Effects of dienogest, a synthetic steroid, on experimental endometriosis in rats

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Abstract

Objective: Dienogest, a synthetic steroid with progestational activity, is used as a component of oral contraceptives and is currently being evaluated clinically for the treatment of endometriosis. The present study was conducted to confirm the effects of dienogest on experimental endometriosis in rats and to elucidate its mechanism of action.

Design: Experimental endometriosis induced by autotransplantation of endometrium in rats.

Methods: Endometrial implants, immune system, and bone mineral were investigated after 3 weeks of medication.

Results: Dienogest (0.1–1 mg/kg per day, p.o.) reduced the endometrial implant volume to the same extent as danazol (100 mg/kg per day, p.o.). Simultaneously, dienogest ameliorated the endometrial implant-induced alterations of the immune system; i.e. it increased the natural killer activity of peritoneal fluid cells and splenic cells, decreased the number of peritoneal fluid cells, and decreased interleukin-1β production by peritoneal macrophages. In contrast, danazol (100 mg/kg per day, p.o.) and buserelin (30 μg/kg per day, s.c.) had none of these immunologic effects. Additionally, combined administration of dienogest (0.1 mg/kg per day) plus buserelin (0.3 μg/kg per day) suppressed the bone mineral loss induced by buserelin alone, with no reduction of the effect on endometrial implants. In vitro studies on dienogest revealed an antiproliferative effect on rat endometrial cells due to inhibition of protein kinase C activity plus a partial progestational effect.

Conclusions: Dienogest appears to be a potent agent with mechanisms of action different from those of danazol and GnRH agonists currently available for the treatment of endometriosis.

European Journal of Endocrinology 138 216–226

Introduction

Endometriosis is an estrogen-dependent disease for which ovariectomy is the most effective treatment. However, many women with endometriosis wish to become pregnant, so medication is the primary treatment for this condition (1). The medications most commonly used include danazol (2), gonadotropin-releasing hormone agonists (GnRH-a) (3), and progestins such as medroxyprogesterone acetate (MPA) (4). Although these agents are relatively effective against endometriosis, they produce a high incidence of adverse reactions. Danazol causes weight gain due to its anabolic/androgenic activity, as well as abnormal lipid metabolism, liver dysfunction, and cerebral thrombosis (5). MPA causes weight gain and thrombosis (6), whereas GnRH-a causes menopause-like symptoms and a decrease in bone mineral density (BMD) attributable to estrogen lack (7). Therefore, the development of a new agent that is safer than those currently available for endometriosis would be highly desirable.

Dienogest, 17α-cyanomethyl-17β-hydroxy-estra-4,9-dien-3-one, is a synthetic steroid that has prominent progestational activity, and it has been clearly shown to lack androgenic, estrogenic, anti-estrogenic, and corticoid-like activities (8). With this hormonal profile, dienogest appears to cause few side-effects in its clinical use. This compound was initially assessed for its contraceptive value, and it is now available in Germany as a low-dose pill in combination with ethinylestradiol. Currently, it is being investigated as an agent for the treatment of endometriosis in Germany, France, and Japan.

In the present study, the effects of dienogest on a rat model of endometriosis were evaluated in comparison with those of danazol and buserelin, and the effectiveness of combined therapy with dienogest plus buserelin was also examined. Both alone and in combination with buserelin, dienogest was effective for the treatment of experimental endometriosis in rats. The mechanism of its effect on endometriosis was also investigated by assessing changes in immune function as well as those in cAMP and protein kinase activity.
Materials and methods

Test drugs
Dienogest (Jenapharm GmbH, Jena, Germany), danazol (Sigma Chemical Co., St Louis, MO, USA), progesterone (Sigma Chemical Co.), and buserelin (Hoechst Japan, Tokyo, Japan) were obtained from the indicated sources. RU-486 was kindly provided by Roussel-Uclaf Co. Ltd (Romainville, France).

Animals
Eight-week-old female Sprague–Dawley rats were purchased from Charles River Japan Inc. (Yokohama, Japan). The animals were maintained at room temperature (23 ± 2°C) and 55 ± 15% relative humidity in a room with a 12 h light/12 h darkness cycle and were given a sterilized solid diet (gamma ray-irradiated CRF-1, Oriental Yeast, Tokyo, Japan) and free access to water during the experimenal period. A vaginal smear test was performed to check the estrous cycle in all rats before use in the experiment. At the end of the study period, the animals were killed by exsanguination under anesthesia with sodium pentobarbital (50 mg/kg i.p.). The guidelines of Mochida Pharmaceutical Co. Ltd were followed for the care and use of the animals in this study.

In vivo studies
Preparation of a rat model of endometriosis At the age of 9 weeks, experimental endometriosis was induced in rats at the proestrous stage of the estrous cycle by autotransplantation of endometrium to a renal subcapsular site (9). After laparotomy under anesthesia with sodium pentobarbital (50 mg/kg i.p.), the left uterine horn was resected, and the endometrium was dissected from the myometrium. Then a 5 × 5 mm fragment of endometrial tissue was transplanted beneath the left renal capsule of the same animal. Two weeks later, a second laparotomy was done to determine the viability of the endometrial implant from the accumulation of fluid, and the dimensions (length, width, and height) of the implant were measured to calculate its volume. Animals with implants having a volume between 20 and 60 mm³ were used in the study.

Effect of various treatments on experimental endometriosis Rats with experimental endometriosis were randomly divided into eight groups: vehicle alone (control; n = 10), dienogest (0.1 mg/kg per day, p.o.; n = 10), dienogest (0.03 mg/kg per day, p.o.; n = 10), danazol (100 mg/kg per day, p.o.; n = 10), danazol (0.1 mg/kg per day, p.o.; n = 10), buserelin (30 μg/kg per day, s.c.; n = 10), and an ovariectomized group (n = 9) treated with vehicle alone. After 3 weeks of treatment, the dimensions of the endometrial implants were measured as described above. Then each implant was excised and fixed in 10% phosphate-buffered formalin for histologic evaluation. The uterus was also removed from each rat and weighed. Moreover, the left femur was removed and fixed in 10% phosphate-buffered formalin for examination of BMD. The volumetric BMD of the distal femur was determined by peripheral quantitative computed tomography (XCT-960A, Norland-Stratec, Fort Atkinson, WI, USA) (10), and the density was calculated separately for the trabecular, cortical, and total bone.

Effect of buserelin or dienogest/buserelin on experimental endometriosis Other rats with experimental endometriosis were randomly divided into three groups: vehicle alone (control; n = 10), buserelin (0.3 μg/kg per day, s.c.; n = 10), and dienogest (0.1 mg/kg per day, p.o.) plus buserelin (0.3 μg/kg per day, s.c.; n = 10). After 3 weeks of treatment, measurement of the endometrial implants, histologic examination of the implants, and assessment of BMD of the left femur were performed as described above. Additionally, peritoneal fluid was recovered from the peritoneal cavity and the spleen was excised for the assay of natural killer (NK) activity.

Measurement of NK activity Mononuclear cell suspensions from the peritoneal fluid and spleen were obtained by centrifugation at 450 g for 30 min at 4°C on a Percoll–Conray gradient (Dai-ichi Chemical Co., Tokyo, Japan), and then the number of cells was adjusted to 1×10⁶/ml. The NK assay was performed according to the method of Blomberg et al. (11). In brief, mononuclear cells were washed in RPMI 1640 supplemented with 10% fetal calf (FCS) (Dainippon Pharmaceutical Co., Osaka, Japan) prior to being combined with the target cells, which were K562 cells (Dainippon Pharmaceutical Co.) prelabeled with europium-DTPA (Sigma Chemical Co.). One hundred microliters (10⁶ cells) of the labeled target cell suspension and 100 μl (10⁴ cells) of the effector cell suspension (effector to target ratio 100:1) were added to each well of a microplate (Nunc, Roskilde, Denmark), and the cells were incubated at 37°C for 4 h under a humidified atmosphere of 5% CO₂–95% air. Then the supernatant was collected and europium fluorescence was determined with a time-resolved fluorometer (DELFIA, Pharmacia Biotech Co., Uppsala, Sweden). NK activity was expressed as the percentage of lysis of target cells, taking the value for cells treated with 2% sodium dodecyl sulfate as 100% and that for cells treated with medium alone as 0%.

Effect of various treatments on NK activity More rats with experimental endometriosis were randomly divided into five groups (n = 7/group): vehicle alone (control), dienogest (0.1 mg/kg per day, p.o.), danazol (100 mg/kg per day, p.o.), buserelin (30 μg/kg per day, s.c.), and 2% sodium dodecyl sulfate.
s.c.), and an ovariectomized group treated with vehicle alone. Additional age-matched animals without endometriosis were used as an intact group \((n = 5)\). After 3 weeks of treatment, measurement of the endometrial implants, recovery of peritoneal fluid, and splenectomy were carried out as described above. Additionally, the volume of the peritoneal fluid was determined according to a modification of the method of Carter et al. \((12)\), and the number of cells in the fluid was counted under a light microscope.

**Effect of dienogest on interleukin-1β (IL-1β) production by peritoneal macrophages** A final set of rats with experimental endometriosis was randomly divided into two groups \((n = 10/\text{group})\): vehicle alone (control) and dienogest \((0.1 \text{ mg/kg per day, p.o.})\). Additional age-matched animals without endometriosis were used as an intact group \((n = 5)\). After 3 weeks of treatment, peritoneal macrophages were recovered for the assay of IL-1β production.

**Isolation of peritoneal macrophages and measurement of IL-1β** Peritoneal macrophages were collected from the peritoneal fluid by the adhesion method \((13)\) and were suspended in medium after adjustment to \(10^5 \text{ cells/ml}\). An aliquot of the cells \((10^5 \text{ in } 0.1 \text{ ml})\) was added to each well of a 24-well plate and incubated at 37°C for 24 h under a humidified atmosphere of 5% CO₂-95% air. Then the medium was centrifuged at 300,000 g for 5 min at 15°C, the supernatant was concentrated with a SMART system (Pharmacia Biotech Co.), and the IL-1β level in the concentrate was measured by a time-resolved-fluoroimmunoassay using the DELFIA system \((14)\). Briefly, rabbit anti-murine IL-1β antibody (Genzyme Co., Cambridge, MA, USA) was added to each well of a 96-well microplate precoated with anti-rabbit IgG antibody and was incubated at room temperature for 1 h with shaking. Then each well was washed with DELFIA washing solution and blocked with Block Ace (Dainippon Pharmaceutical Co.). Next, the concentrated supernatant and europium-labeled murine IL-1β (Genzyme Co.) were added to each well and incubated at room temperature for 1 h. After the wells had been washed with DELFIA washing solution, DELFIA enhancement solution was added; and incubation was continued at room temperature for 5 min with shaking. Finally, europium fluorescence was measured with a DELFIA fluorometer, and the IL-1β content was calculated from the standard curve prepared previously.

**In vitro studies**

**Isolation of rat endometrial cells** Under anesthesia with sodium pentobarbital \((50 \text{ mg/kg, i.p.})\), the uterus was removed from 24 rats and immediately placed into PBS. Each uterus was turned inside out and then incubated for 15 min at 37°C with shaking in Dulbecco’s medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 2% FCS pretreated with charcoal, 0.5% type I collagenase (Nitta Gelatin Co., Tokyo, Japan), and 0.02% deoxyribonuclease I (Sigma Chemical Co.) \((15)\). The uterus was incubated repeatedly in the same medium, and the endometrial cells were collected after each 15 min incubation by filtering them through a nylon mesh filter (Nippon Becton Dickinson Co., Tokyo, Japan). The combined filtrate was then centrifuged at 300 g for 5 min at room temperature. The cell pellet was washed in culture medium (Dulbecco’s MEM supplemented with 2% FCS pretreated with charcoal) and suspended in the same medium after adjustment of the cells to \(5 \times 10^5/\text{ml}\).

**Effect of drugs on the proliferation of rat endometrial cells treated with β-estradiol** One milliliter of endometrial cell suspension \((5 \times 10^5 \text{ cells})\) was added to each well of a 6-well plate and incubated at 37°C for 17 h under a humidified atmosphere of 5% CO₂-95% air. Then 10 μl of 17β-estradiol (Sigma Chemical Co.) solution \((4 \text{ ng/ml})\) were added to each well, and incubation was continued for an additional 8 h. Next, the medium was replaced with medium containing 10 μl of a test agent solution, i.e., dienogest \((3.2 \times 10^{-11}, 3.2 \times 10^{-10}, 3.2 \times 10^{-9}, \text{ and } 3.2 \times 10^{-8} \text{ M as final concentrations})\), danazol \((3.0 \times 10^{-11}, 3.0 \times 10^{-10}, 3.0 \times 10^{-9}, \text{ and } 3.0 \times 10^{-8} \text{ M})\), buserelin \((8.1 \times 10^{-11} \text{ and } 8.1 \times 10^{-9} \text{ M})\), or progesterone \((3.2 \times 10^{-10}, 3.2 \times 10^{-9}, \text{ and } 3.2 \times 10^{-9} \text{ M})\). Then the cells were incubated for 48 h in the absence or presence of RU-486 \((1 \text{ ng/ml}, 100 \mu \text{M of } 5\text{-bromodeoxyuridine (BrdU, Sigma Chemical Co.) solution was added to each well, and incubation was continued at } 37°C \text{ for an additional } 2 \text{ h. The supernatant was collected for the determination of lactate dehydrogenase (LDH) activity, and the cells were washed with PBS (pH 7.4) prior to the measurement of BrdU uptake.})

**Measurement of BrdU uptake** Cells were fixed with methyl alcohol at room temperature for 30 min and then washed with PBS containing 0.1% Tween 20 (washing buffer). Subsequently, a blocking agent solution (Block Ace, Dainippon Pharmaceutical Co.) was added to each well. After preincubation for 30 min at room temperature, the cells were incubated with an anti-BrdU antibody and deoxyribonuclease (Amersham International plc., Buckinghamshire, UK) solution at room temperature for 1 h. After washing of the cells with the washing buffer, the europium-labeled anti-mouse IgG antibody (Pharmacia Biotech Co.) was applied as described above \((16)\). Europium fluorescence was measured with a DELFIA fluorometer, and the BrdU content was calculated from the standard curve prepared previously.

**Measurement of LDH leakage** The incubation medium of cells treated with various agents was centrifuged at 300 g for 5 min at 15°C, and the LDH activity in the supernatant was determined with an MTX LDH-kit.
LDH leakage was assessed from the absorbance at 560 nm. Effect of drugs on the cAMP content, protein kinase A (PKA) activity, and protein kinase C (PKC) activity of rat endometrial cells treated with β-estradiol

The cAMP content and PKA and PKC activities were measured after treatment of endometrial cells with dienogest (3.2×10⁻¹¹, 3.2×10⁻¹⁰, 3.2×10⁻⁹, and 3.2×10⁻⁸ M as final concentrations), danazol (3.0×10⁻¹¹, 3.0×10⁻¹⁰, 3.0×10⁻⁹, and 3.0×10⁻⁸ M), or progesterone (3.2×10⁻¹¹, 3.2×10⁻¹⁰, 3.2×10⁻⁹, and 3.2×10⁻⁸ M) in the absence or presence of RU-486 (1 ng/ml) as described above.

Measurement of cAMP After 100 µl of cAMP tracer solution (Cayman Chemical Co., Ann Arbor, MI, USA) had been added to each well of a 96-well microplate, the solution was decanted; and the microplate was washed with PBS (pH 6.8) containing 0.05% Tween 80 (washing buffer). Then the wells were blocked with Block Ace and washed again with the washing buffer. Next, 10 µl of endometrial cell homogenate (10⁴ cells) and 100 µl of murine anti-cAMP antibody (Cayman Chemical Co.) were added to each well and incubated at 20°C for 2 h with stirring. After discarding the solution and washing the wells with the washing buffer, 100 µl of europium-labeled anti-mouse IgG antibody was added to each well (18). Europium fluorescence was measured with a DELFIA fluorometer, and the cAMP content was calculated from the standard curve prepared previously.

Measurement of PKA and PKC activities The activities of PKA and PKC were measured with a non-radioisotopic protein kinase assay kit (MBL Co. Ltd, Nagoya, Japan) modified for a time-resolved fluororimmunoassay using the DELFIA system (19). Microplate wells coated with a synthetic peptide (PS-peptide: R-F-A-R-K-G-A-L-R-Q-K-N-V) were loaded with 12 µl of endometrial cell homogenate (10⁴ cells) along with 108 µl of a reaction mixture containing 25 mM Tris–HCl (pH 7.0), 3 mM MgCl₂, 0.1 mM ATP, 0.5 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol. For the PKA assay, 2 µM cAMP solution was added to the mixture, whereas 50 µg/ml phosphatidylserine and 2 mM CaCl₂ were added for the PKC assay. After incubation at 25°C for 5 min, the reaction was stopped by the addition of 100 µl of 20% H₃PO₄ solution. Then the wells were washed, 100 µl of mouse anti-phosphorylated PS-peptide antibody (clone 2B9) were added to each well, and incubation was carried out at 25°C for 30 min with shaking. After further washing, 100 µl of europium-labeled anti-mouse IgG antibody was added to each well. Europium fluorescence was measured with a DELFIA fluorometer, and the activities of PKA and PKC were calculated from the standard curves prepared previously. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of phosphorylated peptide per minute.

Statistical analysis

The results of each experiment were expressed as the mean±S.D. Dunnett’s test was used to assess the significance of differences between groups in multiple comparisons. For comparison between two groups, Student’s t-test was used.

Results

In vivo studies

Effect of various treatments on experimental endometriosis At a dose of 0.03 mg/kg, dienogest did not decrease the volume of endometrial implants.
whereas all the other doses tested (0.1, 0.3, and 1 mg/kg) caused a significant decrease. The effect of dienogest was comparable to that of danazol, but less marked than that of buserelin or ovariectomy, both of which also significantly decreased the implant volume compared with that of the control (Fig. 1, upper panel).

On histologic examination (Fig. 2), cysts protruding from the renal surface and covered by the renal capsule were found at the sites of all implants. The size of the cyst observed microscopically reflected the volume of the implant at final laparotomy. The walls of each implant cyst were composed of epithelium and stroma. In the control group, the implants showed histologic features reflecting the estrous cycle-dependent nature of the uterine epithelium. For example, the implants from control rats at the proestrous stage were composed of tall columnar epithelial cells with large, round nuclei and slightly hypertrophic stromal cells (Fig. 2A and B). In the dienogest-treated groups, the implants displayed epithelial cells similar to those of control rats at the proestrous stage (Fig. 2C and D). The implants of danazol-treated rats resembled those of dienogest-treated rats (Fig. 2E and F). In the buserelin-treated rats, the implants were composed of a thin, atrophic epithelium with pyknotic nuclei (Fig. 2G and H). In the ovariectomized rats, the epithelium was even more atrophic than in the buserelin-treated rats, and a sparse nuclear arrangement was noted (Fig. 2I and J), which was similar to that of the uterus during diestrus. The uterine weight in the dienogest-treated rats (192.8±48.9 mg) was comparable to that of the control rats (195.4±44.1 mg), whereas buserelin significantly decreased the uterine weight (74.0±12.5 mg, P<0.01).

None of the doses of dienogest tested had any effect on the BMD of the distal femoral trabecular and cortical bone. On the other hand, danazol, buserelin, and ovariectomy caused a significant decrease in the BMD of trabecular bone in the distal femur. Bone mineral loss increased in the order of danazol<buserelin<ovariectomy (Table 1).

**Effect of buserelin or dienogest/buserelin on experimental endometriosis** A dose of 0.3 μg/kg of buserelin significantly reduced the volume of the endometrial implants. Treatment with dienogest (0.1 mg/kg) plus buserelin (0.3 μg/kg) in combination also significantly decreased the volume of the endometrial implants. The left column of photomicrographs shows cysts protruding from the renal surface and encapsulated by the renal capsule. All the endometrial implants formed cysts. A decrease in the size of the cyst cavity is noticeable in the dienogest (C), danazol (E), buserelin (G), and ovariectomy (I, J) rats. The right column shows high-power views of the cyst walls composed of epithelium and stroma, indicating that they are endometrial tissues. In the control rat (B), tall columnar epithelial cells with large, round nuclei and slightly hypertrophic stromal cells are seen, and the histologic appearance coincides with that in the uterus at the proestrous stage. The dienogest (D) and danazol (F) rats show similar findings, i.e. the epithelium resembles that of the control rat at the proestrous stage. The danazol (F) and buserelin (G) rats show similar findings, i.e. the epithelium resembles that of the control rat at the proestrous stage. In the buserelin (H), the epithelium is atrophic. In the ovariectomized rat (J), the epithelium is more atrophic than in the buserelin-treated rat, and a sparse nuclear arrangement is seen. This is similar to that of the uterus at the diestrous stage. Hematoxylin and eosin stain; ×15 in A, C, E, G, and I; ×250 in B, D, F, H, and J.
decreased the implant volume and tended to reduce it more than buserelin alone, although there was no significant difference between the buserelin and dienogest/buserelin groups (Fig. 1, lower panel). On histologic examination of control rats at the diestrous stage, the epithelium of the implant was atrophic and showed a sparse nuclear arrangement (Fig. 3A and B). In the buserelin-treated rats, the columnar epithelium was low and displayed a dense nuclear arrangement (Fig. 3C and D). In the dienogest/buserelin-treated rats, the epithelium was also low, but it had a sparse nuclear arrangement (Fig. 3E and F). These findings were more similar to those for the control rats at the diestrous stage than to those for the buserelin-treated rats. On bone mineral examination, the buserelin-treated rats showed a significant decrease in the BMD of distal femoral trabecular bone. In contrast, combination therapy with dienogest and buserelin had no perceptible influence on BMD, which remained at the control level (Table 2).

Effect of various treatments on NK activity

The NK activity of peritoneal fluid cells and splenic cells was significantly decreased by renal subcapsular endometrial tissue autotransplantation (Table 3). A dose of 0.1 mg/kg of dienogest stimulated a significant increase in the NK activity of both peritoneal fluid cells and splenic cells, and the NK activity of these animals practically reached that of the intact group. Danazol (100 mg/kg) slightly increased the NK activity of peritoneal fluid cells, but had no positive effect on NK activity in the spleen. Treatment with buserelin or ovariectomy had no effect on the NK activity of cells from the peritoneal fluid or spleen. Thus, NK activity in the buserelin-treated or ovariectomized rats remained at the control level, which was dramatically lower than that in the intact group.

Dienogest also significantly decreased the number of cells in the peritoneal fluid, whereas treatment with danazol, buserelin, or ovariectomy had no such effect. The peritoneal fluid volume remained unchanged in all groups, including the intact one (data not shown).

Table 1 Effect of treatment with dienogest, danazol, buserelin, or ovariectomy on BMD of the distal left femur in rats with experimental endometriosis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg per day)</th>
<th>BMD (mg/cm³)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>702.3 ± 31.6</td>
</tr>
<tr>
<td>Dienogest</td>
<td>0.03</td>
<td>693.8 ± 48.5</td>
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<td></td>
<td>0.1</td>
<td>739.1 ± 65.4</td>
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<tr>
<td></td>
<td>0.3</td>
<td>729.9 ± 84.6</td>
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<tr>
<td>Danazol</td>
<td>100</td>
<td>709.3 ± 49.0</td>
</tr>
<tr>
<td>Buserelin</td>
<td>0.03</td>
<td>678.1 ± 38.5</td>
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<tr>
<td></td>
<td>0.3</td>
<td>639.5 ± 79.3*</td>
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<td></td>
<td>1</td>
<td>608.3 ± 61.3**</td>
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All values are expressed as the mean ± s.d. Data were analyzed by Dunnett’s test. *P < 0.05, **P < 0.01 vs control group.

Effect of dienogest on experimental endometriosis

Figure 3 Representative histologic appearance of experimental endometriosis in a control rat at the diestrous stage (A, B), and in rats given buserelin (0.3 μg/kg per day, s.c.) (C, D) and dienogest (0.1 mg/kg per day, p.o.) plus buserelin (0.3 μg/kg per day, s.c.) (E, F). The control rat at the diestrous stage has epithelium similar to that of the ovariectomized rat (Fig. 2J). In the buserelin rat, the epithelium is low and has a dense nuclear arrangement. It is similar to that in the buserelin (30 μg/kg per day, s.c.) rat (Fig. 2H). In the dienogest plus buserelin rat, the epithelium is also low, but it displays a sparse nuclear arrangement. It is not similar to that seen with buserelin or dienogest (Fig. 2D) monotherapy, but resembles that of the uterus at the diestrous stage. Hematoxylin and eosin stain; ×15 in A, C, and E; ×250 in B, D, and F.
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Effect of dienogest on IL-1b both peritoneal fluid cells and splenic cells. and buserelin significantly enhanced the NK activity of this model were reproducible. Fig. 1 (upper panel), indicating that data obtained with $P < 0.01$, just as occurred in the experiment shown in 4.5 mm$^3$, 6 danazol (13.7 $\pm$ 18.9 mm$^3$), danazol (13.7 $\pm$ 18.2 mm$^3$, $P < 0.01$), buserelin (3.7 $\pm$ 4.5 mm$^3$, $P < 0.01$), or ovariectomy (1.8 $\pm$ 3.3 mm$^3$, $P < 0.01$), just as occurred in the experiment shown in Fig. 1 (upper panel), indicating that data obtained with this model were reproducible.

Additionally, combination therapy with dienogest and buserelin significantly enhanced the NK activity of both peritoneal fluid cells and splenic cells.

Effect of dienogest on IL-1$\beta$ production by peritoneal macrophages A significant increase in IL-1$\beta$ production by peritoneal macrophages from rats with endometriosis was observed compared with the production in the intact group (Fig. 4). Treatment with dienogest (0.1 mg/kg) significantly decreased IL-1$\beta$ production by peritoneal macrophages.

In vitro studies

Effect of drugs on the proliferation of rat endometrial cells treated with $\beta$-estradiol In the absence of RU-486, dienogest ($\approx 3.2 \times 10^{-10}$ M) significantly inhibited BrdU uptake by rat endometrial cells in a concentration-dependent manner (Table 4). Danazol (3.0 $\times$ 10$^{-8}$ M) and progesterone (3.2 $\times$ 10$^{-8}$ M) also significantly suppressed BrdU uptake. However, buserelin (8.1 $\times$ 10$^{-8}$ M) had no inhibitory effect on the uptake. In the presence of RU-486 (1 ng/ml), dienogest ($\approx 3.2 \times 10^{-10}$ M) also significantly inhibited BrdU uptake by rat endometrial cells in a concentration-dependent manner. However, the inhibitory effect of dienogest was slightly reduced by RU-486. On the other hand, the suppressive effects of danazol and progesterone were abolished in the presence of RU-486. Additionally, dienogest, danazol, and progesterone had no positive effect on LDH leakage from the cells under these conditions.

Effect of drugs on cAMP content, PKA activity, and PKC activity of rat endometrial cells treated with $\beta$-estradiol In the absence or presence of RU-486, dienogest ($\approx 3.2 \times 10^{-10}$ M) significantly reduced the PKC activity of rat endometrial cells (Table 5). This inhibitory action was specific to dienogest alone and was not observed with danazol or progesterone. In the absence of RU-486, dienogest, danazol, and progesterone at their maximal concentrations significantly inhibited PKC activity.

<table>
<thead>
<tr>
<th>Table 2 Effect of treatment with buserelin alone or dienogest plus buserelin on BMD of the distal left femur in rats with experimental endometriosis.</th>
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<td><strong>Treatment</strong></td>
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<tr>
<td>Control</td>
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<tr>
<td>Buserelin</td>
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<tr>
<td>Dienogest + buserelin</td>
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</table>

All values are expressed as the mean $\pm$ s.d. Data were analyzed by Student’s test. *$P < 0.01$ vs control group.

Effect of drugs on the proliferation of rat endometrial cells treated with $\beta$-estradiol In the absence of RU-486, dienogest ($\approx 3.2 \times 10^{-10}$ M) significantly reduced the PKC activity of rat endometrial cells (Table 5). This inhibitory action was specific to dienogest alone and was not observed with danazol or progesterone. In the absence of RU-486, dienogest, danazol, and progesterone at their maximal concentrations significantly inhibited PKC activity.

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<th>Table 3 Effect of treatment with dienogest, danazol, buserelin, or ovariectomy alone, or with dienogest plus buserelin on the NK activity of peritoneal fluid and splenic mononuclear cells and the number of peritoneal fluid cells in rats with experimental endometriosis.</th>
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<tr>
<td><strong>Treatment</strong></td>
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<tr>
<td>Control</td>
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<tr>
<td>Dienogest</td>
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<td>Danazol</td>
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<td>Buserelin</td>
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<tr>
<td>Ovariectomy</td>
</tr>
<tr>
<td>Intact</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Buserelin</td>
</tr>
<tr>
<td>Dienogest + buserelin</td>
</tr>
</tbody>
</table>

All values are expressed as the mean $\pm$ s.d. Data were analyzed by Dunnett’s test, except that for the intact group. **$P < 0.01$ vs control. ***$P < 0.01$ vs control or intact group (Student’s t-test). NT, not tested.
models of endometriosis (9, 21, 22). In the present study, we used a rat model of renal subcapsular endometrial tissue autotransplantation, because the good blood supply there ensures a high take rate for the implants. In this model, both danazol and buserelin significantly decreased the volume of endometrial implants, and the histologic findings corresponded to those described previously (22). In addition, this model showed a response to pregnancy (data not shown), which has been demonstrated to reduce endometriosis in humans. Accordingly, this seemed to be a valid model to assess the effect of dienogest compared with those effects of danazol and buserelin, and we were able to demonstrate that dienogest has some promising characteristics as a possible therapeutic agent for endometriosis.

Dienogest significantly decreased the volume of endometrial implants, suggesting its effect on endometriosis. However, the drug did not reduce the uterine weight in this model and had no effect on the release of luteinizing hormone and follicle-stimulating hormone from the pituitary gland at doses effective for implant volume reduction in this model (8), indicating a difference between this drug and GnRH-a in the mechanism of action. It has been shown clinically that surgical ovariectomy or medical ovariectomy with GnRH-a induces bone mineral loss. We therefore evaluated the effect of dienogest, danazol, buserelin, and ovariectomy on BMD. Our results showed that both ovariectomy and buserelin treatment caused a decrease in the BMD of distal femoral trabecular bone. Unexpectedly, danazol resembled buserelin in its action on bone. Although clinical effects of danazol on bone are controversial (23, 24), our finding on BMD is supported further by our observation that danazol increased the

discussion
For investigation of potential endometriosis due to proliferation of the endometrial glands and stroma outside the uterine cavity, experimental rat models have been created by autotransplantation of endometrial tissue into the abdominal wall (20), intestinal mesentery (21), or renal subcapsular space (9). Therapeutic agents such as buserelin, danazol, and leuprolide acetate are reported to be effective in these animal models of endometriosis (9, 21, 22). In the present study, we used a rat model of renal subcapsular endometrial tissue autotransplantation, because the good blood supply there ensures a high take rate for the implants. In this model, both danazol and buserelin significantly decreased the volume of endometrial implants, and the histologic findings corresponded to those described previously (22). In addition, this model showed a response to pregnancy (data not shown), which has been demonstrated to reduce endometriosis in humans. Accordingly, this seemed to be a valid model to assess the effect of dienogest compared with those effects of danazol and buserelin, and we were able to demonstrate that dienogest has some promising characteristics as a possible therapeutic agent for endometriosis.

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Table 5 Direct effect of dienogest, danazol, or progesterone on the cAMP content, PKA activity, and PKC activity of rat endometrial cells treated with \( \beta \)-estradiol.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (M)</th>
<th>Without RU-486</th>
<th>With RU-486 (1 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cAMP content (pmol/10⁴ cells)</td>
<td>PKA activity (mU/10⁴ cells)</td>
</tr>
<tr>
<td>Control (DMSO)</td>
<td>0</td>
<td>0.55 ± 0.01</td>
<td>0.45 ± 0.05</td>
</tr>
<tr>
<td>Dienogest</td>
<td>3.2 \times 10^{-11}</td>
<td>0.55 ± 0.01</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>3.2 \times 10^{-10}</td>
<td>0.55 ± 0.01</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>3.2 \times 10^{-9}</td>
<td>0.55 ± 0.01</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>3.2 \times 10^{-8}</td>
<td>0.33 ± 0.01**</td>
<td>0.23 ± 0.02**</td>
</tr>
<tr>
<td>Danazol</td>
<td>3.0 \times 10^{-11}</td>
<td>0.55 ± 0.01</td>
<td>0.40 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>3.0 \times 10^{-10}</td>
<td>0.55 ± 0.00</td>
<td>0.37 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>3.0 \times 10^{-9}</td>
<td>0.54 ± 0.01</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>3.0 \times 10^{-8}</td>
<td>0.44 ± 0.02**</td>
<td>0.27 ± 0.01**</td>
</tr>
<tr>
<td>Progesterone</td>
<td>3.2 \times 10^{-11}</td>
<td>0.55 ± 0.01</td>
<td>0.42 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>3.2 \times 10^{-10}</td>
<td>0.56 ± 0.01</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>3.2 \times 10^{-9}</td>
<td>0.55 ± 0.01</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>3.2 \times 10^{-8}</td>
<td>0.42 ± 0.02**</td>
<td>0.23 ± 0.03**</td>
</tr>
</tbody>
</table>

All values are expressed as the mean ± S.D. of triplicate determinations. Data were analyzed by Dunnett's test. ** \( P < 0.01 \) vs control. DMSO, dimethylsulfoxide.
production of bone-resorptive cytokines by mouse osteoblasts in vitro (data not shown). These findings suggest that danazol stimulates bone resorption in rats with endometriosis. In contrast, dienogest had no effect on BMD in this model, which would make this drug preferable to avoid this unwanted side-effect associated with the treatment of endometriosis. For the control of adverse effects due to hypoestrogenism induced by GnRH-a, combination therapy with GnRH-a and a gestagen (e.g. MPA) has been proposed for the treatment of endometriosis (25). Because dienogest has progestational activity and no effect on BMD, we also evaluated the effect of a combination of dienogest with buserelin on experimental endometriosis. Dienogest clearly demonstrated a striking protective effect against buserelin-induced bone mineral loss without reducing the potency of the effect on endometrial implants, indicating that dienogest is a promising agent in combination with buserelin for the treatment of endometriosis. Recently, some investigators have reported an increase in the number of activated macrophages (26) and an increase in the levels of IL-1β and tumor necrosis factor-α (27) in the peritoneal fluid of patients with endometriosis, as well as a decrease in these cytokine levels after medical treatment (27, 28). Peritoneal macrophages and macrophage-induced IL-1β may contribute to infertility caused by endometriosis (29). In addition, depressed NK activity has been reported in patients with endometriosis (30) and in a rat model of experimental endometriosis (31). A significant correlation between reduced peritoneal NK activity and the severity of endometriosis was also reported (32). Hence, we examined the NK activity of mononuclear cells from the peritoneal fluid and spleen as well as IL-1β production by peritoneal macrophages in our rat model. We found that dienogest normalized not only the NK activity but also the number of the cells in the peritoneal fluid and IL-1β production by peritoneal macrophages, all of which showed abnormalities in the rats with endometriosis compared with intact rats. These effects of dienogest may combine to improve the peritoneal environment in endometriosis and contribute to diminish pain and infertility due to endometriosis. In contrast, the NK activity in the danazol, buserelin, and ovariec-tomized groups remained lower than in the intact group.

Finally, using rat endometrial cells in vitro, we investigated the mechanism responsible for the effect of dienogest. Dienogest directly inhibited the proliferation of rat endometrial cells without cytotoxicity. Additionally, with RU-486, a progesterone receptor antagonist, the antiproliferative action of dienogest on the cells was somewhat lessened, although it was still prominent. This finding suggests that the inhibitory action of dienogest on proliferation of the cells is dependent on its progestational effect and other actions. On the other hand, the antiproliferative action of danazol and progesterone was completely abolished by RU-486, showing that their inhibitory action was dependent on their progestational effect alone.

In order to investigate the antiproliferative action of dienogest, we then assessed its effect on intracellular signaling systems. Dienogest inhibited PKC activity, and this action was not influenced by RU-486. Therefore, this inhibitory action was clearly different from a progestational effect and may be specific to dienogest. On the contrary, danazol and progesterone had no effect on PKC activity. Moreover, the inhibitory actions of dienogest, danazol, and progesterone on intracellular cAMP content and PKA activity were dependent on their progestational activity.

Because MPA, having progestational activity, is clinically as effective as danazol and has fewer side-effects than danazol (25), we consider that further study is needed to clarify the difference between dienogest and MPA in the treatment of experimental endometriosis. In conclusion, dienogest may be a potent agent for endometriosis with a direct inhibitory action (due to inhibition of PKC activity and an additive progestational effect) on the proliferation of ectopic endometrial tissue. In addition, it normalizes the peritoneal environment, restores NK activity, and suppresses bone mineral loss, all actions which are clearly lacking in the case of danazol and buserelin. Finally, because dienogest clearly differs in its mechanism of action from GnRH-a and danazol, it may be useful in combination with GnRH-a for the treatment of endometriosis.

Acknowledgements
We wish to thank Mr Chihaya Kakinuma, Mr Noritsugu Goda, and Ms Kyoko Motohashi for their technical assistance.

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Received 2 April 1997
Accepted 5 August 1997