Suppression of natural killer cell activity by splenocyte transplantation in a rat model of endometriosis

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BACKGROUND: One immune characteristic of endometriosis is a decrease in natural killer (NK) cell activity. This study was performed to determine whether an abnormal immune reaction in an endometriosis animal model could be transferred to an animal of the same species. METHODS: An endometriosis model was prepared using 8 week old female rats by grafting a small section of one uterine horn onto the mesentery, followed 4 weeks later by removal of the spleen and remaining uterine horn. Splenocytes, that had been depleted of macrophages were injected via the tail vein, and NK cell activity of splenocytes was determined 4 days later. The uterus was simultaneously investigated immunohistochemically for immune cells. There was a control group (untreated; group 1), a control–spleenocyte injection group (group 2), an experimental endometriosis model group (group 3) and an endometriosis model spleenocyte injection group (group 4). RESULTS: Splenocyte NK cell activity was decreased in group 3 to 42.0% of that of group 1 and in group 4 to 38.9%. Immunohistologically, the number of NK cells in groups 3 and 4 markedly decreased to 62.0 and 55.1% of group 1 respectively. CONCLUSION: It was demonstrated that abnormal immunity caused by allograft of immune cells could recur in an endometriosis rat model.

Key words: endometriosis/endometriosis model/natural killer cell activity/splenocyte/transplantation

Introduction
The cellular immune system, particularly natural killer (NK) cells, plays a major role in the onset of autoimmune abnormalities. Mature NK cells migrate to peripheral blood and patrol the body. NK cells play an indirect role by activating macrophages and T cells via production of a variety of cytokines, as well as a direct role consisting of monitoring and modulating tumour cells and infected cells (Roder et al., 1981).

A major immune characteristic of endometriosis is the fact that NK cell activity is decreased (Oosterlynck et al., 1991, 1992; Tanaka et al., 1992; Iwasaki et al., 1993; Kikuchi et al., 1993). It was also reported that NK cell activity is reduced after the addition of serum or ascites from endometriosis patients to NK cells in vitro (Kanzaki et al., 1992; Oosterlynck et al., 1993). It is also interesting to note that the number of NK cells in eutopic endometria was less than that in eutopic endometria (Witz et al., 1994).

A decrease in NK cell activity was also demonstrated using an animal model. One group (Matsubayashi et al., 1995) prepared a model rat and investigated NK cell activity in peripheral blood and ascites. They reported that the formation of endometriosis resulted in a significant decrease in NK cell activity. This suggests that a decrease in NK cell activity may permit the survival or proliferation of endometrial cells in the pelvis.

Abnormal reactions exist in almost every process of the immune response cascade in endometriosis, from recognition and transmission to effect (Haney et al., 1981; Halme et al., 1984; Gleicher et al., 1987; Ota and Igarashi, 1993; Ota et al., 1997, 1999), and excessive responses of the immune system might occur in each or all of these processes. This suggests that immunocompetent cells in autoimmune animals of the same species that have recognized endometriotic tissues could suppress NK cells in the recipient animal after transplantation. We therefore planned this study to demonstrate experimentally in rats whether immune abnormalities caused by allograft of immune cells could recur.

Materials and methods
Animals
An endometriosis model was prepared using 8 week old, 4 day cycle Wistar–Imamichi strain female rats (Omiya, Saitama, Japan). The rats were housed in an environmentally controlled area and main-
tained with water and rat chow ad libitum. A mid-ventral laparotomy was performed aseptically under ether anaesthesia. One uterine horn was ligated at both the uterotubal junction and the cervical end and removed. The excised horn was immersed in sterile culture medium and the endometrium was exposed by cutting lengthwise with a pair of scissors. In this way, three pieces of uterine horn measuring ~3×3 mm were obtained. The section was then grafted to the mesentery near the right side of the reproductive tract using 4–0 nylon surgical suture. The peritoneal cavity was kept moist with copious amounts of saline solution throughout the surgery. Four weeks after the transplant, a laparotomy was performed again. After confirming the formation of an endometrial cyst in the mesentery, the spleen and the remaining uterine horn were extracted and the latter was cut longitudinally. Half of the uterus was fixed in a 10% formalin solution and the other half was rapidly frozen on dry ice to −80°C for preservation after embedding in optimal cutting temperature compound (Sakura Finetek USA, Torrance, CA, USA). The formation of a typical endometrial cyst was reconfirmed histologically by haematoxylin–eosin staining. The study was approved by the Institutional Animal Care and Use Committee of the University of Akita, Akita-city, Japan.

**Preparation of splenocytes for injection**

After being anaesthetized, a laparotomy was performed aseptically to extract the spleen. It was suspended in a phosphate-buffered saline solution (PBS; 0.1 mol/l) and thinly sliced with scissors. After filtering the cell suspension with 40 μm nylon mesh (Cell Strainer; 35-2340; Becton Dickinson, Franklin Lakes, NJ, USA), the splenocyte suspension was washed with PBS. This step was repeated three times. The splenocyte suspension was put in a dish, cultured for 2 h in a 5% CO2 incubator to remove macrophages, centrifuged for 5 min at 2340 g (Becton Dickinson, Franklin Lakes, NJ, USA), then centrifuged for 5 min at 2340 g. The cell suspension was washed with 40 μm nylon mesh (Cell Strainer; 35-2340; Becton Dickinson, Franklin Lakes, NJ, USA) and resuspended in RPMI 1640 medium containing 10% FBS. A volume of 200 μl of cell suspension was then injected into the tail vein using a 27 gauge needle.

Each group was defined as follows. Group 1 (n = 20): control rats that did not receive any treatment; group 2 (n = 10): rats that received splenocyte injection from control rats that did not have any previous treatment (i.e. control splenocyte injection group); group 3 (n = 17): rats with experimental endometriosis established (i.e. control splenocyte injection group); group 4 (n = 13): rats that received injection of splenocytes from rats that had experimental endometriosis (i.e. model splenocyte injection group); and group 5 (n = 5): rats with experimental endometriosis with ectopic endometrial tissue in endometrial cysts for immunohistochemical studies.

**Assay for NK cell activity**

**Preparation of effector cells**

First, the spleen was extracted using the above-mentioned method. Spleens from group 2 or 4 were obtained 4 days after the injection of splenocytes from group 1 or 3 respectively. Spleens were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (IBL, Maebashi, Gunma, Japan) containing 10% fetal bovine serum (SF71107; Cansera, Rex Dace, Ontario, Canada) containing 100 IU/ml of penicillin G and 100 μg/ml of streptomycin sulphate (Gibco, Grand Island, NY, USA) and thinly sliced with scissors. The splenocyte suspension was put in a dish, cultured for 2 h in a 5% CO2 incubator, and was submitted for the measurement of NK cell activity. The splenocyte count was adjusted to 1×10^6 cells/ml. These effector cells were usually >95% viable as assessed by the Trypan Blue dye exclusion test.

**Preparation of target cells**

Human K-562 myeloid leukaemia cells obtained from Dainihon Pharmaceutical Co. (Osaka, Japan) were collected by centrifugation at 220 g for 5 min. Solution containing 50–100 μCi (51Cr) of sodium chromate (New England Nuclear, Boston, MA, USA; sp. act. 350–600 mCi/mg) was added per 1×10^6 cells, the cell suspension was cultured for 1 h at 37°C, and cells were labelled. We knew from a preliminary experiment that labelling by 1 h culture yielded the highest labelling index (maximal release/spontaneous release). PBS solution was then added, and the dishes were gently stirred. The supernatant was discarded after centrifugation at 220 g for 5 min. PBS solution was added again and this step was repeated three times. Finally, RPMI 1640 medium was added to adjust the cell count to 1×10^6 cells/ml.

**Determination of NK activity**

Ten μl of culture solution containing target cells (T; 1×10^6/ml) was pipetted into each well of a microplate. For the maximal release group, 0.2 ml of 1 mol/l HCl was added, while 0.2 ml of RPMI 1640 medium with 10% FBS was added for the spontaneous release group. Thus, the spontaneous release was measured in wells that contained only labelled K-562 cells. The solutions (0.2 ml) containing effector cells (E; 5×10^5/ml) were pipetted for the test release group to give an E:T ratio of 100:1. After centrifugation at 220 g for 5 min, solutions were diluted with PBS again and cultured for 4 h in a 5% CO2 incubator. After completion of culture, 150 μl of conditioned medium was collected from each well and the 51Cr level was measured using a gamma counter. NK cell activity was calculated by: [test (c.p.m.) – spontaneous release (c.p.m.)/maximal release (c.p.m.)] ×100. Activity in each group was expressed by the rate of change relative to the control group, which was set as 100%. The measurements were conducted in triplicate in each sample.

**Staining**

Immunostaining of each antigen was performed using the indirect method. Each section of uteri in groups 1–5 was cut to a thickness of 3 μm, and a portion was submitted for haematoxylin–eosin staining. Antibody localization was determined by the indirect peroxidase method. First, fresh frozen sections were thoroughly air-dried and then fixed by dipping in cold acetone at −20°C for 10 min. In order to suppress non-specific adsorption of proteins, 10% normal swine serum (809-10; Cosin Bio, Sakato, Japan) was added and sections were left to stand at room temperature for 20 min. Each first antibody was added and reacted for 1 h at 37°C. Antibodies used for the staining were as follows: CD4: mouse anti-rat-CD4 monoclonal antibody (MAS 1131: ×200; Harlan Sera-Lab Ltd, Belton, Loughborough, UK); CD8: mouse anti-CD8-α (D-9) IgG2a monoclonal antibody (sc-7970: ×50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); horseradish peroxidase conjugate (4550: ×200; Biosource International, Camarillo, CA, USA) was added and sections were washed with PBS again after reaction for 1 h at 37°C. In order to inhibit endogenous peroxidase, sections were reacted with diaminobenzidine containing 10 mmol/l sodium azide for 5 min at room temperature. Finally, after washing in running water for 20 min, 5% Methyl Green was added for nuclear staining, and sections were reacted for 10 min at room temperature. Normal mouse serum was used instead of the primary antibody for a negative control. For evaluation of staining, eutopic and ectopic endometrial cells were viewed at a magnification of ×400 and the number of positive cells in 10 fields was calculated.
A decrease in Natural killer cell activity in rat model of endometriosis

**Table I. Number of immune cells or macrophages in the eutopic or ectopic endometrium in rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of cells</th>
<th>CD4</th>
<th>CD8</th>
<th>NK</th>
<th>Mf</th>
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<tr>
<td></td>
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<tr>
<td>Group 1 (20)x</td>
<td>CD4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.1 ± 2.4</td>
<td>48.5 ± 3.3</td>
<td>46.3 ± 2.2</td>
<td>64.0 ± 2.4</td>
</tr>
<tr>
<td>Group 2 (10)</td>
<td>CD8</td>
<td>77.8 ± 3.5</td>
<td>60.8 ± 3.4</td>
<td>59.2 ± 2.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>70.0 ± 2.0</td>
</tr>
<tr>
<td>Group 3 (17)</td>
<td>NK</td>
<td>69.8 ± 2.4</td>
<td>51.8 ± 3.6</td>
<td>28.7 ± 2.1</td>
<td>54.6 ± 3.6</td>
</tr>
<tr>
<td>Group 4 (13)</td>
<td>Mf&lt;sup&gt;d&lt;/sup&gt;</td>
<td>65.2 ± 3.5</td>
<td>50.0 ± 3.7</td>
<td>25.5 ± 1.7</td>
<td>46.0 ± 2.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 5&lt;sup&gt;f&lt;/sup&gt; (5)</td>
<td>CD4-positive T cells</td>
<td>54.0 ± 6.4&lt;sup&gt;f&lt;/sup&gt;</td>
<td>48.7 ± 13.1</td>
<td>18.0 ± 5.0</td>
<td>52.7 ± 6.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are means ± SEM.
<sup>b</sup>Between the groups by one-way analysis (P < 0.005).
<sup>c</sup>Between the groups by one-way analysis (P < 0.0001).
<sup>d</sup>Values in parentheses indicate the number of animals examined for the antigen.
<sup>e</sup>Versus group 1 by post-hoc, multiple range test (Scheffe’s F; P < 0.0001).
<sup>f</sup>Versus group 1 by post-hoc, multiple range test (Scheffe’s F; P < 0.005).

Evaluation of each specimen was done by two different observers blinded as to the specimen source. Sections were assigned a number by a first observer, and confirmed by a second observer.

**Statistical analysis**

The results are expressed as the mean ± SEM where applicable. NK cell activities among the groups were compared using the Kruskal–Wallis test with post-hoc, multiple range test (Scheffe’s F); P < 0.005 versus group 1 using the Kruskal–Wallis test (Scheffe’s F).

Evaluation of each specimen was done by two different observers blinded as to the specimen source. Sections were assigned a number by a first observer, and confirmed by a second observer.

**Results**

**Natural killer cell activity**

Splenocyte NK cell activity in group 2 tended to decrease relative to group 1 (Figure 1). Splenocyte NK cell activity was markedly decreased in group 3 to 42.0% of that of group 1 and in group 4 to 38.9% (P < 0.0005). However, the differences between groups 2 and 3, and between groups 2 and 4, were not statistically significant (P = 0.09 and P = 0.06 respectively).

**Variation in number of immune cells and macrophages in eutopic and ectopic endometrium**

There was no significant difference in CD4-positive cells between the four groups with eutopic endometria (Table I). However, in a comparison of the four groups plus group 5, CD4-positive cells decreased significantly in group 5. There was no significant difference in CD8-positive cells between the five groups. There was a marked variation between the groups in terms of NK-positive cells: NK cells increased significantly in group 2 relative to group 1. In contrast, NK cells in groups 3 and 4 decreased to 62.0 and 55.1% of group 1, respectively. The NK cell count decreased even more markedly in group 5 to 38.9% of group 1. The number of macrophages also varied significantly. In group 2, there was no significant difference compared with the control group. On the other hand, the number of macrophages decreased significantly in group 4 relative to group 1. In group 5, the number of cases investigated for the staining was small and there was no significant difference, but the count was about the same as that of group 3.

**Distribution of immune cells or macrophages in endometrium**

CD4, CD8, and macrophages were dispersed in the stromal tissues of the endometrium and were not conglomerated in any slice (Figure 2). In some slices, NK cells tended to be located in the vicinity of the glands, but they did not adhere to glandular cells or the basement membrane. There appeared to be no major difference in their manner of distribution among the groups.

**Discussion**

In the present study, splenocyte NK activity was markedly decreased in a rat model of endometriosis. There have been several reports of a decrease in NK cell activity in an endometriosis rat model. Keenan and colleagues investigated variations in immune cells in endometriosis explants after administering the immune modulator loxoribine, a guanosine analogue, and levamisole, an anthelminthic drug, for 6 weeks in a rat model of endometriosis (Keenan et al., 1999). They reported that NK cell activity tended to decrease in the model group, decreased significantly in the loxoribine group, and in contrast improved to a value close to the control group in the levamisole group. Since endometriosis cannot be induced by a decrease in NK cell activity (Ramey et al., 1996), Keenan suggested that the decrease in theloxoribine group may have...
been because loxoribine caused ectopic endometriotic tissue to atrophy, resulting in a decrease in NK cell migration.

In an endometriosis model rat, it was found that low NK cell activity improved dose-dependently after the administration of danazol, an agent used to treat endometriosis (Matsubayashi et al., 1995). From these findings, they suggested the existence of a humoral factor that regulates the proliferation, activation and/or migration of NK cells in the ectopic endometrium. It is likely that in endometriosis an aberrant immune reaction occurs because of dystopia of the endometrial cells, especially in the pelvic cavity where immunocompetent cells are more easily exposed. As for a possible mechanism, a new antigenicity may be expressed in the transplanted endometrium as a result of cancellation of masking, i.e. expression of antigen or suppression of recognition, caused by the manipulation involved in the transplant of endometrium.

There is no doubt that estrogen plays an important role in the onset and progression of endometriosis. In a rat model of endometriosis (Isaacson et al., 1991), estrogen was administered after oophorectomy to look for variations in the production of complement in eutopic and ectopic endometria. Complement production was greater in the ectopic endometrium than in the eutopic endometrium, and it increased further as a result of estrogen administration. It is interesting to note that increasing the estrogen concentrations clearly reduces the number of NK cells (Garzetti et al., 1993).

It is reported that T or NK cell dysfunction seen in tumour-bearing mice is partially a result of immunosuppressive macrophages induced by the tumour itself (Kono et al., 1996). In tumour-bearing mice, immunosuppressive macrophages are detected in the spleen and in the tumour (Alleva et al., 1993a,b). Therefore, it is thought that they are induced by a disturbance in the immune modulation. Several studies indicate that induced immunosuppressive macrophages inhibit T cells (Tomiioka et al., 1996; Maw et al., 1997). These findings may explain, at least in part, the reduction in the number of CD4-positive cells in the ectopic endometrium. Moreover, there was no significant variation in the number of macrophages in the model rat, but it was significantly reduced in the model splenocyte injection rat. These findings raise the possibility of an increase in immunosuppressive macrophages as a shift in the macrophage subfraction.

Why does NK cell activity decrease in a rat model of endometriosis? One possibility is macrophages. As mentioned already, macrophages are released from the systemic network and present antigens in the abdominal cavity. In endometriosis, binding of antigen-presenting cells and T cells or the local cytokine environment enhances the immune system and presents an inflammatory-like state. Under these circumstances, macrophages might act to inhibit proliferation and activation of NK cells. Eventually, this may cause a decrease in NK cell activity and a reduction in the number of NK cells in the systemic immune system or the spleen.
A second possibility is the involvement of γδT cells, which are found in abundance in epithelia, e.g. skin, small intestine and uterus (Groh et al., 1989; Raulet et al., 1991). These cells act as the first line of defence in tissues exposed to foreign antigens. γδT cells possess killer cell activity, but the cells that have invaded cancer tissues do not exhibit killer cell activity (Seo et al., 1998). We found that γδT cells are markedly increased in the eutopic and ectopic endometria in endometriosis (Oosterlynck et al., 1996a,b). Given the above, an increase in γδT cells in the target tissue may be involved in the inhibition of NK cell activity.

A third possibility is that a decrease in NK cell activity is caused by some immunosuppressive factors. Endometrial cells are injured when NK cells are added to an in-vitro endometrial culture line, but the injury is prevented by the presence of a monoclonal antibody specific to NK cells (Oosterlynck et al., 1991). In addition, it has been reported that NK cell activity in ascites is 2.4 times lower than that in peripheral blood in endometriosis cases (Oosterlynck et al., 1992). These findings suggest that some humoral factors discharged from ectopic endometria into ascites, e.g. glycodecin or ICAM-1, can inhibit NK cell activity by a shielding effect (Koninckx et al., 1998).

By what mechanism can the splenocytes from a rat model of endometriosis decrease NK cell activity in the same species of animal? It has already been reported that the immune cells used in this study can act immunologically on the same species of animal after transplantation (Takabatake et al., 1997a,b). As for the mechanisms, NK cell activity and the number of transplanted splenocytes were lower than those in control animals given splenocytes, so it may simply be a reflection of that. Another possibility is that the composition of immunocompetent cells other than NK cells contained in rat model splenocytes has been altered or a change in their activity affects the immunological state of the recipient animal.

In conclusion, this study revealed that an abnormal immune reaction in an animal model of endometriosis could be transferred to an animal of the same species. A more detailed analysis of immune suppression, including kinetics of macrophages in a rat model and transplanted animals, is needed in the future.

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H. Ota et al.


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