Autocrine stimulation of IGF1 in estrogen-induced growth of endometrial carcinoma cells: involvement of the mitogen-activated protein kinase pathway followed by up-regulation of cyclin D1 and cyclin E

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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 (E_2) treatment at concentrations of 10^{-8} M and 10^{-6} M to estrogen receptor (ER)-positive endometrial carcinoma Ishikawa cells for 24 h resulted in increased cell proliferation by 20% and 28% respectively. The E_2 -induced proliferation was associated with the activation of extracellular signal-regulated kinase (MAPK)3/1 and up-regulation of cyclin D1 and E, which were suppressed by the addition of an MAP2K inhibitor (U0126) or an ER antagonist (ICI 182 780). Then, our screening for estrogen-inducible growth factors identified that IGF1 was up-regulated remarkably by E_2 . Immunoprecipitation using conditioned medium of Ishikawa cells after E_2 treatment confirmed the E_2 -induced secretion of IGF1 protein. Treatment with recombinant IGF1 stimulated cell proliferation in a dose-dependent fashion, in association with MAPK3/1 phosphorylation and up-regulation of cyclin D1 and E. These IGF1-induced responses were suppressed by treatment with MAP2K inhibitor or anti-IGF1 receptor antibody. Immunohistochemical staining confirmed the expression of activated MAPK3/1 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 E_2 -induced proliferation of endometrial carcinoma cells is mediated by the MAPK3/1 pathway via autocrine stimulation of IGF1.

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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 & 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 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 extracellular growth signals such as growth factors and cytokines. After the binding of growth factors to the respective receptors, the conserved RAS/RAF/MAP2K/mitogen-activated protein kinase (MAPK) pathway acts to relay growth stimuli from activated RAS proteins via MAPK kinase 3/1 (MAP2K1/2) to MAPK1/3 kinase or extracellular signal-regulated kinase (MAPK)3/1 (Kolch 2000, Peyssonnaud & Eychene 2001). The MAPK3/1 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). MAPK3/1 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 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 estradiol (E_2)-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 IGF1 by estrogen, we focused on the involvement of IGF1 as an autocrine factor in estrogen-dependent growth of endometrial carcinoma cells.

Materials and 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) 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, ICI 182 780, and an MAP2K inhibitor, U0126, were purchased from Sigma. Anti-IGF1 receptor antibody (Ab-3), which can neutralize the cell surface receptor, was purchased from Oncogene Research Products (San Diego, CA, USA). Before the E_2 or IGF1 stimulation experiments, Ishikawa cells were pretreated with rapamycin (500 ng/ml; Invitrogen) in serum-starved medium (FCS 0.1%) for 24 h to synchronize them to the G1 phase of the cell cycle. Then the cells were

cultured for 24 h without rapamycin in the same medium to wash out the effect of the agent. ICI 182 780, U0126, or anti-IGF1 receptor antibody was added 1 h before the addition of E_2 or IGF1.

Western blotting

Phosphorylation of MAPK3/1 was examined, using a specific antibody that recognizes phosphorylated MAPK3/1 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 phenylmethylsulphonyl fluoride (Sigma), 1 µg/ml aprotinin (Roche Diagnostics), 1 µg/ml leupeptin (Roche Diagnostics), and 20 µg/ml N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Roche Diagnostics). The lysates were centrifuged at 13 000 *g* for 20 min at 4 °C and the supernatants were stored at -80 °C. Extracts equivalent to 50 µg total protein were separated by SDS-PAGE (7.5 or 10% acrylamide) and transferred onto nitrocellulose membranes (Hybond TM-C Super; Amersham Biosciences Inc). 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 h, then incubated with mouse monoclonal antibodies against phosphorylated MAPK3/1 (Cell Signaling Technology, Danvers, MA, USA), cyclin D1 (Progen, Heidelberg, Germany), IGF1 receptor (Oncogene Research Product), ESR1, cyclin E, β -actin (Santa Cruz, St Louis, MO, USA), or rabbit polyclonal antibodies against MAPK3/1 (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 thrice. 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 c.

Total RNA extraction

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

RT-PCR

Estrogen- or IGF1-induced mRNA expression of cyclin D1, cyclin E, and IGF1 was evaluated using a

Figure 1 Estrogen-dependent proliferation, MAPK3/1 activation, and cyclin expression in Ishikawa cells. (a) Ishikawa cells were serum starved for 48 h as described in Experimental procedures, and E_2 was added. Twenty-four hours after E_2 addition, viable cells were measured using a WST-1 assay. E_2 treatment increased the proliferation of Ishikawa cells in a dose-dependent manner with significant differences. (b) E_2 was added to Ishikawa cells after serum starvation and the expression of cyclins and MAPK3/1 was examined using western blotting and RT-PCR. The S-phase fraction was examined using flow cytometry. E_2 (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 MAPK3/1 activation. (c) Effect of an ER antagonist (ICI 182 780) and a MAP2K inhibitor (U0126) on the activation of MAPK3/1. Ishikawa cells were treated with ICI 182 780 (10^{-5} M) or U0126 (10^{-5} M) for 6 h after E_2 treatment, and MAPK3/1 phosphorylation was evaluated using western blotting. Treatment with ICI 182 780 or U0126 suppressed the expression of pMAPK with significant differences. *Versus control, $P < 0.05$, **versus E_2 alone, $P < 0.05$.

Figure 2 Estrogen-induced expression and secretion of IGF1, and IGF1-induced MAPK3/1 activation in Ishikawa cells. (a) E_2 treatment significantly induced the expression of *IGF1* mRNA with a peak at 6 h after treatment. Immunoprecipitation using the conditioned medium of E_2 -treated Ishikawa cells indicated the secretion of IGF1 protein, which peaked 12 h after E_2 addition. These reactions were suppressed ICI 182 780 by 36% for *IGF1* mRNA ($P < 0.05$) and by 49% for IGF1 protein. *Versus control, $P < 0.05$, **versus E_2 alone, $P < 0.05$ (b) Recombinant IGF1 (10 ng/ml) significantly induced the MAPK3/1 activation of Ishikawa cells from 15 to 30 min after the addition. *Versus control, $P < 0.05$ (c) E_2 -induced MAPK3/1 activation was suppressed by anti-IGF1 receptor antibody dose dependently (17% reduction for 50 ng/ml, 57% reduction for 500 ng/ml, $P < 0.05$) 6 h after E_2 stimulation. *Versus control, $P < 0.05$, **versus E_2 alone, $P < 0.05$.

semiquantitative reverse transcription-PCR (RT-PCR) assay. RT was performed using an RNA PCR Kit (Takara Shuzo, Otsu, Japan), with a 1 μ g RNA sample being added to 20 μ l of a reaction mixture consisting of 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l dNTP mixture, 1 U/ μ l RNase inhibitor, 0.25 U/ μ l avian myeloblastosis virus-derived reverse transcriptase, and 0.125 μ mol/l oligo d(T)-adaptor primer. Using a thermal cycler (Gene Amp PCR System

2400-R; Perkin–Elmer, Norwalk, CT, USA), the reaction mixture was incubated at 42 °C for 30 min, heated to 99 °C for 5 min, and then cooled down to 5 °C for 5 min. One microliter of the RT products, containing 50 ng reverse-transcribed total RNA, was amplified by adding 20 µl of a PCR mixture containing 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 2.5 U/100 µl TaKaRa *Taq* DNA polymerase, with 0.2 µmol/l of a set of 21- to 24-mer oligonucleotide primers for cyclin D1, cyclin E, IGF1, 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'-TCTAAGATGAAGGAGACCATC' and antisense, 5'-GCGGTAGTAGGACAGGAAGTTGTT-3'), cyclin E (Takano et al. 2000; sense, 5'-ATGGTATACTTGCTGCTTCG-GCC-3' and antisense, 5'-AGAACGTGGAGCAGGC-GCGCAACT-3'), IGF1 (Hana & Murphy 1994; sense, 5'-CACTGTCACTGCTAAATTCA-3' and antisense, 5'-CTGTGGGCTTGTTGAAATAA-3', or *GAPDH* (sense, 5'-ACGACCACTTTGTCAAGCTC-3' and antisense 5'-GGTCTACATGGCAACTGTGA-3'). The corresponding cDNA fragments were denatured at 94 °C for 30 s, annealed at 55 °C for 1 min, and extended at 72 °C for 1 min. 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 u.v. transilluminator. Each experiment was performed thrice 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 (Gorzelnik et al. 2001, Silver et al. 2006, Murthi et al. 2008). The mean values of results were indicated in Figures 2a and 3c.

Cell cycle analysis

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

Immunoprecipitation

Because the concentration of secreted IGF1 protein in the culture medium was low to detect using western blotting, IGF1 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, USA). The conditioned medium was collected from Ishikawa cells

Figure 3 IGF-induced proliferation and up-regulation of cyclin in Ishikawa cells. (a) After serum starvation of Ishikawa cells for 48 h, recombinant IGF was added and the viable cells were counted using a WST-1 assay. IGF1 stimulated the proliferation of Ishikawa cells in a dose-dependent manner with significant differences. [†]Versus control $P < 0.05$, ^{††} versus IGF-1 10 ng/ml $P < 0.05$. (b) IGF1 induced a stepwise up-regulation of cyclin D1 mRNA with a peak 12 h after the addition, followed by that of cyclin E mRNA. (c) The IGF1-induced up-regulation of cyclin D1 and E 12 h after addition was significantly suppressed by U0126 by 37% and 42% respectively. *Versus control, $P < 0.05$, **versus E₂ alone, $P < 0.05$.

treated with 10⁻⁶ M E₂ at 6, 12, and 24 h after E₂ addition, or from those cells treated with 10⁻⁵ M ICI 182 780. After preclear, 1 ml of the conditioned medium was incubated with anti-IGF1 antibody (BioCarta, San Diego, CA, USA) overnight at 4 °C and IGF1 precipitates were collected for 1 h on protein-A agarose beads. After washing with lysis buffer thrice, precipitates were resuspended in a Laemmli SDS sample buffer and resolved by SDS-PAGE (15% acrylamide). The resolved

immunoprecipitates were electrotransferred onto a nitrocellulose membrane and detected by immunoblotting using an antibody against IGF1.

WST-1 assay

Ishikawa cells were cultured in 96-well plates, with various concentration of E_2 . Cell proliferation was evaluated with Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer's protocol. Each experiment was repeated thrice.

Immunostaining of MAPK3/1 and phosphorylated MAPK3/1

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, MAPK3/1 Antibody and Phospho-MAPK3/1 (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

E_2 stimulates the proliferation of Ishikawa cells in a dose-dependent manner

To evaluate the effect of E_2 on the proliferation of endometrial carcinoma Ishikawa cells, E_2 was added after incubation in serum-starved conditions for 48 h, and viable cells were measured using a WST-1 assay 24 h after E_2 addition. The result indicated that the E_2 treatment at a concentration of 10^{-8} M and 10^{-6} M increased the cell proliferation compare with the control by 20% and 28% respectively ($P < 0.05$; Fig. 1a). Cell cycle analysis showed that the S-phase fraction of E_2 -untreated and E_2 -treated cells after 24 h 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 E_2 , and the expression of cyclin D1 protein was then elevated ~6 h after E_2 treatment. RT-PCR revealed that mRNA expression of cyclin D1 increased 6 h after E_2 stimulation, with a peak at 12 h after E_2 treatment. The expression of cyclin E protein and mRNA appeared 6 h after E_2 stimulation and then increased thereafter (Fig. 1b). These findings indicate that E_2 stimulates cell cycle progression with transcriptional activation of cyclin D1 and E.

E_2 -induced activation of MAPK3/1 in Ishikawa cells

To investigate the involvement of the MAPK3/1 pathway in E_2 -induced proliferation of Ishikawa cells, we examined the activation of this pathway by western blotting using a specific antibody against activated (phosphorylated) MAPK3/1 (pMAPK3/1). E_2 treatment induced the activation of MAPK3/1 2 h after stimulation, which reached a peak 24 h after stimulation. The expression of nonactive MAPK3/1 remained unchanged throughout the experiment (Fig. 1b). When Ishikawa cells were treated with ER antagonist (ICI 182 780) or an MAP2K inhibitor (U0126), the E_2 -induced MAPK3/1 phosphorylation 6 h after the addition of E_2 was significantly reduced by 78% ($P < 0.05$) and 56% ($P < 0.05$) respectively (Fig. 1c). These findings suggest that E_2 -induced activation of the MAPK pathway is involved in E_2 -induced proliferation of Ishikawa cells.

E_2 -induced MAPK3/1 activation is mediated through IGF1 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 TGFA to examine whether the expression of these growth

factors could be induced by estrogen in Ishikawa cells. Consequently, we identified IGF1 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 IGF1 in estrogen-dependent growth of endometrial carcinoma cells. RT-PCR revealed that E₂-induced *IGF1* mRNA expression peaked 6 h after E₂ addition and then disappeared at 24 h, and ICI 182 780 pretreatment significantly decreased the E₂-induced *IGF1* mRNA expression by 36% (Fig. 2a). To identify the engagement of IGF1 in the E₂-induced growth mechanism, the secretion of IGF1 into the culture medium in E₂-treated Ishikawa cells was then examined. The conditioned medium after E₂ with/without ICI 182 780 treatment was collected and immunoprecipitated with anti-IGF1 antibodies. The following immunoblotting revealed that the IGF1 protein in the conditioned medium peaked 12 h after E₂ treatment, and ICI 182 780 treatment reduced the E₂-induced IGF1 secretion 6 h after addition by 49% (Fig. 2a). These findings indicate that E₂ induces IGF1 synthesis and secretion into the culture medium in Ishikawa cells, suggesting a possible presence of an IGF1-mediated autocrine mechanism.

The role of IGF1 in E₂-induced MAPK3/1 activation was then investigated. Western blotting confirmed that Ishikawa cells expressed IGF1 receptors (Fig. 2b), and recombinant IGF1 induced a rapid and transient MAPK3/1 phosphorylation that began 15 min after IGF1 addition with significant differences ($P < 0.05$), and disappeared by 1 h (Fig. 2b). To further confirm the contribution of IGF1 in E₂-induced MAPK3/1 activation, we examined the effect of anti-IGF1 receptor neutralization antibody. When cells were treated with anti-IGF1 receptor antibody, a partial inhibition of E₂-induced MAPK3/1 activation (anti-IGF1 receptor antibody: 50 ng/ml, 17% reduction; 500 ng/ml, 57% reduction, $P < 0.05$) was observed 6 h after the addition of E₂ (Fig. 2c). These results indicated that the E₂-induced MAPK3/1 activation was mediated by an autocrine stimulation of IGF1 in Ishikawa cells.

IGF1 stimulates the proliferation of Ishikawa cells in a dose-dependent manner

Since E₂ treatment activated the MAPK3/1 pathway via an autocrine system of IGF1, we examined whether IGF1 can enhance the proliferation of Ishikawa cells. A WST-1 assay revealed that the addition of recombinant IGF1 at concentrations of 10 ng/ml and 100 ng/ml for 24 h increased cell proliferation compared with the control by 19% and 32% respectively, ($P < 0.05$; Fig. 3a).

IGF1 induces cyclin D1 and E via activation of the MAPK3/1 pathway

To investigate the molecular mechanism of IGF1-induced cell proliferation, we examined the mRNA expression of cyclin D1 and E after IGF1 treatment. RT-PCR revealed that mRNA expression of cyclin D1 increased 2 h after IGF1 stimulation, with a peak at 12 h after E₂ treatment. The expression of cyclin E mRNA increased from 4 h after IGF1 stimulation (Fig. 3b). To investigate whether the activation of MAPK3/1 was involved in the IGF1-induced expression of cyclin D1 and E, we examined the effect of U0126. RT-PCR revealed that treatment with U0126 significantly reduced the IGF1-induced cyclin D1 and E expression 12 h after the addition of IGF1 by 37% for cyclin D1 ($P < 0.05$) and by 42% for cyclin E ($P < 0.05$, Fig. 3c). These findings suggest that IGF1 induced cyclin D1 and E via activation of the MAPK3/1 pathway.

E₂-induced cell proliferation is dependent on the MAPK3/1 pathway via an autocrine mechanism of IGF1

MAPK3/1 dependency in E₂- or IGF1-induced cell proliferation was examined using an MAP2K inhibitor and an antibody against the IGF1 receptor in Ishikawa cells. E₂ treatment for 24 h enhanced the proliferation of Ishikawa cells by 20% (lane 2, Fig. 4) compared with the control with a significant difference ($P = 0.011$), and the

Figure 4 Effect of ICI 182 780, U0126, and anti-IGF1 receptor antibody on E₂- or IGF1-induced proliferation of Ishikawa cells. Ishikawa cells were serum starved for 48 h, and E₂ (10⁻⁶ M) or IGF1 (10 ng/ml) was added. Viable cells were measured 24 h after the addition using a WST-1 assay. ICI 182 780 suppressed the E₂-induced proliferation of Ishikawa cells (lane 3) with a significant difference, but not IGF1-induced proliferation (lane 8). U0126 suppressed both E₂- (lane 4) and IGF1 (lane 9)-induced proliferation. Anti-IGF1 receptor antibody suppressed E₂-induced proliferation (lanes 5, 6). *versus control $P < 0.05$, †versus E₂ alone $P < 0.05$, ‡versus IGF1 alone $P < 0.05$.

E₂-induced proliferation was blocked by the use of ICI 182 780 (lane 3) or U0126 (lane 4). Anti-IGF1 receptor antibody also inhibited the E₂-induced cell proliferation (lanes 5 and 6). IGF1 treatment increased the proliferation of Ishikawa cells by 10% compared with the control after 24 h ($P=0.039$; lane 7). Treatment with ICI 182 780 slightly decreased the IGF1-induced cell proliferation, but the difference was not significant (lane 8). U0126 inhibited the effect of IGF1 on cell proliferation (lane 9). Collectively, these findings suggest that E₂-stimulated proliferation of Ishikawa cells is mediated by MAPK3/1 activation via autocrine stimulation by IGF1.

Immunostaining for MAPK3/1 and phosphorylated MAPK3/1 in normal endometrium and endometrial carcinoma

To investigate the involvement of MAPK3/1 in the growth of endometrial tissues, the immunohistochemical expression of MAPK3/1 and its activation (phosphorylation) were examined in normal and malignant endometrial tissues. The immunoreactivity for MAPK3/1 and phosphorylated (p)MAPK3/1 was observed both in the cytoplasm and nucleus. In normal endometrial glandular cells, the expression of cytoplasmic MAPK3/1 was observed both in the proliferative and secretory phases, whereas that of pMAPK3/1 was

more abundant in the proliferative phase than the secretory phase ($P<0.0001$ in the nucleus, $P=0.0018$ in the cytoplasm; Fig. 5). The expression of MAPK3/1 increased in all cases of endometrial carcinoma examined, and that of pMAPK3/1 increased significantly compared with that in the secretory phase endometrium (versus grade 1: $P<0.0001$ in the nucleus, $P=0.0001$ in the cytoplasm; versus grade 2: $P=0.04$ in the nucleus, $P=0.0084$ in the cytoplasm; versus grade 3: $P=0.003$ 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 pMAPK3/1 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 IGF1 expression and significantly enhanced the cell proliferation of endometrial carcinoma Ishikawa cells. In addition, we have also shown that 1) E₂ induced the production and secretion of IGF1, 2) Ishikawa cells expressed IGF1 receptors, 3) treatment with recombinant IGF1 stimulated the proliferation of Ishikawa cells, and 4) treatment with anti-IGF1 receptor neutralization antibody effectively inhibited both E₂- and IGF1-induced growth of Ishikawa cells. These findings clearly demonstrated the presence of an autocrine loop of IGF1 in estrogen-induced cell proliferation of Ishikawa cells. Previous studies using primary cultures of endometrial carcinoma cells (Reynolds *et al.* 1996) and endometrial carcinoma cell lines (Takeda *et al.* 1996, Reynolds *et al.* 1998) suggested the possible existence of IGF1- or TGFA-mediated autocrine loops; however, the involvement of estrogen stimulation in the autocrine system was not reported. Although Hana & Murphy (1994) reported an increase in *IGF1* mRNA expression after estrogen treatment, the secretion of IGF1 protein was not examined. In breast carcinoma MCF-7 cells, an estrogen-related autocrine loop has been reported to stimulate cell proliferation via ERBB2 and its ligand, heregulin (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 IGF1 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). IGF1-induced cell proliferation was half of that of E₂-induced in this

Figure 5 Immunohistochemical detection of MAPK3/1 and phosphorylated (p)MAPK in endometrial tissues. The expression of MAPK (a, c, e and g) and pMAPK (b, d, f and 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 pMAPK both in the cytoplasm and nucleus was increased in the normal proliferative phase endometrium and endometrial carcinoma compared with that in the secretory phase endometria ($P<0.05$). There was no significant difference in the expression of pMAPK between different histological grades of endometrial carcinoma. The expression of cytoplasmic MAPK did not show marked changes between histological groups. Pro., proliferative phase; Sec., secretory phase. *Significantly different from that of the normal secretory phase endometrium ($P<0.05$).

study, suggesting that the presence of other E₂-induced growth mechanisms than IGF1 cannot fully be denied.

The molecular mechanism of E₂-induced transcription of IGF1 is largely unknown. In the present study, we observed an increase in the expression of *IGF1* mRNA 6 h after E₂ treatment, and this may be consistent with that observed in E₂-administered rat uterus (Murphy & Ghahary 1990). More recently, the expression of IGF1 has reportedly been induced by cyclic AMP-dependent kinase via activation of HS3D located in the IGF1 gene promoter and a transcription factor, CCAAT/enhancer-binding protein δ in rat osteoblasts (Billiard et al. 2001). Kleinman et al. (1995) reported that E₂ sensitized the effects of IGF1 by elevating receptor levels and also decreasing the expression of IGF-binding protein-3, which potentially has an inhibitory function for IGF1. Thus, further studies are needed to clarify the molecular link between estrogen stimulation and up-regulation of IGF1.

Our study also showed that the classical MAPK pathway, MAPK3/1, is involved in estrogen-induced cell proliferation via the IGF1 autocrine loop in endometrial carcinoma Ishikawa cells. Treatment with recombinant IGF1 also induced MAPK3/1 phosphorylation and cell proliferation of Ishikawa cells, which were suppressed by a MAP2K inhibitor, U0126. The activation of MAPK3/1 peaked from 15 to 30 min after the IGF1 stimulation. Earlier studies using fibroblasts indicated that RAS–MAP2K–MAPK3/1 activation is important in IGF1-related cell proliferation (Lu & 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), IGF1 was reported to transmit the signal possibly via the MAPK3/1 pathway (Shen et al. 2004). However, in breast carcinoma MCF-7 cells, the mitogenic signal of IGF1 did not pass through the MAPK3/1 pathway but through the phosphatidylinositol 3-kinase pathway (Dufourmy et al. 1997). In endometrial carcinoma cells, the putative growth factors relaying the signals to the MAPK3/1 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 IGF1 stimulation, MAPK3/1 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, IGF1 stimulation, MAPK3/1 activation, and up-regulation of cyclin D1 and cyclin E in Ishikawa cells. E₂-induced expression of IGF1 was

observed 6 h after E₂ stimulation, and IGF1-induced MAPK3/1 activation was observed as early as 15 min. 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 h after E₂ stimulation, a signal transduction pathway from IGF1 and cyclin D1 via MAPK3/1 seems to be plausible. However, since a weak up-regulation of cyclin D1 mRNA was observed 2 h 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 h 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 h after E₂ stimulation. The molecular mechanism of cyclin E up-regulation remains undetermined; however, the cyclin E gene has multiple E2F1 binding sites, and E2F1 has been reported to activate the expression of cyclin (DeGregori et al. 1995, Ohtani et al. 1995). MAPK3/1-induced up-regulation of E2F1 has also been reported (Berkovich & Ginsberg 2001, Pintus et al. 2003). Collectively, the link between MAPK3/1 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 pMAPK3/1 was increased in both normal proliferative phase endometrium and endometrial carcinoma. The increased expression of pMAPK3/1 in endometrial carcinoma observed in the present study was similar to 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 MAPK3/1 pathway seems to be functioning not only in endometrial carcinoma cells but also in normal endometrial glandular cells *in vivo*. Interestingly, the E₂-induced MAPK3/1 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 et al. (2001) reported that normal endometrial stromal cells secrete IGF1 as a paracrine factor that stimulates the proliferation of neighboring glandular cells, indicating the presence of epithelial–stromal interactions via IGF1 in normal

endometrium. 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 of endometrial stroma, through having the autocrine loop of IGF1 stimulation.

In conclusion, our study demonstrated that activation of the MAPK3/1 pathway plays an important role in E₂-induced growth of endometrial carcinoma cells, and its activation is mediated by IGF1 autocrine stimulation. This result suggests that IGF1 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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