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Effects of Sex Steroids and Growth Factors on Migration and Invasion of Endometrial Adenocarcinoma SNG-M Cells *in vitro*

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Biological effects of sex steroids (estradiol-17β, E2; progesterone, P; medroxyprogesterone acetate, MPA; Danazol, DZ) and growth factors (epidermal growth factor, EGF; transforming growth factor, TGF-α, β) on migration and invasion of endometrial adenocarcinoma SNG-M cells were investigated by haptotactic migration and haptoinvasion assay. The enzymatic degradation of the extracellular matrix by tumor cells was also examined. Tumor cell migration along a gradient of substratum-bound fibronectin was inhibited by 0.1–10 *μM* MPA and DZ, but promoted by 0.1–10 *nM* EGF and TGF-α in a concentration-dependent manner. E2, P and TGF-β did not have any effect on the motility of tumor cells. These effects were also confirmed by wound assay. The invasive activity of SNG-M cells into reconstituted basement membrane (Matrigel) was inhibited by the presence of 0.1–10 *μM* MPA and DZ, but promoted by 0.1–10 *nM* EGF and TGF-α in a concentration-dependent manner. E2, P and TGF-β did not have any effect on tumor cell invasion. The zymography of tumor-conditioned medium showed that the treatment of SNG-M cells with EGF and TGF-α resulted in the increase of the 68, 72 and 92 kDa type IV collagenases (matrix metalloproteinase, MMP-2 and 9). Sex steroids and TGF-β did not have significant effects on MMP-2 and 9. Stromelysin (MMP-3), also secreted by SNG-M cells, was not affected by sex steroids and growth factors. These results suggest that EGF and TGF-α act as positive regulators on the invasion process of endometrial adenocarcinoma cells, which may partly be associated with the induction of type IV collagenase secretion by tumor cells. The inhibitory effects of MPA and DZ on tumor cell invasion may depend at least partly on their inhibitory action on the motility of tumor cells.

Key words: Endometrial adenocarcinoma — Migration — Invasion — Matrix metalloproteinase

In the processes of invasion and metastasis, tumor cells must pass through basement membranes and extracellular matrices (ECM), which consist of adhesive molecules such as fibronectin, laminin, collagen, and other glycoproteins and proteoglycans.[1–5] Tumor cell invasion of ECM is a complex process involving cell attachment, migration, and the degradation of tissue barriers by various proteolytic enzymes secreted by tumor cells. Matrix metalloproteinases (MMPs) are proteolytic enzymes which can degrade native collagens and other ECM components.[6,7] Since their substrates are the major components of ECM, the increased expression of MMPs by malignant tumor cells is believed to play an essential role in invasion and metastasis.[8–10]

Sex steroids and growth factors play an important role in the control of endometrial growth. Estrogens are known mitogens for estrogen-sensitive cells and evidence is accumulating that these steroids are able to induce the synthesis of auto-stimulatory growth factors and their receptors.[11,12] Moreover, recent investigations on endometrial growth revealed that growth factors might influence tumor invasion by affecting the structure of ECM and proteolytic enzymes secreted by tumor cells. Bulletti *et al.*[13] indicated that epidermal growth factor (EGF) and transforming growth factor (TGF)-α might be associated with basement membrane degradation through lysis of laminin and type IV collagen in the endometrium. Various authors[14–17] have also reported that EGF and TGF-α, β appeared to be involved in the degradation and synthesis of ECM components, which in turn would correlate with tumor invasion and metastasis.

In the present study, we investigated the biological effects of sex steroids and growth factors on proliferation, migration and invasion of cultured endometrial adenocarcinoma cells, together with their stimulatory or inhibitory action on MMPs secreted by tumor cells.

MATERIALS AND METHODS

Cell culture SNG-M[18] originating from a metastatic lymphnode of moderately differentiated endometrial ad-

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enocarcinoma was kindly provided by Dr. S. Nozawa, Keio University, Tokyo. SNG-M cells were maintained as monolayer cultures in Ham's F-12 medium (Flow Laboratories Inc., Irvine, Scotland) supplemented with 10% fetal bovine serum (Mitsubishi Chemical Co., Tokyo) at 37°C in a humidified incubator with 5% CO_2 in air. The cells were grown to confluence in 75-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark), washed with phosphate-buffered saline (PBS), and then harvested after a brief treatment with 0.1% trypsin solution containing 0.02% EDTA (Flow Laboratories Inc.). The cell viability was determined by trypan blue dye exclusion prior to use. Tumor conditioned medium (TCM) was prepared from the culture supernatant of the cells. Briefly, confluent monolayers of tumor cells grown in 6 cmϕ plastic dishes (Corning 25010, Iwaki Glass, Tokyo) were rinsed twice with serum-free Ham's F-12 medium and incubated at 37°C for 48 h with 4 ml of serum-free medium. The TCM samples thus obtained were derived from equal numbers of tumor cells, concentrated 5-fold by using Centricon-30 microconcentrators (AMICON, Division of W.R. Grace & Co., Columbia, MA) and then stored at −80°C until use.

Reagents Estradiol-17β (E2), progesterone (P) and medroxyprogesterone acetate (MPA) were purchased from Sigma Chemical Co., St. Louis, MO. Danazol (DZ) was prepared by Tokyo Tanabe Co., Ltd., Tokyo. EGF and TGF-α were purchased from Wakunaga Yakuhin Co., Ltd., Osaka. TGF-β was obtained from King Jozo Co., Ltd., Tokyo. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Wako Pure Chemical Industries, Ltd., Osaka. Purified human fibronectin, gelatin, and casein were purchased from Iwaki Glass. Basement Membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin) was obtained from Collaborative Research Inc., Bedford, MA. All the reagents and media in this study were endotoxin-free.

MTT assay Effects of sex steroids and growth factors on the proliferation of SNG-M cells were examined by MTT assay with some modifications as previously described.[19] Cells (1×10^4) in a volume of 100 μl of growth medium per well were uniformly seeded into 96-well microplates (Nunc), and incubated at 37°C for 24 h. Each well was washed twice with serum-free Ham's F-12 with 0.1% bovine serum albumin (BSA) (Sigma Chemical Co.) and cultured for another 24 h in the same medium. The medium was then removed and replaced with 100 μl of serum-free Ham's F-12 with 0.1% BSA containing various amounts of sex steroids and growth factors. After further incubation at 37°C for 24 or 48 h, 50 μl of MTT dissolved in PBS at a concentration of 2 mg/ml was added to each well, and the plates were incubated at 37°C for 3 h. The medium was then removed, 200 μl of dimethyl sulfoxide was added to each well, and the plates were agitated for 5 min. The absorbance was then read at 570 nm in a scanning spectrophotometer (Hitachi Ltd., Tokyo). All experiments were performed in quadruplicate and repeated three times.

Haptotactic migration assay Tumor cell migration along a gradient of substratum-bound fibronectin was assayed in Chemotaxicell culture chambers (Kurabo, Osaka) according to the methods reported by McCarthy and Furcht[20] with some modifications. Polyvinylpyrrolidone-free polycarbonate filters with 8.0 μm pore size were precoated with 10 μg of fibronectin in a volume of 50 μl on the lower surface, and dried overnight at room temperature under a hood. The coated filters were washed extensively in PBS and then dried immediately before use. Log-phase cell cultures of tumor cells were harvested with 0.1% trypsin containing 0.02% EDTA, washed twice with serum-free Ham's F-12, and resuspended to a final concentration of 5×10^6/ml in serum-free Ham's F-12 with 0.1% BSA. Cell suspension (200 μl) with or without sex steroids and growth factors was added to the upper compartment, and 600 μl of serum-free Ham's F-12 with 0.1% BSA was immediately added to the lower compartment. The chambers were then incubated for 24 h at 37°C in 5% CO_2 in air. The filters were fixed with ethanol, and stained with hematoxylin. The cells on the upper surface of the filters were removed by wiping with a cotton swab. The cells that had migrated to various areas of the lower surface were manually counted under a microscope at a magnification of 400. Each assay was performed in triplicate.

Invasion assay The invasive activity of tumor cells was assayed according to the method reported by Albini *et al.*[21] with some modifications. Briefly, the lower surface of the filters was precoated with fibronectin as described above. Matrigel diluted to 500 μg/ml with cold PBS was applied to the upper surface of the filters (5 μg/filter) and dried at room temperature under a hood. The filters thus prepared were designated Matrigel/fibronectin-coated filters. The following procedures were the same as those of haptotactic migration assay.

Wound assay The motility of tumor cells was estimated by wound assay according to the method reported by Burk[22] with some modifications. Twenty-four-well microplates (Nunc) were precoated with 10 μg/ml of fibronectin in a volume of 300 μl per well, and dried overnight at room temperature under a hood. The coated microplates were washed extensively with PBS and then dried immediately before use. Cells (1×10^5) in a volume of 400 μl of growth medium per well were uniformly seeded into microplates, and incubated at 37°C for 24 h. Each well was washed twice with serum-free Ham's F-12 with 0.1% BSA and cultured for another 24 h in the same medium. The monolayers were then wounded with

a plastic blade and the medium was replaced with 400 μl of serum-free Ham's F-12 with 0.1% BSA containing sex steroids or growth factors. After further incubation at 37°C for 48 h, the cells that had migrated into the cell-free space were observed under a microscope, and the migration distances were measured in triplicate.

Zymograms The gelatinolytic or caseinolytic activity of TCM was examined by electrophoresis in a gelatin or casein-embedded polyacrylamide gel followed by incubation and Coomassie Blue staining, based on the methods described by Heussen and Dowdle.[23] TCMs were mixed with sodium dodecyl sulfate (SDS) sample buffer containing 1 mM phenylmethylsulfonyl fluoride and applied, without heating or reduction, to polyacrylamide gels containing 0.5 mg/ml of gelatin or casein. After electrophoresis, the gels were washed twice with 2.5% Triton X-100 for 60 min to remove the SDS, incubated in the incubation buffer containing 0.15 M NaCl, 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.05% NaN_3 for 48 h, and then stained in 0.1% Coomassie Brilliant Blue.

Receptor assay Estrogen receptor (ER) and progesterone receptor (PR) of SNG-M cells were assayed by the dextran-coated charcoal (DCC) method.[24] Briefly, 2×10^7 cells were homogenized, and aliquots of the cytosol and the nucleus fraction were incubated with increasing concentrations of ^{3}H-estradiol or ^{3}H-R5020 (Amersham,

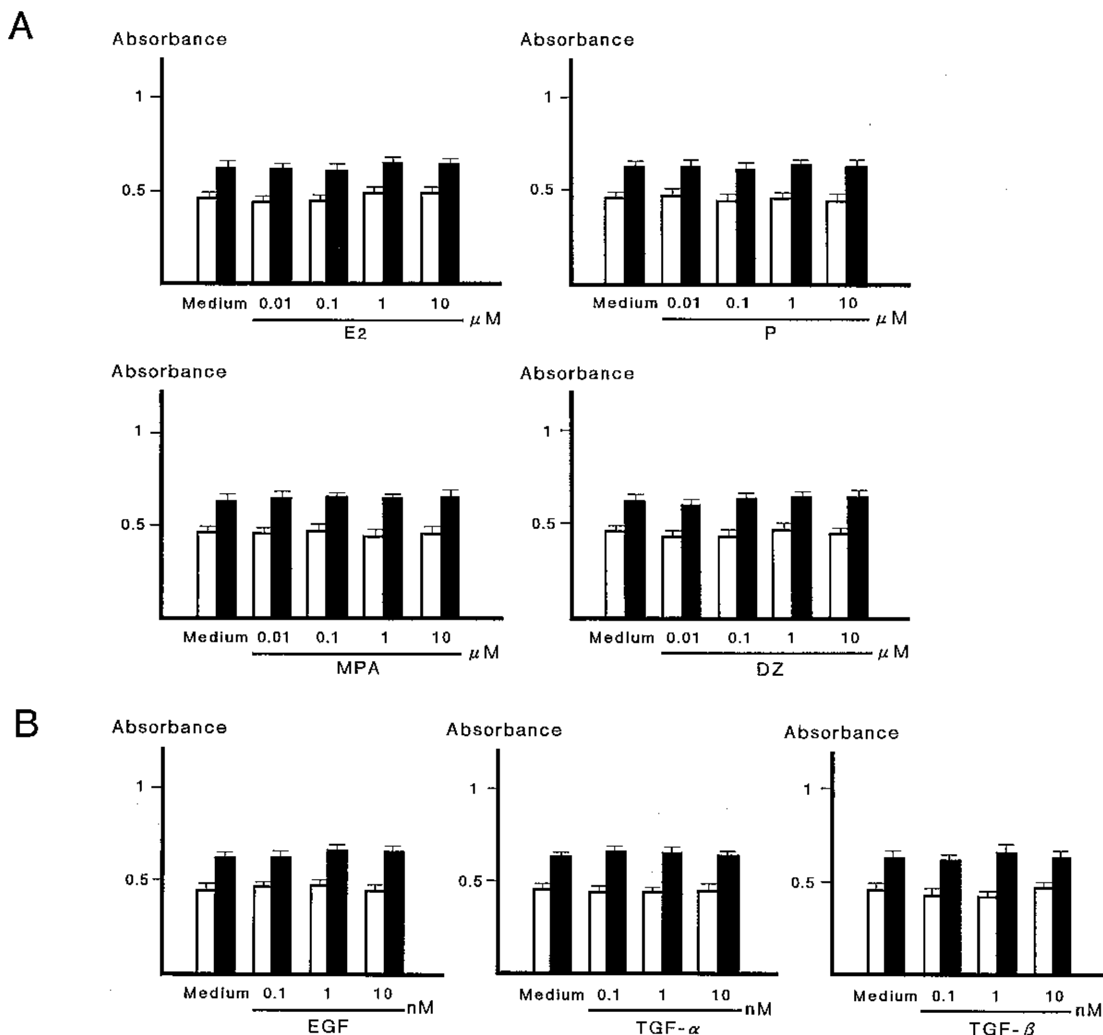


Fig. 1. Effects of sex steroids (A) and growth factors (B) on SNG-M cell growth. Cells (1×10^4) were seeded into 96-well microplates and incubated for 24 h. Each well was washed with serum-free Ham's F-12 with 0.1% BSA and cultured for another 24 h in the same medium. Then, 0.01–10 μM E2, P, MPA and DZ or 0.1–10 nM EGF, TGF-α and TGF-β were added and cell viability was evaluated by MTT assay after a 24-h (□) or 48-h (■) incubation.

Aylesbury, UK). After incubation, receptor-bound steroids were separated by absorption of unbound steroids onto DCC. Bound radioactivity was measured in a liquid scintillation counter (TRI-CARB 300c, Packcard, NY). The total protein content was determined by the Coomassie Blue dye binding method.[25] The results were expressed as the amount of steroid hormone bound specifically to the receptor per mg protein.

Epidermal growth factor receptor (EGFR) of SNG-M cells was assayed as described previously with some modifications.[26] Briefly, 2×10^7 cells were homogenized, and aliquots of the membrane fraction were incubated with increasing concentrations (0.05–5 n*M*) of ^{125}I-EGF (New England Nuclear Inc., Boston, MA) with or without unlabeled EGF (100 n*M*) (Sigma Chemical Co.) for 16 h at room temperature. Radioactivity of the pellets

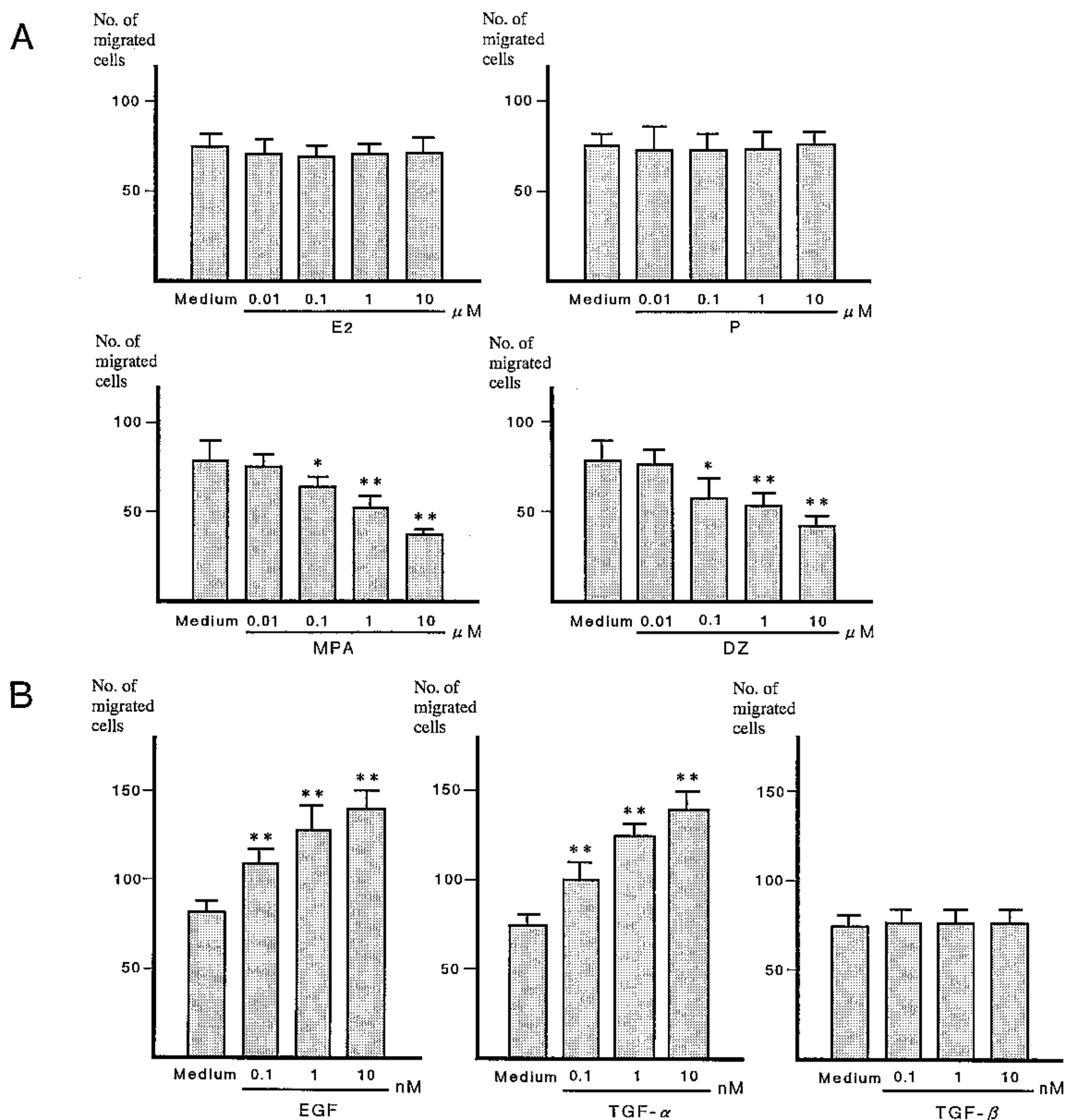


Fig. 2. Effects of sex steroids (A) and growth factors (B) on the haptotactic migration of SNG-M cells. Cells (1×10^6) in Ham's F-12 with 0.1% BSA were seeded with or without 0.01–10 *μM* E2, P, MPA and DZ or 0.1–10 n*M* EGF, TGF-*α* and TGF-*β* into the upper compartment of the Chemotaxicell culture chambers. Filters in chambers were precoated with 10 *μ*g of fibronectin on the lower surface. The migrated cells on the lower surface were counted after a 24-h incubation. * $P<0.05$, ** $P<0.01$ vs. control.

obtained by centrifugation at 2,000g for 20 min at 0°C was measured in a gamma scintillation counter (ANSR, Dainabot Ltd., Tokyo). Scatchard analysis was carried out and EGFR value was expressed as fmoles per mg protein (fmol/mgp).

Statistical analysis The significance of differences between groups was calculated by applying Student's two-tailed t test.

RESULTS

Effects of sex steroids and growth factors on tumor cell growth The effects of sex steroids and growth factors on SNG-M cell growth were studied. As shown in Fig. 1A, E2, P, MPA and DZ at the concentration of 0.01–10 μM had no effect on the growth of SNG-M cells within 48 h. EGF, TGF-α and TGF-β at the concentration of 0.1–10

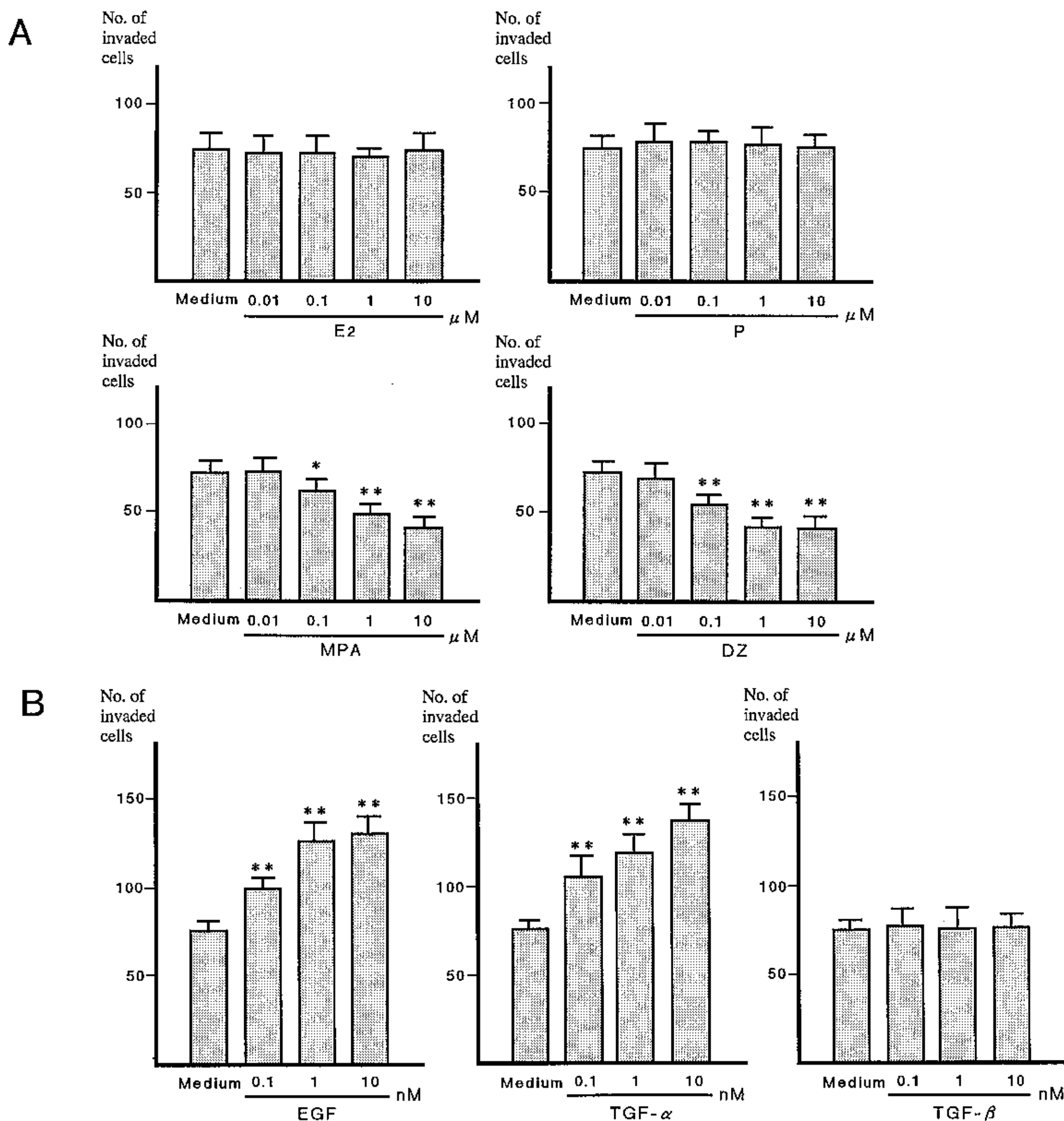


Fig. 3. Effects of sex steroids (A) and growth factors (B) on the invasive activity of SNG-M cells into Matrigel/fibronectin-coated filters. Cells (1×10^6) in Ham's F-12 with 0.1% BSA were seeded with or without 0.01–10 μM E2, P, MPA and DZ or 0.1–10 nM EGF, TGF-α and TGF-β into the upper compartment of the Chemotaxicell culture chambers. Filters in chambers were precoated with 10 μg of fibronectin on the lower surface, and with 5 μg of Matrigel on the upper surface. The invaded cells on the lower surface were counted after a 24-h incubation. * $P<0.05$, ** $P<0.01$ vs. control.

nM also had no effect on the growth of SNG-M cells within 48 h, as shown in Fig. 1B.

Effects of sex steroids and growth factors on tumor cell migration Invasion is a complex process involving cell adhesion, motility and the secretion of different classes of enzymes. Since the invasive activity of tumor cells in an *in vitro* culture system is considered to reflect a combination of the effects of tumor cell motility and enzymatic degradation of ECM components, we first examined the effects of sex steroids and growth factors on tumor cell migration. As shown in Fig. 2A, haptotactic migration of SNG-M cells along a gradient of substratum-bound fibronection was inhibited by 0.1–10 μM MPA and DZ in a concentration-dependent manner. E2 and P did not have any effect on the motility of SNG-M cells. On the other hand, tumor cell migration was promoted by 0.1–10 nM EGF and TGF-α in a concentration-dependent manner, whereas TGF-β did not have any effect on tumor cell motility, as shown in Fig. 2B.

Effects of sex steroids and growth factors on tumor cell invasion As shown in Fig. 3A, the invasive activity of SNG-M cells into reconstituted basement membrane components (Matrigel) was inhibited by the presence of 0.1–10 μM MPA and DZ, whereas E2 and P did not have any effect on tumor cell invasion. However, 0.1–10 nM EGF and TGF-α showed a concentration-dependent promoting effect on the invasive activity of SNG-M cells, whereas TGF-β did not have any effect on tumor cell invasion, as shown in Fig. 3B.

Effects of sex steroids and growth factors on tumor cell locomotion To confirm the biological effects of sex steroids and growth factors on tumor cell migration, we then examined their effects on tumor cell locomotion by wound assay. SNG-M cells were plated on tissue culture plates, wounded and allowed to migrate for 48 h in the presence of 10 μM sex steroids or 10 nM growth factors. As shown in Fig. 4, the locomotion of SNG-M cells into the wounded area was inhibited by MPA and DZ, but remarkably promoted by EGF and TGF-α. E2, P and TGF-β did not have significant effects on tumor cell locomotion.

Effects of sex steroids and growth factors on the degradation of gelatin or casein in zymograms To investigate further the effects of sex steroids and growth factors on the invasion process of SNG-M cells, we examined the enzymatic degradation of gelatin or casein substrates by TCM from SNG-M cells in zymograms. As can be seen in Fig. 5, the gelatin zymography showed that the treatment of SNG-M cells with EGF and TGF-α resulted in an increase of type IV collagenases with molecular weights of 68, 72 and 92 kDa in a concentration-dependent manner. However, sex steroids and TGF-β did not have significant effects on these enzyme levels. The casein zymography revealed that stromelysin secretion by

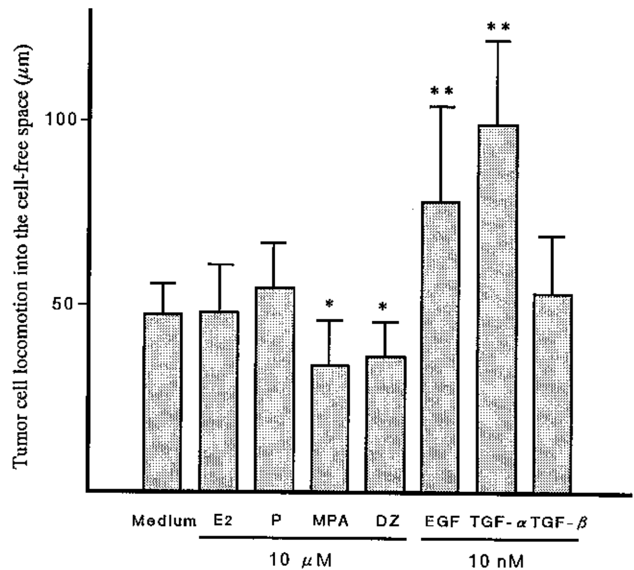


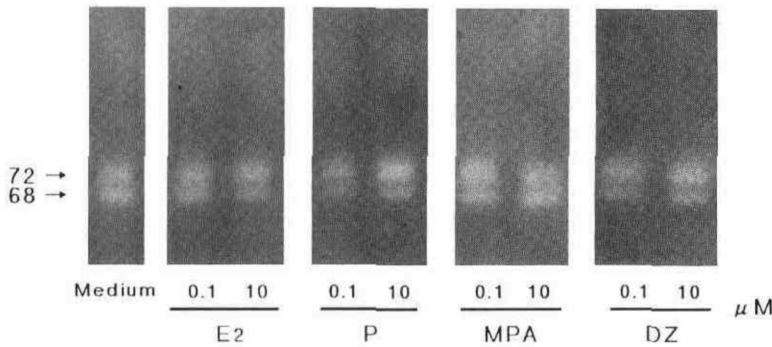
Fig. 4. Effects of sex steroids and growth factors on the locomotion of SNG-M cells. Cells (1×10^5) were seeded into fibronectin-coated 24-well microplates and incubated for 24 h. Each well was washed with serum-free Ham's F-12 with 0.1% BSA and cultured for another 24 h in the same medium. The monolayers were then wounded and allowed to migrate for 48 h in the presence or absence of 10 μM E2, P, MPA and DZ or 10 nM EGF, TGF-α and TGF-β. The cells that had migrated into the cell-free space were observed and the migration distances were measured. * $P<0.05$, ** $P<0.01$ vs. control.

SNG-M cells was not affected by sex steroids or growth factors, as shown in Fig. 6.

ER, PR and EGFR of SNG-M cells SNG-M cells did not contain ER or PR in either cytosol or nuclei. A Scatchard plot of EGF binding to SNG-M cells indicated a single class of binding sites with a dissociation constant of 2.45 nM, and the total binding sites amounted to 405 fmol/mgp.

DISCUSSION

Sex steroids and growth factors play an important role in the proliferation and differentiation of human endometrium. Recent investigations[13, 27, 28] have been focused on autocrine growth factors and malignant characteristics of endometrial adenocarcinoma cells, which are closely related to biological functions of ovarian sex steroids. Bulletti *et al.*[13] and Jasonni *et al.*[28] demonstrated in immunohistochemical studies that E2, P and synthetic progestins might be involved not only in growth control, but also in basement membrane degradation or forma-



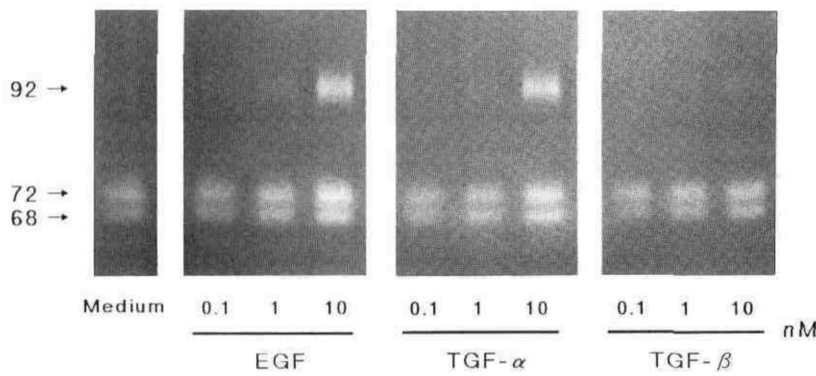
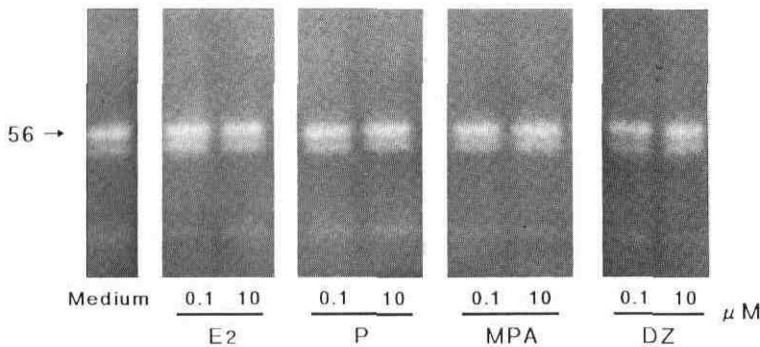


Fig. 5. The gelatinolytic activity of TCM from SNG-M cells. Confluent monolayers of SNG-M cells were cultured for 48 h with or without 0.1 and 10 μM E2, P, MPA and DZ or 0.1–10 nM EGF, TGF-α and TGF-β in serum-free Ham's F-12. TCM was concentrated with a Centricon 30, mixed with SDS-sample buffer, and then immediately applied, without heating or reduction, to gelatin-embedded SDS-polyacrylamide gels. After a 48-h incubation, the gels were stained for proteins with Coomassie Brilliant Blue, and active enzymes were detected as unstained bands. Numbers indicate the molecular weight of the proteinase bands estimated in relation to the migrations of molecular weight standards.



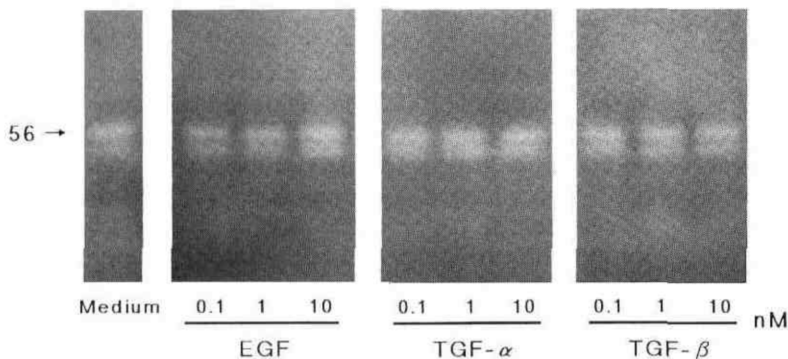


Fig. 6. The caseinolytic activity of SNG-M cells. TCM samples from SNG-M cells were obtained as described in Fig. 5, and applied to casein-embedded SDS-polyacrylamide gels. After a 48-h incubation, the gels were stained with Coomassie Brilliant Blue, and active enzymes were detected as unstained bands.

tion of endometrial adenocarcinoma tissues induced by EGF and TGF-α, β as second mediators of sex steroids. Moreover, Yoshida *et al.*[14] reported that EGF and TGF-α increased the expression of mRNA for several MMPs in human gastric carcinoma cell line, and Yudoh *et al.*[29] demonstrated that EGF stimulated the invasiveness of RCT sarcoma cells through the acceleration of cell adhesion and the degradative cascade of ECM. These previous reports indicate that growth factors are strongly associated with the enzymatic degradation or synthesis of ECM components and the invasion process of tumor cells. However, *in vitro* effects of sex steroids and growth factors on the invasive activity of endometrial adenocarcinoma cells have not been elucidated.

Tumor cell invasion into ECM and basement membranes involves a distinct sequence of events including cell attachment, migration and the degradation of ECM components caused by proteolytic enzymes secreted by tumor cells. Proteolytic enzymes including lysosomal hydrolases,[30–32] collagenases,[33, 34] and in some cases plasminogen activator[35] are elevated in tumor cells with high invasive and metastatic potential. Among various endometrial adenocarcinoma cell lines, SNG-M originating from a metastatic lymphnode was demonstrated to possess high invasive activity and to produce type IV collagenases.[36] We therefore investigated the *in vitro* effects of sex steroids and growth factors on migration and invasion of SNG-M cells, together with the enzymatic degradation of ECM components by TCM obtained from SNG-M cells.

The motility (haptotactic migration) and the invasive activity of SNG-M cells were inhibited by 0.1–10 μM MPA and DZ, but promoted by 0.1–10 nM EGF and TGF-α in a concentration-dependent manner. E2, P and TGF-β did not have any effect on tumor cell migration or invasion under our experimental conditions. Stimulatory or inhibitory effects of sex steroids and growth factors on the motility of SNG-M cells were also confirmed by estimating tumor cell locomotion (wound assay) on tissue culture plates. MTT assay revealed that these results did not depend on the growth-stimulatory or inhibitory effects of sex steroids and growth factors in SNG-M cells. The zymography of TCM showed that the treatment of SNG-M cells with EGF and TGF-α resulted in increased 68, 72 and 92 kDa enzyme levels. It is well known that collagenases exist in both latent and active forms.[6, 7] Three bands of gelatinolytic activity were considered to be the active (68 kDa) and the latent (72 kDa) form of MMP-2, and MMP-9 (92 kDa). However, sex steroids and TGF-β did not have significant effects on these enzyme levels. Stromelysin (MMP-3), also secreted by SNG-M cells, was not affected by sex steroids and growth factors. These results suggest that EGF and TGF-α act as positive regulators, and MPA and DZ act as negative regulators on the invasion process of endometrial adenocarcinoma SNG-M cells, possibly via their stimulatory or inhibitory action on the motility of tumor cells. Moreover, stimulatory effects of EGF and TGF-α on the invasiveness of SNG-M cells were revealed to be closely related to the induction of type IV collagenases secreted by tumor cells.

Biological effects of hormones and growth factors are primarily dependent on the presence of their receptors. We preliminarily examined the motility and the invasive activity of Ishikawa cells[37] as a model which possesses ER, PR and EGFR. However, the number of cells that had migrated or invaded to the lower surface of the membrane was very low (5–10 cells/high power field) under our experimental conditions, and the subsequent procedures could not be carried out. Since many endometrial adenocarcinoma cell lines other than Ishikawa cells do not possess ER and PR, we first investigated the *in vitro* effects of sex steroids and growth factors on migration and invasion of SNG-M cells which were negative for ER and PR, but positive for EGFR. EGF and TGF-α are assumed to act through a common receptor and to share many biological activities.[14] Their stimulatory effects on the invasive activity of SNG-M cells may be mediated by EGFR on the cellular membrane, as indicated by Scatchard plot analysis. However, E2 and P did not have any effect on migration or invasion of SNG-M cells, which may be due to the absence of ER and PR on the cells. It is possible that only EGF and TGF-α act as regulatory factors on the invasion process of cultured endometrial adenocarcinoma cells negative for hormone receptors. On the other hand, Nakajima and Chop[38] indicated that TGF-β inhibited the expression of MMP-1 and 3, but stimulated the production of MMP-2 and 9 in a wide variety of cultured cancer cells of epithelial origin. However, TGF-β did not have any effect on SNG-M cells. Further studies including TGF-β receptor assay are needed to elucidate how TGF-β acts as a regulator of tumor cell migration, invasion and proteolytic enzymes in SNG-M cells and many other endometrial adenocarcinoma cell lines.

The present results suggest that the inhibitory effects of MPA and DZ on tumor cell invasion may depend on their inhibitory action on the motility of tumor cells, because they did not have significant effects on MMPs secreted by tumor cells. Previous investigators[39, 40] demonstrated using clinical materials that MPA and DZ bind to PR and decrease the proliferative activity of endometrial cancer cells. Since SNG-M cells were negative for PR, the biological effects of MPA and DZ on the invasive activity of SNG-M cells may depend on their pharmacological action not mediated by PR. It is noteworthy that urokinase-type plasminogen activator (uPA) plays an important role in the invasion process of en-

dometrial adenocarcinomas.[13] The proteolytic cascade of ECM components is triggered by the uPA-mediated conversion of plasminogen to plasmin and the subsequent activation of procollagenases.[2, 35, 41] When examined with fibrinogen-embedded zymograms, however, MPA and DZ did not have significant effects on the uPA activity of SNG-M cells (data not shown). The reasons for the inhibitory effects of MPA and DZ on tumor cell invasion remain to be established.

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