

# ZD1839 (Iressa) Induces Antiangiogenic Effects through Inhibition of Epidermal Growth Factor Receptor Tyrosine Kinase<sup>1</sup>

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## ABSTRACT

Epidermal growth factor receptor (EGFR) tyrosine kinase is a potential target for anticancer therapy. ZD1839 (Iressa) is a selective inhibitor of EGFR tyrosine kinase. In this study, we investigated the question as to whether the antitumor effect of ZD1839 is partly attributable to antiangiogenic activity and the potential mechanisms involved. Both ZD1839 and SU5416 [a vascular endothelial growth factor (VEGF)-receptor tyrosine kinase inhibitor] inhibited the migration of human umbilical vein endothelial cell cocultivated with EGF-stimulated cancer cells. ZD1839 also inhibited EGF-induced migration and the formation of tube-like structures by human microvascular endothelial cells. Moreover, ZD1839 almost completely blocked EGF-induced neovascularization of mice cornea, and SU5416 partially blocked neovascularization. In contrast, ZD1839 did not inhibit VEGF-induced angiogenesis. However, EGF-induced up-regulation of the angiogenic factors, VEGF and IL-8, was almost completely blocked by ZD1839. The antitumor effects of ZD1839 could, therefore, be mediated in part by the inhibition of tumor angiogenesis through direct effects on microvascular endothelial cells that express EGFR and also through reduced production of proangiogenic factors by tumor cells.

## INTRODUCTION

EGFR<sup>3</sup> is expressed in most cell types with the exception of hematopoietic cells (1). EGF and TGF $\alpha$  are not the only specific ligands for EGFR; heparin-binding EGF, epiregulin, betacellulin, and amphiregulin, and bind to EGFR. Homodimerization of EGFR molecules as well as heterodimerization of EGFR with other EGFR family including HER-2/neu induces ligand-specific activation of a number of intracellular signal transduction cascades such as phospholipase C $\gamma$ , phosphatidylinositol-3'-kinase, small G-proteins, Ras, the Ras GTPase-activating protein, growth factor receptor-binding protein 2, and Src family kinases (2), STAT, and Akt. EGFR, with its mitogenic influence, can contribute to the acquisition/development of malignant phenotypes. Moreover, EGFR activation plays a key role in cell adhesion, cell locomotion, and cell survival, invasion, and angiogenesis, which results in modulation of tumor progression (3).

The development and progression of malignant cancer is closely linked to aberrant EGFR signaling in various tumor types (2). Various mechanisms are likely to be involved in aberrant EGFR signaling: (a) ligand-independent tyrosine kinase activation of EGFR is often caused by the development of the constitutively active receptor mu-

tants, and this mutation is observed in various tumor types such as glioma, non-small cell lung cancer, and carcinomas of prostate, breast, ovary, and stomach (2, 4); (b) overexpression of ligands induces activation of normal EGFR: TGF $\alpha$ , which is overproduced by many different tumor types, enhances EGFR signaling as part of an autocrine loop; and (c) overexpression of wild-type EGFR may result from increased transcriptional or posttranscriptional mechanisms or gene amplification, and this has been associated with invasiveness, frequency of relapse, and prognosis in esophageal cancer, breast cancer, and other cancers (1). EGFR levels in human malignant cells are also critical for limiting sensitivity to several anticancer agents (5–7). EGFR is expected to be a valuable molecular target for development of novel and effective anticancer agents.

Tyrosine-kinase-targeting anticancer agents of the EGFR family that are currently under investigation include antibodies that block ligand binding to the extracellular region of the receptor, EGF/TGF $\alpha$ -toxin conjugates that target the extracellular ligand-binding region of the receptor, and tyrosine kinase inhibitors that act at the level of the intracellular receptor tyrosine kinase (2, 8–10). A recent highlight is the development of quinazoline-derived agents that are specific ATP-competitors of EGFR tyrosine kinase, one representative of which is ZD1839 (or Iressa; Ref. 11). ZD1839 shows antiproliferative activity in various human cancer cell types *in vitro* (12). Tumor growth inhibition *in vivo* by ZD1839 is potentiated by combination with a variety of cytotoxic anticancer agents (12, 13). ZD1839 is currently in Phase II and III clinical trials in cancer patients (11).

EGFR activation is often linked with invasion, metastasis, and angiogenesis; therefore, these processes might be affected by EGFR inhibition with ZD1839 (3). Several studies have shown that blockage of EGFR activation, by either antibodies or tyrosine kinase inhibitors, causes dose-dependent inhibition of production of the angiogenesis-related factors VEGF, TGF $\alpha$ , bFGF, and IL-8 in tumor cells, resulting in the modulation of angiogenesis (14–20). However, it remains unclear whether the blockage of EGFR signaling directly correlates with the inhibition of angiogenesis. In our present study, we used various angiogenesis models *in vitro* and *in vivo* to determine how angiogenesis is modulated by ZD1839.

## MATERIALS AND METHODS

### Materials

ZD1839 was provided by AstraZeneca (Macclesfield, United Kingdom). SU5416, a selective inhibitor of VEGF receptor tyrosine kinase, was obtained from SUGEN (South San Francisco, CA) and also Taiho Pharmaceutical Company. Recombinant human EGF was purchased from PeproTech (London, United Kingdom) and recombinant mouse VEGF was purchased from R&D Systems, Inc. (Minneapolis, MN). Anti-EGFR antibody and anti-phospho-EGFR antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies to ERK 1/2, phospho-ERK 1/2, Akt, phospho-Akt, and phospho-STAT1 were purchased from Cell Signaling Technology (Beverly, MA). Anti-STAT1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Received 10/30/01; accepted 3/5/02.

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<sup>1</sup> Supported by a grant-in-aid on cancer research from Ministry of Education, Culture, Sports, Science and Technology, Japan. "Iressa" is a trademark of the AstraZeneca group of companies.

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<sup>3</sup> The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; bFGF, basic fibroblast growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HUVE, human umbilical vein endothelium; IL, interleukin; STAT, signal transducer and activator of transcription; TGF $\alpha$ , transforming growth factor  $\alpha$ ; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; VEGF, vascular endothelial growth factor; KDR, kinase domain containing receptor; Flk-1, fetal liver kinase 1.

## Cell Culture

Human microvascular endothelial cells were isolated from normal omental tissue that was removed during surgery with informed consent under an institutional review board-approved protocol, and cells were cultured in M199 supplemented with 10% FBS, 60  $\mu\text{g}/\text{ml}$  kanamycin and 60  $\mu\text{g}/\text{ml}$  penicillin (21). We used cells at passage 3–5 because of the responsiveness of the endothelial cells to growth factors such as EGF during serial cultivation (22). HUVE cells purchased from Clonetics Inc (South San Francisco, CA) were cultured according to the manufacturers' instructions. Human vulvar squamous carcinoma A431 cells were cultured in DMEM supplemented with 10% FBS. Human epidermoid cancer cells, KB3-1, were cultured in MEM supplemented with 10% newborn calf serum. The cells were maintained under standard cell culture conditions at 37°C and 5%  $\text{CO}_2$  in a humid environment.

## Cell Migration Assay

For the cell migration assays we used two assay systems with two kinds of primary endothelial cells: HUVE cells, which respond to VEGF, and human microvascular endothelial cells, which respond to EGF.

**Migration Assay under Coculture System.** To assay for migration of human endothelial cells (23–25), A431 cells were grown in the outer chamber and then preincubated with various concentrations of ZD1839 or SU5416 followed by stimulation with 20 ng/ml EGF at 37°C. After 24 h, HUVE cells ( $3 \times 10^5$ ) were suspended in medium supplemented with 0.5% FBS and were seeded in the inner chamber with polycarbonate filters (8  $\mu\text{m}$  pores; Kurabo Inc, Tokyo, Japan) coated with human plasma fibronectin (1.33  $\mu\text{g}/\text{ml}$ ; Life Technologies, Inc.). After a 4-h incubation at 37°C, media in the inner chambers were aspirated, and cells on the upper surface of the filter were removed with a cotton swab. Cells on the lower surface were fixed with methanol and were stained with Giemsa. The stained nuclei were counted. Five fields ( $\times 100$ ) per chamber were counted, and average numbers were determined from assays with three chambers.

**Migration Assay of Human Microvascular Endothelial Cells.** This migration assay was performed with a multiwell chamber as the outer chamber. Polycarbonate filters (8  $\mu\text{m}$  pores) were coated with 1.33  $\mu\text{g}/\text{ml}$  fibronectin for 1 h at 37°C and used as the inner chamber (26). Human microvascular endothelial cells ( $3 \times 10^5$ ) were suspended in M199 containing 0.5% FBS and were seeded in the inner chamber. In the outer chamber, serial dilutions of ZD1839 with or without EGF (20 ng/ml) in the same medium were added. After incubation for 4 h at 37°C, nonmigrated cells on the upper surface of the filter were removed, and the cells that had migrated under the filter were counted. Five fields ( $\times 100$ ) per chamber were counted, and average numbers were determined from assays with three chambers.

## Quantitative Analysis of Tube Formation in Endothelial Cells on Type I Collagen Gel

The tube formation assay was performed as described previously (23, 27–29). In brief, human microvascular endothelial cells were plated onto type I collagen gel in a medium containing 10% FBS. When the cells reached confluence, that medium was replaced with a medium containing 1% FBS, with or without 20 ng/ml EGF and the various doses of ZD1839, and the cells were incubated for an additional 72 h. On the 3rd day, phase-contrast micrographs ( $\times 200$ ) were recorded using a still video camera recorder (R5000H; Fuji, Tokyo, Japan). The total length of the tube-like structures was measured for each field using a Cosmozone image analyzer (Nikon, Tokyo, Japan). Eight random fields were measured for each dish.

## Corneal Micropocket Assay in Mice and Quantification of Corneal Neovascularization

The corneal micropocket assay was performed essentially as described previously (30). Briefly, 0.3  $\mu\text{l}$  of Hydron pellets (IFN Sciences, New Brunswick, NJ) containing human EGF (200 ng) or murine VEGF (200 ng) were prepared and implanted in the corneas of male BALB/c mice. ZD1839 was administered by adding directly to the growth factor/Hydron solution (20 ng/pellet) or by injecting i.p. (50 mg/kg/day) on days 1–6.

SU5416 was administered by i.p. injection (25 mg/kg/day) on days 1–6. On day 7, mice were sacrificed and their corneal vessels were photographed. Images of the corneas were recorded using Nikon Coolscan software with

standardized illumination, contrast, and threshold settings, and were saved on disk. Areas of corneal neovascularization were analyzed using the software package NIH Image 1.61 (31) and were expressed in  $\text{mm}^2$ .

## ELISA of VEGF and IL-8

The concentration of VEGF and IL-8 in the conditioned medium of the human cancer cell lines A431 and KB3-1 and the human microvascular endothelial cells were measured using commercially available ELISA kits (32). These cells were plated in 24-well dishes in a medium containing 10% FBS. When the cells reached subconfluence, the medium was replaced with a serum-free medium for 24 h. The cells were then incubated with various concentrations of ZD1839 for 3 h followed by 20 ng/ml EGF at 37°C. Assays were performed after 24-h incubation with serum-free conditioned medium. Results were normalized for the number of cells and reported as picograms of growth factor/ $10^6$  cells/24 h.

## Western Blot Analysis

A431 or KB3-1 cells were cultured in serum-free medium, and human microvascular endothelial cells were cultured in medium containing 0.5% FBS for 24 h. The cells were then incubated with ZD1839 at concentrations increasing from 0 to 1  $\mu\text{M}$  for 3 h before 20-ng/ml EGF stimulation for 15 min at 37°C. The cells were then rinsed with ice-cold PBS and lysed in Triton X-100 buffer (50  $\mu\text{M}$  HEPES, 150  $\mu\text{M}$  NaCl, 1% Triton X-100, and 10% glycerol containing 1 mM PMSF, 10  $\mu\text{g}/\text{ml}$  aprotinin, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 1 mM sodium vanadate). Cell lysates were subjected to SDS PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA). After transfer, blots were incubated with the blocking solution and probed with anti-EGFR antibody, anti-phospho-EGFR antibody, anti-ERK 1/2 antibody, anti-phospho-ERK 1/2 antibody, anti-STAT1 antibody, anti-phospho-STAT1 antibody, anti-Akt antibody and anti-phospho-Akt antibody, followed by washing. The protein content was visualized using horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL; Amersham).

## Statistical Analysis

Statistical comparisons were performed using Mann-Whitney's test;  $P \leq 0.05$  was considered significant.

## RESULTS

**Inhibition of EGFR Tyrosine Kinase and Activation of ERK by ZD1839.** We examined the effect of ZD1839 on EGFR phosphorylation in human cancer cell lines. EGFR was apparently phosphorylated by exogenous addition of EGF in two cell lines, A431 and KB3-1 (Fig. 1). ZD1839 (0.05  $\mu\text{M}$ ) inhibited the EGF-induced EGFR autophosphorylation by  $>80\%$  and, at 0.5  $\mu\text{M}$ , almost completely blocked the autophosphorylation. ZD1839 at 0.5  $\mu\text{M}$  almost completely inhibited a downstream EGFR signaling transduction pathway, EGF-dependent phosphorylation of ERK 1 and ERK 2 (Fig. 1).

**ZD1839 Inhibits Production of Two Potent Angiogenic Factors, VEGF and IL-8, in Human Cancer Cells.** The blockade of EGFR activation appears to modulate the production of several angiogenic factors in tumor cell lines (14–20). We also examined whether EGF could enhance production of the potent angiogenic factors VEGF and IL-8 in human cancer cells. As shown in Fig. 2, treatment of A431 and KB3-1 cells with EGF enhanced production of VEGF about 3- to 4-fold over the untreated control; ZD1839 at 0.5  $\mu\text{M}$  almost completely inhibited this EGF-induced VEGF production. The production of IL-8 was enhanced more than 10-fold by EGF, and almost completely blocked by ZD1839 at 0.5  $\mu\text{M}$ .

We have previously reported that human microvascular endothelial cells enhance the expression of VEGF, IL-8, and bFGF in response to the cytokine  $\text{TNF}\alpha$  (33, 34). We have observed that EGF also enhanced expression of VEGF, and that ZD1839 blocked the EGF-dependent production of VEGF in human microvascular endothelial cells (data not shown).

**ZD1839 Inhibits Migration by Vascular Endothelial Cells *in Vitro*.** Cell proliferation and migration and tube formation of vascular endothelial cells is requisite for the development of neovasculatures. We constructed a cell migration assay system under cocultivation with cancer cells in a double-chamber model (Fig. 3A). HUVE cells, when alone, demonstrated no enhancement of cell migration in the presence of EGF (Fig. 3B). However, when HUVE and A431 cells were both present, EGF enhanced the migration of HUVE cells. ZD1839 at 0.5  $\mu\text{M}$  or SU5416 at 5  $\mu\text{M}$  almost completely inhibited the cell migration by HUVE cells in response to EGF. Under the coculture conditions, HUVE cells did not respond to EGF, and SU5416 at 5  $\mu\text{M}$  inhibited cell migration completely. ZD1839 thus inhibited HUVE cell migration through blockage of EGF-induced VEGF production from A431 cells (Fig. 3B).

Because human microvascular endothelial cells respond to EGF, we next examined the direct effect of ZD1839 on EGF-induced migration by microvascular endothelial cells. ZD1839, at 0.5–1  $\mu\text{M}$ , dose-dependently inhibited EGF-induced migration by human microvascular endothelial cells (Fig. 3C).

**ZD1839 Inhibits Downstream Signaling of EGFR and Formation of Tube-like Structures by Microvascular Endothelial Cells *in Vitro*.** In the presence of EGF/TGF $\alpha$ , EGFR in human microvascular endothelial cells becomes highly phosphorylated, and the cells

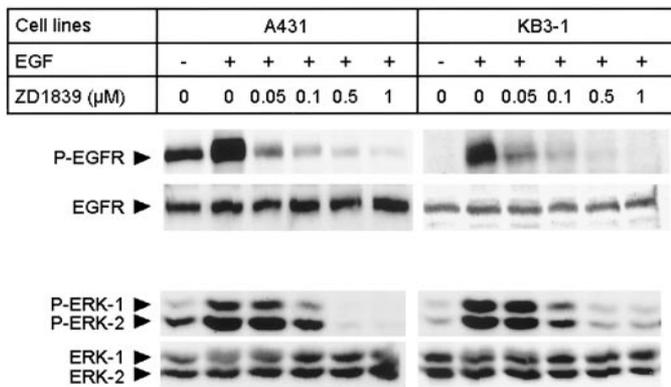


Fig. 1. Dose-dependent inhibition of EGF-induced EGFR autophosphorylation and ERK 1/2 phosphorylation by ZD1839 in human cancer cells. Serum-starved cancer cells (A431 and KB3-1) were treated for 3 h with the indicated concentrations of ZD1839, followed by the addition of EGF (20 ng/ml) for 15 min. Protein extracts were resolved by 7.5% SDS-PAGE and probed with either antibody. EGFR activity was determined using an anti-phospho-EGFR antibody, and the EGFR protein level was evaluated by using an anti-EGFR antibody. ERK 1/2 activity was determined using an anti-phospho-ERK 1/2 antibody, and the ERK 1/2 protein level was evaluated by using an anti-ERK 1/2 antibody.

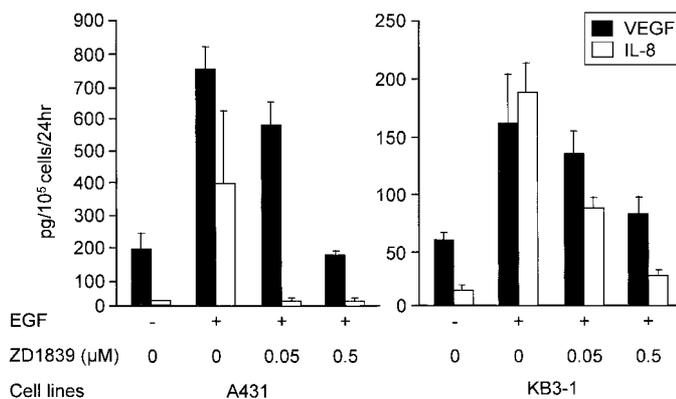


Fig. 2. Inhibition of VEGF and IL-8 protein production by ZD1839 after EGF (20 ng/ml) stimulation of cancer cells for 24 h. VEGF and IL-8 were measured by ELISA. Values are the mean (+SD) of triplicate cultures and have been normalized to account for cell number.

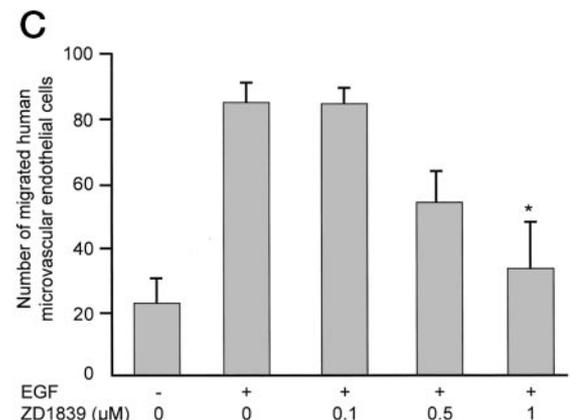
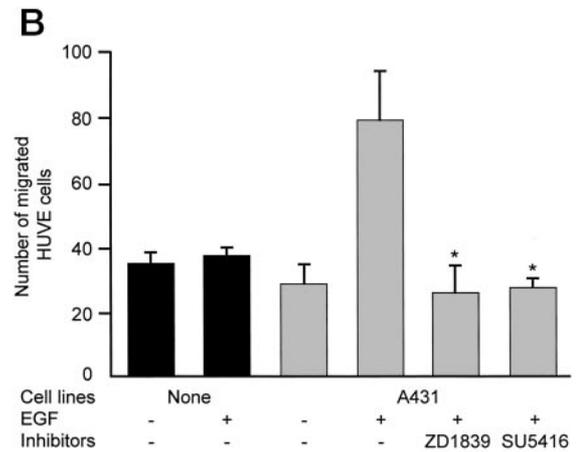
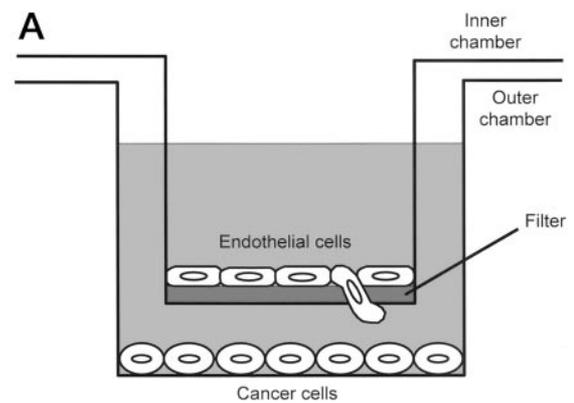


Fig. 3. Migration of HUVE cells under cocultivation with human cancer cells in the absence or presence of EGF, and the effects of ZD1839 and SU5416. In our double chamber assay (A), A431 cells preincubated with ZD1839 (0.5  $\mu\text{M}$ ) or SU5416 (5  $\mu\text{M}$ ) were grown in the outer chamber, then stimulated with EGF. After 24 h, the inner chamber was transferred to the outer chamber, and HUVE cells were placed in the inner chamber. B, after a 4-h incubation, cells that migrated to the lower surface of the filter were counted. Bars, the mean (+SD) of three independent experiments; \*, statistically significant difference ( $P < 0.01$ ) compared with the value obtained in the absence of any drugs. C, dose-dependent inhibition of EGF-induced migration of human microvascular endothelial cells by ZD1839 at 0.5 to 1  $\mu\text{M}$ . Bars, the mean (+SD) of three independent experiments; \*, statistically significant difference ( $P < 0.01$ ) compared with the value obtained from lower ZD1839 doses.

form tube-like structures in collagen gel (22, 24, 27). Consistent with our previous study (22), EGFR in microvascular endothelial cells was markedly phosphorylated by EGF (Fig. 4). ZD1839, at 0.5–1  $\mu\text{M}$ , almost completely inhibited the EGF-dependent EGFR autophosphorylation and, at 0.5  $\mu\text{M}$ , completely inhibited the downstream signaling ERK 1/2 and Akt phosphorylation. Even at 0.05  $\mu\text{M}$ , ZD1839 could inhibit phosphorylation of another EGFR downstream signaling molecule (STAT1).

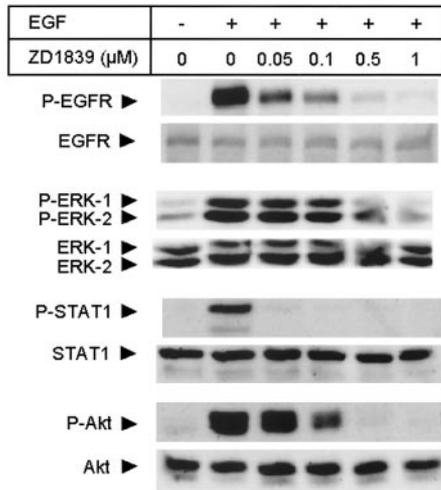


Fig. 4. Inhibition of EGF-induced EGFR autophosphorylation and ERK 1/2, STAT1, and Akt phosphorylation by ZD1839 in human microvascular endothelial cells. Serum-starved endothelial cells were treated for 3 h with ZD1839, followed by the addition of EGF (20 ng/ml) for 15 min. Protein extracts were resolved by 7.5% SDS-PAGE and probed with either antibody. EGFR, ERK 1/2, STAT1, and Akt activity were determined using the corresponding anti-phospho antibody. Immunoreactive proteins were visualized by enhanced chemiluminescence.

Consistent with our previous studies (24, 27), EGF stimulated development of tube-like structures in human microvascular cells 2- to 3-fold over the control. ZD1839, at 0.5–1.0  $\mu\text{M}$ , inhibited the formation of these tube-like structures (Fig. 5). ZD1839 thus appeared

to inhibit both the cell migration and the formation of tube-like structures by microvascular endothelial cells *in vitro*.

**ZD1839 Inhibits EGF-induced Angiogenesis *in Vivo*.** We then examined the effect of ZD1839 on angiogenesis developed in the cornea in mice. EGF as well as VEGF markedly induced angiogenesis in an avascular area of the cornea (Fig. 6). Administration of ZD1839 blocked the EGF-induced neovascularization when the drug was administered not only as pellets but also i.p. Daily i.p. administration of SU5416 also inhibited EGF-induced neovascularization, but its inhibition appeared to be less than that of ZD1839. In contrast, VEGF-induced neovascularization was inhibited by the administration of SU5416 but not of ZD1839 (Fig. 6).

Quantitative analysis, using four mice for each assay, showed >80% inhibition of EGF-induced neovascularization when ZD1839 was administered i.p. or as pellets, and about 60% inhibition of EGF-induced neovascularization was observed after i.p. administration of SU5416 (Fig. 7). There appeared to be no inhibition by ZD1839 of VEGF-induced neovascularization, whereas about 70% inhibition compared with control was observed after i.p. administration of SU5416 (Fig. 7).

## DISCUSSION

Recently, EGFR-targeting anticancer therapeutic agents, such as monoclonal antibodies and small-molecule tyrosine kinase inhibitors have been developed (3, 8–10). ZD1839 is a potent antitumor agent that has been developed to target EGFR tyrosine kinase. Using an angiogenesis model assay system, we demonstrated that ZD1839 could modulate the migration and formation of tube-like structures of

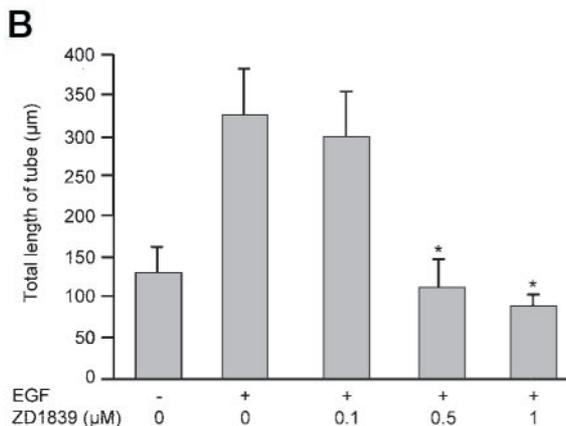
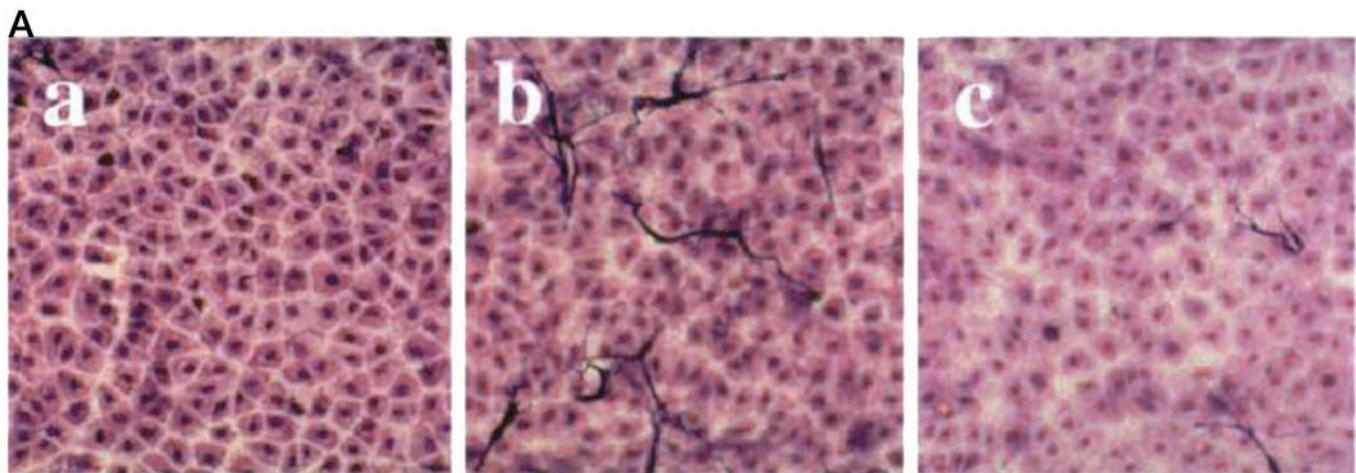


Fig. 5. A, appearance of tube-like structures in type I collagen gel by human microvascular endothelial cells. These cells were plated onto type I collagen gel in medium supplemented with 10% FBS. In A-a, when cells became confluent, the cells assumed a cobblestone-like appearance. In A-b, tube-like structures appeared after a 3-day incubation in medium containing 1% FBS in the presence of EGF (20 ng/ml). In A-c, EGF-induced tubular morphogenesis was inhibited by ZD1839 (0.5  $\mu\text{M}$ ). The cells were fixed and stained briefly in modified May-Gruenwald solution to visualize the tube-like structures ( $\times 400$ ). B, effect of ZD1839 on the formation of EGF-induced tube-like structures. Cells were plated onto type I collagen gel in medium that contained 10% FBS. After the cells became confluent, the medium was replaced with medium containing 1% FBS. The cells were exposed to 20 ng/ml EGF with various concentrations of ZD1839. Tube formation was quantified after additional incubation for 72 h. Columns are mean ( $\pm$ SD) of triplicate experiments. \*, statistically significant difference ( $P < 0.05$ ) compared with the value obtained in the absence of ZD1839.

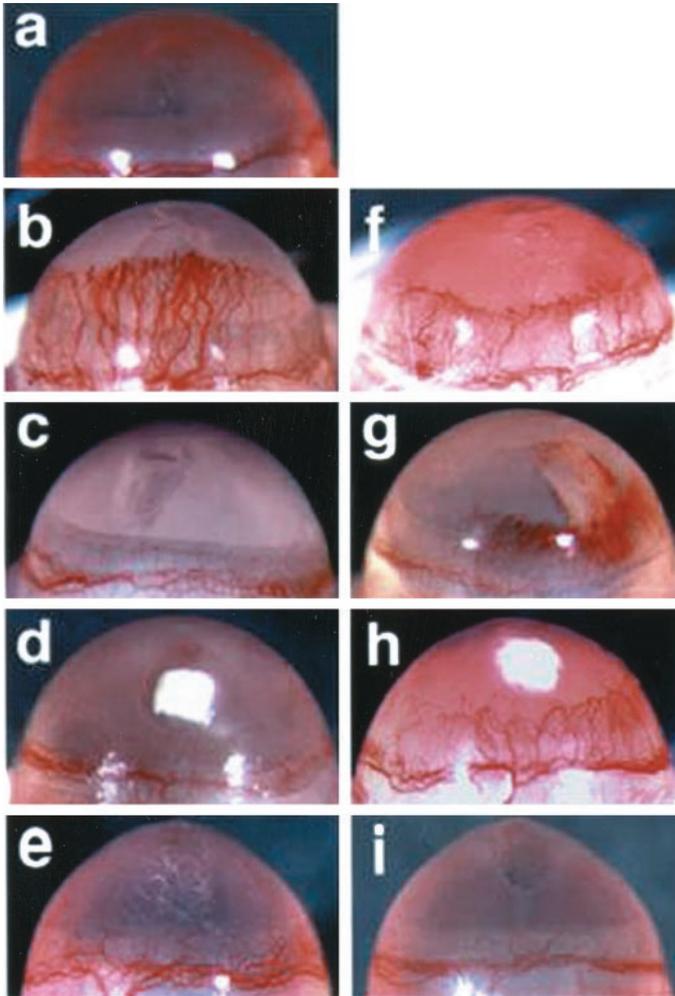


Fig. 6. Effect of ZD1839 on angiogenesis *in vivo*. Angiogenesis induced in mouse corneas by EGF was inhibited by ZD1839 and partially inhibited by SU5416. Hydron pellets containing 200 ng of EGF or 200 ng of VEGF were implanted into the corneas of BALB/c mice. Mice were treated with ZD1839 (50 mg/kg/day, by i.p. injection, or by 20 ng in pellet) or SU5416 (25 mg/kg/day i.p.) on days 1–6. Six days later, vessels in the region of the pellet implant were photographed. Representative photographs of mouse corneas with Hydron pellets containing (a) buffer alone, (b) EGF, (c) EGF with ZD1839 (i.p.), (d) EGF with ZD1839 (pellet), (e) EGF with SU5416 (i.p.), (f) VEGF, (g) VEGF with ZD1839 (i.p.), (h) VEGF with ZD1839 (pellet), (i) VEGF with SU5416 (i.p.), (j) VEGF with SU5416 (pellet).

vascular endothelial cells. We also showed that angiogenesis induced by EGF *in vivo* was blocked by the administration of ZD1839. Therefore, we have demonstrated that ZD1839 can directly modulate angiogenesis *in vitro* as well as *in vivo*.

EGFR activation is expected to modulate various critical processes in the development, maintenance, and spread of malignant tumors, such as invasion, metastasis, and angiogenesis (3, 35). TGF $\alpha$  and EGF are potent inducers of angiogenesis, and coexpression of EGFR and TGF $\alpha$  is closely associated with microvessel density in invasive cancers (36, 37). Various oncogenes such as *H-ras*, *v-src*, *polyoma middle T antigen*, and *fos/jun* can modulate expression of EGFR (38–40), which suggests a close correlation between EGFR activity and malignant states. We have previously reported that EGF/TGF $\alpha$  induces cell migration and formation of tube-like structures by human microvascular endothelial cells (21, 22, 27, 30). Moreover, expression of metalloproteinases and plasminogen activator is enhanced in human microvascular endothelial cells *in vitro* by EGF/TGF $\alpha$  (41–43). Under cocultivation conditions, using an angiogenesis model system with a double chamber, human microvascular endothelial cells form tube-like structures by the production of TGF $\alpha$  from cocultivated

human glioma cells or esophageal cancer cells (24, 44). Hence, either EGF or TGF $\alpha$  can induce various angiogenic stimuli, not only from cancer cells but also from endothelial cells that express EGFR, which supports previous reports that TGF $\alpha$  and EGF are angiogenic factors (37, 45). The EGFR thus appears to play a key role in the angiogenic response to EGF/TGF $\alpha$ . Our present study indicates that the EGF-induced formation of tube-like structures by human microvascular endothelial cells in type I collagen gel is blocked by ZD1839. ZD1839 thus directly inhibits EGF-induced angiogenesis, possibly through the blockade of EGF/TGF $\alpha$ -EGFR signaling.

Phosphorylation of two downstream signaling molecules of EGFR, ERK 1/2 and Akt, was completely inhibited by ZD1839 at 0.5  $\mu$ M in microvascular endothelial cells. ZD1839 (0.05  $\mu$ M) appeared to cause almost complete inhibition of EGF-induced STAT1 phosphorylation. These data suggest that STAT1 phosphorylation is more sensitive to inhibition by ZD1839 than is phosphorylation of either ERK 1/2 or Akt. It remains unclear how the inhibition of STAT1 signaling is associated with the antiangiogenic activity of ZD1839 and the reasons for the sensitivity of this pathway to ZD1839.

The two cancer cell lines (A431 and KB3-1) produced higher amounts of VEGF and IL-8 in response to EGF than the human microvascular endothelial cells. However, we did not observe a significant stimulation of bFGF production by EGF in the cancer cell lines (data not shown). The EGF-dependent production of VEGF and IL-8 was markedly blocked by ZD1839. Consistent with our result, the inhibition of EGFR activation by a specific antibody or an EGFR tyrosine kinase inhibitor has been shown to reduce production of VEGF, bFGF, IL-8, and TGF $\alpha$  in various cancer cell types (14–20). Migration of HUVE cells, which don't respond to EGF, was stimulated when human cancer cells were cocultivated in the presence of EGF, and this EGF-dependent migration was almost completely blocked by ZD1839 and SU5416. Moreover, angiogenesis *in vivo* was markedly induced by EGF, and this angiogenesis was inhibited completely by ZD1839 and partially by SU5416. It has been reported that the IC<sub>50</sub> of SU5416 against VEGF receptor-2 (KDR/F1k-1) tyrosine kinase is  $1.3 \pm 0.8 \mu$ M and against EGFR tyrosine kinase is  $>100 \mu$ M (46). Administration of SU5416, but not ZD1839, inhibited VEGF-induced angiogenesis in mice.

We have previously reported that the inflammatory cytokines TNF $\alpha$  and IL-1 $\alpha$  enhances production of VEGF, IL-8, bFGF, and metalloproteinases from vascular endothelial cells or cancer cells (32, 33, 34, 47), and also that these angiogenesis-related factors appear to play a key role in angiogenesis by TNF $\alpha$  or IL-1 $\alpha$  under autocrine/paracrine control (48). Similar paracrine and/or autocrine controls

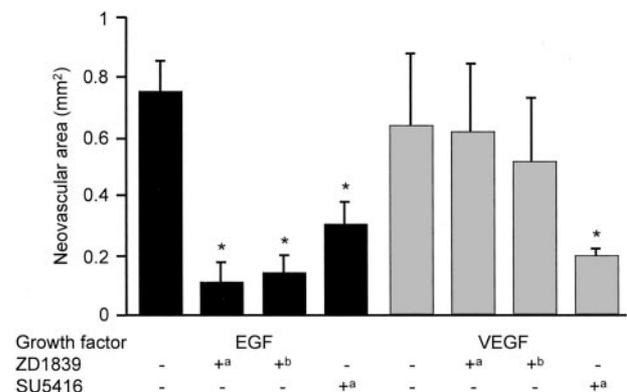


Fig. 7. Quantification of corneal neovascularization in mice after treatment with ZD1839 or SU5416. Neovascular areas developed in mouse cornea as seen in Fig. 6 were quantified as described in "Materials and Methods." Columns are mean ( $\pm$ SD) of three or four independent experiments. \*, statistically significant difference ( $P < 0.05$ ) from value for EGF or VEGF alone; +, drug administered i.p.; +, drug administered in pellet.

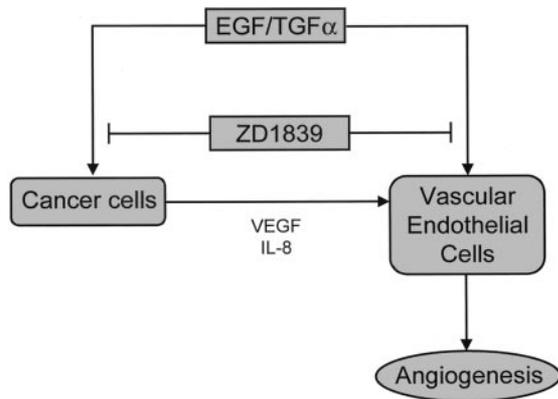


Fig. 8. A model for antiangiogenic effects by ZD1839. EGF/TGF $\alpha$  up-regulates expression of angiogenic factors (VEGF and IL-8) in cancer cells, and these angiogenic-related factors induce angiogenesis by paracrine control. ZD1839 is expected to inhibit EGF/TGF $\alpha$ -induced angiogenesis through direct interaction of vascular endothelial cells and through EGF/TGF $\alpha$ -induced production of angiogenesis-related factors.

could operate through the interaction of EGF/TGF $\alpha$  with EGFR; and VEGF, IL-8, and other angiogenesis-related factors are expected to be involved in EGF-induced angiogenesis (see Fig. 8). The partial inhibition of EGF-induced angiogenesis *in vivo* as well as *in vitro* by SU5416 might be partly attributable to reduced production of angiogenic factors, in particular VEGF. Using various angiogenesis assay models, we found that EGF/TGF $\alpha$ -induced angiogenesis might be attributable to its signaling through direct interaction with vascular endothelial cells, and also to angiogenesis-related factors, such as VEGF, IL-8, and others, that are produced from cancer cells and, possibly, from vascular endothelial cells (Fig. 8).

In conclusion, interaction of EGF/TGF $\alpha$  with EGFR and its family of proteins can affect not only cell proliferation and tumor growth but also invasion, metastasis, and angiogenesis. ZD1839 is, thus, expected to have inhibitory effects on all of these biological processes, not only in cancer cells but also in endothelial cells through inhibition of EGF/TGF $\alpha$ -EGFR signaling. This study demonstrates that ZD1839 shows antiangiogenic activity *in vitro* as well as *in vivo*.

## ACKNOWLEDGMENTS

We thank Drs. K. Takenaka (Fukuoka Municipal Hospital, Fukuoka, Japan), Y. Maehara (Kyushu University, Fukuoka), and T. Okamoto (Kyushu Cancer Center, Fukuoka), for supplying human omentum.

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*Cancer Res* 2002;62:2554-2560.

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