Research Article

Autocrine stimulation of IGF-1 in estrogen-induced growth of endometrial carcinoma cells: Involvement of the MAPK pathway followed by up-regulation of cyclin D1 and cyclin E

Running title: IGF-1 and ERK1/2 in endometrial carcinoma

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ABSTRACT

To examine estrogen-induced growth mechanisms of endometrial carcinoma, we investigated the estrogen-induced activation of the mitogen-activated protein kinase (MAPK) pathway and cell cycle regulators. Estradiol (E2) treatment at concentrations of 10^{-8} M and 10^{-6} M to estrogen receptor (ER)-positive endometrial carcinoma Ishikawa cells for 24 hours resulted in increased cell proliferation by 20% and 28%, respectively. The E2-induced proliferation was associated with activation of extracellular signal-regulated kinase (ERK)1/2 and up-regulation of cyclin D1 and E, which were suppressed by the addition of a MEK inhibitor (U0126) or an ER antagonist (ICI182,780). Then, our screening for estrogen-inducible growth factors identified that insulin-like growth factor (IGF)-1 was up-regulated remarkably by E2. Immunoprecipitation using conditioned medium of Ishikawa cells after E2 treatment confirmed the E2-induced secretion of IGF-1 protein. Treatment with recombinant IGF-1 stimulated cell proliferation in a dose-dependent fashion, in association with ERK1/2 phosphorylation and up-regulation of cyclin D1 and E. These IGF-1-induced responses were suppressed by treatment with MEK inhibitor or anti-IGF-1 receptor antibody. Immunohistochemical staining confirmed the expression of activated ERK1/2 in normal proliferative phase endometria and endometrial carcinomas, indicating the involvement of this pathway in actively proliferating endometrial tissues in vivo. These findings suggest that E2-induced proliferation of endometrial carcinoma cells is mediated by the ERK1/2 pathway via autocrine stimulation of IGF-1.
INTRODUCTION

Endometrial carcinoma is one of the carcinomas known to have a clear relationship between hormonal stimulation and growth regulation (Murphy, 1994). Estrogen stimulates the proliferation of normal and malignant endometrial cells expressing estrogen receptors (ER) (Strauss III & Coutifaris 1999), via various mechanisms including increased ER expression (Granziani et al, 2003), up-regulation of growth factors and/or their receptors (Hana & Murphy, 1994; Kleinman et al, 1995), and induction of proto-oncogene c-fos (Singleton et al, 2003). However, the intracellular molecular machineries underlying the regulation of estrogen-induced cell proliferation are still not fully elucidated.

Cell proliferation is generally evoked by extra-cellular growth signals such as growth factors and cytokines. After the binding of growth factors to the respective receptors, the conserved Ras/Raf/MEK/ERK pathway acts to relay growth stimuli from activated Ras proteins via MAPK/ERK kinase 1/2 (MEK1/2) to p42/44 MAP kinase or extracellular signal-regulated kinase (ERK)1/2 (Kolch, 2000; Peyssonnaux & Eychene, 2001). The ERK1/2 proteins are serine/threonine kinases, that are rapidly activated by phosphorylation and translocated to the nucleus, which appears to be necessary for cell proliferation (Pages et al, 1993). ERK1/2 activation eventually induces the expression of cell cycle regulators such as cyclins, and cyclin-dependent kinases (cdks), which have essential roles to propel cell cycle progression (Sherr, 1995; Nurse, 1997). We have previously reported the cyclical expression of cyclin and cdks in normal cycling endometrial tissues (Shiozawa et al, 1996), as well as the overexpression of these molecules in endometrial carcinoma (Li et al, 1996; Shih et al, 2003), suggesting the involvement of cell cycle regulators in the growth of normal endometrium and in the acquisition of aggressive growth potential of endometrial carcinoma. We also revealed the estrogen-induced transcriptional activation of cyclin D1 via the induction of c-Jun in
normal endometrial cells (Shiozawa et al, 2004).

Nevertheless, the involvement of cyclins in estrogen-induced cell proliferation in ER-positive endometrial carcinoma cells has not been elucidated. The role of the MAPK pathway in estrogen-induced cell proliferation and cell cycle regulator activation also remains undetermined. Therefore in this study, we investigated the involvement of the MAPK pathway and the activation of cell cycle regulators in E2-induced cell proliferation of endometrial carcinoma cells. Then, we screened candidates of growth factors that can be induced by estrogen. Since we identified the up-regulation of insulin-like growth factor (IGF)-1 by estrogen, we focused on the involvement of IGF-1 as an autocrine factor in estrogen-dependent growth of endometrial carcinoma cells.

**Materials & Methods**

**Cell culture**

An ER-positive endometrial carcinoma cell line, Ishikawa, was kindly provided by Dr. Nishida at Kasumigaura Medical Center (Tsuchiura, Ibaragi, Japan). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) with 15% FCS (Biological Industries, Haemek, Israel) and 1% antibiotic-antimycotic solution (Invitrogen). Incubation was carried out at 37°C under 5% CO$_2$ in air. An ER antagonist, ICI182,780, and a MEK inhibitor, U0126, were purchased from Sigma (St. Louis, MO, USA). Anti-IGF-1 receptor antibody (Ab-3), which can neutralize the cell surface receptor, was purchased from Oncogene Research Products (San Diego, CA). Before the E2 or IGF-1 stimulation experiments, Ishikawa cells were pretreated with rapamycin (500 ng/ml) (Invitrogen) in serum-starved medium (FCS 0.1%) for 24 hours to synchronize them to the G1 phase of the cell cycle. Then the cells were cultured for 24 hours without rapamycin in the same medium to wash out the effect of the agent. ICI182,780, U0126, or anti-IGF-1 receptor antibody was added 1 hour before the
addition of E2 or IGF-1.

**Western blotting**

Phosphorylation of ERK1/2 was examined, using a specific antibody that recognizes phosphorylated ERK1/2 in Ishikawa cells treated with various reagents by western blotting. Cells were lysed in a lysis buffer: 50 mmol/L Tris-HCl (pH 8.0), 0.25 mol/L NaCl, 0.5% NP-40, 1 mmol/L PMSF (Sigma), 1 μg/ml aprotinin (Roche Diagnostics, Indianapolis, IN), 1 μg/ml leupeptin (Roche Diagnostics), and 20 μg/ml TPCK (Roche Diagnostics). The lysates were centrifuged at 13,000 x g for 20 minutes at 4°C and the supernatants were stored at -80°C. Extracts equivalent to 50 μg of total protein were separated by SDS-polyacrylamide gel electrophoresis (7.5 or 10% acrylamide) and transferred onto nitrocellulose membranes (Hybond TM-C Super; Amersham Biosciences Inc. Uppsala, Sweden). The membranes were blocked in TBST (0.2 mol/L NaCl, 10 mmol/L Tris, pH 7.4, 0.2% Tween-20) containing 5% nonfat dry milk for 1 hour, then incubated with mouse monoclonal antibodies against phosphorylated ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), cyclin D1 (Progen, Heidelberg, Germany), IGF-1 receptor (Oncogene Research Product), ERα, cyclin E, β-actin (Santa Cruz, St Louis, MO), or rabbit polyclonal antibodies against ERK1/2 (Cell Signaling Technology) in TBST containing 5% nonfat dry milk overnight at 4°C. The membranes were then incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences Inc.) in TBST containing 2% nonfat dry milk. Bound antibodies were detected with an enhanced chemiluminescence system (Amersham Biosciences Inc.). Each experiment was repeated three times. The density of the bands on the gel was quantified by densitometric analysis using a Quantity One Scan System (ATTO, Tokyo, Japan). The mean values of absorbance relative to β-actin were indicated in Figs. 1c, 2b and 2c.
**Total RNA Extraction**

Total RNA was extracted by the acid guanidinium-phenol-chloroform method as described previously (Shiozawa et al., 2004). One microgram of total RNA was treated with 1 U/10 µl DNase I (Life Technologies, Gaithersburg, MD).

**RT-PCR**

Estrogen- or IGF-1-induced mRNA expression of cyclin D1, cyclin E and IGF-1 was evaluated using a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. RT was performed using an RNA PCR Kit (Takara Shuzo, Otsu, Japan), with a 1 µg RNA sample being added to 20 µl of a reaction mixture consisting of 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L dNTP mixture, 1 U/µl RNase inhibitor, 0.25 U/µl avian myeloblastosis virus-derived reverse transcriptase, and 0.125 µmol/L oligo d(T)-adaptor primer. Using a thermal cycler (Gene Amp PCR System 2400-R; PerkinElmer, Norwalk, CT), the reaction mixture was incubated at 42°C for 30 minutes, heated to 99°C for 5 minutes, and then cooled down to 5°C for 5 minutes. One microliter of the RT products, containing 50 ng reverse-transcribed total RNA, was amplified by adding 20 µl of a PCR reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 U/100 µl of TaKaRa TaqDNA polymerase, with 0.2 µmol/L of a set of 21- to 24-mer oligonucleotide primers for cyclin D1, cyclin E, IGF-1, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Primers were synthesized to encompass a specific segment of the cDNA sequence of cyclin D1(Kaneuchi et al, 2003) (sense, 5'-TCTAAGATGACCATC' and antisense, 5'-GCGTACAGGACGAGTTGTT-3'), cyclin E (Takano et al, 2000) (sense, 5'-ATGGTATACCTGCTGCTCGCC-3' and antisense,
5'-AGAACGTGGAGCAGGCGCGCAACT-3'), IGF-1 (Hana & Murphy, 1994) (sense, 5'-CAGTGTGAGCCAGTTTCTAAATCTAC-3' and antisense, 5'-CTGTGGGGCTTGTTGAAATAA-3', or of GAPDH (sense, 5'-ACGACCATTTGTCAAGCTC-3' and antisense 5'-GGTCTACATGGCAACTGTGA-3'). The corresponding cDNA fragments were denatured at 94°C for 30 seconds, annealed at 55°C for 1 minute, and extended at 72°C for 1 minute. After 30 cycles of amplification, the PCR products were analyzed on a 2% agarose gel, and the bands were visualized using ethidium bromide by exposure on a UV transilluminator. Each experiment was performed three times independently. Densitometric analysis was performed using a Quantity One Scan System (ATTO). Gene expression was represented by the relative yield of PCR product from the target sequence to that from the GAPDH gene, because the stable expression of the GAPDH gene under various conditions has been reported (Gorzelniak et al 2001; Silver et al 2006; Murthi et al 2008). The mean values of results were indicated in Figs. 2a and 3c.

Cell cycle analysis

The cell cycle was analysed in E2-treated Ishikawa cells, using a cycleTEST (Becton Dickinson, Franklin Lakes, NJ) and FACScan flow cytometry (Becton Dickinson).

Immunoprecipitation

Because the concentration of secreted IGF-1 protein in the culture medium was low to detect using western blotting, IGF-1 protein secreted into the medium of E2-stimulated Ishikawa cells was examined by immunoprecipitation using a Protein A Immunoprecipitation Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The conditioned medium was collected from Ishikawa cells treated with $10^{-6}$M E2 at 6, 12, and 24 hours after E2 addition, or from those cells treated with $10^{-5}$M ICI182,780. After
preclear, 1 ml of the conditioned medium was incubated with anti-IGF-1 antibody (BioCarta, San Diego, CA) overnight at 4°C and IGF-1 precipitates were collected for 1 hour on protein-A agarose beads. After washing with lysis buffer three times, precipitates were resuspended in a Laemmli SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (15% acrylamide). The resolved immunoprecipitates were electrotransferred onto a nitrocellulose membrane and detected by immunoblotting using an antibody against IGF-1.

**WST-1 assay**

Ishikawa cells were cultured in 96-well plates, with various concentration of E2. Cell proliferation was evaluated with Cell Proliferation Reagent WST-1 (Roche, Basel, Switzerland) according to manufacture’s protocol. Each experiment was repeated three times.

**Immunostaining of ERK1/2 and phosphorylated ERK1/2**

**Histological materials.** Thirty normal endometrial tissue specimens (proliferative phase: 9 cases; secretory phase: 21 cases), and 48 endometrial carcinoma specimens (grade 1: 27 cases, grade 2: 11 cases, grade 3: 10 cases) were subjected to the study. These tissues were used with the approval of the Ethics Committee at Shinshu University School of Medicine after obtaining written consent from the patients.

**Staining procedures.** Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections by the streptavidin-biotin-peroxidase complex method using a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). The primary antibodies, p44/42 MAP kinase Antibody and Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) Monoclonal Antibody (Cell Signaling Technology) were used at a dilution of 1:100. The following procedure was described previously (Shih et al, 2003).
Interpretation of immunohistochemical staining. Positive staining was identified by brown-colored products in the nucleus or in the cytoplasm. All the control slides yielded negative staining. Nuclear staining in each section was evaluated by the percentage of stained cells in 500 arbitrarily selected cells and described as “positivity index (PI)” (maximum score: 100). The PI for cytoplasmic staining was calculated by multiplying the intensity of cytoplasmic staining [strongly positive (=2), weakly positive(=1)] with the matched percentage of nuclear-positive cells among 500 cells (maximum score: 200).

Statistical analysis
Statistical analysis was performed by the Mann-Whitney test (Statview System, Macintosh). Differences were considered significant when p < 0.05.

RESULT
E2 stimulates the proliferation of Ishikawa cells in a dose-dependent manner
To evaluate the effect of E2 on the proliferation of endometrial carcinoma Ishikawa cells, E2 was added after incubation in serum-starved conditions for 48 hours, and viable cells were measured using a WST-1 assay 24 hours after E2 addition. The result indicated that the E2 treatment at a concentration of 10^{-8}M and 10^{-6}M increased the cell proliferation compare to the control by 20% and 28%, respectively (p<0.05, Fig. 1a). Cell cycle analysis showed that the S-phase fraction of E2-untreated and E2-treated cells after 24 hours was 7.6% and 12.7%, respectively (Fig. 1b). Western blot analysis demonstrated that a weak, baseline expression of cyclin D1 was observed before the addition of E2, and the expression of cyclin D1 protein was then elevated approximately 6 hours after E2 treatment. RT-PCR revealed that mRNA expression of cyclin D1 increased 6 hours after E2 stimulation, with a peak at 12 hours after E2 treatment. The
expression of cyclin E protein and mRNA appeared 6 hours after E2 stimulation and then increased thereafter (Fig. 1b). These findings indicate that E2 stimulates cell cycle progression with transcriptional activation of cyclin D1 and E.

**E2-induced activation of ERK1/2 in Ishikawa cells**

To investigate the involvement of the p44/p42MAPK (ERK1/2) pathway in E2-induced proliferation of Ishikawa cells, we examined the activation of this pathway by western blotting using a specific antibody against activated (phosphorylated) ERK1/2 (pERK1/2). E2 treatment induced the activation of ERK1/2 2 hours after stimulation, which reached a peak 24 hours after stimulation. The expression of non-active ERK1/2 remained unchanged throughout the experiment (Fig. 1b). When Ishikawa cells were treated with ER antagonist (ICI182,780) or a MEK inhibitor (U0126), the E2-induced ERK1/2 phosphorylation 6 hours after the addition of E2 was significantly reduced by 78% (P<0.05) and 56% (P<0.05), respectively (Fig. 1c). These findings suggest that E2 induced activation of the MAPK pathway is involved in E2-induced proliferation of Ishikawa cells.

**E2-induced ERK1/2 activation is mediated through IGF-1 synthesis and secretion**

Because the activation of the MAPK pathway has been thought to be evoked by growth factors via their receptors, we performed a preliminary screening of several growth factors including IGF, EGF, and TGF-α, to examine whether the expression of these growth factors could be induced by estrogen in Ishikawa cells. Consequently, we identified IGF-1 to exhibit the most marked estrogen-dependency in terms of the degree of estrogen-induced up-regulation and ER antagonist-induced down-regulation. Therefore, we focused on the involvement of IGF-1 in estrogen-dependent growth of endometrial carcinoma cells. RT-PCR revealed that E2 induced IGF-1 mRNA
expression peaked 6 hours after E2 addition and then disappeared at 24 hours, and ICI182, 780 pretreatment significantly decreased the E2-induced IGF-1 mRNA expression by 36% (Fig. 2a). To identify the engagement of IGF-1 in the E2-induced growth mechanism, the secretion of IGF-1 into the culture medium in E2-treated Ishikawa cells was then examined. The conditioned medium after E2 with/without ICI182, 780 treatment was collected and immunoprecipitated with anti-IGF-1 antibodies. The following immunoblotting revealed that the IGF-1 protein in the conditioned medium peaked 12 hours after E2 treatment, and ICI182, 780 treatment reduced the E2-induced IGF-1 secretion 6 hours after addition by 49% (Fig. 2a). These findings indicate that E2 induces IGF-1 synthesis and secretion into the culture medium in Ishikawa cells, suggesting a possible presence of an IGF-1-mediated autocrine mechanism.

The role of IGF-1 in E2-induced ERK1/2 activation was then investigated. Western blotting confirmed that Ishikawa cells expressed IGF-1 receptors (Fig. 2b), and recombinant IGF-1 induced a rapid and transient ERK1/2 phosphorylation that began 15 minutes after IGF-1 addition with significant differences (P<0.05), and disappeared by 1 hour (Fig. 2b). To further confirm the contribution of IGF-1 in E2-induced ERK1/2 activation, we examined the effect of anti-IGF-1 receptor neutralization antibody. When cells were treated with anti-IGF-1 receptor antibody, a partial inhibition of E2-induced ERK1/2 activation (anti-IGF-1 receptor antibody: 50 ng/ml, 17% reduction; 500 ng/ml, 57% reduction, P<0.05) was observed 6 hours after the addition of E2 (Fig. 2c). These results indicated that the E2-induced ERK1/2 activation was mediated by an autocrine stimulation of IGF-1 in Ishikawa cells.

**IGF-1 stimulates the proliferation of Ishikawa cells in a dose-dependent manner**

Since E2 treatment activated the ERK1/2 pathway via an autocrine system of IGF-1, we
examined whether IGF-1 can enhance the proliferation of Ishikawa cells. A WST-1 assay revealed that the addition of recombinant IGF-1 at concentrations of 10 ng/ml and 100 ng/ml for 24 hours increased cell proliferation compared to the control by 19% and 32%, respectively, (p<0.05) (Fig. 3a).

**IGF-1 induces cyclin D1 and E via activation of the ERK1/2 pathway**

To investigate the molecular mechanism of IGF-1-induced cell proliferation, we examined the mRNA expression of cyclin D1 and E after IGF-1 treatment. RT-PCR revealed that mRNA expression of cyclin D1 increased 2 hours after IGF-1 stimulation, with a peak at 12 hours after E2 treatment. The expression of cyclin E mRNA increased from 4 hours after IGF-1 stimulation (Fig. 3b). To investigate whether the activation of ERK1/2 was involved in the IGF-1-induced expression of cyclin D1 and E, we examined the effect of U0126. RT-PCR revealed that treatment with U0126 significantly reduced the IGF-1-induced cyclin D1 and E expression 12 hours after the addition of IGF-1 by 37 % for cyclin D1 (P<0.05) and by 42 % for cyclin E (P<0.05, Fig. 3c). These findings suggest that IGF-1 induced cyclin D1 and E via activation of the ERK1/2 pathway.

**E2-induced cell proliferation is dependent on the ERK1/2 pathway via an autocrine mechanism of IGF-1**

ERK1/2 dependency in E2- or IGF-1-induced cell proliferation was examined using a MEK inhibitor and an antibody against the IGF-1 receptor in Ishikawa cells. E2 treatment for 24 hours enhanced the proliferation of Ishikawa cells by 20% (lane 2, Fig. 4) compared to the control with a significant difference (P=0.011), and the E2-induced proliferation was blocked by the use of ICI182,780 (lane 3) or U0126 (lane 4). Anti-IGF-1 receptor antibody also inhibited the E2-induced cell proliferation (lane 5
IGF-1 treatment increased the proliferation of Ishikawa cells by 10% compared to the control after 24 hours (p=0.039)(lane 7). Treatment with ICI182,780 slightly decreased the IGF-1-induced cell proliferation, but the difference was not significant (lane 8). U0126 inhibited the effect of IGF-1 on cell proliferation (lane 9). Collectively, these findings suggest that E2-stimulated proliferation of Ishikawa cells is mediated by ERK1/2 activation via autocrine stimulation by IGF-1.

**Immunostaining for ERK1/2 and phosphorylated ERK1/2 in normal endometrium and endometrial carcinoma**

To investigate the involvement of p44/p42 MAPK (ERK1/2) in the growth of endometrial tissues, the immunohistochemical expression of ERK1/2 and its activation (phosphorylation) were examined in normal and malignant endometrial tissues. The immunoreactivity for ERK1/2 and phosphorylated (p)ERK1/2 was observed both in the cytoplasm and nucleus. In normal endometrial glandular cells, the expression of cytoplasmic ERK1/2 was observed both in the proliferative and secretory phases, whereas that of pERK1/2 was more abundant in the proliferative phase than in the secretory phase (p<0.0001 in the nucleus, p=0.0018 in the cytoplasm, Fig. 5). The expression of ERK1/2 increased in all cases of endometrial carcinoma examined, and that of pERK1/2 increased significantly compared to that in the secretory phase endometrium (vs. grade1: p<0.0001 in the nucleus, p=0.0001 in the cytoplasm; vs. grade2: p=0.04 in the nucleus, p=0.0084 in the cytoplasm; vs. grade3: p=0.003 in the nucleus, p=0.0019 in the cytoplasm, Fig. 5). However, there was no significant relationship among the pathological grades of endometrial carcinoma. These findings indicated that the expression of pERK1/2 increased in actively proliferating tissues such as normal proliferative phase endometrium and endometrial carcinoma.
DISCUSSION

The present study demonstrated that E2 induced the up-regulation of IGF-1 expression and significantly enhanced cell proliferation of endometrial carcinoma Ishikawa cells. In addition, we have also shown that, 1) E2 induced the production and secretion of IGF-1, 2) Ishikawa cells expressed IGF-1 receptors, 3) treatment with recombinant IGF-1 stimulated the proliferation of Ishikawa cells, and 4) treatment with anti-IGF-1 receptor neutralization antibody effectively inhibited both E2- and IGF-1-induced growth of Ishikawa cells. These findings clearly demonstrated the presence of an autocrine loop of IGF-1 in estrogen-induced cell proliferation of Ishikawa cells.

Previous studies using primary cultures of endometrial carcinoma cells (Reynolds et al., 1996) and in endometrial carcinoma cell lines (Reynolds et al., 1998; Takeda et al., 1996) suggested the possible existence of IGF-1- or TGF-α-mediated autocrine loops; however, the involvement of estrogen stimulation in the autocrine system was not reported. Although Hana et al. reported an increase in IGF-1 mRNA expression after estrogen treatment, the secretion of IGF-1 protein was not examined (Hana & Murphy, 1994). In breast carcinoma MCF-7 cells, an estrogen-related autocrine loop has been reported to stimulate cell proliferation via HER-2 and its ligand, hereglin (Keshamouni et al., 2002). Therefore, to our knowledge, this is the first report showing the presence of an E2-induced entire autocrine loop mediated by IGF-1 in the cell proliferation of endometrial carcinoma cells. The E2 concentration used in the present study (10^{-6}M) is higher than that of physiological condition, however, elevated E2 concentrations in the tumor microenvironment several times higher than that of the physiological levels have been reported (Ito et al., 2007). IGF-1-induced cell proliferation was half of that of E2-induced in this study, suggesting that the presence of other E2-induced growth mechanisms than IGF-1 can not fully be denied.

The molecular mechanism of E2-induced transcription of IGF-1 is largely
unknown. In the present study, we observed an increase in the expression of IGF-1 mRNA 6 hours after E2 treatment, and this may be consistent with that observed in E2-administered rat uterus (Murphy & Ghahary, 1990). More recently, the expression of IGF-1 was reportedly induced by cyclic AMP-dependent kinase via activation of HS3D located in the IGF-1 gene promoter and a transcription factor, CCAAT/enhancer-binding protein δ (C/EBP δ) in rat osteoblasts (Billiard et al., 2001). Kleinman et al. reported that E2 sensitized the effects of IGF-1 by elevating receptor levels and also decreasing the expression of IGF-binding protein (IGFBP)-3, which potentially has an inhibitory function for IGF-1 (Kleinman et al., 1995). Thus, further studies are needed to clarify the molecular link between estrogen stimulation and up-regulation of IGF-1.

Our study also showed that the classical MAPK pathway, ERK1/2, is involved in estrogen-induced cell proliferation via the IGF-1 autocrine loop in endometrial carcinoma Ishikawa cells. Treatment with recombinant IGF-1 also induced ERK1/2 phosphorylation and cell proliferation of Ishikawa cells, which were suppressed by a MEK inhibitor, U0126. The activation of ERK1/2 peaked from 15 to 30 minutes after the IGF-1 stimulation. Earlier studies using fibroblasts indicated that Ras-MEK-ERK1/2 activation is important in IGF-1-related cell proliferation (Lu K & Campisi, 1992; Jhun et al., 1994; Sasaoka et al., 1996). In experiments using gynecological cancer cell lines, such as a cervical carcinoma cell line (SiHa) and an ovarian carcinoma cell line (OVCR3), IGF-1 was reported to transmit the signal possibly via the ERK1/2 pathway (Shen et al., 2004). However, in breast carcinoma MCF-7 cells, the mitogenic signal of IGF-1 did not pass through the ERK1/2 pathway but through the phosphatidylinositol 3-kinase pathway (Dufourny et al., 1997). In endometrial carcinoma cells, the putative growth factors relaying the signals to the ERK1/2 pathway reported to date were EGF (Tang et al., 2006), keratinocyte growth factor, and fibroblast growth factor-10 (Taniguchi et al., 2003). Accordingly, we provide the first report of a signal transduction...
pathway between E2 stimulation, subsequent IGF-1 stimulation, ERK1/2 activation, and cyclins up-regulation, which leads to the cell proliferation of endometrial carcinoma cells.

The current study demonstrated the time-course between E2 stimulation, IGF-1 stimulation, ERK1/2 activation, and up-regulation of cyclin D1 and cyclin E in Ishikawa cells. E2-induced expression of IGF-1 was observed by 6 hours after E2 stimulation, and IGF-1-induced ERK1/2 activation was observed as early as 15 minutes. The expression pattern of E2-induced cyclin D1 mRNA paralleled that of cyclin D1 protein. Since the significant elevation of cyclin D1 protein expression occurred 6 hours after E2 stimulation, a signal transduction pathway from IGF-1 and cyclin D1 via ERK1/2 seems to be plausible. However, since a weak up-regulation of cyclin D1 mRNA was observed 2 hours after E2 stimulation, another immediate pathway between E2 stimulation and cyclin D1 might also be present, as we demonstrated previously in normal endometrial cells (Shiozawa et al., 2004). Although a weak baseline expression of cyclin E mRNA was observed until 4 hours after E2 stimulation, the expression pattern of E2-induced cyclin E mRNA also correlated with that of cyclin E protein. In our study, up-regulation of cyclin E was also observed 6 hours after E2 stimulation. The molecular mechanism of cyclin E up-regulation remains undetermined; however, the cyclin E gene has multiple E2F-1 binding sites, and E2F-1 has been reported to activate the expression of cyclin (DeGregori et al., 1995; Ohtani et al., 1995). ERK1/2-induced up-regulation of E2F-1 has also been reported (Berkovich & Ginsberg, 2001; Pintus et al., 2003). Collectively, the link between ERK1/2 activation and up-regulation of cyclin D1 and cyclin E may be mediated by various factors.

Finally, we have demonstrated immunohistochemically that the expression of pERK1/2 was increased in both normal proliferative phase endometrium and endometrial carcinoma. The increased expression of pERK1/2 in endometrial carcinoma
observed in the present study was similar with a recent study (Mizumoto et al, 2007). This is not surprising, because the in vivo growth activity of normal endometrial glandular cells in the proliferative phase is as vigorous as that in endometrial carcinoma cells (Shiozawa et al, 1996; Shih et al, 2003). Thus, activation of the ERK1/2 pathway seems to be functioning not only in endometrial carcinoma cells but also in normal endometrial glandular cells in vivo. Interestingly, the E2-induced ERK1/2 activation, as observed in endometrial carcinoma Ishikawa cells, was not observed in cultured normal endometrial glandular cells in vitro (data not shown). Such a discrepancy may be due to the absence of endometrial stroma in the in vitro experiment culturing purified glandular cells (Shiozawa et al, 2001). Pierro E et al. reported that normal endometrial stromal cells secrete IGF-1 as a paracrine factor that stimulates the proliferation of neighboring glandular cells, indicating the presence of epithelial-stromal interactions via IGF-1 in normal endometrium (Pierro et al, 2001). This is consistent with the observation that the activity of cell proliferation of normal glandular cells in vitro is very low when cultured in the absence of stromal cells. All of these findings strongly suggest that endometrial carcinoma cells have acquired the capacity for cell growth independent from endometrial stroma, through having the autocrine loop of IGF-1 stimulation.

In conclusion, our study demonstrated that activation of the ERK1/2 pathway plays an important role in E2-induced growth of endometrial carcinoma cells, and its activation is mediated by IGF-1 autocrine stimulation. This result suggests that IGF-1 and its receptor can be a candidate for molecular targeting for endometrial carcinoma therapy.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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overexpression and lack of p27 correlate positively and cyclin E inversely with a poor


FIGURE LEGENDS

Fig. 1. Estrogen-dependent proliferation, ERK1/2 activation, and cyclin expression in Ishikawa cells. a: Ishikawa cells were serum starved for 48 hours as described in Experimental procedures, and E2 was added. Twenty-four hours after E2 addition, viable cells were measured using a WST-1 assay. E2 treatment increased the proliferation of Ishikawa cells in a dose-dependent manner with significant differences. b: E2 was added to Ishikawa cells after serum starvation and the expression of cyclins and ERK1/2 was examined using western blotting and RT-PCR. The S-phase faction was examined using flow cytometry. E2 (10^{-6}M) treatment increased the S-phase fraction, and induced the up-regulation of cyclin D1, which was followed by the expression of cyclin E and ERK1/2 activation. c: Effect of an ER antagonist (ICI182,780) and a MEK inhibitor (U0126) on the activation of ERK1/2. Ishikawa cells were treated with ICI182,780 (10^{-5}M) or U0126 (10^{-5}M) for 6 hours after E2 treatment, and ERK1/2 phosphorylation was evaluated using western blotting. Treatment with ICI182,780 or U0126 suppressed the expression of pERK with significant differences. *: vs. control, P<0.05, **; vs. E2 alone, P<0.05

Fig. 2. Estrogen-induced expression and secretion of IGF-1, and IGF-1 induced ERK1/2 activation in Ishikawa cells. a: E2 treatment significantly induced the expression of IGF-1 mRNA with, with a peak at 6 hours after treatment. Immunoprecipitation using the conditioned medium of E2-treated Ishikawa cells indicated the secretion of IGF-1 protein, which peaked 12 hours after E2 addition. These reactions were suppressed ICI182,780 by 36 % for IGF-1 mRNA (P<0.05) and by 49 % for IGF-1 protein. *: vs. control, P<0.05, **; vs. E2 alone, P<0.05 b: Recombinant IGF-1 (10 ng/ml) significantly induced the ERK1/2 activation of Ishikawa cells from 15 to 30 minutes after the addition. *: vs. control, P<0.05 c: E2-induced ERK1/2 activation was
suppressed by anti-IGF-1 receptor antibody dose-dependently (17% reduction for 50 ng/ml, 57% reduction for 500 ng/ml, P<0.05) 6 hours after E2 stimulation. *: vs. control, P<0.05, **; vs. E2 alone, P<0.05

**Fig. 3.** IGF-induced proliferation and up-regulation of cyclin in Ishikawa cells. **a:** After serum starvation of Ishikawa cells for 48 hours, recombinant IGF was added and the viable cells were counted using a WST-1 assay. IGF-1 stimulated the proliferation of Ishikawa cells in a dose-dependent manner with significant differences. **b:** IGF-1 induced a stepwise up-regulation of cyclin D1 mRNA with a peak 12 hours after the addition, followed by that of cyclin E mRNA. **c:** The IGF-1-induced up-regulation of cyclin D1 and E 12 hours after addition was significantly suppressed by U0126 by 37% and 42%, respectively. *: vs. control, P<0.05, **; vs. E2 alone, P<0.05

**Fig. 4.** Effect of ICI182,780, U0126, and anti-IGF-1 receptor antibody on E2- or IGF-1-induced proliferation of Ishikawa cells. Ishikawa cells were serum starved for 48 hours, and E2 (10^{-6}M) or IGF-1 (10 ng/ml) was added. Viable cells were measured 24 hours after the addition using a WST-1 assay. ICI182,780 suppressed the E2-induced proliferation of Ishikawa cells (lane 3) with a significant difference, but not IGF-1-induced proliferation (lane 8). U0126 suppressed both E2- (lane 4) and IGF-1-(lane 9) induced proliferation. Anti-IGF-1 receptor antibody suppressed E2-induced proliferation (lane 5, 6).

**Fig. 5.** Immunohistochemical detection of ERK1/2 and phosphorylated (p)ERK in endometrial tissues. The expression of ERK (**a, c, e, g**) and pERK (**b, d, f, h**) in normal endometrium (**a-d**) and endometrial carcinoma (**e-h**). **a** and **b**, **c** and **d**, **e** and **f**, and **g** and **h** are serial sections. **i** shows a graphical representation of the results. The
expression of pERK both in the cytoplasm and nucleus was increased in the normal proliferative phase endometrium and endometrial carcinoma compared to that in the secretory phase endometria (P<0.05). There was no significant difference in the expression of pERK between different histological grades of endometrial carcinoma. The expression of cytoplasmic ERK did not show marked changes between histological groups. Pro.; proliferative phase, Sec. secretory phase. *: significantly different from that of the normal secretory phase endometrium (P<0.05).
Figure 2

(a) IGF-1 mRNA

(b) IGF-1 IR

285x190mm (96 x 96 DPI)
Figure 4

285x190mm (96 x 96 DPI)
Figure 5

ERK1/2

a

b

pERK1/2

c

d

e

f

g

h

i

Positivity index

285x190mm (96 x 96 DPI)