Original Article

Significance of Apoptotic Leucocytes in Peripheral Blood Smears : A Case Control Study

Aparna Narasimha, Harendra Kumar M L, Prasad C S B R, Rupnarayan R

Department of Pathology, Sri Devaraj Urs Medical College, Kolar - 563 101

ABSTRACT

Introduction: *Apoptosis is a carefully governed process that brings about cell death in a regulated manner. The present study was undertaken to evaluate the morphological features of apoptotic leucocytes in peripheral blood smears, confirm their presence by fluorescent stain and to ascertain their clinical significance.*

Material & Methods: *Blood samples were stained with Leishman stain & examined under oil immersion light microscopy. The peripheral smears were also stained with Acridine Orange dye and examined under fluorescence microscope. The percentage of apoptotic leucocytes were determined by counting the number of cells showing features of apoptosis.*

Results: *The morphological changes of apoptosis include contraction of chromatin with nuclear and cytoplasmic budding to form membrane bound apoptotic bodies. Neutrophils were the most common apoptotic leukocytes followed by lymphocytes and eosinophils. Apoptotic neutrophils were more commonly seen in infections whereas lymphocytes were seen in diabetes mellitus and in neoplasia. Fluorescent stain confirmed the presence of apoptotic leucocytes.*

Conclusion: *The presence of apoptotic leukocytes in peripheral blood smears may aid in the differential diagnosis of various disease process. Fluorescent stain (acridine orange) can be used as an additional special technique for detecting apoptotic leukocytes.*

Key words: *Apoptotic leucocytes, Leishman stain, Acridine orange, Fluorescent stain.*

INTRODUCTION

Apoptosis is a carefully governed process that brings about cell death in a regulated physiological context without the release of inflammatory mediators. The morphological changes of apoptosis are defined and include contraction of chromatin with nuclear and cytoplasmic budding occurring to form membrane bound fragments or apoptotic bodies.[1] The enormous turnover rate of leucocytes, programmed cell death as a physiological process in which each step is

Corresponding Author
Dr. Aparna Narasimha,
22, "Moyenvilla", Moyenvilla Road,
Lang ford Town, Bangalore - 25
Tel: 9632140850
E-mail: aparna_patho@yahoo.com

regulated is similar to the process of mitosis and is equally important for leucocytes.[2,3,4] After observing apoptosis in leucocytes during the routine examination of peripheral blood smears, a case control study was undertaken to analyse their clinical significance.

Objectives

1. To evaluate the morphological features of apoptotic leucocytes in peripheral blood smears.
2. To ascertain their clinical significance with respect to age and sex.
3. To confirm their presence by fluorescent stain.

MATERIAL AND METHOD

The material for the present study comprised of 161 cases of both control (50) and study group (111) were obtained from the Hematology laboratory at our institution. Ages and sex matched normal healthy volunteers were taken as controls. Study groups were categorized according to their clinical diagnoses. The patient population included males and females, ranging from newborn to 80 yrs. Clinical details, laboratory findings and therapeutic information were reviewed in all cases. We specifically reviewed the therapeutic history concerning glucocorticoids administration, because of its well documented relationship to apoptosis.[5] The blood samples were collected randomly from controls and study groups using Tripotassium Ethylene Diaminetetraacetic acid (K_3-EDTA) as anticoagulant. The smears were prepared within 1-2 hrs, stained with Leishman stain and examined under oil immersion light microscopy at a final magnification of X 1000. Smears were studied for identification of apoptotic leucocytes. Percentage of apoptotic leucocytes were determined by counting the number of cells showing features associated with apoptosis.

The criteria used for detection of apoptotic leucocytes (AL's) included:

a) *Cell shrinkage* b) *Nuclear cytoplasmic changes*: Peripheral condensation of chromatin along the nuclear membrane, nuclear fragmentation, formation of cytoplasmic blebs, membrane bound apoptotic bodies and cytoplasmic vacuoles c) *Persistence of specific granules and plasma membrane.* [2,6,7]

Conventional nuclear features and cytoplasmic granules were used to categorize leucocytes as neutrophils, eosinophils or lymphocytes.

ALs were arbitrarily semiquantitated by counting the number per 100 cells of neutrophils, lymphocytes and 50 cells of eosinophils. A finding of 1 - 3 ALs were considered as "few", 4 - 5 ALs as "moderate" and 6 or more as "many".[6] Direct smears of both control and study groups were also obtained by finger prick method to rule out anti-coagulant induced artefacts.

The acridine orange stain (HI-media, India) was prepared by dissolving 25mg of the dye in 2ml glacial acetic acid, diluting to 100ml by adding distilled water and stored in amber colored bottle at 4^0C.

Additional direct smears collected were stained with acridine orange dye and examined under fluorescence microscope at a final magnification of X 1000 to confirm the presence of apoptotic leucocytes.

Exclusion Criteria:

a) Patients on chemotherapeutic drugs were not

included in the study.

b) Blood samples drawn earlier than 2 hrs

c) Blood samples without relevant clinical details

Ethical Clearance: This study was ethically cleared by our ethical clearance committee.

Statistical analysis: Descriptive statistical methods like difference in proportions were analysed by using student 't' test. A p value < 0.05 was considered as statistically significant. The statistical test was performed using the Statistical Package for Social Sciences for Windows for personal computers (SPSS).

Fig.1 - Photomicrograph showing apoptotic neutrophils. **(1a & 1c)** - Leishman, X 1000. **(1b & 1d)** Fluorescent stain, X 1000

RESULTS

The present study included 161 cases. The total leucocyte counts in all patients ranged from 4,000 4lakh cells/ cu mm. The study group and the control group included males and females, ranging from newborn to 80 years of age. (Table -1)

Apoptotic leucocytes were easily identified in the peripheral blood smears by their distinct morphological changes by semiquantitative method. (Table 2) In controls, few (1-3) apoptotic neutrophils were observed in newborn 20% (2/10) and in aged individuals above 60 yrs 30% (3/10).

Fig. 2a - Photomicrograph showing apoptotic lymphocyte and eosinophil (Leishman, X 1000) **(2b)** Apoptotic eosinophil. (Fluorescent stain, X 1000)

Apoptotic leucocytes were seen in patients with various clinical conditions. (Table 3)

Infection was the most common finding associated with increased incidence of ALs in most of the clinical conditions seen in about 39.6% cases (44/111) (Table -3).

Apoptosis was observed in all the leucocytes with a relative higher frequency in

Fig.3 - Photomicrograph showing apoptotic lymphocyte along with RBCs showing ring forms of P.falciparum (Field stain, X 1000)

neutrophils. (Table -2) Apoptotic neutrophils (Fig.1) were observed in 63.9% of the cases (71/111) (Table -2) and were associated with infection in 39.4% (28/71), diabetes mellitus in 5.6% (4/71), glucocorticoid administration in 5.6% (4/71), haematologic malignancy in 8.45% (6/71), carcinoma and postoperative states in 14% (10/71), organo-phosphorous (OP) poisoning and chronic obstructive pulmonary disease (COPD) particularly bronchial asthma in 2.81% (2/71) and trauma in 7.04% (5/71). (Table-3) A single case of diabetes mellitus and 6 patients with bronchial asthma showed apoptotic eosinophils 6.30% (7/111). (Table-3) (Fig.2) Apoptotic lymphocytes were seen in 16.21% of cases (18/111) (Table -2). Associated conditions were infections 38.8% (7/18), diabetes mellitus 22.2% (4/18), hematological malignancy, trauma and glucocorticoid administration 11.1% (2/18) each and in organo-phosphorous poisoning 5.5% (1/18). (Table -3) Among infections apoptotic lymphocytes were seen predominantly in malaria (Fig.3) and tuberculosis.

About 15 cases - 9 cases (8.1%) with infections and 6 cases (5.4%) of diabetes mellitus showed the presence of both apoptotic neutrophils as well as lymphocytes. The percentage distribution of various infections in our study is shown in Table -4.

Direct smears stained with acridine orange dye were examined under fluorescent microscopy at a final magnification X 1000. Apoptotic leucocytes and apoptotic bodies were easily identified as bright yellow fluorescence and these findings were consistent with the counts obtained on Leishman stain.

Statistical analysis - The percentage of apoptotic leucocytes in study group were compared with control group by using the Student 't' test- unpaired type 't' = 0.120. p value > 0.05, not statistically significant which implies that there is no significant difference between the study and the control groups.

DISCUSSION

Programmed cell death or apoptosis is a regulated physiological process and is as important as cell production.[8] Cell death occurs by two different mechanisms, namely necrosis and apoptosis. Necrotic cell death is secondary to noxious injury or trauma. Apoptotic cell death occurs during a variety of physiological processes, including normal development, differentiation, and embryogenesis. Necrotic death results in cell lysis, whereas apoptosis leads to cell shrinkage, nuclear pyknosis and chromatin condensation.[2] Necrotic cells appear as smudged cells or basket cells in peripheral blood smears. Apoptotic cells are identified as intact leucocytes with nuclear and cytoplasmic changes under light microscopy.

Various molecular and biochemical changes have been noted during apoptosis which include activation of endonuclease that cleaves DNA into oligonucleosomes, identified as DNA ladders on electrophoresis. Other techniques such as TUNNEL technique and fractin reactivity can also be employed for detecting apoptotic cells but all these techniques are time consuming as well as expensive. [9,10]

In our study we observed ALs in Leishman stained peripheral blood smears during routine examination and ascertained the

Table 1: Age & Sex distribution of cases and controls

AGE GROUPS	CASES			CONTROLS		
	Males No. (%)	**Females No. (%)**	**Total No. (%)**	**Males No. (%)**	**Females No. (%)**	**Total No. (%)**
NB - 1 yr	17 (68 %)	8 (32%)	25 (22.5%)	5 (50%)	5 (50%)	10 (20%)
2 - 19 yrs	9 (60%)	6 (40%)	15 (13.51%)	7 (70%)	3 (30%)	10 (20%)
20 - 39 yrs	11 (44%)	14 (56%)	25 (22.5%)	4 (40%)	6 (60%)	10 (20%)
40 - 59 yrs	12 (52%)	11 (48%)	23 (20.7%)	6 (60%)	4 (40%)	10 (20%)
60 & above	16 (70%)	7 (30%)	23 (20.7%)	8 (80%)	2 (20%)	10 (20%)
TOTAL			111 (100%)			50 (100%)

Abbreviation: NB-newborn

Table 2: Semi-quantitative distribution of apoptotic leucocytes

	Few	**Moderate**	**Many**	**Total**
Apoptotic PMNs	33 (46.4%)	27 (38%)	11 (15.49%)	71 (63.9%)
Apoptotic Lymphocytes	7 (38.8%)	11 (61.1%)	-	18 (16.21%)
Apoptotic Eosinophils	2 (28.57%)	5 (71.42%)	-	7 (6.30%)
Apoptotic PMNs & Lymphocytes	6 (40%) 12 (80%)	4 (27%) 3 (20%)	5 (33%)	15 (13.51%)
Total				111 (100%)

Abbreviation: PMN Polymorphonuclear neutrophils

Table 3: Percentage distribution of apoptotic leucocytes in different clinical conditions

Diagnosis	No. of cases (%)	Neutrophils	Lymphocytes	Eosinophils -	PMN & Lymphocytes
Infections	44 (39.6%)	28 (25.2%)	7 (6.30%)	1 (0.90%)	9 (8.1%)
DM	15 (13.5%)	4 (3.6%)	4 (3.6%)	-	6 (5.4%)
Hae.Malig	8 (7.2%)	6 (5.4%)	2 (1.8%)	-	-
Carcinoma	10 (9%)	10 (9%)	-	-	-
OP poison	3 (2.7%)	2 (1.8%)	1 (0.90%)	-	-
Post-OP	10 (9%)	10 (9%)	-	-	-
COPD	8 (7.2%)	2 (1.8%)	-	6 (5.4%)	-
Trauma	7 (6.3%)	5 (4.5%)	2 (1.8%)	-	-
Glu.Admin	6 (5.4%)	4 (3.6%)	2 (1.8%)	-	-
Total	111 (100%)	71 (63.9%)	18 (16.21%)	7 (6.30%)	15 (13.51%)

Abbreviations:DM diabetes mellitus, Haem.Malig-haematological malignancy, OP organophosphorous poisoning, PMN polymorphonuclear neutrophils, post OP post-operative, COPD chronic obstructive pulmonary disease, Glu. Admin glucocorticoid administration

Table 4: Percentage distribution of various infections

Infections	44 Cases
Bronchopneumonia	7 (15.9%)
Gastroenteritis	5 (11.3 %)
Sepsis	5 (11.3%)
Pulmonary Tuberculosis	11 (25%)
Malaria	6 (13.6%)
Empyma	1 (2.27%)
Peritonitis	1 (2.27%)
Acute Glomerulonephritis	1 (2.27%)
Viral fever	1 (2.27%)
Urinary Tract Infection	3 (6.81%)
Hepatitis	1 (2.27%)
Viral meningoencephalitis	2 (4.5%)

clinicopathologic significance of finding these apoptotic cells. The possibility of anti-coagulant induced artefacts was ruled out as the smears were prepared immediately after addition of anticoagulants. Direct smears obtained by finger prick method served as controls. Evaluation of apoptotic leucocytes in peripheral blood smears has already been done by Shidham *et al.*[6] But they have not used any special stains. So in our study we employed fluorescent dye acridine orange to identify the apoptotic bodies and thus it was helpful in correlating and substantiating our findings.

In normal controls, apoptotic neutrophils were occasionally observed in newborn and in elderly age groups. Apoptosis was observed in all leucocytes with relatively higher frequency in neutrophils (63.9%). The finding of increased numbers of apoptotic neutrophils in the peripheral circulation could be more important as neutrophils are numerically greater and have a reduced life span compared with mononuclear cells.[11] Apoptotic neutrophils were predominantly seen in infectious conditions (39.4%). Among infections, pulmonary tuberculosis showed higher occurrence of apoptotic neutrophils (25%). The activation of circulating polymorphonuclear neutrophils (PMN) from patients with active tuberculosis (TB-PMN) may be associated with induction of apoptosis as compared with PMN from normal individuals. [12]

The higher Fas expression and the lower bcl-2 expression in TB-PMN could contribute to the accelerated rate of spontaneous apoptosis as described by Majewska and coworkers.[13] Moreover, enhanced superoxide anion generation, high TNF-R55 expression, and *MTB*-induced TNF- production.[14] could make TB-PMN prone to undergo spontaneous apoptosis. Perskvist *et al* opined that infection with Mycobacterium tuberculosis causes ROS-dependent alteration of Bax/Bcl-x(L) expression and activation of caspase-3, and thereby induces apoptosis in human neutrophils. [15]

Courtney *et al* have correlated increased circulating peripheral blood apoptotic neutrophils with systemic lupus erythermatosus (SLE) disease activity. [11]

The occurrence of apoptotic lymphocytes is less (16.25%) and when present they were seen in association with diabetes mellitus, infections and in patients on glucocorticoid therapy. Otton *et al* have proposed that the high incidence of infection in poorly controlled diabetic states may be associated with an increased proportion of apoptotic lymphocytes .[16]

Ling-Chu Chang *et al* in their study have concluded that glucocorticoids prolong the survival of bovine blood neutrophils by inhibiting Fas expression via glucocorticoid receptor activation. [17] Significantly increased apoptosis levels were observed in lymphocytes from both P.falciparum and P.vivax infected patients as observed by Spinazzola et al.[18] Among infections we also observed apoptotic lymphocytes in malaria.

The occurrence of apoptotic eosinophil is very rare and in our case it was seen in one case of diabetes mellitus and in chronic asthmatics. Eosinophils are the major pro-inflammatory cells in asthma and their persistence in the airways is probably enhanced due to cytokines

that prolong eosinophil survival by inhibition of apoptosis. A study by Walsh *et al* has reviewed the role of steroids and membrane receptor ligation which play an important role in the induction of eosinophil apoptosis in patients with asthma.[19] Nong *et al* in their study observed that apoptosis of eosinophils were decreased in airways of asthmatic children and inducing eosinophil apoptosis in one of the important mechanism due to inhaled glucocorticoid therapy for asthma.[20] These findings of our study were comparable with that of Shidam and Swami. [6]

To summarize, apoptotic leucocytes can be seen in peripheral blood smears in various age groups and in either sexes. Neutrophils were the most common type ALs observed. Apoptosis can also be seen in lymphocytes but rare in eosinophils. Infection was the most common diagnosis associated with finding of ALs in peripheral blood smears. The finding of apoptotic leucocytes in peripheral blood smears may aid in the differential diagnosis and the frequency of their occurrence may be related to the severity of the disease process.

Our study was limited and applied published morphological features to evaluate apoptotic leucocytes. We recommend further studies applying special techniques such as flow cytometry[20] to evaluate apoptosis with quantitative estimation in relation to the total leucocyte count.

CONCLUSION

1) The presence of apoptotic leucocytes (ALs) in peripheral blood smears may aid in the differential diagnosis of various disease process.

2) Fluorescent stain (acridine orange) can be used as an additional special technique for detecting apoptotic leucocytes.

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REFERENCES

1) Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 1972;26:239-57.

2) Squier MKT, Sehnert AJ, Cohen JJ. Apoptosis in leukocytes. J Leuko Biol 1995;57:2-10.

3) Sachs L, Lotem J. Control of programmed cell death in normal leukemic cells: new implications for therapy. Blood 1993;82:15-21.

4) Abbas AK. Die and let live:eliminating dangerous lymphocytes. Cell 1996:84:655-7.

5) Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous nuclear activation. Nature 1980;284:555-6.

6) Shidam Vinod B, Swami Vanila K. Evaluation of Apoptotic Leukocytes in Peripheral Blood Smears. Arch of Pathol and Lab Med 2000;124:1291-4.

7) Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251-306.

8) Steller H. Mechanisms and gene of cellular suicide. Science 1995;267:1445-9.

9) Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA

fragmentation. J Cell Biol 1992;119:493-501.

10) Suurmeijer AJH, Vander Wijk J, Van Veldhuisen DJ, Yang F, Cole GM. Fractin immunostaining for detection of apoptotic cells and apoptotic bodies in formalin-fixed and paraffin-embedded tissue. Lab Invest 1999;79:619-20.

11) Courtney P A, Crockard A D, Williamson K, Irvine A E, Kennedy R J, Bell A L. Increased apoptotic peripheral blood neutrophils in systemic lupus erythematosus: relations with disease activity, antibodies to double stranded DNA, and neutropenia. Ann Rheum Dis 1995;58:309-14.

12) Mercedes Alemán, Ana García, María A. Saab, Silvia S. de la Barrera, Marta Finiasz, Eduardo Abbate and María C. Sasiain. Mycobacterium tuberculosisInduced Activation Accelerates Apoptosis in Peripheral Blood Neutrophils from Patients with Active Tuberculosis. Am Jr of Respi Cell and Mol Biol 2002;27:583-92.

13) Majewska, E., Z. Sulowska, and Z. Baj. Spontaneous apoptosis of neutrophils in whole blood and its relation to apoptosis gene proteins. Scand. J. Immunl 2000;52:496-501.

14) Aleman, M., M. Beigier-Bompadre, C. Borghetti, S. de la Barrera, E. Abbate, M. Isturiz, and M. C. Sasiain. Activation of peripheral blood neutrophils from patients with active advanced tuberculosis. Clin. Immunol. 2001;100:87-95.

15) Perkvist N, Long M, Stendahl O, Zheng L. Mycobacterium tuberculosis promotes apoptosis in human neutrophils by activating caspase-3 and altering expression of Bax/Bcl-xL via an oxygen-dependent pathway. J Immunol. 2002;168:6358-65.

16) R Otton, FG Soriano, R Verlengia, and R Curi. Diabetes induces apoptosis in lymphocytes. J of Endocrinol 2004;182:145-56.

17) Ling-Chu Chang, Sally A Madsen, Trine Toelboell, Patty S D Weber and Jeanne L Burton. Effects of glucocorticoids on Fas gene expression in bovine blood neutrophils. J of Endocrinol 2004;183:569-83.

18) F. Spinazzola, E. Nicastri, C. Vlassi, P. Ghirga and M. De Marco, et al. Humoral responses against the C-terminal region of merozoite surface protein 1 can be remembered for more than 30 years in persons exposed to Plasmodium vivax. Euro Jr of Clin Microbiol & Inf Diseases 2007; 26:175-9.

19) Walsh GM, Sexton DW, Blaylock MG. Corticosteroids, eosinophils and bronchial epithelial cells: new insights into the resolution of inflammation in asthma. J Endocrinol 2003;178: 37-43.

20) Nong Gm, Li SQ, Yao L, Liu J, Jiang M, Liang XA. Eosinophils apoptosis in asthamatic children. Zhonghua Er Ke Za Zhi 2003;41:278-81.

21) Sgonc R, Wick G. Methods for the detection of apoptosis. Int Arch Allergy Immunol 1994;105:327-32.

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