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# Dexamethasone Inhibits the Growth of a Uterine Cell Line Derived from p53-Deficient Mice

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**ABSTRACT**—UE8 is one of several uterine cell lines established from p53-deficient fetal female mice. UE8 exhibits a typical epithelial morphology in culture; it is strongly positive for cytokeratin, but negative for vimentin. Immunoblot analysis confirmed that UE8 has glucocorticoid receptors. It grows actively in medium supplemented with 3% heat-inactivated, dextran-coated charcoal-treated fetal calf serum. Dexamethazone (DEX) inhibited its growth dose-dependently at concentrations between $10^{-6}$ and $10^{-9}$ M. The inhibition by DEX was further examined in chemically defined medium (CDM): DMEM/F12 containing transferrin (10 μg/ml), selenium ($10^{-8}$ M) and 0.1% bovine serum albumin. DEX at $10^{-6}$ M inhibited the growth of UE8 in CDM or in CDM supplemented with insulin-like growth factor-1 (IGF-1: 10 ng/ml), but there was no inhibition by DEX ($10^{-6}$ M) in the presence of epidermal growth factor (10 ng/ml). Thus, DEX inhibits the growth of a uterine epithelial cell line derived from p53-deficient mice *in vitro*, and also suppresses IGF-1-induced proliferation.

## INTRODUCTION

The mouse uterus has been used widely *in vivo* as a model to study the effects of steroid hormones. In adult mice, estrogen withdrawal by ovariectomy induces a dramatic regression of the epithelium, and the regression is reversed by estrogen replacement. Estrogen-induced uterine growth is blocked by simultaneous administration of glucocorticoids (GCs) which decrease estrogen receptor (ER) levels (Rabin *et al*., 1990). In neonatal mice, the proliferation of uterine luminal cells is not dependent on endogenous estrogen; ovariectomy plus adrenalectomy does not affect DNA synthesis (Ogasawara *et al*., 1983). However, DNA synthesis in the neonatal uterine epithelium is inhibited by dexamethasone (DEX) treatment (Bigsby and Cunha, 1985; Bigsby, 1993; Bigsby and Young, 1993). Thus, GCs may inhibit by more than one mechanism.

GCs are potent modulators of growth in various tissues, including reproductive organs; they may be growth stimulatory for some cell types (Orlowski *et al*., 1990; Langeveld *et al*., 1992; He *et al* ., 1994), whereas they may be growth inhibitory for other cell types (Zendegui *et al*., 1988; Lowe *et al*., 1992; Mouhieddine *et al*., 1996). Investigations of the mechanisms suggested that IGFs associated with the G1 phase of the cell cycle are major targets for GC inhibitory action (Pardee, 1989). Sahlin (1995) reported that DEX attenuates expression of IGF-1 mRNA in the whole uterus; however, the inhibitory effect of DEX in uterine epithelial proliferation has not yet been elucidated.

Establishment of uterine epithelial lines provides useful tools for investigation of the molecular mechanisms of DEX-induced growth inhibition. Recently, we have succeeded in establishing uterine cell lines from p53-deficient mice (Hanazono *et al*., 1997). In the present study, we show that an epithelial cell line (UE8) is positive for glucocorticoid receptor (GR), and that growth of this cell line is inhibited by DEX.

## MATERIALS AND METHODS

### Cell culture and passaging

UE8 was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F12) without phenol red (Sigma, St. Louis, MO, USA) containing 3% (v/v) heat-inactivated fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia), supplemented with penicillin (31 μg/ml, Sigma), streptomycin (50 μg/ml, Sigma) and Fungizone (2.5 μg/ml, Life Technologies, Grand Island, NY, USA). When cells became confluent, they were washed with $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline (PBS) and then incubated in 0.05% (w/v) trypsin-0.53 mM EDTA (Life Technologies) at room temperature for 2 min. Cells were then removed from dishes by pipetting, and one-fortieth of them were passaged. They were continuously cultured and had passaged more than 140 times. Cells used in the present study were at passage between 140 and 160.

### Immunocytochemical staining

Cells in a small volume of suspension were seeded on Lab Tek tissue culture chamber slides (Miles Labs., Naperville, IL, USA) and

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incubated in the medium for 3 days. Cells grown on the slides were fixed in 95% (v/v) ethanol containing 1% (v/v) acetic acid at 0°C for 1 hr. After rinsing with 99% (v/v) ethanol, the slides were placed in 95% ethanol and then immersed in PBS. After pre-incubation in PBS containing 5% (v/v) normal goat serum (NGS; Zymed Lab., San Francisco, CA, USA) and 1% (w/v) bovine serum albumin (BSA; Sigma) to block non-specific binding of antibody, cells were covered with primary antibodies and incubated at room temperature for 1 hr. The primary antisera were anti-cytokeratin polyclonal antibody (1/100; Biomedical Technologies, Stoughton, MA, USA) and anti-vimentin monoclonal antibody (1/10; Boeringer Mannheim Biochemica, Germany). After washing with cold PBS, the slides were again incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG serum (Biosource International, Camarillo, CA, USA) or FITC-conjugated anti-mouse IgG serum (Biosource International) at room temperature for 1 hr. For control slides, incubation with the primary antibodies was omitted. The slides were washed in PBS, and mounted in PBS containing 10% (v/v) glycerol. Immunoreactivity was examined with a fluorescence microscope (BH2-RFK, Olympus Optical Ind., Tokyo, Japan) using a blue light excitation filter system (DM500 + 0515/20EY455).

**Cell and tissue extracts**

When cells became confluent, they were collected in a tube and washed once with PBS. Then 2 ml 0.5 M Tris buffer (pH 7.6) containing 150 mM NaCl, 10 mM EDTA, 20 mM NaF, 0.25 mM PMSF, 1% (v/v) TritonX-100, 1% (w/v) Na-deoxycholate (Sigma) and 0.1% (w/v) sodium dodecyl sulfate (SDS; Sigma) were added to the tube and incubated at 0°C for 20 min before collecting the lysate.

Young (30-day-old) CD-1 (ICR) mice were used. They were given pellet chow (MF; Oriental Yeast Co. Tokyo, Japan) and tap water ad libitum in temperature (22–24°C, RH 50%)- and light (12L-12D)-conditioned room. Mice were killed by cervical dislocation and the thymus was dissected out, minced and suspended in 1 ml 0.08 M Tris buffer (pH 6.9) containing 0.11 M SDS and 0.1 M dithiothreitol (Sigma). The suspension was homogenized in an ice-cold glass homogenizer. The homogenate was sonicated for 10 sec and immediately incubated at 95°C for 5 min. Then the homogenate was centrifuged for 30 min at $2 \times 10^5 \times g$ and the supernatant was collected. Protein concentration of the samples for blotting analysis was determined using BioRad Protein Assay (Bio-Rad, Richmond, CA, USA). The aliquots of all samples were stored at –80°C until analysis.

**Analysis of blotting**

The samples were electrophoresed in SDS polyacrylamide-slab gel and electrophoretically transferred onto polyvinylidine difluoride membrane (Millipore Co., Bedford, MA, USA). Membranes were rinsed in Tris-buffered saline (TBS; pH 7.6) for 15 min. The membranes were blocked with 10% (w/v) skimmed milk (Yukijirushi-Nyuugyou Co., Sapporo, Japan) in TBS-0.05% (v/v) Tween 20 (TBS-T) at 4°C overnight and incubated with anti-human glucocorticoid receptor (1/100; Affinity Bioreagents, Golden, CO, USA) antibody. The antibody was diluted with 5% (w/v) skimmed milk in TBS-T. After washing with TBS-T containing 5% skimmed milk, the membranes were incubated with horseradish peroxidase-conjugated antibody (Biosource International) for 1 hr. Then they were washed twice with TBS-T containing 5% skimmed milk and once with TBS-T, and incubated in a 1:1 mixture of enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, England) reaction solution at room temperature for 1 min. Autographs of chemiluminescence were prepared by exposing the membranes to X-ray film (Kodak) for 30 sec at room temperature.

**Assays of DEX effect on growth**

UE8 cells were trypsinized, collected and washed three times in PBS. Then the cells were plated into 96-multiwell dishes ($5 \times 10^3$ cells/well) in DMEM/ F12 containing 3% (v/v) FCS treated with dextran-coated charcoal (DCCFCS) to remove steroids. DEX (Sigma) was dissolved in absolute ethanol and was added to the medium at concentrations ranging from $10^{-6}$ to $10^{-9}$ M. Control cultures were grown in medium with ethanol vehicle (0.01%; v/v). Media were changed every 2 days. At 1, 3, 5 and 7 days, cells were incubated in 100 μl DMEM/F12 containing 10% (v/v) alamar Blue™ (Biosource International) for 3 hr. Cell numbers per well were estimated by the cellular reduction of alamar Blue™ (alamar Blue assay). Absorbance of the reduced products measured spectrophotometrically by a microplate reader was proportional to the number of cells ($r^2 = 0.99$). Student's *t*-test was used for the statistical analysis.

Effect on growth was also examined in chemically defined medium : DMEM/F12 containing transferrin (Trf: 10 μg/ml, Sigma), selenium (Se: $10^{-8}$ M, Sigma) and 0.1% (w/v) BSA (Sigma). Supplements

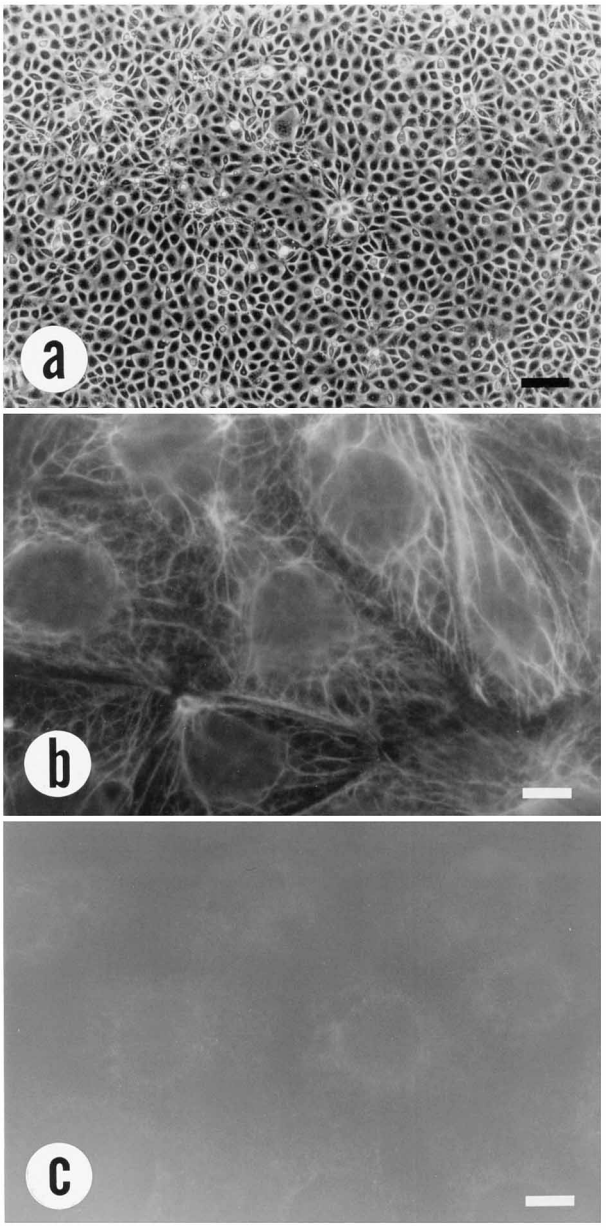


**Fig. 1.** Morphology (**a**) and immunocytochemical staining (**b** and **c**) of UE8 cells. UE8 cells exhibited a typical epithelial form (**a**) and contained a cytokeratin fiber network in the cytoplasm (**b**). The cells did not stain with vimentin monoclonal antibody (**c**). Bars: **a**, 100 μm; **b** and **c**, 10 μm.

were IGF-1 (Toyobo, Osaka, Japan) and EGF (R & D Systems, Minneapolis, MN, USA). EGF and IGF-1 were added to the serum-free medium at 10 ng/ml. DEX was added to the medium at concentrations ranging from $10^{-6}$, $10^{-7}$, or $10^{-8}$ M. Five $\times$ $10^3$ cells were plated into each well and cultured for 3 days. Cell numbers per well were estimated by the alamar Blue assay as described above.

## RESULTS

### Cell morphology and immunocytology

UE8 cells exhibited a typical epithelial polygonal shape in dense confluent monolayer which developed a honeycomb pattern (Fig. 1a).

After 72 hr of culture on Lab Tek tissue culture chamber slides, cells were stained with indirect immunofluorescent methods. The epithelial nature of UE8 was confirmed by specific immunofluorescent detection of cytokeratin fibers (Fig. 1b) forming a network surrounding the nucleus. The cells did not stain with anti-vimentin monoclonal antibody (Fig. 1c).

### Detection of GR

Immunoblot analysis showed the presence of GR in UE8 cells; the blotting profile is similar to that of young mouse thymus (Fig. 2). Although the samples contained degraded form of GR (Carlstedt-Duke *et al.*, 1982), both UE8 and the thymus contained a strong single band between 73 and 127 kDa, which corresponds to the described molecular size (94 kDa: Okret *et al.*, 1984).

### Effect of DEX in medium supplemented with 3% DCCFCS

To examine the effect of DEX on UE8 cell proliferation, growth rates were monitored in medium supplemented with 3% DCCFCS. Cells were cultured with vehicle control or DEX at concentrations ranging from $10^{-6}$ to $10^{-9}$ M. On day 3 of culture, proliferation was significantly inhibited by $10^{-6}$ M DEX: 34% reduction from the control ($p < 0.01$, Fig. 3). On day 5, the inhibitory effect of DEX became prominent at all concentrations: proliferation declined by 45% ($10^{-7}$ M) to 61% ($10^{-6}$ M) from the control ($p < 0.01$). Furthermore, dose-dependent inhibition of proliferation was evident on day 7: the proliferation declined by 21% ($10^{-9}$ M), 40% ($10^{-8}$ M), 48% ($10^{-7}$ M) and 51% ($10^{-6}$ M) from the control ($p < 0.01$).

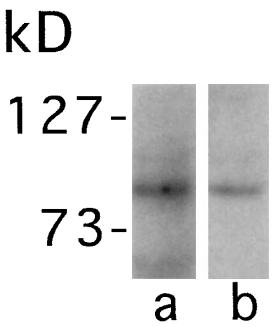


**Fig. 2.** Blotting profile of glucocorticoid receptor. GR was detectable as a single band between 127 and 73 kDa, which corresponds to the described molecular size (94 kDa: Okret *et al.*, 1984). Lane a: UE8 (30 μg/lane); lane b: mouse thymus (30 μg/lane).

### Effect of DEX in CDM

Cells were cultured in CDM with or without EGF (10 ng/ml) or IGF-1 (10 ng/ml) for 3 days. DEX ($10^{-6}$ M) slightly inhibited proliferation in CDM: 8% reduction from the control ($p < 0.01$, Fig. 4A). In this medium, EGF or IGF-1 alone stimulated proliferation 62% (Fig. 4B), and 73% over the control (Fig. 4C), respectively. Although DEX did not inhibit EGF-induced proliferation at any concentrations examined (Fig. 4B), it suppressed IGF-1-induced proliferation: the proliferation declined by 19% ($p < 0.01$, Fig. 4C).

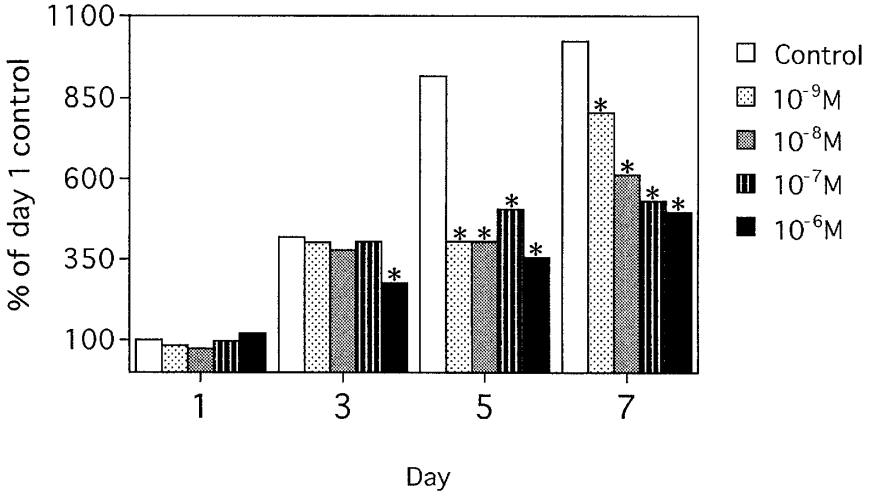


**Fig. 3.** Effect of DEX in medium supplemented with serum. Cells were plated into 96-multiwell dishes ($5 \times 10^3$ cells/well) in DMEM/ F12 containing 3% FCS treated with dextran-coated charcoal. $10^{-6}$ to $10^{-9}$ M DEX was added. At 1, 3, 5 and 7 days, the cell numbers per well were estimated by alamar Blue assay. Data are expressed as % of day 1 control. Standard errors are less than 5%; n = 8; ∗, $p < 0.01$ vs. control.

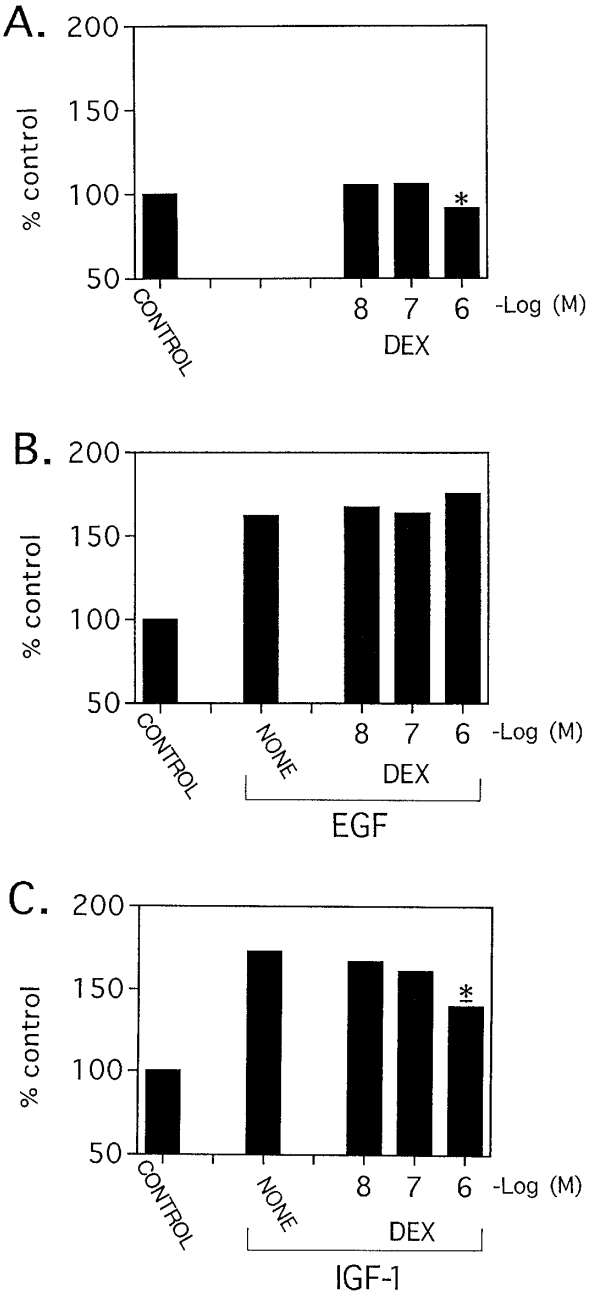


**Fig. 4.** Effects of DEX in CDM. Cells were cultured in CDM (**A**) or in medium supplemented with EGF (**B**; 10 ng/ml) or IGF-1 (**C**; 10 ng/ml) for 3 days. DEX was added at $10^{-8}$, $10^{-7}$ or $10^{-6}$ M. Cells cultured without DEX (**A**) and EGF (**B**) or IGF-1 (**C**) served as controls. None (**B** and **C**) received the ethanol vehicle (0.01%). Data are expressed as % of control (mean ± SEM; n = 8; ∗, $p < 0.01$; **A** vs. control, **C** vs. none, respectively). Standard errors less than 5% are not shown.

## DISCUSSION

In neonatal mice, proliferation of uterine luminal cells is specifically inhibited by DEX administration (Bigsby and Cunha, 1985). Although GR occurs in both epithelium and stroma of the neonatal mouse uterus (Bigsby and Young, 1993), it has not yet been elucidated whether DEX directly inhibits proliferation of epithelial cells without any stromal-epithelial interactions. In the present study, we demonstrated that the newly-established uterine epithelial cell line UE8 possesses GR, and that its proliferation is markedly suppressed by DEX.

Previously Rabin *et al.* (1990) proposed a hypothesis for the inhibitory effect of DEX: DEX might inhibit secretion of an estrogen-dependent autocrine factor which stimulates proliferation. In mouse uterine epithelial cells, EGF is a candidate estrogen-dependent autocrine factor (Huet-Hudson *et al.*, 1990), and prepro-EGF mRNA was detected even in the absence of estrogen stimulation (DiAugustine *et al.*, 1988). As shown in Fig. 4A, the inhibitory effect of DEX was also observed in CDM. This inhibition might result from DEX suppression of EGF production, because addition of EGF completely neutralized the DEX inhibition (Fig. 4B). A similar interaction between DEX and EGF was reported in primary cultures of mouse uterine epithelial cells; glucocorticoid did not inhibit proliferation when EGF was added to the culture medium (Hanazono *et al.*, 1991).

Sahlin (1995) reported that DEX attenuated the estrogen-induced increase of IGF-1 mRNA in the uterus, and proposed that DEX inhibition acts by suppression of IGF-1 production. However, the present study demonstrated that IGF-1 supplement did not reverse the inhibitory effect of DEX; DEX at $10^{-6}$ M proved inhibitory even in IGF-1-supplemented serum-free culture. Interaction between insulin-like growth factor-binding proteins (IGFBPs) and the IGF-1 supplement may be involved in the DEX inhibition. IGFBPs may act as either amplifier or attenuator of IGF effects on target cells (Rechler, 1993). DEX-induced growth arrest of lung alveolar epithelial cells is associated with cellular induction of IGFBP-2 (Mouhieddine *et al.*, 1996). In addition, rat uterine epithelium expresses IGFBP-4 mRNA during diestrus when its proliferation is arrested (Girvigian *et al.*, 1994). Therefore, it is possible in UE8 cells that DEX may induce the production of IGFBPs which bind IGF-1 and prevent it from neutralizing DEX inhibition. Alternatively, DEX might affect the interaction between IGF-1 and its receptor and/or post receptor pathways.

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