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In glomerular cells of puromycin aminonucleoside nephrosis rats both phosphorylated and total STAT3 levels increased during proteinuria

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ABSTRACT

Recent studies showed that JAK/STAT pathway plays role in glomerular damages. The fact that STAT3 could be activated also by oxidative stress make Puromycin Aminonucleoside (PAN) Nephrosis model very appropriate for examination of STAT3 expression changes in glomerular pathology. Along with a control group, three PAN groups sacrificed on different days were formed by the i.p. injection of PAN for 5 consecutive days. Throughout the experiment, 24-hour-urines were collected on specific days and proteinuria levels were monitored. At the end of the experiments, tissue specimens were stained immunohistochemically for both total and phosphorylated STAT3 and evaluated subjectively. They were also examined ultrastructurally in transmission electron microscope. The proteinuria levels did not increase significantly on 5th day but showed a dramatic increase on 10th and 15th days. On 20th and 25th days, urinary protein levels gradually decreased. Ultrastructural examinations showed glomerular damages such as significant decrease in slit pore number, a significant gradual increase in glomerular basement membrane thickness and podocyte hypertrophy on 5th and 15th days; besides significant increase in mesangial matrix. The first significant increases in phosphorylated and total STAT3 levels occurred in 5th day and 15th day groups respectively. These increases diminished in 25th day group. Regarding all the findings, it was deduced that STAT3 is one of the active factors in glomerular pathologies.

1. Introduction

Kidney diseases which can convert to end-stage renal disease (ESRD) if untreated, keeps having a high incidence in the world (Levey et al., 2007). Recent studies showed that the key point lies within the glomerulus where the filtration takes place (Haraldsson et al., 2008). Glomerulus is composed of three cell types all of which are indispensable for proper filtration process: endothelial cells that form the capillaries, mesangial cells that lie within the capillary tuft and podocytes that wrap around the capillaries (Haraldsson et al., 2008). Podocytes which are terminally differentiated cells with a complex ultrastructure thought to be more vulnerable compared to the other two due to its sensitive spot (Tharaux and Huber, 2012). Podocytes have long processes called as foot processes and between them slit pores exist. In these pores slit diaphragm structures are present and they are central to glomerular filtration barrier.

One of the models used for glomerular kidney diseases is Puromycin Aminonucleoside (PAN) Nephrosis model. This model, depending on the application, could mimic minimal change disease (MCD) and focal segmental glomerulosclerosis (Pippin et al., 2009; Shen et al., 2016). PAN leads to foot process disorganization and slit diaphragm breakdown that's why it ends up in deterioration of glomerular filtration barrier and proteinuria (Burford et al., 2017). PAN is thought to be causing oxidative stress in podocytes (Diamond et al., 1986; Wang et al., 2009). However, the exact mechanism of PAN nephrosis could not be clarified.

JAK/STAT pathway was first described in inflammation signalling but later on it was realized that this pathway plays role also in many other cellular activities (Matikainen et al., 1999; Simon et al., 1998). In cancer and metastasis this pathway was found to be involved in inflammation, cell proliferation, apoptosis and epithelial mesenchymal transitions (Teng et al., 2014; Wendt et al., 2014). In addition to many other diseases, recent studies showed that this pathway plays role widely also in kidney damages (Brosius and He, 2015; Chuang and He, 2010). Especially STAT3 was found to be of great importance for glomerular pathology (Lu et al., 2009).

STAT3 firstly studied in mesangial cells of the glomerulus and found to be affected in Thy1 glomerulonephritis (Yanagita et al., 2001). Later

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on it was showed that STAT3 is the key player in HIV-Associated Nephropathy (He et al., 2004). STAT3 also have been studied in Diabetic Nephropathy and Adriamycin Nephrosis models and it was showed that the STAT3 levels had been significantly changed in these pathologies (Lu et al., 2009). However, the specific role of STAT3 in nephrosis is not clear enough and our literature search did not reveal any STAT3 study in PAN. Regarding the fact that the STAT3 can be activated via intracellular oxidative stress (Simon et al., 1998), it would be very purposive to study STAT3 levels in PAN nephrosis model. In this regard we aimed to form a PAN nephrosis model and examine the changes in both total and phosphorylated STAT3 levels.

2. Materials and methods

2.1. Animals and model formation

Twenty-four male Wistar albino rats weighing approximately 120 g were used in the study. They were 6-week-old and obtained from Experimental Animals Reproduction and Research Laboratory (Istanbul University, Turkey). They were housed three to a cage and were fed *ad libitum* with powdered standard rat chow and tap water under standard conditions (20-24oC temperature, 50–60% humidity, 12 h light/dark cycle). The cages were cleaned regularly. The study was conducted in compliance with ethical standards (Istanbul University, Animal Ethics Committee, Nu: 2015/37, 03/03/2015).

The rats were classified as 4 groups *i.e.* six rats per group. The nephrosis model was induced as described by Sasaki et al. (1997). In this model the regimen followed causes a slow but sustained cytotoxicity to glomerular cells. The rats in the control group were injected 1 ml physiological salt solution whereas the rest of the rats were given puromycin aminonucleoside (PA) (sc-200112 A, Santa Cruz; Dallas, Texas, United States) as 1.5 mg/100 g i.p. in 1 ml physiological salt solution for five consecutive days. Control and 5th day group was sacrificed on 5th day. 15th and 25th day groups were sacrificed on 15th and 25th days respectively.

2.2. Biochemical analysis

The rats were transferred to metabolic cages on −1, 5, 10, 15, 20 and 25th day and 24-h urines were collected. Protein amount in urine was measured quantitatively using the modified trichloroacetyl acid (T9159, Sigma-Aldrich; St. Louis, Missouri, United States) method (Choi et al., 1993).

2.3. Light and electron microscopic examinations

Animals were anesthetized and left kidneys were excised through abdomen. The kidneys were transferred to 10% neutral buffered formalin and let them be fixed before embedding in paraffin. Paraffin sections were stained with periodic acid-Schiff (PAS) stain. Mesangial matrix amounts in glomeruli were semi-quantitatively scored regarding PAS-positive material as performed by Raij et al. (1984). Briefly, at least fifty representative glomeruli scattered in the preparation assessed regarding its mesangial matrix expansion. They were all assigned grades from 0 to +4 in proportion to glomerular involvement of mesangial matrix *i.e.* + 1 and +4 grades mean 25% and 100% glomerular involvement respectively. This grading was done by two observers independently and blindly. The grades were summed and divided by the number of glomeruli examined. The final scores of the observers were averaged.

The cortical renal tissue divided into 1 mm3 pieces were transferred to 2.5% glutaraldehyde (Sigma-Aldrich) in a 0.1 M phosphate buffer solution for first fixation. Post fixation was achieved by 1% OsO4 prepared in the same buffer. Later the tissue samples were dehydrated with graded ethanol series (Merck, Kenilworth, New Jersey) and embedded in Araldite medium (10951, Sigma-Aldrich). Semi-thin sections, approximately 1 µm, were cut by glass knives in the ultramicrotome and stained with 1% toluidine blue (prepared with 1% borax in bidistilled water) for observation under binocular light microscope (Olympus BX61). Ultra-thin sections were obtained in 50 nm thickness onto copper grids (300 mesh) with the same microtome, stained with uranyl acetate and lead citrate and they were investigated by transmission electron microscope (Jeol, JEM 1011).

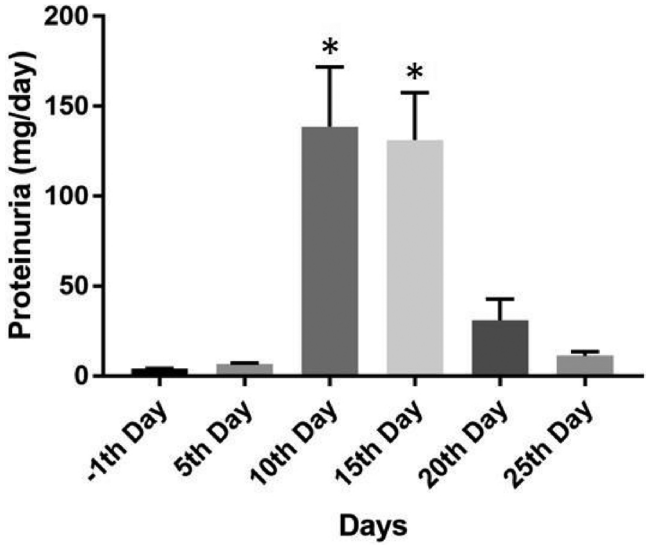

Fig. 1. Proteinuria levels by days. Before the injections protein amounts in the urine was in normal range and did not display a statistically significant increase 5 days after the injections. However, protein excretion in the urine showed a significant increase on 10th and 15th days; and a significant decrease on 20th and 25th days. (*p < 0.05 compared to -1th, 5th, 20th and 25th days.) (N = 24 for -1th and 5th days. N = 12 for 10th and 15th days. N = 6 for 20th and 25th days).

Two morphometric analyses were carried out *i.e.* glomerular basement membrane (GBM) thickness measurement and slit pore (SP) number count in unit length by locating five different peripheral sites from three glomeruli at 20,000X magnification. SP count was held in 7 um length of GBM.

2.4. Immunohistochemical analysis

Immunohistochemical stainings were performed with mouse monoclonal anti-total-STAT3 (sc-8019, Santa Cruz) (tSTAT) and mouse monoclonal anti-phosphorylated-STAT3 (Tyr705) (sc-8059, Santa Cruz) (pSTAT) antibodies. The sections of 5 µm thickness were placed onto slides coated with poly-L-Lysine (P8920, Sigma-Aldrich) then deparaffinized and rehydrated in graded alcohol series. ImmunoCruzTM mouse LSAB Staining System (sc-2050, Santa Cruz) was used and the protocol suggested by the manufacturer was followed. Sections were counter-stained with toluidine blue. The specificity of the stainings were proved via omitting primer antibody step.

Immunoperoxidase staining of the rats' glomeruli was evaluated as performed by Chen et al.(2006). This evaluation has been done by two observers blindedly in all rats of each group in at least fifty representative glomeruli scattered in the preparation. Immunopositivity was graded between 0 to 3; from no staining to strong staining. Then the grades were summed and divided by the number of glomeruli examined.

2.5. Statistical analysis

Values were expressed as mean ± SD. Comparison of means was performed by One Way ANOVA Dunnett T3 test using SPSS21.0 statistical software. $p < 0.05$ was considered statistically significant.