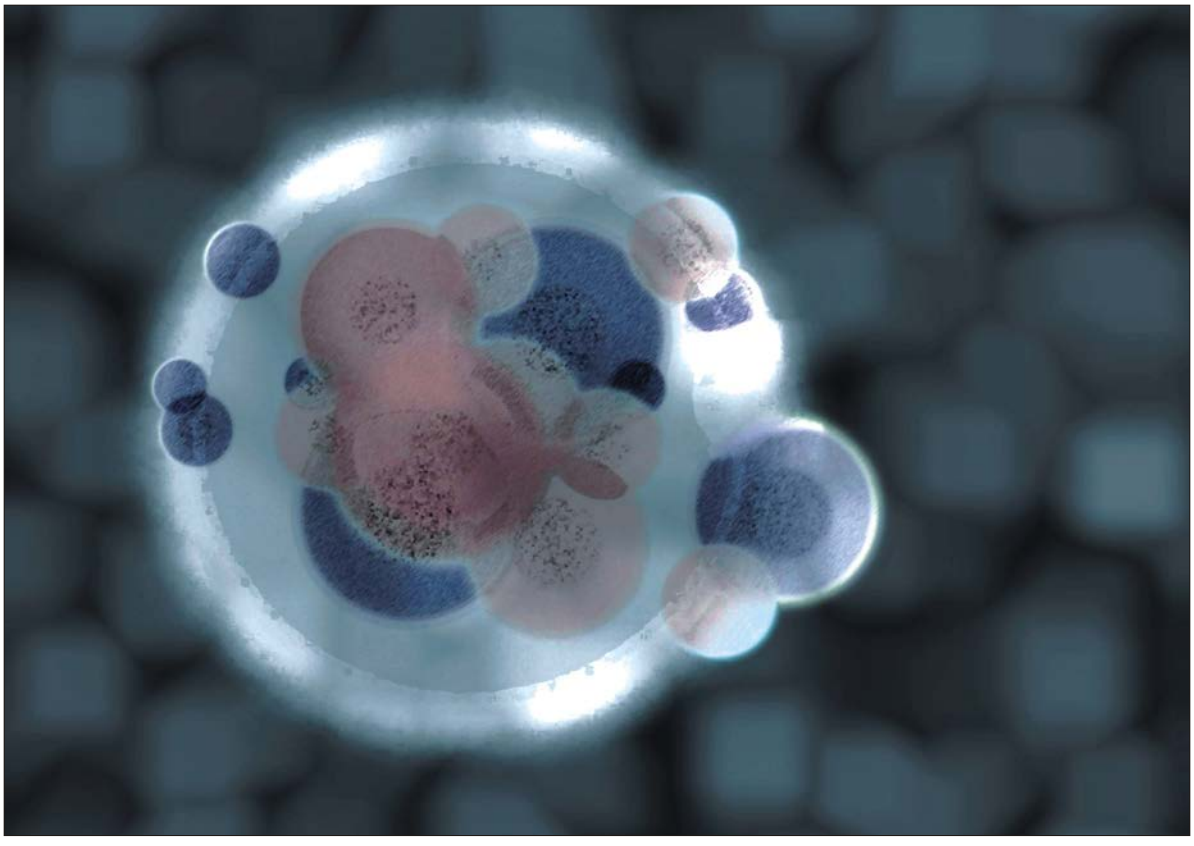

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Apoptotic Markers in Childhood Nephrotic Syndrome

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Abstract: In order to investigate the status and role of serum MMP-9 and urinary annexin V in steroid resistant nephrotic syndrome, 60 children aged between 2 and 15 years were enrolled in this study. Serum MMP-9 and urinary annexin V excretion were measured N patients were examined for apoptotic bodies by DNA *in situ*. Serum MMP-9 was significantly higher in patients than controls (189.1±150.6 and 87±53.7 pg mL^{-1}, respectively, $p<0.0001$). Urinary annexin V was also significantly higher (4.5±0.3 compared to 2.7±0.6 ng g^{-1} creatinine, $p<0.0001$). Apoptosis by DNA *in situ* was positive in 29 children (48%) and positive cases tend to have higher MMP-9 levels ($p = 0.05$). Those with focal segmental glomerulosclerosis had the highest apoptosis rates (69%) and the lowest response to CPA (29%). Responders had higher urinary annexin V ($p = 0.03$) and biopsy evidence of apoptosis ($p = 0.003$) than non-responders. Negative apoptosis by DNA *in situ* predicted response with a likelihood ratio of 2.47. These data confirm the role of annexin V and MMP-9 in apoptosis in nephrotic syndrome. The role of MMP-9 in disease progression and that of TIMPs as adjunctive therapy need further elucidation.

Key words: Apoptosis, annexin V, focal segmental glomerulosclerosis, matrix metalloproteinase, steroid resistant nephrotic syndrome

INTRODUCTION

Apoptosis is a fundamental component of tissue differentiation and development and plays an important role in a variety of diseases. Excessive apoptosis is observed in diseases such as AIDS, Alzheimer's and progressive heart failure. On the other hand, insufficient apoptosis often occurs in tumor growth (Thatte and Dahanukar, 1997) . Many renal diseases are characterized by mononuclear cell infiltrate and inflammatory cells may provide factors that cause parenchymal cell apoptosis (Tawfeek and Zaki, 2005). Nephrotic syndrome is associated with and probably caused by, abnormalities in T lymphocyte function (Zachwieja *et al*., 2003), which may be related to increased apoptosis of circulating T lymphocytes (Zachwieja *et al*., 2002).

Annexins are a superfamily of calcium- and phospholipids-binding proteins (Schiffer *et al*., 2001) which originally evoked interest as mediators of the anti-inflammatory actions of glucocorticoids (Ehrich *et al*., 2007). Annexin V has been reported to possess anticoagulant activity, inhibition of phospholipase A2 as well as regulation of membrane transport, proliferation and signal transduction. Annexin V binds to the phosphatidyl serine-exposing apoptotic cell and can inhibit the procoagulant and proinflammatory activities of the dying cell (Schiffer *et al*., 2001). Children with nephrotic syndrome were reported to have increased T lymphocyte apoptosis, assessed by annexin V (Zachwieja *et al*., 2003; Borzecka *et al*., 2004). Urinary annexin V excretion may be an indicator of apoptosis and renal injury.

The progression of nephrotic syndrome to end stage kidney disease is accompanied by accumulation of Extracellular Matrix Proteins (ECMs). Tissue matalloproteinases (MMPs) are zinc-dependant endopeptidases known for their ability to cleave one or several ECM constituents as well as non matrix proteins. They have a pivotal role in control of signals elicited by matrix molecules (Vu and Werb, 2000; Levick and Brower, 2008) and regulation of cell migration and tissue morphogenesis (Herzog *et al*., 2007).

Based on the well-known ability of MMPs to degrade ECM components and their ability to induce or sustain inflammatory mesangial cell phenotype (Bobkova *et al*., 2008), MMPs are proposed to play an important role in the progression of nephropathies (Ronco and Chatziantoniou, 2008). Moreover (Bauvois *et al*., 2007) suggested that dysregulated renal expression of MMPs and their tissue inhibitors could contribute to tubule-interstitial fibrosis characteristic of progressive primary glomerulopathies.

Matrix metalloproteinase 9, known as gelatinase B, is a member of the MMPs which has been suggested to play a role in the degradation of ECM during inflammation,

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wound healing and angiogenesis (Ohno *et al*., 1997). It is also implicated in the processes of arthritis, cardiovascular diseases, cancer metastasis and renal diseases (Massova *et al*., 1998; Andreini *et al*., 2004).

The aim of this study was to define the status and role of MMP-9 and urinary annexin V excretion in children with Steroid-Resistant Nephrotic Syndrome (SRNS).

MATERIALS AND METHODS

Materials: The present study included 60 children (2-15 years of age) with SRNS in activity constituting the study group and 20 age and sex matching healthy controls. All patients had primary steroid resistance with failure to achieve remission after 6 weeks of daily corticosteroid therapy (60 mg/m^2/day) and were thus medically indicated for renal biopsy. Patients with congenital or secondary forms of nephrotic syndrome and those with elevated serum creatinine were excluded. Patients were enrolled from the Nephrology Clinic and in Patient Departments of Cairo University pediatric Hospitals following informed parent consent during the period of May 2007 till November 2008. The study was approved by the Pediatrics Department Research Committee.

Methods: All patients were subjected to:

- Initial clinical and basic laboratory evaluation
- Quantitation of annexin V in 24 h urine by enzyme-linked immunosorbent assay (ELISA) for human annexin V using BMS252 kit (Bender Medsystems, Vienna, Austria) (Borzecka *et al*., 2004)
- Measurement of serum total MMP-9 by ELISA using Quantikine kit (R and D systems, USA) (Bauvois *et al*., 2007)

Ultrasound-guided core renal biopsy after exclusion of any contraindications. In addition to routine light microscopy, unstained slides from the same specimens were further processed in Medical Biochemistry Department, Cairo University. This was done through identification of apoptotic bodies by DNA *in situ* using TACS. XLTM *in situ* apoptosis detection kit (R and D system, USA).

- Detect apoptosis in renal tissue. This was done through identification of apoptotic bodies by DNA *in situ* using TACS. XLTM *in situ* apoptosis detection kit (R and D system, USA)
- Treatment with oral cyclophosphamide (CPA), in a dose of 2.5 mg/kg/day, together with alternate day prednisone for three months according to the standard protocol
- Assessment of the response to CPA. Responders were defined as those who achieve clinical remission associated with normal protein excretion. Partial responders were defined as having clinical remission and persistent proteinuria below nephrotic range. Failure to achieve clinical remission and/or persistent nephrotic range proteinuria defined resistance
- Repeat assessment of MMP-9 and urinary annexin V at the end of CPA treatment

Serum MMP-9 and urinary annexin V excretion were measured in control subjects for comparison.

Data analysis: Nominal data were expressed as frequency and percentage, while numerical data were expressed as mean and standard deviation. Means were compared using ANOVA and paired sample t-tests, while proportions were compared using Chi square test. Statistical analysis was done using Microsoft Excel 2003 and SPSS statistical package version 11. p-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Pathologically, 35 patients (58%) had focal segmental glomerulosclerosis (FSGS), 19 (32%) had Diffuse Mesangial Proliferation (DMP) and 6 (10%) had minimal change nephropathy (Table 1).

The mean±SD MMP-9 concentration was 87±53.7 pg mL^{-1} in control subjects and 189.1±150.6 pg mL^{-1} in patients before treatment with cyclophosphamide ($p<0.0001$). After treatment, the mean MMP-9 concentration increased to 329.2±172.4 pg mL^{-1} ($p<0.0001$) (Fig. 1). Regarding urinary annexin V, the mean control value was 2.7±0.6 ng g^{-1} creatinine. Patients had significantly higher values; 4.5±0.3 ng g^{-1} ($p<0.0001$), which further increased after treatment (5.2±1.3 ng g^{-1}, $p = 0.0002$).

Table 1: Clinical data of the study group

Parameters*	Patients (SRNS, n = 60)	Controls (n = 20)
Age (years)†	7.6±3.5	8.1±2.1
Male/ Female†	32/28(53/47%)	12/8(60/40%)
Ascites	21(35%)	0
Oliguria	15(25%)	0
Hematuria		
Gross	15(25%)	0
Microscopic	32(53%)	
Systolic BP		
90-95th percentile	9(15%)	0
>95th percentile	24(40%)	0
Diastolic BP		
90-95th percentile	3(5%)	0
>95th percentile	36(60%)	0

*: Data expressed as No. of subjects (%) except for age (Mean±SD). †: Not Significant ($p>0.05$). SRNS: Steroid-Resistant Nephrotic Syndrome. BP: Blood Pressure

Table 2: Response to cyclophosphamide and laboratory findings of the study group according to pathological type

Paramenters	Study group (n = 60)	FSGS (n = 35, 58%)	DMP (n = 19, 32%)	MCNS (n =6 , 10%)	p-value
Response to cyclophosphamide					
Responders	25(42)	10(29)	11(58)	4(67)	0.02
Partial responders	9(15)	5(14)	2(10)	2(33)	
Resistance	26(43)	20(57)	6(32)	0	
Serum MMP-9 (pg/mL)					
Before treatment	189.1±150.6	235.1±198.4	102.8±87.1	202.4±99.7	
p-value		0.002, >0.10	0.002, 0.04	0.04, >0.04	
After treatment	329.2±172.4	331.4±172.6	324.5±154.3	303.1±211.8	
p-value		>0.10	>0.10	>0.10	
Urinary annexin V (ng g^{-1} creatinine)					
Before treatment	4.5±0.3	4.6±0.5	4.3±0.54	4.2±0.32	
p-value		0.05, 0.07	0.05, >0.10	>0.10, 0.07	
After treatment	5.2±1.3	4.9±1.0	4.8±1.1	5.1±1.2	
p-value		>0.10	>0.10	>0.10	
DNA *in situ*	29(48)	24(69)	5(26)	0	0.0008

Numerical data were expressed as Mean±SD. FSGS: Focal Segmental Glomerulosclerosis, DMP: Diffuse Mesangial Proliferation, MCNS: Minimal Change Nephrotic Syndrome, MMP-9: Matrix Metalloproteinase-9

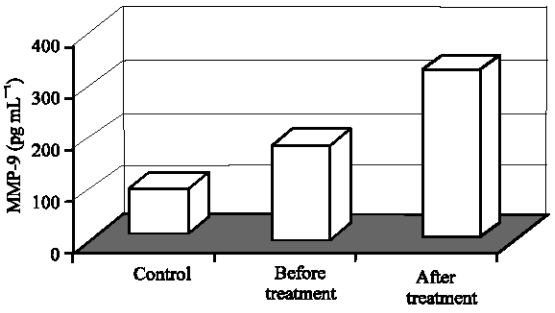

Fig. 1: Mean serum matrix metalloproteinase-9 (MMP-9) concentrations in control subjects, patients with steroid-resistant nephrotic syndrome before and after treatment with cyclophosphamide

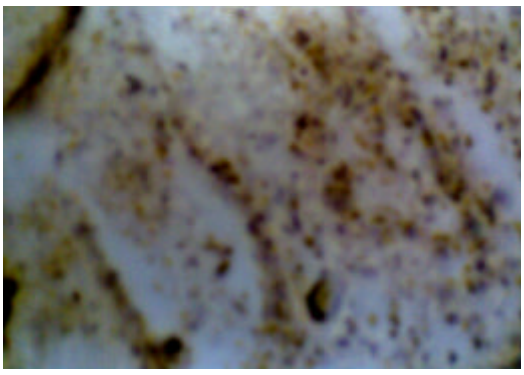

Fig. 2: Photomicrograph of kidney tissue showing positive staining for apoptosis in a case of focal segmental glomerulosclerosis

Twenty nine patients (48%) had positive DNA *in situ* indicating apoptosis in the glomeruli and infiltrating cells of the interstitium (Fig. 2). Table 2 shows the apoptotic markers in patients with different pathological types. Comparing patients who responded to CPA with resistant cases has shown that responsive cases had lower annexin V excretion and DNA *in situ* positivity (Table 3). Negative apoptosis by DNA *in situ* predicted response with 72% sensitivity, 69% specificity and a likelihood ratio of 2.47.

Table 3: Comparison of apoptotic markers between responders to CPA and resistant cases

Parameters	Responders (n = 25)	Resistant (n = 26)	Comparison (p-value)
MMP-9 (pg mL^{-1})	167.70±83.3	208.500±79.7	0.080
Urinary annexin V (ng g^{-1} creatinine)	4.33±0.38	4.590±0.42	0.030
DNA *in situ*	7(28)	18(69)	0.003

Data expressed as Mean±SD, DNA *in situ* are expressed as No. (%)

Table 4: MMP-9 and urinary annexin V according to DNA *in situ* positivity

		DNA *in situ*		
Parameters		Positive	Negative	p-value
MMP-9	Before treatment	263.0±242	163±125	0.05
(pg mL^{-1})	After treatment	396.2±288	321±305	>0.10
Urinary annexin V	Before treatment	4.700±1.4	4.4±1.2	>0.10
(ng g^{-1} creatinine)	After treatment	5.100±1.3	4.9±1.1	>0.10

Data expressed as Mean±SD. MMP-9: Matrix Metalloproteinase-9

Overall, there was no significant correlation between serum MMP9 and urinary annexin V (p>0.10). Table 4 shows the comparison of MMP-9 and urinary annexin V between patients with positive and negative DNA *in situ*.

In the present study, serum MMP-9, urinary annexin V and evidence of apoptosis in kidney biopsy specimens were investigated in 60 children with SRNS. Pathologically, 58% of the study population had FSGS. Consistent with present findings, FSGS is known to be more resistant than the other types of idiopathic nephrotic syndrome. All patients received oral cyclophosphamide for three months and 25 children (42%) achieved complete remission while 26 (43%) were resistant. This comes in

agreement with Ehrich *et al.* (2007). who stated that the rate of complete remission after induction therapy for SRNS due to either FSGS or Minimal Change Nephrotic Syndrome (MCNS) was below 50%. Meanwhile, Hodson and Craing *et al.* (2008) stated that ciclosporin (with or without alternate-day prednisone) and cyclophosphamide (with pulse intravenous corticosteroids) resulted in comparable complete or partial remission rates of about 60%.

Apoptosis plays a central role in maintaining homeostasis in the kidney by deletion of unwanted cells without induction of an inflammatory reaction. More than half of the children with SRNS were positive for apoptosis by DNA *in situ* detection. Those with FSGS had significantly higher rates of apoptosis (69%) compared to those with DMP or MCNS ($p = 0.0008$). Explanation resides in the fact that apoptosis is highly integrated in the pathogenesis of sclerotic lesions. Schiffer *et al.* (2001). stated that podocyte depletion leading to podocyte insufficiency and capillary collapse have been invoked as important steps in the development of FSGS. Apoptosis could constitute a vicious circle leading to more podocyte injury. On the other hand, apoptosis in DMP is not hard to explain given that the accumulation of ECM proteins by itself is an important incentive to apoptosis (Baker *et al.*, 1994; Rost *et al.*, 2002).

We have thus shown that apoptosis is increased in children with SRNS, particularly those with more severe pathology such as FSGS.

This study presented elevation of urinary annexin V in steroid-resistant nephrotic patients compared to controls. These results are in agreement with those reported by Simsek *et al.* (2008). Although, it was reported, based on limited data, that urinary annexin V concentration may be an indicator of renal injury related to the urinary protein level (Matsuda *et al.*, 2000), other reports did not find a correlation between urinary annexin V and protein excretion (Simsek *et al.*, 2008). In the current study, there was a significant elevation of MMP-9 in the study group, which was expected and matching with both experimental animal model and human studies (Krämer *et al.*, 2008; Bauvois *et al.*, 2007).

In children who did not respond to CPA, both urinary annexin V ($p = 0.03$) and the presence of apoptosis in renal biopsy specimens ($p = 0.003$, LH, 2.47) were significantly higher than responders. We could thus demonstrate the relation between increased apoptosis and CPA resistance. The difference in serum MMP-9 concentration between responders and non-responders did not reach significance ($p = 0.08$), while MMP-9 was higher ($p = 0.05$) in patients with biopsy evidence of apoptosis. Nevertheless, we could not demonstrate an association between urinary annexin V and apoptotic bodies in renal tissue.

Both annexin V and MMP-9 increased after treatment with CPA. Although, the increase in annexin V was less marked, it was still statistically significant. Despite that increase in both annexin V (Borzecka *et al.*, 2004) and MMP-9 (Wasilewska and Zoch-Zwierz, 2008). Following specific therapy for nephrotic syndrome were previously reported, this would not be expected given that annexin V is an apoptotic marker and that MMP-9 was higher in those with tissue evidence of apoptosis.

Vu and Werb (2000) stated that MMP-9 can affect cell survival and proliferation both positively and negatively. Although, MMP-9 was reported to induce a pro-apoptotic signal similar to that of fibronectin (Schedin *et al.*, 2000), MMP-9 activity may be required to generate a proliferative or anti-apoptotic signal (Uzui *et al.*, 2000; Chang *et al.*, 2006).

Especially that those with MCNS had higher MMP-9 (despite less apoptosis) compared to those with DMP, it is possible that MMP-9 may have counter-regulatory anti-apoptotic effects. To the same effect, Daniel *et al.* (2001) stated that tissue inhibitors of metalloproteinase (TIMPs) had an anti-inflammatory effect. The role of MMP-9 as a contributor to kidney disease progression and hence the potential use of TIMPs as an adjunct treatment of refractory cases of SRNS is the more important issue which needs further study.

In conclusion, apoptosis was demonstrated in most cases of FSGS presenting as SRNS as well as in some cases of DMP. Serum MMP-9 is elevated in SRNS and increases after treatment with cyclophosphamide. It may possess proliferative anti-apoptotic effect and may contribute to kidney disease progression. The potential therapeutic role of TIMPs needs further elucidation.

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