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Apoptosis as a mechanism of the albumin-induced kidney damage in childhood nephrotic syndrome

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Abstract. *It was shown previously on in vivo studies that proteinuria-induced effects play a crucial role in renal damage in chronic kidney disease (CKD). However, an initial mechanism of irreversible kidney damage in pediatric diseases characterized by chronic proteinuria, i.e. nephrotic syndrome, remains to be unclear.*

The aim of our work was to study the initial mechanism of kidney cells apoptosis development in nephrotic children.

Methods. An examination of renal biopsies of 53 patients (aged 10 to 15 years) with nephrotic syndrome hospitalized in Pediatric Nephrology unit of the Children Clinical Hospital №7 (Kyiv, Ukraine) done. In vitro studies of albumin toxicity performed on rat proximal tubular cells in primary culture (RPTC).

Results. Our study showed that albumin overload in nephrotic children leads to high levels of apoptosis. Its distribution and level varies regarding the level of focal segmental glomerulosclerosis (FSGS). The progression of sclerosis as a sign of irreversible kidney damage is accompanied by gradual increase in expression of proapoptotic factor Bax.

In vitro studies on rat proximal tubular cells in primary culture (RPTC) showed that excessive albumin uptake into rat primary renal cells causes an almost immediate mitochondrial accumulation of the apoptotic factor Bax. We hypothesize that this might be initial pathway leading to kidney cells apoptosis in childhood nephrotic syndrome.

Conclusions. We show that overexpression of apoptotic factor Bax has a place in children with nephrotic syndrome. Thus, chronic influence of albumin is a factor predisposing disturbances in system controlling apoptosis in this cohort of patients. Our data demonstrate that there is a dependence between the Bax overexpression level and the stage of CKD. We show the topologic difference between the Bax levels and FSGS degree. This is an indication that development of glomerular and tubule-interstitial disorders under the influence of proteinuria occurs in specific range. In vitro data demonstrate that albumin overload causes mitochondrial Bax translocation that could be an initial factor in apoptotic pathway activation.

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Апоптоз як механізм індукованого альбуміном пошкодження нирок при нефротичному синдромі у дітей

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Резюме. Раніше у дослідженнях *in vivo* було показано, що ефекти, викликані протеїнурією, відіграють вирішальну роль в пошкодженні нирок при хронічному захворюванні нирок (ХЗН). Однак ініціальний механізм незворотного пошкодження нирок у дітей при захворюваннях, що супроводжуються хронічною протеїнурією, зокрема при нефротичному синдромі, залишається не зрозумілим.

Метою нашої роботи було вивчення ініціального механізму розвитку апоптозу у дітей з нефротичним синдромом.

Методи. Проведено обстеження біоптатів нирок у 53 хворих (віком від 10 до 15 років) з нефротичним синдромом, госпіталізованим до відділення дитячої нефрології дитячої клінічної лікарні №7 (м. Київ, Україна). Експериментальні дослідження токсичності альбуміну, проведені на первинній культурі проксимально-тубулярних клітинах нирок щурів (ПТКНШ).

Результати. Наше дослідження, проведене на біоптичному матеріалі тканин нирок дітей з нефротичним синдромом, показало, що хронічний вплив альбуміну призводить до високого рівня апоптозу. Його розподіл та рівень залежить від рівня фокального сегментарного гломерулосклерозу (ФСГС). Прогресування склерозу, як ознаки незворотного пошкодження нирок, супроводжується поступовим збільшенням експресії проапоптозного фактора Вах.

Дослідження *in vitro*, проведене на первинній культурі проксимально-тубулярних клітин нирок щурів, показало, що вплив високих рівнів альбуміну на ці клітини, викликає майже негайне накопичення проапоптозного фактора Вах навколо мітохондріальних мембран. Ми припускаємо, що це може бути ініціальним процесом шляхом, який веде до апоптозу клітин нирок при нефротичному синдромі у дітей.

Висновки. Виявлено, активація експресії проапоптозного фактора Вах має місце у дітей з нефротичним синдромом. Таким чином, хронічний вплив альбуміну є чинником, що викликає порушення в системі, що контролює апоптоз у цій когорті хворих. Виявлено залежність між рівнем надмірної експресії Вах та стадією ХЗН. Встановлено топологічну різницю між рівнями Вах та ступенем фокально-сегментарного гломерулосклерозу (ФСГС). Це вказує на те, що формування гломерулярних та тубуло-інтерстиційних пошкоджень під впливом протеїнурії відбувається у певному порядку. Дані *in vitro* свідчать, що перевантаження альбуміном викликає мітохондріальну транслокацію Вах, що може бути початковим фактором активації апоптозного сигнального шляху.

Ключові слова: нефротичний синдром, альбумін-індукована токсичність, транслокація Вах.

Introduction. Chronic kidney disease (CKD) is a significant global public health problem with poor prognosis and elevated health care costs [1-3]. Recent clinical guidelines on CKD incorporate albuminuria and the estimated glomerular filtration rate (eGFR) to define and stage CKD [2,3]. CKD results from a variety of causes, including diabetes, glomerulonephritis, hypertension, infections and polycystic kidney disease [4]. Most forms of CKD are progressive and are characterized by disrupted glomerular perm-selectivity, glomerular sclerosis, progressed albuminuria, loss of podocytes and glomerular tubular disconnection [4, 5].

It was shown *in vitro* and *in vivo* that main pathomorphological outcomes that apply to kidney damage in CKD are glomerulosclerosis, vascular sclerosis, tubular-interstitial fibrosis. Adaptive changes of nephrons after the primary injury which can no longer be compensated with time, ultimately lead to irreversible disorders - scarring, sclerosis and further loss of nephrons leading to the end-stage CKD (ES-CKD) formation. Inflammation plays an important role in the development and progression of chronic kidney pathologies and is the primary and persistent violation, which underlies the pathogenesis of others [6]. All mentioned above pathophysiological violations might be accompanied by apoptosis. Apoptosis is programmed cell death that occurs when kidney disease has a place and plays an important role in their physiology. Harmful effects of apoptosis are in fact a source of a large number of kidney cells lost during and/or renal inflammation, scarring, loss of kidney function [7]. The molecular mechanisms of albumin-induced apoptosis and their

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outcomes in nephrotic children are not studies substantially.

The aim of our work was to study the initial mechanism of kidney cells apoptosis development in nephrotic children.

Materials and methods. Patients. An examination of renal biopsies of 53 patients (aged 10 to 15 years) with nephrotic syndrome hospitalized in Pediatric Nephrology unit of the Children Clinical Hospital №7 (Kyiv, Ukraine) was done. All patients were treated accordingly with the local protocols. Among all patients 24 (45.28%) were with hormone-sensitive type of nephrotic syndrome, others – 29 (54.72%) showed hormone-dependent type of nephrotic syndrome. Complex examination other than conventional methods (inspection, monitoring blood pressure, general and biochemical blood tests, determination of daily proteinuria, urinary sediment study and concentration ability of the kidneys, ultrasound of the abdomen etc.), immunohistochemical assessment of apoptosis-dependent glomerular and tubule-interstitial damage were done.

Immunohistochemistry. Renal tissue (3- μ m sections) was deparaffinized and rehydrated prior to processing. Antigen retrieval was carried out by boiling in Citrate Buffer (10mM Citric Acid, 0.05% Tween20, pH 6.0) for 20 min. Sections were treated with Triton X-100 0.3% (Sigma-Aldrich NV/SA, Bornem, Belgium) in PBS for 20min. After three PBS washes, sections were incubated with blocking buffer (5% bovine serum albumin and Triton X-100 0.1% in PBS) for 1 hour. The rabbit polyclonal anti-Bax antibody (Santa Cruz, CA, USA) was applied at a dilution of 1:50 in 5% bovine serum albumin in PBS overnight at 4 C. Following three PBS washes, sections were incubated with a secondary Alexa Fluor 488 goat anti-rabbit IgG (1:500) for 1h at room temperature. Nuclei were counterstained with DAPI. All samples were stained for an identical length of time and under identical conditions. All recordings for each staining were done during one day using identical gain settings. Sections were mounted in Immu-Mount (Thermo Shandon, Midland, Canada) and observed with Leica TCS SP inverted confocal scanning laser microscope using 25X/0.8NA oil-immersion objective. Image analysis was performed using ImageJ software (NIH Image, Baltimore, MD, USA). Three areas in each section were analyzed [8].

Detection of apoptotic cells: Terminal Deoxynucleotidyl (TdT)-Mediated dUTP Nick-End Labeling (TUNEL) assay. An ApopTag Red In Situ Apoptosis Detection kit (Chemicon International, USA) was used to determine the AI, according to the manufacturer's instructions. The kidney tissue cells were mounted in Immu-Mount (Thermo Shandon, Midland, Canada), and the images were recorded with a Zeiss LSM 510 laser scanning confocal microscope using a 25X/0.8NA oil-immersion objective. TUNEL stain was detected using 488 nm excitation and a 510-550 nm band-pass filter. ApopTag was detected using 561nm excitation and a 575 nm long pass filter. Cells were considered

apoptotic when they exhibited ApopTag Red staining and characteristic apoptotic morphology. The AI was calculated as the percentage of TUNEL-positive cells. In each preparation, eight to ten randomly selected areas were examined, and in each area, between 100 and 200 DAPI-stained cells were counted [9].

Cells. RPTC were prepared from kidneys of 20-day-old male Sprague-Dawley rats as described previously. The studies performed in Sweden followed the Karolinska Institutet regulations concerning care and use of laboratory animals and were approved by the Stockholm North ethical evaluation board for animal research. The kidneys were removed and placed in 0.9% NaCl at room temperature. The cortical layers were dissected and placed in Hank's balanced salt (Invitrogen, Grand Island, USA) solution at 37°C and gently mixed using a fire-polished Pasteur pipette. The reaction was stopped by washing the cells twice in a solution containing 1% trypsin inhibitor. After washing, equal volumes of cell suspension were plated on 12-mm glass coverslips in 24-well Petri dishes. The cells were cultured for 3 days in supplemented DMEM (20 mM HEPES, 24 mM NaHCO₃, 10 μ g/ml penicillin, 10 μ g/ml streptomycin, and 10% FBS) on glass coverslips in 5% CO₂ at 37°C. On day two in vitro, when the cells have been shown to maintain most of their proximal tubule characteristics, the cells were exposed to the 0, 5, 10, or 20 mg/mL of fatty acid and endotoxin-free bovine albumin alone (Sigma-Aldrich, St.Louis, USA), with ouabain (Sigma-Aldrich, St.Louis, USA) or with vehicle (PBS) for 8 or 18 h [9].

Bax translocation assessment. RPTC were cultured as described previously. On day two in vitro, when they have been shown to maintain most of their proximal tubule characteristics, the cells were exposed to the mitochondria-targeted green fluorescent protein Cell-Light® Mitochondria-GFP BacMam (Life Technologies, Grand Island, USA) overnight in the incubator. On day three in vitro, RPTC were incubated with 0, 2.5 or 10 mg/mL of albumin with or without ouabain (5 nM) or vehicle (PBS) for 0-8 h. In another set of experiments, the cells were treated with 10 mg/mL albumin for 0, 15, 30 or 45 min. For Bax immunostaining, the cells were fixed in 4% PFA, washed once with cold PBS and treated with Triton X-100 (Sigma-Aldrich NV/SA, Bornem, Belgium). The mouse monoclonal anti-Bax [6A7] Ab primary antibodies (Abcam, Cambridge, UK) were applied overnight at 4°C. The controls were subjected to the same treatment, but the primary antibody was omitted. The secondary Alexa Fluor 546 goat anti-mouse IgG IgG (Invitrogen, Grand Island, NY, USA) was applied for 1 h at room temperature. The cells were mounted and observed using a Zeiss LSM 510 laser scanning confocal microscope and a 63X/1.4NA oil objective. Analysis of the Bax translocation to the mitochondria was performed with the Matlab image processing toolbox.

Statistics. Statistical analysis was done using the method of variation statistics (STATISTICA 6.0) and

nonparametric statistical approaches (Mann-Whitney test). Results are presented as Mean \pm SEM. $P < 0.05$ was considered as statistically significant.

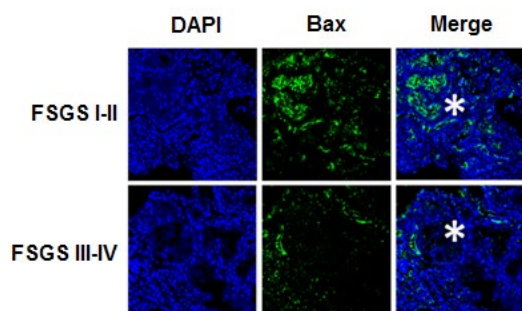
Results. Patients characteristics. Characteristics for participating patients given in Table 1.

Table 1

Group characteristics for participating patients

Characteristics	Value
Age, years	11.09 \pm 3.46
Body mass index	21.5 \pm 1.3
Sex (male/female)	29/24
Normal BP/AH	21/32
Hormone-sensitive type of nephrotic syndrome/ hormone-dependent type of nephrotic syndrome	24/29
Average disease course, years	9.09 \pm 2.16

BP – blood pressure, AH - arterial hypertension.

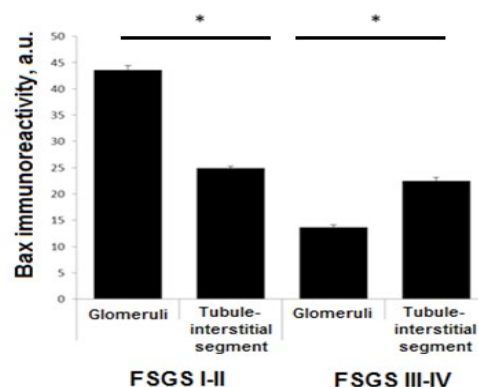


* - glomeruli, * $p < 0.05$.

Fig. 1. Topical characteristic of the Bax expression in different stages of FSGS. DAPI: visualization of nuclei; Bax: Bax immune signal in kidney tissue.

Apoptosis level in kidney tissue of nephrotic children. Results of the apoptosis level analysis in kidney sections of biopsy material of nephrotic children with morphological form of chronic glomerulonephritis focal segmental glomerulosclerosis revealed the presence of high level of apoptotic cells. Moreover, it is shown that in sclerotic glomeruli with the level of glomerulosclerosis I-II st. the majority of apoptotic cells localized in glomeruli. In case of glomerulosclerosis IV st. a high

Proapoptotic factor Bax expression in kidney tissue of patients with nephrotic syndrome. We have analyzed the levels of expression and localization of proapoptotic factor Bax in patients with morphological variant of nephrotic syndrome – focal segmental glomerulosclerosis. Stages of FSGS were determined by level of glomerular sclerotic area. Level of sclerosis corresponding to $\leq 25\%$ of the glomerular area was assumed as I stage of FSGS, II stage of FSGS - 25-50%, III stage – 50-75% and IV stage – 75-100%. Analysis of Bax expression in kidney biopsies from children with focal segmental glomerulosclerosis show the presence of high level of Bax expression in both glomerular and tubule-interstitial segments. Higher level of immune signal was recorded in glomeruli as compared to tubule-interstitial segment in FSGS I-II stages (43.57 ± 0.88 a.u. vs 24.9 ± 0.41 a.u., $P < 0.01$). When complete glomerular sclerosis presents a high level of Bax was documented in the surrounding tubule-interstitial segment (13.7 ± 0.42 a.u. vs 22.5 ± 0.65 a.u., $P < 0.01$) (Figure 1).



level of apoptosis detected in surrounding tubule-interstitial segment (Fig. 2, A).

Quantitative analysis showed that in case of FSGS I-II st. the apoptotic index (AI) in glomeruli: $22.29 \pm 0.86\%$, in tubule-interstitial segment: $9.43 \pm 0.59\%$ ($p < 0.01$). At high level of glomerulosclerosis (IV st.) distribution of apoptotic cells was different. High AI found in tubule-interstitial component: $29.27 \pm 1.18\%$, in glomeruli AI detected at level $4.7 \pm 0.54\%$ ($p < 0.001$) (Fig. 2, B).

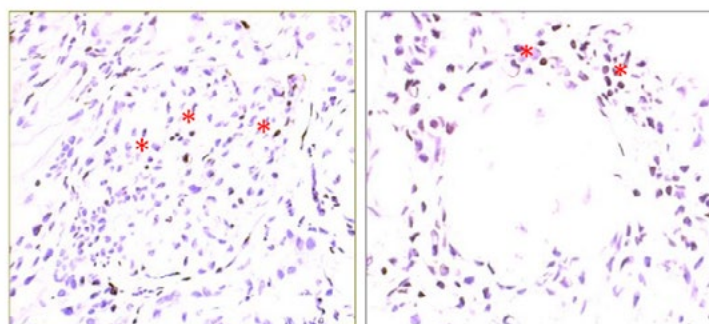
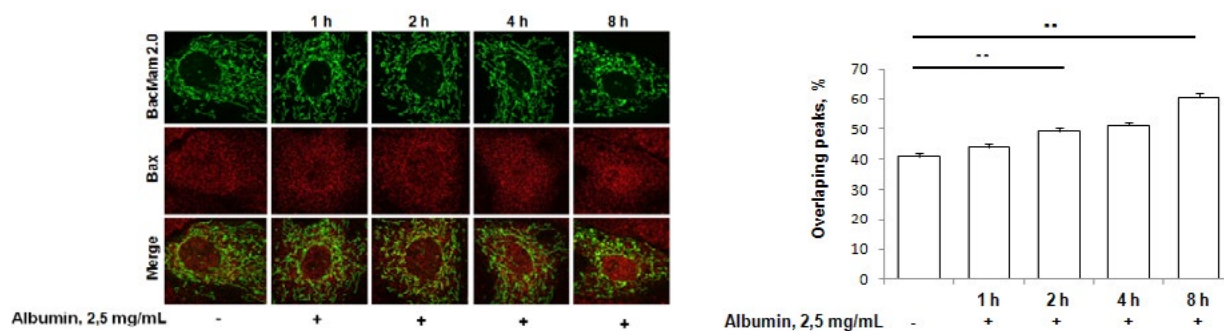


Fig. 2. Apoptosis level in kidney tissue of nephrotic children. A: TUNEL staining; B: quantitative analysis of apoptosis.

Albumin overload induces Bax translocation to mitochondria. In this study we used an albumin to induce apoptosis in RPTC. To further characterize mitochondrial involvement in albumin toxicity, we performed time sequence studies, monitoring Bax and mitochondria co-localization in albumin-exposed rat proximal tubule. Concentration of albumin 2.5 mg/mL

used in this experiment. The co-localization of immunolabeled Bax and mitochondria expressing GFP was analyzed in confocal micrographs (Fig. 3). Co-localization increased in a time-dependent manner, and the increase was significant after 2 hours incubation with 2.5 mg/mL albumin.



** $p < 0.01$. Statistical analysis was performed using the Mann-Whitney U test. Experiments were repeated four times

Fig. 3. Immunofluorescence staining of proapoptotic factor Bax (red) in RPTC incubated with 2.5 mg/ml albumin. Cells were transfected with mitochondrial marker BacMam 2.0 (green). Number of co-localized Bax/mitochondrial peaks was counted.

Discussion. Proteinuria is the hallmark of glomerular diseases, and the magnitude of proteinuria is an established adverse prognostic factor in a variety of progressive nephropathies [10]. Ongoing proteinuria has been proposed to lead to tubular injury primarily via excessive tubular cell uptake of filtered proteins and/or protein-bound substances such as fatty acids. Albumin, the most abundant protein in the glomerular filtrate, is endocytosed by proximal tubule cells and can cause direct tubule cell injury in animals and cell culture models [10, 11]. Several mechanisms remain under investigation in these experimental settings, proposed [12]. There is, however, a paucity of mechanistic information in pediatric diseases characterized by chronic proteinuria, i.e. nephrotic syndrome.

The relationship between the two cardinal pro- and anti-apoptotic members of the Bcl2 family of proteins, Bax and Bcl-xL, plays a crucial role in determining the balance between cell life and death [13]. We demonstrated that in vitro cellular uptake of albumin causes rapid accumulation of Bax around the mitochondria, resulting in the disabling of the mitochondrial membrane and initiation of the apoptotic process. These in vitro data may be crucial in further understanding of the apoptosis initiation in patients.

Here we show that overexpression apoptotic factor Bax have a place in children with nephrotic syndrome meaning that chronic influence of albumin is a factor predisposing disturbances in system controlling apoptosis in this cohort of patients. Our data demonstrate that there is a dependence between the Bax overexpression level and the stage of CKD. We show the topologic difference between the Bax levels and FSGS degree. This is an indication that development of glomerular and tubule-interstitial disorders under the influence of proteinuria occurs in specific range.

Conclusions. We show that overexpression of apoptotic factor Bax has a place in children with nephrotic syndrome. Thus, chronic influence of albumin is a factor predisposing disturbances in system controlling apoptosis in this cohort of patients. Our data demonstrate that there is a dependence between the Bax overexpression level and the stage of CKD. We show the topologic difference between the Bax levels and FSGS degree. This is an indication that development of glomerular and tubule-interstitial disorders under the influence of proteinuria occurs in specific range. In vitro data demonstrate that albumin overload causes mitochondrial Bax translocation that could be an initial factor in apoptotic pathway activation. Further study of the molecular mechanisms, i.e. specific caspases-3, -8 activation, fibrosis markers levels in children with nephrotic syndrome has a great importance for understanding the nature of the disease progression.

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Authors' contributions.

Ie.A. Burlaka: hypothesis generation, references collection, material collection, experimental work proceeding, data analysis, manuscript writing and submission

I.V. Bagdasarova: hypothesis generation, references analysis, material collection arrangement.

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