Insulin-Like Growth Factor–I Stimulates Proliferation of Mouse Uterine Epithelial Cells in Primary Culture (44152)

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Abstract. **Estrogens stimulate proliferation and differentiation of uterine epithelial cells *in vivo*. Mitogenic action of estrogens may be mediated by growth factors such as insulin-like growth factor–I (IGF-I). This study was designed to determine whether IGF-I and insulin affect proliferation of uterine epithelial cells obtained from 3- to 4-week-old immature female mice in a serum-free culture system. The epithelial cell number on Day 5 in culture was significantly increased by adding IGF-I (10 and 100 ng/ml) or insulin (100 and 1000 ng/ml) to the culture media, indicating that IGF-I is more effective than insulin in inducing the epithelial growth. The epithelial DNA synthesis was significantly stimulated by IGF-I (1 and 10 ng/ml), suggesting that both the epithelial proliferation and their detachment from substratum are stimulated by 1 ng/ml of IGF-I, but that the former is more accelerated than the latter by 10 ng/ml of IGF-I. These results demonstrate that both IGF-I and insulin directly stimulate the growth of uterine epithelial cells, and suggest that insulin may act *via* IGF-I receptors. IGF-I immunoreactivity was detected in the cytoplasm of the cultured cells, indicating that the cells synthesize IGF-I. Estradiol-17β (E_2) at lower concentrations (0.001–0.1 n*M*) tended to increase the number of epithelial cells, while E_2 at higher concentrations (1 to 100 n*M*) did not affect it. It is highly probable that IGF-I produced in endometrial cells induces their proliferation by an autocrine or paracrine mechanism.** [P.S.E.B.M. 1997, Vol 215]

The growth of uterine endometrial cells is regulated by estrogens and progestins (1), but mitogenic activity of estrogens has not been demonstrated in cultures of epithelial cells (2, 3). Several growth factors such as epidermal growth factor (EGF), transforming growth factor–α (TGFα), and heparin-binding epidermal growth factor-like growth factor (HB-EGF) are considered to mediate estrogen action in the uterus (4–11). In fact, estrogen increased EGF, TGFα, and HB-EGF synthesis in the uterus. Moreover, EGF and TGFα stimulated the growth of uterine epithelial cells (4, 12), and blockage of EGF action with specific antibodies against EGF decreased the estrogen-induced uterine growth (4).

Insulin-like growth factor–I (IGF-I) is detected in the luminal and glandular epithelium, stroma, and myometrium in the rat and mouse uterus (13–15), and in the human and primate uterus (16, 17). IGF-I synthesis is stimulated by treatment with estrogen in the rat and mouse uterus (14, 15). Consequently, IGF-I may also mediate the actions of estrogens on the uterine epithelial growth. However, as far as we know, action of IGF-I on the growth of uterine epithelial cells had not been analyzed *in vitro*. Therefore, the present study was planned to clarify the effect of IGF-I on the proliferation of mouse uterine epithelial cells in primary serum-free culture. Effects of estrogen on the proliferation of the epithelial cells was also studied.

Materials and Methods

Animals. Three- to four-week-old female mice of the ICR strain (CLEA Japan Inc., Osaka, Japan) were used in the present study. They were maintained in a temperature-controlled room and were fed *ad libitum*. All experiments were performed under protocols following the Guidelines of Animal Experimentation, Faculty of Science, Okayama University.

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Isolation of Uterine Epithelial Cells. Uterine epithelial cells were isolated following the method of Tomooka *et al.* (12). Briefly, female mice were sacrificed by cervical dislocation, and uteri were dissected out. The uterine horn was longitudinally cut into two parts to expose the endometrial surface. The tissue fragments were kept in 0.5% trypsin (Difco, Detroit, MI) in Hanks' solution containing 20 mM HEPES and 0.3% bovine serum albumin at 4°C for 1 hr, and then at 37°C for 1 hr. Trypsin was inactivated by soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) at 37°C for 15 min. The epithelial and stroma tissues were separated by gentle pipetting. The epithelial tissue fragments were collected, and were separated from fibroblastic cells using a self-generated Percoll gradient (Pharmacia, Uppsala, Sweden). Collected epithelial fragments were further treated by gentle pipetting to obtain smaller fragments. The cell number was determined by counting the nuclei with a hemocytometer after epithelial cell suspension was treated with 0.1 *M* citric acid solution containing 0.1% crystal violet at 37°C to lyse the cells and to stain their nuclei (18). The cell viability was assessed by trypan blue exclusion test, and was usually more than 90% in each study.

Preparation of Collagen Gel Substratum for Culture. Type I collagen solution was prepared from rat tail collagen fibers according to the method of Imagawa *et al.* (19). Neutralized and osmolarity-adjusted collagen solution was added to a 12-well culture plate (Falcon, NJ), and allowed to form gel by incubating at 37°C.

Serum-Free Culture of Isolated Epithelial Cells. An 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium without phenol red and calcium was prepared and was supplemented with $CaCl_2$ (0.1 m*M*), bovine serum albumin (fraction V, 1000 mg/l, Sigma), hydrocortisone (100 μg/l), triiodothyronine (400 ng/l), transferrin (10 mg/l), glucagon (10 ng/l), parathormone (200 ng/l), and sodium selenite (5 μg/l). EGF and recombinant human IGF-I were obtained from Sigma and Amersham (Buckinghamshire, United Kingdom), respectively. Insulin (Sigma) was stocked at concentration of 10 mg/ml in sterile 1% acetic acid solution. Estradiol-17β (E_2, Sigma) was initially dissolved in sterile ethanol and was stocked at concentration of 10 μ*M* (0.03% ethanol). Working solutions of insulin and E_2 were reconstructed just before use by diluting stock solutions with culture media.

The isolated epithelial cells were seeded at the cell density of 2.5×10^4 cells/cm^2 in collagen gel–coated 12-well plates and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. After 1-day preculture, IGF-I, insulin, and E_2 were added to the culture media. The culture medium was changed every 2 days. Independent experiments were carried out at least three times for each study.

Determination of the Number of Epithelial Cells. The number of cultured epithelial cells was determined by a modification of the tetrazolium assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a substrate (MTT assay [20]). The amount of formazan after 4 hr of incubation at 37°C was spectrophotometrically determined by measuring absorbance at 540 nm and was used to estimate cell numbers in a well. Cell numbers were expressed as a percentage by regarding the maximal cell number as 100%.

Determination of the Proliferating Cells. DNA-synthesizing cells were detected using a cell proliferation kit (Amersham). Epithelial cells were seeded on a poly-L-lysine–coated slide glass. 5-Bromo-2′-deoxyuridine (BrdU) was added into the culture medium (3 mg/ml), and cultures were continued for 5 hr with IGF-I (1 and 10 ng/ml). The BrdU-containing cells were detected immunocytochemically. The percentage of BrdU-labeled cells was calculated.

Immunocytochemical Analysis of Epithelial Cells. Epithelial cells were cultured on the collagen-coated glass slide in the medium containing insulin (100 ng/ml) and EGF (10 ng/ml). The cells were fixed by pre-chilled methanol (–20°C) for 30 min and dried overnight at room temperature. For cytokeratin or vimentin detection, the cells were treated with 0.05% trypsin in PBS for 15 min at 37°C, then incubated with pkk-1 mouse anticytokeratin antibody (Labsystems, Finland) or with antivimentin antibody (Organon Teknika, NC) for 72 hr at 4°C. For IGF-I detection, the fixed cells were incubated with rabbit anti-human IGF-I antiserum (UB2-495, NIDDK) for 72 hr at 4°C. Cytokeratin–, vimentin–, and IGF-I–immunoreactivity was visualized using ABC kit (Vector Laboratories, Burlingame, CA). For controls, PBS was applied instead of the antiserum.

Statistics. Statistical analysis was carried out by analysis of variance and Duncan's multiple range test.

Results

Observation of Cultured Epithelial Cells. Uterine epithelial and stromal tissues were easily separated by treatment with trypsin and gentle pipetting. Most epithelial cells attached to the collagen gel substratum within a few hours after seeding. Phase contrast microscopy revealed that attached cells were flattened, forming polygonal sheets, a characteristic of epithelial cells in culture. Immunocytochemical analysis showed that the cultured cells expressed cytokeratin (Fig. 1), but not vimentin (data not shown), indicating that they were derived from the epithelial cells, not from the stromal cells. The epithelial cells in the central part of the cell sheet became smaller than those in the peripheral areas, suggesting that the cells proliferate in the central area of a cell sheet.

The Growth of Epithelial Cells. The number of cells was measured by using MTT assay. The amount of formazan produced (the optical density observed at 540 nm) was well correlated with the number of cells in culture (Fig. 2). In the optimal culture condition (including insulin 100 ng/ml and EGF 10 ng/ml), the cell number increased 1.8-fold in 7 days (Fig. 3). After 7 days in culture, the cells became semiconfluent (data not shown). Therefore, in the following experiments, the number of the cells were deter-

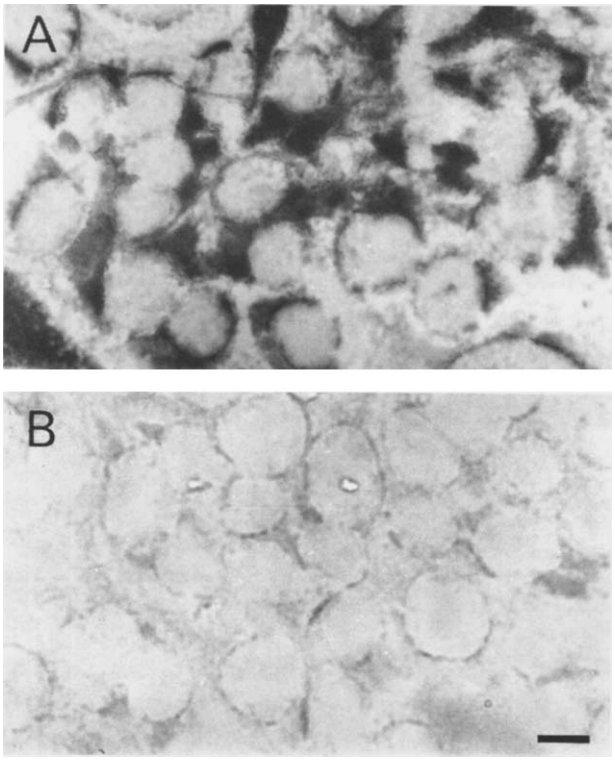

Figure 1. Immunocytochemical detection of cytokeratin in uterine epithelial cells on Day 3 of culture stained with anticytokeratin antibody (A), and control (B). In Panel A, cytoplasm of the epithelial cells was strongly stained, but not in the nucleus. Bar, 10 μm.

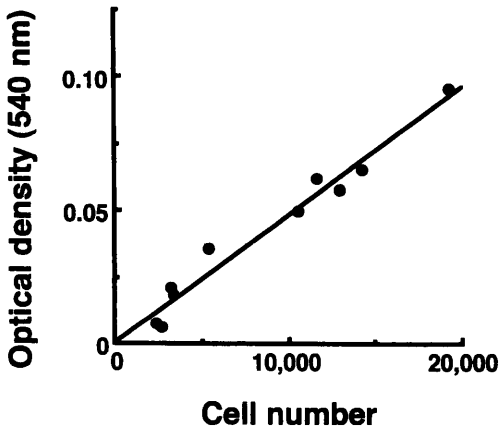

Figure 2. Correlation of MTT assay with the cell number. After the MTT assay was finished, cells on collagen gels were fixed with methanol, stained with Giemsa's solution, and dried. The cell number was counted, and the relationship between OD_{540} and the cell number was examined. Correlation coefficient (r) was 0.965 in this assay.

mined on Day 5 in culture when there was enough space for the cells to proliferate.

Effects of IGF-I and Insulin on the Growth of Epithelial Cells. Preliminary studies showed that the maximal epithelial proliferation was obtained when 10 ng/ml of EGF was added to the serum-free medium (data not shown). When added to EGF-supplemented media, the epithelial cell number was significantly increased by IGF-I (10

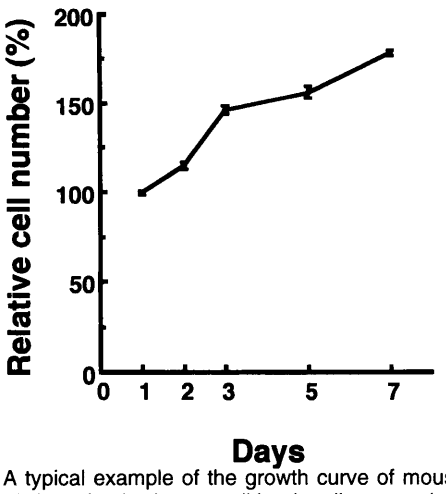

Figure 3. A typical example of the growth curve of mouse uterine epithelial cells in optimal culture condition (medium containing insulin 100 ng/ml and EGF 10 ng/ml). Each point shows mean of three culture wells. Bars, SEM.

and 100 ng/ml) or insulin (100 and 1000 ng/ml) in a dose-dependent manner (Figs. 4 and 5). Even in the absence of EGF, IGF-I and insulin increased the number of epithelial cells (data not shown).

To clarify the mechanism of IGF-I–induced epithelial growth, replicating cells were labeled with BrdU, and were immunocytochemically detected. The percentage of replicating cells was significantly increased by IGF-I (1 and 10 ng/ml) (Fig. 6). This indicates that IGF-I stimulates DNA synthesis of the epithelial cells. As shown in Figures 4 and 6, 1 ng/ml of IGF-I significantly increased the percentage of replicating cells but did not significantly affect the epithelial cell number on Day 5 in culture, indicating that the increase in DNA synthesis did not necessarily correlate with the increase in cell number.

Effects of Estrogens on the Growth of Epithelial Cells. When supplemented with insulin (100 ng/ml), E_2 at 0.001–0.1 nM tended to increase the number of epi-

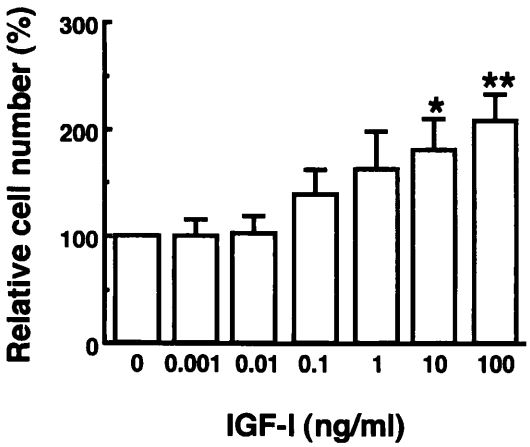

Figure 4. Effect of IGF-I on the number of mouse uterine epithelial cells on Day 5 of culture. Each column shows mean of four independent experiments. Bars, SEM. $*P < 0.05$; $**P < 0.01$, versus control (IGF-I 0 ng/ml).

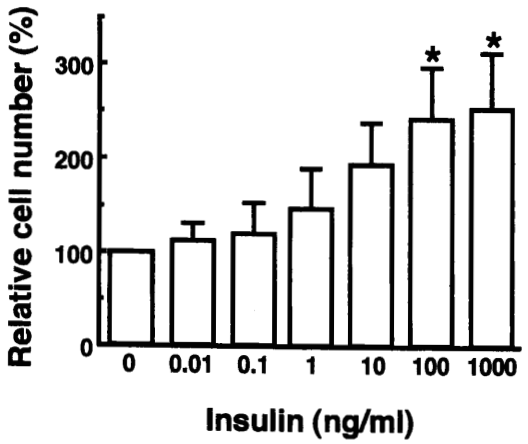

Figure 5. Effect of insulin on the number of mouse uterine epithelial cells on Day 5 of culture. Each column shows mean of three independent experiments. Bars, SEM. *$P < 0.05$ versus control (IGF-I 0 ng/ml).

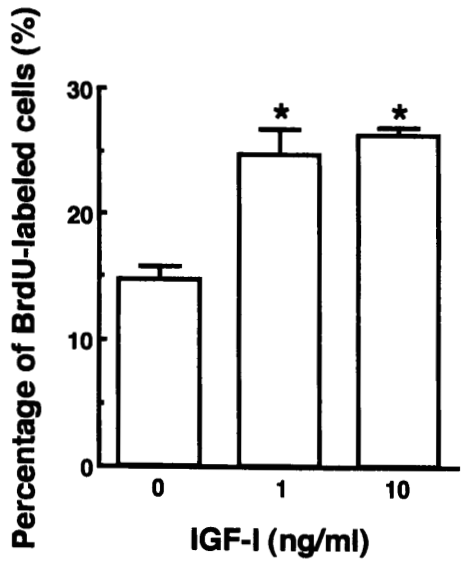

Figure 6. Effect of IGF-I on the percentage of BrdU-labeled mouse uterine epithelial cells. Each column shows mean of three culture wells. Bars, SEM. *$P < 0.01$ versus control.

thelial cells on Day 5 in culture, while E_2 at 1 to 100 n*M* did not affect it (Fig. 7).

Observation of IGF-I–Containing Cells. Immunocytochemical analysis with specific antisera showed that most epithelial cells contained IGF-I immunoreactive materials in their cytoplasm in culture, indicating that the cells not only respond to the mitogenic activity of IGF-I, but also synthesize/store IGF-I in their cytoplasm (Fig. 8).

Discussion

In the present study, we found that IGF-I and insulin dose-dependently stimulate the growth of mouse uterine epithelial cells *in vitro*. Beck and Garner (21) had reported that IGF-I stimulated the DNA synthesis of rat uterine cells in *vitro,* but they did not identify the cell types examined.

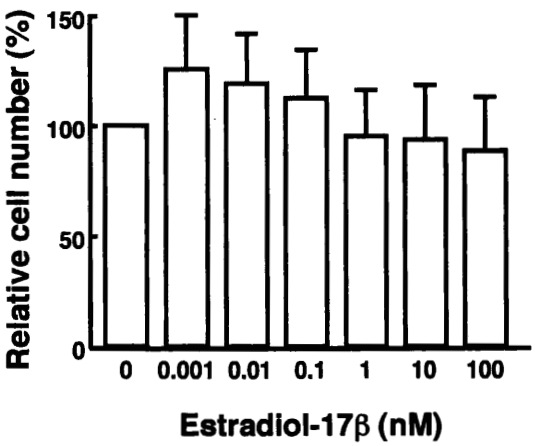

Figure 7. Effect of estradiol-17β (E_2) on the number of mouse uterine epithelial cells with insulin (100 ng/ml) on Day 5 of culture. Each column shows mean of four independent experiments. Bars, SEM.

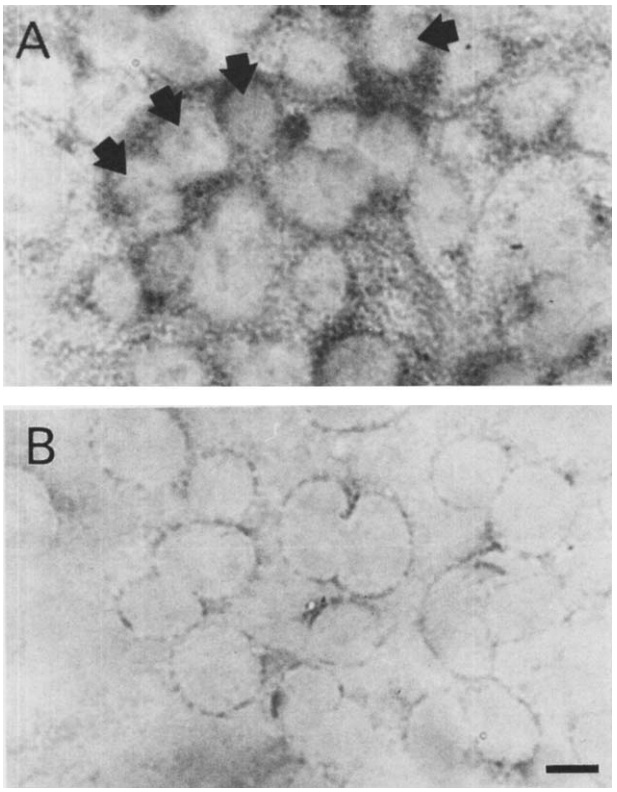

Figure 8. Immunocytochemical detection of IGF-I in mouse uterine epithelial cells on Day 3 of culture stained with anti–IGF-I antiserum (A), and control (B). In Panel A the cytoplasm most of the epithelial cells showed IGF-I immunoreactivity in the cytoplasm. Arrows indicate the cell nucleus. Bar, 10 μm.

Judging from their dissociation method, one can surmise that they obtained fibroblastic stromal cells, and they found that IGF-I was mitogenic for the cells. In this study, we found that the cells in culture were cuboidal in shape and formed monolayer cell sheets. In addition, most of them expressed cytokeratin, but not vimentin. These observations clearly indicate that the cells cultured in the present study consisted entirely of epithelial cells. Therefore, we con-

cluded that IGF-I directly acts on the uterine epithelial cells to stimulate their DNA synthesis and their proliferation.

Growth promoting action of insulin on uterine epithelial cells has been reported in the mouse (22) and the guinea pig (23). Consistent with these reports, we found in the present study that insulin stimulated the growth of uterine epithelial cells. When IGF-I and insulin were compared, IGF-I was more effective than insulin in inducing epithelial growth, because their growth was stimulated by 10 ng/ml of IGF-I but not by 10 ng/ml of insulin. It has been reported that insulin of a higher concentration can bind to type I IGF-I receptors in several types of cells (24). Therefore, the growth-promoting action of insulin observed in the present and previous reports may be through type I IGF-I receptors.

When the cells were treated with 1 ng/ml of IGF-I, the percentage of DNA-replicating cells was significantly increased, but the total cell number was not significantly affected. It is possible that some replicating cells did not proliferate in culture, but it is more probable that IGF-I stimulates their proliferation to increase their number but that more cells detach from the substratum in response to IGF-I to decrease it, resulting in no change in the total cell number. When the cells were treated with higher concentration of IGF-I (10 ng/ml), the cells may proliferate more rapidly to overcome the number of cells detaching from the substratum, resulting in an increase in cell number. This suggests that the number of epithelial cells detaching from the substratum by differentiation/apoptosis/necrosis must be taken into account to accurately measure the growth of epithelial cells *in vitro*. Further studies are needed to clarify the mechanism of cell detachment from the substratum in culture.

IGF-I binding sites were predominantly detected in myometrial smooth muscle cells, particularly in the outer longitudinal smooth muscle rather than in endometrial stroma or epithelial cells in the rat uterus (25). In human and ovine uterus, IGF-I type I receptor mRNAs are detected in the luminal and glandular epithelium and stromal cells (16, 26). In the mouse uterus, localization of type I IGF-I receptor mRNA has not been reported so far as we know. The present findings demonstrate that the uterine epithelial cells respond to IGF-I, and indicate the presence of IGF-I type I receptors in the cells.

IGF-I immunoreactivity was detected in cultured epithelial cells under serum-free condition, indicating that IGF-I was synthesized in these cells. Several studies had already reported that IGF-I was synthesized in the luminal and glandular epithelial cells, stromal cells, and myometrial cells in the rat and mouse uterus (13–15). The present study demonstrated that even epithelial cells had the ability to synthesize and probably secrete IGF-I. The present findings together with those previous reports strongly support the hypothesis that IGF-I is locally synthesized within uterine tissues and that it acts on the epithelial cells by an autocrine or paracrine mechanism.

Estrogens stimulate the expression of IGF-I and type I IGF receptor in the mouse uterus (15, 25, 27–29). Estrogen-induced IGF-I expression has primarily been detected in the epithelial cells, but not in the stromal cells, in ovariectomized mice (15). Thus, IGF-I has been supposed to be another mediator of estrogen action to induce proliferation of uterine epithelial cells. Mitogenic activity of estrogens has not been detected *in vitro* (2, 3), but our present study suggests that estrogens have a quite weak, yet direct growth-promoting action on the uterine epithelial cells. This estrogen-induced growth may be mediated through IGF-I, since estrogens stimulate expression of IGF-I (27).

In conclusion, IGF-I and insulin stimulated the growth of the mouse uterine epithelial cells in primary serum-free culture. It is highly probable that IGF-I produced in endometrial cells controls uterine epithelial growth by an autocrine or paracrine mechanism.

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