Epidermal Growth Factor and Transforming Growth Factor-α Stimulate the Proliferation of Mouse Uterine Stromal Cells

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ABSTRACT—Growth factors produced in the uterine endometrium are considered to be involved in the proliferation of the mouse uterine stromal cells induced by estradiol-17β (E_2) and progesterone (P). The effect of epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), one of EGF-related growth factors, on the proliferation of mouse uterine stromal cells was studied in a serum-free culture. The growth of the uterine stromal cells was measured by MTT assay. EGF was found to increase the number of uterine stromal cells in a dose-dependent manner. The DNA-replicating cells were investigated using the immunocytochemical detection of bromodeoxyuridine (BrdU)-labeled cells. EGF and TGF-α increased the percentage of BrdU-labeled cells in a dose-dependent manner. Administration of the combination of E_2 (10^{-9} M) and P (10^{-7} M) for 2 days increased the percentage of BrdU-labeled cells 2.3-fold. The stimulatory effect of EGF, TGF-α and the combination of E_2 and P on DNA replication in the uterine stromal cells was repressed by RG-13022 (10^{-5} M, the inhibitor of the EGF receptor tyrosine kinase). RT-PCR analysis of EGF-receptor-, TGF-α-, and EGF-mRNA was carried out in the cultured uterine stromal cells, and revealed the expression of those mRNAs. These data supported the hypothesis that uterine endometrial stromal growth induced by sex steroids required the EGF family of ligands such as EGF and TGF-α, both produced in the stromal cells, acting for DNA synthesis through EGF receptors.

Key words: uterus, endometrium, EGF, TGF-α, mouse

INTRODUCTION

The mammalian uterine endometrium consists of epithelial cells and stromal cells. The proliferation and differentiation of both the uterine endometrial epithelial and stromal cells are regulated by estrogen and progestin. Estrogen stimulates the proliferation of luminal and glandular epithelial cells in the uterus, whereas the proliferation of stromal cells is induced by a combined treatment of estradiol and progesterone (Huet-Hudson *et al.,* 1989). Much evidence has suggested that the steroid hormone-induced growth of uterine endometrial cells is mediated by an autocrine or paracrine action of polypeptide growth factors synthesized within uterine tissues (Cooke *et al.,* 1998). Several reports have indicated that estrogen-induced proliferation of uterine epithelial cells is mediated by epidermal growth factor (EGF) (Iguchi *et al.,* 1985; Tomooka *et al.,* 1986; Nelson *et al.,* 1991; Uchima *et al.,* 1991; Shiraga *et al.,* 2000), while other reports have shown that transforming growth factor-α (TGF-α, one of the EGF-related growth factors) (Nelson *et al.,* 1992), insulin-like growth factor-I (IGF-I) (Murphy *et al.,* 1987; Shiraga *et al.,* 1997) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) (Zhang *et al.,* 1994) are implicated in the proliferation of the uterine epithelial cells. As reported above, the regulatory mechanism of the proliferation of uterine epithelial cells has been extensively studied. However, the molecular mechanism that regulates the sex steroid hormone-induced proliferation of uterine stromal cells is not clear.

Immunoreactive EGF has been localized in the neonatal and adult mouse uterine stromal cells (Falck and Forsberg, 1996). TGF-α and its mRNA are also detected in both uterine epithelial cells and stromal cells (Tamada *et al.,* 1991). Both EGF and TGF-α are known to bind to EGF receptors. EGF-receptor mRNA has been detected in the mouse uterine stromal cells (Das *et al.,* 1994; Tong *et al.,* 1996). These findings imply that EGF and EGF-related growth factors act on the uterine stromal cells in a paracrine manner. Therefore, the present study was aimed at clarifying the involvement of EGF and TGF-α in the estradiol and

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progesterone-induced proliferation of uterine stromal cells, since in our culture system, treatment with a combination of estradiol and progesterone stimulated the proliferation of the mouse uterine stromal cells.

MATERIALS AND METHODS

Animals

Twenty one- to twenty three-day-old female mice of the ICR strain (CLEA Japan Inc., Osaka, Japan) were used in the present study. They were housed in a temperature-controlled animal room, and were given a commercial diet of CA-1 (CLEA Japan Inc. Osaka, Japan) and tap water *ad libitum*. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation, Faculty of Science, Okayama University, Japan.

Uterine stromal cell isolation

Uterine stromal cells were isolated according to the method of Ross *et al.* (1993). Briefly, uterine horns were removed from the mice and longitudinally cut to expose the endometrial luminal surface. The tissue fragments were treated with 0.1% trypsin (w/v, Type III, Sigma Chemical Co., St. Louis, MO, USA) at 4°C for 1 hr, and then at 37°C for 55 min. Trypsin was inactivated by 0.1% soybean trypsin inhibitor (STI, w/v, Sigma Chemical Co.) at 37°C for 10 min. The epithelial tissues were separated from the stromal tissues with forceps under a stereoscopic microscope. The endometrial stromal and myometrial tissues separated from the epithelial tissues were incubated with 0.1% trypsin and 1 mM EDTA at 37°C for 1 hr. Then they were treated with 0.1% STI at 37°C for 10 min. After gentle pipeting and DNase I (Sigma Chemical Co.) treatment, the stromal cells separated from the myometrium were collected by centrifugation. The cell viability was assessed by trypan blue dye exclusion test and was usually more than 95% in each study. All chemicals were dissolved in Ca^{2+}- and Mg^{2+}-free Hanks' solution containing 20 mM HEPES and 0.3% bovine serum albumin (BSA, w/v, Fraction V, Sigma Chemical Co.).

Uterine stromal cell culture

The isolated uterine stromal cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium without phenol red (DME/F12 medium, Sigma Chemical Co.) containing 2% dextran-coated charcoal-treated fetal bovine serum (v/v, Gibco BRL, Gaithersburg, MD, USA) (DC-FBS) (Lagace *et al.,* 1980) for 1 day. After a 1-day pre-culture, the cells were cultured in DME/F12 medium supplemented with BSA (1 g/l), hydrocortisone (100 µg/l), triiodothyronine (400 ng/l), transferrin (10 mg/l), glucagon (10 ng/l), parathormone (200 ng/l), sodium selenite (5 µg/l) and insulin (100 µg/l). Insulin was stocked at a concentration of 10 µg/ml in sterile 1% acetic acid solution. E_2 (Sigma Chemical Co.), P (Sigma Chemical Co.) and RG-13022 (BIOMOL Research Laboratories, Inc., PA, USA), a specific inhibitor of tyrosine kinase of EGF receptor (Yoneda *et al.,* 1991), were initially dissolved in a small amount of sterile ethanol. They were all diluted with the culture medium, and the final ethanol concentrations were less than 0.01%. Mouse EGF was obtained from Sigma Chemical Co., and human TGF-α was obtained from Amersham Pharmacia (Uppsala, Sweden).

The isolated uterine stromal cells were seeded in 24-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ, USA) at a density of $1-2\times10^4$ cells/ml per well for the determination of the cell number and the DNA-replicating cells, and in 9-cm tissue culture plates (Becton Dickinson) at a density of 2×10^6 cells per plate for RNA extraction. The culture was continued at 37°C in humidified atmosphere of 5% CO_2 and 95% air.

Immunocytochemical analysis of uterine stromal cells

Uterine stromal cells were cultured on a poly-L-lysine-coated slide glass. The cells were fixed by pre-chilled 100% methanol (–20°C) for 20 min and dried at room temperature. For vimentin or cytokeratin detection, the cells were treated with 0.01% trypsin in 0.01 M PBS for 5 min at 37°C, then incubated with mouse anti-vimentin antibody (Sigma Chemical Co.) or pkk-1 mouse anti-cytokeratin antibody (Organon Teknika, NC, USA) overnight at 4°C. Vimentin-, and cytokeratin-immunoreactivity were visualized using ABC kit (Vector Laboratories, Burlingame, CA, USA).

Determination of the number of uterine stromal cells

The number of cultured stromal cells was determined by a modification of the tetrazolium assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Chemical Co.) as a substrate (MTT assay; Alley *et al.,* 1988). The amount of formazan accumulated after 4-hr incubation at 37°C was spectrophotometrically determined by measuring absorbance at 540 nm and was used to estimate the number of the cells in a well. The amount of formazan (the optical density observed at 540 nm) was well correlated with the number of cells in culture (Shiraga *et al.,* 1997). The cell number was expressed as a percentage by regarding the minimal cell number as 100%.

Determination of the proliferating cells

DNA-replicating cells were immunocytochemically detected using a cell proliferation kit (Amersham Pharmacia). Uterine stromal cells were seeded on a poly-L-lysine-coated slide glass. Bromodeoxyuridine (BrdU, 3 mg/ml) was added to the culture medium, and the culture was continued for 5 hr. About one thousand stromal cells were observed from each slide. The percentage of BrdU-labeled cells to total cells was calculated.

Primers

Primers of EGF receptor, TGF-α and EGF were designed based upon the mouse sequence and were obtained from the Gibco BRL Custom Primers, Life Technologies Asia Pacific (Yokohama, Japan). The primer sequences and the location on the nucleotide sequences were as follows: EGF receptor 5' (GTGTGAAGAAGTGCCCCCGAAAC, 931–953), EGF receptor 3' (AACGACCGCCAAAGAAAACTGACC, 1359–1382), TGF-α 5' (AGCCAGAAGAAGCAAGCCATCACT, 367–390), TGF-α 3' (CTCATTCTCGGTGTGGGTTAGCAA, 845–868). EGF 5'(AGATGAGTGTGTGCTGGCTAGATC, 2987–3010), and EGF 3' (TCCGAGTCCTGTAGTAGTAAGTCC, 3529–3552).

cDNA synthesis and PCR amplification

Total RNA (2 µg) in a final volume of 20 µl was subjected to reverse transcription (RT) using Superscript II Reverse Transcriptase (Gibco-BRL) with random primers according to the manufacturer's instructions. Each PCR was performed using primer pairs specific for EGF-R, EGF and TGF-α as described above. PCR was performed using Takara Taq (Takara Shuzo Co.) and a thermal cycler (Gene Amp PCR System 9700, Applied Biosystems, Branchburg, NJ, USA). The conditions for PCR were as follows: after activation of DNA polymerase by a 20-sec incubation at 95°C, 35 cycles (for EGF-R cDNA) and 40 cycles (for EGF and TGF-α cDNA) of reactions, including denaturation for 30 sec at 95°C and extension for 1 min at 60°C, followed by an additional extension for 10 min at 60°C. A 10-µl aliquot of each reaction was electrophoresed on 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet rays.

The size of the resulting DNA fragment following PCR amplification was expected to be 452 base pairs (bp) for EGF-R, 502 bp for TGF-α and 566 bp for EGF. The cDNA fragments were subcloned into pGEM3zf (+), and sequenced using fluorescent primers and an automated DNA sequencer.

Statistics

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

RESULTS

Immunocytochemical analysis of cultured uterine stromal cells

Uterine stromal cells were attached to a substratum within a few hours after seeding. Phase contrast microscopy revealed that the stromal cells in the culture were flattened, and were mostly fibroblast-like in appearance (Fig. 1A). Immunocytochemical study showed that the cultured cells expressed vimentin (a marker for stromal cells) (Fig. 1B), but not cytokeratin (a marker for epithelial cells) (Fig. 1C). These observations indicate that the cultured cells consisted of uterine endometrial stromal cells.

The growth of uterine stromal cells

After a 1-day pre-culture in the medium containing 2% DC-FBS, the uterine stromal cells were cultured in the serum-free medium. Under the serum-free conditions, the uterine stromal cells continued to grow until Day 5 in culture, and the number increased 4.5-fold (Fig. 2).
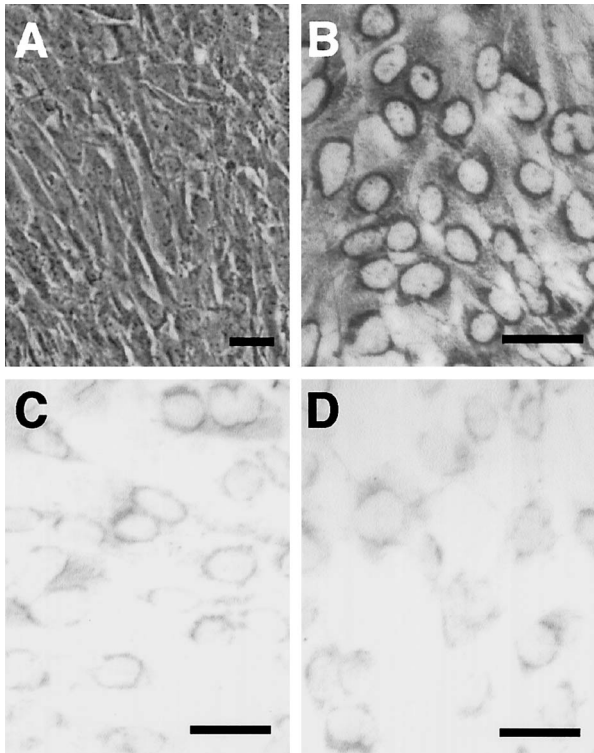

Fig. 1. Immunocytochemical detection of vimentin and cytokeratin in the cultured mouse uterine stromal cells. A: Cultured uterine stromal cells are observed by the phase contrast microscope. Bar=50 μm; B: Cultured cells were stained with anti-vimentin antibody. Bar=20 μm; C: Cultured cells were stained with anti-cytokeratin antibody. Bar=20 μm; D: Cultured cells were stained without those antibodies as controls. Bar=20 μm.

Effect of RG-13022 on the incorporation of BrdU to the nucleus of uterine stromal cells

The nucleus of DNA-replicating stromal cells was labeled with BrdU, which was detected immunocytochemically. To examine whether EGF receptor-signaling was involved in the growth of the uterine stromal cells, the stromal cells were treated with RG-13022, an inhibitor of the EGF receptor tyrosine kinase, for 2 days, starting on Day 1
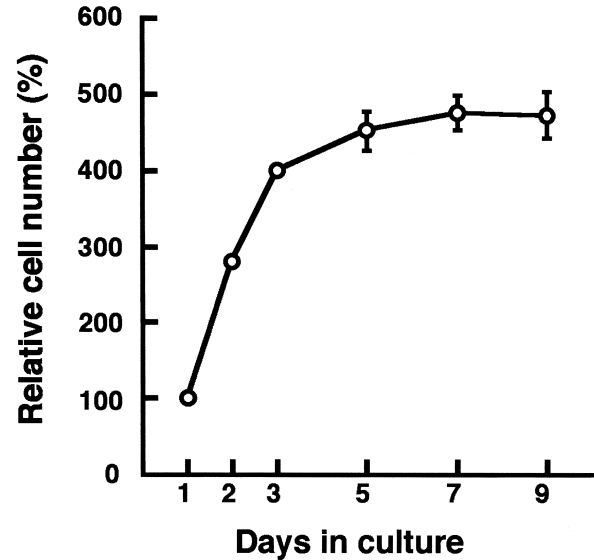

Fig. 2. A typical example of the growth curve of cultured uterine stromal cells. After a 1-day pre-culture in DME/F12 medium containing 2% DC-FBS, the stromal cells were cultured in serum-free DME/F12 medium. Each point represents the mean (±S.E.M.) of three independent experiments.
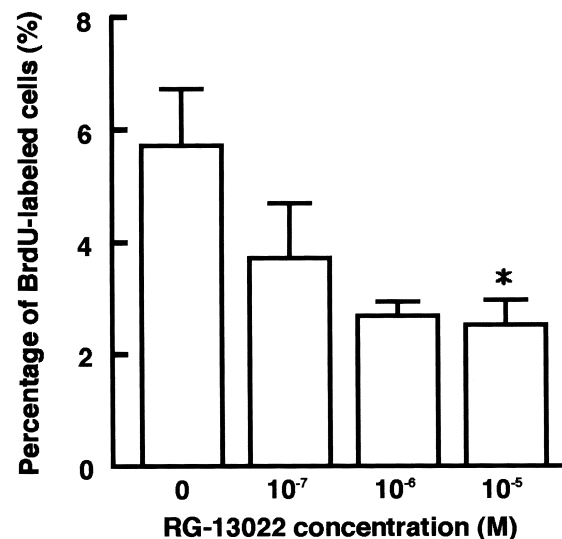

Fig. 3. Effect of RG-13022 on the percentage of BrdU-labeled mouse uterine stromal cells. Stromal cells were treated with RG-13022 (0, 10^{-7}, 10^{-6}, 10^{-5} M) for 2 days. RG-13022 treatments were started on Day 1 in culture. Each column represents the mean (±S.E.M.) of three independent experiments. *$p < 0.05$, compared with control values.

in culture (Fig. 3). RG-13022 treatment decreased the percentage of BrdU-labeled stromal cells in a dose-dependent manner, and in the following experiments, 10^{-5} M RG-13022 was used.

Effect of EGF on the number and DNA replication of uterine stromal cells

Uterine stromal cells were cultured with various concen-

trations of EGF (0.1 to 10 ng/ml) for 6 days. EGF increased the number of uterine stromal cells in a dose-dependent manner (Fig. 4). When uterine stromal cells were treated with EGF (10 ng/ml) from Day 5 in culture, they had increased 1.3-fold in the number over the control level at Day 7 in culture. This increase continued, 1.5-fold at Day 9, and remaining 1.6-fold at Day 11 (Fig. 5). EGF increased the percentage of BrdU-labeled stromal cells in a dose-
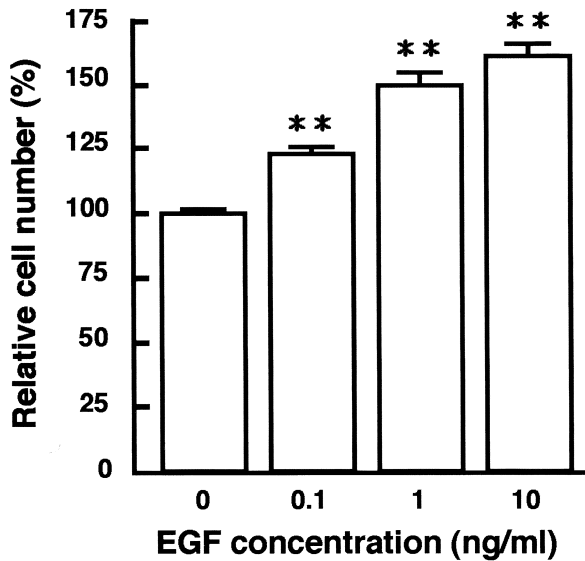

Fig. 4. Effect of EGF on the number of the mouse uterine stromal cells. Stromal cells were treated with EGF (0, 0.1, 1, 10 ng/ml) for 6 days. EGF treatments were started on Day 5 in culture. Each column represents the mean (±S.E.M.) of three independent experiments. **p<0.01, compared with control values.

Fig. 6. Effect of EGF on the percentage of BrdU-labeled mouse uterine stromal cells. Stromal cells were treated with EGF (0, 0.1, 1, 10 ng/ml) for 2 days. EGF treatments were started on Day 5 in culture. Each column represents the mean (±S.E.M.) of three independent experiments. **p<0.01, compared with control values.
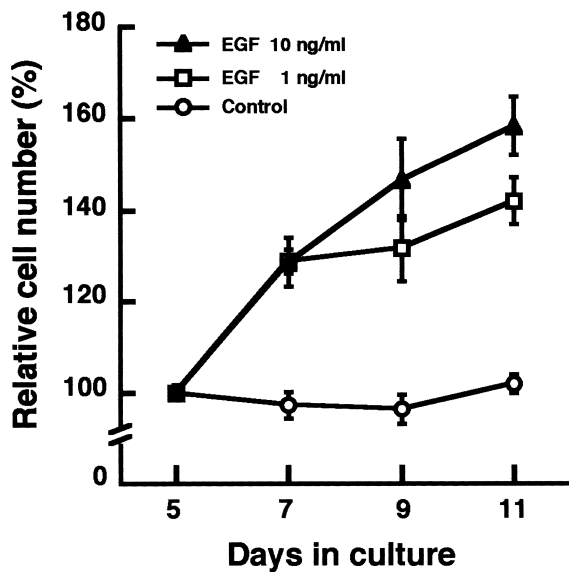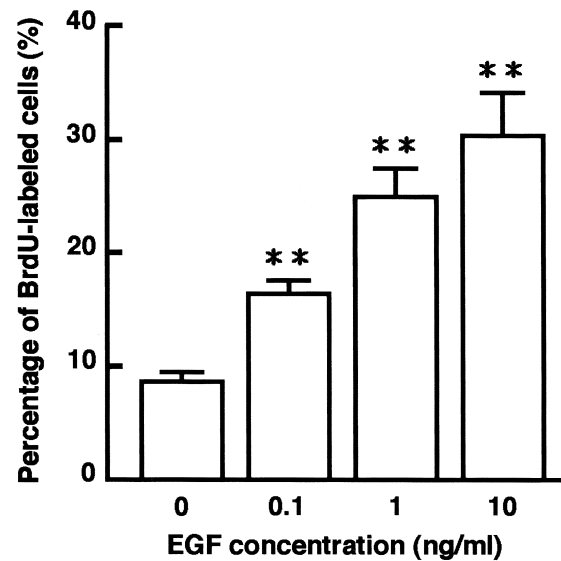

Fig. 5. Effect of EGF on the number of mouse uterine stromal cells in time course. EGF (0, 1, 10 ng/ml) treatments were started on Day 5 in culture. Experiments were replicated independently 3 times. Each point represents the mean (±S.E.M.)
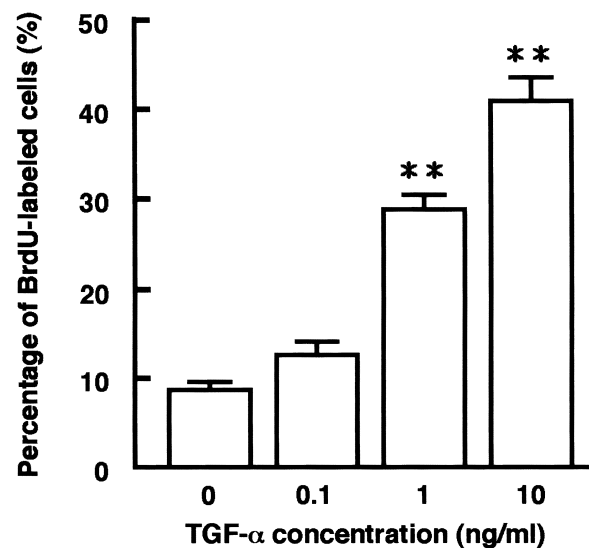

Fig. 7. Effect of TGF-α on the percentage of BrdU-labeled mouse uterine stromal cells. Stromal cells were treated with TGF-α (0, 0.1, 1, 10 ng/ml) for 2 days. TGF-α treatments were started on Day 1 in culture. Each column represents the mean (±S.E.M.) of three independent experiments. **p< 0.01, compared with control values.

dependent manner (Fig. 6).

Effect of TGF-α on the DNA replication of uterine stromal cells

Uterine stromal cells were cultured with various concentrations of TGF-α (0.1 to 10 ng/ml) for 6 days. TGF-α increased the percentage of BrdU-labeled stromal cells in a dose-dependent manner (Fig. 7).

Effect of RG-13022 on the DNA replication of uterine stromal cells induced by exogenous growth factors and sex steroids

E_2 (10^{-9} M) or P (10^{-7} M) treatment did not increase the number of BrdU-labeled cells (data not shown), whereas the combination of E_2 and P significantly increased the number of BrdU-labeled cells. Similarly, EGF and TGF-α caused the number of BrdU-labeled cells to increase. To clarify how EGF receptor signaling is implicated in mediating the action of E_2 and P, the uterine stromal cells were treated with RG-13022 (10^{-7} M) in the presence of the combination of E_2 and P. The RG-13022 treatment nullified the E_2 and P-induced DNA replication of the uterine stromal cells as well as the EGF- or TGF-α-induced DNA-replication (Fig. 8).

Detection of EGF-receptor-, TGF-α-, and EGF-mRNAs in cultured uterine stromal cells

RT-PCR was performed to evaluate EGF receptor-, TGF-α-, and EGF-mRNA expression in the cultured uterine stromal cells using primer pairs for the amplification of EGF receptor cDNA (Fig. 9, lane 1), TGF-α cDNA (Fig. 9, lane 3)
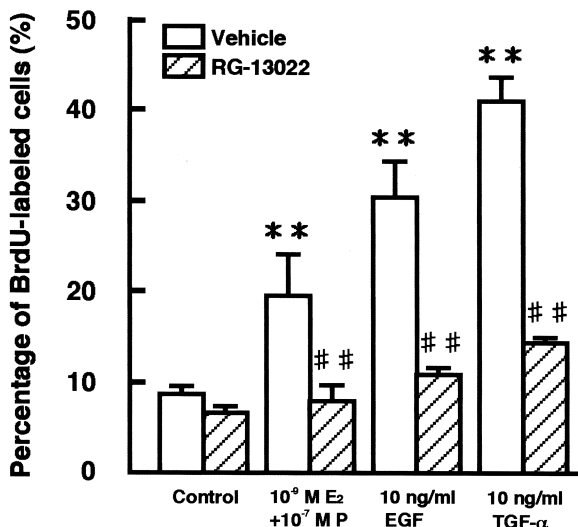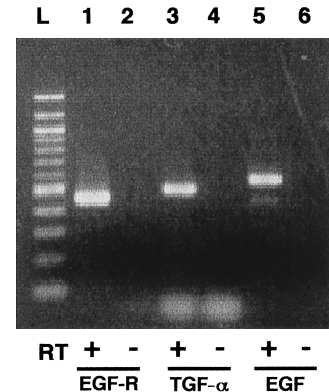

Fig. 9. Electrophoretic analysis of RT-PCR products. One μg of total RNA extracted from cultured uterine stromal cells was reverse-transcribed and amplified by PCR using primers for the amplification of EGF receptor (lane 1), TGF-α (lane 3) and EGF (lane 5) as described in Materials and Methods. For negative control, RNA samples (without RT) were amplified using primers for EGF receptor (lane 2), TGF-α (lane 4) and EGF (lane 6). The one-tenth aliquot of each reaction was electrophoresed, and stained with ethidium bromide. The sizes of the RT-PCR products were determined by comparison with 100 bp DNA Ladder as markers. TGF-α-, EGF- and EGF-receptor-amplified cDNA bands were 502, 566 and 452 bp, respectively. L: 100-bp ladder.

and EGF cDNA (Fig. 9, lane 5). These amplifications resulted in single bands in the ethidium bromide-stained gels. From the size of the RT-PCR products, it was concluded that the 452-bp product, 502-bp product and 566-bp product were amplified cDNAs of EGF receptors, TGF-α, and EGF, respectively. The sequencing analysis revealed that the amplified cDNA fragments were cDNAs coding parts of the mouse EGF receptor, TGF-α and EGF, respectively (data not shown). To exclude the possibility of genomic contamination, RNA samples were amplified by PCR without RT, and no PCR products were found (Fig. 9, lanes 2, 4 and 6).

DISCUSSION

The uterine stromal cells continued to grow under the serum-free condition. RG-13022 treatment inhibited the proliferation of the uterine stromal cells even in the absence of EGF and TGF-α under serum-free conditions, which suggests the involvement of EGF-receptor signaling in the growth of uterine stromal cells. In addition, we demonstrated the synthesis of EGF and TGF-α in the uterine stromal cells and their stimulatory effect on DNA replication of uterine stromal cells. From these findings, we concluded that endogenous EGF-related growth factors were involved in the regulatory mechanism of the growth of mouse uterine stromal cells.

In our culture system, the uterine stromal cells ceased to increase in number on Day 5 in culture. However, when the medium was treated with EGF beginning on Day 5, the uterine stromal cells started to increase in number, indicat-

Fig. 8. Effect of the association of RG-13022 and sex steroid hormones, growth factors on the percentage of BrdU-labeled mouse uterine stromal cells. Stromal cells were treated with E_2 (10^{-9} M) and P (10^{-7}), EGF (10 ng/ml), TGF-α (10 ng/ml) in the absence of 10^{-5} M RG-13022 (open columns) or the presence (hatched columns) for 2 days. All treatments were started on Day 1 in culture. Each column represents the mean (±S.E.M.) of three independent experiments. **p<0.01, compared with control values. ##p< 0.01, compared with vehicle-treated groups.

ing that the cessation of the growth of uterine stromal cells was partly due to the depletion of endogenous growth factors, probably EGF-related growth factors.

In the adult ovariectomized mouse uterus, the proliferation of stromal cells is induced by a combination of E_2 and P (Huet-Hudson *et al.*, 1989). In the present study treatment with E_2 or P failed to stimulate DNA replication in the uterine stromal cells, whereas the combination of E_2 and P stimulated DNA replication *in vitro*. The proliferation of the uterine stromal cells induced by the combination of E_2 and P in a serum-free culture is quite consistent with the proliferation of uterine stromal cells induced by E_2 and P *in vivo*.

The DNA replication that had been enhanced by the combined treatment with E_2 and P was nullified by an RG-13022 treatment. This finding suggests that the action of E_2 and P on the DNA replication of uterine stromal cells requires the EGF-receptor signaling. Therefore, EGF-related growth factors are considered to mediate the action of the combination of E_2 and P through the EGF receptor. This hypothesis is supported by a previous report that in response to estrogen treatment DNA synthesis in uterine endometrial stromal cells of EGF-receptor knockout mice is depressed compared with that of wild type mice, suggesting that EGF-receptor signaling is required in estrogen-induced proliferation of uterine endometrial stromal cells (Hom *et al.*, 1998).

EGF and EGF mRNA levels are increased by estrogen in the mouse uterus (DiAugustine *et al.*, 1988; Falck and Forsberg, 1996). The TGF-α mRNA level is also increased by estrogen in the adult ovariectomized rat uterus (Borgundvaag *et al.*, 1992) and in the immature mouse uterus (Nelson *et al.*, 1992). Immunoreactive TGF-α protein is secreted at high levels into the mouse uterine luminal fluid after estrogen treatment (Nelson *et al.*, 1992). As mentioned above *in vivo* analysis of EGF and TGF-α expression in the uterus has been well reported. However, physiological growth factors which are involved in steroid hormone-induced proliferation of the stromal cells remains to be determined. Interestingly, in a study of pituitary growth, we found that TGF-α is a mediator of estrogen-induced proliferation of mammotrophs, since treatment of mouse pituitary cells with antisense TGF-α oligodeoxynucleotide or anti-TGF-α antibodies inhibited estrogen-induced proliferation of mammotrophs (Oomizu *et al.*, 2000; Takahashi *et al.*, 2002; Sharma *et al.*, 2003). These results altogether suggest that estrogen-induced growth of various tissues is controlled by a common mechanism of an autocrine or paracrine action of growth factors.

The stromal cells do not proliferate when progesterone receptor antagonists are used to block the receptor function (Cullingford and Pollard, 1988; Rider and Psychoyos, 1994) or when anti-progesterone antibodies are administered during early pregnancy (Rider *et al.*, 1986). Progesterone is essential for the growth of uterine stromal cells. The increase in the progesterone receptor level is evoked by estrogen (Aronica and Katzenellenbogen, 1991). The mech-

anism of cooperative action between E_2 and P needs to be clarified.

In conclusion, we found that estradiol and progesterone co-operatively stimulated the proliferation of uterine stromal cells, and that EGF-receptor ligands were involved in the steroid-induced growth of uterine endometrial stromal cells.

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