Geget (2006) 6 : 69 - 76


Original Article

# Apoptosis of Peripheral Blood Lymphocytes in Primary Nephrotic Syndrome

**Ehsan Mowafy, Mohamed Hazem Gouda, Magdy El-Bordiny* and Yossra Abdalla**

*Departments of Pediatrics and Clinical Pathology*, Faculty of Medicine, Alexandria University, Egypt*

## ABSTRACT

**Background:** Apoptosis is a highly programmed form of cell death that has significant role in such physiological processes as normal cell turnover, as well as many pathological conditions .Several studies implicates altered lymphocyte function in the mechanisms underlying idiopathic nephrotic syndrome. An increased apoptosis rate may also contribute to the functional abnormalities of T-cells already found in these patients.

**Objectives:** The objectives of the present work were to investigate the phenotypic characteristics of T-lymphocyte subsets in newly diagnosed nephrotic children and after induction of remission. This study also aimed at detecting the percentage of apoptotic lymphocytes in the peripheral blood of the same group of patients.

**Methods:** The study was carried out on 30 selected children. Twenty children with first attack nephrotic syndrome suggestive of minimal change type and ten healthy children of comparable age as a control group. All children were subjected to history taking, clinical examination and laboratory investigations including complete blood picture, blood urea, serum creatinine, total serum proteins, serum cholesterol, serum C3, complete urine analysis and 24 hours urine for proteins. All children were also further subjected to specific laboratory tests to study peripheral blood lymphocytes including detection of lymphocytes subsets by three color flow analysis using monoclonal antibodies CD3 and CD4 and also determination of apoptotic lymphocytes in the peripheral blood by flow cytometry using annexin-v-flourescein isothiocynate (FITC) and propidium iodide (PI).

**Results:** There was no statistically significant difference between nephrotic patients at presentation and control group as regards percentage of CD3 and CD4 lymphocytes in peripheral blood ($p = 0.89$ and 0.30 respectively). Also, the percentage of apoptotic lymphocytes in peripheral blood was significantly higher in the patient group at presentation of nephrotic disease than after induction of remission and than the control group ($p = 0.000$ and 0.000 respectively). Finally, the percentage of apoptotic lymphocytes was significantly higher at remission of the disease than the control group ($p = 0.001$).

**Conclusions:** In the present study increase in the apoptosis rate of CD3+ lymphocytes was found. This could be a crucial point in the pathogenesis of nephrotic syndrome but clearly more prospective studies of large groups are needed with the analysis of the apoptosis rate within different subsets of lymphocytes. It is very difficult at the moment to explain why the lymphocyte apoptosis is increased in nephrotic syndrome. This might result from impaired production of and dysregulation in the synthesis of different cytokines, which are essential for the normal life span of lymphocytes such as interleukin (IL)-2 and TNF-α. Another explanation for the abnormalities found in our study may be reduced anti-oxidant defense in patients with nephrotic syndrome which contribute to an increase in the apoptosis rate of circulating lymphocytes.

## INTRODUCTION

Apoptosis, the programmed cell death, is a natural physiological process which is genetically controlled. It is the ability of the cell to carry out its own death through activation of an internally encoded suicide

program[1]. It occurs for two reasons: to remove unnecessary cells as a part of the normal development or as a defense mechanism to get rid of cells that bear mutation[1].

Apoptotic cell death can be distinguished from necrotic cell death which is a pathological form of cell death resulting from acute cellular injury[2]. Apoptosis can be induced by growth factor withdrawal, DNA damage and activation of death receptors e.g. Fas receptor[2]. It is regulated by a variety of genes; the most important of which are P53 gene and bcl-2 family[3].

Apoptosis triggered by ischemia, exogenous toxins or endogenous mediators of damage may be the initial insult capable of causing renal diseases[4]. The correct knowledge of the contribution of apoptosis in different renal pathologies is required for the design of therapeutic strategies[4].

Apoptosis of peripheral blood lymphocytes is accompanied by loss of membrane phospholipid asymmetry, resulting in exposure of phosphatidylserine (PS) at the cell surface. Expression of PS at the cell surface plays an important role in the recognition and removal of apoptotic cells by macrophages[5].

Annexins are calcium dependant phospholipid binding proteins. Fluorescin labeled purified annexin V has been developed as a biochemical tool for detection of apoptotic cells. It binds to negatively charged phospholipids that are exposed on the cell surface during apoptosis. Apoptotic lymphocytes strongly stain with annexin V whereas normal cells are annexin V negative[6].

The significance of the immune system in the pathogenesis of minimal change nephrotic syndrome was suggested to some years ago, but until now the precise nature and cause of the disease have not been established[7]. Several studies of lymphocytes subpopulations and lymphocyte functions have been made in children with nephrotic syndrome to identify an abnormal T-cell clone and to determine its role in the disease. Following the work of Shalhoub in 1974[8], many authors have described disturbances in the distribution of CD4+ and CD8+ cells and their role in the pathogenesis of lipoid nephrosis, but an explanation of these abnormalities is still lacking[9-11].

The occurrence of apoptosis has been demonstrated in several renal diseases[4]. However, there are numerous unanswered questions regarding the precise role of apoptosis in renal damage, the extracellular and the intracellular factors that induce or prevent apoptosis in the kidney. Also, the possible therapeutic value of the modulation of renal cell apoptosis in kidney diseases has not been adequately investigated yet.

## AIM OF THE WORK

The objectives of the present work were to investigate the phenotypic characteristics of T-lymphocyte subsets in newly diagnosed nephrotic children and after induction of remission. This study also aimed at detecting the percentage of apoptotic lymphocytes in the peripheral blood of the same group of patients.

## SUBJECTS AND METHODS

The study was carried out on 30

selected children. Group I twenty children with first attack of nephrotic syndrome suggestive of minimal change type according to criteria of ISKDC[12]. Steroid resistant cases were excluded. Also, ten healthy children of comparable age as a control group. In nephrotic children, blood sampling was done twice; the first sample was taken at presentation of acute attack of the disease (group Ia) and the second one was taken after induction of remission (group Ib), using a modified long term steroid protocol as follows: prednisone 60 mg/kg/day daily divided doses for one month then same treatment as single dose day by day for 6 weeks then gradual tapering.

All children were subjected to history taking, clinical examination and laboratory tests including complete blood picture, blood urea, serum creatinine, total serum proteins, serum cholesterol, serum C3, complete urine analysis and 24 hours urine for proteins. All children were also further subjected to specific laboratory investigations including determination of lymphocytes subsets by three color flow analysis using monoclonal antibodies CD3 and CD4[13] and also detection of apoptotic lymphocytes in the peripheral blood by flow cytometry using annexin-v-flourescein isothiocynate (FITC) and propidium iodide (PI)[14]. The last test was done at the first presentation of NS and after induction of remission in nephrotic patients and only once in the control children. Written consent was taken from all children's families.

**Statistical Methods**

The results were tabulated and analyzed using the appropriate statistical methods with the level of statistical significance at ($p \leq 0.05$). Values were expressed as percentage or mean ± SD.

**RESULTS**

The age of nephrotic children in the present study ranged between 2 to 6 years with a mean of 3.75 ± 1.16. They were 11 males and 9 females. Their urine analysis showed heavy proteinuria (≥ 1.7 gm/24 hrs) and absence of microscopic hematuria. The results of the study are demonstrated in table 1 to 3.

Table 1 shows the means and standard deviations of the laboratory data in nephrotic children at presentation and the control group. There was no statistically significant difference between patients and control as regards total leukocytic count, serum creatinine and serum C3. On the other hand, total serum proteins, serum albumin, serum cholesterol and 24 hours urine proteins were significantly higher in nephrotic patients than in control group.

Table 2 shows the comparison between nephrotic patients at presentation and control group as regards percentage of CD3 and CD4 lymphocytes in peripheral blood. There was no significant statistical difference between patients and control group as regards both lymphocytes subpopulations. ($p = 0.89$ and 0.30 respectively)

Table 3 shows the comparison between nephrotic patients at presentation, after induction of remission and the control group as regards the percentage of apoptotic lymphocytes (CD3 + and Annexin V + lymphocytes) detected in peripheral blood

by flow cytometry. The percentage of apoptotic lymphocytes was significantly higher in the patient group at presentation of nephrotic disease than after induction of remission and than the control group (p = 0.000 and 0.000 respectively). Also, it was significantly higher at remission of the disease than the control group (p = 0.001).

**Table 1: Laboratory data expressed as means and standard deviations observed in nephrotic children at presentation and control group.**

| | **Patient group (n = 20)** | **Control group (n = 10)** | **p value** |
|---|---|---|---|
| **WBCs count ($\times 10^3$/cc)** Mean ± SD | 7.51 ± 1.06 | 7.16 ± 1.39 | p = 0.455 |
| **Lymphocyte count (%)** Mean ± SD | 34.15 ± 16.55 | 63.3 ± 5.46 | p = 0.000* |
| **Serum creatinine (mg/dl)** Mean ± SD | 0.54 ± 0.19 | 0.41 ± 0.07 | p = 0.053 |
| **Serum C3 (g/L)** Mean ± SD | 1.33 ± 0.2 | 1.13 ± 0.41 | p = 0.091 |
| **Total serum protein (g/dl)** Mean ± SD | 3.25 ± 0.34 | 6.55 ± 0.34 | p = 0.000* |
| **Serum albumin (g/dl)** Mean ± SD | 1.31 ± 0.19 | 4.03 ± 0.32 | p = 0.000* |
| **Serum cholesterol (mg/dl)** Mean ± SD | 348.95 ± 39.17 | 154.5 ± 16.17 | p = 0.000* |
| **24 hrs urine for protein (gm/24 hrs)** Mean ± SD | 2.93 ± 0.87 | 0.09 ± 0.02 | p = 0.000* |

**Table 2: Comparison between nephrotic patients at presentation and control group as regards percentage of CD3 and CD4 lymphocytes in peripheral blood.**

| | Patient group (n = 20) | Control group (n = 10) | Statistical test |
|---|---|---|---|
| **CD3%** | | | |
| Range | 51-76 | 51-83 | t = 0.138 |
| Mean | 67.75 ± 7.07 | 67.30 ± 10.77 | p = 0.891 |
| **CD4%** | | | |
| Range | 40-62 | 35-62 | t = 1.040 |
| Mean | 52.30 ± 6.43 | 49.20 ± 9.85 | p = 0.307 |

**Table 3: Comparison between nephrotic patients at presentation, after induction of remission and control group as regards the percentage of apoptotic lymphocytes in peripheral blood.**

| CD3+ANNEXIN V+ (%) | Patient Group (n = 20) | | Control (group II) (n = 10) |
|---|---|---|---|
| | At presentation (group Ia ) | At remission (group Ib) | |
| Range | 3.3-15.1 | 1.2-5 | 1-3.8 |
| Mean | 7.35 ± 2.7 | 3.09 ± 0.88 | 1.86 ± 0.95 |
| **t-test with control group** | t = 6.258 p = 0.000* | t = 3.521 p = 0.001* | |
| **t-test between presentation and remission in patient group** | t = 6.113 p = 0.000* | | |

## DISCUSSION

The nephrotic syndrome (NS) is the most common chronic renal disease of childhood. It is characterized by proteinuria, hypoproteinemia, edema and hyperlipidemia[7].

Apoptosis is a highly programmed form of cell death that has significant role in such physiological processes as normal cell turnover, as well as many pathological conditions. It undoubtedly is involved in morphogenesis and a variety of pathophysiological conditions of the kidney, ranging from cyst formation to inflammation and glomerular or interstitial scaring[15].

Nephrotic syndrome is accompanied by

and probably related to abnormal T-lymphocyte function. Decreased stimulation of survival factors and increased levels of "dead signals" may lead to the malfunction of many cells; including lymphocytes[16].

In our work, lymphocyte subsets have been studied using color flow cytometry; our results revealed that there was no significant difference in total T-lymphocyte (CD3+) and in (CD4+) in acute untreated attacks of nephrotic syndrome in comparison with control group. Similar results were reported by Bagga and his collogues in their study[17].

On the other hand, other results were obtained by Kopayashi and his collogues[11] They found that in MCNS, CD4+Leu8+ (suppressor-inducer) and CD3+CD25+ (IL-2 receptor positive T-cells) were significantly increased, and CD4+Leu8- (helper-inducer) and CD8+CD11+ (suppressor) were significantly decreased in comparison with normal healthy children.

In our study, no change in lymphocytes subset count was observed. This can be explained by the fact that the immune system and disturbed T-lymphocytes function, rather than lymphocytic count, may be implicated in the pathogenesis of childhood nephrotic syndrome. This was proved previously by Hewitt IK and his collogues[18].

Other studies implicates altered lymphocyte functions in the mechanisms underlying idiopathic nephrotic syndrome[19]. An increased apoptosis rate may also contribute to the functional abnormalities of T-cells already found in these patients.

In the present study we investigated the rate of apoptosis within T-lymphocyte subpopulations. Apoptotic lymphocytes in the peripheral blood of the patients were detected by flow cytometry using annexin-V-flourescein isothiocynate (FITC) kit described by Koopman and his colleagues[5]. Propidium iodide was also used to exclude necrotic cells from the analysis. It was found that the percentage of annexin-V-(FITC)-positive CD3 cells in the peripheral blood of the first attack of minimal change nephrotic children is 7.35%, and 3.09% after induction of remission with steroids and 1.86% in controls. These changes were statistically significant.

Our results coincide with those reported by Zachwieja J and his collogues[16]. Also, similar results were reported by Borzecka H and his collogues[20]. The study comprised 26 nephrotic children; in the acute phase before institution of the therapy, the percentage of apoptotic T lymphocytes was significantly higher than in the control.

It is very difficult at the moment to explain why the lymphocyte apoptosis is increased in nephrotic syndrome. We can speculate that two mechanisms may be involved in this phenomenon. First, increased apoptosis of CD3 lymphocytes might result from impaired production of and dysregulation in the synthesis of different cytokines, which are essential for the normal life span of lymphocytes. Withdrawal of the positive signals which are needed for survival may promote apoptosis[1]. This has been observed in cases of impaired interleukin (IL)-2 productions which plays a central role in both cellular and humoral immune response[21]. Also, increased production of the molecules that bind specific receptors on the cell surface

and signal the cell to begin the apoptosis program (death activators) can also influence the number of T-lymphocytes. This is the case with TNF-α, injury cytokine, which is secreted mainly by TH-2 cells[22].

Another explanation for the abnormalities found in our study may be reduced anti-oxidant defense in patients with nephrotic syndrome. The anti-oxidant system consist of many different components which defend tissues against free radical attach[23]. In children with nephrotic syndrome there are many abnormalities within the anti-oxidant system such as low anti-oxidant enzyme activity and low total anti-oxidant status which may lead to increased levels of oxidant within cells. Oxidants may damage the heparan sulfates on glomerular endothelial cells, and within the basement membranes, leading to proteinuria[24].

In support to this theory, Zachwieja and his collogues[25] have reported that in patient with nephrotic syndrome, reduced anti-oxidant defense contribute to an increase in the apoptosis rate of circulating lymphocytes. They found that total anti-oxidant status (TAS) is significantly lowered compared with levels in healthy children; also they found differences in glutathione reductase and glutathione peroxidase activity in children with nephrotic syndrome compared with controls.

In the present study increase in the apoptosis rate of CD3+ lymphocytes was found. This could be a crucial point in the pathogenesis of nephrotic syndrome but clearly more prospective studies of large groups are needed with the analysis of the apoptosis rate within different subsets of lymphocytes.

## REFERENCES


1. **Schanper, H. (1994):** Apoptosis: death as "vital" biological function. Pediatr. Nephrol.; 8: 377-382.
2. **Schwartz, L.; Smith, S.; Jones, M.; et al. (1993):** Do all programmed cell deaths occur via apoptosis? Proc. Natl. Acad. Sci.; 90: 980-984.
3. **Oda, E.; Ohki, R.; Murasawa, H.; et al. (2000):** Noxa, a BH-3 only family member of BCL-2 family and candidate mediator of P53-induced apoptosis. Science; 288: 1053-1085.
4. **Alberto, O.; Silvia, G.; Corina, L.; et al. (1996):** Apoptosis in renal diseases. Frontiers in Bioscience; 1: 30-47.
5. **Koopman, G.; Reutelingsperger, C.; Kuijten, G.; et al. (1994):** Annexin V for flow cytometric detection of phosphatidylserine expression on B-cells undergoing apoptosis. Blood; 84: 1415-1420.
6. **Hawkins, T.; Das, D.; Young, B.; et al. (2002):** DT40 cells laking the $ca^{2+}$-binding protein annexin 5 are resistant to $ca^{2+}$-dependant apoptosis. Cell Bio.; 99: 8054-8059.
7. **Bergstein, J. (2004):** Conditions particularly associated with proteinuria. In: Behrman RE, Kliegman RM, Nelson WE (eds). Nelson Textbook of pediatrics; 17th edition. Philadelphia, London, Toronto: WB Saunders Company; 1751-1757.
8. **Shalhoub, R. (1974):** Pathogenesis of lipoid nephrosis: a disorder of T-cell function. Lancet ii; 556-560.
9. **Fiser, R.; Arnold, W.; Chalton, R.; et al. (1991):** T-lymphocyte subsets in nephrotic syndrome. Kidney Int.; 40: 913-16.
10. **Hulton, S.; Shah, V.; Byrne, M.; et al. (1994):** Lymphocytes subpopulation, interleukin-2 and interleukin-2 receptor expression in childhood nephrotic syndrome. Pediatr. Nephrol.; 8: 135-139.
11. **Kobayashi, K.; Yoshikawa, N.; Nakamura, H.; et al. (1994):** T-cell subpopulation in childhood nephrotic syndrome. Clin. Nephrol.; 41: 253-258.
12. **International Study of Kidney Disease in Children (1978):** Nephrotic syndrome in children. Prediction of histopathology from clinical and laboratory characteristics at time of diagnosis. Kidney Int.; 13: 159.
13. **Shortman, K. and Wu, L. (1996):** Early T-

lymphocyte progenitors. Ann. Rev. Immunol.; 14-29.

14. **Van-Engelant, M.; Nieland, L.; Ramaekers, S.; et al. (1998):** Annexin V affinity assay: A review on apoptosis detection system based on phosphatidylserine exposure. Cytometry; 31: 1-9.
15. **Mene, P. and Amore, A. (1998):** Potential role of apoptosis in renal diseases. Nephrol. Dial. Transplant.; 13: 36-43.
16. **Zachwieja, J.; Dworacki, G.; Bobkowski, W.; et al. (2002):** Increased apoptosis of peripheral blood lymphocytes in children with nephrotic syndrome. Pediatr. Nephrol.; 17: 197-200.
17. **Bagga, A.; Vasudev, A.; Moudgil, A.; et al. (1996):** Peripheral blood lymphocyte subsets in idiopathic nephrotic syndrome of childhood. Indian J. Med. Res.; 104: 292-295.
18. **Hewitt, I.; House, A.; Potter, J.; et al. (1992):** Altered in vitro lymphocyte response in childhood nephrotic syndrome. Pediatr. Nephrol.; 6: 464-466.
19. **Inage, Z.; Wada, N.; Kikkawa, M.; et al. (1990):** Suppressor T-lymphocyte dysfunction in MCNS: Role of H2 histamine receptor-bearing suppressor T-lymphocytes. Clin. Nephrol.; 33: 20-24.
20. **Borzecka, H.; Sikora, P.; Majewski, M.; et al. (2004):** Apoptosis of peripheral blood T lymphocytes in nephrotic children. Annales Universitatis Mariae Curie-Sklodowska (Med); 59 (1): 253-256.
21. **Dohi, K.; Morita, H.; Ogawa, S.; et al. (1991):** Production of interleukin 2 (IL-2) and responsiveness to IL-2 of peripheral blood lymphocyte subsets in nephrotic syndrome. Kidney Int.; 40: 913-916.
22. **Suranyi, M.; Guasch, A.; Hall, B.; et al. (1993):** Elevated levels of tumour necrosis factor-alpha in the nephrotic syndrome in humans. Am. J. Kidney Dis.; 21: 251-259.
23. **Gwinner, W.; Landmesser, U.; Brandes, R.; et al. (1997):** Reactive oxygen species and antioxidant defense in puromycin amino-nucleoside glomerulopathy. J. Am. Soc. Nephrol.; 8: 1722-1731.
24. **Fydryk, J.; Jacobson, E.; Kurzawska, O.; et al. (1998):** Antioxidant status in children with steroid-sensitive nephrotic syndrome. Pediatr. Nephrol.; 12: 751-754.
25. **Zachweija, J.; Bohkowski, W.; Zaniew, M.; et al. (2003):** Apoptosis and antioxidant defense in nephrotic syndrome. Pediatr. Nephrol.; 18: 1116-1121.