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N-Acetylneuraminic acids (nana): a potential key in renal calculogenesis

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Abstract *N*-Acetylneuraminic acids (NANA) promote binding of calcium ions to macromolecules and cells, increase the intrinsic viscosity of glycoproteins and facilitate gel formation in water. Since these properties are crucial in urinary calculogenesis, we evaluated NANA levels in urine and serum as well as their expression in kidney tissues. Using a modified thiobarbituric acid assay, the evaluation of free and bound NANA in 24-h urine samples revealed a ratio of 1.87 in 33 non-stone-formers but a reversed ratio of 0.84 in 41 recurrent calcium oxalate stone-formers. Time kinetics revealed a gradual rise in NANA expression until 48 h of culture and a significantly higher release into supernatants of papillary renal epithelial cells (REC) when compared with cortical REC. To examine NANA distribution in kidney tissues, paraffin-embedded biopsies from five normal and six stone-forming kidneys were labeled with the biotinylated NANA-specific lectins *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA). Immunohistochemistry revealed intense luminal MAA reactivity of distal tubular REC and collecting ducts in 96.7% and 91.5% of normal and stone-forming kidneys respectively. By contrast, there was a marked difference between normal and stone-forming kidneys for SNA reactivity (17.7% vs 95%) at the same locations. Finally, the glycocalyx of recurrent stone-formers showed altered sialylglycoside linkages [α(2,6) instead of α(2,3)] that may indicate an altered REC function. Given the calcium-binding potential of NANA, their increased local concentration within the glycocalyx layer in the distal nephron may either initiate stone formation or facilitate attachment of microcrystals to REC.

Key words Renal stone formation · Idiopathic calcium oxalate lithiasis · *N*-acetylneuraminic acids · Thiobarbituric acid assay · Lectins

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Introduction

Following reports that about 64% of the organ matrix of urinary stones is protein [21] and that urinary proteins are selectively associated with calcium oxalate (CaOx) crystals [24, 31, 40, 46], urinary protein chemistry has in recent years become the main arena of urinary stone research. Nevertheless, the chemical nature and functional role of urinary stone proteins is still poorly understood [14, 35]. However, there are also other substances with interesting properties that might be important in stone formation. In the present study, we focused on a class of substances that are part of both the uromucoid and the stone matrix. They are collectively referred to as "sialic acids" and, in humans, predominantly take the form of *N*-acetylneuraminic acids (NANA). NANA are negatively charged sugars and mainly occur as non-reducing terminal residues of carbohydrate chains of glycoproteins or glycolipids in biological fluids and cell membranes. They appear to facilitate binding of calcium ions to macromolecules and cells, increase the intrinsic viscosity of all glycoproteins, and facilitate gel formation in water [6, 12, 37].

It was repeatedly hypothesized that these substances may be relevant in urinary stone formation [3, 19, 28, 29, 34], and clinical observations such as the higher incidence of urinary stones in patients with sialolithiasis [27] underline the need for further research into their role in stone formation. More recently, it was shown that

intratubular microliths are embedded in a chemically unidentified gelatinous substance on the surface of renal epithelial cells (REC) [7, 17]; and that microcrystallites, in an unknown mechanism, are bound to REC membranes during urinary stone growth [15, 20, 45]. Crystal-cell interaction is considered a key factor in urinary stone formation, as crystal retention apparently is a precondition of stone growth [10, 43]. Nevertheless, no hard data exist on the pathogenetic potential of NANA in calculogenesis.

The objective of the present study was to obtain reliable information on a possible role of NANA in renal stone formation by evaluating their origin, production, localization and distribution patterns not only in urine but also in serum and renal tissue specimens of CaOx stone-formers as compared with normal controls.

Patients and methods

Patients

NANA were investigated in 24-h urine collections and sera of idiopathic recurrent CaOx stone-formers ($n = 41$) and healthy individuals ($n = 33$). Recurrent stone-formers were defined as patients with at least three stone episodes over the previous 3 years. None of the patients had stones or urinary tract infection at the time of urine collection, and none were on drugs. Only patients with normal renal function (serum creatinine < 1.2 mg%) and without metabolic disorders, as confirmed by analysis of 24-h urine samples, were included. Sex and age distribution were similar among the patients (26 women, 15 men; mean age 55.8 ± 14.5 years) and healthy controls (20 women, 13 men; mean age 52.5 ± 17.5 years). The 24-h urines and sera were collected and stored without preservatives at −20°C until biochemical analysis. Hemolytic or icteric sera were excluded.

Tissues

Fresh renal tissue specimens were obtained from recurrent stone-formers treated by open surgery ($n = 6$) or from kidneys removed for small, peripheral renal cell cancer ($n = 5$). All tissue samples were from areas with a macroscopically healthy appearance, and their microscopically normal condition was confirmed histologically. The two groups were age- and sex-matched and all patients had normal renal function.

Cell culture

Immediately after nephrectomy, sterile renal tissue was separated macroscopically into papillary and cortical tissue compartments, and both were minced into 3-mm^3 tissue fragments. Enzymatic digestion was done in RPMI 1640 (Gibco BRL Life Technologies, Gaithersburg, USA) containing 10% fetal calf serum (Gibco), 200 U/ml type I collagenase (Sigma, St. Louis, Mo.) and 100 U/ml type I DNAse (Sigma) under stirring for at least 3 h at 37°C, resulting in a heterogeneous cell suspension. Large cell clusters and debris were removed by short segmentation and gradient centrifugation over a 20% Percoll gradient for 10 min at 60g. Cells were then cultured for 24 h in RPMI 1640 + 10% fetal calf serum in high densities (1×10^6/ml) to avoid contamination by fibroblasts and to allow attachment to the plastic surface. Primary cultures of papillary and cortical REC were trypsinized using trypsin EDTA solution (Gibco), split in a 1:3 ratio and fed using serum-free WAJC 404 Base powder (Irvine Scientific, Santa Ana, Calif.) medium supplemented with select trace inorganics (Irvine Scientific), 1.176 g/l $NaHCO_3$, 2.5% prolactin (Sigma), 0.1% epidermal growth factor (Collaborative Research, Bedford, Mass.), 0.1% premix, pituitary extract, and 1 ng/ml cholera toxin (Sigma). Cells were split twice a week using Hanks, buffered salt solution plus 674 U/ml type I collagenase (Sigma). The epithelial origin of cultured cells was confirmed by staining of ethanol-fixed cells using anti-cytokeratin 18.

For the demonstration of NANA production by REC derived from papillary and cortical tissue segments, in vitro triplicates of 1×10^6 cells were cultured in 300 µl WAJC 404 using 24-well plates (Falcon, Becton Dickinson, Plymouth, UK), and supernatants harvested after 1, 3, 6 and 48 h of culture. Supernatants were then centrifuged for 10 min at 330g, sterile filtered using Spin × tubes (Costar, Cambridge, Mass.) to avoid contamination by cellular debris, and frozen at −70°C. To measure the amount of cellular NANA, adherent cell layers were refloated in WAJC and double-distilled, ionized water (aq. bid.), frozen and thawed up (37°C) five times before testing. In some experiments, cells were serially diluted and supernatants as well as cells layers harvested after 5 days of culture. Cells, supernatants and pure medium were handled under the same conditions as the cell cultures and kept at −70°C until analysis [9]. Experiments were performed several times and analyzed in triplicate.

Measurement of NANA with thiobarbituric acid

NANA in urines, sera, cells, supernatants and control medium were estimated based on thiobarbituric acid using a previously described method [44] with slight modifications. One hundred microliter volumes of samples, standards (NANA from Serva, Heidelberg, Germany) and the following blanks were used: 0.9% sodium chloride for serum samples, aq. bid. for urines and cell suspensions dissolved in aq. bid., as well as the appropriate medium for supernatant estimation. Total NANA were evaluated by hydrolysis using 100 µl 0.1 M sulfuric acid (Merck, Darmstadt, Germany) for 60 min at 80°C. Bound NANA were evaluated by addition of 100 µl aq. bid. for 60 min at room temperature. Further treatment was performed using 100 µl 0.2 M sodium metaperiodate (Merck) in 9 M orthophosphoric acid (Merck) for 25 min at room temperature and 1 ml 10% (w/v) sodium metaarsenite (Sigma) in 0.5 M sodium sulphate (Merck) plus 0.1 M sulfuric acid. Then 3 ml 0.6% (w/v) thiobarbituric acid (Merck) was mixed and heated in 0.5 M sodium sulfate for 15 min at 100°C, followed by 5 min ice-bath cooling. Samples were treated by 2 ml cyclohexanone (Merck), mixed and centrifuged (7000 *g*) for 10 min at 2°C, and the cyclohexanone phase measured immediately after centrifugation at 532 nm and 549 nm with a Shimadzu UV 120-01 absorbance spectrophometer (Shimadzu, Kyoto, Japan).

Results were calculated according to Warren, excluding possible interferences of deoxyribose [1, 26, 44]. Total NANA (hydrolyzed samples) were reduced by bound NANA (samples without hydrolyzation) to calculate free NANA contained in the samples.

The modified NANA assay showed an imprecision of CV 3.4% (within run) or 4.9% (day to day; $n = 10$; standard: 100 µl/ml). Since very low concentrations of NANA were expected in cell suspensions and supernatants [38, 41], we investigated the limits of detection of the modified assay by adding NANA in varying concentrations to aq. bid. or medium and estimating the recovery of NANA in samples with and without hydrolysis. The detection limits were found to be < 1 ng/ml, compared with 3.7 ± 9.3 ng/ml in the literature [13, 26]. The method was considered adequately specific and more sensitive than the enzymatic test [13, 26].

To exclude any interference by cell culture media, sample absorptions were tested in different media. No sample absorption was measured when using serum-free WAJC 404 or RPMI; very high absorption resulted when using Eagle's Minimum Essential Medium (Gibco) substituted with fetal calf serum (data not shown). Thus, all experiments were performed in serum-free media.

Lectins

For immunohistochemistry, directly biotin-labeled lectins (EY Laboratories, San Mateco, Calif.) were used. The hemagglutinating

Table 1 Levels of *N*-acetylneuraminic acids (NANA) in 24-h urine and serum of healthy individuals (controls) and recurrent stone-formers (RSF) Values are means ± SD; NS, not significant; Student's *t*-test (significance $P < 0.05$)

	n	NANA in urine						*n*	NANA in serum		
		Total		Bound		Free			Total	Bound	Free
		mg/l	mg/day	mg/l	mg/day	mg/l	mg/day		mg/l	mg/l	mg/l
Controls	33	82 ± 28	126 ± 40	32 ± 16	47 ± 19	51 ± 7	79 ± 31	28	755 ± 268	748 ± 263	7 ± 3
RSF	41	88 ± 31	186 ± 44	51 ± 26	107 ± 40	37 ± 12	79 ± 22	30	677 ± 145	674 ± 143	3 ± 1
		NS	$P < 0.005$	$P < 0.05$	$P < 0.005$	$P < 0.05$	NS		NS	NS	$P < 0.05$

lectin from *Sambucus nigra* (SNA) binds with high affinity to glycoconjugates containing α(2,6)-linked NANA, while isomeric structures containing terminal α(2,3) linkage are recognized by the immobilized leucoagglutinin from the seeds of *Maackia amurensis* (MAA) [5, 22, 30, 36, 39]. The lectin from *Laburnum alpinum* (LAA) is specific for *N*-acetylglucosamine (GlcNAc), and the presence of a terminal NANA does not inhibit lectin binding. LAA was used as a non-NANA-specific lectin in order to control NANA-specific lectin binding.

Immune staining and lectin peroxidase staining

Tissue samples were fixed immediately after surgery in 10% formaldehyde in 0.02 M phosphate, 0.15 M sodium chloride, pH 7.2, using standard methods. Paraffin-embedded tissues were cut and dried at 56°C for 5 min, dewaxed in xylene for 10 min and rehydrated. Endogenous peroxidase was blocked using 0.3% H_2O_2 in methanol for 30 min. Slides were then immersed in TRIS-buffered saline (0.15 M sodium chloride, 0.05 M TRIS-HCL, pH 7.6) and incubated with either biotinylated MAA, SNA or LAA lectins, or incubation buffer alone for 1 h at room temperature. After repeated washes using phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA), sections were incubated with streptABComplex/HRP (Dakopatts, Glostrup, Denmark) developed in 3,3-diaminobenzidine. Counterstaining was performed with hematoxylin, and dried sections were then coverslipped in Aquamount (BDH, Pool, UK).

Semiquantitative analysis of luminal surface binding of NANA-specific lectins

Hematoxylin-counterstained and routine hematoxylin-eosin stained sections were compared to identify the various microanatomical parts of the renal tubular system. Tubular epithelial cells were separately examined for their luminal membrane peroxidase positivity. Using ×630 magnification, 500 cells of proximal tubules, distal tubules and collecting ducts were counted per section. Data of intermediate tubules were based on 100-cell counts.

Statistical analysis

Statistical analysis of urine and serum samples was performed using Student's *t*-test (significance level $P < 0.05$). REC culture experiments were performed in triplicate; data are given as the mean ± standard deviation (SD). Positive luminal surface expression of NANA-specific lectins is given in percent and SD.

Results

NANA in urines and sera of stone-formers and controls

Results are listed in Table 1. There was no statistical difference between the two groups regarding total NANA concentration. The 24-h excretion levels, by contrast, were significantly elevated among stone-formers due to their higher volumes of urine (2388 ± 760 vs 1564 ± 637 ml; $P < 0.005$). Concentrations of free NANA in urine were significantly lower among recurrent stone-formers than among healthy individuals ($P < 0.05$). Bound NANA, by contrast, were significantly increased in stone formers as compared with healthy individuals ($P < 0.05$). The ratio of free to bound NANA was 0.84 ± 0.37 in stone-formers and

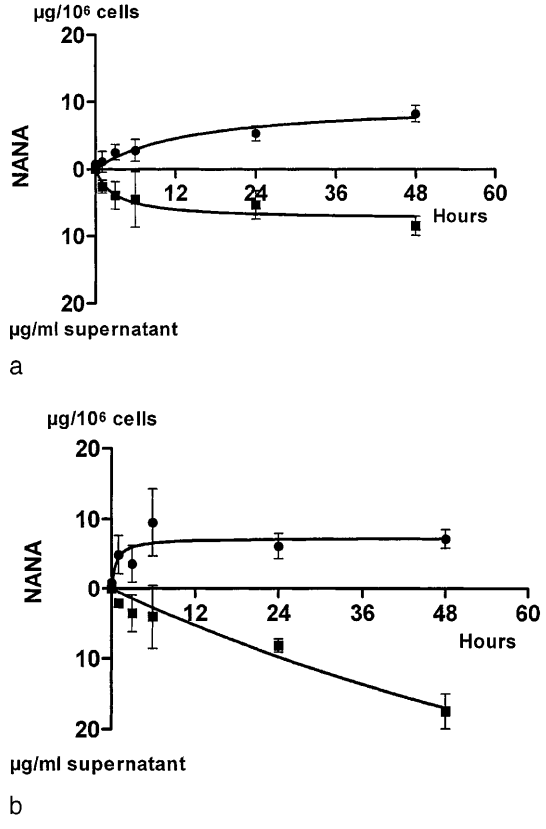

Fig. 1a,b Mean concentrations of *N*-acetylneuraminic acids (NANA) in cell lysates (per 10^6 cells) and in supernatants (per ml) during a 48-h time kinetic assay. **a** Cultures of renal epithelial cells (REC) from the cortical region. **b** Cultures of REC from the papillary region. Nonlinear regression analysis (function: $Y = B_{max} \cdot X/(K_d + X)$, where Y is NANA concentration (*upper hyperbola* cell lysates, *lower hyperbola* supernatants) X is observation time, $B_{max} = 7.3$ and $K_d = 0.66$ for cell lysates, and $B_{max} = -64.3$ and $K_d = 134$ for supernatants

1.87 ± 1.02 in controls. This reversed ratio was observed for both concentration and 24-h excretion. Likewise, the sera of the stone-formers exhibited significantly lower free NANA levels than the control sera ($P < 0.05$), whereas no apparent difference was seen between the two groups for bound serum NANA.

Demonstration of NANA production in REC culture

NANA production by REC was analyzed in vitro using REC derived from kidneys without calculi cultured over several passages under NANA-free and serum-free conditions. Therefore, NANA uptake from the culture medium as well as outgrowth of contaminating non-epithelial cells was kept to a minimum. Serial dilutions of REC were cultured for 5 days, and supernatants and cells were then analyzed for their NANA content. NANA were detectable in both the supernatants and cell lysates, and the quantity of NANA correlated with the number of cells per assay (Table 2). We further compared NANA production by REC from papillary and cortical regions, revealing a gradual rise in NANA

Table 2 NANA production by renal epithelial cells in vitro

Cell no.	Cellular (ng/ml)	Supernatant (ng/ml)
1×10^6	6.46	7.93
0.5×10^6	4.26	4.79
0.25×10^6	3.04	2.94
0.12×10^6	2.75	2.52
0.06×10^6	2.19	2.11

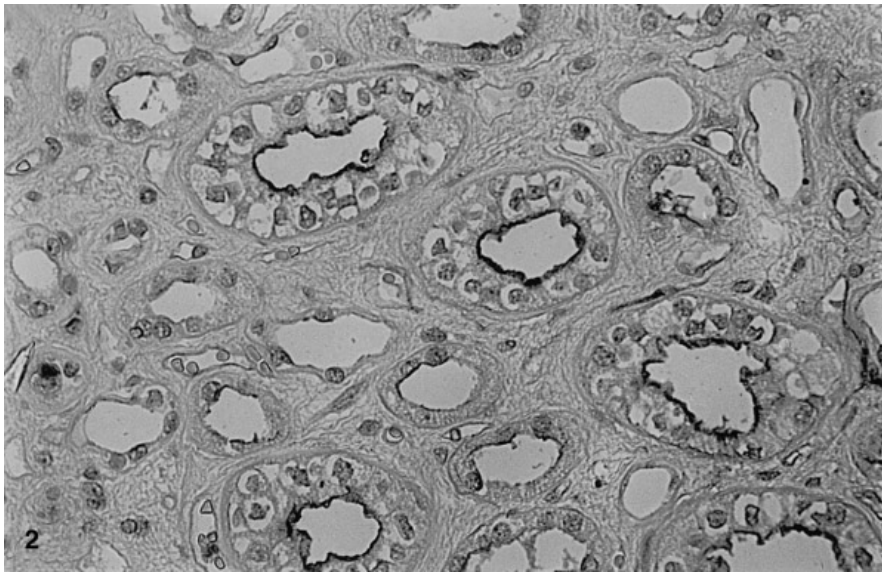

Fig. 2 NANA expression in renal tissues of recurrent calcium oxalate stone-formers. Immunoreactivity of the NANA layer at the luminal surface with biotinylated *Sambucus nigra* lectin (SNA) is shown. Magnification ×400

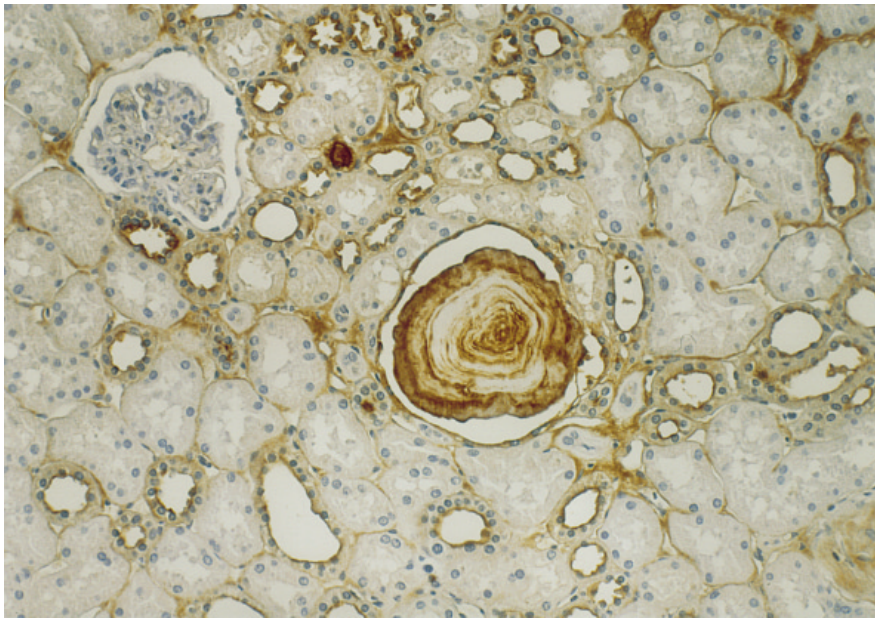

Fig. 3 Immunoperoxidase staining with biotinylated SNA: microlith surrounded by a NANA-positive film

production until 48 h of culture (Fig. 1). Papillary REC in the same patients released higher amounts of NANA into the supernatant than cortical REC, although both cell types showed a similar amount of NANA within cell lysates (Fig. 1).

Surface expression of NANA in renal tissue and semiquantitative analysis

Using peroxidase staining with biotinylated MAA and SNA, we identified the microanatomical localization of NANA within human kidney tissues. NANA were found to be expressed predominantly at the luminal surface of renal tubules (Fig. 2). In addition, we observed microliths surrounded by a NANA-positive film and concentric immunoreactive layers within microliths (Fig. 3).

SNA yielded characteristic binding patterns within the various anatomical regions of the kidney, although these patterns differed in healthy individuals and recurrent stone-formers. SNA immunoreactivity in healthy individuals was more abundant within the cortex than in the papillary region (Fig. 4a, b). In contrast, tissues from stone-formers revealed a reversed picture, with SNA immunoreactivity predominantly found within papillary areas (Fig. 4c, d).

The lectin binding patterns were further analyzed by semiquantitative analysis in order to evaluate the number of REC exhibiting luminal surface expression within different parts of the nephron. Table 3 shows the different amounts of reactivity of both lectins in stone-formers versus healthy controls. MAA was shown to be abundantly expressed within the distal parts of the nephron without any significant difference between normal and stone-forming kidneys. By contrast, luminal SNA distribution within the nephron was significantly altered in stone-formers: while the abundance of SNA in normal kidneys decreases from proximal to distal, SNA in stone-formers is inversely distributed, showing its highest amount within the distal nephron. This suggest a different chemical composition of the glycocalyx layer, since the relationship between MAA and SNA varies in normal and stone-forming kidneys.

Discussion

In their pioneering reports on stone matrix characteristics, Keutel et al. [19] and Boyce [3] concluded that enzymatic dissociation of NANA is the first step in the transformation of uromucoid into mineralizing stone matrix [28]. Later, NANA were shown to be present in the uromucoid and the stone matrix, and were supposed to be passively deposited [29, 34]. In a recent study, Knörle et al. [23] demonstrated a role of NANA in maintaining the function of Tamm-Horsfall glycoprotein in the precipitation of calcium oxalate and in the formation of renal stones. However these studies, although emphasizing the potential importance of NANA metabolism in stone formation, lack detailed information on the distribution of NANA within the three main compartments, i.e. urine, serum and in particular in renal tissue.

The first main finding in our study was the significant difference in urinary excretion profiles of NANA between recurrent stone-formers and non-stone-formers. In spite of elevated total NANA excretion, NANA concentrations were not significantly higher among stone-formers, since stone-formers show significantly higher urinary volumes. However, stone-formers did show a reversed ratio of free to bound NANA, both in concentration and in total excretion.

The concentration of free NANA in the urine was lower in stone-formers than in healthy individuals. In addition, the concentration of free NANA in stone-formers was substantially higher in urine than in serum, which cannot be explained by glomerular filtration nor as a result of reduced free serum NANA. Bound urinary NANA, by contrast, was significantly elevated in stone-formers. These abnormal urinary NANA excretion profiles, however, were obtained in idiopathic recurrent stone-formers without any measurable metabolic disorders in their 24-h urine samples. Therefore, our results can not be extrapolated to patients with metabolic disorders. Presumably, the cause of this reversed NANA excretion pattern in stone-formers may originate from an increased metabolism at the REC level.

Our results are in contrast to a recent report by Van Aswegen et al. [42]. These authors concluded that stone-formers excrete less NANA than healthy men [42]. Some major points, however, argue against this conclusion: (i) the given urinary NANA concentrations markedly exceed reference values [13]; (ii) it appears problematic to calculate total daily excretion from those concentrations, as stone-formers and healthy individuals show considerable differences in urinary volume; and (iii) the attempted sialidase-based interpretation appears contradictory because the reduced bound NANA segment is not matched by an elevated free NANA segment despite simultaneously elevated urinary sialidase activity.

Table 3 Positive luminal surface expression (% positive cells) of NANA-specific lectins (*MAA maackia amurensis*, *SNA Sambucus nigra*)

	Proximal tubules		Intermediate tubules		Distal tubules and collecting ducts	
	Controls	RSF	Control	RSF	Controls	RSF
MAA	5.5 ± 2.6	14.9 ± 10.3	93.5 ± 3.5	98.8 ± 1.1	96.7 ± 2.2	91.5 ± 5
SNA	56 ± 3.6	13.9 ± 10.9	32.5 ± 2.3	96.7 ± 1.1	17.7 ± 2.7	95 ± 4.2

Our second main finding was that REC, similar to other cell types [41], produce NANA and release them into supernatants, thus demonstrating the renal origin of NANA. Furthermore, papillary REC release higher amounts than cortical REC, while cell lysates from the two regions did not show significant differences in this respect. Since we primarily focused on NANA as a product of REC, we have so far limited our in vitro assays to cell cultures from non-stone-formers. Studies on cell-crystal interactions in renal cell culture models from stone-formers are certainly an important next step for future research.

Finally, we studied the pattern of expression of NANA and different NANA linkages to carbohydrate residues within renal tissues. We identified a predominantly luminal NANA expression. We were able to differentiate between NANA isomers based on glycosidic linkages, which are known to define their properties: e.g., $\alpha(2,3)$ linkages are hydrolyzed much faster than $\alpha(2,6)$ bonds [6].

The two NANA-specific lectins SNA and MAA showed typical distribution patterns both within the various parts of the nephron, and between normal control tissue and stone-bearing kidneys. A decrease in membrane NANA on glomerular epithelial cells may be responsible for increased permeability of the glomerular capillary filter in human glomerular disease [2, 18]. Likewise, alterations of the NANA content in the tubular glycocalyx could cause changes in tubular membrane functions. Several authors [11, 25] have suggested that an altered quality of the glycocalyx may modulate the uptake of calcium oxalate crystals by adjacent epithelial cells. In addition, the luminal surface of REC, which is coated by a mucous layer composed of glycosaminoglycans and other substances, may serve as a shield that effectively inhibits adherence of bacteria and other substances to the epithelium [32].

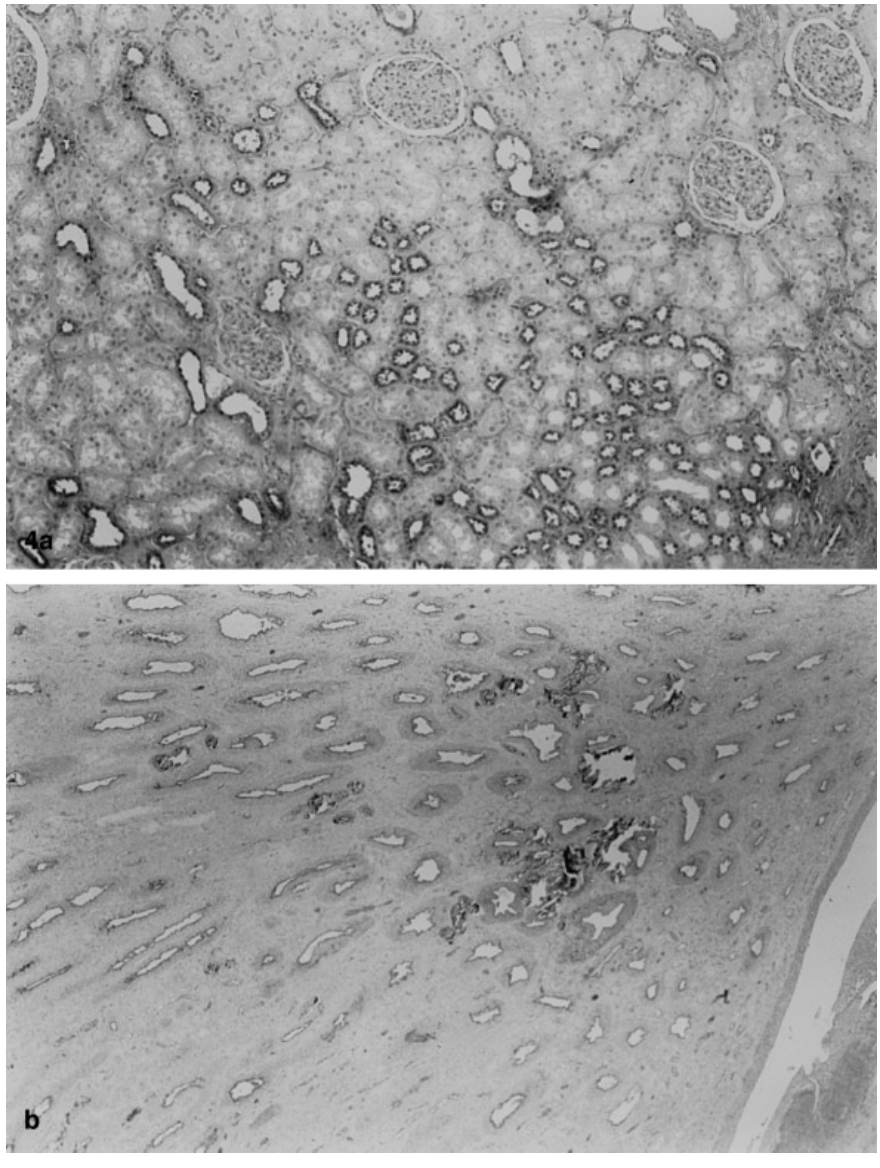

Fig. 4 Different immunoperoxidase staining patterns with biotinylated SNA in renal tissues of healthy individuals (**a** cortical region, **b** papillary region) and recurrent calcium oxalate stone-formers (**c** cortical region, **d** papillary region). Magnification ×100

The rationale for these working hypotheses is the properties of NANA as demonstrated in other biological systems. It was shown that positively charged chrysotile fibers bind to negatively charged NANA residues on red blood cell membranes, causing clustering of membrane proteins and increased cell permeability to different ions [4]. There is evidence that microcrystallites are bound to kidney epithelial membranes during urinary stone growth [8, 15, 16, 20, 45]. Riese et al. [33] used a rat model to study the relationship between cell membrane polarity and CaOx crystal binding. From their results they concluded that loss of epithelial membrane polarity may result in an enhanced capacity to bind CaOx. Our results suggest that an altered membrane polarity could be caused by changes in its NANA content.

On the basis of the different composition of the REC glycocalyx in stone-formers as compared with normal individuals, further investigations should clarify the impact of NANA on tubular membrane function as a main component in cell-crystal interaction and thus in renal stone formation.

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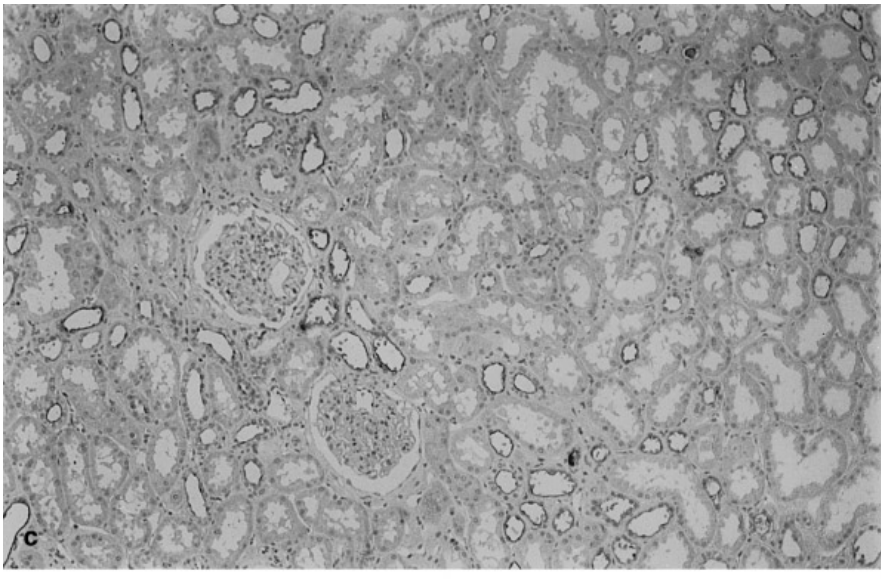

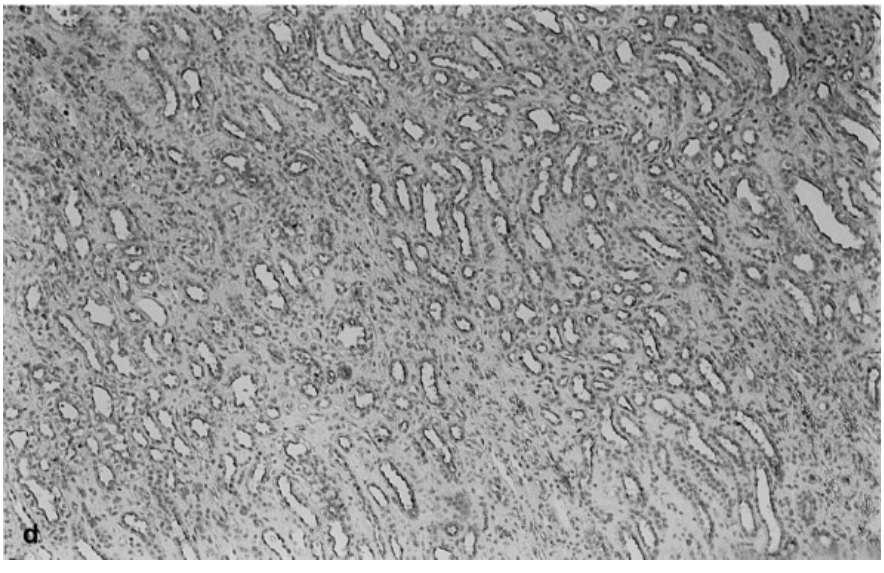

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