Difference Expressions CD34+ in Acute Myeloid Leukemia Cell Culture in the Administration of Cytarabine-Daunorubicine Dose Standards

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ABSTRACT

The cure rate for patients with Acute Myeloid Leukemia (AML) is 20-75%. Standard-dose cytarabine (SDAC)-daunorubicine gives a remission rate of ± 60%, and the case of relapse is frequently found. In-vivo CD34+ expression is a reliable and straightforward test that must evaluate AML patients' response to predict the response of chemotherapy induction phase accurately. Differences in in-vitro CD34+ expression are expected to be able to predict chemosensitivity in AML patients. An experimental post-test-only control group study was conducted from May to December 2019, and 8 AML subjects were found. Peripheral Blood Mononuclear Cells (PBMC) were isolated from peripheral blood samples of patients with AML collected in EDTA tubes. The PBMC isolated from peripheral blood were divided into two groups, and each group contained 106 PBMC cells in culture media. The control group (without treatment) and the SDAC-daunorubicine group were incubated for 4 hours at 37^0C with a 5% $CO2$ atmosphere. The expression of CD34+ was measured using FACSCalibur™, while CD34+ percentage was calculated with CellQuest™ software. The percentage of CD34+ in the control, SDAC + DNR, showed a significant difference with $p < 0.001$. This study showed a significant difference between the control group and the group administered with the standard dose of cytarabine-daunorubicine with $p < 0.001$. The average CD34+ expression in the SDAC-DNR treatment group was higher than in the control group. CD34+ markers cannot be used as predictors of chemosensitivity in the administration of chemotherapy.

Keywords: Acute myeloid leukemia, CD34+, cytarabine-daunorubicine, flow cytometry

INTRODUCTION

Acute Myeloid Leukemia (AML) is the most common malignancy of the bone marrow. It is characterized by a proliferation of immature leukemic cells or blasts, which fail to differentiate, leading to eventual bone marrow failure if left untreated. While the median of five-year survival is 25%, there is significant variability in the disease subtype. It can range from 15% to 10% for patients with poor-risk AML to 90% for patients with Acute Promyelocytic Leukemia (APL).[1] According to Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN), in 2012, the mortality of AML is estimated at 23.865-26.5461/year worldwide. GLOBOCAN forecasts that there will be 351,965 incidences of acute leukemia worldwide until 2012. The mortality rate is 265.461 worldwide with an Average Standardized Rate (ASR) of 3.4 per 100.000.[2] The number of AML patients based on immunophenotyping and examinations from June 2012 to June 2017 at Dr. Soetomo Hospital, Surabaya

was 238 patients. According to hospital statistical data of the Hospital Information System (SIRS) in 2007, patients with leukemia (7.42%) rank fifth after breast cancer (16.85%), cervical cancer (11.78%), liver and bile duct cancer (9.69%). Research at Sardjito General Hospital from January 2000 to December 2009 showed that the percentage of Acute Lymphoid Leukemia (ALL) was 40.6%, while Acute Myeloid Leukemia (AML) was 13.9%.[3] Use of daunorubicin (DNR) and cytarabine (Ara-C) induction chemotherapy in the treatment of adult patients with AML (but not APL) are considered suitable for intensive chemotherapy.[4] Standard induction chemotherapy based on the cytarabine (Ara-C) and anthracyclines in 60-80% younger adult patients is performed to achieve Complete Remission (CR).[5] This study aimed to analyze differences in the percentage of in-vitro CD34+ expression measured with the flow cytometry method on AML cells after administration of cytarabine (SDAC)-daunorubicine at standard dose. The difference in the percentage of in-vitro CD34+

expression was expected to predict chemosensitivity in AML patients in Dr. Soetomo Hospital, Surabaya.

METHODS

Eight newly diagnosed patients with AMLs (1 AML-M1, 4 AML-M2, and 3 AML-M4) based on the French American-British classification were enrolled. The study was conducted from May to December 2019 using a post-test-only control group design. Peripheral blood samples were obtained before specific antileukemic therapy and after informed consent by the patient as a part of diagnostic procedures. Two hematologists independently reviewed slides at the Department of Internal Medicine, Division of Hematology and Oncology of Dr. Soetomo Hospital. Blasts were isolated on the mononuclear layer by density gradient centrifugation in Ficoll-Hypaque (Pharmacia, Sweden) and cultured in RPMI 1640 (Gibco, BRL) containing 20% fetal calf serum (Gibco, BRL) for 24 hours at 37^0C with 5% $CO2$ atmosphere.[6] It consisted of 15 mg/mL bovine serum albumin, 7.8 mg/mL cholesterol, 1 mg/mL insulin, 2-mercaptoethanol, 2 mL Glutamine, 100 U/mL Penicillin and 100 mg/mL Streptomycin in iscove modified dulbecco medium.[6] This step was performed after 24 hours followed by 4 hours of incubation using cytarabine (Cytosine β-D-arabinofuranoside; Sigma-Aldrich, USA) dissolved in RPMI. The required concentration was 2 μM cytarabine equivalent to a routine dosing regimen with an Ara-C plasma concentration of 0.5-1 g/m2. Daunorubicine (Tokyo Chemical Industry, Japan) 0.4 μM according to the protocol in previous studies.[7-9] For each sample, 1x106 cells were acquired, and cells were gated for live blasts for all event areas. Data were then analyzed using Cellquest™ software (Becton Dickinson, USA). A statistic the paired T-test was used to analyze the difference of results among samples. Also, the Shapiro-Wilk test was used to determine the data normality.

The Ethics Committee approved this study of Dr. Soetomo Hospital, Surabaya, with ethical clearance number 1152/KEPK/V/2019.

RESULTS AND DISCUSSION

The research subjects were recruited from May to December 2019. The research subjects agreed to participate in the study and signed informed consent. The study subjects comprised eight patients with one patient AML-M1, four patients with AML-M2, and three patients with AML-M4.

Data normality test using the Shapiro-Wilk test showed that all CD34+ data were normally distributed ($p > 0.05$). Therefore, the difference of CD34+ expression between treatments was analyzed using analysis of variants with subjects. The study of variants with subjects showed significant differences in CD34+ expressions between treatments ($p < 0.05$) (Table 2). The Least Significance Different (LSD) test results showed that CD34+ SDAC + DNR was different from the control (Table 3).

Table 2. The difference of CD34+ expressions using the analysis of variance of the same subject

CD34+ (%)	N	Mean±SD (CD34+)	p-value
Control	8	56.68±17,673	
SDAC + DNR	8	92.06±5,373	< 0.001

Note: SDAC: Standart Dose Cytarabine (%), DNR: Daunorubicine (%)

Table 3. The LSD test results of CD34+ expressions between groups

Difference in CD34+	N	Difference Mean±SD	p-value
SDAC+DNR with control	8	35.38±14,320	< 0.001

Note: SDAC: Standart Dose Cytarabine (%), DNR: Daunorubicine (%)

This study showed a younger age range of AML patients of 39.8 4,015. However, a study O'Donnel *et al.* in the United States showed that AML patients ranged in age from 65 to 74 years. It was shown that there was a tendency of an increased number of patients with AML aged <60 years.[10] A research in Indonesia by Rahmadin *et al.* in M. Djamil Hospital, Padang in 2017 retrospectively suggested that AML

Table 1. Hemoglobin, leukocyte, and thrombocyte of patients

Parameter	n	Mean±SD	Median (min-max)
Hb (g/dL)	8	6.75±2.28	6.3 (3.6–9.7)
WBC (10^3/μL)	8	35.84±31.13	26.64 (12.08–108,37)
PLT (10^3/μL)	8	41.75±31.972	34 (12–97)
Blast peripheral blood smear (%)	8	34.25±8.729	30 (22–43)

Note: Hb: Hemoglobin (g/dL), WBC: White Blood Cell (103/μL), PLT: Platelet (103/μL), SD: Standard Deviation

patients' prevalence with an age range of 20-59 years reached 85.71% in 35 research subjects.[11] The most common types of AML subtypes in this study were 50% AML-M2, followed by 37.5% AML-M4 and 12.5% AML-M1. In respect of the distribution of subtypes, AML-M2 (25%) was the most subtype of AML-M1 types, followed by AML-M4 subtypes (20%).[12] This study found significant differences between the control group and the group treated with SDAC-daunorubicine with p < 0.001. This study showed that the average administration of SDAC-daunorubicine in cell culture led to a higher percentage of cells with CD34^{+} than the percentage of cells with CD34^{+} in the control group (Figure 1). In-vivo studies of regiments showed a complete remission rate of 60-80% in younger patients and 45-60% in older patients (aged ≥ 60 years).[4] Cytarabine-daunorubicine was associated with a higher response rate than conventional 7+3 chemotherapy with cytarabine plus daunorubicin.[13] A study by Costello *et al.* on CD34^{+}/CD38 leukemia progenitor cell population showed a lower expression of Fas/Fas-L and Fas, which induced apoptotic function to the in vitro population of CD34^{+}/CD38^{+}cells.[14] However, this study only evaluated CD34^{+} expression as a marker of chemosensitivity. The difference in results between in-vitro and in-vivo studies was because there were changes in cell morphology in this study. It enabled the detection of cytoplasmic fragments containing CD34^{+} as events with CD34^{+}.
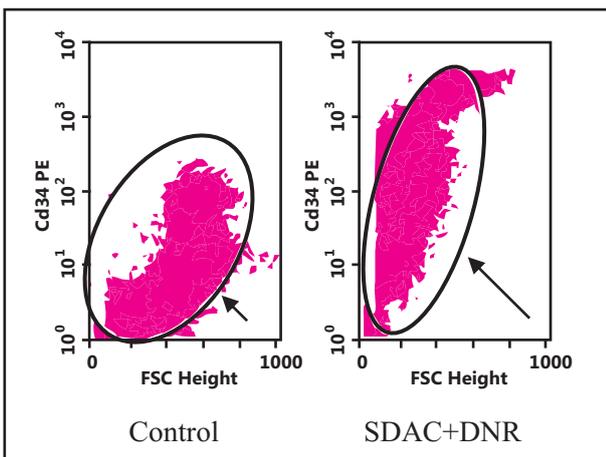
CD34^{+} was shifted to the left, indicating morphological changes of cell fractions or cells, which was read as an event, suggesting that the CD34^{+} expression could not be used as a marker. Chemotherapy can cause changes in cell morphology. Membrane damage and compaction of the nucleus caused cytoplasmic fragments containing CD34^{+} detected as an event, giving rise to a false high of CD34^{+} expression. The increase in the apoptosis process causes changes in cell morphology, including condensation of the nucleus, changes in the cytoplasm, and the appearance of blebs on the membrane.[15]

CONCLUSION AND SUGGESTION

In-vitro research cannot describe the provision of therapy in-vivo. Based on this study's results, the administration of in-vitro therapy regimens does not always match the results in-vivo study. Further research was needed to assess chemotherapy's effect on leukemia cells using different markers as a predictor of chemosensitivity in the provision of in-vitro therapeutic chemotherapy.

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Figure 1. FSC scatter pattern between the control group and the treatment group

The FSC scatter on flow cytometry in SDAC + DNR treatment was previously compared with the control group. It was found that the cell population with

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