cellular debris and CaOx crystals. Also, in the interstitial tissue, positive cells occurred in clusters. In the interstitium, the presence of crystals leads unmistakably to cell damage and cell loss and, as a result, to local cell proliferation. In the tubular system and in the interstitium, we could not correlate the presence of positive cells with the occurrence of crystals. All crystals were dissolved because of the low pH during the incubation in citric acid buffer in the microwave oven. Crystal ghosts were regularly encountered in the lumen of the tubular system.

S. Eneström: What are the drawbacks of using PCNA-antibodies for evaluation of cell proliferation, which are minimized using the MIB-1 antibody to Ki-67?
Authors: Whereas the expression of PCNA is maximally elevated in late G_1 and S-phases of cycling cells, Ki-67 is expressed throughout the cell-cycle. As such, the Ki-67 is a sensitive marker for proliferation. Anti Ki-67 (or MIB-1) antibodies could not be used in our studies, however, since they were generated against human nuclear antigens and are not reactive with rat tissue. Another drawback of the Ki-67 antigen is that non-proliferating cells may retain this antigen for a considerable period of time, suggesting that also the Ki-67 may not

be always a reliable marker of cell proliferation. van Dierendonck [56] has discussed the reliability of the Ki-67 and PCNA antigens as markers for cell proliferation.

R.L. Hackett: Localization of THP is discussed only in the relation to the cortex. In other EG models, crystals and stones are often located in the papillae. What was the THP-staining pattern in your rat model?
Authors: In the papillae, THP was regularly found in the thin part of the loop of Henle, in collecting tubules, and on the calyceal side of the papillary crystals. In the kidneys of the CID-fed rats without renal crystals, THP was only demonstrable in the TAL.

S. Eneström: Does the variability in morphological lesions between the animals with renal crystals not make it difficult to draw conclusions regarding the effect of CaOx crystals on the tissue?
Authors: We studied two groups of CID-fed rats: rats without and rats with renal crystals. Since, in both groups, the urinary oxalate concentrations were comparable, we draw the following conclusions: (1) the fact that no damage was observed in the CID-fed rats without renal crystals, established that the retained crystals, and not the high urinary oxalate concentration, are responsible for the presence of renal lesions (e.g., morphological damage, and changes in THP- and staining patterns); and (2) the seriousness of these renal lesions is increased if the number of retained crystals is higher.

S. Eneström: Please discuss the role of crystal matrix protein (31 kDa glycoprotein) found in the organic extract of calcium oxalate crystals as it is an important determinant of calcium oxalate stone disease.
Authors: It has recently been shown that rat crystal matrix proteins, as obtained by demineralization of urinary crystals, contain osteopontin, albumin, and trace amounts of prothrombin-related proteins [45]. Also, in humans, osteopontin is a major matrix component of calcium oxalate monohydrate stones (> 100 μg/100 mg stone [47]). The significance of osteopontin as urinary inhibitor and as chemoattractant of mononuclear cells has been discussed elsewhere in this paper.

P.S. Chandhoke: What was the method of urine collection and how was the urine processed before chemical analysis?
Authors: We kept the rats individually in metabolic cages and we collected the urines on ice without any preservative for 24 hours. For each urine, the volume and the pH were determined.

P.S. Chandhoke: Could you explain why there seems to be an inverse relationship between urinary calcium concentration and the extent of nephrolithiasis. If vitamin D_3 increases intestinal absorption of calcium, and is partly responsible for the hypercalciuria necessary for crystal formation, what is the reason that calcium concentration is less in CID rats than in control rats (Table 1)?
Authors: There appears an inverse relationship between urinary calcium excretion and the extent of nephrolithiasis. It is, however, not the calcium excretion itself but the steep increase of free oxalate that causes that the solubility product of CaOx is surpassed. Precipitation of the CaOx crystals decreases the urinary concentration of free calcium. This decrease is most obviously noticed in the calcium concentration, since, compared with oxalate, the urinary concentration of this ion is low. The question whether the administration of vitamin D_3, which has been reported to enhance the intestinal absorption of calcium, did not compensate for this decrease in urinary calcium deserves further attention. An explanation for this phenomenon may be that, because of the diet, the rats consume less food. Recently, we investigated the effect of the EG concentration in the drinking water on the renal deposition of CaOx crystals; we noticed a decrease in body weight at EG concentrations of 0.8% and higher [11].

P.S. Chandhoke: What is the effect of warming the slide in a microwave oven on the tissue and the antigen-antibody reaction?
Authors: Microwave treatment is commonly used for tissue fixation and for enhancement of immunohistochemical staining. It improves the immunoreactivity, related to temperature and micro diffusion. Compared with protease digestion, it is clearly superior. Since the same results are obtained by boiling of the sections in buffer, the antigens seem to be recovered by the generated heat. As pointed out by Shi *et al.* [52], microwave heating does not damage alcohol-fixed paraffin sections. Although it is conceivable that cross-links induced by paraformaldehyde fixation are altered, our morphological observations did not have any indications that microwave treatment is harmful.

P.S. Chandhoke: In Table 1, there seems to be an increased inhibitory activity for crystal growth in rats with renal crystals compared to those rats without crystals and control rats. What does this mean?
Authors: Although these differences are statistically not significant ($p = 0.073$), these results suggest that the rats with renal crystals try to prevent a further crystal deposition by increasing the urinary crystal growth inhibition. Further studies are required to confirm this.

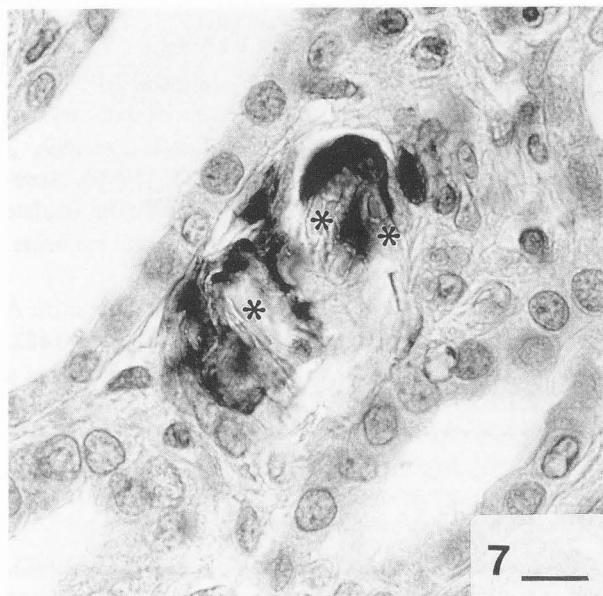**E. Nouwen:** Glomerular and tubular lesions are likely

Figure 7. Detail of the renal cortex of a CID-fed rat, showing macrophages (arrows) around CaOx crystals. A number of crystals were phagocytosed (*). The macrophages were identified with an anti-ED1 monoclonal antibody. Bar = 10 μm.

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to affect renal function. Several urinary parameters related to crystal formation have been determined. Did you also measure renal functional parameters such as serum creatinine?

Authors: In the accompanying paper [11], we characterized the vitamin D-EG rat model by measuring various urinary parameters. In that study, we found that at an EG concentration of 0.5% and higher, the urinary excretion of creatinine was slightly decreased.

E. Nouwen: Were the crystals and morphological lesions uniformly or focally distributed in the kidney?

Authors: The lesions were mostly focally distributed. Since the intratubular and the interstitial crystals were the cause of these lesions, their severity varied according to the amount of renal crystals.

E. Nouwen: Is the observed enlargement of the intercellular spaces between the epithelial cells of the collapsed tubules not just a tissue artefact? Was it also regularly seen in paraformaldehyde-fixed, paraffin-embedded, or Lowicryl-embedded tissue?

S. Eneström: How was it possible to detect widened intercellular spaces between tubular epithelial cells without electron microscopy?

Authors: Previously, we have described the ultrastructural changes in the tubular epithelium after crystal dep-

osition. The enlargement of the intercellular spaces mentioned in this paper could microscopically be noticed in 1 μm thick sections (Epon and Lowicryl) of glutaraldehyde- or paraformaldehyde-fixed tissue (Fig. 3). This enlargement was only observed in the kidneys of CID-fed rats. The affected tubules were closely situated to the damaged (sclerotic) glomeruli, suggesting that both were invariably part of the same nephron. The epithelial cells of these tubules looked viable and a number of these cells were even in mitosis.

S. Eneström: Was there tubulo-interstitial nephritis and, if so, which type of interstitial cells increased in number?

E. Nouwen: The contribution of inflammatory cells to kidney damage and regeneration becomes more and more recognized. Were inflammatory cells, such as lymphocytes, granulocytes, and macrophages, involved in the observed increase in interstitial cell number?

Authors: In the kidneys with renal crystals, the interstitium was enlarged due an increase of the interstitial matrix and an increase of the number of mononuclear cells. As such, there is an interstitial nephritis. The mononuclear cells derive, in part, from the blood. The CaOx crystals present in the renal tubular system and the renal interstitium are coated with a glycoprotein layer, of which THP and osteopontin are important components. Both inhibitors are reported to attract neutrophils, monocytes and macrophages and to enhance subsequent crystal adhesion and phagocytosis [46, 53]. Besides THP and osteopontin, various secretory products of leukocytes have been identified in the crystal matrix, including neutrophil elastase, calprotectin, and α_1-antitrypsin [50, 54, 55]. In preliminary immuno-cytochemical experiments using anti-ED1 antibodies, we showed that most interstitial crystals are surrounded by macrophages (Fig. 7; see also reference [9]). Recently, three other cell types have also been recognized in the renal cortex of rats, viz., fibroblasts, dendritic cells and lymphocytes [48]. Evaluation of the roles of these cells in the processing of interstitial crystals may be an important direction of research in the near future.

S. Eneström: Were interstitial giant cells observed in the interstitium?

Authors: In the interstitium, we regularly observed clusters of nuclei around CaOx crystals, but electron microscopy is required to determine whether we have to do with a cluster of mononuclear cells or with a multinucleated giant cell. The occurrence of giant cells around CaOx crystals has been described earlier by us [9]. As discussed elsewhere in this paper, we also found that macrophages are involved in phagocytosis of CaOx crystals and it is likely that part of these giant cells derive

from macrophages. The occurrence of giant cells has also been described in kidney biopsies of hyperoxaluric patients [49, 57] and, after local administration of CaOx crystals, also in the skin, thyroid, peritoneum, and bone marrow [44, 51]. These giant cells partly were derived from macrophages and partly from epithelial cells. Thus, the next question is whether the giant cells observed in the renal interstitium are derived from macrophages or from tubular cells. For this purpose, specific immunocytochemical markers are needed which can discriminate between both cell types at the ultrastructural level.

E. Nouwen: Was there any qualitative or quantitative difference observed in the presence/formation of calcium oxalate crystals in the TAL, where THP is produced and where its concentration can be suspected to be the highest, compared to other tubular nephron segments? If not, how can the presence of crystals in nephrons segments upstream of THP production site be explained?
Authors: Without obvious differences in size or number, CaOx crystals were encountered throughout the tubular system, including the TAL. These crystals were coated with THP. The fact that crystals (and THP) were also encountered in the proximal tubules and, sometimes even in glomeruli, is probably related to obstruction further down the nephron, causing a retrograde urinary flow towards the glomerulus. The fact that THP is synthesized by the epithelial cells of the TAL and is normally absent from the proximal segments of the nephron, strongly suggests that tubular obstruction by cellular debris and crystals plays an important role in the pathogenesis of nephrolithiasis.

E. Nouwen: What evidence do the authors have to conclude that the crystals actually move by some kind of "transepithelial transport" through the basement membrane? It appears to me that they could as well be formed directly in the interstitium, or that they could have reached the interstitial compartment at places were the basement membrane was disrupted; alternatively, they might represent relics resorbed atrophic tubuli.
Authors: In a previous study [9], we observed that large CaOx crystals adhere to the tubular epithelium and are overgrown by neighbouring tubular epithelial cells. The newly arrived epithelial cells form a new basement membrane on the crystal surface. Although it is likely that the interstitial crystals are formed *in situ*, this may be an important mechanism, in which luminal crystals appear in the interstitium.

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