Selective Inhibition of the Epidermal Growth Factor Receptor by ZD1839 Decreases the Growth and Invasion of Ovarian Clear Cell Adenocarcinoma Cells

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ABSTRACT
The mechanism that regulates the growth of ovarian clear cell adenocarcinoma (CCA) are not well understood. We investigated the role of several growth factors that bind to membrane tyrosine kinase receptors and added them to the ovarian CCA cell lines KK, RMG-1, and HAC-II to evaluate their effect on growth and cellular invasion. Epidermal growth factor and transforming growth factor-α significantly stimulated the growth and invasion of CCA cell lines in vitro. ZD1839, an epidermal growth factor receptor-tyrosine kinase inhibitor, decreased the growth and invasion of CCA cell lines in vitro and in vivo inhibited the growth of xenografts of the CCA cell line RMG-1. Severe combined immunodeficient mice bearing RMG-1 xenografts treated with ZD1839 survived for longer than the untreated control group. From these findings, we conclude that ZD1839 may offer a new and effective treatment for ovarian CCA.

INTRODUCTION
CCA of the ovary is commonly resistant to chemotherapy (1, 2) and consequently remains one of the most difficult tumors to treat in the field of gynecologic oncology (3). Optimal treatment of CCA is believed to be complete resection of the tumor; however, complete resection is difficult to accomplish in patients with advanced disease. An effective treatment modality for these tumors, especially in advanced cases, is urgently needed. Despite the need to understand the mechanisms of growth and metastasis of CCA, which would enable us to establish new treatment modalities, very little information is available to date. Low expression of p53 and cyclin A proteins and high expression of p21 and cyclin E proteins in resected ovarian CCA specimens were reported by Shimizu et al. (4). Also, loss of CD44 splice control in CCA and the relationship to metastatic potential was reported recently by Sancho-Torres et al. (5). These reports provided some information about specific aspects of CCA, but give little information that could lead to the development of new treatments.

We demonstrated previously that ER-α is absent from clinically resected ovarian CCA, compared with its frequent presence in SAC and endometrioid adenocarcinoma specimens (6). Ovarian cancer genesis involves activation of several growth factor genes after stimulation of ER (7, 8), and this may explain how the growth of CCA is regulated by some growth factors in an estrogen-independent manner. There have been reports that growth factors such as EGF and TGF-α stimulate the growth in ovarian SAC (9). However, almost no reports concerning the effect of growth factors in CCA have been published.

We have investigated the role of several growth factors on the growth and invasion of ovarian CCA cell lines, as well as the potential for treatment of this type of tumor using ZD1839 (Iressa), a potent and selective inhibitor of EGFR tyrosine kinase activity (10).

MATERIALS AND METHODS
Culture Conditions. Three human ovarian CCA cell lines, KK (11), RMG-1 (12), and HAC-II (13) were grown as monolayer cultures in DMEM (Nissui, Tokyo, Japan) supplemented with 10% FCS (Mitsubishi Chemicals Co., Tokyo, Japan). The cultures were incubated under stable growth conditions at 37°C in a humidified atmosphere containing 5% CO2.

Clinical Specimens. Twenty-eight ovarian CCA patients who had been treated initially in the Department of Obstetrics and Gynecology, Toyama Medical and Pharmaceutical University Hospital (Toyama, Japan), from 1982 to 1999 were registered in this study. The median age of these patients was 51.0 years (range, 32–65 years; mean, 51.9 years). Nine cases (32.1%) were associated with concomitant endometriosis. They were treated with abdominal total hysterectomy and bilateral salpingo-oophorectomy, and pelvic and paraaortic lymphadenectomy was performed in some cases when intra-abdominal stage was more than T2.

Immunohistochemical Staining. We performed immunohistochemical staining by the strepto-avidin biotin method using an immunohistochemical staining kit (Nichirei Co., Tokyo, Japan) after a high temperature, antigen unmasking procedure (121°C for 15 min) and blocking of intrinsic peroxidase. The antibodies used were rabbit polyclonal anti-EGF antibody Z-12 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse
monoclonal anti-TGF-α antibody (Oncogene Science Inc. Cambridge, MA), and rabbit polyclonal anti-EGFR antibody (Santa Cruz Biotechnology), each at a concentration of 1:100 and incubated at 4°C overnight. Diaminobenzidine was used as the staining substrate. The result was interpreted as positive when >10% of the tumor cells appeared positively stained under the high-power field of the microscope. Immunohistochemical examination for EGF, TGF-α, and EGFR was also performed on the three ovarian CCA cell lines cultured on glass slides by the same procedure.

Reagents. Recombinant EGF was purchased from Wakanaga Pharmaceutical Co. (Tokyo, Japan). Recombinant human TGF-α and VEGF were purchased from R&D Systems (Minneapolis, MN). Human bFGF, PDGF-aa, and PDGF-bb were from Oncogene Science Co. (Cambridge, MA). SCF, Flt3-ligand, and HGF were from Genzyme Co. (Cambridge, MA). IGF-I was purchased from Biomedical Technologies Inc. (Stoughton, MA). Mouse anti-EGF-R antibody (EGF Receptor Ab-3, GR13) was purchased from Calbiochem Co. (San Diego, CA). In our preliminary experiments, the addition of 1 µg/ml anti-EGF-R antibody inhibited ~75% of EGF-R phosphorylation of RMG-1 after the addition of 10 µg of EGF. ZD1839 was kindly provided by AstraZeneca (Macclesfield, Cheshire, United Kingdom). Stock solutions were prepared in DMSO and diluted to appropriate concentrations in culture medium before addition to the cells. An equivalent dilution of DMSO without the inhibitor served as a control.

Treatment of Cultured CCA Cells. CCA cells (5 × 10⁵ cells/ml) were suspended in DMEM supplemented with 2% FCS, and 100 µl of the suspension were added to each well (500 cells per well) of a 96-well flat culture plate (Corning Inc., Corning, NY). Growth factor (EGF, TGF-α, bFGF, PDGFA, PDGFbb, SCF, Flt3-ligand, HGF, VEGF, or IGF-I) was added to the CCA cell suspension at a concentration of 10 ng/ml. The effect of increasing dose (0.1, 1.0, 10, or 100 ng/ml) was also investigated for EGF, TGF-α, and bFGF. In experiments to determine the effects of inhibiting EGFR, mouse monoclonal anti-EGFR antibody (100 µg/ml) or ZD1839 (0.1, 1.0, 10, or 100 µg/ml) was added to cells containing EGF or TGF-α (10 ng/ml). Cells were incubated for 72 h at 37°C under 5% CO₂.

Cell Proliferation Assay. Cellular growth was measured by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI). Cell proliferation assay solution (20 µl) was added to each well and incubated for 3 h at 37°C under 5% CO₂. The absorbance at 490 nm (A₄₉₀) was obtained for each well with a microplate reader (TOSOH Corp., Tokyo, Japan). The cell number for each well was calculated from the A₄₉₀ for wells containing a standard number of cells. We performed each experiment in triplicate using three consecutive wells, and the mean value was used. Cellular growth after administration of each cytokine was expressed as a relative growth, which was a fold number compared with the cellular number of the control, which had no cytokine added. The cell number after incubation with each cytokine in invasion assay or EGFR inhibitor was compared with controls (no cytokine).

Invasion Assay. The method used in this study has been described previously (14, 15). Briefly, the upper compartment of the chamber containing filters with 8.0-µm pores (Becton Dickinson Labware, Franklin Lakes, NJ) was coated with 10 µg/50 µl growth-factor-free Matrigel (Becton Dickinson) and 1 µg/10 µl fibronectin (Nakarai Tesk Co., Tokyo, Japan) on the lower surface, then was left to polymerize and dry overnight. Cells were incubated with cytokines and/or EGFR inhibitor at concentrations described above. After incubation for 9 h, the cells that had passed through the Matrigel and fibronectin to the lower surface were stained with hematoxylin and counted.

Phosphorylated EGFR Quantification. EGF (10 ng/ml) and ZD1839 (0.01, 0.1, 1.0, 10 µg/ml) were added to RMG-1 cells. Cells were collected by a cell scraper after 4 h of incubation, then washed twice with cold PBS. The cell number was recorded at this time. The concentration of phosphorylated EGFR was measured using an active EGFR EIA Kit (Takara Biomedicals, Tokyo, Japan) following the manufacturer’s instructions and expressed as the amount of phosphorylated EGFR (fmol/ml) per 1 × 10⁷ cells.

In vivo Assay of RMG-1. RMG-1 xenografts were transplanted into SCID mice, and when the size of the tumor reached ~70 mm³, ZD1839 (5, 50, 500 µg/kg) was injected i.p. once a week for 4 weeks. Both control and ZD1839-administered groups included 5 mice each, and the experiment was repeated twice. Although this compound was used as an o.p. compound in many studies, we used this drug as i.p. administration because many human ovarian cancers have been known to disseminate intraperitoneally. Because data for i.p. administration of mouse were not available at the initial time of the study, the concentration of ZD1839 was determined according to the peak plasma concentration of canine when 5 mg of ZD1839 was administered i.v. Also, because of safety considerations, we decided to administer ZD1839 weekly. Control animals were injected with DMSO (equal volume as animals receiving ZD1839 500 µg/kg). Tumor size was monitored twice weekly by measuring the shorter diameter and longer diameter, then volume was calculated from the following equation: Tumor volume = (longer diameter) × (shorter diameter)²/2. Tumor volume from treated mice was compared with that of the control group with a modification to the methods by Houchens et al. (16).

Statistical Analysis. Two-sided Student’s t tests were performed to evaluate the cellular growth after administration of each cytokine; P < 0.05 was considered significant. ANOVA statistical analysis was performed to evaluate dose dependency. All statistical analyses were performed with StatView (version 4.5 for Macintosh; Abacus Concepts, Berkeley, CA).

RESULTS

Effects of Cytokines and Anti-EGFR Antibodies on CCA Growth. The growth of KK cells after the addition of EGF, TGF-α, bFGF, PDGFaa, PDGFbb, SCF, Flt3-ligand, HGF, VEGF, or IGF-1 (10 ng/ml) is shown in Fig. 1. Cellular growth was significantly increased after the addition of EGF (P = 0.05), TGF-α (P = 0.01), and bFGF (P = 0.001). A similar effect was observed in RMG-1 and HAC-II cell lines (data not shown). Dose-dependent growth acceleration of the RMG-1 cell lines was observed after the addition of EGF, TGF-α, and bFGF(Fig. 2). The acceleration of cellular growth by EGF and TGF-α was completely neutralized by addition of anti-EGFR antibody (Fig. 2).

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Effects of Cytokines on Cellular Invasion of CCA Cells.

The cellular invasion of KK cells after the addition of the growth factors (10 ng/ml) is shown in Fig. 3. Cellular invasion was significantly increased after the addition of EGF, TGF-α, and bFGF. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \) versus control.

Amount of Phosphorylated EGFR after Addition of EGF and ZD1839. It has been shown previously that ZD1839 is a potent inhibitor of tyrosine-phosphorylation of EGFR in many human tumor cells (10). In this study, a marked increase of phosphorylated EGFR was observed after the addition of EGF at 10 ng/ml to RMG-1 cells. Treatment with ZD1839 decreased EGF-stimulated phosphorylation of EGFR in a concentration-dependent manner; complete inhibition was observed after the addition of 10 \( \mu \)g/ml ZD1839 (Fig. 5).

Inhibition of CCA Cellular Growth and Invasion by ZD1839. A dose-dependent decrease of KK cellular growth (Fig. 6A) and invasion (Fig. 6B) was observed after the addition of ZD1839. Growth and invasion of RMG-1 and HAC-II cells were similarly inhibited by the addition of ZD1839 (data not shown).

In Vivo Inhibition of CCA Cellular Growth by ZD1839. A dose-dependent reduction in tumor growth was observed after administration of ZD1839 compared with the controls (control versus 5 \( \mu \)g/kg ZD1839, \( P = 0.0008 \); control versus 50 and 500 \( \mu \)g/kg ZD1839, \( P < 0.0001 \); Fig. 7). Approximately 40 days after the administration of ZD1839, the tumor volume in the ZD1839-treated group was \( \sim 50\% \) of that in the control group.
Treatment with ZD1839 was well tolerated, as determined by the maintenance of body weight (data not shown). Survival of mice in each group was also compared: ZD1839-treated mice survived longer than the mice in the control group (P < 0.01; Fig. 8).

**DISCUSSION**

Dramatic evolution of anticancer chemotherapy, especially after the introduction of platinum-containing chemotherapeutic regimens and the recent launch of paclitaxel for ovarian cancer, has meant that therapeutic effect, prognosis, and quality of life for ovarian cancer patients have improved (10, 16–20). Ovarian CCA, however, has eluded an effective treatment modality because of its chemoresistant nature (3). Poor prognosis of ovarian CCA compared with ovarian SAC has become evident in recent years (21, 22). Although the development of a new treatment approach to this tumor is crucial, little information on its biological characteristics has been available thus far.

The role of the ERs, particularly ER-α, in the carcinogenesis or progression of surface epithelial ovarian cancer has been revealed in recent years (23–25). However, our previous study disclosed no expression of ER-α in ovarian CCA (6), thus estrogen-independent stimulation was suspected for the regulation of growth or carcinogenesis of ovarian CCA. Signals from ER-α via an estrogen-responsive enhancer element induce a genetic activation of several growth factors such as EGF or TGF-β (7, 8). Our investigation was performed to clarify the relation of growth factors to the growth or metastatic regulation of ovarian CCA. EGF and TGF-β were revealed to be potent stimulators of the growth and invasion of ovarian CCA cell lines. In ovarian SAC, Kurachi et al. (9) reported stimulating effects of EGF and TGF-β on the growth of elected cell lines. The presence of EGFR has been reported in 20–70% of clinically resected ovarian SAC specimens (26–33). In our study, EGFR was detected in 17 of 28 (60.7%) clinically resected ovarian CCA specimens. EGF and TGF-β were localized in 57.1 and 78.6% of the cases. Coexpression of EGFR and EGF or TGF-β was completely (100%) matched. An in vitro assay clarified that growth of the ovarian CCA cell lines was promoted by addition of EGF or TGF-β through EGFR. Signals from ER-α via an estrogen-responsive enhancer element induce a genetic activation of several growth factors such as EGF or TGF-β (7, 8). Our investigation was performed to clarify the relation of growth factors to the growth or metastatic regulation of ovarian CCA. EGF and TGF-β were revealed to be potent stimulators of the growth and invasion of ovarian CCA cell lines. In ovarian SAC, Kurachi et al. (9) reported stimulating effects of EGF and TGF-β on the growth of elected cell lines. The presence of EGFR has been reported in 20–70% of clinically resected ovarian SAC specimens (26–33). In our study, EGFR was detected in 17 of 28 (60.7%) clinically resected ovarian CCA specimens. EGF and TGF-β were localized in 57.1 and 78.6% of the cases. Coexpression of EGFR and EGF or TGF-β was completely (100%) matched. An in vitro assay clarified that growth of the ovarian CCA cell lines was promoted by addition of EGF or TGF-β through EGFR. These results suggest that the stimulation of EGF or TGF-β through EGFR is essential for the growth of ovarian CCA. EGF and TGF-β also stimulated cellular invasion. From these findings, we conclude that the growth and invasion of ovarian CCA are under the control of EGF or TGF-β through EGFR, as is also true for ovarian SAC.

Growth inhibition of human cancer cells by anti-EGFR antibodies has been reported in several cancer cell lines (34, 35);
however, the effect of EGFR-TKI is reportedly greater than EGFR antibodies (36). Also EGFR-TKI has been reported to be dependent on the receptor number (37). Recent studies have demonstrated the function of ZD1839, which inhibits the phosphorylation of EGFR after its ligand (EGF, TGF-α/H9251) binds to EGFR (10). Administration of ZD1839 with several anticancer agents such as cisplatin, carboplatin, paclitaxel, docetaxel, doxorubicin, etoposide, and topotecan showed strong tumor-growth inhibition in in vitro and in vivo experiments using human ovarian (OVCAR3), breast (ZR-75–1, MCF-10A ras), and colon (GEO) cancer cell lines that coexpress EGFR and TGF-α/H9251 (10). In our study, three CCA cell lines that coexpress EGFR, EGF, and TGF-α/H9251 were used. ZD1839 inhibited the growth and invasion of these cell lines dose dependently. The antitumor effects of ZD1839 coadministered with several anticancer agents, however, should be further investigated for the CCA cell lines. An inhibitory effect of TGF-α-mediated growth in several ovarian cancer cell lines (PE01, PE04, SKOV-3, and PE01CDDP) was reported by Simpson et al. (38); an EGFR-TKI completely inhibited TGF-α-stimulated tyrosine phosphorylation of EGFR and reduced phosphorylation of another tyrosine kinase type of membrane receptor, the ErbB-2 receptor. The authors concluded that EGFR-TKI can inhibit the growth of ovarian carcinoma cells in vitro, a finding that was consistent with inhibition of tyrosine phosphorylation at the EGFR (38). In our study, a similar result was obtained; ZD1839 inhibited the phosphorylation of EGFR in CCA cell lines after EGF bound to EGFR in vitro. Consequently, ZD1839 inhibits the growth of CCA cell lines both in vitro and in vivo and also inhibits cellular invasion in vitro. Similar observations using several types of EGFR-specific phosphorylation inhibitors in human pancreatic (39), head and neck (40), prostate (41), breast (42), bladder (43), and brain (44) cancer have been reported recently.

Strikingly, only very minor side effects of ZD1839, such as skin rash, were observed in a Phase I study (45). Phase II and III studies of this compound are ongoing in Europe, the United States, and Japan. We used this compound because ovarian CCA cells are stimulated by EGF and TGF-α/H9251 through EGFR by an autocrine mechanism. ZD1839 strongly inhibited the growth and invasion of three CCA cell lines in a dose-dependent manner. In addition, ZD1839 inhibited the growth of RMG-1 xenografts and led to prolonged survival of ZD1839-treated mice compared with controls, although dose-dependent prolongation of the survival was not observed. Because we administered the compound once a week for 4 weeks, the survival data were demonstrated as a result of the administration duration of only the first 21 days and led to the lack of dose-dependent prolongation of survival. However, we think that the most important point is that SCID mice with ZD1839 administration survived longer than controls without ZD1839 administration. These findings strongly indicate that ZD1839 would be a candidate for further clinical studies to establish a new treatment modality for ovarian CCA for which no alternative treatment is currently available.

REFERENCES


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