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ORIGINAL RESEARCH

Dienogest, a synthetic progestin, inhibits the proliferation of immortalized human endometrial epithelial cells with suppression of cyclin D1 gene expression

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ABSTRACT: Dienogest is a specific progesterone receptor agonist with potent oral endometrial activity and is used in the treatment of endometriosis. In this study, we examined the direct effects of dienogest on the proliferation of human endometrial epithelial cells using an immortalized cell line. 5-Bromo-2'-deoxyuridine incorporation into the cells was inhibited by dienogest and by progesterone (P_4) in dose-dependent fashion at concentrations of 10^{-8} mol/l or higher. To identify the target genes of dienogest and P_4, we screened the expression of 84 genes related to cell cycle regulation by real-time polymerase chain reaction after 6 h of treatment at a concentration of 10^{-7} mol/l. Results showed that only cyclin D1 expression was significantly down-regulated, although expression of the other genes did not significantly change after dienogest or P_4 treatment compared with the control. In a time-course study during the first 24 h after drug treatment, dienogest and P_4 each produced a lasting decrease in the expression of cyclin D1 mRNA, followed by a decrease in cyclin E1 mRNA but not an increase in the expression of cell cycle inhibitor genes (p21, p27 and p53). These findings suggest that dienogest directly inhibits the proliferation of human endometrial epithelial cells with suppression of cyclin D1 gene expression.

Key words: cell proliferation / cyclin D1 / dienogest / endometrial epithelial cells / progesterone

Introduction

Progesterone (P_4) plays a key role in female reproduction via progesterone receptor (PR). Progestins are a class of compounds that mimic the activity of P_4. Progestins have been administered in response to gynecological concerns such as contraception (Sitruk-Ware, 2006), hormone replacement therapy (Campagnoli *et al*., 2005), endometriosis (Surrey, 2006) and endometrial hyperplasia and neoplasia (Lopez de la Osa Gonzalez, 1994). Although a wide variety of progestins have been designed, progestins classified as first-, second- or third-generation have androgenic activity that yields adverse effects (Sitruk-Ware, 2004a, b).

Dienogest (17α-cyanomethyl-17β-hydroxy-estra-4,9-dien-3-one) is a fourth-generation progestin with potent oral progestational activity without any systemic androgenic activity (Sitruk-Ware, 2004a, b; Sasagawa *et al*., 2008). Dienogest has been used in a contraceptive pill and in hormone replacement therapy (Kuhl, 1996; Foster and Wilde, 1998). In addition, dienogest has been reported to be highly effective in the treatment of endometriosis (Moore *et al*., 1999; Cosson *et al*., 2002; Schindler *et al*., 2006; Harada *et al*., 2009), a chronic and recurrent disease characterized by the presence of glandular epithelium and stroma outside the uterine cavity (Child and Tan, 2001; Schweppe, 2001; Valle and Sciarra, 2003). The effects of dienogest on endometriosis are associated not only with antiovulatory activity (Irahara *et al*., 2007), but also with a direct effect on proliferation or cytokine production in stromal cells from eutopic and ectopic endometrial tissues (Okada *et al*., 2001; Horie *et al*., 2005; Fu *et al*., 2008). However, it is still unclear whether dienogest has any direct effect on endometrial epithelial cells contributing to its therapeutic effect on endometriosis.

Endometrial epithelial cells are known to differ from stromal cells in their morphological response to endogenous and exogenous sex hormones (Noyes *et al*., 1950; Deligdisch, 2000) and in gene expression

pattern during the menstrual cycle (Matsuzaki *et al*., 2004; Yanaihara *et al*., 2004, 2005). Sex hormones regulate endometrial epithelial proliferation via a paracrine mechanism mediated by stromal cells in mice (Kurita *et al*., 1998), although, in humans, endometrial epithelial cells are directly regulated by sex hormones (Shiozawa *et al*., 2001; Blauer *et al*., 2005; Kurita *et al*., 2005). Therefore, it is important to investigate the direct antiproliferative effects of dienogest and its mechanism in human endometrial epithelial cells to examine the pharmacological influence on endometriosis. It has been reported that in the red lesions of peritoneal endometriosis found to be active endometriotic foci, higher mitotic activity was observed in the glandular epithelium than in other lesions (Nisolle *et al*., 1993). Moreover, the expression of aromatase and cyclooxygenase-2, which are believed to associated with the pathological abnormalities of endometriosis (Attar and Bulun, 2006), was abundant in ectopic endometrial epithelial cells compared with stromal cells (Ota *et al*., 2001; Matsuzaki *et al*., 2006). These findings suggest an important role for epithelial cells in the pathogenesis of endometriosis. However, because they do not grow as well in primary culture and exhibit a limited ability to grow in subculture, few investigations have examined direct hormonal effects on epithelial cells. Recently, several immortalized cell lines have been established from human eutopic or ectopic endometrial epithelial cells. Zeitvogel *et al*. (2001) established cell lines from peritoneal endometriotic biopsies by transformation with SV40 T-antigen. Human endometrial epithelial cell lines immortalized with normal morphological and functional characteristics, including responsiveness to sex-steroid hormones, have also been successfully established by transfection with TERT (Hombach-Klonisch *et al*., 2005) or E6/E7/TERT (Kyo *et al*., 2003). These cell lines may be an important tool for the study of the biology of human endometrial epithelial cells and the pathology of endometrial diseases, such as endometriosis, endometrial hyperplasia and neoplasia.

In the present study, we therefore examined the direct effects of dienogest on the *in vitro* proliferation of the human endometrial epithelial cell line, which was established by transfection with E6/E7/TERT (Kyo *et al*., 2003). Furthermore, to examine the pharmacological pathway underlying the anti-proliferative effects of dienogest on cells, we also analyzed the expression of human cell cycle regulator genes by real-time polymerase chain reaction (PCR) to identify the target genes of dienogest.

Materials and Methods

Cells cultures

We used EM-E6/E7/TERT, EM-PR and T-47D cell lines. The EM-PR cell line, with stable PR expression, was transfected with the PR type B gene into EM-E6/E7/TERT cells, which is an immortalized human endometrial epithelial cell line (Kyo *et al*., 2003). The parent cell line can be purchased as a component of the 'Endometrial Glandular Epithelial Cell Culture Kit' from Wako Pure Chemical Industries, Ltd. (https://www.e-reagent.com/cgi-bin/gx.cgi/AppLogic+ufg280disp_pr.ufg280disp_Main?code=W01PRCHEM01&next=1). EM-PR cells stably expressing PR were established by retroviral infection of PR-B expression vector (MSCVbsd-PRB) into EM-E6/E7/TERT. Infection was performed in the presence of polybrene. After initial drug selection, pool of the transfected cells was used for the experiments. Although the parent cells, EM-E6/E7/TERT, constitutively express weak levels of PR, EM-PR cells were confirmed to express PR stably. We used EM-PR cells in the present study because it was necessary to analyze the pharmacological action and molecular mechanism of drugs via PR. The human breast cancer cell line T47D was obtained from the American Type Culture Collection (Manassas, VA, USA). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) containing 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen). The cells were incubated at 37°C in 95% air/5% CO_2.

Characterization of EM-PR cells

Immunocytochemistry

We analyzed the expression of PR protein. EM-PR cells, EM-E6/E7/TERT cells and T47D cells were seeded onto the Lab-Tek™ chamber slide (Thermo Fisher Scientific, Waltham, MA, USA) with DMEM supplemented with 10% FBS containing antibiotics, and were then cultured overnight. The next day, cells were fixed with 10% buffered formalin. Cells were then incubated with Dako target retrieval solution, high pH (Dako, Glostrup, Denmark), at 95°C for 40 min to activate the epitope. These samples were incubated with monoclonal mouse anti-human PR (clone PgR636; Dako) at a 1:800 dilution or with negative control antibody for 30 min at room temperature. After incubation with a horseradish peroxidase-labeled anti-mouse goat polyclonal antibody, the immune complex was visualized with 3,3′-diamnobenzidine tetrachloride using a DakoCytomation EnVision kit (Dako). Hematoxylin was used for nuclear counter stain.

Transfection and PR activity reporter assays

We used the luciferase reporter pGRE-Luc (Takara Bio, Ohtsu, Japan) for PR, which was constructed of glucocorticoid responsive element (GRE) sequences. Such GRE sequences mediate progesterone responses (Schule *et al*., 1988). As an internal standard, a Renilla luciferase plasmid, pRL-CMV vector (Promega, Madison, WI, USA), was used. The Nucleofector™ device (Amaxa Biosystems, Cologne, Germany) and a nucleofection buffer system (Nucleofector kit V, Amaxa Biosystems) along with program T-030 were utilized for gene delivery. Approximately 1×10^6 cells and 10 μg plasmid DNA (9.1 μg GRE-Luc and 0.9 μg pRL-CMV) were used for electroporation. After transfection, the cells were seeded into 96-well culture plates in phenol red-free DMEM/Ham's F-12 (DMEM/F12) medium (Invitrogen) supplemented with 10% inactivated and dextran-coated charcoal-treated FBS (DCC-FBS) (HyClone Laboratory, Logan, UT, USA) containing antibiotics. After the cells were cultured for 4 h, P_4 (Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO), diluted with phenol red-free DMEM/F12 with 10% DCC-FBS containing antibiotics, and then added to each well. The final concentration of DMSO in the medium never exceeded 0.1%. After 20 h of incubation, the medium was removed and the induced luciferase activity was measured using a Dual-Glo™ luciferase assay system (Promega) according to the manufacturer's instructions. Luminescence was measured with a multilabel counter (Wallac 1420 ARVO sx, PerkinElmer, Boston, MA, USA) and relative light units (RLU) were calculated by dividing the firefly luciferase activity by the Renilla luciferase activity in each well. PR activities were calculated by setting the maximum activity as 100% and the control as 0%.

5-Bromo-2′-deoxyuridine uptake assay

The effect of dienogest on the proliferation of EM-PR cells and EM-E6/E7/TERT cells was evaluated by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA. The BrdU incorporation was detected using the DELFIA® cell proliferation kit (PerkinElmer) according to the manufacturer's protocol. Briefly, cell cultures were allowed to proliferate to confluence in DMEM (Sigma Aldrich) supplemented with 10% FBS (Invitrogen) containing 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen), and then cells were pre-incubated in phenol red-free DMEM/F12

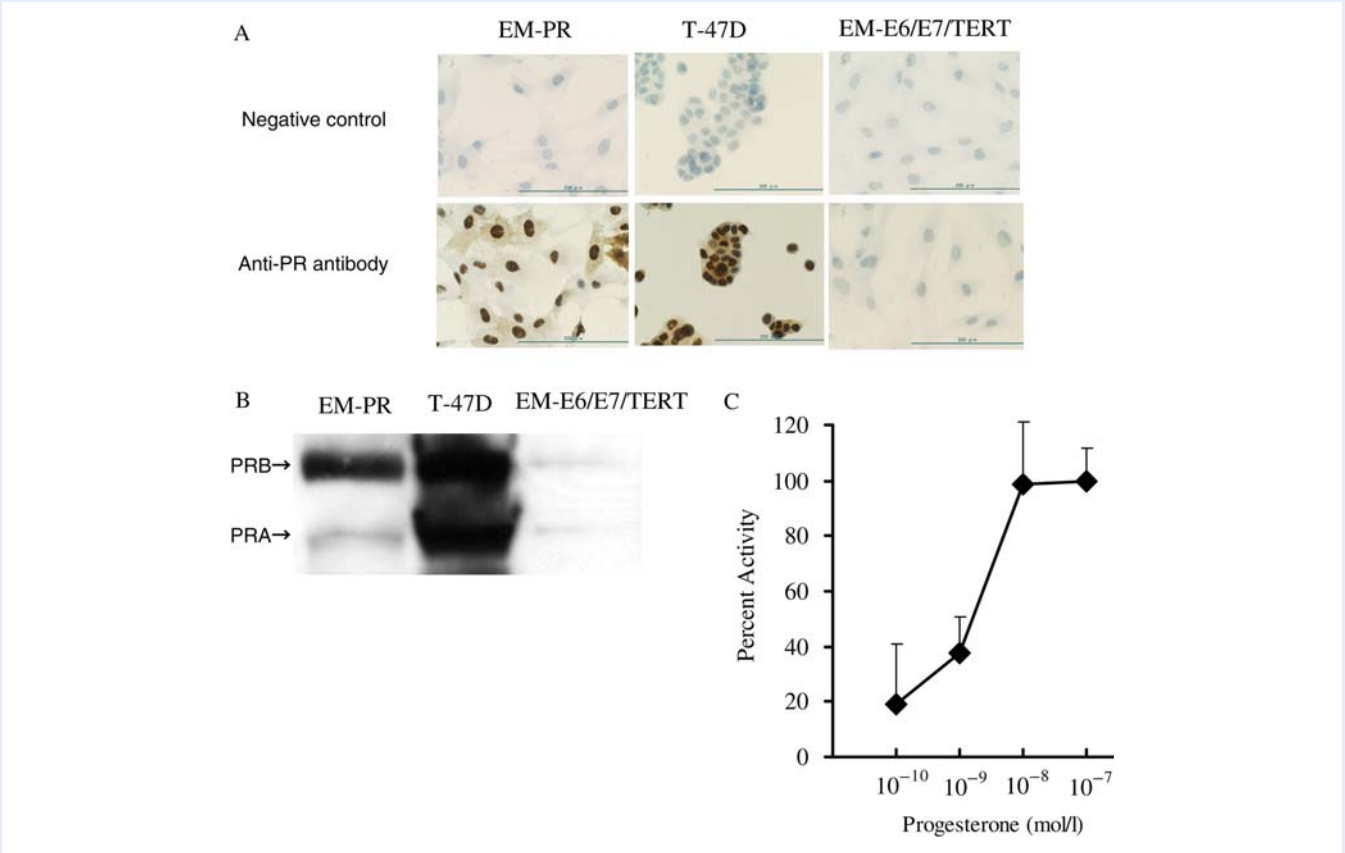

Figure 1 Characterization of EM-PR cells.

Expression of PR proteins was examined by immunocytochemical (**A**) and western blot (**B**) analysis in EM-PR cells and EM-E6/E7/TERT. PR-positive T-47D cells were used as a positive control. Scale bars, 200 μm. Transactivation of PR was examined by reporter gene assays (**C**) in EM-PR cells transfected with GRE-Luc. After transient transfection, the cells were incubated for 4 h and were then incubated for 20 h in medium containing various concentration of P_4 (10^{-10} to 10^{-7} mol/l) or 0.1% DMSO as a vehicle control. The percent activity was calculated by setting the maximum activity as 100% and the activity of the control as 0%. Plots and vertical bars represent the mean ± SEM of five wells.

medium (Invitrogen) with 0.1% DCC-FBS (HyClone Laboratory) containing antibiotics for 3 days of serum starvation. The EM-PR cells were harvested and suspended in phenol red-free DMEM/F12 medium supplemented with 0.1% DCC-FBS containing antibiotics and seeded into 96-well culture plates at a density of 3×10^3 cells per well. After the cells were cultured in phenol red-free DMEM/F12 medium supplemented with 0.1% DCC-FBS overnight, dienogest supplied by Bayer Schering Pharma and P_4 (Sigma Aldrich) were dissolved in DMSO, diluted with phenol red-free DMEM/F12 with 1% DCC-FBS containing antibiotics, and then added to each well. The final concentration of DMSO in the medium never exceeded 0.1%. Blank wells were supplemented with 0.1% DCC-FBS containing 0.1% DMSO. During the last 4 h of the 24-h culture period, 10 μmol/l of BrdU solution was added and incubated at 37°C. After removing the culture medium, the cells were fixed with ethanol. The cells were treated with europium-labeled anti-BrdU mouse monoclonal antibody, and the time-resolved fluorescence of europium bound to the cells was measured with a multilabel counter.

Measurement of lactate dehydrogenase leakage

Lactate dehydrogenase (LDH) leakage was measured to examine the cytotoxic effects of drug treatment on the cells. After EM-PR cells were treated as described above, the conditioned medium was collected. The amount of LDH activity in the medium was measured by a colorimetric assay with tetrazolium salts in conjunction with diaphorase using the CytoTox 96® non-radioactive cytotoxicity assay kit (Promega). Absorbance was measured at 490 nm with a multilabel counter.

Measurement of cellular caspase 3/7 activity

Cellular caspase 3/7 activity was measured to examine the effects of drug treatment on cell apoptosis. After EM-PR cells were treated as described above, caspase 3/7 activity in the cell lysate was measured by a luminometric assay with a synthetic substrate of luciferase using the Caspase-Glo® 3/7 assay kit (Promega). Luminescence was measured with a multilabel counter and expressed as RLU.

RNA extraction, reverse transcription and real-time quantitative PCR of 84 human genes related to cell cycle regulation

We examined the expression of 84 human genes ABL1, ANAPC2, ANAPC4, ARHI, ATM, ATR, BAX, BCCIP, BCL2, BIRC5, BRCA1, BRCA2, CCNB1, CCNB2, CCNC, CCND1 (cyclin D1), CCND2, CCNE1 (cyclin E1), CCNF, CCNG1, CCNG2, CCNH, CCNT1, CCNT2, CDC2, CDC16, CDC20, CDC34, CDK2, CDK4, CDK5R1,

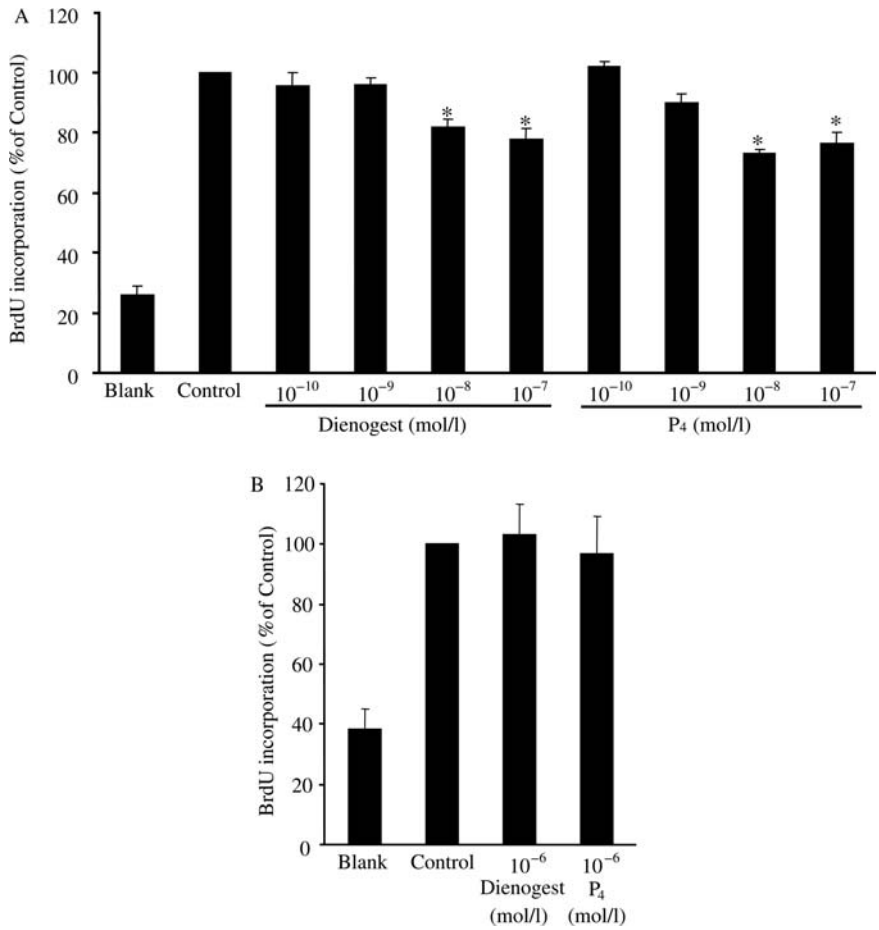

Figure 2 The effects of dienogest and P_4 on BrdU incorporation into EM-PR cells (**A**) or EM-E6/E7/TERT parent cells (**B**).

The cells were placed in phenol red-free DMEM/F12 medium supplemented with 0.1% DCC-FBS overnight and were then incubated for 24 h in phenol red-free DMEM/F12 medium supplemented with 1% DCC-FBS containing various concentration of P_4 or dienogest [10^{-10} to 10^{-7} mol/l (A) or to 10^{-6} mol/l (B)]. Blank wells were supplemented with 0.1% DCC-FBS containing DMSO. The effect of DMSO as a vehicle control was assigned a potency of 100%. Columns and vertical bars represent the mean ± SEM of six (A) or four (B) separate experiments. Asterisks indicate a significant difference by Dunnett's test (*$P < 0.05$ versus control).

CDK5RAP1, CDK6, CDK7, CDK8, CDKN1A (p21), CDKN1B (p27), CDKN2A, CDKN2B, CDKN3, CHEK1, CHEK2, CKS1B, CKS2, CUL1, CUL2, CUL3, DDX11, DNM2, E2F4, GADD45A, GTF2H1, GTSE1, HERC5, HUS1, KNTC1, KPNA2, MAD2L1, MAD2L2, MCM2, MCM3, MCM4, MCM5, MKI67, MNAT1, MRE11A, NBS1, PCNA, RAD1, RAD17, RAD51, RAD9A, RB1, RBBP8, RBL1, RBL2, RPA3, SERTAD1, SKP2, SUMO1, TFDP1, TFDP2, TP53 (p53) and UBE1 related to cell cycle regulation by real-time quantitative PCR array analysis. EM-PR cells were seeded into a 100-mm culture dish at a density of 7×10^5 cells per dish in 10 ml of culture medium containing 0.1% DCC-FBS and cultured overnight. The next day, after the cells were treated with or without dienogest or P_4 (10^{-7} mol/l) in the presence of 1% DCC-FBS for 6 h, total RNA was extracted from the cell lysate using the RNeasy® Micro kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cDNA was prepared by reverse transcription using the RT^2 PCR Array First Strand kit (SuperArray, Frederick, MD, USA) following the manufacturer's instructions. PCR array analysis of a set of the 84 genes (Human Cell Cycle RT^2 Profiler™ PCR array, SuperArray) was performed using the ABI-Prism 7500 Fast Real-time PCR Systems (Applied Biosystems, Foster, CA, USA). In brief, a total volume of 25 μl of PCR mixture, which contained cDNA equivalent to 10 ng RNA in SuperArray RT^2 qPCR Master Mix solution (SuperArray), was loaded in each well of the PCR array. cDNA amplification with PCR was performed under the following conditions: 10 min at 95°C for one cycle and 15 s at 95°C followed by 1 min at 60°C for 40 cycles. ABI Sequence Detection System 1.3.1 software (Applied Biosystems) was used to determine the threshold cycle (Ct) value. All mRNA Ct values for each sample [Ct (sample)] were normalized by the Ct value of the housekeeping glyceraldehyde-3-phosphate dehydrogenase [Ct (GAPDH)] in the same sample as follows: ΔCt (sample) = Ct (sample) − Ct (GAPDH). The relative mRNA level was expressed as the value of $2^{-\Delta Ct\ (sample)}$.

Time course of cyclin D1, cyclin E1, p21, p27 and p53 gene expression

We analyzed the time course of the expression of cyclin D1, cyclin E1, p21, p27 and p53 genes by real-time quantitative PCR. EM-PR cells and EM-E6/E7/TERT cells were seeded into six-well plates at a density of 9×10^4 cells per well in 3 ml of culture medium containing 0.1% DCC-FBS and cultured overnight. The next day, after the cells were

treated with or without dienogest or P_4 (10^{-7}mol/l) in the presence of 1% DCC-FBS for 0, 6, 12 and 24 h, total RNA was extracted as described above. cDNA was prepared using the SuperScript™ III First-Strand Synthesis System (Invitrogen). The primer/probe sets for cyclin D1 (Assay ID Hs00277039_m1), cyclin E1 (Assay ID Hs00233356_m1), p21 (Assay ID Hs00355782_m1), p27 (Assay ID Hs00153277_m1) and p53 (Assay ID Hs00153349_m1) of TaqMan® Gene Expression assays and the primer/probe set for GAPDH [Human GAPDH Endogenous Control (VIC/TAMRA Probe, Primer Limited)] were purchased from Applied Biosystems. Each PCR reaction mixture contained cDNA equivalent to 10 ng RNA and one target primer/probe, and amplification by PCR was performed under the following conditions: 20 s at 95°C for one cycle and 3 s at 95°C followed by 30 s at 60°C for 40 cycles. ABI Sequence Detection System 1.3.1 software (Applied Biosystems) was used to determine Ct values. All mRNA Ct values were normalized by the housekeeping GAPDH Ct value as described above. Data were expressed as the ratio of $2^{-\Delta Ct\ (sample)}$ value of each time point compared with time 0 h.

Western blot analysis

We analyzed the expression of PR and cyclin D1 and E1 proteins by western blot analysis. Cells were seeded into 150-mm culture dishes at a density of 1.4×10^6 cells per dish in 15 ml of culture medium containing 0.1% DCC-FBS, and were cultured overnight. The next day, before (PR and cyclin D1 and E1 proteins) or after treatment with or without dienogest (10^{-7} mol/l) or P_4 (10^{-7} mol/l) in the presence of 1% DCC-FBS for 24 h (cyclin D1 and E1 proteins), cells were washed twice with ice-cold PBS and harvested with trypsin. After washing with ice-cold PBS twice, cells were incubated in radioimmunoprecipitation assay lysis buffer (1× TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide) containing 1% phenylmethanesulphonylfluoride (serine protease inhibitor), 1% protease inhibitor cocktail and 1% sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and were then incubated on ice for 30 min. After centrifugation at 10 000 rpm for 10 min at 4°C, the supernatant was collected as whole cell lysates. Whole cell lysates (20 μg/lane) were then separated by 5–20% SDS-PAGE and transferred to PVDF membranes (ATTO, Tokyo, Japan). After blocking at room temperature for 1 h, membranes were incubated with anti-cyclin D1 (1:1000 dilution) (DCS6, Cell Signaling Technology, Danvers, MA, USA), anti-cyclin E1 (1:1000 dilution) (HE12, Cell Signaling Technology), anti-PR (1:200 dilution) (H-190, Santa Cruz Biotechnology) and anti-β-actin (1:1000 dilution) (C4, Santa Cruz Biotechnology) antibody overnight at 4°C, followed by washing with 0.05% Tween/PBS. Subsequently, membranes were incubated with horseradish peroxidase-linked anti-mouse or rabbit IgG antibodies (1:2000 dilution) (Cell Signaling Technology) at room temperature for 1 h, followed by washing with 0.05% Tween/PBS. Protein-antibody complexes were detected using the ECL Plus western blotting detection system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Fluorescent signals from each band were quantified using ImageQuant analysis (Typhoon 9410, GE Healthcare Bio-Sciences) and were normalized against levels of β-actin with the corresponding treatment.

Statistical analysis

Values are the mean ± SEM. The significance of findings in the BrdU incorporation experiment was examined by Dunnett's *t*-test, and those of the LDH leakage, caspase 3/7 activity, PCR array analysis and the time-course of gene expression experiments were analyzed by Student's *t*-test or Aspin–Welch's *t*-test.

Results

As shown in Fig. 1A and B, both EM-PR and T-47D cells expressed PR protein on immunocytochemistry and western blot analysis. On the other hand, PR protein expression in the parent cells, EM-E6/E7/TERT, was slight. EM-PR, T-47D and EM-E6/E7/TERT cells expressed both PR type A and B proteins (Fig. 1B). Moreover, progesterone at physiological concentrations induced transactivation of PR in EM-PR cells, as shown in reporter gene assays (Fig. 1C). Therefore, EM-PR cells expressed functional PR and were useful as a stable human epithelial cell system for the analysis of progestational effects and functions.

The effects of dienogest and P_4 on the proliferation of EM-PR cells and EM-E6/E7/TERT parent cells were examined by measuring BrdU incorporation. As shown in Fig. 2A, dienogest and P_4 at concentrations of 10^{-8} and 10^{-7} mol/l induced significant suppression of serum-mediated increase in BrdU incorporation into DNA 24 h after treatment of EM-PR cells. On the other hand, in EM-E6/E7/TERT parent cells, dienogest and P_4 did not suppress serum-mediated increases in BrdU incorporation into DNA at 24 h after treatment, even at a concentration of 10^{-6} mol/l (Fig. 2B). The effects of dienogest and P_4 on LDH leakage from EM-PR cells were examined in conditioned culture medium. As shown in Fig. 3A, dienogest and P_4 at a concentration of 10^{-7} mol/l did not change LDH activity in conditioned culture medium for 24 h after treatment. The effects of dienogest and P_4 on cellular caspase 3/7 activity of EM-PR cells were examined. As shown in Fig. 3B, dienogest and P_4 at a concentration of 10^{-7} mol/l did not change cellular caspase 3/7 activity for 24 h after treatment.

The effects of dienogest on the expression of 84 human genes related to cell cycle regulation of EM-PR cells were examined by real-time PCR at 6 h after treatment. Dienogest and P_4 at a concentration of 10^{-7} mol/l significantly reduced cyclin D1 mRNA levels when compared with controls, but expression of the other 83 genes did not significantly change (data not shown). We therefore examined the

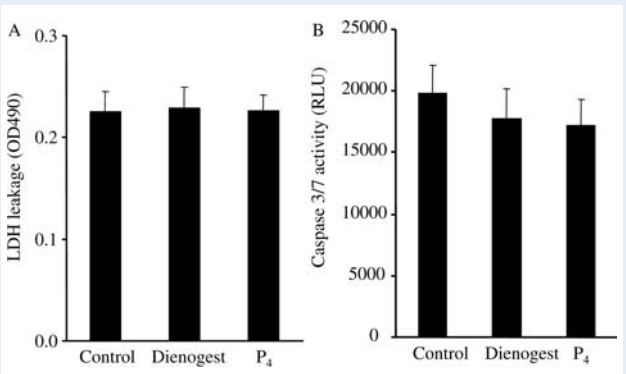

Figure 3 The effects of dienogest and P_4 on LDH leakage (**A**) and cellular caspase 3/7 activity (**B**) of EM-PR cells.

The cells were placed in phenol red-free DMEM/F12 medium supplemented with 0.1% DCC-FBS overnight and were then incubated for 24 h in phenol red-free DMEM/F12 medium supplemented with 1% DCC-FBS containing one of the following agents: DMSO as a vehicle control, P_4 (10^{-7} mol/l) or dienogest (10^{-7} mol/l). Columns and vertical bars represent the mean ± SEM of four separate experiments.

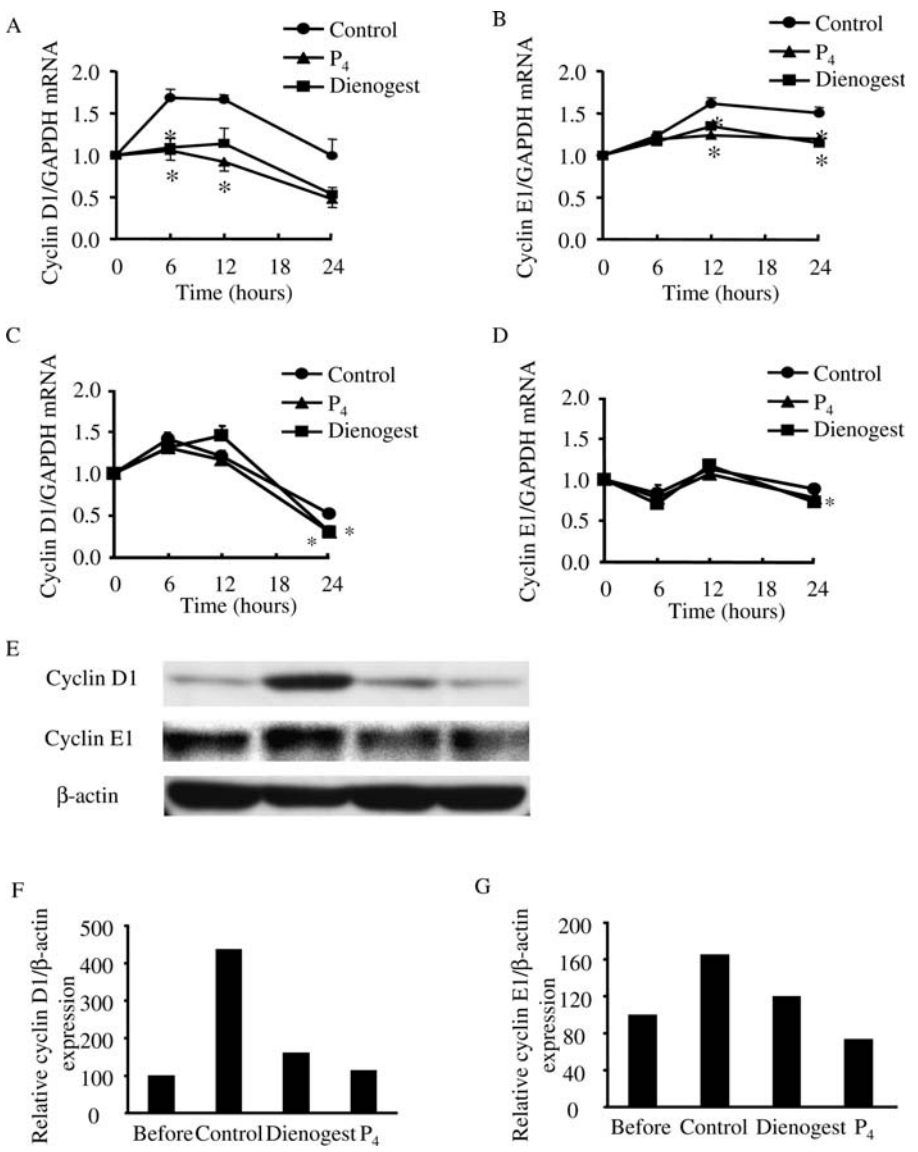

Figure 4 The effects of dienogest and P_4 on the expression of cyclin D1 and cyclin E1 mRNA were measured quantitatively with real-time PCR analysis and normalized to that of GAPDH in EM-PR cells (**A** and **B**) or EM-E6/E7/TERT parent cells (**C** and **D**).

Cyclin D1 and cyclin E1 proteins were also analyzed by western blot analysis (**E**) and the density of the blots were measured quantitatively and normalized to that of β-actin in EM-PR cells (Cyclin D1, **F** and Cyclin E1, **G**). The cells were placed in phenol red-free DMEM/F12 medium supplemented with 0.1% DCC-FBS overnight and were then incubated for 0, 6, 12 or 24 h in order to evaluate mRNA levels, or for 24 h to evaluate protein levels in phenol red-free DMEM/F12 medium supplemented with 1% DCC-FBS containing one of the following agents: DMSO as a vehicle control, P_4 (10^{-7} mol/l) or dienogest (10^{-7} mol/l). Values are the ratio of relative mRNA level normalized to GAPDH. Plots and vertical bars represent the mean ± SEM of three separate experiments (A and B) or one experiment in triplicate (C and D). Asterisks indicate a significant difference by *t*-test (*$P < 0.05$ versus time-matched controls). Values are the ratio of relative protein level normalized to β-actin compared with time 0 h (F and G). Columns and vertical bars indicate the representative value of intensity of fluorescent signals from each band.

time-related effects of dienogest and P_4 on the mRNA expression of cyclin D1 and other important genes related to cell cycle regulation during the G1 phase (cyclin E1, p21, p27 and p53) by real-time quantitative PCR during a 24-h period. Cyclin D1 and cyclin E1 are cell cycle regulators that accelerate cell cycle progression. Dienogest and P_4 significantly decreased both the expression of cyclin D1 mRNA, as compared with controls, at 6 h after treatment (Fig. 4A), and that of cyclin E1 mRNA after 12 h (Fig. 4B) in EM-PR cells. On the other hand, dienogest (10^{-7} mol/l) and P_4 (10^{-7} mol/l) showed only slight changes up to 24 h after treatment in EM-E6/E7/TERT parent cells (Fig. 4C and D). Moreover, we examined the effects of dienogest and P_4 on the protein expression of cyclin D1 and E1 at 24 h after treatment by western blot analysis. Dienogest and P_4 decreased the expression of both cyclin D1 and E1 at the protein level in EM-PR cells (Fig. 4E–G). The cyclin-dependent kinase inhibitors p21, p27 and p53 are cell cycle regulators that inhibit cell cycle progression. Dienogest and P_4 did not change the expression of p21 and p53 mRNA when compared with controls up to 24 h after

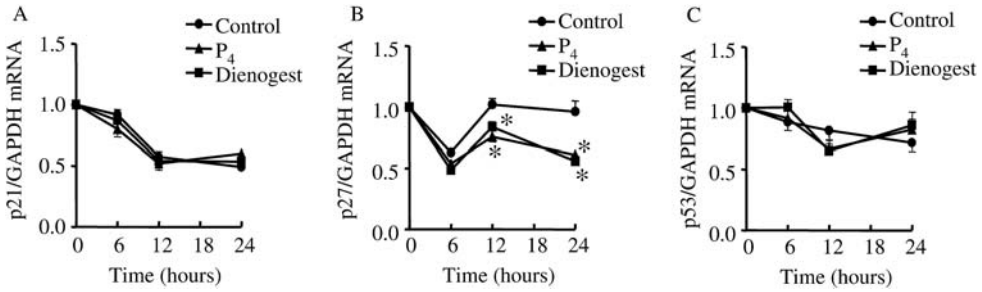

Figure 5 The effects of dienogest and P_4 on the expression of p21 (**A**), p27 (**B**) and p53 (**C**) mRNA were measured quantitatively with real-time PCR analysis and normalized to that of GAPDH in EM-PR cells.

The cells were placed in phenol red-free DMEM/F12 medium supplemented with 0.1% DCC-FBS overnight and were then incubated for 0, 6, 12 and 24 h in phenol red-free DMEM/F12 medium supplemented with 1% DCC-FBS containing one of the following agents: DMSO as a vehicle control, P_4 (10^{-7} mol/l) or dienogest (10^{-7} mol/l). Values are the ratio of relative mRNA level normalized by GAPDH compared with time 0 h. Plots and vertical bars represent the mean ± SEM of three separate experiments. Asterisks indicate a significant difference by *t*-test (*$P < 0.05$ versus time-matched control).

treatment (Fig. 5A and C), but decreased the expression of p27 after 12 h (Fig. 5B) in EM-PR cells.

Discussion

In the present study, we demonstrated the direct antiproliferative effects of dienogest without the induction of apoptosis or cytotoxicity on an immortalized human endometrial epithelial cell line expressing functional PR. Therefore, it is suggested that the antiproliferative effect of dienogest on human endometrial epithelial cells is dependent on its progestational effect via PR. The concentrations of dienogest (10^{-8} and 10^{-7} mol/l) that exhibited antiproliferative effects on cells were within the range of serum dienogest concentrations at the dosage (2 mg/day) used to treat endometriosis (Foster and Wilde, 1998). Growth suppression of endometrial epithelial cells by progestins has been explained by some ideas such as down-regulation of estrogen receptors (Lessey *et al*., 1988), induction of steroid-metabolizing enzymes (Mustonen *et al*., 1998) and decreased estrogen receptor transactivation via PR type A (Giangrande and McDonnell, 1999). However, in this study, we investigated the direct anti-proliferative effects of dienogest and P_4 on human endometrial epithelial cells stimulated with serum being free from estrogens. Our findings indicate that dienogest and P_4 have anti-proliferative effects on human endometrial epithelial cells that are unrelated to anti-estrogenic action. It has been reported that the effect of dienogest in the reduction of serum estradiol concentrations is less than that by GnRH agonists, but dienogest is as effective as GnRH agonist in the relief of pain symptoms associated with endometriosis (Harada *et al*., 2009). The estrogen-independent antiproliferative effects of dienogest on endometrial epithelial cells may contribute to the effectiveness against endometriosis without causing hypoestrogenic side effects such as bone loss and hot flashes. On the other hand, it was also suggested that the response to P_4 is different between eutopic and ectopic endometrium due to some quantitative or qualitative differences in PR expression, indicative of progesterone-resistance (Bulun *et al*., 2006). It has also been reported that endometriotic tissue does not show the same morphological changes as endometrial tissue during the menstrual cycle (Schweppe, 2007). However, pharmacologically effective doses of progestins in the treatment of endometriosis were known to induce progestational changes in both endometriotic and endometrial tissue, causing decidualization and atrophy (Crosignani *et al*., 2006). There was sufficient PR expression to induce progestional effects in endometriotic tissue, although these required greater than pharmacological doses of P_4. Dienogest had therapeutic effects on endometric lesions (Moore *et al*., 1999; Cosson *et al*., 2002; Momoeda *et al*., 2007). In addition, it was reported that dienogest showed anti-proliferative effects on both human eutopic and ectopic endometrial stromal cells (Okada *et al*., 2001; Fu *et al*., 2008). Therefore, the pharmacological effects of dienogest include direct inhibition of human endometriotic epithelial cell proliferation. These anti-proliferative effects of dienogest on endometrial components thus contribute to its therapeutic effects on endometriosis.

The mechanism underlying the antiproliferative effect of dienogest on endometrial cells has been poorly understood. Both dienogest and P_4 have PR agonistic activity (Sasagawa *et al*., 2008). In this study, dienogest had pharmacological responses similar to those P_4 in EM-PR cells. Dienogest and P_4 did not show antiproliferative effect on the parent cells EM-E6/E7/TERT, having low PR expression. It thus appears that the antiproliferative effect of dienogest is dependent on its progestational effect via PR.

Moreover, in this study, dienogest inhibited serum-stimulated proliferation of cells by attenuating cyclin D1, followed by cyclin E1, both of which are the key regulators of cell cycle progression from the G1 phase to the S phase via PR, without affecting any other cell cycle regulator genes. Several investigators have shown that P_4 and progestins inhibit proliferation in human endometrial epithelial normal and malignant cells and breast cancer cells via an increase in the expression of cyclin-dependent kinase inhibitors, including p21 and p27 (Groshong *et al*., 1997; Shiozawa *et al*., 2001; Kawaguchi *et al*., 2006). However, it has been reported that progestins inhibit the proliferation of breast cancer cells with suppression of cyclin D1 expression and that the antiproliferative effect of progestins was depressed in cells overexpressing cyclin D1 (Musgrove *et al*., 2001). Our findings indicate that cyclin D1 is the regulator responsible for the antiproliferative effect of dienogest on human endometrial epithelial cells.

PR exist as two major isoforms transcribed from the same gene by initiation at two distinct promoters, PR-A and PR-B, which have overlapping and distinct functional activities (Gadkar-Sable *et al*., 2005). The expression pattern of PR subtypes in endometriotic tissue is controversial. Some studies have indicated an increase in PR type A expression in endometriotic tissue (Attia *et al*., 2000; Igarashi *et al*., 2005), although others have shown PR type B expression to be predominant (Misao *et al*., 1999; Bukulmez *et al*., 2008). PR subtype expression and the contribution of each PR subtype to the pathogenesis of endometriosis are unclear. In this study, EM-PR cells transfected with the PR type B gene expressed both isoforms of PR protein. It is unclear whether the anti-proliferative effect of dienogest in EM-PR cells was mediated by PR-A or PR-B. Therefore, additional studies to identify the molecular mechanisms through both PR isoforms, and the pathways responsible for regulation between PR and cyclin D1 are needed to clarify the direct anti-proliferative effects of dienogest on endometrial cells.

In summary, we demonstrated that dienogest inhibits the proliferation of human immortalized endometrial epithelial cells with suppression of cyclin D1 levels. These findings suggest that the pharmacological mechanism of dienogest in treating endometriosis includes a direct anti-proliferative effect on human ectopic endometrial epithelial cells.

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