

Expression of keratinocyte growth factor and its receptor in human endometrial cancer in cooperation with steroid hormones

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Abstract. The keratinocyte factor (KGF) and its receptor (KGFR) are implicated in tissue development and repair. We studied the expression and functions of KGF and KGFR in association with estrogen and progesterone in human endometrial tissues and cells. In non-cancerous human endometrial tissues in the secretory phase, a strong immunoreactivity of KGF in glands, stromal cells, and smooth muscle cells of spiral arteries was detected; however, in proliferative-phase tissues, the immunoreactivity of KGF or KGFR was weak or absent. Most of the 32 endometrioid adenocarcinoma cases showed positive KGF and KGFR stainings (90.6 and 71.9%, respectively). We then studied, using Ishikawa well-differentiated human endometrial cancer cell line that expresses estrogen receptor (ER) and progesterone receptor (PR), the expression of KGF and KGFR in conjunction with estrogen and progesterone, and observed that the KGFR expression of Ishikawa cells was upregulated by estrogen and that this upregulation was markedly enhanced by the coadministration of progesterone. We also observed that KGF administration to cells, with KGFR upregulated expression, stimulated ERK1/2 phosphorylation and cell adhesion to fibronectin. The implications of the hormone-stimulated KGF-KGFR expressions in the regulation of cell behavior associated with human endometrial cancer are discussed.

Introduction

Endometrial cancer is one of the most common malignancies of the female genital tract (1). The changes in the structure

and function of the endometrium during the menstrual cycle are regulated by the coordinated action of steroid hormones and locally produced growth factors. One of the main causes of endometrial cancer is considered to be prolonged exposure of endometrial cells to unopposed estrogen without progesterone (2). Progesterone modulates the growth promotion effect of estrogen (3). Also, growth factors play roles in tumor development.

Most endometrial cancers are histologically classified as adenocarcinomas, the most common of which are of the endometrioid type; their degree of differentiation is graded according to their degree of 'solid proliferation' in tumor tissues (1). Tumor grade is closely associated with the expression of steroid hormone receptors. Tissues with low-grade cancer express estrogen receptors (ERs) and progesterone receptors (PRs) and such cancer has a relatively good prognosis (4).

The keratinocyte growth factor (KGF), also known as fibroblast growth factor-7 (FGF-7), is a member of the FGF family of heparin-binding polypeptides that was originally isolated from human embryonic lung fibroblasts (5). KGF is expressed in normal stromal cells of various organs including the lung, skin, mammary gland, stomach, bladder, digestive tract and male and female reproductive tracts such as the seminal vesicle (6), prostate (7) and endometrium (8), and implicated in organ and tissue development (9). KGF transcripts are generally detected in mesenchymal cells adjacent to the epithelium where KGF acts predominantly on epithelial cells in a paracrine manner (9). KGF also stimulates the growth of hair follicles (10) and the thickening of the mucosa of the gastrointestinal tract (11).

KGF binds to a cell surface KGF receptor, KGFR, also known as FGF receptor type II (FGFR-2IIIb) (12). Because FGFR-2IIIc and KGFR are derived from the same *fgfr-2* gene by the alternative splicing of the carboxyl-terminal half of the third Ig-like region of the extracellular domain, they are homologous in their intracellular domains and most of their extracellular domains. KGFR localizes mainly in epithelial cells, whereas FGFR-2IIIc localizes mainly in mesenchymal cells (13). KGF mRNA is predominantly expressed in stromal cells of the human endometrium, whereas KGFR mRNA is predominantly expressed in epithelial cells (14).

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KGF and KGFR expressions have been detected in a wide range of cancer tissues, and the mode of KGF effect on tissue cell behaviors, such as cell growth, motility, invasion and differentiation, depends on the types of organ and tissue from which tumors originate (15). KGF stimulates the growth of cancer cells derived from scirrhous gastric tumors (16) and enhances the invasiveness of poorly differentiated gastric cancer cells (17).

We previously reported that KGFR expression is associated with cell differentiation in uterine cervical cancers (18) and colorectal cancer tissues (19). In prostate cancer, KGFR introduction into cancer cells induces cell differentiation (20) and results in the KGF-dependent inhibition of proliferation (21).

KGF is an essential mediator of steroids in various organs (6-8) and in cancers derived from them (22). KGF stimulates the motility of ER-positive breast cancer cells but not that of ER-negative cells, and has an inhibitory role in apoptosis induction (23). Moreover, it downregulates ER and PR expressions in breast cancer cells and increases cancer resistance against tamoxifen, an ER agonist, used for the endocrine therapy of breast cancer (22).

The effects of steroid hormones on KGF and KGFR expression levels have been observed in the endometrium of various species, including pigs (24), monkeys (8) and humans (14,25). Progesterone administration increases KGF expression levels in the monkey and mouse endometria (8). In the human endometrium, KGF expression level is increased during the luteal phase of the menstrual cycle, whereas KGFR mRNA expression level is increased by estrogen (25).

KGF and KGFR transcripts are detected in endometrial cancer cells (26) and KGF administration stimulates the proliferation of endometrial cancer cells (27).

A report on the intracellular signal transduction pathways associated with the KGF and KGFR systems has shown that ERK1/2 phosphorylation is a key process in the mitogen-activated protein kinase (MAPK) cascade following receptor activation by KGF (28). MAPK and ERK1/2 activations are associated with cell proliferation. Furthermore, MAPK activation is closely related to cell adhesion to the extracellular matrix (ECM) (29) that is implicated in the modulation of cell behavior and differentiation.

Previous studies have shown KGF involvement in modulating cell behavior such as adhesion to the ECM and migration (30), in addition to cell proliferation (16,27) and differentiation (31). In our recent study, we reported that transduction of the *kGF* gene into colorectal cancer cells enhances cell adhesion to ECM with the concomitant upregulation of ERK1/2 phosphorylation (32).

In this study, we examined KGF and KGFR expression in the normal human endometrium in different phases of the menstrual cycle and in the tissues of an endometrioid adenocarcinoma in association with ER and PR expression. We also investigated the effects of steroid hormones on the expression and functions of KGF and KGFR of a well-differentiated human endometrial cancer cell line, Ishikawa. Furthermore, we showed that the changes in cell behavior via the ERK1/2 signal transduction pathway are induced after the interaction of a ligand with KGFR.

Materials and methods

Materials. The chemicals and reagents used were purchased from their respective manufacturers as follows: CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit from Promega (Madison, WI, USA); accutase from Innovative Cell Technologies, Inc. (San Diego, CA, USA); Isogen from Nippon Gene (Tokyo, Japan); Takara RNA PCR kit (AMV) Ver. 3.0 from Takara Biotech (Tokyo, Japan); RNeasy minikit from Qiagen GmbH (Hilden, Germany); transcriptor first-strand cDNA synthesis kit and LightCycler FastStart DNA Master SYBR Green I from Roche Diagnostics GmbH (Mannheim, Germany); goat polyclonal anti-FGF-7 antibodies and recombinant human KGF (rhKGF) from R&D Systems Inc. (Westerville, OH, USA); rabbit polyclonal anti-ERK1/2 antibody from Cell Signaling Technology, Inc. (Danvers, MA, USA); rabbit polyclonal anti-phosphorylation-specific ERK1/2 (p-ERK, Tyr 204) antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); Immobilon P transfer membrane from Millipore Japan (Yonezawa, Japan); M-PER Mammalian Protein Extraction reagent and Super Signal West Dura chemiluminescent substrates from Pierce (Rockford, IL, USA); SERVA Blau G from Serva Electrophoresis GmbH (Heidelberg, Germany); Histofine Simple Stain Max PO (M), (G) or (R) kit from Nichirei Biosciences, Inc. (Tokyo, Japan); goat anti-rabbit IgG-HRP antibody from American Qualex (San Clemente, CA, USA); Alexa 488 goat anti-rabbit IgG from Molecular Probes, Inc. (Eugene, OR, USA); mouse monoclonal anti-ER α antibody (1D5) and mouse monoclonal anti-PR (PgR636) antibody from Dako Japan Co., Ltd. (Kyoto, Japan); vectashield mounting medium containing 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) from Vector Laboratories, Inc. (Burlingame, CA, USA); 35-mm glass-bottom dish from Matsunami Glass Ind., Ltd. (Osaka, Japan); Silane-coated slides and a malinol mounting medium from Muto Pure Chemicals Co., Ltd. (Tokyo, Japan). All the other chemicals and reagents used were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Patients and tissues. Our study included 32 primary endometrioid adenocarcinoma patients (median age, 57 years; range, 32-84 years) treated at the Department of Obstetrics and Gynecology, Nippon Medical School from January 2004 to June 2006. In accordance with the International Federation of Gynecology and Obstetrics (FIGO) surgical staging system (1988), the patients were classified as follows: stage IA, n=2; stage IB, n=10; stage IC, n=8; stage IIB, n=1; stage IIIA, n=6; stage IIIC, n=4; and stage IVB, n=1 (including peritoneal invasion).

In accordance with the histopathology degree of differentiation of FIGO 1988, the tumors were classified histologically as follows: grade 1, n=14; grade 2, n=11; and grade 3, n=7. Because the depth of myometrial invasion is associated with other prognostic factors such as tumor grade, the primary tumors were classified as follows: a) limited to endometrium, n=2; b) invasion to <1/2 of myometrium, n=14; and c) invasion to >1/2 of myometrium, n=16. During celiotomy, peritoneal fluid samples were obtained for cytological tests. Endometrial non-cancerous

tissues were obtained from 5 normal proliferative-phase and 5 secretory-phase endometria of patients who underwent endometrial curettage or hysterectomy for adenomyosis and leiomyoma.

This study was approved by the Ethics Committee of Nippon Medical School in accordance with the principles embodied in the Declaration of Helsinki 1975. All the treatments and clinical research were conducted with informed consent of the patients.

Immunohistochemistry. Paraffin-embedded tissue sections (3.5 μm thick) were subjected to immunostaining with a Histofine Simple Stain Max PO (M), (G) or (R) kit. After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 min. For ER and PR, the heating method was used for antigen retrieval according to the manufacturer's recommendation. The sections were incubated with the appropriate antibody for 16 h at 4°C [1:50 dilution for the anti-KGF antibody, 1:1,000 dilution for the anti-KGFR antibody (in-house) (33), 1:50 dilution for the anti-ER α antibody and 1:100 dilution for the anti-PR antibody], using phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). Bound antibodies were detected with a Simple Stain Max PO (M), (G) or (R) reagent using diaminobenzidine tetrahydrochloride (DAB) as the substrate, and the sections were counterstained with Mayer's hematoxylin. Negative control studies were performed by omitting the primary antibodies.

Evaluation of staining results. Immunohistochemical stainings for KGF and KGFR were evaluated as positive when >30% of the cytoplasm and/or membrane of the tumor cells is stained, regardless of staining intensity. A normal endometrium was used as the positive control. For the evaluation of ER and PR stainings, the quickscore, or the semiquantitative immunoreactive score (IRS), was applied (34). On the basis of the percentage of tumor cells showing nuclear reactivity, 6 classes were defined (class 1, <4% of positive cells; class 2, 4-19%; class 3, 20-39%; class 4, 40-59%; class 5, 60-79%; and class 6, >80%); mean staining intensity was evaluated separately in 4 classes (0, negatively stained; 1, weakly stained; 2, moderately stained; and 4, strongly stained). By multiplying the scores, an IRS within 0-18 was obtained. A normal endometrial gland was used as the positive control. Two investigators (A.I. and T.I.) separately evaluated all the specimens in a blind manner.

In situ hybridization. A 304-bp *Bam*HI-*Sal*I cDNA fragment, corresponding to nucleotides 461-764 of the human KGF cDNA sequence, was subcloned into the pGEM-T vector and the result was confirmed by sequencing. Probes were labeled with digoxigenin (DIG)-UTP using the DIG RNA-labeling kit. *In situ* hybridization was performed as previously described (35). Tissue sections were deparaffinized and incubated at room temperature (RT) for 20 min in 0.2 M HCl and then at 37°C for 15 min in PBS with 100 $\mu\text{g}/\text{ml}$ proteinase K. The sections were then postfixed for 5 min in PBS with 4% paraformaldehyde (PFA), and incubated twice for 15 min each in PBS with 2 mg/ml glycine at RT

and once in 50% (V/V) formamide/2X SSC for 1 h at 42°C prior to the initiation of hybridization. Hybridization was performed in a moist chamber for 16 h at 42°C. The sections were then washed sequentially with 2X SSC for 20 min at 42°C and with 0.2X SSC for 20 min at 42°C. For immunological detection, a DIG nucleic acid detection kit was used. The sections were washed briefly with buffer 1 (100 mM Tris-HCl and 150 mM NaCl, pH 7.5), incubated with 1% (w/v) blocking reagents in buffer 1 for 60 min at RT, and with alkaline-phosphatase-conjugated polyclonal sheep anti-digoxigenin Fab fragment containing 0.2% Tween-20 at 1:2,000 dilution for 60 min at RT. The sections were then washed twice for 15 min at RT with buffer 1 containing 0.2% Tween-20, equilibrated with buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) for 2 min, and incubated with a staining solution containing nitroblue tetrazolium and X-phosphate in a dark box for 1 h. After the reaction was stopped with TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), the sections were mounted in an aqueous mounting medium.

Cell culture. The human endometrial cancer cell line Ishikawa (clone 3-H-12, No. 129) (36) was kindly provided by Dr M. Nishida (Kasumigaura National Hospital, Ibaraki, Japan). The cells were grown in phenol red-free RPMI-1640 (Gibco-Invitrogen) medium containing 5% charcoal-treated heat-inactivated fetal bovine serum (FBS, JRH Biosciences, Inc., Lenexa, KS) supplemented with 200 U/ml penicillin and 200 $\mu\text{g}/\text{ml}$ kanamycin at 37°C under a humidified 5% CO₂ atmosphere and were used within 10 passages. For Western blot and RT-PCR analyses, the cells (2x10⁶ cells) were seeded onto a 100-mm culture dish and grown in 7 ml of the phenol red-free RPMI-1640 medium with 5% charcoal-treated FBS in the presence of 10 nM 17- β estradiol (E₂) alone or both 10 nM 17- β estradiol (E₂) and 1 μM medroxyprogesterone acetate (P₄) (E₂+P₄) in combination with 10 ng/ml recombinant human KGF (rhKGF).

Cell growth kinetics. Cell growth was analyzed using the CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (MTS-based assay) method in accordance with the manufacturer's instructions. Briefly, the cells were seeded at 5,000 cells/well in 96-well microplates with or without E₂+P₄ in the culture medium in combination with 10 ng/ml rhKGF and incubated for 24, 48, or 72 h at 37°C in a humidified 5% CO₂ atmosphere. MTS/phenazine methosulfate solution at a vol/vol ratio of 19:1 was added to each well 4 h before the termination of incubation. The absorbance at 490 nm was then measured using a spectrophotometer.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the cells using Isogen according to the manufacturer's protocol, and the cDNA synthesis and PCR were performed using a Takara RNA PCR kit. The primers used for the detection of KGF mRNA corresponded to nucleotides (nts) 765-784 and nts 905-927 of the human KGF cDNA (163 bp, accession no. M60828), namely, (5'-TTG-TGG-CAA-TCA-AAG-GGG-TG-3') and (5'-CCT-CCG-TTG-TGT-GTC-CAT-TTA-GC-3'), respectively. The primers used for the detection of KGFR

mRNA were (5'-GCT-CTG-TTC-AAT-GTG-ACC-GA-3') and (5'-TGA-AGA-GAG-GCG-TGT-TGT-TA-3') that corresponded to nts 1394-1413 and nts 1761-1780, respectively, of KGFR cDNA (387 bp, accession no. M80634). For the detection of β -actin mRNA (218 bp, accession no. X00351), as the positive control, the following primer pairs were used: for nts 222-241, (5'-AAG-AGA-GGC-ATC-CTC-ACC-CT-3'), for nts 420-439, (5'-TAC-ATG-GCT-GGG-GTG-TTG-AA-3').

Western blotting. Protein extraction was carried out according to the protocol that uses M-Per Mammalian Protein Extraction reagent. Briefly, the cells were solubilized in M-Per reagent with Protease Inhibitor Cocktail for Mammalian Tissues. The lysates were centrifuged for 10 min at 13,000 rpm to pellet cell debris. The supernatants were collected, and protein concentration was measured by the Bradford method. Equal amounts of protein for all the samples were loaded and run on a 10 or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrophoretically blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore Japan, Tokyo, Japan). The blots were blocked for 1 h with 5% skim-milk in Tris-buffered saline (TBS) containing 0.2 M Tris-HCl, 150 mM NaCl, and 0.05% Tween-20. The membrane was then incubated overnight (O/N) at 4°C with anti-KGF antibody (1:1,000), rabbit polyclonal anti-KGFR/FGFR-2 IIIb antibody (1:3,000), anti-ERK1/2 antibody (1:1,000), anti-p-ERK, Tyr 204 antibody (1:500), or anti- β -actin antibody (1:5,000), followed by O/N incubation at 4°C. After a 30-min washing in TBS, the blots were incubated with 1:5,000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at RT. Immunoreactive products were visualized using a Super Signal West Dura Chemiluminescence substrate and quantified using Chemi Doc XRS System and Quantity One Software (Bio-Rad Laboratories, Inc., Tokyo, Japan).

Quantitative confocal laser microscopic immunofluorescence assay. Ishikawa cells (1×10^5) in 2 ml of phenol red-free RPMI-1640 medium containing 5% charcoal-treated FBS and supplemented with E_2 alone or E_2+P_4 were cultured in 35-mm glass-bottom dishes for 72 h. Then the cells were fixed in ethanol:acetone (1:1) solution for 10 min at 4°C. The fixed cells were treated O/N at 4°C with an anti-KGFR/FGFR-2 IIIb antibody (1:200) in PBS containing 1% BSA that was also used for the Western blot analysis. The cells were washed with PBS and then incubated with FITC-conjugated anti-rabbit IgG. One hour after incubation, the cells were washed with PBS and mounted with Vectashield H-1200 containing DAPI.

The fluorescence intensity of the dye was determined using the confocal laser scanning microscope Digital Eclipse C1 TE2000-E (Nikon Insteck Co., Ltd., Tokyo, Japan) with a blue diode (excitation, 405 nm; emission, 450/35 nm), an argon laser (excitation, 488 nm; emission, 515/30) and helium-neon lasers (excitation, 543 nm; emission, 605/75 nm). Fluorescence images were acquired and analyzed using the confocal microscope Digital Eclipse C1 control software EZ-C1 (Version 2.30, Nikon Insteck). For the quantitative fluorescence analysis of the conjugated FITC, confocal

images were acquired using the same parameters (gain and laser power), exported in TIFF format and analyzed with the MetaMorph software version 7 (Molecular Devices, Sunnyvale, CA), using the region measurement tool, and average intensity was calculated from the images. Ten images were selected randomly from each group, and the average intensity of FITC per cell was calculated by dividing the average intensity in each image by the number of cells in the same image that was determined from the number of nuclei stained with DAPI using the Manually Count Object Tool.

Cell attachment assay. One hundred microliters each of fibronectin (Invitrogen Corp., Carlsbad, CA, USA) or type-IV collagen (Sigma-Aldrich Corp.) solution at concentrations of 0-20 μ g/ml (based on serial dilution) was added into the wells of 96-well microplates followed by O/N incubation at 4°C for coating the wells. The residual culture area was blocked with 1% BSA in PBS at RT for 3 h.

The cells cultured in the medium supplemented with E_2+P_4 for 48 h were harvested with Accutase, resuspended in serum-free medium and further incubated for 1 h at 37°C for cell recovery. Then, 100 μ l of each cell suspension was seeded at 4×10^4 cells/well with or without 10 ng/ml rhKGF followed by incubation for 1 h at 37°C in a CO₂ incubator to investigate the effects of rhKGF on cell attachment. Non-adherent cells were removed by gentle washing with a serum-free medium and the relative number of attached cells was determined by MTS-based assay.

Statistical analysis. All values are expressed as mean \pm SEM. Group comparison was carried out by one-way analysis of variance (ANOVA). All the groups were analyzed simultaneously with a Bonferroni-Dunn test. For immunohistochemistry, Fisher's exact test was used to analyze the correlation between KGF or KGFR expression and clinicopathological features. The correlation between KGF or KGFR expression and the IRS score of ER or PR expression was analyzed by Student's t-test. A difference with $p < 0.05$ was considered statistically significant. Computations were performed using the StatView version 5.0 software package (Abacus Concepts, Berkeley, CA, USA).

Results

KGF, KGFR and KGF mRNA in human endometrial tissues. First, we investigated KGF and receptors in human endometrial tissues from patients with a regular menstrual cycle, using immunohistochemical staining. In non-cancerous proliferative-phase endometrial tissues, in which estrogen is the dominant factor for tissue development, the immunoreactivity of the anti-KGF antibody was found to be weak or absent (Fig. 1A). The KGFR stains were negative or weak in most of the luminal and glandular epithelia of the proliferative-phase tissue (Fig. 1B). In secretory-phase endometrial tissues, in which both estrogen and progesterone function concomitantly, intense KGF immunoreactivity was detected in glands, stromal cells, and smooth muscle cells of the spiral arteries (Fig. 1C). KGFR staining was detected in glands of secretory-phase tissues (Fig. 1D).

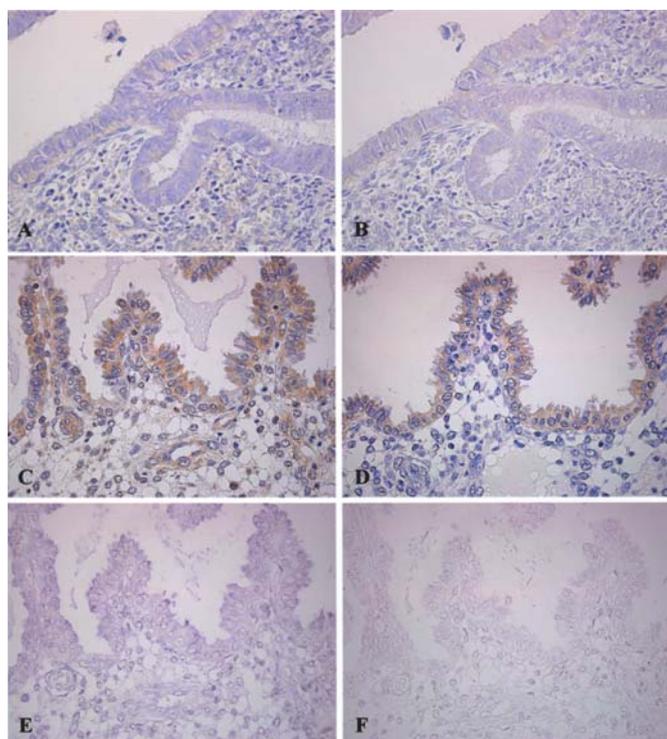


Figure 1. Immunohistochemical analyses of KGF and KGFR expressions, and *in situ* hybridization of KGF in human endometrial tissues. In proliferative-phase endometrial tissues, KGF immunoreactivity was weak or absent (A), and KGFR immunoreactivity was negative or weak in the luminal and glandular epithelia (B). In secretory-phase endometrial tissues, intense KGF immunoreactivity was found in glands, stromal cells, and smooth muscle cells of the spiral arteries (C), and KGFR immunoreactivity was detected in the glands (D). In the section hybridized with KGF antisense probe, KGF mRNA was found to be expressed in the cytoplasm of both stromal and epithelial cells of secretory-phase human endometrium (E). In the section hybridized with KGF sense probe, no positive cells are found (F). Original magnification, x600.

To confirm whether apparent expression of KGF proteins results from *de novo* translation in secretory-phase endometrial epithelial cells, we applied *in situ* hybridization to the detection of KGF mRNA in the cells. In Fig. 1E, it is shown that KGF mRNA was actually expressed in the cytoplasm of both the stromal cells and epithelial cells of the secretory-phase human endometrium. However, the sense probe showed no positive signals (Fig. 1F).

Steroid hormone receptors, KGF and KGFR in endometrial cancer tissues. We investigated the modes of expression of steroid hormone receptors, KGF and KGFR in human endometrial cancer tissues using immunohistochemical staining. It was observed that ER was localized in the nuclear region of the cancer cells, more frequently in the cells forming glandular structures than in the cells of solid nests of endometrial cancer tissues (Fig. 2A and B). PR was also detected in the nuclear region of the cancer cells, with patterns similar to those of ER (Fig. 2C and D).

Next, we investigated the KGF or KGFR expression of human endometrial tumor tissues in relation to histological grades. In endometrial cancer tissues, immunohistochemical staining revealed positive KGF immuno-

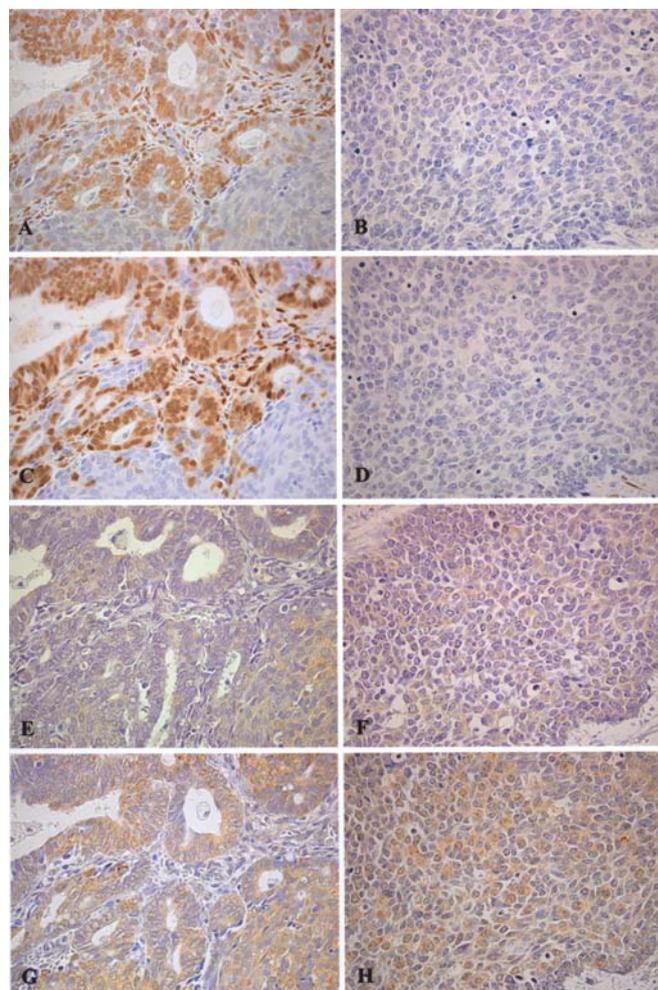


Figure 2. Immunohistochemical detection of ER, PR, KGF and KGFR in tissues of human endometrioid adenocarcinoma. ER staining was localized in the nucleus of the tumor cells forming glandular structures (A), and cells forming solid nests were ER-negative (B). PR staining was detected in the nucleus of the tumor cells, with patterns similar to those of ER staining (C and D). KGF staining was detected in the cytoplasm of the cancer cells that form either glandular structures (E) or solid nests (F), and KGFR staining was detected in the cytoplasm or membrane of the cancer cells, with a distribution pattern similar to that of KGF staining (G and H). KGF and KGFR were detected in squamous/morular cells of G1 and G2 cases (E and G). Original magnification, x600.

reactivity in the cytoplasm of the tumor cells that form either glandular structures or solid nests (Fig. 2E and F). KGFR was detected in the cytoplasm and/or membrane of the cancer cells with a distribution pattern similar to that of KGF (Fig. 2G and H). KGF- and/or KGFR-positive cells were also detected in squamous/morular cells of G1- and G2-grade tumors (Fig. 2E and G). Among the tissue specimens from the 32 endometrial cancer patients, positive KGF staining was detected in 90.6% and positive KGFR staining was detected in 71.9% of the specimens (Table I).

The numbers of patients that showed ER and PR expressions in the tumor cell nucleus were 28 and 30 of the 32 patients (87.5 and 93.8%), respectively. ER immunoreactivity evaluated by the Q-score was in the range of 0-18, mean 6 ± 5 , and PR immunoreactivity was in the range of 0-18, mean 9 ± 5 . A high ER score tended to correlate with a

Table I. Clinicopathological features and KGF or KGFR expression in endometrial cancers.

Variables	n	KGF		KGFR	
		n (%)	P-value	n (%)	P-value
Age (years)					
≤50	10	9 (90)	>0.99	9 (90)	0.21
>50	22	20 (91)		14 (63)	
Histological grade					
G1 or G2	25	23 (92)	0.54	19 (76)	0.37
G3	7	6 (86)		4 (57)	
FIGO stage					
I or II	21	18 (86)	0.53	14 (67)	0.44
III or IV	11	11 (100)		9 (82)	
Myometrial invasion					
a or b	16	15 (94)	>0.99	14 (88)	0.11
c	16	14 (86)		9 (56)	
Lymph node metastasis					
Negative	26	23 (88)	>0.99	19 (73)	0.56
Positive	4	4 (100)		2 (50)	
Unknown	2	2 (100)		2 (100)	
Peritoneal wash cytology					
Negative	29	26 (90)	>0.99	20 (69)	0.54
Positive	3	3 (100)		3 (100)	

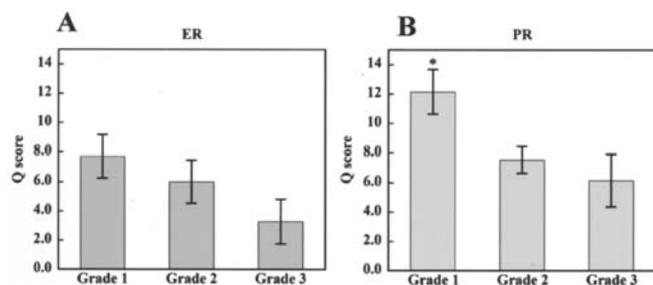


Figure 3. Relationship between mode of ER or PR expression of and histological grade of endometrioid adenocarcinoma tissues. (A) Relationship between tumor grade and ER Q-score. The tumor tissues with high ER Q-score showed low histological grades ($p=0.23$). (B) Relationship between tumor grade and PR Q-score. The high PR Q-score correlated significantly with the low histological grade of the tumor ($p=0.03$).

low histological grade of a tumor ($p=0.23$), and a high PR score correlates significantly with a low histological grade of a tumor ($p=0.03$) (Fig. 3).

No significant relationships were found between KGF or KGFR immunoreactivity and clinicopathological features: patient age, FIGO staging, pathological grades, depths of myometrial invasion and pelvic lymph node metastasis, and the peritoneal wash cytology. KGF and KGFR were detected in most of the endometrial cancers regardless of histological grade (Table I).

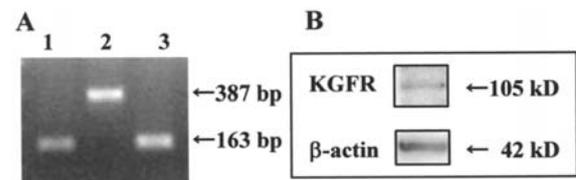


Figure 4. KGF and KGFR expressions of Ishikawa cells. (A) RT-PCR analysis of KGF and KGFR mRNAs: lane 1, KGF; lane 2, KGFR; and lane 3, β -actin (control). (B) Western blot analysis of KGFR: upper panel shows KGFR and lower panel shows β -actin.

KGF and KGFR expressions in endometrial cancer cells in vitro. The cell line Ishikawa is derived from tissues of a well-differentiated human endometrial cancer and this cell line expresses ER and PR. Both 163-bp and 387-bp bands that correspond to KGF mRNA and KGFR mRNA, respectively, were detected in Ishikawa cells by RT-PCR analysis (Fig. 4A). The results of the Western blot analysis of Ishikawa cells indicated the presence of a 105-kDa band that corresponds to KGFR in the cells (Fig. 4B), however, only a small amount of KGF was detected in the cells (data not shown).

Steroid hormones and KGF or KGFR expression. Having confirmed the KGFR expression of Ishikawa cells, we determined if KGFR expression is regulated by steroid hormones

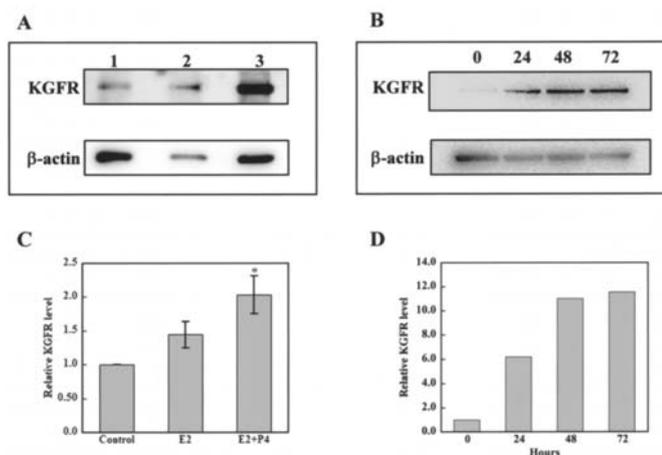


Figure 5. Western blot analysis of KGFR expression of Ishikawa cells treated with E_2 alone or with both E_2 and P_4 (E_2+P_4). E_2 alone (lane 2) or E_2+P_4 (lane 3) increased KGFR expression levels in the cells cultured for 72 h (A and C). The KGFR expression levels of the cells cultured in E_2+P_4 increased with cultivation time (B and D). Data are presented as mean \pm SEM (n=3). *p<0.05.

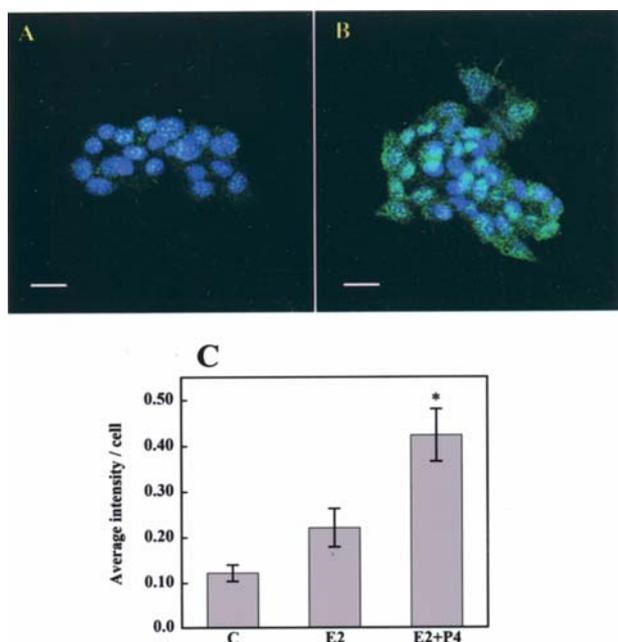


Figure 6. Quantitative immunofluorescence laser microscopy of KGFR expression of Ishikawa cells. (A) Immunohistochemical staining of cells cultured in the medium supplemented with E_2 alone. (B) Immunohistochemical staining of cells cultured in the medium supplemented with E_2+P_4 . (Bar, 20 μ m). KGFR fluorescence staining is localized in the cytoplasm or nuclear region of the cells. The apparent fluorescence intensities of the staining are higher in the cells cultured with E_2+P_4 than in those cultured with E_2 alone. (C) Quantitative analysis of fluorescence intensities of cells cultured in the medium alone (control), column 1; with E_2 , column 2; and with E_2+P_4 , column 3. Data are presented as mean \pm SEM (n=3). *p<0.05.

in the cells. Result of the Western blot analysis of the cells cultured in the medium supplemented with E_2 alone or with both E_2 and P_4 (E_2+P_4) are shown in Fig. 5. The administration of E_2 alone into the culture medium increased

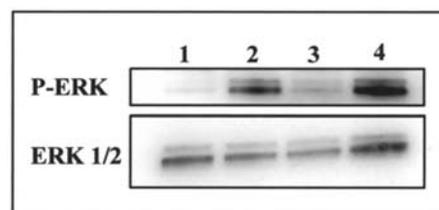


Figure 7. Western blot analysis of ERK phosphorylation in Ishikawa cells. Ishikawa cells precultured in the medium with E_2+P_4 were incubated with rhKGF for 60 min and ERK phosphorylation was analyzed by Western blotting. Control medium, lane 1; medium with KGF, lane 2; medium with E_2+P_4 , lane 3; medium with E_2+P_4 and KGF, lane 4. Upper panel, phosphorylated ERK (p-ERK); lower panel, ERK1/2.

the KGFR expression level in the cells and the coadministration of P_4 with E_2 markedly upregulated the KGFR expression level (Fig. 5A). Quantitative analysis of the data showed that the KGFR expression level increased by approximately 2-fold in the E_2+P_4 -treated cells compared with the control culture (Fig. 5C). Fig. 5B and D confirm that the KGFR expression level of the cells cultured in E_2+P_4 increased with cultivation time.

In addition to the Western blot analysis, we used quantitative immunofluorescence laser microscopy to confirm the upregulation of the *in situ* KGFR expression of Ishikawa cells by steroid hormones. The cells cultured in the presence of either with E_2 alone or with E_2+P_4 were subjected to the analysis.

The quantitative immunofluorescence microscopy of the cells also demonstrated that either E_2 alone or E_2+P_4 significantly upregulated the *in situ* KGFR expression of the cells (Fig. 6A and B, respectively). The data show that the *in situ* KGFR expression level increases by 2- or 4-fold in the cells treated with E_2 or E_2+P_4 , respectively (Fig. 6C).

KGFR, ERK signal transduction pathway and cell behavior. Ishikawa cells that had been cultured for 48 h in the medium supplemented with E_2+P_4 were further incubated with rhKGF for 60 min, and then subjected to Western blot analysis. In Fig. 7, it is shown that the rhKGF administration markedly stimulated the phosphorylation of ERK proteins. The rhKGF-stimulated ERK phosphorylation was more prominent in the cells precultured with E_2+P_4 than in the control cells (data not shown).

We investigated the changes in cell behavior as a result of the activation of the signal transduction cascade of the cells. First, we investigated the modes of expression of secretory-phase-specific proteins of the endometrium, such as glycodefin and the leukemia inhibitory factor (LIF) in the cells. Ishikawa cells were cultured in the medium supplemented with E_2 or E_2+P_4 in combination with rhKGF, and the amount of glycodefin or LIF mRNA in the cells was measured using a real-time PCR method. The amount of glycodefin or LIF mRNA was negligible as a result of rhKGF administration (data not shown).

Next, we examined the growth potency of Ishikawa cells that were incubated in the culture medium containing E_2 or E_2+P_4 in combination with rhKGF. As shown in Fig. 8,

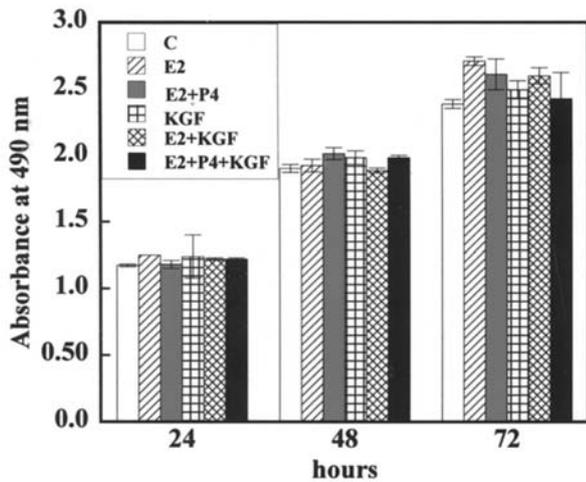


Figure 8. Growth of Ishikawa cells. Cell growth was determined by MTS-based assay and presented as mean \pm SEM (n=3).

rhKGF administration induced no change in the growth kinetics of the cells despite the presence of steroid hormones in the culture medium.

Because KGFR activation via ligand binding induced no changes in the synthesis of the characteristic proteins or the growth rate of the cells, we then examined the effects of KGFR activation on the adhesion activities of the cells to ECM proteins.

Ishikawa cells treated with E_2+P_4 to upregulate KGFR expression were further incubated with or without rhKGF, and the adhesion activities of the cells for fibronectin or the type-IV collagen substrate were examined. As shown in Fig. 9A, the number of cells that adhered to fibronectin increased in a concentration-dependent manner and the rhKGF administration to the reaction medium markedly up-regulated the adhesion activities of the cells. The adhesion activities of the cells for type-IV collagen were very low and rhKGF did not stimulate cell attachment to the collagen substrate (Fig. 9B).

Discussion

By the immunohistochemical analysis of the surgical specimens of a normal human endometrium from the non-cancerous patients with a regular menstrual cycle, both KGF and KGFR were detected more strongly in secretory-phase tissues than in proliferative-phase tissues. This result is consistent with that of a study of monkey endometrial tissues, which indicates that KGF expression is progesterone-dependent (8).

KGFR mRNA expression was reported to be highest in cell extracts of late-proliferative phase endometrial tissues as determined by biochemical analysis (25). In our study, KGFR staining was found to be weak or absent in the normal human endometrial tissues in the proliferative phase. The endometrial tissues that we obtained were mainly in the early-proliferative phase to the mid-proliferative phase and further examinations are required to confirm the result of biochemical analysis of KGFR expression in the human menstrual cycle.

Because KGF and KGFR are expressed in the tissues of low-grade endometrial cancer, their expressions are likely to be regulated by steroid hormones in low-grade tumors. On the other hand, because significant KGFR expression was observed in the tissues of patients with G3-grade endometrial cancer, in which no ER or PR expression was observed, it is probable that KGF and KGFR expression in high-grade endometrial cancer cells are regulated by other factors.

We also reported in this study that KGF is expressed not only in stromal cells but also in epithelial cells of the normal human endometrium in the secretory phase. Our report is in agreement with the report on describing KGF expression in the porcine endometrium (24). These reports suggest that KGF functions in either the autocrine or paracrine mode in the normal human endometrium. In our immunohistochemical examination of endometrial cancer tissues, the expression of not only KGFR but also KGF was detected in about two-thirds and nine-tenths of the specimens of endometrial cancers, respectively. A similar result on KGFR

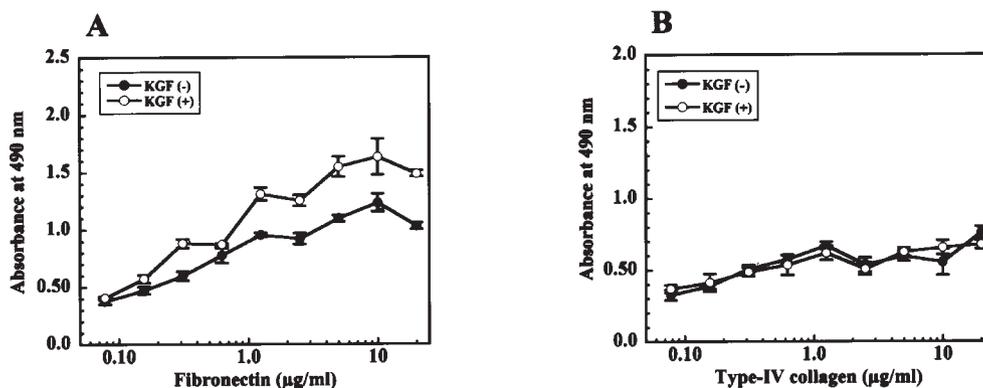


Figure 9. Effect of KGF on adhesion of Ishikawa cells to ECM substrates. (A) Cell adhesion to fibronectin. (B) Cell adhesion to type-IV collagen. Cells cultured for 48 h in medium supplemented with E_2+P_4 were subjected to attachment assay in the presence (open circle) or absence (solid circle) of rhKGF. The number of cells attached to fibronectin increased in a concentration-dependent manner. rhKGF administration to the reaction medium upregulated markedly the adhesion activities of the cells (A). The adhesion activities of the cells to type-IV collagen were very low and rhKGF did not stimulate cell attachment to the collagen substrate (B). Data are presented as mean \pm SEM (n=4). *p<0.05.

expression was reported previously (37); however, in that report, KGFR expression correlated with the advance of tumor stage and myometrial invasion. We showed in this study that there are no relationships between KGF or KGFR expression and clinicopathological features. The reason behind the differences in the relationship between KGFR expression and clinicopathological features is still unknown and further examinations are required to elucidate the differences.

The localizations of both KGF and KGFR were detected in the glandular epithelial cells of endometrial cancer tissues, and that, in most of cancer tissues, KGF-positive epithelial cells coexpressed KGFR. This finding suggests that KGF functions in the autocrine mode in endometrial cancers. KGF has been detected in the epithelial cells of other tissues and cancers, such as prostate hyperplasia, prostate cancer (38), and breast cancer (39,40) by immunohistochemical analysis and these results support our hypothesis.

Although the KGF-induced stimulation of endometrial cell growth has been reported (27), no growth promotion effect of KGF was detected on Ishikawa cells in our study. The growth promoting effect of KGF is known to vary according to cell type, as has been reported in the case of gastric (16,17) and breast cancers (23). The endometrial cell line examined in a previous study was derived from a moderately differentiated endometrial cancer, whereas the cell line Ishikawa was derived from a well-differentiated endometrial cancer that expresses both ER and PR. This suggests that the effect of KGF on cell proliferation varies in endometrial cancer according to the degree of differentiation.

The KGFR expression of Ishikawa cells was slightly upregulated by E₂ and was markedly enhanced by the coadministration of P₄. The data support our hypothesis that KGFR expression is regulated by steroid hormones in human endometrial cells.

In this study, we successfully applied a quantitative image analytic method to the determination of the *in situ* KGFR expression of the cells (32). The digital fluorescence images of each cell were acquired using a confocal laser microscope system and analyzed using an image analysis software. The results correlated well with the data obtained by Western blot analysis. Because the procedure is simple and sensitive and requires fewer cells than conventional assay methods, it is applicable to cells that are difficult to grow or in situations where a sufficient number of cells can not be obtained for the assay, such as primary-cultured cells.

The histological grades of the tumor are closely associated with the expression of steroid hormone receptors in endometrioid adenocarcinoma. Previous studies have shown that growth factors are involved in modulating the differentiation of cancer cells, and that KGF is associated with tumor cell differentiation in several types of cancer (18-21). KGF is also a modulator of steroid hormones and the receptors of organs (6-8) and cancers derived from them (22).

Fibronectin is one of the major ECM components in endometrial stroma (41) and ECM regulates cell behavior and differentiation, including those of tumor cells (42). We demonstrated that KGF administration into cells, in which KGFR expression has been upregulated by the steroid hormones enhances cell adhesion to fibronectin.

ERK1/2 phosphorylation is the key step in the signal transduction pathway that causes changes in the modes of cell growth and in cell behavior. In this study, we demonstrated that KGF induced ERK1/2 phosphorylation of endometrial cancer cells. It is consistent with previous reports on other cell lines (e.g., endometrial cancer cell line, porcine trophoblast cell line and colorectal cancer cell line), indicating the involvement of the MAPK pathway in the KGF-KGFR signal transduction pathway. We demonstrated that KGF-KGFR interactions induce the activation of the ERK1/2 signal transduction pathway, which result in the enhanced adhesion of cells to the fibronectin substrate implying that KGF and KGFR are involved in modulating the development of endometrial cancer cells by enhancing their adhesion to ECM, in association with steroid hormones and their receptors.

The interactions of cells to ECM cause changes in cell behavior, such as the induction of cell differentiation and the cessation of cell growth (43). Progesterone administration into endometrial cancers may, therefore, prevent cancer cell growth in accordance with KGF-KGFR interactions and the enhanced adhesion of cells to ECM.

In conclusion, the KGFR expression of endometrial cancer cells is upregulated by E₂ and markedly enhanced by P₄ coadministration. KGF-KGFR interactions followed by the activation of the ERK1/2 signal transduction pathway of the endometrial cells seem to be implicated in the regulation of cell behavior, particularly in the cell adhesion to ECM. Further studies are required to elucidate the role of KGF-KGFR interaction in cooperation with steroid hormones in the development of endometrial tumors and tissues.

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