Rom J Morphol Embryol 2017, 58(3):823–830

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

ORIGINAL PAPER

The relationship between glomerular function and podocyte structure of pre-proteinuria and acute nephrosis in puromycin aminonucleoside-induced rat models: a comparative electron microscopic study

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Abstract

Puromycin aminonucleoside (PA) has been generally utilized as model of podocyte injury followed by massive proteinuria, severe damage on endocytotic activity of epithelial cells and postmodification of endocytosed compounds. However, total PA nephrosis (PAN) mechanism cannot be understood. We aimed to study glomerular function, foot process degeneration and transport pathways of podocytes in pre-proteinuria and acute PAN rats. Eighteen male Wistar albino rats were divided into three groups: control, pre-proteinuria and acute nephrosis groups (*n*=6). PA was injected into pre-proteinuria group for three times and acute group for nine times. Proteinuria levels in urine, creatinine and albumin levels in blood were detected 24 hours after PA injections. Renal cortex samples were prepared for transmission electron microscopy. Proteinuria levels in acute group significantly elevated, whereas creatinine clearance, serum albumin levels and urine volumes diminished compared to control and pre-proteinuria groups. In pre-proteinuria group, hypertrophy and structurally rich cytoplasm were detected only within podocytes. Acute group had various protein absorption granules secreted from podocyte cytoplasm to the urinary space through exocytosis after lysosomal digestion; but not observed in pre-proteinuria group. The number of slit pores in pre-proteinuria group decreased, particularly related to fusion of foot processes, subsequently leading to proteinuria. We concluded that foot process fusion begins prior to development of proteinuria although their serum albumin and creatinine clearance levels do not differ significantly. Additionally, we suggested that in acute PAN, first affected glomerular cells could be podocytes and there could be a correlation between glomerular function and number of slit pores.

Keywords: proteinuria, puromycin aminonucleoside nephrosis, transport, foot process fusion, ultrastructure.

Introduction

The leakage of plasma proteins into the urine, which is called as proteinuria, is a result of glomerular filtration barrier (GFB) dysfunction. Clarification of the pathogenic mechanisms underlying proteinuria is one of the most important themes in the field of nephrology. Which structure within the glomerular barrier does represent the primary filter for retaining plasma proteins is a question that has been argued for the past three decades [1–4].

The visceral glomerular epithelial cells (podocytes) participate in the formation of GFB along with the endothelial cells of glomerular capillary and glomerular basement membrane (GBM). GBM significantly contributes to hydraulic resistance and macromolecular permeability properties of the glomerulus [5, 6]. Thereby, the plasma proteins are held within the capillary lumen because of the high selectivity function and charge property of GFB.

The podocytes are highly differentiated cells that form multiple interdigitating foot processes around the capillary loop. The neighboring foot processes derived from different cell bodies were connected by a continuous membrane-like structure called the slit-membrane [7, 8] and they play role as a final barrier of GFB for retaining plasma proteins [5, 6, 9, 10]. Increasing evidence suggests that podocytes are a key determinant in the maintenance of the permselective function of the glomerular capillary [11–15].

Puromycin aminonucleoside (PA) is widely used as an inducer of podocyte injury models characterized by severe proteinuria [7, 16–18]. In rat glomeruli with PA nephrosis (PAN), histological changes similar to human nephrotic syndrome with minimal change disease and focal segmental glomerulonephritis could be demonstrated [7]. PA is known to interfere with endocytotic activity of the epithelial cells and affects the post-modification of endocytosed compounds in the cells but the total PAN mechanism could not be fully understood.

In this paper, we aimed to study the relationship between glomerular function and ultrastructure of the podocytes, assessing pre-proteinuria and acute groups of PAN-induced rats. Moreover, we also ultrastructurally investigated the protein transport pathways in podocytes,

ISSN (print) 1220–0522 ISSN (online) 2066–8279

which cause high levels of proteinuria in progressing acute nephrosis in PAN-induced rats.

Materials and Methods

Experimental design

Eighteen young male Wistar albino rats weighing 90–120 g (from Laboratory of Experimental Animals Reproduction and Research, Cerrahpaşa Medical School, Istanbul University, Turkey) were housed in individual cages in a temperature- and humidity-controlled room, with a 12-hour light/dark cycle. They were fed with standard rat chow and had free access to tap water. Rats were divided into three groups with one control and two experimental groups (*n*=6). Group I served as control and was injected with 1 mL isotonic sodium chloride. The other two experimental groups (pre-proteinuria and acute nephrosis groups) were subcutaneously injected with 1.67 mg puromycin aminonucleoside (Sigma Chemical Co., St. Louis, MO, USA) per 100 g body weight in 1 mL isotonic sodium chloride (Table 1). Proteinuria developed in all PA-injected rats at 6th day after the 5th injection (Table 2). According to these parameters, we constituted two experimental groups: group II, the 'pre-proteinuria group', was sacrificed on day 4 after 3rd injection; group III, the 'acute nephrosis group', was sacrificed on day 10 after 9th injection (Table 1). All rats were sacrificed under ether anesthesia at the end of the study according to the regulation of the Animal Ethics Committee of Istanbul University.

Table 1 – ***Injection intervals and amounts of all groups***

Groups	Injection intervals	Injection amount	Total injection Nos.	Sacrification day
Group I: Control	Daily	1 mL isotonic NaCl	9	10th day
Group II: Pre-proteinuria	Daily	1.67 mg PA per 100 g body weight in 1 mL isotonic NaCl	3	4th day
Group III: Acute nephrosis	Daily	1.67 mg PA per 100 g body weight in 1 mL isotonic NaCl	9	10th day

PA: Puromycin aminonucleoside.

Biochemical assays

The urine of all rats was collected in metabolic cages for 24 hours and protein level was measured in the collected urine by the modified trichloroacetic acid (TCA) method [19] (Table 2). All animals were weighed and then their blood was taken for serum albumin and creatinine (Jaffe) [20] assays under ether anesthesia before sacrification (Table 3). Serum albumin levels were measured in the clinical chemistry analyzer (Architect C8000, Abbott, Illinois, USA) using reagents purchased from the same manufacturer.

Table 2 – ***Values of proteinuria in acute nephrosis group / 1–9 injections (inj.)***

Rats (*n*=6)	Proteinuria [mg/24 h]									
	Before inj.	*1 inj.*	*2 inj.*	*3 inj.*	*4 inj.*	*5 inj.*[a]	*6 inj.*	*7 inj.*	*8 inj.*	*9 inj.*
Mean±SD	5.04±2	4.91±1.4	5.25±1.4	6.74±3.2	11.04±5.2	14.60±2.8	15.44±4.2	20.50±6.4	36.24±40	91.34±91*

[a]Proteinuria increased after 5th injection; SD: Standard deviation; *p<0.0025.

Table 3 – ***All groups: proteinuria, serum albumin, creatinine clearance, weight, and urine volume***

Groups		Proteinuria [mg/24 h]	Serum albumin [mg/mL]	Creatinine clearance [mL/min]	Weight [g]	Urine volume [mL]
Control	*At start*	3.12±2.4	3.23±0.1	0.55±0.8	105±13.78	14.6±6.31
	Last	4.87±3				
Pre-proteinuria	*At start*	4.59±3.7	3.02±0.15	0.49±0.8	100±15.8	13.6±2.25
	>3 inj.	5.57±2				
Acute nephrosis	*At start*	5.04±2	2.5±0.63**	0.38±0.28**	101±0.8	9±4.35*
	>9 inj.	91.34±91*				

All data are presented as mean ± SD (median) and analyzed by Dunn's Multiple Comparison Test following Kruskal–Wallis ANOVA. For all groups, their own control protein excretion (at start) is shown. SD: Standard deviation; ANOVA: Analysis of Variance; *p<0.0025, **p<0.05.

Statistics

Proteinuria, serum albumin, creatinine clearance, weight and urine volume data of all groups were given as mean ± standard deviation (median) and compared by Kruskal–Wallis Analysis of Variance (ANOVA) followed by Dunn's Multiple Comparison Test. In all comparisons, statistical significance was defined as p<0.05 and p<0.0025 (Table 3).

Transmission electron microscopy (TEM)

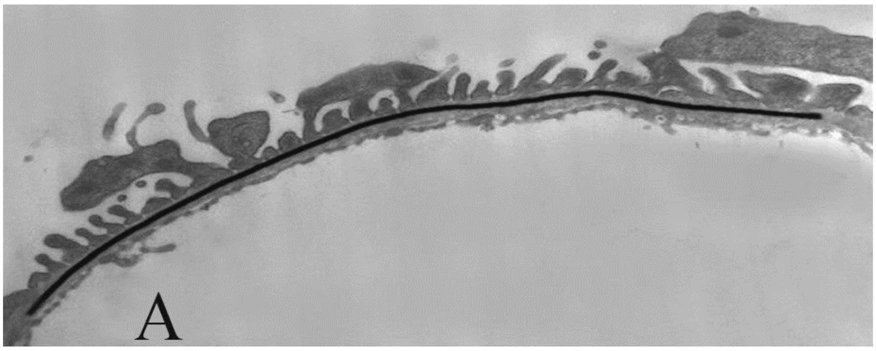
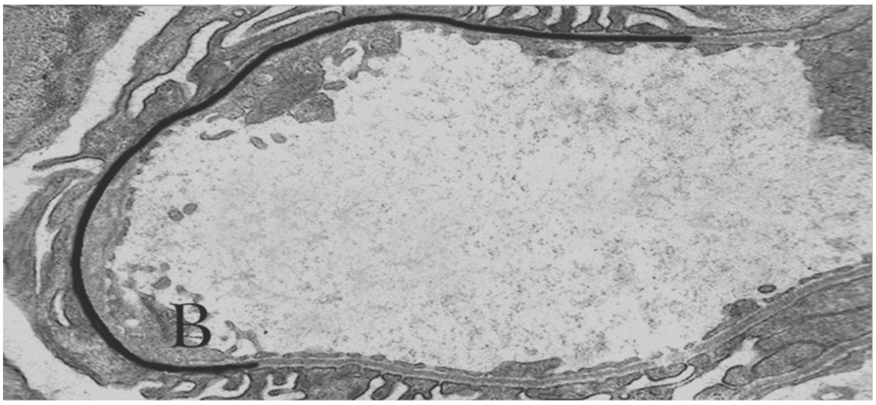
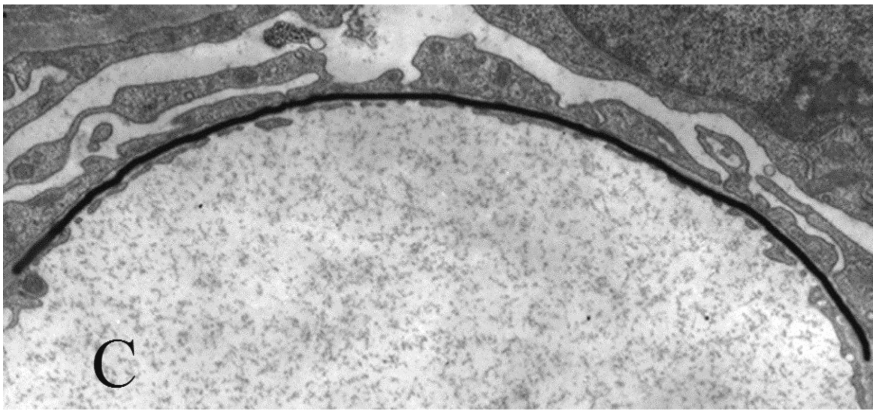
The left kidney cortex was cut into 1-mm^3 pieces for TEM analysis. They were first fixed in 4% glutaraldehyde (Sigma, G5882, USA) in a 0.1 M phosphate-buffered saline (PBS), post-fixed by 1% OsO_4 prepared in the same buffer solution, dehydrated with graded ethanol (Merck, Germany) and embedded into Araldite medium (G4901 Sigma Chemical Co., St. Louis, MO, USA).

Ultra-thin sections were obtained in 50 nm thickness onto copper grids (300 mesh) with the ultramicrotome (Reichert UM 2 and UM 3, Austria), stained with uranyl acetate and lead citrate and were investigated by the TEM (Zeiss EM 9 and EM 10, Oberkochen, Germany).

Morphometric study

Three peripheral areas of three cortical glomeruli from each electron microscopy (EM) samples of all groups were analyzed morphometrically on foot process images and the number of filtration slits in 10 μm GBM zone was calculated by the TEM (Zeiss EM 9 and EM 10, Oberkochen, Germany) [21] (Table 4).

Table 4 – ***Morphometric analysis of number of slit pores in glomeruli three groups. Slit pore numbers were calculated in 6 cm pericapillary GBM of three peripheral regions. Black lines in electron micrographs (×6000) are given to evaluate 10 µm GBM regions***

Groups	Electron micrographs of the groups	No. of slit pores (10 µm GBM)
Control		32.68±0.62
Pre-proteinuria		16.3±1.11*
Acute nephrosis		6.05±1.54**

GBM: Glomerular basement membrane. *p<0.001, **p<0.05.

Results

Urine and blood levels

In control and experimental groups, proteinuria (mg/24 h), serum albumin (mg/mL), creatinine clearance (mL/min) levels, urine volume (mL) and weight (g) are shown in Table 3. In all PAN-induced rats, proteinuria increased suddenly and significantly after the 5th injection (6th day). Proteinuria levels in control and 'pre-proteinuria group' were not significantly different from each other (Table 3). Their serum albumin and creatinine clearances were also not significantly different. However, in the acute group, proteinuria significantly increased up to ~18 times (p<0.0025). Serum albumin and creatinine clearance levels decreased significantly (p<0.05). The weights of the rats in pre-proteinuria and acute groups were not significantly different from each other (Table 3).

Electron microscopic findings

The electron micrographs of the glomeruli of control group, pre-proteinuria group and acute group were shown on Figures 1a, 1b and 1c, respectively. The pre-proteinuria group had hypertrophic podocytes and structurally rich cytoplasm but there was not any protein absorption granule (PAG) inside the cytoplasm (Figure 1b). Unlike our control and pre-proteinuria groups, the most distinctive feature observed in the acute nephrosis group was the presence of PAGs in large number and different sizes inside the cytoplasm of podocytes (Figure 1c).

When the mitochondria of podocytes were closely inspected, it was noted that in pre-proteinuria group proliferated mitochondria were smaller in size compared to control group (Figure 1b). In acute group, proliferation of mitochondria increased but along with that some mitochondria was found to be highly hypertrophied and degenerated (Figure 1c).

Numerous small invaginations, progressively growing endocytotic vesicles (Figure 2a) and PAGs formed by fusion of these vesicles were seen under the cell membrane facing GBM of podocytes (Figure 2) in the acute group. Phagosomes including PAGs were also observed with various residual bodies containing electron dense materials in different concentrations (Figure 2). Residual bodies were tightly enveloped by widespread microfilament bundles, with an electron dense appearance (Figure 2, c–h). Some electron micrographs showed that these residual bodies secreted its electron-dense materials to the urinary space by exocytosis (Figure 2, f–h).

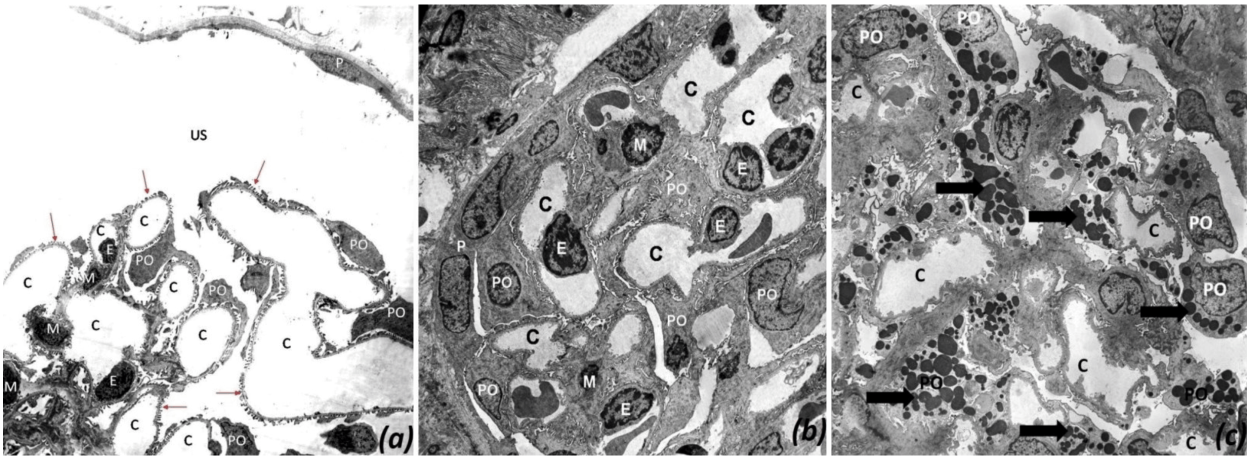


Figure 1 – ***TEM micrographs of a region from renal corpuscle in: (a) The control group (×1450); (b) The pre-proteinuria group, hypertrophic podocytes (POs) are remarkable (×1850); (c) The acute group, protein absorption granules (arrows) inside cytoplasm of podocytes (×2000). US: Urinary space; P: Parietal layer; PO: Podocyte; C: Capillary lumen; E: Endothelium; M: Mesangial cell. TEM: Transmission electron microscopy.***

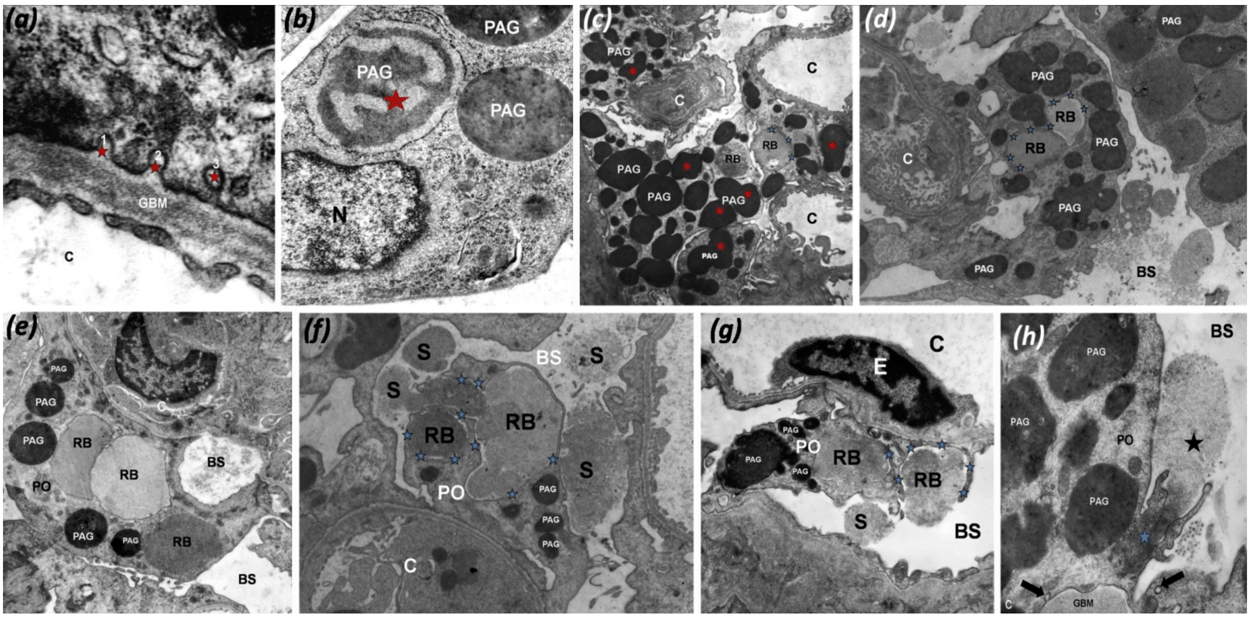


Figure 2 – ***TEM micrographs from renal corpuscles of the acute group: (a) Developmental stages (1, 2 and 3) of endocytotic vesicles (red stars) invaginated from basal cell membrane facing glomerular basal membrane (GBM) of foot processes (×60 000); (b) PAGs and lysis in one of these PAG (red star) are observed in podocyte (N) cytoplasm (×31 500). (c) PAGs and RBs in hypertrophic podocytes (×6300). Some groups of PAGs are formed by fusion of small PAGs (red stars). Around the RBs, dense microfilaments (blue stars) are remarkable; (d) Increased number of PAGs, groups of RBs and their stages of formation are observed inside the cytoplasm of hypertrophic podocytes (×10 000). RBs are remarkable with a homogenous appearance and around with dense microfilament bundles (blue stars) before exocytosis; (e) PAGs and RBs with different formation stages inside hypertrophic PO cytoplasm (×6600); (f) Exocytosis in PO with microfilament bundles (blue stars) covering RBs and exocytotic substance (S) secreted to BS (×10 600); (g) Exocytosis in PO. Microfilament bundles (blue stars) covering the RB and exocytotic substance (S) secreted to BS are seen (×10600); (h) Secreted material (black star) during exocytosis from PO cytoplasm. Microfilaments (blue star) around an exocytotic vesicle, endocytotic vesicles (arrows) and PAGs are observed (×31 500). PO: Podocyte; PAG: Protein absorption granule; RB: Residual body; C: Capillary lumen; BS: Bowman's space; E: Endothelium. TEM: Transmission electron microscopy.***

In the experimental groups (pre-proteinuria and acute groups), the number of filtration slits were progressively decreased when compared to control group (Table 4). Their EM images designated that this decrease may result from foot process fusion (Figures 3 and 4). In the acute group, foot process fusion started with narrowed filtration slits and positioning of filtration slit membrane in an upper position (Figure 4), proceeded with the appearance of electron dense bridges between foot processes at the slits to form tight junctions between foot processes (Figure 4b), until they are completely fused (Figure 4, b–d). Electron-dense areas formed by increased microfilaments were observed with foot process fusion (Figure 4, b–d). These fusion regions have many microfilament bundles and these fused foot processes spread on long distances of GBM (Figure 4e).

In the acute group, rats with a high-proteinuria level had rare but remarkable locally nude GBM regions because of foot process loss in glomeruli (Figure 5). On the other hand, some podocytes showed pseudocystic degenerations and in urinary space, some had apoptotic characteristics (Figure 5a).

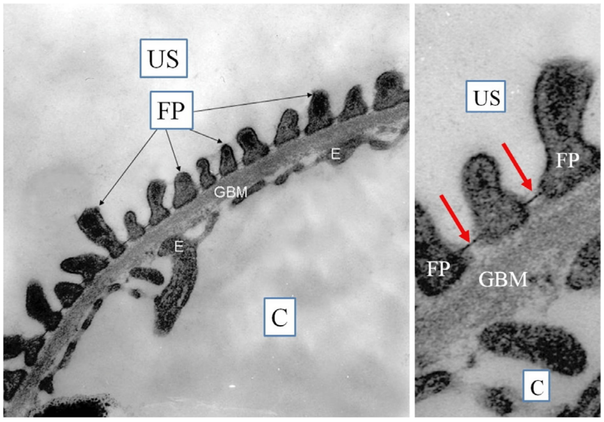


Figure 3 – ***A section of glomerular filtration barrier in the control group (×9500 with a magnified part in ×20 000). FP: Foot process; Red arrow: Membrane of filtration barrier; GBM: Glomerular basal membrane; E: Fenestrated endothelial cell; C: Capillary lumen; US: urinary space.***

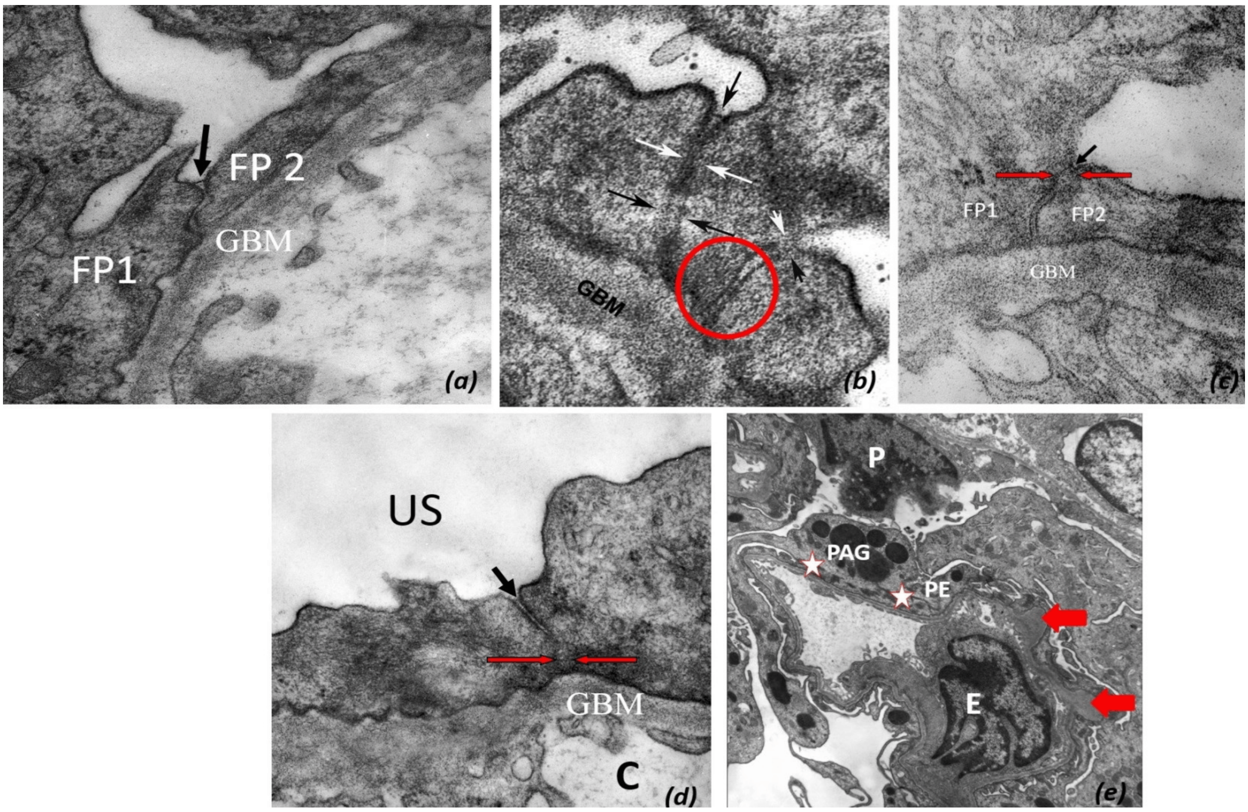


Figure 4 – ***Fusion steps of foot processes in the acute group: (a) Narrowed filtration slit region between two foot processes (FP1 and FP2), with apical dislocation of filtration slit membrane (black arrow) (×20 000); (b) Narrowed (white arrow) and fused (black arrow) filtration slit regions with apical dislocation of filtration slit membrane (black arrow). Repeated electron dense bridges between foot processes (red circle) and fusion sites are shown (small black and white arrows) (×31 500); (c) Narrowed filtration slit region between two foot processes (FP1 and FP2), with apical dislocation of filtration slit membrane (black arrow), fusion of foot process regions (red arrow) and narrowed filtration space in the lower region (×20 000); (d) Two fused foot processes (red arrow) (FP1 and FP2) with apical dislocation of filtration slit membrane (black arrow) (×31 500); (e) Extremely fused and effaced foot processes have dense microfilament bundles (stars). Enlarged regions (arrows) invaginated into foot processes on GBM and locally attached regions between podocyte (P) and parietal layer (PE) are observed (×6000). GBM: Glomerular basal membrane; US: Urinary space; C: Capillary lumen; E: Endothelium; PAG: Protein absorption granule.***

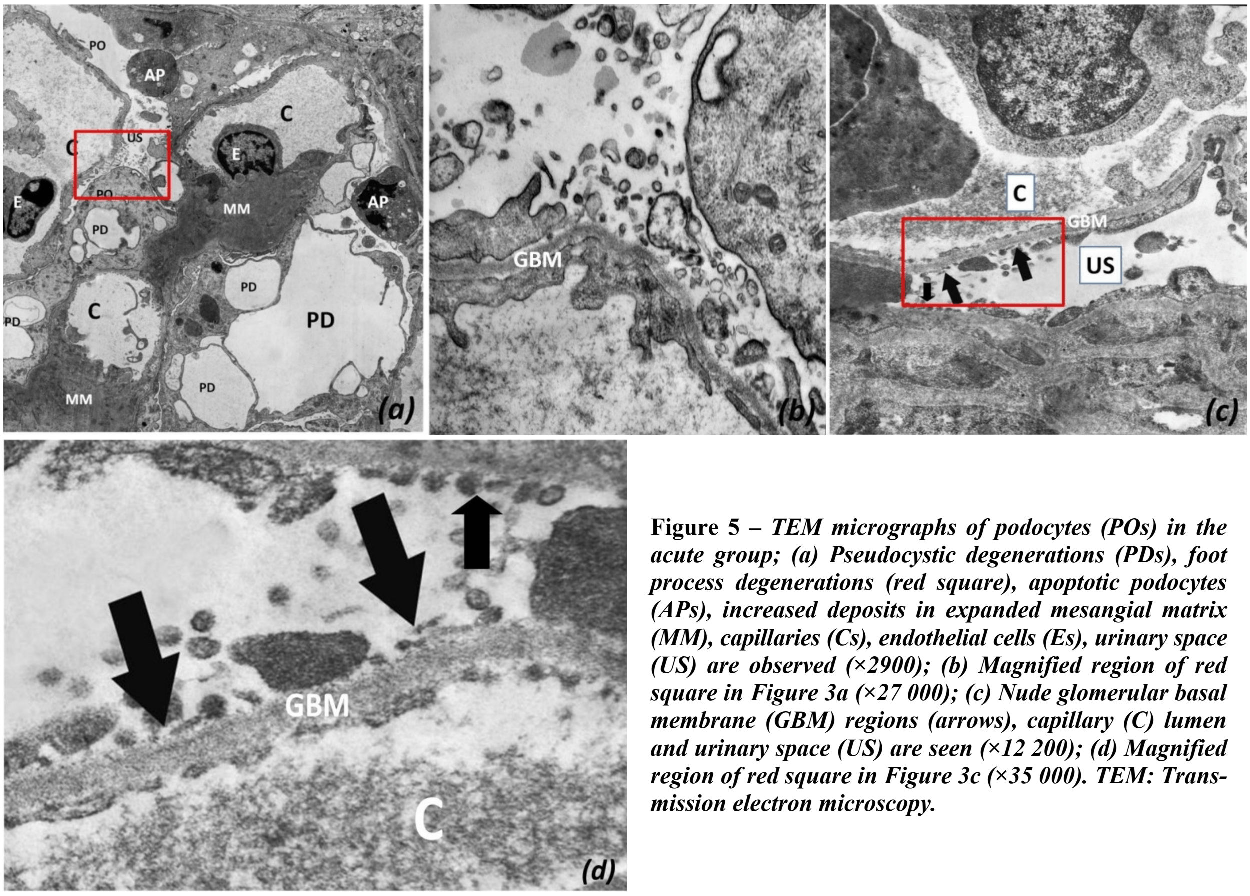


Figure 5 – *TEM micrographs of podocytes (POs) in the acute group; (a) Pseudocystic degenerations (PDs), foot process degenerations (red square), apoptotic podocytes (APs), increased deposits in expanded mesangial matrix (MM), capillaries (Cs), endothelial cells (Es), urinary space (US) are observed (×2900); (b) Magnified region of red square in Figure 3a (×27 000); (c) Nude glomerular basal membrane (GBM) regions (arrows), capillary (C) lumen and urinary space (US) are seen (×12 200); (d) Magnified region of red square in Figure 3c (×35 000). TEM: Transmission electron microscopy.*

Discussion

Podocyte injury models followed by a massive proteinuria are usually induced by using PA. However, there has not been any studies about pre-proteinuria stage of the PA nephrosis, yet. Therefore, this is the first study which clarifies the cell type that is affected first within the glomerulus of kidneys of PAN rats, included a pre-proteinuria group. In this group, none of the cells except podocytes showed a pathology and they displayed significant hypertrophy and structurally rich cytoplasm. Thus, it is thought that the first cells affected upon PA application are podocytes.

We showed that foot process damage observed in PA nephrosis stems from foot process fusion and it arises before the occurrence of proteinuria, thereby not being a result but a pre-step in the development of proteinuria. In our acute group, we also observed highly fused areas together with tight junctions, which may be accountable for the decrease in number of filtration slits and urine volume. Some studies have also suggested that the decrease in the total length of filtration-slit membranes diminishes hydraulic permeability [1, 14], and that filtration slits are narrowed and form junctions in pathological conditions [13, 22, 23]. Similarly, some researchers have found that the effacement/fusion of foot processes occurred before the onset of proteinuria, and the extent of effacement/fusion correlates with the quantity of urinary protein [24]. In our experimental group, foot process fusion started with narrowed filtration slits and upper positioning of filtration slit membrane, preceded with the appearance of electron dense bridges at the slits to form tight junctions between foot processes, until they are completely fused. We also considered that the apical dislocation of slit diaphragm is the first step of foot process fusion and it is completed with foot process fusion. We showed the loss of foot process membranes and a complete fusion. The processes of the foot process effacement/fusion and loss leading to naked GBM, reorganization of actin cytoskeleton, and the apical dislocation of the slit diaphragm are main characteristics of damaged podocytes, which is concomitant with proteinuria [15, 22, 23, 25–27]. Our conclusions supported some studies with experimental animal models characterized by human glomerular disease and proteinuria proposing that there is a relationship between proteinuria and decreased number of slit-pore because of foot process fusion/effacement [3, 7, 10].

On the other hand, we observed electron-dense areas formed by increased microfilaments in pre-acute and acute groups with foot process fusion. It has been proposed that fused foot processes contain dense microfilaments and the contractile structure of foot processes controls the enlargement of GBM [2, 7]. In accordance with our findings, Shirato *et al.* have showed microfilament increase at podocytes by forming “Masugi nephritis” [25]; and Whitesite *et al.* have demonstrated that fibrillary structures aggregate at the beginning, and then completely disaggregate with the progression of proteinuria at the podocyte cytoskeleton of PAN rats [26].

In renal disease models of experimentally high proteinuria and human renal disease, it is emphasized that an endocytotic pathway through the podocyte is generally effective on protein transport from glomerular capillary lumen to urinary space [1, 2, 4, 6–10, 23, 25, 26, 28].

Tracking techniques (ferritin, dextran, gold, florescent isothiocyanide) in nephrotic rats demonstrated that some proteins could be invaginated by endocytotic vesicles of the podocyte cell body and protein transport follows a lysosomal process, as well filtration from the slit-diaphragm of podocytes [9]. Furthermore, some of the studies have supported that naked GBM areas due to the foot process disruption are responsible from the increased proteinuria [1, 4, 29, 30]. In this study, we tried to clarify this issue in an experimental model of acute PAN, which causes high proteinuria levels in rats.

In this study, inside hypertrophic podocytes of acute nephrosis group, the most remarkable structures were electron-dense PAG in different number and sizes, occasionally generated the multi-lobed granular structures by fusion. Researchers reported that the presence of these absorption granules is one of the morphological indicators of the proteinuria [1, 9, 23]. Some studies have shown that protein intake through podocytes is an endocytotic way *via* plasma membrane facing the basal side of the podocyte foot process; these vesicles accumulating in intracytoplasmic vacuoles (phagosome) have increasing concentrations of materials and generate PAGs gaining an electron dense appearance [9, 25, 28]. During these concentration processes, PAGs gain the lysosomal enzymes by fusion with the primary lysosomes and form secondary lysosomes. Because of lysis of their materials, less concentrated and heterogeneous residual bodies appear and are covered with the dense microfilament bundles. These residual bodies known with these morphological characteristics excrete their materials to urinary space with exocytosis way [9, 25, 28]. In our study, we often distinguished electron dense PAGs and their formation stages, residual bodies covered with the microfilament bundle and some images during their full secretion process in acute PAN group. Finally, we considered that an intra-podocytic pathway related to endocytosed proteins may be responsible for the high proteinuria levels in our experimental rat group.

In another experimental study with rats, it was indicated that PAGs, frequently observed on the 7th day of the PAN treatment, were significantly less on the 12th day of the treatment [8]. It was also shown that PAN damaged the endocytotic activity of epithelial cells and affected PAG fusion progress. Whiteside *et al.* reported that at the first aggregations on the podocytic cytoskeleton of PAN-induced rats and by an increase in proteinuria, the cytoskeleton was totally disaggregated and separated from the basal membrane [26]. We also supported these researchers in such that intrapodocytic PAG and electron dense microfilaments excessively diminished and large pseudocystic structures occurred in two rats with high proteinuria. Thus, we considered that no new PAGs developed due to the decreased endocytotic activity of podocytes because of decreased microfilament intensity; residual bodies could not progress in the cytoplasm due to diminished microfilaments, dissolved their contents, growing and forming the pseudocystic structures; the membranous wall of these structures ruptured and released their contents in the urinary space. Moreover, podocytes of these two rats showed more mesangial matrix increase in the areas with large pseudocystic degenerations.

In addition, we observed foot process losses in some places and locally nude external face of GBM regions in some acute rats. We considered that these regions could be responsible for increased permeability against proteins in these rats. Nude GBM has been also observed in experimental models of glomerular diseases [10], more likely to have an important role in proteinuria pathogenesis. Several researchers also established the relationship between proteinuria progression and nude GBM regions appeared because of foot processes separation from GBM [4, 7, 8, 26, 28]. However, some researchers established that these nude regions occurred at the beginning of proteinuria [4, 8, 10] and some that these appeared at a later stage [9]. In this study, we did not observe any PAG and foot process loss in the pre-proteinuria group, despite presenting some nude GBM regions in severe proteinuric rats. Thus, we considered that this kind of lesions could be observed in severe proteinuria conditions.

The energy demand of podocytes is high due to their critical functions such as maintaining highly organized cytoskeleton, producing extracellular proteins and transcytosis. Many recent studies tried to explain the PA effect on mitochondria and stated that mitochondria of podocytes become activated upon an injury to be able to protect and repair podocyte functions [31, 32]. Consistent with the literature, in our research, the profile of mitochondria in pre-proteinuria group showed that mitochondria proliferated and became smaller due to endocytic activity. In acute group, as the exocytotic activity followed the endocytosis and energy demand increased, the morphology of mitochondria indicate the continuation of new mitochondria biogenesis but along with that some mitochondria showed degeneration and hypertrophy.

Conclusions

In this study, we suggested the first cells within the glomerulus affected by PA application could be podocytes. We concluded that foot process fusion begins prior to the development of proteinuria although their serum albumin and creatinine clearance levels do not differ significantly. Additionally, we suggested that in acute PAN, there could be a correlation between glomerular function and number of slit pores. Moreover, PAGs are secreted with exocytosis to urinary region after lysosomal digestion in acute group but not observed in pre-proteinuria, in spite of decreased number of foot process. Rare nude regions of glomerular basal membrane could be responsible for high proteinuria in acute PAN.

Conflict of interests

All authors stated that there is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgments

This study was conducted in Department of Histology and Embryology, Cerrahpaşa Medical School of Istanbul University, Turkey.

The concept and design of research belongs to IS and MP. Animal experiments were carried out by SK and MU and transmission electron microscopic works were conducted by IS. HAS and ZBG carried out the biochemical studies. ODD, HIS and EYS performed data analysis, interpretation and writing the article, supervised by IS.

The authors thank to Dr. Bülent Uruluer, Azize Gümüşyazıcı and Ercüment Boztaş, the technicians of the Laboratory of Electron Microscopy, Department of Histology and Embryology, Cerrahpaşa Medical School of Istanbul University.

This work was supported by Scientific Research Project Coordination Unit of Istanbul University [Project Number: 1707/15082001].

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Received: June 6, 2017 *Accepted: November 8, 2017*