Effect of Danazol on NK Cells and Cytokines in the Mouse Uterus

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Abstract. Danazol, which has been used as a medicine for endometriosis, has a valid effect in pretreatment of patients receiving in vitro fertilization and embryo transfer, although its reproductive mechanism remains unclear. BALB/c mice were subcutaneously injected with danazol for 2 weeks. Blood and uteri were collected and cytokines were assayed. Following danazol treatment, an increase in pregnancy ratio was evident that was accompanied by up-regulation in serum macrophage-colony stimulating factor (M-CSF). RT-PCR analysis revealed that expression of M-CSF and Ly49, a phenotypic marker of natural killer (NK) cells, was up-regulated in the uterus of the danazol-treated mice. In immunohistochemical analysis, M-CSF and Ly49, together with α5 integrin, were clearly detected in the endometrium of the danazol-treated mice with very similar pattern of localization. These results suggest that danazol has an effect to promote pregnancy that induces recruitment of NK cells and a concomitant increase in the expression of M-CSF and α5 integrin in the uterus.

Key words: Integrin α5, Danazol, Ly49 antigen, Macrophage-colony stimulating factor, Uterine natural killer cells
called decidual NK cells in humans and uterine NK (uNK) cells in mice, can produce cytotoxic proteins, such as pore-forming protein (perforin), granzyme B and Fas ligand [8]. Additionally, decidual NK and uNK cells can produce various cytokines, such as macrophage-colony stimulating factor (M-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) and leukemia inhibitory factor (LIF) [9, 10], which are known to play crucial roles for successful implantation through interactions with related factors, such as integrins [11]. Generally, the cytotoxic activity of uNK cells is strictly controlled in the normal pregnant uterus [9].

Regulation of NK cell activity is dependent on the interaction between the major histocompatibility complex (MHC) class I molecules on target cells and the Ly49 antigens, which are a specific receptor for mouse NK cells [12]. Ly49 is characterized by a C-type lectin-like extracellular domain and composes a superfamily (including 23 subtypes, named as Ly49A–W) that is expressed in NK-cell populations and responsive to various types of MHC molecules [13]. Recognition of self-MHC molecules by Ly49 (except Ly49D and H) induces the inhibitory signal that regulates the cytotoxic activity of NK cells [14]. In the mouse pregnant uterus, uNK cells are believed to be regulated by the Ly49-MHC inhibitory system [15]. Disorder of the analogous inhibitory mechanism in the human uterus is suspected as an important cause of recurrent spontaneous abortion [16]. On the other hand, helper T cells can enhance NK cell cytotoxicity generally through secretion of T helper (Th) type-1 cytokines, such as interferon (IFN)-γ and interleukin (IL)-2. Interestingly, it is known that the Th-1 cytokine-dominant condition, which is induced by the effect of IL-12 [17], is responsible for unidentified recurrent spontaneous abortion; conversely, a successful pregnancy requires Th-2 cytokines (IL-4, IL-10, etc.) [18].

As stated above, successful pregnancy is closely influenced by the specificity of the cytokine profile derived from NK and helper T cells. To assess the effect of danazol on reproduction, it is necessary to understand the movement of cytokines and related factors following danazol treatment. In the present study, immunological conditions were examined in danazol-treated mice in association with uNK cells and their cytotoxicity.

### Materials and Methods

**Mice and treatments**

BALB/c mice purchased from Japan SLC (Hamamatsu, Japan) were used for the experiments. The mice were bred with free access to water and food under the conditions of controlled temperature (22 ± 2°C), humidity (50 ± 10%), and light (12-h light/dark cycle) in the Laboratory Animal Center, Osaka Medical College. All mice were received humane care as outlined in the Osaka Medical College Guidelines for the Care and Use of Laboratory Animals. After 1 week of acclimatization to the breeding environment, female virgin mice at 8 weeks of age were treated with danazol (Mitsubishi Pharma Corporation, Osaka, Japan) at 0, 0.75, 7.5 and 75 mg/kg (body weight) diluted with cottonseed oil (Nacalai Tesque, Kyoto, Japan) containing 10% ethanol via subcutaneous injection on the back for 14 consecutive days. For the control treatment, mice were treated with only cottonseed oil containing 10% ethanol in the same manner as the danazol treatment. During the treatments, vaginal smears were collected each day and used for monitoring of the estrous cycle following Giemsa staining. Sera, uteri and ovaries were collected three hours (day 0), or 4 days or 8 days after the final treatment and immediately frozen in liquid nitrogen or fixed with 10% neutral-buffered formalin. The optimal dose for danazol treatment was evaluated by the degree of influence on the estrous cycle and the numbers of mature ovarian follicles and corpora lutea, which were examined using paraffin sections stained with H and E.

In addition, some of the danazol-treated mice and control mice were paired with normal male BALB/c mice in the evening 4 days after treatment. Detection of vaginal plug at the next morning was regarded as a successful copulation and defined as day 0 of pregnancy. At day 5 of pregnancy, implantation sites were observed by visualization following the injection of 0.1 ml Evans blue (10 mg/ml) via the tail vein. The numbers of pregnant females and implantation sites per uterus were counted. The pregnancy rate was calculated from the number of pregnant females and the number of plug-confirmed females.

**Serum cytokines**

Serum cytokines were examined for IFN-γ, IL-4,
IL-12 and M-CSF using a sandwich ELISA (DuoSet® and Quantikine M® kits, R&D Systems, Minneapolis, MN, USA) in unmated mice after days 0, 4 and 8 of treatment. Cytokine measurements were carried out following the manufacturer’s instructions. Briefly, serum samples were reacted with capture antibody coated on 96-well plate. Subsequently, biotin-conjugated detection antibody was added, and the specific binding was detected using streptavidin-horseradish peroxidase and substrate solution. Optical density was measured at a wavelength of 450 nm using a Model 450 Microplate Reader (Bio Rad, Hercules, CA, USA) and the concentration was calculated from a standard curve that was prepared from a standard protein assay.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

To examine the expression level of cytokines and the frequency for NK cell appearance, RT-PCR analysis was carried out on the uteri collected from unmated mice after day 0 of danazol treatment. The primer sequences and references for M-CSF, GM-CSF, LIF, and Ly49 [19–21] are summarized in Table 1. The expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Total RNA was extracted from homogenized frozen uteri using TRIzol® LS reagent (Invitrogen, Carlsbad, CA, USA), and treated with DNase I (Invitrogen) for digestion of contaminated DNA. The amount of RNA was measured using a SmartSpec Plus spectrophotometer (Bio Rad) and diluted uniformly. RNA was mixed with Oligo (dT)12-18 primer (Invitrogen) and heated at 70 C for 5 min. Subsequently, cDNA was synthesized by RT reaction with MMLV RNase (H- point mutant (Promega, Madison, WI, USA) and dNTP (Takara Bio, Ohtsu, Japan) at 42 C for 1 h and 90 C for 5 min. The reaction was stopped at 4 C. Then, PCR reaction on cDNA was carried out with specific primers and Ex Taq™ DNA polymerase (Takara Bio), using a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA); pre-incubation at 95 C for 5 min; and 30–35 cycles at 94 C for 15 sec, 56–60 C (corresponding to the primer Tm point) for 30 sec, 72 C for 1.5 min, and finally at 72 C for 7 min. The reaction was stopped at 10 C. The PCR products were fractionated using 1.0% agarose gel and stained with ethidium bromide.

Histological studies

Based on RT-PCR analysis, some immunological molecules were further examined at the histological level. Immunohistochemical analysis was carried out using paraffin sections that were prepared from uteri collected after day 0 of danazol treatment. Hydrated sections were incubated at 4 C overnight with a primary antibody; rabbit polyclonal anti-M-CSF antibody (H-300), goat polyclonal anti-Ly49 antibody (V-17), and goat polyclonal anti-α5 integrin (C-19), all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Detection of the primary antibody was conducted using a Vectastain® Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. Briefly, sections were incubated with a biotinylated secondary antibody and then avidin/biotin-conjugated horseradish peroxidase. The peroxidase activity was detected with diaminobenzidine (DAB; Vector Laboratories) or 3-amino-9-ethylcarbazole (AEC; Vector Laboratories). Sections were briefly counterstained with hematoxylin.

Statistical analysis

Statistical differences between the danazol-treated and control mice in each experiment were evaluated by the Student’s t-test or chi-square test. Significant and most significant differences were determined when values were P<0.05 and P<0.01, respectively.
Results

Effects of danazol on mouse reproductive physiology

The estrous cycle was disturbed and prolonged following danazol treatment in a dose-dependent manner (Table 2). High doses of danazol prolonged the estrous cycle especially in the metestrous and/or diestrous stages, and 75 mg/kg of danazol fully stopped the estrous cycle in all treated mice. Danazol treatment also influenced the ovarian cycle. There was little influence on the number of mature follicles; however, the number of corpora lutea decreased following treatment with 7.5 and 75 mg/kg of danazol. A dose of 7.5 mg/kg of danazol was considered an appropriate dose to gently change the internal conditions of the uterus, through hormonal effect.

Interestingly, 7.5 mg/kg of danazol showed an improvement effect for pregnancy (Table 3). Compared with controls, the pregnancy rate was significantly increased in the danazol-treated mice. The number of implantation sites at day 5 of pregnancy was not clearly altered. The period for successful copulation was significantly prolonged after danazol treatment.

Effects of danazol on cytokines and NK cells

Serum IFN-γ, IL-12, and IL-4 could not be detected by ELISA in both the control and danazol-treated mice. Expression of GM-CSF and LIF was barely detectable in the uteri of both the control and danazol-treated mice. A significant increase was observed in the serum level of the M-CSF after danazol treatment compared with the controls (Fig. 1A). The increase in serum M-CSF was maintained for as long as 8 days. Analogous to serum levels, RT-PCR analysis showed that transcription of M-CSF was clearly detected in 40% of uteri following danazol treatment, although this was absent in the controls (Fig. 1B).

Transcription of Ly49A was detected and increased in the uteri of danazol-treated mice compared with the controls (Fig. 2A and B). While Ly49C was found in the uteri of both the treated and control mice (Fig. 2A), the expression level was significantly enhanced in the danazol-treated mice (Fig. 2B). Immunohistochemical analysis revealed that there were Ly49-positive cells at the endometrial epithelium and the stroma in the control uteri (Fig. 3A). The number of Ly49-positive cells was increased in the danazol-treated mice, showing abundant accumulation to the epithelium (Fig. 3B). A similar expression pattern was observed in the immunohistochemistry of M-CSF, which showed a clear increase in positive reaction in the endometrium of danazol-treated mice (Fig. 3C and D).

Additionally, in the danazol-treated mice, a

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<th>Table 2. Effects of danazol on mouse reproductive physiology</th>
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<td>Length of the estrous cycle (day)</td>
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<tr>
<td>No. of mature ovarian follicles</td>
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No. in parenthesis: no. of mice used. *P<0.05 and **P<0.01 vs. 0 mg/kg, respectively.

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<th>Table 3. Effects of danazol on mouse fertility</th>
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<td>Control</td>
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<td>Pregnancy rate (%)</td>
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No. in parenthesis: no. of mice used. *P<0.05 vs. control. *7.5 mg/kg of danazol.
The strong positive reaction was detected for α5 integrin in the cytoplasm of epithelial cells, although it was weak in the controls (Fig. 3E and F).

Discussion

Our analysis of danazol-treated mice showed an increase in the pregnancy rate, indicating the clinical value of danazol in the pretreatment of IVF-ET. However, the danazol-treated mice needed more than 3 extra days to achieve successful copulation after treatment compared with the controls. It seems that an adjustment period, equivalent to an estrous cycle, is required for preparation of conception. This supports the clinical protocol of IVF-ET, which is performed after a 1 month stationary period after danazol pretreatment [4].

Danazol treatment enhanced the expression levels of uterine Ly49A and/or Ly49C, and this indicates that many NK cells were recruited locally.
in the uterus. Generally, cytotoxicity in Ly49A- and Ly49C-positive NK cells is regulated via recognition of H-2\textsuperscript{d} antigen [22], and interestingly, the H-2\textsuperscript{d} antigen is a specific MHC haplotype for the BALB/c mice used in the present study. Together with the results of Ly49 immunohistochemistry, this suggests that low cytotoxic uNK cells gather in the endometrium after danazol treatment. It has been considered that there is a close relationship between the cytotoxic activity of NK cells and the occurrence of unidentified recurrent spontaneous abortion [6]. An examination of the phenotype of NK cells in aborted patients showed specific changes, such as a
decrease in low cytotoxic CD16−CD56bright NK cells, and conversely, an increase in high cytotoxic CD16+CD56dim NK cells [5]. Additionally, it has been clarified that in the normal uterus of the pregnant mouse, low cytotoxic uNK cells display chemotactic activity in the presence of some extracellular matrix proteins, such as fibronectin and laminin [8]. Danazol is thought to have a reproductive effect relating to recruitment of NK cells, which have a low cytotoxic phenotype, in the endometrium.

The balance between Th-1/Th-2 cytokines has a strong influence on the functional maturation and cytotoxicity of NK cells [17, 18]. In the present study, however, notable expression of Th-cytokines could not be detected in either the danazol-treated mice or controls, indicating no relevance between the effect of danazol and functional regulation of helper T cells. On the other hand, clear elevation of M-CSF was observed in sera and uteri following danazol treatment. M-CSF has conventionally been considered to be a prime candidate factor for an implantation initiator [23]. In the female reproductive tract, M-CSF is derived from the endometrium and trophoblast, and interestingly, NK cells in the human and mouse uterus can also produce M-CSF [9, 10]. These reports support our results and indicate a close correlation between Ly49-positive NK cells and M-SCF production in the uterus. We believe that at the very least, elevation of the amount of uterine M-CSF is dependent on the accumulation of uNK cells. Danazol may influence the phenotype and function of uNK cells, inducing a decrease in cytotoxic activity and M-CSF production in the uterus.

M-CSF in the uterus is known for its contribution to successful implantation by enhancing expression of cell adherent molecules, such as fibronectin and α5 integrin [24]. It is noteworthy that the α5β1 subunit of integrin plays a crucial role in embryo-endometrial interaction, and its up-regulation is observed in human decidua cells and migrating trophoblasts during the first trimester [25, 26]. Recently, a notable clinical report showed that danazol pretreatment applied to IVF-ET enhanced local expression of the αvβ3 subunit of integrin in the endometrium of inexplicably infertile patients [27]. Together with our results, we believe that the danazol-induced improvement in pregnancy rate can be accounted for by enhanced accumulation of uNK cells in the endometrium, resulting in up-regulation of local M-CSF and some types of integrins, such as the α5β1 subunit. These effects will be beneficial to create an implantation window in the uterus.

The results of the present study suggest that the reproductive effect of danazol is based on the improvement of immunological conditions in the form of chain-reactive induction of cytokines and integrins in the endometrium. Therefore, danazol may be suitable in cases of infertility caused by immunological disorders, such as patients with autoimmune diseases and endometriosis. Further investigation of the danazol effect will assist in understanding the pathological mechanism in inexplicably infertile patients, which, in return, will improve the quality of treatment for human reproduction.

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**References**

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