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Ultrastructural Study of Podocyte Alterations inPodocytopathies

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ABSTRACT

Introduction: Ultrastructural alterations in podocytes are an important diagnostic and prognostic marker of nephropathies. However, the biomedical understanding of the detected submicroscopicalteratuons in podocytes, such as effacement of cytopodia and naked glomerular basement membrane, remains controversial.

The aim of this study was to investigate the relationship between ultrastructural changes in podocytes (cytopodiaeffacement andnaking of glomerular basement membrane) with a number of clinical and laboratory indicators of renal dysfunction using the example of podocytopathies.

20 patients (11 men, 9 women) were examined. Patients with podocytopathies were represented by: focal segmental glomerulosclerosis - 8 anddisease of minimal changes- 12.

Materials and methods: All patients underwent standard laboratory and instrumental studies: total serum cholesterol. mmol / l;blood serum protein. g / l; serum creatinine. mmol / l; urea. mmol / l; serum albumin g / l; glomerular filtration rate according to CKD-EPI. ml / min / 1.73 m²; daily protein loss g / day

Light microscopy changes: Glomerulosclerosis were assessed quantitatively: glomeruli completely sclerosed and / or with focal segmental sclerosis were considered. Quantitative ultrastructural stereological analysis was performed by evaluating the cytopodial width (FPW) and the degree of naked glomerular membrane (% NGBM) of the glomeruli.

Results: In podocytopathies. the largest number of sclerosed glomeruli was observed in focal segmental glomerulosclerosis. which was accompanied by the lowest daily proteinuria and glomerular filtration rate. Quantitative values of the cytopodia width were associated with the level of daily protein loss ($r = 0.34$. $p > 0.05$) and serum albumin level ($r = -0.53$, $p < 0.05$) in patients with nephrotic syndrome. Correlation analysis of the values of the cytopodia width and %naked glomerular basement membrane in patients with podocytopathies revealed a statistically significant negative interrelation of these morphometric parameters. ($r = -0.54$, $p = 0.012$).

Conclusions: In patients with podocytopathy a correlation between the severity of nephrotic syndrome and proteinuria / hypoalbuminemia on the one hand and the cytopodia width on the other was found. The revealed negative relationship between the width of the cytopodia and the%of the naked glomerular basement membrane in patients with podocytopathiescould be a consequence of the early stages of podocytes damage, accompanied by transient naked glomerular basement membrane.

KEYWORDS

Podocytopathies, Podocyte, Cytopodia, Nephrotic Syndrome, Proteinuria, Podocytopathy,Naked.

Introduction

According to the present views. R. Colvin, A. Chang et al. 2019 (“Diagnostic pathology of the kidney” 3rd edition. - 2019. Elsiever Philadelphia. USA. - Section 2.- Glomerular disease: Podocytopathies, - PP 48-86.) podocytopathies (PP) includedisease of minimal changes (DMC), metabolic obesity syndrome (MS) and primary collapsing, secondary focal segmental glomerulosclerosis (FSGS).Generally,these pathologies according to the WHO Classification (ICD-10) are documented in two pathological forms: DMC and FSGS. Severe proteinuria with nephrotic syndrome (NS) are mostoften theirmainclinicalmanifestations [1,7,27]. At the cellularlevel, podocytes were found to be themain components involved in the development of massive proteinuria in these diseases [2,3]. At the same time itwas revealed submicroscopically [4] that the foot process effacement of podocyteswithfollowed destruction and desquamation from the surface of the glomerular basement membrane (GBM) lead to its “naked”.Thefootprocess effacement of podocytes with the following destruction and desquamation from the surface of the glomerular basement membrane (GBM) was found to lead to its “naking”.

The authors associate these alterations with damage to cytoskeletal proteins, in particular,synaptopodin and actin, as a result of increased activity of calcineurin (serine-threonine-phosphatase),that can be counteracted by clinically used calcineurin inhibitors [5]. Timely detection of podocyte damage is essential for prognosis of the disease, in particular ofglomerulosclerosis development of [6, 7].However, it has been shown that foot process effacement of podocytes and naked GBM not always correlate with the severityof proteinuria [8, 9].

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This work was carried out to further clarify the problem of the possible relationship between alterationsof the podocytes in PP with a number of clinical and laboratory indicators of the glomerular filter state. To ensure adequate accuracy and reliability of the data, quantitative stereological analysis of a number of ultrastructural alterations in podocytes, in particular, the cytopodia or foot processes (CP) width and % naked glomerular basement membrane (% NGBM) was used.

Materials and Methods

The group under study consisted of 20 patients (11 men, 9 women). Depending on the morphologicalvariant of nephropathy, the patients were divided into the following groups: focal segmental glomerulosclerosis (FSGS) – 8,disease of minimal changes(DMC) - 12. All patients when combined formed a group of patients with PP (whole group).

The clinical course of the nephropathy (azotemia, the presence of arterial hypertension and its degree, the duration and nature of the urinary syndrome) was analyzed in all patients and standard laboratory and instrumental studies were performed, including total serum cholesterol(Tchol), mmol / l;blood serum protein (GPr), g / l; serum creatinine (Cr), mmol / l; urea (urea), mmol / l; serum albumin (AS) g / l; glomerular filtration rate (GFR) according to CKD-EPI, ml / min / 1.73 m²; daily loss of protein, g / day (24hPr).

In addition, according to the clinical data, but irrespective of their sex, the patients were divided into 2 groups: persons with (NS +)and without (NS-) nephrotic syndrome.

For pathohistological examination, samples of the renal parenchyma were immediately fixed in 4% paraformaldehyde solution in phosphate buffer (pH 7.4) for 24 h at room temperature. After standard processing (dehydration, impregnation, embedding), 3-4 μm serial sections (ESM-350, ERMA Japan)were prepared from paraffin blocks and stained with hematoxylin and eosin, Schiff's reagent (PAS),trichromal stain (Masson), according to Jones, according to Veigert, Congo red. The study was carried out in a light microscopically (Carl Zeiss Imager Z 2; Germany). Pathological alterations were assessed quantitatively. The total number of glomeruli with completely sclerosed glomeruli and / or with focal segmental sclerosis was taken into account. The latter wasconsidered as manifestations of the so-called glomerulosclerosis (GS).

Immunolabeling on cryostat sections was performed by direct fluorescence using FITC-labeled antibodies (DAKO, Denmark) to IgA, IgM, IgG, C3c, C1q, fibrinogen, as well as kappa (κ) and lambda (λ) light chains. Biopsies were fixed for ultrastructural analysis in a standard aldehyde fixative solution, then in osmium tetroxide and embedded in epoxy resins. Ultrathin sections were contrasted with uranyl acetate and lead citrate andwere analyzed in a transmission electron microscope (JEM 7A,Japan). The studies were carried out on the basis of the Laboratory of Electron Microscopy of the Department of Pathology of the Research Center and the Laboratory of Clinical Immunology and Morphology of the Research Institute of Nephrology of First Saint State Medical University named after academician I.P. Pavlov.

Measurements of the podocytes cytopodiawidth(FPW) were carried out in accordance with the known method [9]. In the present study, 5-10 fields of one or two glomeruli from each patient were photographed at an original magnification of x10000. The study ofelectronogramswith CP was performed using the Image J Software (NIH 1997). FPW arithmetic values were calculated by the formula: FPW = π/4 x (Σ GBM lenght / Σ foot process) for each patient and group as a median with interquartile range. The number of CPs on the glomerular basement membrane was counted. CP was defined as any cytoplasmic segment of the podocytes adjacent to the basement membrane of the glomerulus between two filtration pores or slit diaphragms (Figure 1A). The obtained electronograms were used to determine the percentage of the “naked” GBM. The “naked” zone was defined as the area of the GBM outer surface,where there was no podocytic processes more than 25-30 nm wide and a slit diaphragm between the corresponding to thefootsurface. “Naked” (%)GBM was calculated by the formula: %NGBM = Σ GBM length (La) / Σ lengths of naked areas (Lm) x 100, similar to the method used by E.JWeil 2011 in the study of desquamation podocytes (Fig. 1B). Arithmetic values were calculated for each patient and, accordingly, for each group as a median with an interquartile range.

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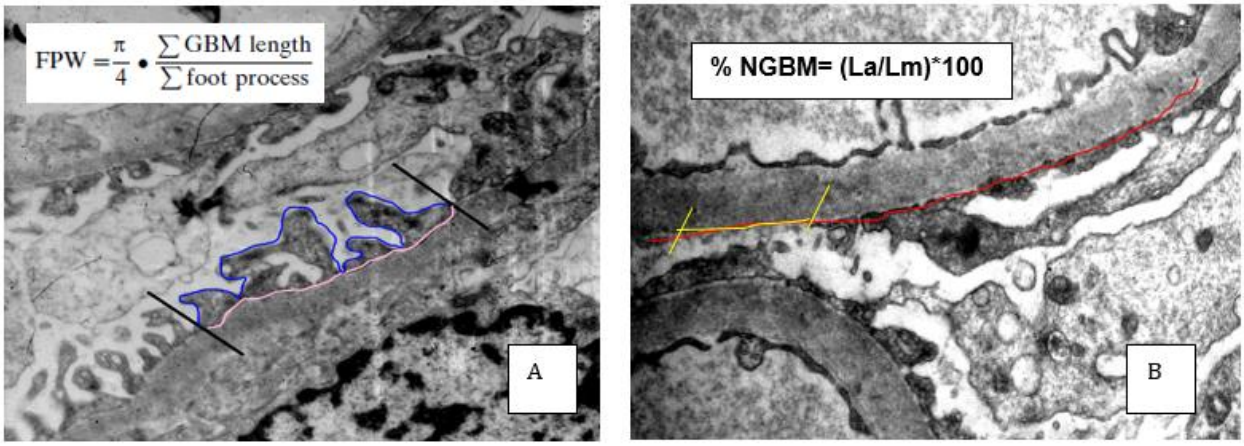

Figure 1. Evaluation of the width of the podocyte cytopodialeffacement areas (FPW)and %NGBM by a quantitative method

A. Red line - measurement of the length of the basement membrane filtration surface (GBM length),Blue line - detection of podocytes cytopodia (foot process).

B. Red line - measurement of the length of the basement membrane filtration surface the (GBM length),Yellow line - detection of the naked GBM.

Image J Software (NIH 1997), 10.000 magnification.

The data were statistically processed using standard software packages for applied statistical analysis (Statistica 7.0 for Windows). Standard methods for parametric and nonparametric statistics were used. The data in the tables areasan average value with standard deviation or median and interquartile range in incorrect distribution. For a comparative analysis between the groups, the Kruskal-Walis and Mann-Whitney U Test tests were used at p besides <0.05, respectively. correlation analysis by means of Spearman Rank order Correlations with the calculation of the "R" coefficient at p <0.05.

Results

Table 1 shows the results of the main clinical and laboratory parameters in the nosological groups of patients.

Table 1. The results of the main clinical and laboratory parameters in nosological groups of patients

Parameter	1 - DMC(n=12)	2-FSGS (n=8)	The whole group (n=20)
Sex man/woman	6/6	5/3	12/8
Age, years	35.5(25.-47.5)	39.5(25.5-61.0)	36.5(25.0-50.0)
Totalserumcholesterol, (mmol/l)	8.06 (6.94-10.01)	7.15 (6.2-12.3)	7.4 (6.6-10.0)
Blood serum protein, (g/l)	53.0(45-60.5)	55.0(39.5-66)	53.0(42.5-62.0)
Serum albumin, (g/l)	20.8(14.9-27.6)	27.15 (11.05-36.50)	22.76±10.59
GFRby CKD-EPI, 61-120 ml/min/1.73 m^2	111.5 (69.50-125.74)	81.09 (46.60-108.23)	99.68 (51.8-123.6)
Protein daily loss, (g/day)	9.67 (4.2-10.34)	6.07(3.39-11.47)	7.79 (3.82-10.37)
Urea, (mmol/l)	4.35 (3.35-4.95)*	5.60(5.05-9.05)*	4.85(3.7-6.5)
Creatinine,(mmol/l)	0.072 (0.056-0.084)	0.086 (0.076-0.146)	0.081 (0.060-0.092)
Nephroticsyndrome %	75%	62%	70%

* statistically significant differences between groups

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Men predominated in PP groups of patients, - 55.0% of the total number of all patients. There was no significant age difference between the groups. The mean age for the entire cohort of patients was 36.5 years. Daily proteinuria was lower in FSGS. The highest serum albumin level wasregistered in FSGS, while the GFR in these patients was the lowest [10]. The highest total cholesterol level was observed in the DMCgroups of patients. It should be noted that the data obtained are not statistically reliable.

As expected, the number of patients with NS + / NS- was 14/6 in PN (Table 2). In the NS- group, the ratio of nosologies was 50/50%, and in the NS + group, patients with DMC prevailed, 64.28%, respectively. Changes in clinical and laboratory parameters in the groups corresponded to the presence or absenceof NS in patients (Table 2).

Table 2. The results of the main clinical and laboratory parameters in the groups of patients with (NS +)and without(NS-) nephrotic syndrome

Parameter	**NS+ (n=14)**	**NS- (n=6)**
Sex, male/female	6/8	3/3
Age, years	38 (25-48)	29.5 (24-57)
Total serum cholesterol(3.8—7.02 mmol/l)	8,86 (6,82-10,92)	6,9 (5,69-7,59)
Blood serum protein (65—85 g/l g/l)	50 (39-60)	62 (57-65)
Serum albumin, (40—50 g/l)	19 (12.4-24)*	35.15 (29.2-39) *
GFRbyCKD-EPI, 61-120 ml/min/1.73 m^2	99,68 (71.18-122.72)	84.58 (51,7-124,58)
Urea (mmol /l)	4.75(3.7-6,0)	4.95(4.5-8.2)
Creatinine(mmol/l)	0.07(0.05-0.08)	0,08(0.07-0.13)
Daily protein loss (50-100 mg/day.)	10.18 (6.24-12.5) *	3.82 (2.34-5.21) *

* statistically significant differences between the groups

Light microscopy. In PP, the largest number of sclerosed glomeruli, i.e. those with GS were registerted inFSGS. GS value was significantly (at $p < 0.05$) higher than in DMC. The grouping of patients into the NS + and NS- groups did not reveal a significant difference in the number of GS between the groups, although the absolute GS in the NS- group was higher (Table 3).

Glomerulosclerosis in the combined group of PP patient had a correlation dependence with urea ($r = 0.56$, $p < 0.05$) and GFR ($r = -0.44$, $p < 0.05$) (Table 4). No connections between GS and daily proteinuria and / or AS levels was found. There were also no statistically significant correlations between GS, FPW ($r = -0.29$, $p > 0.05$) and% NGBM ($r = -0.43$, $p > 0.05$) (Table 4).

Table 3. Results of the main structural parameters in nosological groupsand in patients with nephrotic syndrome (NS +) and without it (NS-)

Parameter	**DMC(n=12)**	**FSGS (n=8)**	**NS+ (n=14)**	**NS- (n=6)**
Glomerulosclerosis	0.00 (0.00-0.00) *	0.275 (0.121-0.33) *	0.00 (0.00-0.10)	0.155 (0-0.33)
FPW (nm)	1425.58 (696 -1842)	1547,5 (1040 -2049)	1577.5 (872 -2325)	990.5 (718 -1480)
%NGBM	0.19 (0-0.524)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.304 (0-0.488)

* statistically significant differences between the groups

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Electron Microscopy

In FSGS, the cytopodia width did not differ from the patients in the DMC group. However, in patients with FSGS, the value of the naked glomeruli basement membranes was somewhat less than in the DMC group, although it was statistically not significant (Table 3). Analysis of the FPW statistical valuesand% NGBM in the groups of patients with nephrotic syndrome and without it revealed differences in the values of FPW and% NGBM, although the differences between the groups of patients were not statistically significant at p <0.05 (Table 3).

Correlation analysis in DMC and FSGS patients showed a tendency towards a negative relationship between the FPW and% NGBM values in the corresponding patients. However, these data were also statistically unsignificant.
Correlation analysis in the combined (whole) group showed that FPW quantitative values were statistically significant associated with AS level of patients (r = -0.53, p <0.05) (Table 4) and had a positive tendency with values of 24hPr and cholesterol at p> 0.05. The % NGBM in the combined group had a negative tendency with 24hPr level (r = -0.44, p> 0.05). The opposite dynamics of the relationships between FPW and% NGBM with a number of indicators of renal dysfunction (AS, urea, total serum protein, total serum cholesterol) was registered (Table 4).

Table 4. The results of the correlation analysis of clinical, laboratory and structural indicators of renal dysfunction in whole groupof PP

	24hPr	Cr	Urea	AS	Tchol	GPr	GFR	GS	FPW	%NGBM
GS	**-0.03**	**0.38**	**0.56***	**0.13**	**-0.12**	**-0.05**	**-0.44***	**1.00**	**0.29**	**-0.43**
FPW	**0.34**	**-0.03**	**0.24**	**-0.53***	**0.36**	**-0.48**	**-0.12**	**0.29**	**1.00**	**-0.54***
%NGBM	**-0.44**	**-0.24**	**-0.33**	**0.19**	**-0.05**	**0.13**	**0.22**	**-0.43**	**-0.54***	**1.00**

* statistically significant differences between the groups

Correlation analysis of FPW and% NGBM values among PP patients (whole group) confirmed a statistically significant (r = -0.54, p <0.012) negative dependence of these morphometric parameters characterizing the morphofunctional state of podocytes at the ultrastructural level (Fig-2).

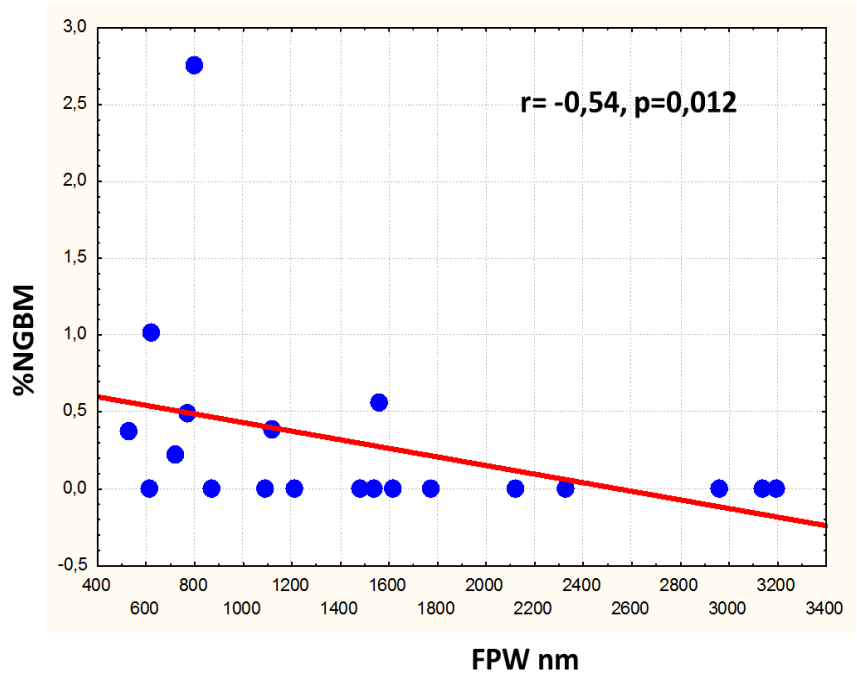

Figure 2. Correlation of % naked glomerular basement membranes (% NGBM) and podocyte cytopodia width (FPW) in a group of patients with only podocytopathies (DMC + FSGS)

Abscissa: podocyte cytopodia width (FPW) in nanometers.
Y-axis: % naked glomerular basement membranes (% NGBM).

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Discussion

According to R. Colvin, A. Chang et al. 2019 ("Diagnostic pathology of the kidney" 3rd edition.-2019. Elsiever Philadelphia, USA. - Section 2.- Glomerular disease: Podocytopathies. - PP 48-86.) Podocytopathies include: disease of minimal alterations (DMC), metabolic syndrome / obesity (MS) and primary, collapsing, secondary focal segmental glomerulosclerosis (FSGS). At the same time, according to the WHO Classification (ICD-10), pathomorphological PP forms are DMC and FSGS diseases,with the main manifestation as NS [1,7,27]. That's why patients with pathomorphological patterns of DMC and FSGS as PP were used in our study.

Podocytes are considered postmitotic cells. Their function depends on their fine specialization, due to their unique cytoskeleton structure. Particularlry,the podocytes peform four different functions: regulation of selective glomerular permeability [11], structural support of glomerular capillaries together with mesangial cells [12], together with endothelial and mesangial cells - remodeling of the glomerular basement membrane (GBM) [13], endocytosis of proteins filtered into the primary urine [14]. Podocyte function depends on their complex multicomponent architectonics, which includes (1) a slit diaphragm complex, (2) a cellular cytoskeleton with actin at the base, and also associated proteins and adhesion molecules, (3) the microenvironment of podocytes, including GBM, to which they are attached [15]. The process of cytopodia effacement in PP was one of the ultrastructural manifestations of podocyte dysfunction studied in this study.

It has already been shown that the actin cytoskeleton undergoes reorganization, condensing in a narrow strip of cytoplasm adjacent to the plasma membrane, which is accompanied by a change in the structure of the slit diaphragms [2]. Indicated in PP alterations are accompanied clinically by proteinuria, often with nephrotic syndrome [9].

These alterations are consistent with our data (Table1), particularly, the most significant proteinuria and nephrotic syndrome (Table 2) were found in the patients withDMC than in FSGS. For the assessmentthe degree of foot process effacement [17], quantitative determination of FPW havebeen used, since the method is considered as the most convincing criteriaan alteration of podocytes.

As aresult, it was revealed thatthe differences in FPW values between the studied groups were insignificant, in contrast to other works. The combined extra-nosological correlation analysis revealed relationship between the severity of FPW, the level of AS ($r = -0.53$, $p < 0.05$) and 24hPr ($r = 0.34$, $p > 0.05$) (table 4) in patients with PP, and this is consistent with the observations of some, although not all, the authors [17], being in thecontradiction with others [9].
Despite these differences, our results are likely to be quite convincing, since they coincide with the widely recognized concept of a direct relationship between the severity of damage to the cytoskeleton of podocytes, on the one hand, and the degree of proteinuria, on the other. The triggering mechanism of this damage in nephroticlevel proteinuria, in the majority of cases of immune and non-immune glomerulopathies, is the effacement of podocyte cytopodia [5], which, however, does not exclude other interpretations that require special studies.

Podocytes cytopodiaeffacement is,apparently, a reversible process in cases of stopping the exposure of pathogenic factors. However, in prolonged exposure, they increase and become irreversible resulting in glomerulosclerosis [18].
In the present study no correlations were found betweenGS, FPW, and also with % NGBM.

However, the relationship between GS and GFR was found, which seems to be quite natural, since both indicators reflect the number of nephrons that have lost their function and thus can serve as an important predictor of end-stage renal disease [19]. The nakingparts of the basement membrane, (accompanied by desquamation of podocytes, by glomeruli podocytopenia with massive proteinuria) areconsidered to be one of the stages of damage to podocytes with a high probability of glomeruloslerosisdevelopment [20, 21].

This data prompted us toevaluate% NGBM besides FPW, considering the data that excessive proteinuria in a number of diseases can develop without cytopodiaeffacement [21]. The results showed that differences in% NGBM in patients were not observed regardless of their grouping, both in terms of nosological and syndromic characteristics. Besides, no statistically significant correlation between% NGBM and GS severity was registered. These data somewhat contradict the results of other researchers [22], since in the studiesanalysed by the authors,the material was

obtained only from patients with diabetes mellitus (in our PP study), and there were significant differencies with our methodology. In particular, in our study, separate areas of naked basement membranes with a relatively intact structure of the podocyte body were considered. Whilethe samples with total desquamation of visceral glomerular epithelium were taken into account in the investigations of the authors cited above.

The relationship of % NGBM with 24hPr and AS (when compared with FPW data) served a reason to perform a correlation analysis of quantitative values of FPW and % NGBM (Table 4). The results demonstrated the presence of a significant negative correlation between FPW and % NGBM in the combined group of patients with PP.

As a hypothesis explaining the negative correlation between FPW and % NGBM, it is possible to assumed that between the podocyte CP or a number of adjacent podocytes, appears membrane areas not covered with a slit diaphragm, the so-called areas of transient naked areas,at the earliest stages of damage (“stage 1”),. In the case of the transition to the “stage 2” [2]it is occuredthe CP effacement activation with “spreading” and hypertrophy of podocytes. As a result these zones (transient naked areas) decrease in size.The revealed alterationsseem to reflect the structural modification of podocytes in the form of CP effacement that is a preventive - adaptive cell reaction to the possibility of desquamation from the GBM surface under stress and the action of damaging factors [2].

In experiments simulating massive proteinuria with nephrotic syndrome, the fact of podocytes desquamation was established [23]. Similar alterations were observed in patients with idiopathic FSGS, and also in amyloidosis, diabetes, and a number of other pathologies [20]. However, podocytes desquamation is practically not documented in the disease of minimal alterations and in hereditary nephrotic syndrome, i.e. in conditions with severe nephrotic syndrome and effacement of podocytes CP [24].

Considering the results of the analyzedresearchs, we suppose that the severity of pathological alterations, such as FPW and % NGBM, is not a consequence of proteinuria, but primarily depends on the pathogenesis of each specific disease, which is consistent with the data of other authors [9]. The data our study showed that the decisive factors in nephron damage are the severity of clinical manifestations, the time of the onset and dynamics of symptoms. Our methodology based on the previously published methodological techniques, can be considered as an original if it is regarded as a whole, although the achievements of our predecessors is also gratefully taken into account [25, 26].

Conclusions

Thus, the results of the present study revealed a correlation between the severity of nephrotic syndrome and proteinuria / hypoalbuminemia on the one hand and FPW on the other (1), between GFR and the number of sclerosed glomeruli (2). And a negative relationship between FPW and % NGBM in the patients with podocytopathies was established (3).

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