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Original Article

CD4⁺ T-cell, CD8⁺ T-cell, CD4⁺/CD8⁺ ratio, and apoptosis as a response to induction phase chemotherapy in pediatric acute lymphoblastic leukemia

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Abstract

Background Acute lymphoblastic leukemia (ALL) is a neoplastic disease resulting from somatic mutation in the lymphoid progenitor cells, often occurring in children aged 2-5 years, predominantly in males. Results from the induction phase of chemotherapy are used to measure success, but the failure remission rate is still high. Increased apoptosis of cancer cells, as induced by CD4⁺ and CD8⁺ T-cells, is an indicator of prognosis and response to chemotherapy.

Objective To assess for correlations between CD4⁺, CD8⁺, or CD4⁺/CD8⁺ ratio to the chemotherapy induction phase response (i.e., apoptosis) in pediatric ALL patients.

Methods This observational analytical cohort study was done in 25 pediatric ALL patients. Whole blood (3 mL) with EDTA anticoagulant were used to measure absolute counts of CD4⁺, CD8⁺, and CD4⁺/CD8⁺ ratio. Peripheral blood mononuclear cells (PBMC) were examined for apoptosis. The principle of CD4⁺, CD8⁺ examination was based between antigens on the surface of the leukocyte in the blood with fluorochrome labeled antibodies in the reagents, while the principle of apoptosis examination was FITC Annexin V will bond with phosphatidylserine that moves out of the cell when the cell undergoes apoptosis, then intercalation with propidium iodide (PI). All examination were detected by flow cytometry BD FACSCalibur.

Results Subjects were 25 newly-diagnosed, pediatric ALL patients (64% males and 36% females). Most subjects were 3 years of age (20%). Numbers of CD4⁺ and CD8⁺ cells, as well as CD4⁺/CD8⁺ ratio were significantly decreased after chemotherapy. However, apoptosis was not significantly different before and after chemotherapy (P=0.689). There were significant negative

correlations between apoptosis and CD4⁺ (P=0.002; rs=-0.584), and CD8⁺ (rs=-0.556; P=0.004), before chemotherapy. In addition, CD4⁺-delta and apoptosis-delta also had a significant positive correlation (rs=0.478; P=0.016). However, no correlation was found between the CD4⁺/CD8⁺ ratio and apoptosis, before or after chemotherapy.

Conclusion There are significantly lower mean CD4⁺, CD8⁺, and CD4⁺/CD8⁺ ratio after chemotherapy than before. Also, there are significant correlations between CD4⁺-delta and apoptosis-delta, as well as between apoptosis and CD4⁺, CD8⁺, and CD4⁺/CD8⁺ ratio, before chemotherapy. The CD4⁺, CD8⁺, and CD4⁺/CD8⁺ ratio can be used to predict apoptosis before chemotherapy. In addition, CD4⁺-delta can be used to predict apoptosis-delta as a response to induction phase chemotherapy in pediatric ALL. [Paediatr Indones. 2017;57:138-43 doi: <http://dx.doi.org/10.14238/pi57.3.2017.138-43>].

Keywords: ALL; CD4⁺; CD8⁺; apoptosis

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Acute lymphoblastic leukemia (ALL) is a neoplastic disease resulting from somatic mutation in the lymphoid progenitor cells in the bone marrow, often occurring in children aged 2-5 years, and predominantly in males. Diagnosis of ALL is based on finding $\geq 25\%$ lymphoblasts of blood smear or bone marrow aspiration (BMA) evaluation. Outcome criteria was made as follows, $< 5\%$ lymphoblasts was belonging to remission, and $> 5\%$ was belonging to non remission, including partial remission.¹⁻⁵

Failure of apoptosis and the immune response causes uncontrolled growth of cancer cells. The progression of ALL has been correlated with quantitative and qualitative (function) host immune responses. Cluster differentiation 8⁺ (CD8⁺) T-cell are an effector cell resulting an apoptosis tumor cells by cytotoxic or cytolytic effects. Anti-tumor mechanisms of CD4⁺ T-cells are not fully understood. The CD4⁺ T-cells have no cytotoxic or cytolytic effects. Many cytokines produced by CD4⁺ T-cells are needed for the development of CD8⁺ effector cells. Tumor necrosis factor (TNF) and interferon-gamma (IFN-gamma) secreted by activated CD4⁺ T cells will induce expression of major histocompatibility complex I (MHC I) and induce CD8⁺ T-cell activity to lyse tumor cells.⁶⁻¹⁰ The induction phase of chemotherapy is usually used as a measure of successful chemotherapy, but remission failure rates are still high.¹⁰ Increased apoptosis of cancer cells induced by CD4⁺ or CD8⁺ T-cells can be used as one indicator of prognosis and response to induction phase chemotherapy in pediatric ALL patients.⁹ The aim of this study was to assess for correlations between apoptosis and CD4⁺, CD8⁺, and CD4⁺/CD8⁺ ratio, before and after the induction phase of chemotherapy in pediatric ALL patients.

Methods

This observational, analytical, cohort study included 25 new cases of pediatric ALL patients who were diagnosed based on bone marrow aspiration at the Department of Pediatrics, Airlangga University Medical School/Dr. Soetomo Hospital, Surabaya, from July to December 2016. The inclusion criteria were new cases of pediatric ALL aged 1 month to 16 years

who had never received chemotherapy, and planned to undergo induction phase chemotherapy regularly for 6 weeks. The exclusion criteria were ALL patients with a history of HIV/AIDS or had infections as indicated by fever and other signs. Healthy patients control were obtained from healthy volunteers aged 1 month to 16 years who had investigated no history of certain diseases and seem good condition during the examination was done. This study was approved by the Ethics Committee of our institution and written informed consent was obtained from all patients' parents.

Clinical characteristics including age, sex, hemoglobin (Hb) level, platelet count, and leukocyte count were obtained at diagnosis (before chemotherapy) and after induction phase chemotherapy. Whole blood (3 mL) with EDTA anticoagulant were used to measure absolute counts of CD4⁺, CD8⁺, and CD4⁺/CD8⁺ ratio. Reagents used for CD4⁺, CD8⁺ examination was BD Multitest CD3FITC/CD8PE/CD45PerCP/CD4APC. The principle of CD4⁺, CD8⁺ examination was to detect bonds between antigens on the surface of the leukocyte present in the blood with fluorochrome labeled antibodies present in the reagents. Peripheral blood mononuclear cells (PBMC) were examined for apoptosis. The FITC Annexin V and propidium iodide (PI) were used for apoptosis examination. The principle of apoptosis examination was FITC Annexin V will bonds with phosphatidylserine that moves out of the cell when the cell undergoes apoptosis, then intercalation with propidium iodide (PI). The FITC Annexin V can identify early apoptosis, late apoptosis and necrosis based on nucleus changes. Propidium Iodide (PI) was used to distinguish late apoptosis and necrosis with early apoptosis. All examination were detected by flow cytometry BD FACSCalibur performed before and after induction phase chemotherapy, at the Department of Clinical Pathology, Airlangga University Medical School/Dr. Soetomo Hospital, Surabaya.

Comparison of Hb levels before and after induction phase chemotherapy was evaluated by paired T-test, while leukocyte counts, platelet counts, CD4⁺, CD8⁺ cells, CD4⁺/CD8⁺ ratio, and apoptosis before and after induction phase chemotherapy were evaluated by Wilcoxon Signed Rank test. Correlations of absolute count of CD4⁺, CD8⁺ cells, CD4⁺/CD8⁺ ratio with apoptosis were evaluated by Spearman's

correlation test. CD4⁺ -delta was defined as difference between number of CD4⁺ before and after induction phase chemotherapy, as well as CD8⁺ -delta, CD4⁺/CD8⁺ -delta, and apoptosis delta. Results with P values ≤ 0.05 were considered to be statistically significant.

Results

During the study period, 41 new cases of pediatric ALL patients fulfilled the inclusion criteria, but 16 patients dropped out, of whom 11 patients died and 5 patients did not complete the induction phase of chemotherapy. Hence, our cohort study of 25 new cases of pediatric ALL patients consisted of 16 males (64%) and 9 females (36%), with a 1.78:1 ratio (Table 1). Many of our subjects were 3 years of age (20%). Leucocyte count was significantly lower

(P=0.014) but platelet count was significantly higher (P=0.000), between before and after induction phase of chemotherapy. Mean numbers of CD4⁺ and CD8⁺ cells, as well as CD4⁺/CD8⁺ ratio were significantly decreased after induction phase chemotherapy. Mean numbers of CD4⁺ before induction phase chemotherapy was 3,060.24 (SD 4660.03), after induction phase chemotherapy was 887.64 (1531.33). Mean numbers of CD8⁺ before induction phase chemotherapy was 3,084.76 (SD 4535.51), after induction phase chemotherapy was 1,647.28 (SD 3644.99). Mean numbers of CD4⁺/CD8⁺ ratio before induction phase chemotherapy was 1.12 (SD 0.67), after induction phase chemotherapy was 0.65 (SD 0.61). However, apoptosis did not significantly decrease after chemotherapy (P=0.689), before induction phase chemotherapy was 18.19 (SD 19.82) and after induction phase chemotherapy was 14.09 (SD 10.85) (Table 2).

Table 1. Clinical characteristics of study subjects before and after induction phase chemotherapy

Characteristics	N=25	P value
Gender, n		
Male	16	
Female	9	
Age, n		
1-5 years	13	
6-10 years	7	
≥ 10 years	5	
Leucocyte count, x10 ³ µL		0.014*
Before		
Mean (SD)	52.68 (120.45)	
Median (range)	8.81 (1.20 – 585.00)	
After		
Mean (SD)	7.58 (10.78)	
Median (range)	5.12 (0.79 – 56.00)	
Hb concentration, g/dL		0.165
Before		
Mean (SD)	10.30 (3.13)	
Median (range)	9.48 (5.00 – 16.00)	
After		
Mean (SD)	11.35 (2.02)	
Median (range)	11.30 (7.60 – 15.40)	
Thrombocyte count, x10 ³ µL		0.000*
Before		
Mean (SD)	62.26 (54.17)	
Median (range)	42.6 (5.00 – 204.00)	
After		
Mean (SD)	190.33 (119.07)	
Median (range)	177.00 (18.80 – 447.00)	

*significant if P ≤ 0.05, Before=before induction phase chemotherapy, After=after induction phase chemotherapy

Table 2. Comparison between CD4⁺, CD8⁺ cells, CD4⁺/CD8⁺ ratio, and apoptosis, before and after induction phase chemotherapy in pediatric ALL patients

Variables	Time	Mean (SD)	Median (range)	P value
CD4 ⁺ , cells	Before	3,060.24 (4660.03)	1751.00 (210.0 – 23,016.0)	0.000*
	After	887.64 (1531.33)	199.00 (4.0 – 5,966.0)	
CD8 ⁺ , cells	Before	3,084.76 (4535.51)	1820.00 (341.0 – 18,541.0)	0.004*
	After	1,647.28 (3644.99)	423.00 (52.0 – 17,859.0)	
CD4 ⁺ /CD8 ⁺ ratio	Before	1.12 (0.67)	1.03 (0.33 – 2.91)	0.004*
	After	0.65 (0.61)	0.44 (0.06 – 2.31)	
Apoptosis	Before	18.19 (19.82)	10.83 (0.26 – 80.58)	0.689
	After	14.09 (10.85)	10.34 (2.86 – 49.17)	

*significant if P ≤ 0.05, Before=before induction phase chemotherapy, After=after induction phase chemotherapy

Table 3. Comparison of apoptosis between pediatric ALL patients and healthy children before and after induction phase chemotherapy

Variables	Apoptosis		P value
	Mean (SD)	Median (range)	
ALL patients (before chemotherapy)	18.19 (19.82)	10.83 (0.26-80.58)	0.683
Healthy control patients	16.21 (3.52)	17.73 (12.19-18.71)	
ALL patients (after chemotherapy)	14.09 (10.85)	10.34 (2.86-49.17)	0.316
Healthy control patients	16.21 (3.52)	17.73 (12.19-18.71)	

Table 4. Correlation between apoptosis and CD4⁺, CD8⁺ cells, and CD4⁺/CD8⁺ ratio before and after induction phase chemotherapy

Variables	Correlation with apoptosis before chemotherapy		Correlation with apoptosis after chemotherapy	
	r _s	P value	r _s	P value
CD4 ⁺ cells	- 0.584	0.002	0.081	0.701
CD8 ⁺ cells	- 0.556	0.004	- 0.105	0.619
CD4 ⁺ /CD8 ⁺ ratio	- 0.117	0.579	0.040	0.849

A comparison of apoptosis was done in ALL patients and healthy control subjects. We found no significant differences in apoptosis between the two groups either before or after chemotherapy (Table 3).

We also assessed for correlations between apoptosis, before and after chemotherapy, and CD4⁺, CD8⁺ absolute counts, and CD4⁺/CD8⁺ ratio. There were significant, negative correlations between apoptosis and CD4⁺, as well as apoptosis and CD8⁺, before chemotherapy. However, there was no significant correlation between CD4⁺/CD8⁺ ratio and apoptosis, before or after chemotherapy (Table 4).

There was a significant correlation between CD4⁺-delta and apoptosis-delta after induction phase chemotherapy. However, we observed no significant correlation between apoptosis-delta and CD8⁺-delta or CD4⁺/CD8⁺-delta (Table 5).

Discussion

Acute lymphoblastic leukemia is a hematological malignancy that often occurs in children, and comprises 25–30% of all pediatric malignancies. The highest incidence is in 2–5-year-olds, and predominantly in boys. Our cohort study of 25 new cases of pediatric ALL patients consisted of 16 (64%) males and 9 (36%) females, with 1.78 : 1 ratio. Many subjects were 3 years of age (20%) (Table 1).

There were significant differences in leukocyte (P=0.014) and thrombocyte (P=0.000) counts, before and after induction phase chemotherapy. Uncontrolled lymphocyte proliferation and defective apoptosis in ALL patients caused leukocytosis dominated by lymphoblasts. Infiltration of hematopoietic cells by leukemic cells accumulated in the bone marrow

causes anemia and thrombocytopenia.^{1,13} After chemotherapy, leukocyte count decreased, while Hb level and thrombocyte count increased. Evaluation of bone marrow aspiration after induction phase chemotherapy showed that all the patients were in remission, as determined by leukemic cell clearance from the bone marrow, mainly in the first 2 weeks after induction phase chemotherapy.¹³

Mean CD4⁺, CD8⁺ cells, and CD4⁺/CD8⁺ ratio were significantly decreased after induction phase chemotherapy (Table 2). Verma *et al.* reported a significant decrease in B cells, T cells, and NK cells approximately 2 weeks after chemotherapy.¹⁴ Decreased CD4⁺/CD8⁺ ratio after chemotherapy in this study (P=0.004) was due to the larger decrease in CD4⁺ cells than that in CD8⁺ cells. Recovery of CD4⁺ cells after chemotherapy was slower than recovery of CD8⁺ cells. Chemotherapy decreases the CD4⁺ and CD8⁺ cells by increasing regulatory T cells (T reg), which suppress the immune response by downregulating IL-2, and upregulating IL-10 and TGF-beta. Although IL-10 and TGF-beta are strong, immunosuppressive factors, IL-2 is an important immune regulating factor. It is produced by T helper cells, which increase T cell proliferation, NK cell activity, and B cell antibody secretion. Past studies have shown that IL-2 concentration in ALL patients is low.^{15,16}

After chemotherapy, apoptosis was not significantly decreased (P=0.689). In contrast, Laane *et al.* found an increase in apoptosis after chemotherapy.¹⁷ Firstly, our results may have been caused by: 1) sampling time outside the window of maximal effect on apoptosis time (24 - 72 hours). Liu *et al.* reported a significant increase in apoptosis of lymphoblasts > 24 hours after induction phase chemotherapy in pediatric ALL patients, rather than in the early hours after chemotherapy.¹⁸ Secondly, chemotherapy can cause necrosis, so lymphoblasts may not have been detected as apoptotic bodies.¹⁹ Third, the subjects may have had chemotherapy drug resistance, although 100% of patients entered remission,²⁰ and fourth, anti-apoptotic proteins levels (e.g., BCL-2, BCL-XL, etc.) in the patients were higher than pro-apoptotic proteins levels (e.g., BAX, BOK, BCL-Xs, BID, BAD, or Noxa).¹⁸ Lymphoblast cells in ALL patients are more fragile than in healthy children, thus, apoptosis in ALL patients was not significantly

higher than apoptosis in the healthy control group (Table 3).

There was a negative, significant correlation between apoptosis and CD4⁺ and CD8⁺ cells before chemotherapy (Table 4). This finding indicates that greater numbers of CD4⁺ and CD8⁺ cells result in decreased apoptosis. We noted that not only the number of CD4⁺ and CD8⁺ cells, but their function, was also important in immune response. The number and function of CD4⁺ and CD8⁺ cells before chemotherapy were correlated with subjects' response to chemotherapy and improved survival.¹⁰

Apoptosis had no significant correlation with CD4⁺/CD8⁺ ratio, either before or after chemotherapy. Similarly, Dewyer *et al.* suggested that CD4⁺/CD8⁺ ratio did not correlate with tumor response in induction phase chemotherapy.²¹ No correlation between CD4⁺ or CD8⁺ cells and apoptosis after induction phase chemotherapy, may have been due to decreases of CD4⁺, CD8⁺ cells, and apoptosis after chemotherapy.

There was a significant correlation between CD4⁺-delta (number of CD4⁺ cells before chemotherapy minus the number of CD4⁺ cells after chemotherapy) and apoptosis-delta (Table 5). We found that decreased CD4⁺ cells lead to decreased apoptosis. The CD4⁺ cells had no cytotoxic or cytolytic effect on tumor cells, but many cytokines produced by CD4⁺ cells are needed for the development of CD8⁺ into effector cells.⁶⁻¹⁰

The limitations of this study included the lack of an extended time to recognize the possibility of future relapse or resistance to chemotherapy drugs, apoptosis sample preparation was not accompanied by a specific marker for lymphocytes, so a series of monocytes may have been included, and the sampling time among patients after chemotherapy varied, potentially effecting the decreased apoptosis.

Comparing the values before and after induction phase of chemotherapy we conclude that: 1) CD4⁺ and CD8⁺ count cells not significantly higher, 2) there is no difference in apoptosis, 3) there was a negative correlation between apoptosis and CD4⁺ and CD8⁺ cells before induction phase chemotherapy in pediatric ALL patients, but no correlation after induction phase chemotherapy, 4) there is no correlation between CD4⁺/CD8⁺ ratio and apoptosis, 5) there is a significant correlation between CD4⁺-delta and

apoptosis-delta, after induction phase chemotherapy. CD4⁺ and CD8⁺ cells, and CD4⁺/CD8⁺ ratio can be used to predict apoptosis before chemotherapy, while CD4⁺-delta may be useful to predict apoptosis after induction phase response to chemotherapy in ALL patients.

Suggestions for future research also include determining the best time of sampling after chemotherapy (24 hours, 36 hours, 48 hours, or 72 hours) to obtain results with significantly increased apoptosis, compared to what our study showed, and to assess the profile of the percentage of T reg and expression of cytokines in ALL patients before and after chemotherapy.

Conflict of Interest

None declared.

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