Expression of Renal Crystallization Inhibitors in Experimental Nephrolithiasis

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The expression of renal inhibitors of crystallization (Tamm-Horsefall protein, osteopontin, bikunin) in experimental nephrolithiasis was studied in rats receiving 1% ethylene glycol solution for drinking for 3 weeks. The expression of Tamm-Horsefall protein increased, while osteopontin and bikunin expression decreased in experimental nephrolithiasis.

Key Words: *experimental nephrolithiasis; crystallization inhibitors; expression*

Drug correction of oxalate nephrolithiasis, the main form of urolithiasis, remains a pressing problem of [7].

Human urine is a complex colloid system with physicochemical properties favoring the formation of concrements in the kidneys [3]. However, urolithiasis does not develop in all individuals, because human organism has certain defense mechanisms preventing stone formation. It is assumed that protein macromolecules expressed in renal tissues and preventing aggregation, adhesion, and nucleation of calcium minerals play the key role in lithogenesis inhibition. Due to these properties, these molecules are called crystallization inhibitors [4]. They are Tamm-Horsefall protein (THP), osteopontin (OPN), and bikunin (BCN). It has been found that these proteins loose their inhibitory effects in disease or even become crystallization stimulators [4]. Detection of the causes of these events would promote understanding of nephrolithiasis pathogenesis and search for new effective methods for drug prevention of urolithiasis.

We studied the pathomorphology of THP, OPN, and BCN expression in the kidneys in experimental oxalate nephrolithiasis.

MATERIALS AND METHODS

The study was carried out on 30 outbred male rats (200-250 g) fed standard diets. The animals were divided into 2 groups. Group 1 animals (reference) received tap water for drinking over 3 weeks. Group 2 rats received 1% ethylene glycol (low molecular diatomic alcohol, one of its metabolites in the organism is oxalate ion) for 3 weeks in order to induce nephrolithiasis. Long ethylene glycol treatment leads to accumulation of oxalate ions, which, excreted in high amounts with the urine, create conditions for the formations of renal concrements and development of nephrolithiasis [2].

After 3 weeks, the rats of both groups were decapitated by cervical dislocation under ether narcosis in accordance with the Helsinki Declaration World Medical Association requirements (2000). The kidneys were analyzed.

Calcium compound depositions were detected by silver impregnation by Kossa's method, with the reaction control (0.1% hydrochloric acid) [9]. The depositions and distribution of calcium deposits, their mean size and location in renal tissues were evaluated. The biosynthetic activity and total functional viability of cells were evaluated by safranine O (T) polychromatic staining as described previously [8]. The connective tissue elements were detected and the connective tissue maturity was evaluated by fibrin staining by the MSB (Marcius-Scarlett-Blue) method modified by D. D. Zerbino [5].

The expression of THP, OPN, and BCN was studied by indirect two-step streptavidin biotin method

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with the reaction specificity control. After standard deparaffination and rehydration procedures, blocking of endogenous peroxidase was carried out as recommended by the antibody manufacturer (Santa Cruz).

Antigenic specificity was restored by pretreatment of sections plunged in citrate buffer (pH 6.0) in a microwave oven at 600 W, 3×7 min [1].

Antibodies to THP (G-20: sc-19554; 1:80), OPN (P-18: sc-10593; 1:80), and BCN (M-17: sc-21600; 1:80; Santa Cruz) served as the first antibodies.

The reaction product was visualized by goat ABC Staining System: sc-2023 (Santa Cruz) and diaminobenzidine.

Morphometric studies were carried out using ImageJ 1.43 and AxioVision 3.1 software. The expression was evaluated by diaminobenzidine staining intensity using ImageJ image analyzer. For more convenient interpretation of the results, the data were calculated by the formula:

$$E\%=100-\frac{100\times D_x}{256}$$

where E% is expression percentage, 256 is the maximum staining intensity, and D_x is diaminobenzidine staining intensity. The data are presented as the mean (X) and error in the mean (m) and are considered significant at $p<0.05$. The differences between the groups were evaluated by Dann's and Mann–Whitney's tests (SigmaStat 3.5, Systat Software, Inc.).

RESULTS

Three-week ethylene glycol consumption led to manifestation of morphological signs characteristic of oxalate nephrolithiasis. Elements of renal tissue restructuring appeared: hydropic degeneration of the epithelium and its desquamation, dilatation of the tubular and collection tubular lumen, lymphohistiocytic infiltration of the interstitium. Significant calcium deposits (21.4±3.40 per visual field) were found in the renal medulla, mainly at the base and middle third of the renal papilla, the mean size of these deposits was 16.5±0.60 μ. Large microliths (30-35 μ) were found in 10% observations, they closed the lumen of collection tubules (Fig. 1, *a*). Connective tissue growth with the formation of peritubular and perivascular fibrosis was found perifocally. Functional activity of collection tubular epithelium sharply decreased.

No signs of this kind were detected in the reference group. The histological picture of the renal sections generally corresponded to normal. No calcium depositions were verified histochemically.

Immunohistochemical study showed changes in the expression of the studied proteins in renal tissues. During week 3 of oxalate nephrolithiasis formation, manifest expression of THP was found in the cytoplasm and membranes (mainly apical membranes) of the distal tubular epitheliocytes in the renal cortical matter and Henle's loop thick compartment cells in the external medullary zone (Fig. 1, *b*). Similar location of THP expression was found in intact animals. The renal expression of THP increased significantly (by 9.4%) in animals with experimental nephrolithiasis in comparison with the reference group ($p<0.05$). In addition, in intact rats slight expression of THP was seen in the interstitium over the entire area of the renal papilla, while in nephrolithiasis it was focused mainly in its middle third, where the calcium deposits were mainly located. On the other hand, no THP expression was detected near rather large microliths blocking the collection tubules.

The expression of OPN in the epitheliocyte cytoplasm in the nephron tubules, collection tubules, and transitional epithelium of the pelvicaliceal system of intact rats was moderate. The expression of OPN reduced significantly (by 6%; $p<0.05$) after 3 weeks of ethylene glycol consumption in comparison with intact animals without appreciable topological difference between the groups. In addition, moderate expression of OPN was found in the protein cylinders in the collection tubule lumen in the inner zone of the renal medulla. No direct relationship between the formation of calcium microliths and OPN expression was detected (Fig. 1, *c*).

The topological characteristics of BCN expression were largely similar to those of OPN: moderate expression of cytoplasmic location in the nephron tubular and collection tubular epithelium, no expression in the interstitium. However, there were some differences. Signs of slight expression of BCN in the renal papilla interstitium (in sites of predominant deposition of calcium) were detected during week 3 of nephrolithiasis simulation, though no direct topological relationship with the microliths was detected (Fig. 1, *d*). In addition, a statistically significant (5.5%; $p<0.05$) reduction of BCN expression was detected during week 3 of ethylene glycol treatment.

The experiment demonstrated a clear-cut picture of oxalate nephrolithiasis development in rats drinking ethylene glycol during 3 weeks.

The expression of THP in normal rats and in rats with nephrolithiasis was detected predominantly on the apical membrane of cells in the ascending compartments of Henle's loop and in the distal tubules. This was in good agreement with published data according to which THP was located mainly in the thick ascending compartment of Henle's loop [4]. On the other hand, the expression of THP increased by almost 10% after 3 weeks of ethylene glycol consumption, which was somewhat unexpected. Previous studies de-

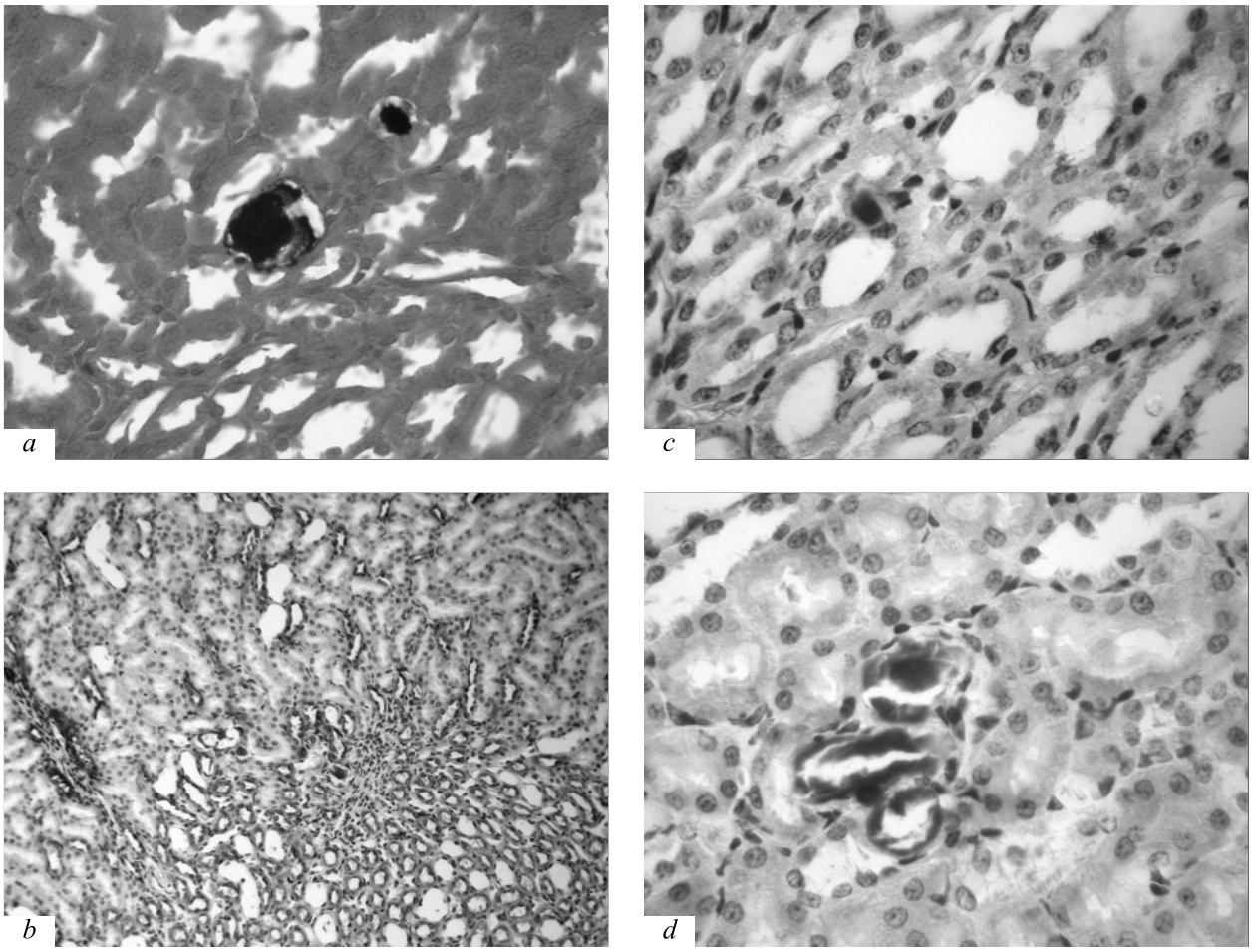

Fig. 1. Experimental nephrolithiasis on day 21. *a*) microliths in collection tubule lumen. Kossa's staining, ×400; *b*) manifest expression of THP by epitheliocytes in the distal tubules and thick part of Henle's loop. No perifocal expression of the protein near microliths, ×100; *c*) moderate expression of OPN near calcium microlith in collection tubule lumen, ×400; *d*) moderate expression of BCN in the cytoplasms of collection tubule epitheliocytes. No topological relationship with microliths, ×400.

tected reduction of urinary excretion of THP in nephrolithiasis, but only THP excretion and not its cell expression, and not in absolute figures, but relative to creatinine excretion [4]. Discussing the results, we had to admit that some authors regarded THP as a lithogenesis stimulus [6]. From this standpoint, increase of its expression at the peak of experimental disease detected in our experiment was somewhat justified. However, the opinion on the inhibitory effects of THP predominates in modern publications, though there are in fact no practical data on increase of THP expression in experimental nephrolithiasis. Presumably, the increase of THP expression during week 3 of nephrolithiasis development is an adaptive mechanism aimed at protection of the kidneys from the toxic effects of oxalate ions and their calcium salts.

Analysis of renal expression of OPN also gave contradictory results. Some authors noted an increase of OPN expression in rats with experimental nephrolithiasis [11,13]. Our experiments demonstrated its significant reduction. Presumably, attenuation of OPN expression in experimental nephrolithiasis reflects exhaustion of OPN-dependent defense mechanisms after lasting prevention of crystallization processes in the kidneys. This hypothesis is indirectly confirmed by clinical observations demonstrating a significant reduction of OPN levels in the urine of nephrolithiasis patients [12,14].

As for renal expression of BCN, our data agree with some sources and disagree with others. We found BCN mainly in the cytoplasm, while some authors demonstrated apical location of this protein [4]. In addition, we found that BCN expression decreased in rats after 3 weeks of ethylene glycol consumption. On the one hand, it seemed quite logical and could be regarded as a component of the oxalate nephrolithiasis pathogenesis, on the other hand, some authors demonstrated significant (by several times) increase of renal expression of BCN mRNA under similar experimental conditions. Importantly that positive growth was observed only after week 8 of ethylene glycol consump-

tion by rats [10]. Rather many authors obtained similar results – by topological characteristics and by the time course of expression [4].

Hence, experimental nephrolithiasis induced in rats by 3-week consumption of ethylene glycol was associated with an increase of THP expression and a decrease of OPN and BCN expression in the kidneys. Despite the contradictions in our results, we consider them demonstrative and justified. The increase of THP expression should be presumably regarded as a compensatory reaction aimed at inhibition of lithogenic processes. Reduction of OPN and BCN expression, in turn, may indicate exhaustion of these proteins' activities developing by the end of week 3 after lasting control of the factors provoking lithogenesis. These regularities can be regarded as characteristic signs of experimental oxalate nephrolithiasis development.

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