ZD1839, a Selective Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor, Shows Antimetastatic Activity Using a Hepatocellular Carcinoma Model

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Abstract
The epidermal growth factor receptor (EGFR) is highly expressed in many human tumors and provides a new target for anticancer drug development. EGFR-targeted agents have shown promising antitumor activity in preclinical and clinical trials. However, little is yet known about the effect of these new agents on tumor metastasis. Here, we investigate the effects of ZD1839 (Iressa), a selective EGFR tyrosine kinase inhibitor, on the metastatic properties of murine hepatocellular carcinoma CBO140C12. ZD1839 inhibited not only cell growth but also epithelial growth factor-induced chemotactic migration and production of active matrix metalloproteinase-9 in vitro. In mice, orthotopic implantation of a fragment of CBO140C12 tumor into the liver resulted in the formation of a solitary tumor nodule and intrahepatic metastasis. ZD1839, given p.o., inhibited growth of the implanted tumor and intrahepatic metastasis by ~50%. These results indicate that EGFR signaling plays an important role in tumor metastasis and that ZD1839 is effective at inhibiting intrahepatic metastasis.

Introduction
The EGFR is a M170,000 cell surface glycoprotein with tyrosine kinase activity: a activation of the EGFR-TK initiates a cascade of intracellular signaling events (1). High expression of EGFR has been observed in many human tumors, including lung, colon, breast, head and neck, ovarian, bladder, and liver, and has been shown to correlate with advanced tumor stage and poor clinical prognosis (2, 3). The EGFR signaling pathway is associated with metastatic properties, including cell motility, adhesion, and invasion in vitro (4, 5). However, it remains unclear whether EGFR-targeted therapies are effective at inhibiting tumor metastasis in vivo.

EGFR is an attractive target for novel anticancer therapy (6). Two therapeutic approaches are currently targeting EGFR in clinical studies: monoclonal antibodies and small molecule EGFR-TKIs. ZD1839 (Iressa) is a p.o. active, selective EGFR-TKI that blocks signal transduction pathways implicated in proliferation and survival of cancer cells and other host-dependent processes promoting cancer growth (7). ZD1839 shows antitumor activity and prolongs survival in vivo in nude mice when given in combination with various anticancer drugs (8, 9). It remains unclear whether EGFR-targeted therapies are effective at inhibiting tumor metastasis in vivo.

In this study, we evaluated the effects of ZD1839 on various tumor metastasis models in vitro and in vivo using hepatocellular carcinoma to clarify the impact of the EGFR signaling pathway on tumor metastasis.

Materials and Methods
Materials. ZD1839 was kindly provided by AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom). It was dissolved in DMSO for the in vitro study and in 0.5% (w/v) hydroxypropylmethylcellulose solution for use in vivo. EGF and HGF were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and Genzyme/Techné (Minneapolis, MN), respectively. Caspase-3 and phospho-EGFR, phospho-ERK, and phospho-JNK antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Cell Lines. The CBO140C12 murine hepatocellular carcinoma cell line was kindly provided by Dr. K. Ogawa (Department of First Pathology, Asahikawa Medical College, Asahikawa, Japan) and maintained in DMEM:F-12 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS, 300 mg/liter L-glutamine, and 2 g/liter glucose. Mice. Five-week-old specific pathogen-free female B6C3F1 mice were purchased from Japan SL(C (Hamamatsu, Japan). The mice were maintained under specific pathogen-free conditions and used according to institutional guidelines.

Cell Proliferation Assay. CBO140C12 cells (1 × 10^4 cells/well) were seeded in 50 µl of medium containing 1% FBS in 96-well plates. Cells were allowed to adhere for 2 h...
and then equal volume of medium containing ZD1839 was added. Antiproliferative activity was determined by Cell Counting kit (Dojindo, Kumamoto, Japan). BrdUrd incorporation was determined by Cell Proliferation ELISA BrdU (Roche, Mannheim, Germany) according to the manufacturer’s instructions.

**Western Blot Analysis.** CBO140C12 cells were cultured in medium containing 0.5% FBS for 24 h. After indicated treatment, the cells were then rinsed with ice-cold PBS and lysed in sample buffer [25 mM Tris-HCl (pH 6.8), 5% w/v glycerol, 1% w/v SDS, 0.05% w/v bromphenol blue]. Cell lysates were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were incubated with Block Ace (Dainipponseiyaku, Suita, Japan) and probed with anti-EGFR antibody and antiphospho-EGFR antibody before washing. The protein content was visualized using horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham). Experiments were performed at least twice, and a representative experiment is shown.

**Cell Migration Assay.** The chemotactic migration of CBO140C12 cells was measured using Transwell cell culture chambers (Costar, Cambridge, MA) as previously described (10) with some modifications. The filter’s lower surface was precoated with 3 μg of gelatin (Sigma). A CBO140C12 cell suspension (2 × 10^3/100 μl) was added to the upper compartment of the chamber; chemoattractants and ZD1839 were added to the lower compartment. After a 12-h incubation period, the filters were fixed, and cells that had migrated to the lower surface were manually counted under a microscope at ×200 magnification.

**Gelatin Zymography.** Gelatin zymography was performed as previously described (11) with some modifications to assess secretion of MMP-9. Briefly, to prepare tumor-conditioned medium, CBO140C12 cells were allowed to grow to subconfluence in 3.5-cm tissue culture dishes in DMEM:F-12 containing 10% FBS. After several washes with serum-free DMEM:F-12, the medium was replaced with DMEM:F-12 containing 0.1% BSA plus EGF and ZD1839, and the cultures were incubated for an additional 12 h. The conditioned media were concentrated using Centricon (Millipore) according to the manufacturer’s instructions and applied to SDS-polyacrylamide gels (7.5% w/v) copolymerized with gelatin (0.1% w/v) and incubated at 37°C for 24 h. Enzyme-digested regions were quantified by NIH image 1.62.

**Intrahepatic Metastasis Model by Orthotopic Implantation of Tumor Fragments.** Orthotopic implantation of CBO140C12 tumor into mouse liver was performed as described previously (12). ZD1839 (75 mg/kg) or vehicle was administered p.o. to the mice five times a week, starting on the third day after tumor implantation; the mice were sacrificed on day 21 to weigh the primary tumor and count the metastasized colonies.

**Statistical Analysis.** Statistical comparisons were performed using Mann-Whitney’s test or Student’s two-tailed t test. P < 0.05 was considered to be significant.

**Results**

**Effect of ZD1839 on Proliferative Activity of CBO140C12.** ZD1839 has a known effect on tumor cell proliferation and apoptosis (6). We first examined the effect of ZD1839 on the growth of CBO140C12. ZD1839 did not affect the growth of CBO140C12 cells during a 12-h incubation (Fig. 1A), but the inhibition of tumor growth was apparent by 12 days. EGF-induced chemotaxis of CBO140C12 was induced by treatment with EGF and completely inhibited by 0.1 μM ZD1839 (Fig. 2). ZD1839 also inhibited downstream caspase-3 (Fig. 1D), indicating antiproliferative and apoptotic activities of ZD1839 in CBO140C12 cells.

**Inhibition of EGFR Signaling by ZD1839.** We next examined the effect of ZD1839 on EGFR phosphorylation in CBO140C12 cells. EGFR tyrosine phosphorylation (Y1068) was induced by treatment with EGF and completely inhibited by 1 μM ZD1839 (Fig. 2). ZD1839 also inhibited downstream EGFR signaling transduction pathways, EGF-dependent phosphorylation of ERK and JNK.

**Effect of ZD1839 on the Metastatic Properties of CBO140C12.** To investigate the influence of ZD1839 on tumor metastasis in vitro, we examined the effect of ZD1839 on EGF- or HGF-induced chemotaxis (Fig. 3). When chemoattractants were added to the lower compartment of the Transwell chamber, chemotactic migration of CBO140C12 cells was apparently stimulated. EGF-induced chemotaxis of CBO140C12 cells was completely inhibited by 1 μM ZD1839. In contrast, HGF-induced chemotaxis was not affected by...
ZD1839, indicating that ZD1839 specifically targets EGF-induced changes and does not have a general effect on cell motility and cytoskeletal changes. Next, we investigated the effect of ZD1839 on the secretion of MMP-9, which is believed to play an important role in tumor invasion. Zymographic analysis showed that EGF-induced production of MMP-9 in CBO140C12 cells was completely inhibited by 1/50 M ZD1839 (Fig. 4). Taken together, these results indicate that ZD1839 inhibits the EGF-induced metastatic properties of CBO140C12 cells in vitro.

**Effect of ZD1839 on Metastasis in Vivo.** To evaluate the therapeutic efficacy of ZD1839 in vivo, we used the spontaneous intrahepatic metastasis model, which is considered to contain all steps of tumor metastasis (12). After orthotopic implantation of a tumor fragment, ZD1839 (75 mg/kg) was administered p.o. five times/week until sacrifice on day 21. First, we established an ex vivo model by which we could expect status of CBO140C12 tumor in ZD1839-treated mice. The cultured CBO140C12 cells were stimulated by 20% of serum collected at 4 h after administration on day 21. Although EGFR phosphorylation was not detected in both groups (data not shown), phospho-ERK and phospho-JNK levels in CBO140C12 cells treated with serum from ZD1839-treated mice were lower than vehicle-treated mice, suggesting that ERK and JNK in CBO140C12 tumor are being depressed when ZD1839 is administrated p.o. (Fig. 5). Moreover, administration of ZD1839 significantly reduced not only the volume of the implanted tumor but also the number of tumor colonies in the liver compared with the vehicle-treated controls, without causing loss of body weight (Fig. 6).

**Fig. 4.** Effect of ZD1839 on EGF-induced MMP-9 in CBO140C12 cells by gelatin zymography. CBO140C12 cells were cultured in EGF and/or ZD1839 for 12 h. The conditioned medium was analyzed using gelatin zymography. The data were shown with mean ± SD of two experiments. *, P < 0.05 and #, P < 0.05 compared with 5 and 50 ng/ml EGF, respectively. Similar results were obtained in three independent experiments.
Discussion

EGFR autocrine/paracrine pathways contribute to a number of processes important to the development and progression of cancer, including cell proliferation, apoptosis, angiogenesis, and metastatic spread. Our in vitro results using ZD1839 support previous reports that the selective EGFR-TKIs ZD1839, OSI-774, AG1478, and PD153035 inhibit cell proliferation and metastasis-associated processes, including tumor invasion, adhesion, and migration in vitro (6, 13–15). Here, we demonstrate that EGFR-TKI ZD1839 inhibits tumor metastasis in an in vivo model.

ZD1839 has been regarded as an anticancer drug that blocks cell cycle progression and induces apoptosis (8, 16). Culture with ZD1839 for 72 h reduced the number of CBO140C12 cells in vitro by ~50% (Fig. 1). In addition, ZD1839 reduced primary tumor growth in vivo (Fig. 6). Therefore, we cannot rule out the possibility that inhibition of intrahepatic metastasis by ZD1839 might be because of inhibition of tumor growth. However, we reported previously that anticancer drugs such as cisplatin and doxorubicin could significantly inhibit primary tumor growth, whereas intrahepatic metastasis was not affected (17). This result indicates that inhibition of intrahepatic metastasis needs a number of specific metastatic processes to be targeted in addition to inhibition of tumor growth. In this study, we showed that ZD1839 has antimetastatic action in CBO140C12 cells, including inhibition of chemotactic migration and MMP-9 production. These observations suggest that ZD1839 could inhibit intrahepatic metastasis, at least in part, by inhibition of the metastatic properties of tumor cells.

ZD1839 is also known to have antiangiogenic activities in host cells and tumor cells (18, 19). ZD1839 inhibits the production of autocrine/paracrine growth factors from tumor cells involved in autonomous local growth and angiogenesis (18) and the migration of normal endothelial cell lines on tumor angiogenesis model (19). Given that tumor angiogenesis provides channels through which cancer cells can metastasize (20), its antiangiogenic effect might be one of antimetastatic mechanisms of ZD1839 in vivo. However, metastasis is a complex cascade, so additional studies are necessary to clarify the precise antimetastatic mechanisms of ZD1839.

In conclusion, we have demonstrated that ZD1839 inhibits not only cell proliferation but also EGF-induced metastatic properties of tumor cells in vitro and intrahepatic metastasis in vivo. These findings carry two implications. One, that ZD1839 is effective in inhibiting metastasis; in addition to its anticancer effect, the antimetastatic effect of ZD1839 will have wide applications in the clinical setting, including in combination with other anticancer agents that do not demonstrate this antimetastatic effect and potential for use in early-stage disease in an adjuvant setting, and two, that EGFR signaling plays a crucial role in tumor metastasis in vivo. Revealing an in vivo mechanism of ZD1839 on inhibitory effect of intrahepatic metastasis is necessary to emphasize the importance of EGFR signaling. Histological analysis...
might provide a novel insight into understanding the mechanism. Additional in vivo studies using other specific EGFR inhibitors such as EGFR monoclonal antibodies and other EGFR-TKIs are required to support these findings.

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References
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