Danazol effects on human endometrial cells *in vitro*

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Summary

Although danazol has been reported to inhibit endometrial cell growth *in vitro*, it is difficult to accept that this is through direct inhibition of danazol on endometrial cells. This is because local danazol therapy improves endometriotic signs and symptoms without any disturbance in ovulation or the menstrual cycle. We have re-evaluated the effects of danazol on human endometrial cells by using normal human endometrial stromal cells and two cell lines derived from highly-differentiated endometrial adenocarcinomas. Danazol is difficult to dissolve in aqueous solutions, and undissolved danazol significantly inhibited endometrial cell growth even at less than 100 ng/ml. At 500 ng/ml of fully dissolved danazol, which is the therapeutic dose, danazol solution inhibited leukemic cell growth but not endometrial cell growth. Therefore, the action of danazol may be not an inhibitory effect but rather a regulatory function of endometrial cell growth.

Key words: Endometriosis; Danazol; Endometrium; Endometrial stroma cell.

Introduction

Endometriosis occurs only in menstruating women. Both GnRH agonist therapy and oophorectomy can make endometriotic lesions atrophic. This means that endometriosis is a hormone-dependent disease, but it does not mean that estrogen by itself could never be considered as the cause. However, the pathogenesis of endometriosis remains unclear. The standard dose of danazol for endometriotic patients is 400 mg to 800 mg per day per os, inhibiting production of ovarian estrogen and the LH-FSH surge at the pituitary gland and causing dysovulation and ammenorrhea [1-9]. Thus, oral administration of danazol inhibits production of endogenous estrogen, which is believed to be the main mechanism by which danazol prevents endometriosis. However, local administration of low dose danazol, such as by vaginal danzol suppository [10-13], cervical injection of danazol suspension [14], or use of the intrauterine danazol ring [10], can improve endometriotic signs and symptoms without causing any menstrual disorders. From these clinical results, certain direct actions of danazol on endometriotic tissues have been thought to exist, but the mechanism of the direct action has not been made clear. Local danazol therapy does not inhibit either production of endogenous estrogen or ovulation [10, 12, 13]. To understand how local danazol therapy is pharmacologically effective for endometriosis could clarify the actual cause or pathogenesis of endometriosis.

There have been several reports of direct actions of danazol on endometrial cells [15, 16, 17, 18, 19]. In these reports, the direct effects of danazol were examined on the highly-differentiated endometrial adenocarcinoma cell line Ishikawa [15], surgically excised endometrial carcinoma cells [16], or normal human endometrial cells [17, 18, 19]. The respective investigators all concluded that danazol works on the cell directly by inhibiting proliferation. However, this is not fully convincing because a small dose of intrauterine danazol has an effect on endometriosis without disturbing normal ovulatory function [10]. If danazol inhibits endometrial cell growth directly, patients would complain of ammenorrhea due to atrophic endometrial cells. Therefore, we find a distinct discrepancy between the reported clinical effects of local danazol therapy and the *in vitro* inhibitory effect of danazol on endometrial cell growth. In the present study, we re-evaluated and clarified this discrepancy in *in vitro* studies using normal human endometrial stromal cells and two cell lines derived from highly-differentiated endometrial adenocarcinoma.

Material and Methods

Cell Lines and Cell Culture

As representative endometrial epithelial cells, two highly-differentiated human endometrial adenocarcinoma cell lines, HHUA [20] (obtained from Riken Cell Bank, Tsukuba, Japan) and Ishikawa [15] (courtesy of Dr. Nishida, Tsukuba University Japan), were used in the study. Normal human endometrial stromal cells were established by *in vitro* primary culture of discarded cells from tissue samples obtained for endometrial cancer screening from women with normal menstrual cycles with their informed consent. Three human premyelocytic leukemia cell lines, HL60 [21], U937 [22], and THP-1 [23], were examined in this study as positive controls for the direct effect of danazol. All cells were cultured in Opti-MEM medium (GIBCO-BRL) / 5% fetal bovine serum / 100 U/ml Penicillin (GIBCO-BRL) / 100 µg/ml Streptomycin (GIBCO-BRL).

Preparation of Diluted Danazol Solution

Dilution-A: Stock danazol solution (10 mg/ml of danazol in 99.5% ethanol) was diluted directly with a culture medium and then added to the cell cultures. Because danazol is less soluble in aqueous solutions, danazol prepared this way made the culture media white and turbid, and unsoluble danazol crystals were found in the cultures.

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Dilution-B: In order to dissolve danazol thoroughly in the culture media, the same stock danazol solution was diluted as follows with ethanol to make 100 μg/ml danazol in ethanol, then with the culture medium to make 5 μg/ml danazol in 5% ethanol, and finally stepwise with culture medium containing 5% v/v ethanol. One ninth volume of the stepwise diluted solution was added to each cell culture so that all cultures contained 0.5% v/v ethanol. Ethanol was also added to the control cell cultures for a final 0.5% v/v ethanol concentration. Using Dilution-B, neither insoluble danazol crystals nor white turbidity was found in cell cultures.

Cell Proliferation Assay

Cell proliferation was measured using a non-RI colorimetric cell proliferation assay kit, XTT (Boehringer-Mannheim). Briefly, cells at log phase were detached with 0.25% trypsin/1 mM EDTA solution (GIBCO-BRL), cultured in a 96-well tissue culture plate at 100 μl/well (5,000 cells/well) overnight, and on the following day the danazol solution in 0.5% v/v ethanol was added to each cell culture. Danazol effects on cell proliferation of endometrial cells were examined on the cells cultured both short-term (2-day culture) and long-term (14-day culture). Danazol solution prepared as Dilution-B was administered to those cell cultures every other day and cell proliferations were assayed on the second day or 14th day. More than three assays were performed for each experiment to verify the results. Statistical analyses were performed by Student's t-test or ANOVA depending on the protocol. Significance was set at $p<0.05$. Figures represent means±SEM of 5 experiments.

Results

Effects of danazol prepared as Dilution-A (Fig. 1)

Cell proliferations of HHUA and endometrial stromal cells were suppressed dose-dependently by danazol in the 2-day culture. However, danazol crystals were microscopically detected in the culture containing more than 100 ng/ml of danazol, though cell growth was strongly suppressed.

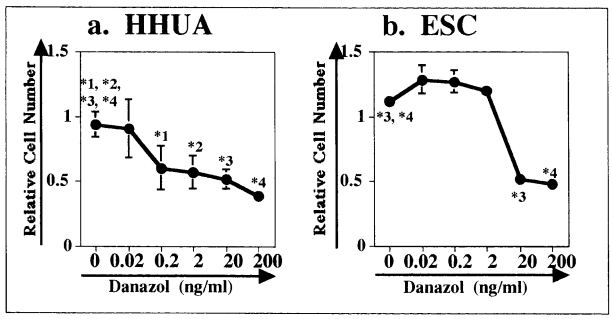

Figure 1. — *Endometrial cell growth suppressed by danazol prepared as Dilution-A.*
a. HHUA cells. b. Normal human endometrial stromal cells (ESC). Relative cell numbers are expressed as absorbance at 450 nm by the cell proliferation assay kit and represent the mean ± SEM of 5 experiments (the error bars of some of the points lie within the size of the symbols). *1, *2; $p<0.05$. *3, *4; $p<0.01$.

Effects of danazol prepared as Dilution-B (Fig. 2)

Danazol as Dilution-B did not show any effect on endometrial cell growth for 2 days, although danazol solution as Dilution-B suppressed cell growth of the three leukemic cell lines (Fig. 2). Danazol at a concentration of 100-500 ng/ml – which showed no effect on endometrial cell growth in this study – is reported to coincide with the concentration of serum danazol found in patients who are administered danazol orally (400-800 mg/day). Danazol crystals could not be detected microscopically in these cell culture plates.

Danazol effect on cell proliferation of endometrial cells cultured long-term (Fig. 3)

In order to examine cell growth of endometrial cells stimulated repetitively with danazol as Dilution-B, danazol solution was administered to those cell cultures

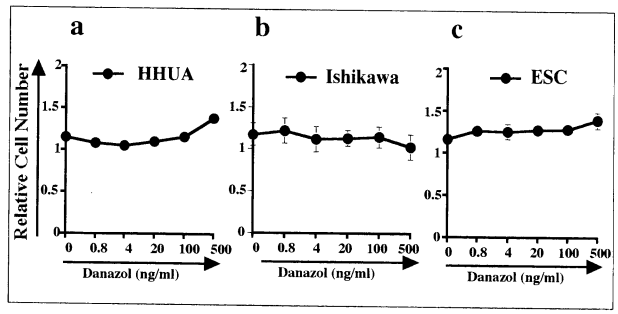

Figure 2. — *Effect on short-term endometrial cell growth by danazol prepared as Dilution-B.*
a. HHUA cells. b. Ishikawa cells. c. Normal human endometrial stromal cells (ESC). Those cells were cultured 2 days after addition of danazol. Relative cell numbers are expressed as absorbance at 450 nm by the cell proliferation assay kit and represent the mean±SEM of 5 experiments (the error bars of some of the points lie within the size of the symbols). No significant effect of danazol on cell proliferation was found in these 3 human endometrial cells.

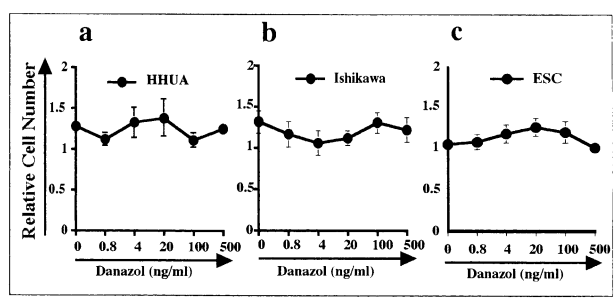

Figure 3. — *Long-term effect on endometrial cell growth by danazol prepared as Dilution-B.*
a. HHUA cells. b. Ishikawa cells. c. Normal human endometrial stromal cells (ESC). Fresh danazol solution was added to the cultured cells every other day for 14 days. Relative cell numbers are expressed as absorbance at 450 nm by the cell proliferation assay kit and represent the mean±SEM of 5 experiments (the error bars of some of the points lie within the size of the symbols). No significant effect of danazol on cell proliferation was found in these 3 human endometrial cells.

every other day and 14 days after their cellular proliferations were assayed. As shown in Fig. 3, danazol as Dilution-B did not show any effect on cell proliferations of HHUA, Ishikawa, or endometrial stromal cells within the 14 days.

Discussion

Our results suggest that growth suppression of endometrial cells may not be a result of a direct action of danazol on endometrial cells. Although it has been reported in previous studies that danazol suppressed in vitro both endometrial epithelial cell growth [15, 16, 17, 18] and endometrial stromal cell growth [19], we found no growth suppression of endometrial cells under a danazol concentration of 500 ng/ml. However danazol suppressed leukemic cell growth dose-dependently at this same concentration. Moreover, these non-cytotoxic effects of danazol on endometrial cells were found in cells cultured both short-term and long-term. Danazol concentrations in serum and peritoneal fluid have been reported to be below 5 ng/ml in endometriotic patients who received danazol vaginal suppositories and whose endometriotic symptoms were improved, without any apparent disturbance of endogenous estrogen production or ovulation [10, 12, 13].

These clinical facts indicate that danazol causes some non-cytotoxic effects on endometrial cells but without growth-suppressive effects. Despite the indication of some direct non-cytotoxic effects of danazol on endometrial cells, danazol might regulate homeostasis of endometrial, danazol might regulate homeostasis of endometrial tissues within the range of normal physiological variation if serum estradiol is within normal limits.

Danazol is also considered to play a role on autoimmunity as an immunomodulatory drug [24, 25, 26, 27], because oral danazol improves symptoms associated with certain autoimmune diseases and decreases serum autoantibody levels [28, 29, 30]. Danazol is also thought to have some regulatory effects on local endocrine systems because it can bind to steroid receptors [31, 32, 33, 34, 35] and suppress activity of certain steroid synthetases [36, 37, 38]. These are indications that danazol may regulate the proliferation and differentiation of endometrial tissues indirectly as a result of effects on the immune or local endocrine systems.

Endometrial stromal cells produce various growth factors to support proliferation and differentiation of the endometrial epithelium. Surrey and Halme reported that danazol inhibited endometrial stromal cell proliferation [19]. However the present study showed no apparent inhibitory effect of the danazol solution, which was prepared as Dilution-B not as Dilution-A, on cell proliferation of endometrial stromal cells. Accordingly, indirect growth inhibition of endometrial stromal cells cannot be a main mechanism of local danazol therapy.

From our findings, it can be said that the main pharmacological mechanism of local danazol therapy may be balancing proliferation and differentiation rather than inhibiting cell growth directly. We are currently investigating novel intracellular signals in endometrial cells stimulated with danazol. We postulate that these signals regulate endometrial epithelial cell growth. The mechanism of local danazol therapy may be an indirect modulatory effect on endometrial cell growth via the immune or endocrine systems. Further analyses on the effects of local danazol therapy will help clarify the actual pathogenesis of endometriosis.

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