Violations in apoptosis control system in children with nephrotic syndrome

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Abstract
Proteinuria is a marker of kidney damage which reflects the loss of selectivity of the filtration barrier. The objective of this paper was to study the topical features of factors controlling apoptosis activity levels in kidney tissue in children with nephrotic syndrome. 53 patients aged 10-15 years with active stage of nephrotic syndrome were included in the study. Immunohistochemical examination of proapoptotic factor Bax, antiapoptotic factor Bcl-xL levels, apoptosis evaluation in kidney biopsy specimens were done. Analysis of the level of proapoptotic factor Bax levels in kidney slices obtained from children with morphological forms of nephrotic syndrome, focal segmental glomerulosclerosis, showed the presence of high levels of Bax in both glomerular and tubular-interstitial segments. However, higher immunosignal was recorded in glomeruli with FSGS-I st. compared to tubular segment. When complete glomerular sclerosis observed high levels of Bax are localized in the surrounding tubule and interstitial segment. Levels of antiapoptotic factor Bcl-xL levels were studied. In kidney sections a presence of a certain level Bcl-xL in both glomeruli, tubuli and interstitium was found. Higher immune signal was recorded in tubule-interstitial segment as compared to glomeruli with FSGS-I st. When complete glomerular sclerosis occurred relatively high immune signal of Bcl-xL is localized in the surrounding tubule-interstitial segment with almost complete absence of glomeruli. The results of analysis of the level of apoptosis in sections of kidney biopsy material from children with morphological form of nephrotic syndrome focal segmental glomerulosclerosis revealed the presence of a high level of apoptotic cells. Moreover, we show that in sclerotic glomeruli with glomerulosclerosis level II-III the majority of apoptotic cells are localized in glomeruli. Quantitative analysis of apoptosis levels in kidney sections of patients with FSGS-I-II st. revealed apoptotic index (AI) in glomeruli at level 22,29 ± 0,86%, in the tubule-interstitial component - 9,43 ± 0,59% (p<0,01). With FSGS III-IV st. high AI was found in tubule-interstitial component - 29,27 ± 1,18%, in glomeruli - 4,7 ± 0,54% (p<0,001). Thus, progression of glomerulosclerosis in studied pathology accompanied by increased activity of proapoptotic factor Bax and simultaneous reduction of antiapoptotic factor Bcl-xL. The dependence of levels of topical Bax and Bcl-xL expression on stages of FSGS indicate the step-dependent manner of glomerular and interstitial injuries development under the influence of proteinuria.

Keywords: nephrotic syndrome, glomerulosclerosis, apoptosis, Bax, Bcl-xL, immunostaining.

1. Introduction
The incidence of children with chronic kidney disease (CKD) is from 1.5 to 3.0 cases per million of population. The main causes of CKD in childhood are developmental abnormalities (congenital anomalies of the kidney and urinary tract), focal segmental glomerulosclerosis (FSGS), hemolytic uremic syndrome (HUS), immune-complex diseases, inherited abnormalities, Alport syndrome etc [1, 2]. Main pathomorphological outcomes that apply to kidney damage in CKD are glomerulosclerosis, vascular sclerosis, tubule-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 [2]. 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. Renal histology in chronic renal pathologies including nephrotic syndrome is characterized by typical signs of inflammation i.e. infiltration with white blood cells, hyperemia, fibrosis etc. In addition to inflammation, fibrosis has a role in nephrotic syndrome. These disorders are accompanied by activation of the renin-angiotensin-aldosterone system, oxidative stress, endothelial dysfunction and others [1-3]. 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 \cite{4, 5}. The molecular mechanisms underlying irreversible renal damage in children with nephrotic syndrome depending on apoptosis activation might be a potential therapeutic issue in CKD treatment.

2. Materials and 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) was done. All patients were treated accordingly the 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. Immunohistochemical determination of the apoptosis controlling factors (Bax, Bcl-xL) was performed using material of kidney biopsies of children with nephrotic syndrome and its morphological form focal segmental glomerulosclerosis (FSGS). Briefly, sections were washed from paraffin, dehydrated. As primary antibodies polyclonal anti-Bcl-xL (Santa Cruz, CA, USA, dilution 1: 200), anti-Bax (Santa Cruz, CA, USA, dilution 1: 200) were applied. As secondary antibodies Alexa Fluor 546 and Alexa Fluor 488 Ab (Invitrogen, USA, dilution 1: 500) were used. The nuclei of cells were labeled using 4,6-diamino-2-fenilindol (DAPI, Sigma) in concentration 1,5 mg/mL. DAPI was added to phosphate buffer (pH 7.4) during the final wash. Before microscopy slides were covered with cells Immu-Mount (Thermo Shandon, Midland, Canada).

TUNEL test to determine the level of apoptosis biopsy material was performed on formalin-fixed paraffin sections 5 μm thick. Kidney sections were treated with proteinase K (20 mg/ml) for 20 minutes at 37 °C. Endogenous peroxidase activity was blocked kidney slices by incubation in 0.3% H2O2 in phosphate buffer (pH 7.4) for 10 minutes. To determine apoptosis a Peroxidase in Situ Apoptosis detection Kit (Chemicon International, UK) has been used. Sections were counterstained with Harris hematoxylin (Richard Allan Scientific, USA). Images were obtained using Zeiss LSM 510 inverted scanning confocal microscope and x40/1.4 NA oil-immersion objective. Images were processed using the Image J software (NIH, USA). Optical slice thickness was 1-2 microns.

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 ± SEM. P<0.05 was considered as statistically significant.

3. Results
3.1 Expression of proapoptotic factor Bax 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 ≤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).

![Fig 1: Topical characteristic of the Bax expression in different stages of FSGS. DAPI - visualization of nuclei; Bax - Bax immune signal in kidney tissue. * = glomeruli, * p<0.05.](image)

3.2 Expression of antiapoptotic factor Bcl-xL in kidney tissue of patients with nephrotic syndrome
Important role in the development of apoptosis plays a ratio of factors Bcl-xL/Bax. The analysis of the antiapoptotic factor Bcl-xL expression in sections of renal biopsy material from children with morphological forms of nephrotic syndrome focal segmental glomerulosclerosis revealed the presence of certain level Bcl-xL in both glomeruli and tubule-interstitial segment. However, higher intensity immune signal was recorded in tubule-interstitial segment as compared to glomeruli with FSGS stage II (25.29 ± 0.55 a.u. vs 8.71 ± 0.8 a.u., P<0.01). When complete glomerular sclerosis occurred relatively high level of immune signal of Bcl-xL was detected.
in surrounding tubule-interstitial segment and was almost complete absent in glomeruli (19.57 ± 1.02 a.u. vs 6.81 ± 0.31 a.u., P<0.01) (Figure. 2).

3.3 Apoptosis in kidney tissue of patients with nephrotic syndrome

We detected a high level of apoptotic cells in kidney biopsy material from children with nephritic syndrome. Moreover, we documented that in sclerotic glomeruli with glomerulosclerosis level II-III apoptotic cells are localized predominantly in glomeruli (Fig. 3A). When complete glomerular sclerosis occurs high level of apoptosis detected in the surrounding tubule-interstitial segment (see Fig. 3B). Quantitative analysis showed that in glomeruli with FSGS I-II stage an apoptosis in glomeruli was detected at level 22.29 ± 0.86%, in tubule-interstitial segment - 9.43 ± 0.59% (p<0.01). In glomeruli with high level of sclerosis distribution of apoptotic cells was different. High apoptosis index (AI) was found in the tubule-interstitial component - 29.27 ± 1.18%, in the glomeruli AI was 4.7 ± 0.54% (p<0.001) (see Fig. 3C).

4. Discussion

Control and regulation of apoptosis signaling pathways exist under the influence of proteins from Bcl-2 family. Bcl-2 is involved in the regulation of mitochondrial membrane permeability and contains both proapoptotic and antiapoptotic proteins. The Bcl-2 family includes antiapoptotic proteins Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-W, BAG and proapoptotic - Bcl-10, Bax, Bak, Bid, Bad, Bik, Blk. Shift in the balance of activity of either proapoptotic factors or antiapoptotic factors leads to an excessive apoptosis [6, 7]. The imbalance between the process of cell death and cell proliferation leads to structural and functional violations in kidneys. For example, the prevalence of cell proliferation leads to their excessive accumulation that it turns leads to neoplasia, which is characteristic of proliferative forms of glomerular pathologies. Increased levels of cell death particularly by apoptosis is a cause of irreversible loss of certain functions due to the inability of cell renewal. Apoptosis is regulated by extracellular and intracellular molecular regulators which are members of the relevant signaling pathways. Cell death usually occurs in response to changes in cell microenvironment in which there is no specific factors (survival factors) or presence of proapoptotic factors has place. Initial factors in this case may be surrounding cells, mediators and extracellular matrix components [7, 8]. The process of cell death begins with an activation of intracellular factors in response to proapoptotic stimuli from cell microenvironment. The presence of Fas ligand on the cell surface is a determinant
of cell sensitivity to Fas-induced apoptosis. Mesangial cells, proximal tubular kidney cells, fibroblasts expressing cell receptor Fas.

Mitochondria is a key player in apoptosis development which has not connection with the death receptor. Mitochondria-dependent disorders can promote cell death independently of death receptor activation. Mitochondrial changes during apoptosis include: 1) the disappearance of mitochondrial transmembrane potential gradient ($\Delta \Psi_m$) in connection with the opening transient pores; 2) release of specific proteins such as cytochrome c, AIF, and SMAC/Diablo, from intermembrane mitochondrial space into the cytosol, where they participate in the effector phase of apoptosis - direct caspase activation \(^{10}\). The main mechanisms of the antiapoptotic factors that belong to Bcl-2 family are the following. First of all, they inhibit caspases activation in apoptosis. Bcl-xL inhibits complex formed by caspase-9, Apaf-1 and cytochrome C, which ultimately prevents caspase-3 activation. The second scenario is closing the VDAC and prevention of mitochondrial apoptogenic factors such as cytochrome c and AIF release into the cytoplasm \(^{9,11}\).

In present study we show that progression of glomerulosclerosis in children with nephrotic syndrome is accompanied by increased activity of proapoptotic factor Bax and a simultaneous reduction in expression of antiapoptotic factor Bcl-xL. Revealed dependence of topology of Bcl-xL levels on FSGS degree indicates that development of glomerular and tubule-interstitial disorders under the influence of proteinuria occurs in specific range. However, we have not detected any differences in Bax, Bcl-xL expression and levels of apoptosis between patients with hormone-sensitive and hormone-dependent type of nephritic syndrome.

Proteinuria is a marker of kidney damage, reflecting the loss of selectivity of the filtration barrier. Moreover, proteinuria is an important prognostic factor in development and progression of kidney damage due to activation of apoptosis, inflammation, fibrosis \(^{13}\). High concentrations of protein in ultrafiltrate cause proximal tubular cell apoptosis. Apoptosis in turn induces inflammatory processes. Activated in proximal tubular cells apoptosis leads to tubular atrophy, atubular glomeruli formation \(^{12,13}\). Formation of atubular glomeruli determines the degree of renal function loss on one hand and the progression of tubule-interstitial damage on other \(^{4,5}\). Further study of the molecular mechanisms of apoptosis in proteinuric kidney disease in children and development of novel approaches to their correction is a promising research field in terms of prevention kidney function impairment.

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6. References