Decreased expression of killer cell inhibitory receptors on natural killer cells in eutopic endometrium in women with adenomyosis

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BACKGROUND: Increased expression of killer cell inhibitory receptors (KIRs) has been found on natural killer (NK) cells in peritoneal fluid in women with endometriosis. In this study, we tried to measure the expression of KIRs on NK and T cells in women with adenomyosis, in an attempt to find the possible role of KIRs in the development of adenomyosis. METHODS: A total of 10 women with adenomyosis (study group) and 12 women with uterine myoma (control group) were included in this study. The expression of KIRs, including NKB1, GL183, EB6 and CD94, on NK and T cells in myometrium and endometrium was examined by flow cytometry. RESULTS: There was a decreased expression of NKB1 and GL183 on NK cells in the endometrium, but not in the myometrium, in women with adenomyosis. However, the expression of KIRs on T cells, either CD4⁺ or CD8⁺, was not different in either myometrium or endometrium between women with and without adenomyosis. CONCLUSIONS: The expression of KIRs on NK cells was decreased in eutopic endometrium in women with adenomyosis. It may be a compensatory effect in which the NK cytotoxicity is activated in order to eradicate the abnormal endometrial cells that might exit of the eutopic site of the endometrium.

Key words: adenomyosis/endometrium/killer cell inhibitory receptor

Introduction

Natural killer (NK) cells are known to kill virally infected or tumour cells while sparing normal self cells (Trinchieri, 1989; Moretta et al., 1994). This ability was found to depend on the interaction between killer cell inhibitory receptors (KIRs) expressed on NK cells and major histocompatibility complex (MHC) molecules expressed on normal cells, which leads to the inhibition of NK cell function (Gumperz et al., 1996; Lanier and Phillips, 1996; Rouas-Freiss et al., 1997). On the contrary, failure to express MHC molecules may render tumour or virus-infected cells susceptible to NK-mediated lysis (Ljunggren and Karre, 1990; Moretta et al., 1992, 1996).

The decreased NK cell activity in peripheral blood and peritoneal fluid of women with endometriosis has been well established in recent years (Oosterlynck et al., 1991; Ho et al., 1995). It is thought to promote implantation of the endometrium as a tissue graft (Lefkowitz et al., 1988), and its cause is probably due to overexpression of KIRs. Our previous study demonstrated that increased expression of NKB1 and EB6 was found on NK cells in peritoneal fluid in women with advanced stage endometriosis (Wu et al., 2000). Another study showed a similar result, where the proportion of KIR2DL1⁺ NK cells was increased in peritoneal fluid and peripheral blood in women with endometriosis (Maeda et al., 2002). Moreover, the endometriotic tissue could also affect NK cells by an unknown mechanism to impair the NK cytotoxicity. Our previous studies demonstrated that NK cytotoxicity in endometriosis could be affected by either cytokines or T cells (Ho et al., 1996a, 1997). The KIRs expressed on T cells might also play a role in the regulation of NK cytotoxicity.

In contrast to endometriosis being characterized by ectopic endometrium in the peritoneal cavity, adenomyosis is defined as the presence of endometrial glands within the myometrium. The only difference between adenomyosis and endometriosis is the site of ectopic endometriotic tissues, i.e. within or outside of the uterus. In this study, we tried to measure different kinds of KIR expression on NK and T cells in different parts of the uterus, as well as different expression of KIRs between women with and without adenomyosis, in an attempt to find the possible role of KIRs in the development of adenomyosis.
**Materials and methods**

**Subjects and specimens**

This study consisted of 10 women who suffered from adenomyosis (study group) and 12 women in whom uterine myoma was found (control group). These women underwent hysterectomy, via either the abdominal or vaginal route, at our hospital due to intolerable symptoms. All the participating women were of pre-menopausal status, and they were free from recent infection and obvious clinical immunological diseases, such as systemic lupus erythematosus, rheumatoid arthritis and Hashimoto thyroiditis. The diagnosis of adenomyosis was made by histopathological examination without exception, and the study protocol was approved by the institutional review boards at our hospital.

Peripheral venous blood, myometrium and endometrium were obtained immediately after the uterus was removed from the women in both groups. In the study group, the myometrium was acquired from the tissue where there is coarsely trabeculated and diffusely hypertrophied myometrium stippled with foci of ectopic endometrium, while in the control group, the myometrium was obtained from tissue other than uterine myoma. This tentative grouping determined by the naked eye was then found to be fully consistent with the final diagnosis provided by the pathologists. The cervical tissue was only derived in women without adenomyosis.

The aspirated blood was collected in glass tubes containing heparin, and was processed within 30 min. Peripheral blood mononuclear cells (PBMCs) were isolated by layering over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuging at 800 g for 20 min. The isolated PBMCs were washed twice with RPMI-1640 medium (Life Technologies, Inc., Grand Island, NY) to remove residual Ficoll-Hypaque solution, and were reconstituted to a final cell concentration of 1–2 × 10⁶ cells/ml. The viability of PBMCs was verified with a trypan blue exclusion test. Viability was typically more than 95%.

Myometrial, endometrial and cervical tissues were collected aseptically and separately in a tissue flask containing RPMI-1640. Contaminated blood was removed by washing with RPMI-1640. Tissues were cut into tiny pieces (0.5 mm³) with a surgical knife, and were suspended in 5 ml of RPMI-1640. The suspensions were ground and passed consecutively through different sized mechanical sieves (sieve size 150, 300 and 400), and were overlad on discontinuous (100%/50%/30%) Percoll gradients (Sigma Chemical Co., St Louis, MO). After centrifuging at 800 g for 45 min, mononuclear cells were obtained from the interface of 100 and 50% Percoll solution, and were then reconstituted to a final cell concentration of 1–2 × 10⁶ cells/ml. The viability of mononuclear cells was verified with a trypan blue exclusion test. Viability was typically more than 90%.

**Immunophenotypic analysis with three-colour flow cytometry**

The methods have been described in detail previously (Yang et al., 2000). In brief, monoclonal antibodies (mAbs) conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were obtained from the interface of 100 and 50% Percoll solution, and were passed consecutively through different sized mechanical sieves suspended in 5 ml of RPMI-1640. The suspensions were ground and passed consecutively through different sized mechanical sieves (sieve size 150, 300 and 400), and were overlad on discontinuous (100%/50%/30%) Percoll gradients (Sigma Chemical Co., St Louis, MO). After centrifuging at 800 g for 45 min, mononuclear cells were obtained from the interface of 100 and 50% Percoll solution, and were then reconstituted to a final cell concentration of 1–2 × 10⁶ cells/ml. The viability of mononuclear cells was verified with a trypan blue exclusion test. Viability was typically more than 90%.

All values are expressed as mean ± SD. As the data were not normally distributed, the Mann–Whitney U-test for non-parametric data was used to compare the difference between the groups. A P-value < 0.05 was considered statistically significant.

**Results**

Ten women with and 12 women without adenomyosis were recruited for this study. The median age was 44 years (range 38–49) for women with adenomyosis and 45 years (range 37–51) for those without adenomyosis. Among them, five out of 10 women with adenomyosis and six out of 12 women without adenomyosis were in the follicular phase of their menstrual cycle, while the others were in the luteal phase. Since our previous study (Ho et al., 1996b) revealed that the NK cell populations and the NK cell activation markers were similar in different phases of the menstrual cycle, the following data are analysed without regard for the phase of the menstrual cycle.

The mean number of total lymphocytes per g of tissue was 2.6 (range 0.1–6.5) × 10⁶ in the myometrium and 1.9 (0.7–4.4) × 10⁶ in the endometrium in women with adenomyosis, similar to those numbers obtained in the myometrium [1.8 (0.3–5.4) × 10⁶] and endometrium [2.5 (0.1–7.5) × 10⁶] in women without adenomyosis. The mean lymphocyte number in the cervix was 1 (0.3–3.4) × 10⁶ in women without adenomyosis.

Among myometrium, endometrium and cervix in women without adenomyosis, there was no difference in the fraction of NK and T cells. The MFI of CD94 was significantly lower in the endometrium in women with adenomyosis, similar to those numbers obtained in the myometrium [1.8 (0.3–5.4) × 10⁶] and endometrium [2.5 (0.1–7.5) × 10⁶] in women without adenomyosis. The mean lymphocyte number in the cervix was 1 (0.3–3.4) × 10⁶ in women without adenomyosis.

Among myometrium, endometrium and cervix in women without adenomyosis, there was no difference in the fraction of NK and T cells. The MFI of CD94 was significantly higher in the subpopulations of CD56⁺ (114.5 ± 99) and CD56⁺CD94⁺ (175.1 ± 113) cells in the endometrium, compared with those in the myometrium (38.5 ± 29.9 and 94.2 ± 48.5) and cervix (44.6 ± 33.8 and 84.8 ± 38). The MFI of GL183 in the CD56⁺GL183⁺ subpopulation in endometrium was 157.4 ± 97.8, much higher than that in...
the cervix (80.9 ± 52). On the other hand, NKB1 and EB6 were similarly expressed on NK cells in different parts of the uterus (Table I). The difference was also not prominent in the expression of various kinds of KIRs on CD4+ and CD8+ T cells in different compartments of the uterus (Table II).

In the comparison between women with and without adenomyosis, NK cell populations were not different. The MFI of NKB1 in the CD56+ subpopulation (3.2 ± 1), as well as the percentage of CD56+NKB1+ in CD56+ cells (12.2 ± 12.7%), in eutopic endometrium in women with adenomyosis were significantly lower than those in women without adenomyosis (5.8 ± 2.8 and 26.1 ± 10.4%, respectively). The MFI of GL183 in the CD56+ subpopulation (8.3 ± 2) in endometrium in women with adenomyosis was also lower than that in women without adenomyosis (27.4 ± 23.6). On the other hand, the KIR expression on NK cells was not different in the myometrium and peripheral blood between women with and without adenomyosis (Table III). Also, the expression of KIRs on T cells, either CD4+ or CD8+, was similar in endometrium, myometrium and peripheral blood between those with and without adenomyosis (Table IV).

### Table I. KIR expression on NK cells in different portions of the uterus from women with uterine myoma (n = 12)

<table>
<thead>
<tr>
<th></th>
<th>Myometrium</th>
<th>Endometrium</th>
<th>Cervix</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK cells (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKB1</td>
<td>22.9 ± 16.6</td>
<td>11.6 ± 9.9</td>
<td>19.3 ± 16.7</td>
</tr>
<tr>
<td>% CD56+NKB1+</td>
<td>5.8 ± 2.8</td>
<td>5.8 ± 2.8</td>
<td>8.6 ± 11.5</td>
</tr>
<tr>
<td>% CD56*GL183+ cells</td>
<td>22.8 ± 12.1</td>
<td>26.1 ± 10.4</td>
<td>29.3 ± 20.9</td>
</tr>
<tr>
<td>% CD56*EB6+ cells</td>
<td>73.3 ± 37.8</td>
<td>71.1 ± 44.2</td>
<td>55.5 ± 40.8</td>
</tr>
<tr>
<td>GL183</td>
<td>15.2 ± 9.3</td>
<td>27.4 ± 23.6</td>
<td>21.7 ± 21.9</td>
</tr>
<tr>
<td>% CD56*GL183+ cells</td>
<td>45.5 ± 18</td>
<td>46.3 ± 21.1</td>
<td>52.9 ± 17.7</td>
</tr>
<tr>
<td>% CD56*EB6+ cells</td>
<td>100.8 ± 57.1</td>
<td>157.4 ± 97.8</td>
<td>80.9 ± 52.4</td>
</tr>
<tr>
<td>EB6</td>
<td>6.3 ± 3.7</td>
<td>5.2 ± 3.2</td>
<td>7.9 ± 4.5</td>
</tr>
<tr>
<td>CD8+ cells</td>
<td>31.2 ± 24</td>
<td>27 ± 19.7</td>
<td>33 ± 18.4</td>
</tr>
<tr>
<td>CD94</td>
<td>26.5 ± 8.1</td>
<td>36.2 ± 26.1</td>
<td>28 ± 7.5</td>
</tr>
<tr>
<td>CD94</td>
<td>38.5 ± 29.9</td>
<td>114.5 ± 99.3</td>
<td>44.6 ± 33.8</td>
</tr>
<tr>
<td>% CD56*CD94+ cells</td>
<td>67.5 ± 17.1</td>
<td>67.7 ± 23.6</td>
<td>73.7 ± 16.4</td>
</tr>
<tr>
<td>% CD56*CD94+ cells</td>
<td>94.2 ± 48.5</td>
<td>175.1 ± 113</td>
<td>84.8 ± 38.6</td>
</tr>
</tbody>
</table>

The myometrium was obtained from tissue other than uterine myoma in these women. KIR = killer cell inhibitory receptor; NK = natural killer cell; MFI = mean fluorescence intensity. All values are mean ± SD.

aCalculated as the mean fluorescence intensity of NKB1 on CD56+ cells.

bCalculated as the percentage of CD56+NKB1+ in CD56+ cells.

cCalculated as the mean fluorescence intensity of NKB1 on CD56+ cells.

**Table II.** Mean fluorescence intensity of KIRs on T cells in different portions of the uterus (n = 12)

<table>
<thead>
<tr>
<th></th>
<th>Myometrium</th>
<th>Endometrium</th>
<th>Cervix</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+NKB1+</td>
<td>28.9 ± 21.9</td>
<td>31.3 ± 24.1</td>
<td>22.3 ± 17.4</td>
</tr>
<tr>
<td>CD4+GL183+</td>
<td>2.4 ± 0.8</td>
<td>2.1 ± 0.7</td>
<td>2.6 ± 1.4</td>
</tr>
<tr>
<td>CD4+EB6+</td>
<td>2.6 ± 0.7</td>
<td>2.5 ± 0.8</td>
<td>4.1 ± 3.8</td>
</tr>
<tr>
<td>CD4+CD94+</td>
<td>2.9 ± 0.9</td>
<td>2.4 ± 1</td>
<td>3.6 ± 1.9</td>
</tr>
<tr>
<td>CD8+NKB1+</td>
<td>2.6 ± 0.7</td>
<td>2.9 ± 1.9</td>
<td>3.6 ± 3.7</td>
</tr>
<tr>
<td>CD8+GL183+</td>
<td>2.9 ± 2.5</td>
<td>2 ± 0.6</td>
<td>2.9 ± 2.1</td>
</tr>
<tr>
<td>CD8+EB6+</td>
<td>3.1 ± 1.2</td>
<td>4.5 ± 5.9</td>
<td>3.3 ± 2</td>
</tr>
<tr>
<td>CD8+CD94+</td>
<td>4 ± 3.8</td>
<td>2.4 ± 0.9</td>
<td>3.5 ± 1.3</td>
</tr>
<tr>
<td>CD8+CD94+</td>
<td>8.7 ± 5.9</td>
<td>6.3 ± 3.2</td>
<td>8.6 ± 3.8</td>
</tr>
</tbody>
</table>

KIR = killer cell inhibitory receptor. All values are mean ± SD.

aCalculated as the mean fluorescence intensity of NKB1 on CD4+ T cells.

**Discussion**

The functions of uterine NK cells are not yet well known. Because they are present in abundance at the time of implantation, they may play a role in the implantation process and the subsequent orderly growth and development of the placenta (King et al., 1998). Previous results revealed that uterine NK cells have a similar repertoire of KIRs to those found on NK cells in the blood (Verma et al., 1997). These uterine NK cell KIRs have identical structural characteristics in the extracellular domains, transmembrane region and cytoplasmic tail to those of peripheral blood NK cell receptors, indicating that the two cell populations may function in the same way. However, the proportions of NK cells expressing a particular KIR and the antigenic density are not the same between peripheral blood and uterine NK cells (Verma et al., 1997).

Although heterogeneity in expression of KIRs between blood and uterus was found, there has not been any report describing KIR expression in different parts of the uterus. Our results revealed that there was an increased CD94 expression on NK cells in the endometrium compared with that in the myometrium and cervix. This might imply that the NK activity is by nature depressed in the endometrium compared with that in the myometrium and cervix because the increased expression of KIR generally represented a decreased NK activity (Wu et al., 2000). This naturally depressed NK cytotoxicity in the endometrium might therefore be one of the possible mechanisms that account for the development of endometriosis and/or adenomyosis in women of reproductive age. Without performing functional studies using class I HLA-expressing target cells, however, we cannot confirm that the increased expression of CD94 could inevitably result in inhibition of NK activity in this study.

We also demonstrated decreased expression of NKB1 and GL183 on endometrial NK cells in women with adenomyosis compared with that in women without adenomyosis. It may be a compensatory effect, in which the NK cytotoxicity is
activated in women with adenomyosis in order to eradicate the abnormal endometrial cells that might exit the eutopic site of the endometrium. It also implies that the ‘abnormal’ endometrial cells, rather than the impaired NK cell function, account for the development of adenomyosis. The different immunological expression of KIRs in the eutopic endometrium is supported by a previous report (Braun et al., 2002), in which a reduction in apoptosis of endometrial cells was found in the eutopic endometrium in women with endometriosis due to reduced macrophage trafficking into the eutopic endometrium. However, GL183 recognizes both inhibitory and activatory forms of KIRs, which could result in either inhibition or activation of the NK-mediated cytolytic activity (Moretta et al., 1995). Since western blotting and reverse transcription (RT)–PCR were not performed in this study, the possibility remains that inhibitory as well as activatory forms of GL183 may be decreased. Similarly, an alternative explanation for the decreased NK cytotoxicity seen in endometriosis is that there may be decreased expression of activatory receptors rather than increased inhibitory KIR expression. As K562 target cells generally lack class I MHC expression (Rouas-Freiss et al., 1997), any changes in the levels of inhibitory or activatory KIRs should not necessarily affect NK cytotoxicity once K562s were employed as the target cells.

Our results did not reveal different KIR expression on the myometrial NK cells between women with and without adenomyosis. This unlike the finding of an increased KIR expression on the peritoneal NK cells in women with endometriosis (Wu et al., 2000; Maeda et al., 2002). As a result, the local immunological appearance in response to the eutopic endometrium might be different between adenomyosis and endometriosis.

Unlike the different expression of KIRs on NK cells, we demonstrated that KIRs were expressed similarly on both CD4+ and CD8+ T cells among various uterine tissues and between women with and without adenomyosis (Tables II and IV). In agreement with previous reports (Moretta et al., 1996; Mingari et al., 1996), we also found that KIRs were expressed more frequently on CD8+ T cells than on CD4+ T cells.
cells. One reasonable explanation for the advantage of this event is that the CD8+ T cells have NK-like activity, and would thus be deleterious to normal cells if they did not express KIRs. Their defective expression could be involved in autoimmune diseases caused by autoreactive cytotoxic T cells (Mingari et al., 1998).

In conclusion, we demonstrated a decreased expression of KIRs on NK cells in eutopic endometrium in women with adenomyosis compared with that in women without adenomyosis. It might be a compensatory effect in which the NK cytotoxicity is activated in order to eradicate the abnormal endometrial cells that might exit the eutopic site of the endometrium.

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