子宮內膜異位症病人腹水中之自然殺手細胞功能與其細胞表面的抑制性接受體之關係

The relationship between natural killer cell cytotoxicity and killer inhibitory receptor (KIR) in peritoneal fluid of women with endometriosis

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一、中文摘要

子宮內膜異位症是生育年齡婦女最常見的疾病之一，不但會引起骨盆腔疼痛，也是不孕症最常見的原因。子宮內膜異位的原因目前仍不清楚。不過骨盆腔內的自然殺手細胞變性被認為是主要原因。目前我們找出44**位病人，其中**11**位對照組婦女，**11**位輕度子宮內膜異位病人，**22**位重度子宮內膜異位病人，腹水經由腹腔鏡手術取得。藉針對不同白血球表現型的單株抗體和三色流體細胞儀，分析這類病人周邊血液和骨盆腔中白血球的次族群，了解自然殺手細胞抑制性接受體的表現情形，最後再利用**chromium^{51}**釋出細胞毒殺試驗，來測試這些白血球的細胞毒殺功能。結果得知，不論輕度或重度，子宮內膜異位症的腹水中，自然殺手細胞之毒殺能力均比對照組降低，其中自然殺手細胞上的**NKB1 and EB6**兩種自然殺手細胞抑制性接受體均呈現異常升高現象。尤其**NKB1**這種接受體，在嚴重期的子宮內膜異位症比輕微的病人更升高。以上的結果顯示，自然殺手細胞之毒殺能力下降，有可能為其細胞表面之抑制性接受體過度活躍所致，可能為子宮內膜異位症的致病原因之一。**
關鍵詞：細胞毒殺試驗、子宮內膜異位症、自然殺手細胞抑制性接受體、腹水

二、英文摘要

Malfunction of peritoneal natural killer cells (NK) may result in endometriosis. The present study was designed to determine whether the decrease in NK cytotoxicity is at early and advanced stages of endometriosis and is due to the increase in the NK inhibition receptors. A total of 44 women (controls, N=11; women with early stage endometriosis, N=11; and women with advanced stage, N=22) were included in this study. NK cytotoxicity was determined by assay of ^{51}Cr release against K562 cells and the expression of killer cell inhibitory receptors (KIR, including NKB1, GL183, and EB6) in NK cells was examined by flow cytometry. Women with endometriosis showed a decrease in peritoneal NK cytotoxicities against K562 at early and advanced stages of endometriosis. The expression of KIR (NKB1 and EB6) was significantly elevated in the peritoneal NK cells of women with advanced stage endometriosis compared with controls. KIR (NKB1) was also significantly increased in peritoneal NK cells of women with advanced stage endometriosis compared with those of women with early stage endometriosis. The results of this study suggest that the decrease in peritoneal NK cytotoxicities against K562 is observed and this disease may be partially due to the increased expression of KIR on these NK cells.
Key words: cytotoxicity, endometriosis, killer cell inhibitory receptors (KIR), peritoneal fluid

三、計畫緣由與目的

Oosterlynck *et al.'s* finding of

defective natural killer (NK) cell activity in patients with endometriosis has led to the suggestion that impaired clearance of ectopic endometrial tissue by NK cells may contribute to the development of the disease. Our previous study also demonstrated that NK cytotoxicity is impaired in the peritoneal fluid (PF) of women with advanced endometriosis and that the recovery of peritoneal NK activity could be achieved by treatment with gonadotropin releasing hormone analogue. We have also shown that decreased interferon-gamma levels and inactivation of $CD4^+$ T lymphocytes can explain the impaired NK cytotoxicity in endometriosis. However, whether this association is indeed a cause or an effect of endometriosis remains controversial.

NK cells can express killer cell inhibitory receptors (KIR) that recognize major histocompatibility (MHC) class I antigens on the target and signal inhibition of cytotoxicity. They may engage their targets using a variety of receptors, including CD2, CD16, CD69, or lectin-like receptors related to those which may inhibit NK cytotoxicity. In addition, NK cells have also been found to express a particular KIR in individuals who do not possess the relevant class I ligand, indicating that these receptors may be capable of allogeneic recognition. Our previous studies suggested that NK cytotoxicity in endometriosis could be affected by either cytokines or T cells. It is also possible that endometriotic tissue itself could affect KIR of NK cells by an unknown mechanism to impair the NK cytotoxicity.

Our previous study demonstrated that the percentage of NK cells in the PF and their activation markers such as CD69, CD25, and HLA-DR were similar in women with and without endometriosis in spite of the decrease in NK cytotoxicities noted in the PF of women with advanced stage of endometriosis. It is of interest to determine whether peritoneal NK cells also express KIR and their relationship with the decreased NK cytotoxicity and the severity of endometriosis. In this study, we investigated whether there is any difference in the level of KIR expression of NK cells from peripheral blood (PB) and PF between women with and without endometriosis.

Peritoneal fluid was obtained from women who were either undergoing laparoscopy for endometriosis or for tubal ligation at the Division of Reproductive Medicine, Department of Obstetrics and Gynecology, National Taiwan University Hospital were enrolled in the study. They have problem of dysmenorrhea associated with infertility or not. The main purpose of the controls coming to our hospital was for tubal ligations due to multiparity. 33 women with endometriosis, including early (stage I and stage II, N=11) and advanced (stage III and stage IV, N=22) stages comprised the study subjects. 11 fertile women without evidence of endometriosis or inflammation, who underwent laparoscopic tubal ligations served as the control group. All PF was aspirated into sterile heparinized tubes at room temperature from the Douglas pouch immediately after insertion of the trocar to minimize the risk of contamination with blood. The PF specimens were transferred immediately to the laboratory and processed within 30 minutes, including centrifugation of the PF (400 *g*, 10 minutes). The pellet of PF was resuspended in RPMI-1640 medium, and peritoneal fluid mononuclear cells (PFMCs) were isolated by layering over Ficoll-Hypaque and centrifuged at 800 *g* for 20 minutes. The isolated PFMCs were washed twice with RPMI medium to remove residual Ficoll-Hypaque solution. Six mL of peripheral venous blood was drawn into a heparin-rinsed tube and separated by a similar procedure. Peripheral blood mononuclear cells (PBMCs) were washed three times with RPMI medium using the same procedures of isolation and purification used for PFMCs.

Erythroleukemic (target) cells (K562) were labeled with 100 μCi ^{51}Cr per 10^6 cells/mL in 5% CO_2 and at 37°C for 4 hours.

The cells were then washed three times with RPMI-1640 medium at 4°C and resuspended to a concentration of $5x10^4$/mL in a culture medium (RPMI+10% fetal calf serum). Effector cells (100 μL of mononuclear cell suspension from peripheral blood or peritoneal fluid, $2x10^6$/mL) were plated in triplicate in U-bottom 96-well plates to give a range of effector-target (E:T) cell ratios of 40:1, 20:1, 10:1, and 5:1 for PBMC. Minimum isotope release was determined by spontaneous release in media, whereas maximum release was determined by lysis of target cells with 100 μL 1% Triton X100. After incubation for 4 hours at 37 °C, the supernatant was removed and counted in a gamma counter. The percentage of specific chromium release was calculated according to the formula:

NK cell lysis (%) = (test - spontaneous counts) / (maximum - spontaneous counts) x 100%

The lytic unit was calculated by computing the percent specific lysis for all measured E:T ratios (at least 4 different ratios) and to fit a curve to the measured points on the graph.

The following monoclonal antibodies (mAb) were used for immunophenotypic analysis: FITC-anti-CD45/PE-anti-CD14, control γ_1 FITC/γ_{2a} PE, FITC-anti-CD3 /PE-anti-CD19, FITC-anti-CD3 /PE-anti -CD56, FITC-anti-CD3 /PE-anti-NKB1/ PerCP-anti-CD56, FITC-anti-CD3/PE-anti -GL183/PerCP-anti-CD56, FITC-anti-CD3 /PE-anti-EB6/PerCP-anti-CD56. Tri-color flow cytometry was performed with an argon-ion laser at 15 mW and 488 nm excitation. Triggering was set on the forward scatter channel and the threshold was adjusted to exclude debris.

Leucogate was used to measure the proportion of lymphocytes in the sample being studied without any scatter gates. The Simultest control (mouse IgG1 FITC+IgG2a PE) was used for background control. The gate was set around the lymphocytes ($CD45^+CD14^-$) to exclude other cells from analysis, and 10,000 lymphocytes were evaluated. For three color analyses, the region of positive CD56-PerCP staining was selected, and then the instrument was set to read CD3-FITC cells from forward scatter and KIR-PE (including NKB1, GL183, EB6; Table 1) from side scatter as previously described. The density of surface markers was expressed as the mean fluorescence intensity (MFI) of cells stained with specific monoclonal antibodies of KIR.

All results were expressed as mean ± SD. The Kruskal-Wallis test was used to test the differences of various levels of KIR and NK cytotoxicities among the three groups. The between-group differences were compared using the Mann-Whitney *U*-test. Results were considered statistically significant if P was less than 0.05. Pearson correlation test was used to detect the presence of a correlation between KIR and cytotoxicities of NK cells.

四、結果與討論

Peritoneal NK cytotoxicities against K562 were significantly reduced in endometriosis samples compared with controls (LU_{20} 8.0±4.0, 4.6±3.6 for controls and patients with advanced endometriosis, P=0.011). For early stage endometriosis, reduced PF NK cytotoxicities against K562 were noted when compared to controls (LU_{20} 4.7±2.7 vs. 8.0±4.0, P=0.028). These results extended our previous results to show that the NK cytotoxicities against K562 were impaired in the early and advanced stages of endometriosis. Comparison in PB NK cells, no significant difference was noted among the three groups.

The mean fluorescence intensity (MFI) of KIR on PB NK cells did not show any significant difference. The expression of KIR on PF NK cells from women with early stage endometriosis elevated but this elevation did not reach statistical significance compared with that of controls. However, on PF NK cells, the MFI of NKB1 from women with advanced endometriosis was higher than that of controls and patients with early stage endometriosis (advanced

endometriosis 183±56 vs. controls 97±45, P<0.001; advanced vs. early endometriosis 131±92, P=0.007) (Table). The MFI of EB6 on NK cells was also significantly increased in patients with advanced stage of endometriosis compared with controls (82±45 vs. 53±14, P=0.021).

Table. MFI of KIR expression in PB and PF NK cells of women with endometriosis and controls.

	Control	Endometriosis	
MFI of KIR	(N=11)	early (n=11)	advanced (n=22)
PB NK			
EB6	107±60	93±35	108±62
GL183	234±70	284±105	261±135
NKB1	197±91	295±141	281±159
PF NK			
EB6	53±14[a]	62±27	82±45[a]
GL183	246±80	319±88	345±223
NKB1	97±45[b]	131±92[c]	183±56[bc]

Data as mean±SD, [a]P=0.021, [b]P<0.001, [c]P=0.007 respectively by Mann-Whitney *U* test.

Endometriosis may be the result of insufficient disposal of retrograde tissue by NK cells. NK cytotoxicity is usually defined by the ability to lyse the K562. There is some evidence that reduced NK cytotoxicity is specifically against endometrial cells, especially autologous endometrial cell antigens. In our previous study, we investigated some activation markers on NK cells including CD69, CD25, HLA-DR, and found no difference between women with and without endometriosis. However, the NK cytotoxicities in PFMC were reduced in patients with advanced endometriosis. This led us to examine whether other systems such as inhibitory receptors might inhibit the cytotoxicities of NK cells. NK cells can use a number of different receptors (NK receptors, such as CD2, CD69) to identify their targets, and activate KIRs to produce signal inhibition of their cytotoxicity.

In this study, increased KIR (NKB1 and EB6) expression was found on PF NK cells in women with advanced stage endometriosis. This increased expression may be due to the regurgitated endometrial cells in the pelvic cavity. Larger amounts of retrograde menstruation will induce more KIR activation leading to increased impairment of NK cytotoxicity in the peritoneal cavity. In this study, the severity of endometriosis was correlated with NK cytotoxicities. We also noted a more significant increase of KIRs (NKB1 and EB6) on PF NK cells in women with advanced endometriosis. However, we failed to demonstrate a correlation between the expression of any single particular KIR examined and NK cytotoxicities in the present study. Perhaps, the inhibitory effect involves the cumulative interaction of several KIRs with activation markers of NK cells.

五、計畫結果自評

We accomplish this study on the basis of the plan that we presented previously. This study did not clarify whether the decrease in cytotoxicity and KIR expression on peritoneal NK cells is the cause or the result of endometriosis. Further study to examine the NK cytotoxicities against K562 transfected with class I HLA antigens of women with or without endometriosis is needed to understand the role of peritoneal NK cells in the pathophysiology of endometriosis.

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