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# ICI 182,780 Stimulates the Growth of a Uterine Cell Line Derived from p53-Deficient Mice

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**ABSTRACT**—UE8 is a uterine epithelial cell line established from p53-deficient fetal female mice. UE8 exhibits a typical epithelial morphology in culture and is strongly positive for cytokeratin, but negative for vimentin in immunocytochemistry. UE8 shows an active growth in a phenol red free 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 supplemented with 3% heat-inactivated and dextran-coated charcoal-treated fetal calf serum. Both immunocytochemistry and immunoblot analyses confirmed that UE8 was negative for the estrogen receptor. Diethylstilbestrol added to the medium at concentrations between  $10^{-6}$  and  $10^{-8}$  M had no significant effect on the proliferative rate, and estradiol-17 $\beta$  at  $10^{-6}$  M was slightly inhibitory. Unexpectedly, however, the "pure anti-estrogen" ICI 182,780 at  $10^{-6}$  M significantly enhanced cell proliferation. This is the first observation that the "pure anti-estrogen" ICI 182,780 stimulates the growth of uterine epithelial cells without estrogen receptors.

## INTRODUCTION

The development of pure anti-estrogens without estrogenic activity may be important in the relief of a variety of clinical problems. For this purpose, suitable *in vitro* models are needed to estimate the quality of the proposed agents. MCF-7 (Osborne *et al.*, 1987) and Ishikawa (Nishida *et al.*, 1985) cells, whose proliferation was stimulated by estrogen, have been utilized to estimate the quality of anti-estrogens with conflicting results. For example, tamoxifen is an antagonist for the growth of MCF-7 cells (Katzenellenbogen *et al.*, 1987; Wakeling *et al.*, 1989; Pratt and Pollak, 1993), but it is a full agonist for the growth of Ishikawa cells (Kleinman *et al.*, 1996). Therefore, a variety of cell lines may be necessary to estimate with certainty the side effects of newly-developed reagents.

The mouse uterus is a particularly sensitive indicator of the agonist activity of anti-estrogens (Clark and Markaverich, 1982; Furr and Jordan, 1984). However, its usage is hampered by failure to establish estrogen-sensitive cell lines except for the m-M116 cells established from a leiomyoma of a CaBP9k/Tag transgenic mouse (Blin *et al.*, 1996). Recently, we have succeeded in establishing uterine cell lines from p53-deficient mice (Hanazono *et al.*, 1997). Throughout the characterization of these cell lines, we found that one of the lines

showed a proliferative response to the pure anti-estrogen ICI 182,780.

## 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 without phenol red (DMEM/F12; Sigma, St. Louis, MO, USA) containing 3% (v/v) heat-inactivated fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia), supplemented with penicillin (31  $\mu$ g/ml, Sigma), streptomycin (50  $\mu$ g/ml, Sigma) and Fungizone (2.5  $\mu$ g/ml, Life Technologies, Grand Island, New York, USA). When cells became confluent, they were washed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline (PBS) and then incubated in trypsin solution at room temperature for 2 min. After the incubation, cells were removed from dishes by pipetting, and one-fortieth of them were passaged. They were continually cultured and passaged more than 140 times. Cells used in the present study were at passage between 140 and 160.

### Immunocytochemical staining

The method of immunocytochemical staining was described previously (Hanazono *et al.*, 1997). In brief, cells in a small volume of cell suspension were seeded on Lab Tek tissue culture chamber slides (Miles Labs., Naperville, IL, USA) and incubated in the medium with estradiol-17 $\beta$  (E<sub>2</sub>,  $10^{-8}$  M) 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 ethanol (99%; v/v), 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

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antibody, cells were covered with anti-estrogen receptor (ER) monoclonal antibody (AER314, 1/50; NeoMarkers, Fremont, CA, USA), and incubated for 8 hr. After washing, cells were incubated with FITC-conjugated anti-mouse IgG serum (Biosource International, Camarillo, CA, USA). On control slides, incubation with the primary antibody was omitted. Immunoreactivity was examined with a fluorescence microscope (BH2-RFK, Olympus Optical Ind., Tokyo, Japan).

#### Cell and tissue extracts

When cells became confluent in a 100-mm dish, cells were collected in a tube and washed once in 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 phenylmethylsulfonyl fluoride (Sigma), 1% (v/v) TritonX-100 (Sigma), 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.

An adult CD-1 female mouse was killed by cervical dislocation and the uteri were dissected out. The tissues were 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) (Bentvelsen *et al.*, 1996). 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. 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). Aliquots of all samples were stored at -80°C until analysis.

#### Analysis of blotting

The samples were electrophoresed on SDS-polyacrylamide-slab gel and electrophoretically transferred onto polyvinylidene difluoride membrane (Millipore, 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 for overnight and incubated with anti-ER antibody (1/50; NeoMarkers). The antibody was diluted with 5% (w/v) skimmed milk in TBS-T, which was also used to wash the membranes. They were incubated with horseradish peroxidase-conjugated antibody (Biosource) for 1 hr. Then they were washed twice with TBS-T containing 5% skimmed milk and once with TBS-T, and reacted with an enhanced chemiluminescence detection reagent (ECL; Amersham, Buckinghamshire, England) at room temperature for 1 min. Autographs of chemiluminescence were prepared by exposing the membranes for 30 sec to X-ray film (Kodak) at room temperature.

#### Effects of estrogens and ICI 182,780 on growth

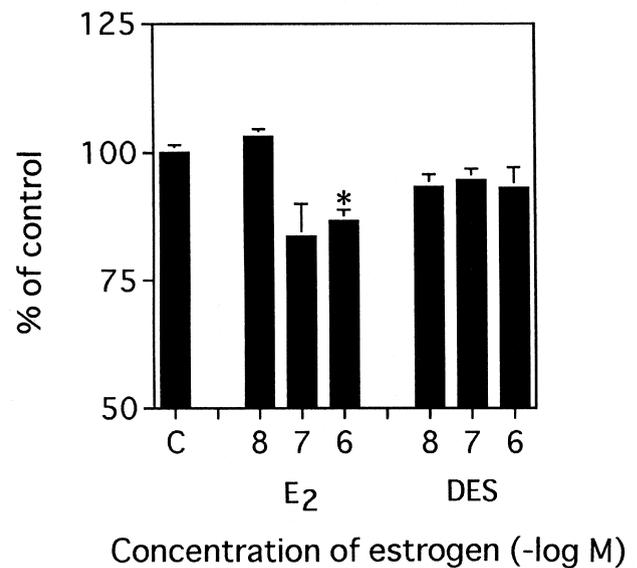
UE8 cells were trypsinized, collected and washed three times in PBS. Then the cells were plated on 96-well dishes ( $5 \times 10^3$  cells/well) in DMEM/ F12 containing 3% (v/v) FCS treated with dextran-coated charcoal (DCCFCS) to remove steroids. ICI 182,780 (a generous gift from Dr. C. Mackay, ICI Pharmaceuticals, Cheshire, England) was added to the medium at  $10^{-6}$  or  $10^{-7}$  M, and the cells were cultured for 1, 3 and 5 days. Media were changed every 2 days.  $E_2$  and diethylstilbestrol (DES; Sigma) were dissolved in absolute ethanol and added to the medium at  $10^{-6}$ ,  $10^{-7}$ , or  $10^{-8}$  M. Ethanol vehicle (0.01%; v/v) was added to control cultures. Cells were cultured for 3 days, and then they were incubated in 100  $\mu$ l DMEM/F12 containing 10% (v/v) alamar Blue™ (Biosource) for 3 hr; cell numbers per well were estimated by the amount of dye reduction. The absorbance of the reduced products, which was measured spectrophotometrically by a microplate reader, was proportional to the number of cells ( $r^2 = 0.99$ ). Student's *t*-test was used for statistical analysis.

## RESULTS

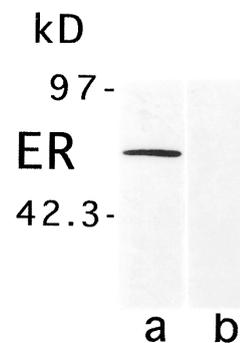
### Sensitivity to estrogens and detection of ER

To examine the effect of estrogens on UE8 cell proliferation, growth rates were monitored in 3% DCCFCS with increasing concentrations of  $E_2$  or DES. As shown in Fig. 1, these estrogens had no stimulating effect on UE8 cells.  $E_2$  slightly inhibited cell proliferation at  $10^{-6}$  M.

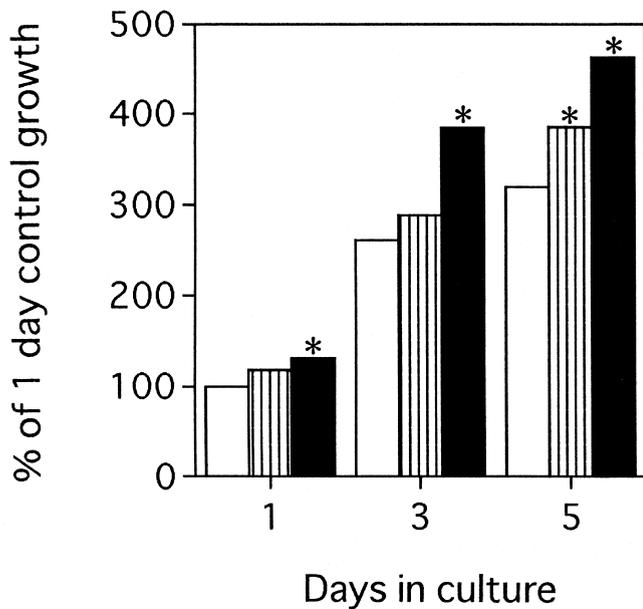
Immunocytochemistry did not reveal ER in UE8 cells (data not shown), and this result was further confirmed by Western blotting. An intact mouse uterus as a positive control contained ER as a strong single band at 66 kDa. (Fig. 2, lane a), but extract of UE8 cells did not contain any detectable immunoreactive band (Fig. 2, lane b).



**Fig. 1.** Estrogen sensitivity of UE8. To determine estrogen stimulation of UE8 cell proliferation, growth rates were monitored in DMEM/ F12 containing 3% DCCFCS. Cells were cultured for 3 days with increasing concentrations of  $E_2$  or DES ( $10^{-8}$ ,  $10^{-7}$  and  $10^{-6}$  M). Control cultures (C) were grown in medium with ethanol vehicle (0.01%). Cell numbers per well were estimated by alamar Blue assay. Data are expressed as % of control growth (the mean  $\pm$  SEM;  $n = 5$ ). \*,  $p < 0.01$  vs. control.



**Fig. 2.** Blotting profile of estrogen receptor (ER). Lane a: extract of mouse uterus (30  $\mu$ g). Lane b: extract of UE8 (30  $\mu$ g). ER in mouse uterus was detectable as a single band, but UE8 did not contain any specific immunoreactive band.



**Fig. 3.** Effect of ICI 182,780 in DCCFCS-supplemented medium. Cells were plated on 96-well dishes ( $5 \times 10^3$  cells/well) in DMEM/ F12 containing 3% DCCFCS. ICI 182,780 was added at  $10^{-6}$  M (solid bars) or  $10^{-7}$  M (striped bars). Control cultures (open bars) were grown in medium with ethanol vehicle (0.01%). Media were changed every 2 days. At 1, 3, 5 days, cell numbers per well were estimated by alamar Blue assay. Data are expressed as % of day 1 control growth (mean  $\pm$  SEM; n = 5). \*, p < 0.01 vs. control value for each day.

#### Sensitivity to ICI 182,780

ICI 182,780 was first tested on the cells in medium containing 3% DCCFCS. Proliferation was slightly stimulated by ICI 182,780 at  $10^{-7}$  M: 20% over the control (p < 0.01, Fig. 1) on day 5 of culture. A higher concentration ( $10^{-6}$  M) resulted in further growth stimulation: 31% on day 1, 47% on day 3 and 45% on day 5 over controls (p < 0.01, Fig. 3).

#### DISCUSSION

The mouse uterus is a particularly sensitive indicator of agonist activity of anti-estrogens (Clark and Markaverich, 1982; Furr and Jordan, 1984) and has been widely used *in vivo* to estimate activity of estrogen antagonists. Establishment of cell lines from the uterus may allow to determine whether an antagonist acts as a pure anti-estrogen or not. Furthermore, cell lines are critical for investigation of the molecular mechanisms of antagonist action. In the present study, we reported that the proliferation of UE8 cells was stimulated by a "pure anti-estrogen" ICI 182,780, but inhibited by  $E_2$  at  $10^{-6}$  M. However, both immunocytochemical and immunoblot analyses revealed no detectable ER in these cells. The same results were obtained from another line UE2 which was co-established with UE8 (data not shown). This is the first observation that the "pure anti-estrogen" ICI 182,780 can stimulate the growth of uterine cells *in vitro*.

Our present observations raise an interesting question: whether the proliferation of UE8 cells was stimulated through

ER. The growth of ER-positive MCF-7 cells is inhibited by ICI 182,780, but that of ER-negative BT-20 cells is not affected by the anti-estrogen (Wakeling and Bowler, 1992). Although neither immunocytochemistry nor immunoblot analysis demonstrated that UE8 cells have detectable ER, it is possible that the monoclonal antibody employed in the present study might be unable to recognize ER, because the p53-deficiency may cause conformational changes due to a greatly increased rate of accumulation of genetic mutations (Kastan *et al.*, 1992). In LNCaP prostate tumor cells, a mutated steroid receptor was reported: the cells have a point mutation in the androgen receptor at the androgen-binding domain, and their proliferation was stimulated not only by androgens but also by anti-androgens, estrogens and progesterone (Veldscholte *et al.*, 1990, 1992). Therefore, it is possible that the anti-estrogen stimulates proliferation through mutated ER which lost immunoreactivity to the anti-ER antibody. As shown in Fig. 2,  $E_2$  at  $10^{-6}$  M caused growth inhibition of UE8 cells; such inhibition is also reported in primary cultures of mouse uterine epithelial cells (Uchima *et al.*, 1991), even though DES had no effect on the proliferation.

Alternatively, the anti-estrogen (or its possible contaminants) may stimulate the proliferation of UE8 cells without involving ER; DES or its metabolites can have effects through some macromolecules of ER-negative cells (Barrett *et al.*, 1981). ICI 182,780 or its metabolites may also stimulate the proliferation of UE8 cells through a non-ER mechanism. In addition, Kuiper *et al.* (1996) cloned a novel rat ER (ER $\beta$ ), which has low homology of its N-terminal with that of the previously cloned ER (recently renamed ER $\alpha$ ). They reported that MCF-7 cells do not contain ER $\beta$ , whereas two types of ER ( $\alpha$  and  $\beta$ ) are expressed in rat and human uterus (Kuiper *et al.*, 1997). Although additional studies are needed to clarify the mechanism how the anti-estrogen stimulates cell proliferation without the ER, UE8 is an unique cell line to investigate estrogen-regulated proliferation.

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