The inhibitory effect of dienogest, a synthetic steroid, on the growth of human endometrial stromal cells in vitro

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Dienogest is a synthetic steroid that has been used as a progestogen in contraceptive pills and is currently being studied for its possible clinical use in the treatment of endometriosis. In this study, we investigated the direct effects of dienogest in differentiation and proliferation of human endometrial stromal cells (ESC) in vitro. After 12 days in the presence of oestradiol (10⁻⁸ mol/l) plus dienogest (10⁻⁶ mol/l), cultured ESC underwent morphological differentiation and produced prolactin, a typical marker for decidualization. By using Northern blot analysis and radioimmunoassay, it was shown that treatment of ESC with oestradiol (10⁻⁸ mol/l) plus dienogest (10⁻⁹ to 10⁻⁶ mol/l) led to an increase in the levels of prolactin mRNA and prolactin production in a dose-dependent manner. Additionally, RU-486, a progesterone receptor antagonist, almost completely inhibited dienogest-induced prolactin production. As shown by the thymidine uptake method, there was a dose-dependent inhibition of ESC proliferation with dienogest (P < 0.01, control versus concentrations > 10⁻⁷ mol/l). The significant inhibition of ESC proliferation by dienogest (10⁻⁷ mol/l) was partially reversed by RU-486 (10⁻⁶ mol/l). In summary, dienogest directly acts on endometrial tissue in progestogenic response, such as decidualization, increased prolactin production and growth retardation. These data imply that dienogest exerts direct effect in suppressing growth of endometriotic implants.

Key words: decidualization/dienogest/endometrial stromal cells/endometrium/proliferation

Introduction

Endometriosis is a complex gynecological disorder, with varied and diverse symptomatology, including pelvic pain, dysmenorrhea, dyspareunia and infertility. Various drugs have been employed in the medical treatment of endometriosis (Lessey, 2000; Minjarez and Schlaff, 2000). The medications most commonly used include danazol (17β-hydroxy-2,4, 17α-pregnadien-zo-yno[2,3-djsoxazole] gonadotrophin-releasing hormone (GnRH) agonists and progestins. Although these agents are relatively effective against endometriosis, they produce a high incidence of adverse reactions. Due to its anabolic/androgenic activity, danazol leads to side-effects including abnormal lipid metabolism, liver dysfunction, and cerebral thrombosis (Barbieri, 1990; Kauppila, 1993). Progestin causes weight gain, lethargy, depression, and thrombosis (Moghissi, 1984; Metzger and Luciano, 1989), whereas GnRH agonists cause profound hypo-oestrogenaemia associated with hot flushes and reductions in bone mineral density (Judd, 1992; Adashi, 1994). Thus, new agents with a higher efficacy and fewer side-effects would be highly desirable.

Dienogest (17α-cyanomethyl-17β-hydroxy-estra-4, 9-dien-3-one), is a synthetic steroid that combines properties of both the 19-nortestosterone derivatives and the progesterone derivatives (Figure 1), as well as having unique pharmacodynamic and pharmacokinetic properties (Kuhl, 1996; Foster and Wilde, 1998). Dienogest was known as STS 557 and has been shown to have high progestational activity according to an assay by secretory transformation of oestrogen-primed endometrium in immature rabbits (Oettel and Kurischko, 1980). Dienogest has moderate affinity (~10% of that of progesterone) for the progesterone receptor in human uterine tissue in vitro, (Kaufmann et al., 1983; Oettel et al., 1993; Kuhl, 1996). However, the binding affinities of dienogest to the androgen receptor are low and there is no or nearly no affinity to the oestrogen, aldosterone or glucocorticoid receptors (Oettel et al., 1993; Kuhl, 1996). Although slight oestrogenic effects of dienogest have been detected in animal models (Koch, 1984), these did not occur with clinically relevant doses in women (Foster and Wilde, 1998). This peculiarity appears to be responsible for the fact that dienogest has no androgenic properties and even some antiandrogenic properties (Kuhl, 1996; Katsuki et al., 1997a; Foster and Wilde, 1998). This compound was initially assessed for its contraceptive value, and it is now available in combination with ethinyl oestradiol.
in Germany as a low-dose pill. With this hormonal profile, dienogest appears to cause few side-effects in its clinical use (Kuhl, 1996; Foster and Wilde, 1998). In female rats and female monkeys, dienogest showed no effect on the bleeding time, coagulation, fibrinolysis and platelet aggregation, in contrast with medroxyprogesterone acetate and danazol (Katsuki et al., 1998a; Nobukata et al., 1999). Recent studies have been conducted to confirm the effects of dienogest on experimental endometriosis induced by autotransplantation of endometrium in rats (Katsuki et al., 1998b). Dienogest significantly decreased the volume of the endometrial implants, suggesting its effect on endometriosis. Furthermore, dienogest was highly effective on endometriotic lesions and symptoms, showing an objective endoscopic and subjective symptomatic improvement in 80 and 83% respectively (Köhler et al., 1987, 1989). Currently, it is being investigated in Germany, France and Japan as an agent for the treatment of endometriosis. However, the direct effects of dienogest on human endometrium have not been consistently identified.

Decidualization is a dramatic morphological and functional differentiation of the human endometrium during the progesterone-dominant secretory phase of the menstrual cycle and pregnancy. Recently, an in-vitro model of human decidualization has been developed. In this model, human endometrial stromal cells (ESC) cultured in the presence of progesterone undergo morphological differentiation and produce decidual proteins such as prolactin and insulin-like growth factor binding protein-1 (IGFBP-1) (Irwin et al., 1991; Tahanelli et al., 1992). We and others have used this model and have found that progesterone enhanced macrophage colony-stimulating factor (M-CSF), tissue factor, plasminogen activator inhibitor type 1, tissue inhibitor of metalloproteinase, and interleukin-15 (Schatz and Lockwood, 1993; Hatayama et al., 1994; Higuchi et al., 1995; Zhang and Salamonsen, 1997; Krikun et al., 1998; Okada et al., 2000). In this study, we investigated the direct effects of dienogest on proliferation and differentiation of ESC cultured in vitro.

**Materials and methods**

**Isolation and culture of ESC**

ESC were purified from the proliferative phase endometrium and cultured as described previously (Hatayama et al., 1994; Okada et al., 1999, 2000). Briefly, tissue samples were washed with Dul-becco’s modified Eagle’s medium (DMEM)/Ham’s F-12 medium (Gibco BRL, Grand Island, NY, USA) and minced into small pieces of <1 mm². The tissues were then incubated for 2 h at 37°C in DMEM/F-12 medium containing 1 mg/ml collagenase (Wako Pure Chemical Co. Ltd, Osaka, Japan) and 0.005% deoxyribonuclease (DNase) type I (Boehringer Mannheim GmbH, Mannheim, Germany). After subsequent pipetting, the cell suspension was diluted with 2-volume DMEM/F-12 medium and placed in a centrifugation tube (Corning Glass Works, Corning, NY, USA), where it remained upright for 10 min at unit gravity. The supernatant, excluding the lowermost 2 ml, was transferred into a new tube to collect suspended single cells. After repeating this procedure several times, the cell suspension was washed three times and used as a source of ESC. The viability, determined by dye exclusion, was >90%. Two million viable ESC were cultured in 75 cm² flasks in DMEM/F-12 medium supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT, USA), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL) at 37°C in humidified atmosphere of 5% CO₂ in air. The culture medium was replaced 30 min after plating to reduce epithelial cell contamination. The purity of ESC was determined by morphology and by immunohistochemical staining as described previously (Inoue et al., 1996) with markers specific to ESC (vimentin), epithelial glands (cytokeratin), endothelial cells (factor VIII), or leukocytes (CD45). These antibodies were produced from Dako (Kyoto, Japan) and cells were transferred to Lab-Tek chamber slides (Nunc Naperville, IL, USA) for immunohistochemical staining. Initially, the purified fraction contained approximately 1–2% endothelial cells, 2–3% epithelial cells, 1–2% leukocytes, 1–2% macrophages, and 95% ESC by immunohistochemistry. The proportion of vimentin-positive cells in confluent ESC was >99% by immunohistochemical staining.

**Steroid hormone treatment**

To remove the effect of endogenous steroid hormones, FCS to be used in the cell culture was treated as follows. One hundred ml FCS mixed with 0.25 g activated charcoal (Sigma Chemical Co., St Louis, MO, USA) and 0.025 g dextran (clinical grade; Sigma) was stirred at 56°C for 30 min and centrifuged to separate the dextran-coated charcoal pellet. The supernatant was then subjected to the same treatment at 37°C and the dextran-coated charcoal-stripped (DCS) FCS was filtered through a 45 µm sterilization unit (Corning) and stored at −20°C.

After 1–2 passages when ESC were nearly confluent, cells were plated in 75 cm² flasks for Northern blot analyses and 6-well plates for radiomunooassay. ESC were cultured until confluent and the media were replaced with Phenol Red-free DMEM/F-12 supplemented with 10% DCS-FCS. After 48 h, ESC were washed and were cultured in DCS-FCS media supplemented with progesterone (Sigma), oestrogen (17β-oestradiol) (Wako), danazol (Sigma), dienogest (Mochida Pharmaceutical Co., Shizuoka, Japan), RU-486 (Sigma), or DMSO (Wako) as a vehicle control. The culture media were changed every 3 days.

**RNA extraction and Northern blotting**

Total RNA was prepared from cultured cells by the acid guanidinium-phenol-chloroform method using TRIzol Reagent (Gibco BRL). Total RNA (20 µg) was separated in a 1.2% formaldehyde gel and transferred to Hybond-N+ nylon membrane (Amersham Corp., Arlington Heights, IL, USA). The probe was labelled by the multi-prime DNA labelling system (Amersham). Human prolactin probe (Okada et al., 1999) and human S26 probe (Okada et al., 2000), which recognizes the mRNA-binding human ribosomal protein RNA, were prepared as described previously. Hybridization was done at 42°C for 18 h in 5Xstandard saline-phosphate-EDTA (SSPE)/
5×Denhardt’s solution/50% formamide/0.5% sodium dodecyl sulphate (SDS)/100 µg/ml salmon sperm DNA. The filters were washed at room temperature in 2×standard saline citrate (SSC)/0.1% SDS, following by 0.1×SSC/0.1% SDS at 50°C, and then autoradiographed. The membranes were deprobed and rehybridized with the human S26 probe as an internal control, because its expression level is virtually constant in many tissues (Vincent et al., 1993). The mRNA levels were calculated after normalization to S26 mRNA expression on the basis of the hybridized signal as measured in a BAS 2000 Bioimage Analyzer (Fujix, Tokyo, Japan). Each value indicates the mean ± SD for four cultures obtained from four patients.

Prolactin assay by radioimmunoassay

The prolactin concentration in the culture medium was measured by radioimmunoassay using a commercial kit (Daiichi Radioisotope Lab., Tokyo, Japan). The detection limit was 1.0 ng/ml. Prolactin values were standardized on the basis of the number of cells counted at the time when the culture media were collected for the prolactin assay. Studies were performed with five cultures obtained from five patients, each in triplicate wells.

Cell proliferation assay

ESC proliferation was measured by a [methyl-3H]thymidine incorporation assay. ESC were cultured for 2–4 days until sub-confluence in 75 cm² flasks and were subsequently cultured to 6-well plates. ESC (5×10⁴ cells) in 6-well plates were made quiescent by incubation in the presence of Phenol Red-free DMEM/F-12 supplemented with 0.1% DCS-FCS. After 48 h of serum starvation, the medium was removed and replaced with control medium of Phenol Red-free DMEM/F-12 supplemented with 1% DCS-FCS containing the control vehicle or steroids. After 20 h of incubation, [methyl-3H]thymidine (1 µCi/well) (Amersham) was added 4 h before harvest. ESC were washed three times with ice-cold PBS (phosphate-buffered saline). After the addition of ice-cold 10% TCA (trichloroacetic acid) for 10 min on ice, ESC were washed three times with ice-cold 5% TCA. The acid-insoluble fractions were dissolved in 0.5 N NaOH for 10 min on ice, and then HCl and TCA were added to a final concentration of 0.5 N and 10% respectively. Cell lysates filtrated through the GF/C filters (Whatman, Maidstone, UK) and the filters were washed three times with ethanol. The GF/C filters were transferred to scintillation vials and the incorporation of [³H]thymidine was measured with the use of a liquid scintillation counter (TRI-CARB 4530; Packard, Osaka, Japan). The control medium could accelerate thymidine uptake by ESC after serum starvation (data not shown). Studies were performed with five cultures obtained from five patients, each in triplicate wells.

Statistical analysis

Data are expressed as mean ± SD. Results were analysed with a statistical software package, StatView II version 4.0 (Abacuss Concepts, Inc., Berkeley, CA, USA). Differences in the measured parameters across the different groups were statistically assessed using analysis of variance with repeated measurements, followed by Fisher’s protected least significant difference, multiple range test. A level of P < 0.05 was considered statistically significant.

Results

Decidualization of ESC was determined by morphology and prolactin production. Morphological studies showed that ESC cultured with oestradiol remained fibroblastic in appearance throughout the culture period (Figure 2A). In contrast, after 12 days in the presence of oestradiol (10⁻⁸ mol/l) plus dienogest (10⁻⁶ mol/l), spindle-shaped ESC were transformed into large polygonal cells, resembling decidual cells in vivo (Figure 2B). We evaluated whether oestradiol plus dienogest regulated the expression of prolactin mRNA using the cultured ESC. Figure 3 shows patterns of prolactin mRNA expression alongside the control of unregulated S26 mRNA, as assessed by Northern blot analysis. The level of prolactin mRNA was undetectable in samples obtained from ESC after 12 days of culture in the presence of oestradiol (10⁻⁸ mol/l) alone. In contrast, oestradiol (10⁻⁸ mol/l) plus progesterone (10⁻⁶ mol/l) or oestradiol (10⁻⁸ mol/l) plus dienogest (10⁻⁶ mol/l) caused a significant and similar increase in prolactin mRNA (Figure 3). The prolactin concentrations in the culture medium as measured by radioimmunoassay also showed the same results as the mRNA experiments (Figure 4). Treatment of ESC with dienogest (10⁻⁸ to 10⁻⁶ mol/l) led to an increase in the levels of prolactin mRNA and prolactin production in a dose-dependent manner (Figures 3 and 4). Additionally, RU-486,
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Figure 4. Prolactin (PRL) production by human endometrial stromal cells (ESC) exposed to the following agents for 12 days: oestradiol (E2) plus progesterone (P), oestradiol plus dienogest, and oestradiol plus dienogest plus RU-486. Radioimmunoassay was used to measure prolactin production during the last 3 days of a 12 day culture period. The effect of oestradiol (10^{-8} mol/l) plus progesterone (10^{-6} mol/l) is assigned a potency of 100%. Columns and vertical bars represent the mean ± SD of five separate experiments. Significantly different values: *P < 0.01 versus oestradiol (10^{-8} mol/l) plus progesterone (10^{-6} mol/l).

Figure 5. Dose-dependent inhibition of proliferation of human endometrial stromal cells (ESC) in culture by dienogest as assayed by the thymidine uptake method. ESC were placed in Phenol Red-free Dulbecco’s modified Eagle’s medium/Ham’s F-12 supplemented with 0.1% dextran-coated charcoal-stripped fetal calf serum for 48 h and were then incubated for 24 h in control medium with various concentrations of dienogest (10^{-11} to 10^{-5} mol/l). The effect of dimethylsulphoxide as vehicle control is assigned a potency of 100%. Columns and vertical bars represent the mean ± SD of five separate experiments. Value significantly different (*P < 0.01 versus control).

an antiprogestin compound that exerts a high affinity for the progesterone receptor, almost completely inhibited the dienogest-induced prolactin production (Figure 4).

We also determined whether dienogest could affect the proliferation of ESC. There was a concentration-dependent inhibition of cell proliferation with dienogest (P < 0.01 versus control) when concentrations were >10^{-7} mol/l (Figure 5). Figure 6 shows that a significant inhibition of ESC proliferation by dienogest (10^{-7} mol/l) was partially reversed by RU-486 (10^{-6} mol/l). The addition of danazol at a concentration of 10^{-7} mol/l in the culture medium had no inhibitory effect.
endometriosis tissues, because there exist some differences in genesis and its antiangiogenic action may be involved in its mediated via the progesterone receptor of ESC. The effects 1993).

Figure 6. Effects of RU-486 on the dienogest-mediated inhibition of proliferation of human endometrial stromal cells (ESC) as assayed by the thymidine uptake method. ESC were placed in Phenol Red-free Dulbecco’s modified Eagle’s medium/Ham’s F-12 supplemented with 0.1% dextran-coated charcoal-stripped fetal calf serum for 48 h and then were incubated for 24 h in control medium containing the following agents: dimethylsulphoxide as vehicle control, dienogest, dienogest plus RU-486, RU-486 alone, or danazol. Columns and vertical bars represent the mean ± SD of five separate experiments. Significantly different values: *P < 0.01 versus control.

on the [3H]thymidine incorporation by ESC, but the higher concentration of danazol (10⁻⁵ mol/l) significantly suppressed proliferation (Figure 6). Progesterone at concentrations >10⁻⁷ mol/l also significantly inhibited the growth of ESC in a dose-dependent manner (data not shown).

Discussion

In this study, the possible direct effect of dienogest on differentiation and proliferation on ESC was examined in vitro. ESC can be decidualized in vitro with progesterone. This in-vitro model has provided an opportunity to investigate production and regulation of prolactin, which was produced by ESC with progesterone or dienogest. The binding affinities of dienogest for human, rat and rabbit progesterone receptors have been reported to be as low as 10, 11 and 17%, respectively, of that of progesterone (Kauffman et al., 1983; Oettel et al., 1993; Kuhl, 1996; Katsuki et al., 1997a). However, we found that ESC cultured in the presence of dienogest underwent apparent morphological differentiation and produced prolactin, a typical marker for decidualization. This action of dienogest on cultured ESC was similar to that of progesterone. Furthermore, RU-486 as a progesterone receptor antagonist almost completely inhibited dienogest-induced prolactin production. Our results indicate that the effect of dienogest might be mediated via the progesterone receptor of ESC. The effects of dienogest on endometrium may differ from those for endometriosis tissues, because there exist some differences in the expression of steroid receptors between endometrial and endometriosis tissues (Fujimoto et al., 1999; Misao et al., 1999). However, the present study using eutopic endometrial cells should help to understand the mechanism of the effect of dienogest in endometriosis treatment.

The question that we asked was whether dienogest might directly inhibit growth and proliferation of ESC. Our data clearly demonstrate a direct inhibition of proliferation of ESC by dienogest as assessed by the thymidine uptake method. ESC were growth-arrested by serum starvation and subsequently cultured in the condition medium. We have previously shown that the cultured ESC expressed nuclear receptors for the steroid hormones progesterone, androgen and oestrogen (Iwai et al., 1995). This model allowed us to investigate the mitogenic effects of steroid hormones. Indeed, the investigations have attempted to demonstrate the direct effects of danazol, progestin and GnRH agonists on proliferation of ESC in vitro (Rose et al., 1988; Surrey and Halme, 1992). The addition of dienogest in the culture medium inhibited the growth of ESC in a concentration-dependent manner and this inhibition by dienogest is the same as that caused by progesterone. Danazol also inhibited the growth of ESC, although this inhibition was less than that induced by dienogest. The adequacy of the present experiments is supported by reports that danazol (10⁻⁷ mol/l) had a direct inhibitory effect on the growth of ESC in culture (Rose et al., 1988).

Progestins have been found to suppress the growth of ESC in the presence of serum (Surrey and Halme, 1992). On the other hand, progesterone has been shown to have no effect on ESC proliferation (Bhargava-Periwal et al., 1996), although the experimental conditions were not clearly stated. Other investigations have demonstrated that progesterone stimulates the proliferation of cultured ESC to a greater degree than cells grown in control medium alone, as indicated by measurement of cell number after 9–15 days culture (Irwin et al., 1989, 1991). However, the possibility exists that the stimulatory effects of progesterone depend on some growth factors produced with decidualization of ESC after 9–15 days of treatment with progesterone. Indeed, progestin is suggested to regulate an autocrine growth control loop in the endometrium that involves IGF, IGF receptor and IGFBP (Frost et al., 1993).

The pharmacokinetics of a single oral dose of dienogest had been assessed in female volunteers (Foster and Wilde, 1998). Maximum serum dienogest concentrations were reached within ~2 h and the mean maximum serum concentrations were 0.9×10⁻⁷, 1.7×10⁻⁷, 3.2×10⁻⁷, 6.8×10⁻⁷ mol/l after 1, 2, 4, 8 mg therapeutic doses respectively (Foster and Wilde, 1998). Since dienogest (10⁻⁷ mol/l) had a significant inhibitory effect on the [3H]thymidine incorporation by ESC, dienogest may directly inhibit the proliferation of ESC at therapeutic doses. In view of the role of dienogest in the growth characteristics of ESC, the study on the effect of metabolites of dienogest may be important. However, the metabolites of dienogest generally show less affinity for the progesterone receptor compared with the parent compound (Oettel et al., 1993).

Dienogest is also suggested to be an antagonist of angiogenesis and its antiangiogenic action may be involved in its therapeutic effects on endometriosis. Angiogenesis is essential
to the advancement of pelvic endometriosis (Healy et al., 1998; Hyder and Stancel, 1999; McLaren, 2000). Topical dienogest treatment has been shown to dose-dependently inhibit embryonic angiogenesis (Nakamura et al., 1999), while oral administration of dienogest can significantly suppress angiogenesis induced by S-180 mouse tumour cells in the mouse dorsal air sac assay (Nakamura et al., 1999).

At present the precise mechanism by which dienogest acts to inhibit ESC proliferation is unknown. However, it is very interesting to note that the antiproliferative action of dienogest is partially reversed by the effect of RU-486. This finding suggests that the inhibitory action of dienogest on proliferation of ESC is dependent on both its progestational effect and other actions. A recent study demonstrated that dienogest has an antiproliferation action that is markedly different from that of progestins (Katsuki et al., 1997b). Although the human endometrial carcinoma HEC-88nu expresses oestrogen receptor but not progesterone receptor, oestradiol-stimulated tumour growth of HEC-88nu cells was suppressed by dienogest. Thus, this antiproliferative action of dienogest may act by receptors other than the progesterone receptor, and it is probable that dienogest has novel activity additional to its progestational effects. Furthermore, in-vitro studies on dienogest have revealed an antiproliferative effect on rat endometrial cells due to the inhibition of protein kinase C activity as well as the progestational effect (Katsuki et al., 1998b).

In conclusion, in this study we examined the possible role of dienogest in differentiation and proliferation of ESC. ESC cultured in the presence of dienogest underwent morphological differentiation and produced prolactin. The results of our in-vitro experiments demonstrate a direct inhibitory effect of dienogest on ESC proliferation. Therefore, it is suggested that dienogest is a potent agent for treating endometriosis by inhibiting proliferation of ectopic endometriotic tissues in vivo.

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