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RESEARCH

COMPARISON OF PERIPHERAL BLOOD ACTIVATED NK CELL PERCENTAGE BEFORE AND AFTER INDUCTION PHASE CHEMOTHERAPY IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

(Perbandingan Persentase Sel NK Teraktivasi Darah Tepi Sebelum dan Sesudah Kemoterapi Tahap Induksi di Pasien Leukemia Limfoblastik Akut Anak)

Syntia TJ[1], Endang Retnowati[1], Yetti Hernaningsih[1], I Dewa Gede Ugrasena[2], Soeprapto Ma'at[1]

ABSTRAK

Leukemia Limfoblastik Akut (LLA) adalah keganasan sel progenitor limfoid yang berasal dari sumsum tulang dan ditandai proliferasi leukosit. Kejadian LLA masih tinggi, sehingga perlu diteliti peran sel NK dalam melawan leukemia. Tujuan penelitian adalah untuk mengetahui perbedaan persentase sel NK teraktivasi sebelum dan sesudah pengobatan induksi dan hubungan persentase sel NK teraktivasi sebelum pengobatan induksi dengan keluaran kemoterapi pasien LLA anak. Penelitian analitik observasional dengan rancang bangun cohort prospektif. Subjek penelitian 27 pasien di Ruang Rawat Inap Hemato-Onkologi Anak RSUD Dr. Soetomo Surabaya, antara bulan Maret–Juli 2016. Metode memeriksa flowcytometry menggunakan alat BD FACS Calibur™ reagen Fast Immune CD56FITC/CD69PE/CD45 Per CP No.katalog.5055879. Analisis statistik dengan uji Wilcoxon Signed Rank dan regresi logistik. Terdapat perbedaan bermakna rerata persentase sel NK teraktivasi sebelum pengobatan induksi 0,57% (SB 0,53%) dan sesudahnya 2,01% (SB 1,86%) p=0,000. Menunjukkan peningkatan bermakna sel NK teraktivasi sesudah pengobatan induksi. Kenasaban sel NK teraktivasi sebelum pengobatan induksi dengan keluaran kemoterapi berkurangnya gejala penyakit (remisi) dan meninggal R=0.723 berarti kenasabannya kuat. Peningkatan persentase sel NK teraktivasi sesudah pengobatan induksi disebabkan kerja kemoterapi meningkatkan hasil MICA/B dan kerja activating receptors sel NK (NKG2D) yang bersifat sitotoksik yang kuat. Persentase sel NK teraktivasi sebelum pengobatan induksi yang rendah disebabkan mekanisme menghilangnya tumor di LLA. Terdapat perbedaan bermakna persentase sel NK teraktivasi sebelum dan sesudah pengobatan induksi. Hasilnya dapat menjadi peramal keberhasilan pemberian kemoterapi LLA anak. Persentase sel NK teraktivasi sebelum kemoterapi tahap induksi yang tinggi berpengaruh kuat terhadap keluaran kemoterapi berkurangnya gejala penyakit dan sebaliknya bila rendah berpengaruh terhadap kemungkinan yang bersangkutan meninggal. Diperlukan hasil jangka panjang sampai selesai dalam pengelolaan pemberian pengobatan terkait.

Kata kunci: *LLA anak, sel NK teraktivasi, keluaran kemoterapi*

ABSTRACT

Acute Lymphoblastic Leukemia (ALL) is a malignancy of the lymphoid progenitor cells from the bone marrow, which characterized by leukocytes proliferation. The incidence of ALL is still high, thus the examination about the role of NK cell against leukemia cells is needed. The study is aim to know the difference of activated NK cell percentage before and after chemotherapy induction and the correlation between activated NK cell percentage before chemotherapy induction with the outcome of chemotherapy in ALL by analyzing. This research was an analytical observational study with cohort prospective design. The subjects consist of 27 patients who were treaded in the Hemato-Oncology Pediatrics Ward at Dr. Soetomo Hospital Surabaya in between March–July 2016. The examination was done by BD FACS Calibur™ with the reagen from Fast Immune CD56FITC/CD69PE/CD45 Per CP No.catalog.5055879 using a flow cytometry. The statistical analysis used Wilcoxon Signed Rank Tes and logistic regression. A significant improvement was shown, the mean of activated NK cell percentage before chemotherapy induction was 0.57% (SD 0.53%) and after chemotherapy induction was 2.01% (SD 1.86%) with p=0.000. A strong correlation was shown between the activated NK cells before chemotherapy induction with an outcome of chemotherapy if high persentage related with remission and low percentage related with died (R=0.723). Increased activated NK cell percentage after chemotherapy induction was due to the chemotherapy

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effect which increased MICA/B production and the role of NK cell activating receptors (NKG2D) having a high cytotoxicity. A significant difference of activated NK cell percentage before and after chemotherapy induction was shown. The results can be used as a predictor of successful chemotherapy and as a prognostic factor. Activated NK cell percentage before chemotherapy strongly influenced the outcome of chemotherapy, if high percentage related with remission and low percentage related with the death. So in this case a long-term observation until complete chemotherapy management is needed.

Key words: ALL, activated NK cell, outcome of chemotherapy

INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) is a malignancy of lymphoid progenitor cells derived from bone marrow leukocytes and characterized by the proliferation of leukocytes and the manifestation of abnormal cells in the peripheral blood.[1] The peak age of incidence is between 3–5 years. Acute lymphoblastic leukemia is more common in male than female.[2] The incidence of the disease in the United States based on The Surveillance, Epidemiology and End Result (SEER, 2006) Program of National Cancer Institute provides information on ALL statistics was ±3,930 new cases each year.[3] Acute Lymphoblastic Leukemia in Indonesia was ±2.5–4.0 per 100,000 children. The new cases of ALL in Indonesia was ±2000 to 2300 each year.[4] The new cases of ALL children at Dr. Soetomo Hospital Surabaya in 2006 were as many as 82 children; remission 33 patients (48.5%), non-remission 10 patients (14.7%) and 25 patients died (36.8%).[5] The high rate of resistance of drugs around 9.3 cases/106 children per year, remains a significant problem.[6] ALL patients who died is still high, one of which caused by the failure of the treatment. The differences response of chemotherapy influencies to patients outcomes.[6]

This study observed the immunity cellular response especially role of NK cell in ALL pediatric of this to analyze that there were correlated with the outcome of chemotherapy induction phase, because of this treatment was aimed to eradicatie about 99% leukemia cells and return to the normal hematopoiesis system. The chemotherapy induction phase was administered for six (6) weeks. The chemotherapy outcome was determined by a percentage of lymphoblasts in the bone marrow by microscopic morphological examination induction therapy regiment based on the BFM/COG (The Berlin-Frankfurt - Munster/a Children's Oncology Group) included vincristine, anthracycline, corticosteroid and L-asparaginase.[7]

The NK cell has an important component role as an effector mechanism against leukemia cells with spontaneuos cytotoxicity to all various types of cell targets in the eliminate on of cancer by inhibiting cell proliferation and angiogenesis, triggering apoptosis, stimulating adaptive immune system and increased processing as well as antigen presentation.[7] The role of NK cells in hematological malignancies was first published by Costelloetal,[8] that there was a significant increase of NK cells activated in patients with hematological malignancies after getting chemotherapy.[8] Following this, another study which made the NK cell as a candidate for a new strategy of immunotherapy. So another study is needed to compare the activated percentage of NK cells ($CD56^+CD69^+$) after chemotherapy induction phase associated with the patients outcome at this time has not been studied at Dr. Soetomo Hospital Surabaya.[9] This study will helps the clinicians overcome the problem in the immunity of patients and improve the treatment success rate of ALL patients. The aim of the study was to know the difference of activated NK cell percentage before and after chemotherapy induction and the correlation between activated NK cell percentage before chemotherapy induction with outcome of chemotherapy in ALL. The benefits of this study were to provide the examination of NK cells that could be used as a predictor of successful drug chemotherapy, that determining the prognosis of pediatric ALL and to provide the suggestion for the clinicians to develop of NK cells as an immunotherapy.

METHODS

This type of study was an analytical observational study with a cohort prospective design. The sampling was done in the Hemato-Oncology Pediatric Ward at Dr. Soetomo Hospital Surabaya and the processing of the samples were carried out in the Laboratory of Clinical Pathology, Dr. Soetomo Hospital Surabaya between March–July 2016. The calculation of the sample size was at least 15 people. The inclusion criteria were ALL patients aged between 1 month-8 years newly diagnosed and who received the same protocol treatment of chemotherapy and the parents/guardians agreed to participate in this study. The exclusion criteria was ALL patients who have been treated with steroids and cytostatics earlier. And the

drop out criteria were patients who withdrew from the study. The statistical analysis used Wilcoxon Signed Rank Tes to comparing the activated percentage NK cells and logistic regression to the related activated NK cells with chemotherapy outcomes.

RESULTS AND DISCUSSION

The study involved 30 ALL pediatric patients before the induction therapy and after the induction phase of remission obtained in 19 patients, three (3) patients were dropped out and eight (8) patients died. The total study subjects were 27 patients, mostly male 18 children (66.7%) and females 9 children (33.3%). The age of patients were about 1–14 years, the mean and standard deviation were of 6.59 years and 4.34 years (see Table 1).

The most incidence was ≤ 5 years of age and the ratio of males and females was 2 : 1, according to Widiaskara in 2005[5] males suffer more of ALL. In males and females can undergo chromosomal translocations in leukemia resulting in excessive de regulation of gene expression or form a new gene fusion, aneuploidy and specific gene mutations. A factor which affect the prognosis of males patients more worse than females, is that incidence of Philadelphia translocation is often found in males. Another factor is the difference metabolism of lymphoblast in the maintenance phase of chemotherapy regimens among male with female. This study showed that the largest age group of the first time was diagnosed as ALL was ≤ 5 years, as according Widiaskara study.[5] Age is a significant prognostic factor in ALL pediatric patients. In patients with aged ≤ 5 years or age between 2~5 years showed that the survival rate was two times greater than the comparation to the age ≤2 years or ≥10 years.

The results of hemoglobin levels, number of leukocytes and platelet counts in 27 patients on examination before and after induction phase can be seen in Table 2.

The mean of hemoglobin levels before induction therapy was 6.98 (g/dL) and the mean after that was 8.06 (g/dL). So that the average increased, but not significantly different (p=0.449). Presumably because leukemic cells accumulation in the bone marrow lead to disruption of blood cell formation and the effects of induction chemotherapy drug suppressing the growth of stem cells and induced apoptosis of young hematopoietic cells.

The mean number of leucocytes before induction therapy was 98,993 ($\times 10^6$/mL), while the average number of leucocytes after induction therapy was 4,116 ($\times 10^6$/mL), so that the average decreased significantly (p=0.000). The children with the number of leukocytes ≥50,000 ($\times 10^6$/mL) were at a high risk category for experienced non-remission, relapse and died. The children with high leukocytes early in the disease before induction therapy, have a high progressive proliferation of blast cells. Acute lymphoblastic leukemia ediatric patients with leukocyte count ≥50,000 ($\times 10^6$/mL) are major risk factor for Tumor Lysis Syndrome (TLS) and infection. Tumor lysis syndrome greatest risk was during the induction phase of chemotherapy for rapid tumor destruction and excessive at the same time.

The results mean of platelets after induction therapy was 134,037 ($\times 10^3$/mL), whereas the mean platelet count prior to induction therapy was 69,409 ($\times 10^3$/mL) so that the average had increased significantly (p=0.001). From the 27 ALL patients studied only 19 patients had complete data for the activated NK cells percentage before and after the induction phase (see Table 3).

Table 1. The characteristics of the subjects

The sample characteristics	Frequency	%
Gender		
Males	18	66.7%
Females	9	33.3%
Age (year)		
The average ± SD	6.59 ± 4.34	–
<5 year	14	51.9%
5–10 year	5	18.5%
>10 year	8	29.6%
Range (min - max)	1 s/d 14	–

Table 2. The profile of hemoglobin levels, number of leucocytes and platelets

Data	Before induction phase	After induction phase	
	Range The average ±SD	Range The average ±SD	p
Hemoglobin	4.20–10.20	0.0–14.40	0.449
(g/dL)	6.98±1.68	8.06±5.39	
Leucocytes	1850–595000	0–9900	0.000 *
($\times 10^6/\mu$L)	98 993±136569	4.116±3.171	
Platelets	30000–204000	0–230000	0.001
($\times 10^3/\mu$L)	69 409±40988	134 037±90506	

Tabel 3. The number of activated NK cells before and after induction therapy

Time of examination	N	Activated NK cells percentage (%)			
		$\bar{x}$	SD	Minimum	Maximum
Before induction therapy	27	0.45	0.48	0.04	2.13
After induction therapy	19	2.01	1.86	0.21	6.06

Table 4. The normality test of the activation of NK cells percentage

Data	Group	Value of Shapiro Wilks	p-value	Information
Activated NK cells percentage (%)	Pre induction	0.705	0.000	Not normally
	Post induction	0.766	0.000	Not normally

Table 5. The comparison of the activated NK cell percentage before and after chemotherapy induction

Time of examination	n	Activated NK cells percentage (%)				p
		$\bar{x}$	SD	Minimum	Maximum	
Before induction therapy	19	0.57	0.53	0.07	2.13	0.000 *
After induction therapy	19	2.01	1.86	0.21	6.06	

* Significant at $\alpha = 0.05$ (Wilcoxon Signed Rank Test)

Normality test of comparing to difference of the activated NK cell percentage before and after chemotherapy induction study by Shapiro Wilk, showed that the data obtained were not normally distributed (see Table 4).

The comparison analysis of the activated NK cells percentage before and after chemotherapy induction was done by using Wilcoxon Signed Rank Test, p=0.000. The significant differences of activated NK cell percentage before and after chemotherapy induction were shown (see Table 5).

The activated percentage NK cells after chemotherapy induction phase increased significantly compared to before induction therapy. The increased of activated NK cell ($CD56^+CD69^+$) percentage after chemotherapy induction phase can be used as a predictor of successful drug chemotherapy in ALL children. The theories of Burmester and Pezzutto[14] about the effect of chemotherapy on the activity of NK cells, showed that chemotherapy can kill cancer cells to become debris that act as cancer antigen and captured by APC. Cancer antigen will stimulate the activity of effector cells, including NK cells, macrophages, dendritic cells, TCD4 and TCD8 cytotoxic cells. Another mechanism of action of chemotherapy against NK cells was by increasing DNAM ligand-1 regulation and increasing employment activating receptors (NKG2D) which have a strong cytotoxicity.

The study carried out by Shi[16] suggested that the group of demethylating agents (azacitidine and decitabine) and members of the immune-modulatory drugs (thalidomide and lenalidomide) improved recognition of activating receptors (NKG2D and NKp46) against the ligands. The histone deacetylase inhibitors (trichostatin) group increased the production of MICA/B,ULBP-2 is ligand of activating receptors (NKG2D). The group of histamine dihydrochloride All-trans retinoic acid monoclonal antibody (Rituximab) increased the affinity Fc receptor (FcgRIII) through ADCC process against cancer cells during recognition causing degranulation by releasing perforin protein cytolytic and granzyme B, so the target cell would undergo apoptosis and necrosis. Bortezomib (proteasome inhibitor) caused the degradation of proteins and increased expression of NKG2D. Dounorubicin and doxorubicin increased the production of MICA/B, ULPBI-3, PVR, Nectin-2 and increased expression of DNAM-IL Group Tyrosine kinase inhibitor (Imatinib) also served to increase activating the expression of NKG2Dreceptors.

On examination of 27 patients obtained results with chemotherapy outcomes as follows:

Table 6. Distribution of chemotherapy outcome

Outcome	Frequency	%
Remission	19	70.4
Died	8	29.6

The result of chemotherapy outcome were 19 patients (70.4%) in remission and 8 patients (29.6%) died.

The significant difference of the activated NK cell percentage before chemotherapy induction related with the remission outcome compared to those who died was shown ($p<0.05$).

The regression logistic analysis showed that activated NK cells percentage before induction therapy strongly influenced the chemotherapy outcome ($p=0.027$). The magnitude of correlation between activated NK cells percentage before chemotherapy induction phase with chemotherapy outcomes was remission and deat obtained from regression coefficient (R)=√ 0523=0723, which means a strong correlation category. Patients with the activated NK cells percentage before chemotherapy induction phase which has a high chances of remission and patients with the activated NK cells percentage before chemotherapy induction phase has a low result. So the chemotherapy outcome was death, these results could be used as prognostic determinant in ALL pediatric patients.

There was a correlation between the activated NK cells percentage were higher on before chemotherapy induction phase has the chance of remission, can give the clinician's suggestion on the development of NK cells as an immunotherapy. According to Burke *et al.*[9] strategy to increase NK cell activity against leukemia cells by increasing endogenous activation of NK cells and how adoptive transfer. Increased endogenous activation of NK cells by way of an adjuvant such as TLR ligand aims to redistribute endogenous cells NK cells from the related population in the blood (CD56-CD16+) are strong cytotoxins to lymph nodes that populations of NK cells are cytotoxins weak (CD56+CD16-). The provision of chemotherapy also served to increase the activation of endogenous NK cells as mephalan, doxorubicin, orbortezomib to improve ligands regulation of NKG2D on leukemia.

In this study, there were eight (8) patients ALL who died (29.6%) possible due to higher risk infection and decrease the immune system. This study showed that patients with activated NK cell percentage were low prior to induction therapy greatly affected the outcome of chemotherapy died ($r=0.723$). In the research of Baier *et al.*,[15] explained that the mechanism of tumor occurs ALL escape. This mechanism affects the decrease in production of MHC class 1 chain-related protein A/B (MICA/B), thereby weakening the signal in triggering caspase so DNA target cells did not undergo apoptosis. Other tumor escape mechanism

Table 7. The comparison result of activated NK cell percentage before chemotherapy induction related with chemotherapy outcome remission and died

Outcome	n	Activated NK cells percentage (%)				p
		$\bar{x}$	SD	Minimum	Maximum	
Remission	19	0.57	0.53	0.07	2.13	0.002 *
Died	8	0.16	0.08	0.04	0.25	

* Significant at $\alpha = 0.05$ (Mann-Whitney Test)

Table 8. The result of analysis regression logistic about influencing the activated of percentage NK cells before induction therapy related chemotherapy outcome

Variable	B	P	R2
NK cells pre induction	-13.11	0.027	*0.523
Constant	2.43	0.069	

* Significant at $\alpha = 0.05$

is a mutation of the gene Major Histocompatibility Complex class 1 (MHC class 1) thereby disrupting the regulation of MHC class 1 result able to recognize inhibitory NK cell receptors that become active and target cells are not killed. In addition ALL remove the tumor derived soluble factor (TDSF) which contribute to triggering the formation of an immunosuppressive compound is VGEF, IL-10, TGF-β and prostaglandin E. TDSF significantly inhibit cytotoxicity NK cells.

Other factors are the resistance to chemotherapy is a phenomenon when tumor cells become insensitive to chemotherapy drugs. The mechanism of chemotherapy resistance in general to include cancer; efflux pump chemotherapy drugs due to overexpression of trans-membrane transporter protein (P-gp); the enzyme polymorphism pemetabolisme drugs such as enzyme thymidylate synthase polymorphisms and metilentetrahidrofolat reductase; defects in the apoptotic pathway because overexpression of anti-apoptotic proteins (Bcl-2) and a defect in the pro-survival pathway through the activation of NF-Kb.7 specific mechanisms in ALL include the existence of a set of gene expression associated with; resistance to a single chemotherapeutic agent; cross-resistance and minimal residual disease (MRD). The results of examination of the percentage of activated NK cells (CD56 + CD69 +) in healthy people were as follows:

The activated NK cells percentage (CD56+CD69+) in 6 healthy individuals ranged from 0.12 to 0.67% with an average of 0.42% and a standard deviation of 0.16%. The results of the examination activated NK cells percentage (CD56+CD69+) is low this indicated the absence of stimulation of NK cell activation in healthy people. The purpose of examination activated NK cells percentage in healthy people to determine the range normal value of NK cells. In accordance to the study carried out by Johanis[19], which shown that the normal range of activated NK cells obtained by examining 10 healthy individuals ranges between 0.95–1.67%, with the average of 0.50% and a standard deviation of 0.49%.

CONCLUSIONS AND SUGGESTIONS

The study conclusions based on the results and discussion are as follows: There are significant differences in the activated NK cells percentage before and after chemotherapy induction phase. These results can be used as a predictor of successful drug chemotherapy in ALL pediatric patients; The activated NK cells percentage before chemotherapy induction phase has astrongly influenced to chemotherapy outcome. Patients with the activated NK cells percentage before chemotherapy induction phase

Table 9. The activated NK cells percentage in normal patients

No	Name	Age	The activated NK cells percentage (%)
1.	An.Ag	12	0.47
2.	An.F	11	0.12
3.	An.Al	13	0.37
4.	An.J	11	0.51
5.	An.S	12	0.38
6.	An.T	13	0.67
	Mean		0.42
	SD		0.16

which has a high result, so the chemotherapy outcome was remission and patients with the activated NK cells percentage before chemotherapy induction phase has a low result so the chemotherapy outcome was death.

Those results could be used as prognosis determinant in ALL pediatric patients. It is suggested a long-term studies should be done to complete the management of chemotherapy and necessary to be done studies about activated NK cells in vitro by culture Peripheral Blood Mononuclear Cell (PBMC) for better results.

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