Lymphokine activated killer cells from peripheral blood mononuclear cells of endometriosis of patients improve cytotoxicity to endometriosis cell culture

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Abstrak

Latar belakang: *Untuk menilai peningkatan imunitas selular terhadap biakan sel endometriosis dari sel LAK hasil perangsangan Sel Mononuklir Darah Tepi (SMDT) penderita endometriosis dengan IL-2.*

Metode: *Studi ini merupakan penelitian kuasi-eksperimental pra dan pasca-perlakuan dengan menggunakan pembanding (kontrol). Dilakukan pemeriksaan fenotip CD3+ CD4+, CD3+ CD8+ dan CD56+ sel efektor dari SMDT endometriosis dan kontrol. Dilakukan pula uji sitotoksisititas SMDT penderita endometriosis dan kontrol terhadap lini-sel Daudi, K562, dan biakan sel endometriosis dengan menggunakan 51 Chromium pada teknik teraradioimun (radioimmunoassay, RIA)*

Hasil: *Pada pemeriksaan fenotip SMDT dari 10 pasien endometriosis dan 6 pasien kontrol pada sebelum dan sesudah perangsangan dengan IL-2 ditemukan bahwa sebelum perangsangan dengan IL-2 ditemukan CD3+CD4+, CD56+ pada kelompok endometriosis lebih rendah daripada kelompok kontrol (p>0,05); fenotip CD3+ CD8+ pada kelompok endometriosis lebih tinggi daripada kelompok kontrol. Setelah perangsangan dengan IL-2 ditemukan CD3+CD8+, CD56+ dari SMDT kelompok endometriosis lebih tinggi daripada sebelum perangsangan dengan IL-2 dan ditemukan perbedaan yang bermakna (p < 0,05). Pada uji sitotoksisitas ditemukan peningkatan bermakna (p < 0,05) sitotoksisitas sel efektor baik pada SMDT endometriosis maupun SMDT kontrol terhadap sasaran (target) lini-sel Daudi dan K562 setelah perangsangan IL-2. Sitotoksisitas sel efektor baik pada SMDT endometriosis maupun SMDT kontrol terhadap sasaran biakan sel endometriosis setelah perangsangan IL-2 tampak meningkat*

Kesimpulan: *Sel LAK hasil perangsangan SDMT penderita endometriosis dengan IL-2 meningkatkan imunitas selular terhadap biakan sel endometriosis. (**Med J Indones 2011; 20:87-93**)*

Abstract

Background: To assess the increased cellular immunity of Peripheral Blood Mononuclear Cells (PBMC) derived LAK cells from endometriosis patients towards endometriosis cell cultures after stimulation with IL-2.

Methods: This study is a quasi-experimental study of pre and post treatment using controls. Phenotype evaluation of CD3+CD4+, CD3+CD8+ and CD56+ effector cells of PBMC from endometriosis patients and controls was performed. Cytotoxicity test of PBMC from endometriosis patients and control towards Daudi, K562 cell line and endometriosis cell cultures using 51Chromium release assay was also carried out.

Results: Phenotype evaluation of PBMC from endometriosis patients (n=10) and controls (n=6) were done prior to and after IL-2 stimulation. Before IL-2 stimulation, CD3+CD4+, CD56+ from endometriosis group (n=10) tend to be lower than control (n=6) whereas CD3+CD8 + were higher in endometriosis group than controls. After IL-2 stimulation, CD3+ CD8+, CD56+ of PBMC from endometriosis group were significantly increased (p <0.05).Cytotoxicity test revealed a significant increase (p <0.05) in both PBMC's effector cells from endometriosis and control group towards target cells, Daudi, and K562 cell lines after IL-2 stimulation. PBMC's effector cells cytotoxicity from both endometriosis and control towards target endometriosis cell cultures were also elevated after IL-2 stimulation.

Conclusion: LAK cells derived IL-2 stimulated PBMC from endometriosis patients increased cellular immunity towards endometriosis cell cultures. (**Med J Indones 2011; 20:87-93**)

Key words: *Daudi cell line, endometriosis, endometriosis cell cultures, K562 cell line, LAK cells, NK cells*

Endometriosis is an endometrial-like benign disorder which grows outside the uterine cavity, and commonly triggers chronic inflammatory reaction.[1-3] Chronic pelvic pain, infertility, and menstrual disorders are among the most common symptoms related to endometriosis. Endometriosis associated internal genitalia adhesion is often ended up with hysterectomy. Recurrence rate after surgery and hormonal treatment is 33.3 to 40.3%. Furthermore, endometriosis could disrupt women's productivity and is also related with a high cost treatment.[2, 3]

Endometriosis is estimated to affect 10 to 15% women in reproductive age. It is estimated that about 5-21% of patients with endometriosis are hospitalized due to pelvic pain.[2] In 2005, around 170 million or 10% of total world population of women in reproductive age were associated with endometriosis while in

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Indonesia, an estimation of 6.1 million women were with this disorder.[4,5] Since first described by Austrian Karl Freiherr von Rokitansky (1860), various theories have been proposed to explain the pathogenesis of endometriosis, including involvement of immune system.[4,6-8] Immune related theory in endometriosis is probably most attractive when by connected with other theories thus might be potential to develop a strategy in endometriosis therapy.

The presence of immunological defects in endometriosis, especially impairment of natural killer (NK) cell function and activity has been widely described.[9] Decreased activity of NK cells is a qualitative defect thus not caused by lack of NK cell numbers.[10] Decrease in NK cell activity resulted in disruption of endometrial clearance including those in the reversed flow into the peritoneal cavity.[11, 12] Decreased activity of NK cells in peripheral blood mononuclear cells (PBMC) has also been described in patients with endometriosis.[13,14]

NK cells cytotoxicity can be enhanced after stimulation of interleukin (IL)-2 into lymphokine activated killer (LAK) cells. LAK cells are leukocytes stimulated tumor killer cells which are associated with non restricted major histocompatibility complex (MHC) antigens.[15] The cytotoxic capacity of LAK cells can be determined by using Daudi, K562 cell lines, and endometrial cell culture. Destruction of K562 cell line would only be caused by NK cells while Daudi cell line is a target of cytotoxic T and LAK cells can destruct both of two cell lines.[9,16]

Oosterlynck, et al[17] found an elevation of PBMC's cytotoxic activity after stimulation with IL-2 for three days on endometrial cultured and uncultured cells, taken from the uterus of endometriosis patients controls and non endometriosis controls. To our knowledge, there is no endometriosis study using IL-2 stimulated LAK cells from PBMC's endometriosis patients with IL-2 against endometriosis cell cultures, this study is different from study done by Oosterlynck, et.al. who used target cells endometrial carcinoma (AN3CA and RL95) and endometrial cells.[16, 18]

METHODS

This study is a pre and post-treatment quasi experiment versus controls, assessing the role of LAK cells from IL-2 stimulated PBMC of endometriosis patients on endometriosis cell cultures. Samples were collected from PBMC of endometriosi and non endometriosis patients (controls).

Samples for this study were taken from dr. Cipto Mangunkusumo General Hospital Jakarta, and Fatmawati Hospital Jakarta, from November 2009 to June 2010.

Samples were processed and assessed in Makmal Integrated Immunoendocrinology Faculty of Medicine, University of Indonesia (MTIE-FMUI) and the INSERM U976 Saint-Louis Hospital, Paris VII, Paris, France. Approval for the study was obtained from the Institutional Review Board of the Faculty of Medicine, University of Indonesia.

Subjects and samples

The subjects of this study were women undergoing laparoscopy for endometriosis treatment (n=10) and control (n=6) in the Division of Reproductive Immunoendocrinology, Department of Obstetrics and Gynecology, dr. Cipto Mangunkusumo Hospital Jakarta, and Fatmawati Hospital Jakarta. None of the patients received any hormonal treatment during the 3 months prior to the intervention. Endometriosis scoring was determined according to the revised American Fertility Society Classification. Endometriosis was diagnosed as class III (n = 2) and class IV (n = 8). The diagnosis was further confirmed by histopathology examination. Endometriosis cells were obtained from endometriosis cysts taken from each patient after laparoscopy.

Venous blood samples were taken from healthy volunteers (n=6) and endometriosis patients (n=10) for evaluation of NK cells, cytotoxic T cells, PBMC and LAK cells. Blood samples were collected into heparinized tubes and delivered to the laboratory. All subjects signed a consent form approved by the FMUI. PBMC were isolated by Ficoll-Hypaque gradient centrifugation. PBMC were then recovered, washed in phosphate buffered saline (PBS), counted, and assessed for viability in a trypan blue dye (0.2% (v/v) in PBS), and promptly used for flow cytometry and Chromium Release Assay (CRA).

Cell targets

K562, a human chronic myelogenous leukemia cell line, was cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium, CM) at 37°C, 5% CO_2. Daudi, a space human lymphoma cell line, and primary endometriosis culture were cultured in culture medium and used as targets for NK cell, cytotoxic T cell, PBMC and LAK cell assays. Target cells were harvested on day4, washed in PBS and counted in a trypan blue dye prior to examination. K562 and Daudi cell lines were kindly provided by INSERM.

LAK cell generation

PBMC were cultured in the complete medium (CM) described above. The CM was additionally supplemented with 100 IU/mL of recombinant human IL-2 (kindly

provided by INSERM). Cells were cultured in triplicate in a 24 wells plate at 37°C, 5% CO_2 for 4 days. On day 4, LAK cells were harvested, counted, and evaluated for cytotoxic activity towards Daudi, K562 cell lines and endometriosis cells.

^{51}Cr-release assay

K-562, Daudi cell lines and endometriosis cells were labeled with 100 μCi of ^{51}Cr mCi/mg (Perkin Elmer, Billerica, MA 400-1200Ci) for 1h at 37°C and used as target cells. The labeled cells were washed twice, resuspended in complete medium, and counted for the viable cells. Cells were co-incubated at a ratio 1:20; 1:5 between target to effector (T:E) cells. NK-cell, T-cell, PBMC and LAK-cell assays were done with $5x10^3$ labeled target cells per well in triplicate using 96-wells V-bottom microtiter plates.

The final volume of culture medium with 10% FCS was 200 μl in each well. After 4 h of incubation, 100 μl of medium was removed from each well in a γ counter to determine ^{51}Cr release. Radioactivity was measured by Trilux Micro Beta Automatic Gamma Counter. This procedur was performed at INSERM U976 Saint-Louis Hospital, Paris VII, Paris, France.

The percentage of cytotoxic activity was calculated using the formula:
% specific lysis = (sample cpm - spontaneous cpm)/ (maximal cpm-spontaneous cpm) × 100%.

Immunoflurescence Assay

In parallel to measurement of cytotoxicity, phenotypic analyses were performed with Ficoll/hypaque peripheral blood leukocyte gated on lymphocytes. For immunofluorescence experiment, cells were simultaneously incubated for 30 min with a PE-conjugated mAB, PCy5-conjugated mAB and FITC-labeled BY55 mAB. Indirect immunofluorescence assay was perfomed using a PE-conjugated goat anti mouse IgM from Beckman Coulter. Analysis was which performed on a FACScan microfluorometer (Beckman Coulter FC50) after compensation among PE, PCy5 and FITC. This

procedur was performed at INSERM U976 Saint-Louis Hospital, Paris VII, Paris, France.

Data analysis

Data was recorded on a special form and analyzed statistically using SPSS 12.0. Descriptive data was displayed to see the phenotype characteristics of CD3+CD4+, CD3+CD8+, CD56 and cytotoxicity of NK cells, cytotoxic T cells, and PBMC's LAK cells from endometriosis patients and controls towards Daudi, K562 cell lines and endometriosis cell cultures. Normality test was performed using Kolmogorov-Smirnov, univariate analysis and followed by bivariate analysis. Normally distributed data was tested using unpaired t-test. Pre-and post-treatment data was analyzed using a parametric test (pair test). On the contrary, abnormal data pattern was analyzed using Mann-Whitney test on unpaired groups and Wilcoxon test for pre-and post-treatment pairs.

RESULTS

In this study, samples consisted of PBMC from endometriosis patients (n=10; 2 moderate and 8 severe stage), and control (n=6). Before stimulation with IL-2 on PBMC from endometriosis and controls, CD3+CD4+ (helper T cells), CD56+ (NK cells) phenotype in endometriosis group was lower than the control group although there was no significant difference. Furthermore, the CD3+CD8+ (cytotoxic T cells) of endometriosis group was higher than the control group without any significant difference. It appears that the ratio of CD4+(helper T cells) to CD8+ (cytotoxic T cells) in the endometriosis group is less than one (Table 1).

Cytotoxicity test before IL-2 stimulation revealed that cytotoxic T cells in endometriosis group were lower than the ineffective control group. Moreover, the NK cells in endometriosis group were significantly lower than those in control group. The cytotoxicity of PBMC from endometriosis group was also lower compared to the control group. It appears that even though the toxicity was lower than control, PBMC from the endometriosis group could react effectively towards endometrial cell cultures before IL-2 stimulation (Table 2).

Table 1. Characteristics of controlled PBMC and endometriosis phenotype

Phenotype	Endometriosis			Controlled			p
	n		Median (min-max)	n		Median (min-max)	
CD3+	10	74,4	(43,50-92,01)	6	72,9	(70,56-84,00)	0,871
CD3+CD4+	10	27,8	(22,59-58,81)	6	42,8	(29,55-58,82)	0,104
CD3+CD8+	10	31,1	(14,50-46,14)	6	26,4	(20,25-41,09)	0,745
CD56+	10	11,7	(4,50-35,44)	6	12,4	(7,78-29,43)	0,588

Mann Whitney test; phenotype unit (%); CD3+ (T cells); CD3+CD4+ (helper T cells); CD3+CD8+ (cytotoxic T cells); CD56+ (NK cell); significant if p<0,05

The phenotypes of CD3$^+$CD4$^+$, CD3$^+$CD8$^+$and CD56$^+$ from both endometriosis and control group were increased after stimulation with IL-2. Elevation of CD3$^+$CD4$^+$(helper T cells) and CD3$^+$CD8$^+$ (T cytotoxic cells) after stimulation with IL-2 were significantly higher (p = 0.005 and p =0.007, respectively) than before treatment. It has also been found that the ratio of CD4$^+$ (helper T cells) to CD8$^+$ (T cytotoxic cells) in PBMC from endometriosis group was greater than 1after stimulation with IL-2 (Table 3, Figure 1, Figure 2, and Figure 3).

The cytotoxicity of LAK cells from endometriosis group towards Daudi, K562cell lines, and endometriosis cell cultures was increased after IL-2 stimulation. It seems that cytotoxic T cells from endometriosis group could response effectively towards Daudi cell line after IL-2 stimulation by activation into LAK cells. Likewise, cytotoxic T cells in control group could also react towards Daudi cell line after IL-2 stimulation with higher efficiency than the endometriosis group. Furthermore, NK cells in endometriosis group could respond more potently towards K562 cell line after IL-2 stimulation. However, its toxicity was lower than control group.

Table 2. Cytotoxicity test with 51Cr 1:20 and 51Cr 1:5 calibration on PBMC in endometriosis and control groups before to stimulation with IL-2 on Daudi, K562 cell lines and endometriosis cell cultures

Target cell	^{51}Cr	Endometriosis		Control		p
		n	Median (min-max)	n	Median (min-max)	
Daudi cell line	1:20	10	2.4 (0.90-3.90)	5	6.0 (1.80-9.30)	0.125
	1:5	10	1.7 (0.10-3.50)	5	2.5 (0.60-5.60)	0.297
K562 cell line	1:20	10	3.4 (0.50-8.20)	5	24.1 (1.30-38.20)	0.050*
	1:5	10	2.9 (0.90-7.50)	5	9.7 (0.80-18.30)	0.066
Endometriosis cells	1:20	6	11.8 (9.20-28.00)	4	21.7 (13.50-28,30)	0.136
	1:5	6	9.1 (0.00-22.50)	4	17.4 (12.10-22.00)	0.286

Mann Whitney test; Cytotoxicity test in %; cytotoxiticity value is effective if more than 10%, and ineffective if less than 10%); significant if p<0,05; PBMC = peripheral blood mononuclear cell
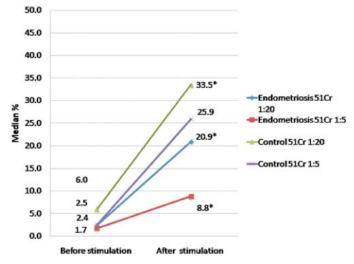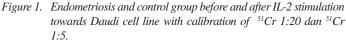
Table 3. PBMC phenotype characteristic groups before and after stimulation with IL-2

n	Phenotype	Before stimulation with IL-2		After stimulation with IL-2		p
		Median (min-max)		Median (min-max)		
Control group:						
6	CD3$^+$	72.9	(70.56-84)	73.1	(63.62-88.61)	0,753
6	CD3$^+$CD4$^+$	42.8	(29.55-58,82)	62.6	(33.4-70.26)	0.028*
6	CD3$^+$CD8$^+$	26.4	(20.25-41.09)	27.8	(13.77-40.82)	0.917
6	CD56$^+$	12.4	(7.78-29.43)	15.1	(4.88-33.48)	0,173
Endometriosis group:						
10	CD3$^+$	74.4	(43.50-92.01)	81.4	(70.78-96.63)	0.007*
10	CD3$^+$CD4$^+$	27.8	(22.59-58.81)	48,5	(29.14-65.78)	0.005*
10	CD3$^+$CD8$^+$	31.1	(14.50-46.14)	39,3	(27.28-59.63)	0.007*
10	CD56$^+$	11.7	(4.50-35.44)	15,4	(7.04-42.20)	0.007*

Wilcoxon test; phenotype unit (%); CD3$^+$ (T cells); CD3$^+$CD4$^+$ (helper T cells); CD3$^+$CD8$^+$ (cytotoxic T cells); CD56$^+$ (NK cells); significant if p<0,05

Table 4. Cytotoxicity test with 51Cr 1:20 and 51Cr 1:5 calibration in PBMC endometriosis group before and after stimulation with IL-2 against Daudi line-cells, K562 line-cells, and cultured endometriosis cell

Target cell	^{51}Cr	Before stimulation with IL-2		After stimulation with IL-2		p
		n	Median (min-max)	n	Median (min-max)	
Daudi line-cell	1:20	10	2.4 (0.90-3.90)	10	20.9 (5.60-33.50)	0.005*
	1:5	10	1.7 (0.10-3.50)	9	8.8 (2.60-24.10)	0.011*
K562 line-cell	1:20	10	3.4 (0.50-8.20)	10	25.3 (3.60-38.60)	0.005*
	1:5	10	2.9 (0.90-7.50)	10	12.1 (1.80-28.50)	0,005*
Endometriosis cell	1:20	6	11.8 (9.20-28.00)	6	16.7 (9.60-28.70)	0.345
	1:5	6	9.1 (0.00-22.50)	6	13.1 (1.91-18.82)	0.917

Wilcoxon test; Toxicity value in %; Cytotoxicity value if effective if more than 10%, and ineffective if less than 10%); Significant if p<0,05; PBMC = peripheral blood mononuclear cell

Figure 1.   Endometriosis and control group before and after IL-2 stimulation towards Daudi cell line with calibration of ^{51}Cr 1:20 dan ^{51}Cr 1:5.
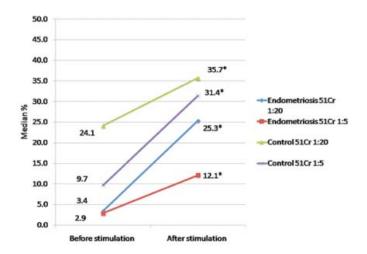

Figure 2. PBMC cytotoxicity and endometriosis LAK cells group and the control before and after stimulation with IL-2 towards K562 cells-line with calibration of 51Cr 1:20 and 51Cr 1:5.
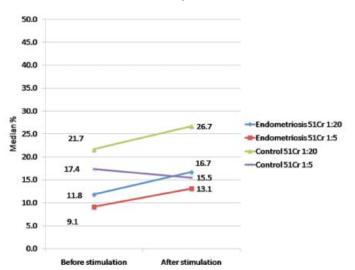

Figure 3. Cytotoxicity of PBMC and LAK cells in endometriosis group before and after IL-2 stimulation towards endometriosis cell cultures labeled with calibration of ^{51}Cr 1:20 and ^{51}Cr 1:5.

DISCUSSION

Endometriosis is an endometrial-like benign disorder which grows outside the uterine cavity, and could trigger chronic inflammatory reaction.[1-4] Theories have been proposed to explain the pathogenesis of endometriosis. Immune related theory was one of the most attractive theories which could interrelate with other theories. Thus, immune factors may play an important role to prevent and overcome endometriosis regardless its pathogenesis. Endometriosis associated immunological defect, particularly in NK cells function and activity, has been widely reported. In this study we demonstrate a defect in PBMC's NK cells from endometriosis group before IL-2 stimulation towards K562 cell line. Impairment of NK cells function from endometriosis group was also supported by the fact that cytotoxicity of LAK cells after IL-2 stimulation was lower than control group.

Impairment of NK cells function could be repaired by stimulating PBMC from endometriosis patients with IL-2 to activate them into LAK cells. Study by Roussel[19] showed an increased proliferation of CD3+ after IL-2 stimulation related to the formation of LAK cells. Furthermore, Oosterlynck et al[17] did not find any significant difference between the endometriosis (n=13) and control group (n=5) without IL-2 stimulation.

Difference in immune response is also affected by genetic factors. Hsieh et al[20] reported an association of homozygous polymorphism of IL-2 receptor beta (IL-2Rbeta)-627*C with endometriosis. Moreover, Kitawaki et al[21] demonstrated that the genotype of killer cells immunoglobulin-like receptors (KIRs) is correlated with susceptibility towards endometriosis. Our study found a significant increase of the phenotype CD3+CD4+ (helper T cells), CD56+ (NK cells) after IL-2 stimulation in PBMC of endometriosis group. There was also elevation in LAK cell cytotoxicity from endometriosis group towards Daudi, K562 cell lines and endometriosis cell cultures after IL-2 stimulation. From this study, we assume that IL-2 can stimulate activation of PBMC into LAK cells which are more potent in cytotoxicity and not associated with MHC antigens.[15]

In this study, the increase of LAK cells cytotoxicity from endometriosis group is lower than control. Szyllo et al[22] reported that IL-2 concentration in PBMC from endometriosis patients was higher (8.3±7.13) than control (2.8 ±1.28) after stimulation with phytohemagglutinin (PHA). Oosterlynck et al[17]stated that there is no intrinsic abnormality found in lymphocyte cells of endometriosis patients since they are still capable to respond after IL-2 stimulation. Tsudo et al[23] described an important role of p75 peptide as receptor of IL-2 on large granular lymphocytes in the activation of these cells into LAK cells. Disruption

in affinity between p75 and IL-2 would cause a decrease in proliferation response after IL-2 stimulation in patients with leukemia. Therefore, the role of p75 in pathogenesis of endometriosis should be further elaborated.

In conclusion, defect of cellular immunity in endometriosis could be repaired by stimulating PBMC from endometriosis patients with IL-2 into LAK cells. Activation of PBMC into LAK cells after IL-2 stimulation in endometriosis group resulted in increased cellular immunity towards endometriosis cell cultures.

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