

Research Article

Fas-Ligand and Granzyme-b Levels in Children with Nephrotic Syndrome

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ABSTRACT

There is an urgent need to develop alternative to kidney biopsy in children to diagnose nephrotic syndrome. Increased apoptosis plays a central role in the development of nephrotic syndrome. The aim of this study was to evaluate the blood levels of two markers of apoptosis, Fas-ligand (Fas-l) and granzyme-b in children with nephrotic syndrome. Thirty children with biopsy-proven nephrotic syndrome as well as twenty four healthy controls were evaluated in this study. Clinical and laboratory data of included cases and controls were collected. Serum Fas-ligand and granzyme-b levels were measured using specific enzyme-linked immunosorbent assay (ELISA). The results of the current study showed that children with nephrotic syndrome had significantly higher serum levels of Fas-ligand (183.29 ± 43.34 pg/ml) and granzyme-b (151.68 ± 30.47 pg/ml) than healthy control subjects (Fas-l = 99.28 ± 7.20 pg/ml, and granzyme-b = 86.10 ± 5.68 pg/ml) ($P=0.000$). A significant increase in Fas-ligand levels in Focal segmental glomerulosclerosis cases than that of minimal changes nephrotic syndrome cases ($p=0.004$) was found. Granzyme-b levels were not significantly differing between the two groups of nephrotic syndrome cases. In conclusion, markers of the extrinsic pathway of kidney apoptosis as Fas-ligand and granzyme-b are potential novel biomarkers in children with nephrotic syndrome that could be used as non invasive alternative to kidney biopsy in assessment of those children.

Keywords: Fas ligand - Granzyme-b - Nephrotic syndrome- Children - Apoptosis

INTRODUCTION

Nephrotic syndrome (NS) is caused by different problems that damage the kidney. This can occur from: cancer, diseases such as diabetes, systemic lupus erythematosus (SLE) amyloidosis, genetic, immune disorders, infections, such as strep throat, hepatitis, or mononucleosis and the use of certain drugs. It can occur with kidney problems such as: Focal segmental glomerulosclerosis (FSGS) and minimal changes glomerulonephritis (MCGN)^{1,2}.

The vast majority of childhood NS is caused by minimal change diseases (MCD) or (FSGS). MCD, though often protracted, is a benign disease, whereas FSGS has a persistent progression to end-stage renal disease (ESRD) in most cases³ and is the most common glomerular lesion in children that leads to either dialysis or transplantation⁴. As the initial clinical presentation of these two lesions is similar and no specific clinical or laboratory parameters exist to distinguish lesions, often only a pathologic evaluation of renal tissue will establish this critical distinction⁵. However, in early stages, critical for intervention even a thorough pathologic evaluation may be indecisive⁶. More recently it has been advocated that it is not histology but nonresponse to corticosteroid and cyclosporine (CsA) treatment that is a decisive indicator of

progressive disease⁷. So, in the context that even renal biopsy might not be able to differentiate between MCNS and FSGS in early stages of the disease⁶, there is an urgent need to develop non invasive assessment tool in those children to diagnose the cause of NS since its diagnosis has significant therapeutic and prognostic implications. Nephrotic syndrome is one of the autoimmune lymphoproliferative syndromes⁶. It affects lymphocyte apoptosis⁴. Apoptosis is programmed cell death that occurs when kidney disease plays an important role in their physiology. Harmful effect 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. The molecular mechanisms underlying irreversible renal damage in children with NS depending on apoptosis stimulation might be of potential therapeutic implication⁸. Apoptosis induced by inflammatory cytokines, Fas-Ligand, perforin and granzymes⁹. This condition is usually caused by mutations in fas gene and other genes including fas ligand and granzyme gene¹⁰ Fas ligand (Fas-l or cd 95) is a type II transmembrane protein that belongs to the tumour necrosis factor (TNF) family its binding with its receptors lead to apoptosis. Fas ligand/receptor interactions play an important role on the regulation of the immune system and

the progression of cancer⁵. This pathway has been stressed recently as an important mechanism involved in cell death in glomerulonephritis, because glomerulocyte express Fas and Fas ligation of Fas receptor by the antibody results in massive kidney cell death in mice¹¹.

Granzymes (Gzms) are a group of five cellular enzymes that activate programmed cell death. GzmA and GzmB are the most abundant inside the cell. GzmB acts mainly by activating the caspase pathway¹². Granzyme b is a serine protease most commonly found in the granules of cytotoxic lymphocytes (CTLs), natural killer cells (NK cells) and cytotoxic T cells. It is secreted by these cells to mediate apoptosis in target cells¹³. Granzyme-b has shown to be included in inducing inflammation by stimulating cytokine release and is also involved in extracellular matrix remodelling. Elevated levels of granzyme-b are also implicated in a number of autoimmune diseases several skin disease, and type I diabetes. Among the cytotoxic effectors, Fas ligand tended to show co-expression with TGFβ1, while granzyme-b and perforin were expressed in all steroid-resistant cases⁷. So, the main goal of the present work was to evaluate levels of Fas-ligand and granzyme-b as markers of increased apoptosis in children with NS and to determine their utility as novel biomarkers and non-invasive tool for predicting the progression of pediatric NS as well as to investigate its correlation with clinical and laboratory indices of these conditions in children.

SUBJECTS AND METHODS

This is a case control study that included 30 children with NS recruited from Pediatric Nephrology Clinic, Cairo university Children's Hospital, and 24 healthy children as control subjects. Inclusion criteria of NS cases were the presence of nephrotic-range proteinuria (protein excretion of more than 40 mg/m²/h), edema, hypoalbuminemia (serum albumin <3 gm/dl), and hypercholesterolemia (serum cholesterol >200 mg/dl) at assessment. Nephrotic syndrome cases with impaired kidney function and those with ESRD on dialysis were excluded from the current study. Ethical considerations: Informed consent was obtained from included children's guardians. The study had been approved by the Ethical committee of National Research Centre (NRC), Egypt. Each included NS patient was subjected to full medical history taking focusing on frequency of relapses, response to steroid therapy, and need of other immunosuppressive or cytotoxic drugs. Physical examination of all included cases and control children focusing body mass index (BMI) calculation by the formula [BMI= weight (kg) / height (m²)], and blood pressure measurement. Renal biopsies of included patients were reviewed and NS cases were categorized into two subgroups based on their pathological findings [12 patients (40 %) with MCNS & 18 patients (60%) with FSGS]. Laboratory investigation were done for all cases and controls including complete blood picture using Coulter Counter T890 (Coulter Counter, Harpenden, UK) and serum levels of urea, albumin, total protein, creatinine, cholesterol and calcium, using the automated clinical chemistry analyzer (Olympus AU 400 analyzer). Soluble Fas-ligand and Granzyme-b assay was done by in vitro

ELISA kit (Ray Biotech, inc., USA)¹⁴ in the laboratory of NRC in Egypt.

Statistical Analysis

Statistical package for social science (SPSS) program version 16 was used for analysis of data which was represented as mean ±SD. Student's t-test for quantitative independent variables was used for analysis of difference between two groups. Correlation between quantitative variables was done using Pearson's bivariate test. In all tests P<0.05 was considered statistically significant.

RESULTS

Patients group were 16 males and 14 females, their age ranges between 4 and 15 years. All NS cases were on steroid therapy at assessment. 46% of NS cases were steroid resistant NS (failed of response after 4 weeks of daily oral steroid therapy of 60 mg/ m² /day followed by three pulses of methylprednisolone). Fifteen children (50%) of the studied NS cases were on cytotoxic drugs in addition to steroid therapy. Nine patients had frequent relapsing NS (had two consecutive relapses or two of four relapses in any 6 month period). Twenty seven patients were on calcium channel blocker antihypertensive therapy.

Table (1) demonstrated clinical lab investigations for all patients and controls. There were significant increase in cholesterol (p=0.009), calcium (p=0.000), albumin (p=0.001), protein (p=0.029), urea (p=0.003) and haemoglobin levels in patients vs. controls (p=0.000). There were significant differences in granzyme-b (p=0.000) and Fas-ligand (p=0.000) in patients versus control.

Table (2) showed a significant increase in FSGN as regards to MCNS (p=0.004) while granzyme-b levels were not significantly differ between the two groups (p=0.419).

Table (3) showed the correlation between granzyme- b, Fas-ligand and different variables. We found a significant correlation between fas-ligand and platelets (p=0.016). Also, there was a significant correlation between granzyme-b and creatinine levels (p=0.03).

DISCUSSION

Nephrotic syndrome (NS) is one of autoimmune glomerulonephritis disease. This disorder is caused by the defective Fas mediated apoptosis Fas/Fas-l system is a key regulating system responsible for activation of apoptosis in various cell types including cellular constituents of the cell wall^{15,16}. Granzyme-b is a serine protease found in the granules of cytotoxic lymphocytes and natural killer cells (NK). It is secreted by these cells to mediated apoptosis in target cells¹². NS is diagnosed on clinicolaboratory basis, but its definite diagnosis is made by renal biopsy¹⁷, what makes innovation of non invasive tests for diagnosis of pediatric NS mandatory. In the present study 30 patients with NS as well as 24 healthy controls were subjected to full history taking, and physical examination. Pathology review of NS cases was done. Laboratory investigation and analysis for Fas-ligand and granzyme-b were done for cases and controls.

Serum levels of Fas -Ligand in NS patients were significantly higher than that of control subjects. The mean

Table 1: General characteristics of the studied groups.

	NS (No=30)	Controls (No.=24)	p value
Age (years)	9.9±3.88	10±4.51	0.14
Gender (Male/Female)	Male=16 (53.33%) Female=14 (46.67%)	Male=12 (50%) Female=12 (50%)	
BMI (kg/m ²)	17.1±4.8	20.6±1.44	0.162
SBP (mmHg)	98.66±6.66	95.54±9.70	0.16
DBP (mmHg)	64.66±6.67	61.55±10.10	0.17
Cholesterol(mg/dl)	271.29±82.12	173.29±14.20	.009
Calcium,(mg/dl)	8.70±0.55	10.72 ±0.50	.000
Albumin(g/dl)	2.94±1.02	4.81±0.31	.001
Protien(g/dl)	5.10±1.42	6.48 ±0.32	.029
Urea(mg/dl)	34.57±19.35	7.80±0.99	.003
Creatinine(mg/dl)	0.62±0.47	0.72 ±0.12	.595
Hematocrite (%)	33.92±2.16	38.11±2.18	.004
Platelets [$\times 10^3/\text{mm}^{-3}$]	378.86±157.48	314.00±6.60	.298
White blood cell [$\times 10^3/\text{mm}^{-3}$]	9.31±3.05	8.10±0.86	.332
Hemoglobine(g/dl)	10.58±0.50	14.07±0.65	.000
Granzym-b(pg/ml)	151.68±30.47	86.10±5.68	.000
Fas-ligand(pg/ml)	183.29±43.34	99.28±7.20	.000

BMI (body mass index), SBP (systolic blood pressure), DBP (diastolic blood pressure).

P was significant if <0.05

Table 2: Comparison between FSGS and MCNS as regards to apoptotic markers.

	FSGS (N=18)	MCGN (N=12)	p-value
Granzyme -b (pg/ml)	161.40 ± 36.83	158.00±62.55	0.419
Fas -ligand (pg/ml)	145.95±26.34	90± 1.09	0.004

FSGS; focal segmented glomerulonephritis, MCGN; Minimal change glomerulonephritis.

P was significant if <0.05

serum Fas- Ligand level was (183.29±43.34pg/ml) in the patients, while that of the control subjects was (99.28±7.20pg/ml) (p=0.000). Many authors reported the increase level of Fas-Ligand with many diseases as SLE¹⁸, diabetic¹⁹ and in non-alcoholic steatohepatitis²⁰. In addition, the elevated level of the Fas-Ligand during the disease activity is correlated to organ damage²¹. The mean serum Fas-Ligand level observed during the disease activity (relapse of NS) in the present study in FSGS cases (164±51.3) was significantly higher than that of MCNS cases (90±1) (p=0.004). This difference could be attributed to the phenotypic and functional changes in the NK cells during the disease²². We found a significant correlation between serum Fas-ligand level and platelet count (p=0.016). Since platelets were previously reported to play an important role in intercellular communication and as mediator of inflammation and immunomodulation²³, their strong positive correlation with serum Fas-ligand levels supports the later role in immune mediated inflammatory process of NS.

Reduction of apoptotic bodies' clearance from phagocytic/macrophage system during the disease results in an increased apoptotic burdens that in turn results in hyper activation of the immune system cells⁸. A positive correlation that approaches the statistical significance between Fas ligand levels and cholesterol levels was

reported in the current study (p=0.073) which was in accordance with Pedigo et al,²⁴. Tumour necrosis factor (TNF) causes inflammation and some activation and is associated with altered cholesterol homeostasis that could explain this finding. We found that serum granzyme-b levels are significantly elevated in NS cases compared to control group. The mean granzyme-b level was 151.68±30.47 pg/ml in the patients, while it was (86.10±5.68) pg/ml in the controls (p=0.000). Granzyme-b levels were insignificantly elevated in FSGS cases (mean=161.4±29.8) than those of MCNS cases (mean=158.00±62.55) (p=0.419). There was significant correlation between granzyme-b and serum creatinine (p=0.03). In rat studies, it has been shown that granzyme/perforin-mediated pathway plays a role in crescentic glomerulonephritis and that antiperforin antibodies can be used in treatment²⁵. Zeybek et al. studied urine granzyme b levels in active stage of MCNS, remission cases and control subjects; they found granzyme b activity was neither high nor low in study and control groups. They owed their findings to the probability that during active MCD the NK cell activity only remains at the level of decreased zeta chain expression and there is no need for granzyme b production because of absence of crescent formation²⁶. Since 60% of our included NS cases had FSGS, Zeybek et al. study findings cannot be applied

Table 3: correlations between Fas-ligand and granzyme-b and different variables in NS patients (n=30).

	Fas l		Granzyme- b	
	R	P value	r	P value
Hb(g/dl)	-0.398	0.254	-0.08	0.86
Ht (%)	0.175	0.63	0.498	0.14
WBCS [$\times 10^3/\text{mm}^3$]	-0.173	0.633	-0.341	0.33
Platelets [$\times 10^3/\text{mm}^3$]	0.731	0.016	-0.145	0.69
Cholesterol(mg/dl)	-0.589	0.073	-0.415	0.23
Albumin(g/dl)	0.066	0.855	0.114	0.75
Urea(mg/dl)	-0.115	0.753	0.05	0.89
Creatinine(mg/dl)	0.095	0.773	0.675	0.03
Calcium(mg/dl)	0.326	0.359	-0.064	0.86
Protein(g/dl)	0.339	0.339	-0.55	0.1
Fas-ligand(pg/dl)	1	1	0.866	0.89
Granzyme-b(pg/dl)	0.866	0.061	1	1

Hg; hemoglobin, Ht; hematocrite, WBCS; White blood cell counts.

P was significant if <0.05

to our studied cases. The previous findings suggest that apoptosis signalled through the Fas-ligand and granzyme-b pathway appears to play important role in the pathogenesis of autoimmune NS. The regulation of this pathway may alter the clinical expression of the disease. Their strong role in pathogenesis of NS not only offers a diagnostic tool of the disease, but also of prognostic and therapeutic implications.

CONCLUSION

Markers of the extrinsic pathway of kidney apoptosis as Fas-l and granzyme-b are potential novel biomarkers that might be used as non invasive assessment tools of children with NS especially those resistant for corticosteroid therapy. These two new biomarkers may improve the diagnostic accuracy of pediatric NS being incriminated in its pathogenesis and could be targets of therapeutic intervention of this illness. Future studies are needed to validate our findings and to assess change in Fas-l and granzyme-b levels with disease progression and in response to therapeutic intervention.

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