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Original Article

Apoptotic cell identification: An *in-vivo* study during induction treatment of childhood acute lymphoblastic leukemia

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ABSTRACT

Background Acute lymphoblastic leukemia (ALL) in children has high cure rate but it can cause death due to the side effects of treatment or to the disease itself. Thus the evaluation on response of treatment is important and may predict the prognosis. Since apoptosis can be induced by chemotherapy, it is thought that the number of leukemic cells that undergo apoptosis may reflect drug sensitivity and cytoreduction rate, thus it may correlate with prognosis.

Objective To detect apoptotic cells in peripheral blood of children with ALL during the first week of treatment.

Methods We conducted a cross sectional study on 58 children with newly diagnosed ALL treated in Department of Child Health, Sardjito Hospital, Yogyakarta. Apoptotic cells were detected on smears of buffy coat made from peripheral blod and stained with May-Grunwald Giemsa. The apoptotic cells viewed under light microscope within 12 time points during 7 days after treatment started.

Results Apoptotic cells were identified in 3 of 58 patients with index range of 4.2% to 36.2%.

Conclusion Apoptotic cells can be detected in peripheral blood with simple method. The explanation of why not all blood smears viewed showed these cells need further study. It may due to the methods or the apoptotic process itself. **[Paediatr Indones 2006;46:195-198]**.

Keywords: *apoptotic cells detection, peripheral blood, treatment response, childhood acute lymphoblastic leukemia*

Acute lymphoblastic leukemia (ALL) is the most common malignancy found in children in Indonesia as well as in the world.[1] Although it has a high cure rate (80-90%), if it is not treated, it can be fatal.[2] In developing countries the mortality rate is still high, due to problems in diagnosis and limited access to chemotherapy. The diagnosis of ALL is based on the presence of leukemic cells (lymphoblasts) in circulation and definitely by the finding of more than 25% lymphoblasts in the bone marrow. At diagnosis, it is estimated that a number of 10^{12} lymphoblasts develops and burdens the body, while at the end of the induction phase about 10^{10} leukemic cells still persist.[3] The higher the rate of cytoreduction, the more successful the regimen eradicates the malignancy, as has been proven by the response to one week monotherapy and by minimal residual disease studies.

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Many studies have shown a correlation of early response to treatment with the prognosis of childhood ALL.[4-12] Unfortunately, studies to measure residual disease or apoptotic cells as response to treatment in ALL need laboratory facilities such as flowcytometer, or PCR and those are not always available in leukemia treatment centers in Indonesia.[13-14] To solve this problem, we used a simpler, cheaper and reproducible method to detect leukemic cells that undergo apoptosis during the induction phase of treatment. The basic idea is that apoptotic leukemic cells have a specific morphology that could be viewed under light microscope.

Apoptosis or programmed cell death is a series of genetically controlled events which results in the removal of unwanted cells without tissue disruption. It can be activated by relevant stimuli and it is characterized by distinct morphological and biochemical features. Morphologically, apoptotic cells will present with nuclear chromatin condensation, budding of the plasma membrane and the production of membrane-bound apoptotic bodies. Since apoptosis is a way of cell death that can be induced by chemotherapy,[15] it is thought that the number of leukemic cells that undergo apoptosis may reflect drug sensitivity and its role in cytoreduction may correlate with prognosis. Previous studies have shown that apoptotic leukemic cells could be identified in the circulation during ALL treatment and this has a correlation with leukemic cell reduction.[16-17] The purpose of this study was to detect apoptotic cells in peripheral blood of children with ALL during the first week of treatment.

Methods

We conducted a cross sectional study on 58 children with ALL between 0-15 years old treated with Wijaya Kusuma ALL (WK-ALL) 2000 protocol in Department of Child Health, Sardjito Hospital, Yogyakarta from March 1999 to May 2001. The WK-ALL 2000 protocol was designed to meet the Indonesia situation, adopted from the experience of childhood ALL treatment in US and Europe consisted of 2 risk-based group i.e., standard-risk (SR) and high-risk (HR) patients. Patients whose WBC >50,000/ml, age <1 year or >10 years and showed involvement of any site in the body (mediastinal, testicle, central nervous system) at the time of diagnosis were classified into HR patients. The inclusion criteria were children with newly diagnosed ALL (L_1 or L_2), without prior treatment for ALL, agreed to participate in this study. Informed consent was obtained from the parents or guardians.

Specimens of peripheral blood were taken during the first week of the 6 weeks remission induction phase consisted of one dose of intrathecal methotrexate adjusted to the age, dexamethasone 6 mg/m^2/day in SR and HR patients plus 1 dose of daunorubicin for HR patients. At the first day of treatment, a blood smear was made at 3 hourly intervals and repeated every 24 hours for the second to seventh day. The smears were made from buffy-coat prepared by centrifugation of finger tip blood in heparinized TerumoÒ capillary tubes at 2,500 rpm. Slides were stained with May-Grunwald Giemsa (Merck). Apoptotic cells were identified as small cells with nuclear condensation or apoptotic body formation (**Figure 1**). The apoptotic cells were calculated as apoptotic index, i.e., the percentage of apoptotic cells among 200 mononuclear cells counted under light microscope.

Results

Apoptotic cells were identified in 3 patients at different time point of studies stated before (**Table 2**). The apoptotic cells were observed at a range of 4.2% to 36.2%. Two patients showed a sharp decrease of WBC in line with the decrease of lymphoblasts and increase of apoptotic cells percentage after 7 days treatment.

TABLE 1. SUBJECT CHARACTERISTICS

Characteristic	Number	%
Sex		
Male	35	60
Female	23	40
Age at diagnosis (years)		
0-1	1	2
1-10	52	90
10-15	5	9
FAB classification		
L1	55	95
L2	3	5
Risk-group		
High risk (HR)	27	47
Standard risk (SR)	31	53

TABLE 2. BLASTS CELLS NUMBER REDUCTION AND APOPTOTIC INDEX

No	Identity	FAB risk	d0-d7		Apoptotic index		BMP
			WBC (/ul)	Blast(%)			d0 (%)
1	Male, 2 yr	L1-SR	11,400–3,600	70-16	h9	= 8.4	99
2	Male, 14 yr, 4 mo	L1-HR	93,600-12,200	89-61	h24	= 4.2	89
					h120	= 5.4	
					h144	= 36.2	
3	Male, 4 yr, 9 mo	L1-SR	2,400–2,110	11-0	h3	= 11.8	100

FAB=French-American-British classification for acute leukemia; Risk=patients risk- grouping, SR=standard risk and HR=high risk; d0-d7=peripheral blood before and day 7 after treatment started, WBC=white blood cell number, Blast=blast number as percentage of WBC; Apoptotic index=percentage of apoptotic cells in 200 mononuclear cells counted, h9=9 hours after treatment; BMP d0=blast number in bone marrow before treatment started

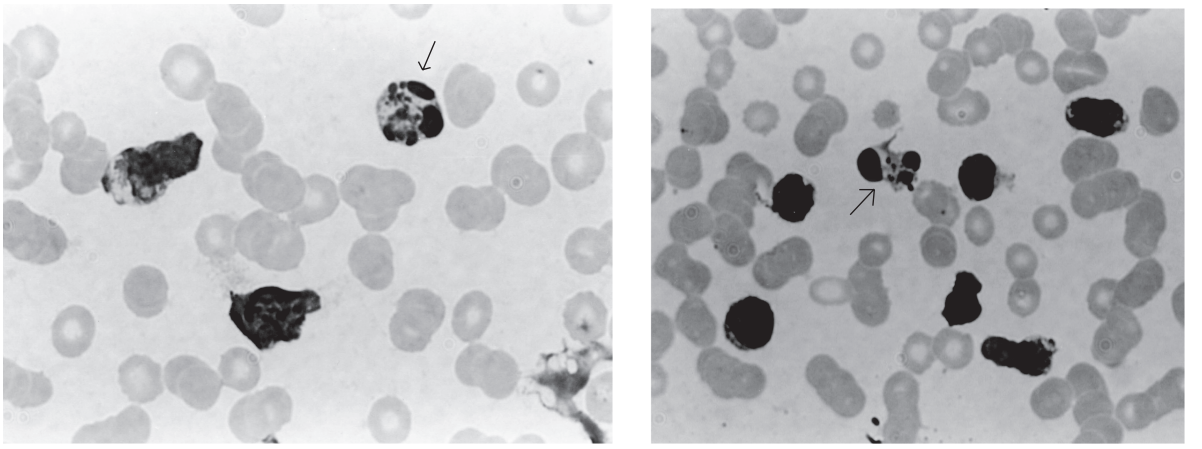

FIGURE 1. LEUKEMIC CELLS (LYMPHOBLAST) UNDERWENT APOPTOSIS AT 9 HOURS (A) AND 3 HOURS (B) AFTER TREATMENT STARTED IN PATIENT 1 AND 3, RESPECTIVELY.
NOTE THE SMALLER SIZE WITH NUCLEAR CONDENSATION AND APOPTOTIC BODY FORMATION.

Discussion

Until now only few studies had been performed to identify apoptosis during treatment in childhood ALL, especially using simple methods. Matsubara *et al*,[16] found sharp declines of WBC and blast populations during chemotherapy, but failed to identify apoptotic cells in the circulation using May Grunwald-Giemsa staining. Tsangaris *et al*,[17] used nuclear staining with ethidium bromide under fluorescent microscop and found apoptotic cells in the circulation in various percentages, 24 hours after treatment. In comparison to the methods used by Tsangaris *et al* (1996),[17] our method was simpler, using light microscop; May Grunwald-Giemsa staining was available easily in all hospitals, without fractionation process and no more reagents and facilities needed.

Our study shows that apoptotic cells could be identified morphologically in the circulation, with the buffy-coat as a better source of mononuclear cells or lymphoblasts than that of direct smear of a blood drop. During the first week of ALL treatment only sporadical apoptotic cells were recognizable in the peripheral blood, although the number of leukemic cells decreased sharply. The explanation of why we didn't find those cells in all smears or in the other 55 patients is still unclear. It may come from the situation where the apoptotic cells have been rapidly removed by phagocytes from the circulation or maybe our methods were not sensitive enough to detect small numbers of those cells in the circulation. Another possibility is there is certain machinery that 'prevent' apoptosis detection *in vivo*.

In conclusion, our results showed that apoptotic cells can be detected with simple method from circulation, especially on slides obtained from buffy coats. However, in most patients no apoptotic cells were seen, though the number of leukemic cells in the circulation decreased rapidly. Apparently, cell death through apoptosis is a process that takes place out-

side the circulation, or apoptotic cells are rapidly taken out from the circulation.

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