Inhibition of Growth Factor Production and Angiogenesis in Human Cancer Cells by ZD1839 (Iressa),[1] a Selective Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor[2]

Fortunato Ciardiello,[3] Rosa Caputo, Roberto Bianco, Vincenzo Damiano, Gabriella Fontanini, Sabina Cuccato, Sabino De Placido, A. Raffaele Bianco, and Giampaolo Tortora

Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Università degli Studi di Napoli "Federico II," 80131 Naples [F. C., R. C., R. B., G. P., V. D., A. R. B., G. T.], and Divisione di Anatomia Patologica, Dipartimento di Oncologia, Università di Pisa, 56100 Pisa [G. F., S. C.], Italy

ABSTRACT

The transforming growth factor-α/epidermal growth factor receptor (TGF-α-EGFR) autocrine pathway, which is involved in the development and the progression of human epithelial cancers, controls, in part, the production of angiogenic factors. These angiogenic factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), are secreted by cancer cells to stimulate normal endothelial cell growth through paracrine mechanisms. ZD1839 (Iressa) is a p.o.-active, selective EGFR-tyrosine kinase inhibitor (TKI) in clinical trials in cancer patients. In this study, we evaluated the antiangiogenic and antitumor activity of ZD1839 in human colon (GEO, SW480, and CaCo2), breast (ZR-75-1 and MCF-7 ADR), ovarian (OVCAR-3), and gastric (KATO III and N87) cancer cells that coexpress TGF-α and EGFR. ZD1839 treatment determined a dose- and time-dependent growth inhibition accompanied by the decrease of VEGF, bFGF and TGF-α production *in vitro*. Treatment of immunodeficient mice bearing well-established, palpable GEO xenografts with ZD1839 determined a cytostatic dose-dependent tumor growth inhibition. Immunohistochemical analysis of GEO tumor xenografts after ZD1839 treatment revealed a significant dose-dependent reduction of TGF-α, bFGF, and VEGF expression in cancer cells and of neoangiogenesis, as determined by microvessel count. Furthermore, the antitumor activity of ZD1839 was potentiated in combination with the cytotoxic drug paclitaxel in GEO tumor xenografts. Tumor regression was observed in all mice after treatment with ZD1839 plus paclitaxel, and it was accompanied by a significant potentiation in inhibition of TGF-α, VEGF, and bFGF expression with a few or no microvessels. Furthermore, 6 of 16 mice bearing well-established, palpable GEO xenografts had no histological evidence of GEO tumors at the end of treatment with ZD1839 plus paclitaxel. These results demonstrate that the antitumor effect of ZD1839 is accompanied by inhibition in the production of autocrine and paracrine growth factors that sustain autonomous local growth and facilitate angiogenesis, and that this effect can be potentiated by the combined treatment with certain cytotoxic drugs, such as paclitaxel.

INTRODUCTION

Growth factors regulate cancer development through several mechanisms. These include uncontrolled cell growth attributable to the autocrine production of growth factors by cancer cells and stimulation of tumor neovascularization as a result of paracrine stimulation of normal endothelial cells by angiogenic growth factors secreted by cancer cells (1, 2).

Tumor angiogenesis, the process leading to the formation of new blood vessels within the tumor mass, plays a central role in cancer cell survival, in tumor growth, and in the development of distant metastasis (2). In this respect, the formation of new blood vessels is essential for providing an adequate oxygen and nutrient supply to the growing tumor mass and for initiating metastatic spread (2). The development of blood vessels is regulated by the production of several growth factors and growth inhibitors (3). Different growth factors, such as bFGF,[4] VEGF, and TGF-α, have been identified as positive regulators of angiogenesis and are secreted by cancer cells to stimulate normal endothelial cell growth through paracrine mechanisms (4–6). VEGF is a potent and specific mitogen for endothelial cells that activates the angiogenic switch *in vivo* and enhances vascular permeability (6). Enhanced expression of VEGF has been observed in human cancers including colorectal, breast, non-small cell lung, and ovarian cancers and is directly correlated with increased neovascularization as measured by MVC

Received 11/28/00; revised 2/20/01; accepted 2/28/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Iressa is a trademark of the AstraZeneca group of companies.

[2] Supported by grants from the Associazione Italiana per la Ricerca sul Cancro and from the Consiglio Nazionale delle Ricerche Target Project on Biotechnologies.

[3] To whom requests for reprints should be addressed, at Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli Federico II, Via S. Pansini 5, 80131 Napoli, Italy. Phone: 39-081-7462061; Fax: 39-081-7462066; E-mail: fortunatociardiello@yahoo.com.

[4] The abbreviations used are: bFGF, basic fibroblast growth factor; IC_{50}, 50% inhibitory concentration; EGF, epidermal growth factor; EGFR, EGF receptor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; MAb, monoclonal antibody; TKI, tyrosine kinase inhibitor; CM, conditioned medium; MVC, microvessel count.

Downloaded from clincancerres.aacrjournals.org on February 25, 2021. © 2001 American Association for Cancer Research.

within the tumor (7, 8). Moreover, the density of microvessels in the areas of most intense neovascularization has been demonstrated to be an independent prognostic marker in breast and non-small cell lung cancer patients (9–11).

The TGF-α-EGFR autocrine pathway plays a key role in the development and the progression of human epithelial cancers (12). Overexpression of TGF-α and/or EGFR has been detected in the majority of human carcinomas. This overexpression has been associated with resistance to cytotoxic drugs and to hormone therapy in human breast cancer and is generally an indicator of poor prognosis (12). For these reasons, the blockade of the TGF-α-EGFR autocrine pathway has been proposed as a target for anticancer therapy (13).

Experimental evidence has been provided for a link between EGFR signaling and angiogenic mechanisms. In fact, it has been shown that EGF and TGF-α can up-regulate the production of VEGF in human cancer cells (14, 15). Furthermore, we and others have suggested recently that treatment with anti-EGFR blocking MAbs determines an antitumor effect *in vivo* that is attributable both to the direct blockade of the EGFR-dependent mitogenic pathway and, at least in part, to the inhibition of the secretion of various paracrine growth factors, including TGF-α, VEGF, bFGF, and interleukin 8, that stimulate the migration, proliferation, and functional differentiation of intratumor endothelial cells (16–18).

A promising approach for the therapeutic blockade of EGFR signaling in human cancer has been developed recently with the discovery of low molecular weight compounds that inhibit the ligand-induced activation of the EGFR tyrosine kinase activity necessary for all receptor-activated intracellular signaling (19, 20). Among various quinazoline-derived agents that have been tested as anticancer drugs *in vitro* and in preclinical models (20), ZD1839 (Iressa) is a p.o.-active, selective EGFR-TKI that blocks signal transduction pathways implicated in proliferation and survival of cancer cells (13) and is currently in clinical trials in cancer patients.

In the present study, we tested the effect of ZD1839 treatment on the production of TGF-α, VEGF, and bFGF in several human cancer cell lines, including ovarian, breast, colon, and gastric cancers, that express both EGFR and TGF-α. Furthermore, the antiangiogenic activity of ZD1839 was evaluated *in vivo* in nude mice bearing well-established GEO colon cancer xenografts. Because we have shown recently that ZD1839 significantly potentiates the antitumor activity of some chemotherapeutic agents, such as paclitaxel (21), we also evaluated whether this activity was accompanied by an antiangiogenic effect *in vivo*.

MATERIALS AND METHODS

Materials. ZD1839 was provided by AstraZeneca (Macclesfield, United Kingdom). Paclitaxel was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines. GEO human colon cancer, OVCAR-3 human ovarian cancer, and ZR-75-1 human breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). MCF-7 ADR human breast cancer cells were kindly provided by Dr. G. Zupi (Istituto Nazionale per la Ricerca sul Cancro "Regina Elena," Rome, Italy). Human gastric (KATO III and N87) and colon (SW480 and CaCo2) cancer cell lines were obtained from Prof. B. Jansen (University of Vienna, Vienna, Austria). All cancer cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), 100 UI/ml penicillin, 100 μg/ml streptomycin, and 4 mM glutamine (ICN, Irvine, United Kingdom) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

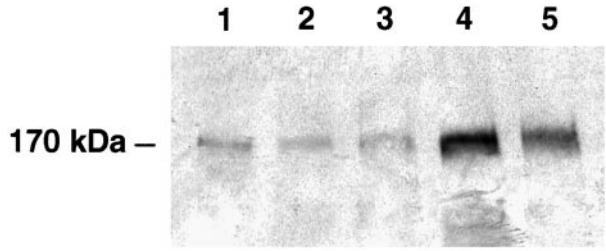

Fig. 1 Western blot analysis of EGFR expression in human cancer cell lines. *Lane 1*, GEO cells; *Lane 2*, ZR-75-1 cells; *Lane 3*, N87 cells; *Lane 4*, MCF-7 ADR cells; *Lane 5*, SW480 cells. Total cell proteins (50 μg) were fractionated through 7.5% SDS-PAGE, transferred to nitrocellulose filters, and incubated with a specific antihuman EGFR MAb. Immunoreactive proteins were visualized by enhanced chemiluminescence.

Western Blot Analysis. Total cell protein extracts (50 μg of total proteins/lane) were resolved by electrophoresis using 7.5% SDS-PAGE precast gels (Bio-Rad Laboratories, Milan, Italy), transferred to nitrocellulose filters, and incubated with an antihuman EGFR MAb (Transduction Laboratories, Lexington, KY). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, Buckinghamshire, United Kingdom), as described previously (21).

Growth in Soft Agar. Cells (10^4 cells/well) were suspended in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson, Lincoln Park, NJ) and treated each day for 5 days with different concentrations of ZD1839 (range, 0.01–2.5 μM). After 10–14 days, cells were stained with nitro blue tetrazolium (Sigma Chemical Co.), and colonies >0.05 mm were counted as described previously (21).

Evaluation of TGF-α, VEGF, and bFGF Secretion. The concentration of TGF-α, VEGF, or bFGF in the CM obtained from the various human cancer cell lines was measured using commercially available sandwich ELISA kits and according to the manufacturers' instructions. The ELISA kits for VEGF and for bFGF were purchased from R&D Systems, Inc. (Minneapolis, MN). The ELISA kit for TGF-α was purchased from Oncogene Research Products (Cambridge, MA). GEO cells were plated in 60-mm dishes (Becton Dickinson) and treated for 4 days with different concentrations of ZD1839. Assays were performed using 24-h-collected, serum-free CM. Results were normalized for the number of producing cells and reported as pg of growth factor/10^6 cells/24 h.

GEO Xenografts in Nude Mice. Female BALB/c athymic (*nu+/nu+*) mice, 4–6 weeks of age, were purchased from Charles River Laboratories (Milan, Italy). The research protocol

Downloaded from clincancerres.aacrjournals.org on February 25, 2021. © 2001 American Association for Cancer Research.

Table 1 EGFR, TGF-α, bFGF, and VEGF expression in human cancer cell lines

EGFR expression was determined by Western blotting. EGFR relative levels were determined as compared with EGFR levels in GEO cells. GEO cells possess ~40,000 binding sites/cell as determined by ^{125}I-labeled EGF binding assay (23). TGF-α, bFGF, and VEGF secretion was determined on 24-h-collected serum-free CM by using specific ELISA kits, as described in "Materials and Methods." Results were normalized for the number of producing cells and reported as pg of growth factor/10^6 cells/24 h.

Cell line	EGFR (relative expression)	TGF-α (pg/10^6 cells/24 h)	bFGF (pg/10^6 cells/24 h)	VEGF (pg/10^6 cells/24 h)
ZR-75-1	0.6	285 ± 30	195 (±10)	85 ± 5
MCF-7 ADR	6.2	580 ± 25	255 ± 35	75 ± 5
OVCAR-3	3.7	680 ± 35	645 ± 55	55 ± 4
GEO	1	405 ± 15	710 ± 55	150 ± 8
SW480	3.5	480 ± 20	600 ± 65	400 ± 12
CaCo2	0.6	255 ± 15	ND[a]	110 ± 8
KATO III	1	340 ± 25	ND	180 (±8)
N87	0.4	360 ± 35	380 ± 40	145 (±20)

[a] ND, not detectable.

was approved, and mice were maintained in accordance with the institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimatized at the University of Naples Medical School Animal Facility for 1 week prior to being injected with cancer cells. Mice were injected s.c. with 10^7 GEO cells that had been resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA). After 7 days, when established tumors of approximately 0.2–0.3 cm^3 in diameter were detected, 6 mice/group were treated i.p. on days 1–5 of each week for 2 weeks with ZD1839 at the indicated daily doses. To determine the effects of the combination of ZD1839 and paclitaxel, additional groups of 16 mice were treated i.p. on days 1–5 of each week for 3 weeks with ZD1839, 3.75 mg/mouse/day, in combination with 20 mg/kg paclitaxel on day 1 of each week for 3 weeks. Tumor size was measured using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as reported previously (21).

Immunohistochemical Analysis. Immunocytochemistry was performed on formalin-fixed, paraffin-embedded tissue sections (5 μm) of GEO xenografts as reported previously (16). After overnight incubation with the appropriate primary antibody at 4°C, sections were washed and treated with an appropriate secondary biotinylated goat antibody (1:200 dilution; Vectastain ABC kit; Vector Laboratory, Burlingame, CA), washed, reacted with avidin-conjugated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide, as described previously (22). The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted. The following antibodies were used: an anti-Ki67 MAb (clone MIB1; DBA, Milan, Italy) used at 1:100 dilution; an anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) used at 1:50 dilution; an anti-bFGF rabbit polyclonal antibody (Santa Cruz Biotechnology) used at 1:200 dilution; and an antihuman TGF-α mouse MAb (Ab-2; Oncogene Science, Manhasset, NY) used at 1:100 dilution. To determine the percentage of positive cells, at least 1000 cancer cells/slide were counted and scored (22). Both the percentage of specifically stained cells and the intensity of immunostaining were recorded, as reported previously (16, 22–24). New blood vessels were detected as described by Weidner *et al.* (9), using a MAb raised against the human factor VIII-related antigen (Dako, Milan, Italy) at the dilution of 1:50 and stained with a standard immunoperoxidase method (Vectastain ABC kit). Each slide was scanned at low power (×10–100), and the area with the higher number of new vessels was identified (hot spot). This region was then scanned at ×250 (0.37 mm^2). Five fields were analyzed, and for each of them, the number of stained blood vessels was counted. For individual tumors, MVC was scored by averaging the five field counts (22, 24).

RESULTS

We first evaluated the expression of EGFR in a series of eight human epithelial cancer cell lines of different origin, including breast (ZR-75-1 and MCF-7 ADR), ovarian (OVCAR-3), colon (GEO, SW480, and CaCo2), and gastric (KATO III and N87) cancers by Western blotting. Different levels of EGFR were observed in all cancer cell lines (Fig. 1 and Table 1). Furthermore, TGF-α, the major ligand for EGFR in human cancers (12), was measured in the CM collected from these cells. As shown in Table 1, TGF-α was secreted by all human cancer cell lines tested with levels ranging from 255 pg/10^6 cells/24 h (Caco2 cells) to 680 pg/10^6 cells/24 h (OVCAR-3 cells). Collectively, these data suggest that a TGF-α-EGFR autocrine growth pathway is active in all of these cancer cell lines. We next determined the effects of ZD1839 on the growth of these cell lines in soft agar. Treatment with ZD1839 caused a dose-dependent inhibition of colony formation with an IC_{50} ranging from 0.15 μM (MCF-7 ADR cells) to 0.9 μM (N87 cells; Table 2).

The endogenous levels of two major angiogenic growth factors, bFGF and VEGF, were also measured in the CM collected from the eight human cancer cell lines. As illustrated in Table 1, both bFGF and VEGF were secreted by the cell lines tested, except for CaCo2 and KATO III cells, in which no detectable bFGF was found. To determine the effects of blocking EGFR activation on the production of autocrine growth factors and paracrine angiogenic growth factors by these human cancer cells, CM obtained from the various cell lines, which were treated for 5 days with different concentrations of ZD1839, were collected and analyzed for the presence of TGF-α, bFGF, and VEGF. As shown in Fig. 2*A*, a dose-dependent inhibition of the secretion of TGF-α was observed, with an ~50% reduction at doses of ZD1839 that were comparable with growth-inhibi-

Downloaded from clincancerres.aacrjournals.org on February 25, 2021. © 2001 American Association for Cancer Research.

Table 2 Effect of ZD1839 on growth of human cancer cell lines in soft agar

IC_{50}s were calculated by performing dose-response experiments. Cancer cells were treated with different concentrations of ZD1839 (range, 0.05–2.5 μM) each day, for a total of 5 days. Colonies were counted after 10–14 days.

Cell line	ZD1839 IC_{50} (μM)
ZR-75-1	0.2
MCF-7 ADR	0.15
OVCAR-3	0.25
GEO	0.4
SW480	0.3
CaCo2	0.6
KATO III	0.2
N87	0.9

tory IC_{50} in the soft agar assay in each cancer cell line. To determine whether there was a time-dependent effect of the ZD1839 treatment on the inhibition of TGF-α secretion, GEO and OVCAR-3 cancer cells were treated with 0.5 μM ZD1839 for 1, 3, and 5 days, respectively (Fig. 2*B*). A reduction in TGF-α levels in the CM was observed after 24 h of treatment, reaching a maximum between 3 and 5 days of treatment in both cell lines. A similar dose-dependent and time-dependent inhibition in the secretion of bFGF and VEGF was observed in the cancer cell lines tested (Figs. 3 and 4).

We have studied previously the effect of novel therapeutics including agents that are able to block the activation of the EGFR, such as MAbs C225 and ZD1839, in nude mice bearing human GEO colon cancer xenografts (16, 21). In this respect, ZD1839 treatment produced a dose-dependent cytostatic inhibition of GEO tumor growth (21). Because GEO cells form moderately differentiated adenocarcinomas that express the EGFR and various autocrine and paracrine growth factors including TGF-α, bFGF, and VEGF when injected s.c. in immunodeficient mice (16), we have evaluated the effect of ZD1839 treatment on the production of these growth factors *in vivo*. Therefore, nude mice bearing established GEO xenografts (0.25 cm^3 in volume) were treated i.p. daily (days 1–5) for 2 weeks with different doses of ZD1839 (Table 3). ZD1839 inhibited GEO tumor growth in a dose-dependent manner with a parallel reduction in tumor cell proliferation, as assessed by Ki67 nuclear staining. Immunohistochemical evaluation of the expression of TGF-α, bFGF, and VEGF, which was performed on GEO tumors at the end of the 2 weeks of treatment, revealed a marked and dose-dependent reduction in the percentage of positive GEO cells for all three growth factors (Table 3). Tumor-induced vascularization was quantified by immunohistochemistry as MVC in the areas of most intense neovascularization, using an anti-factor VIII related-antigen MAb (16, 22, 24). ZD1839 treatment substantially reduced MVC in a dose-dependent manner.

We have demonstrated previously that ZD1839 treatment significantly enhances the antitumor activity of the cytotoxic drug paclitaxel in nude mice bearing GEO xenografts. Previous studies have also suggested that paclitaxel treatment may affect tumor neovascularization *in vivo* (25). We therefore tested the effect of the combined treatment with these two drugs. Nude

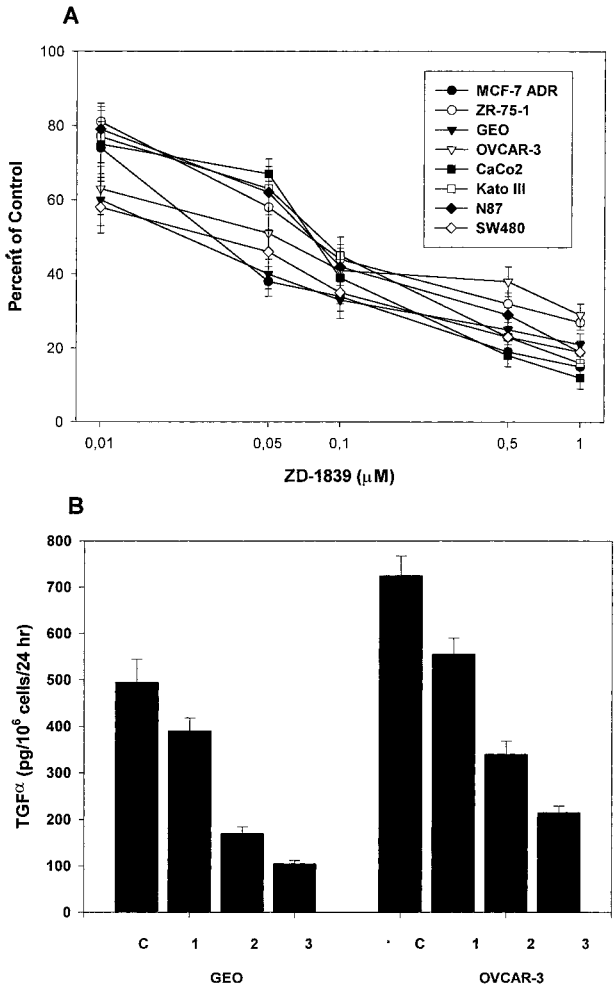

Fig. 2 *A*, dose-dependent inhibition by ZD1839 treatment of TGF-α secretion in the CM collected from the indicated human cancer cell lines. Cancer cells were treated with the indicated concentrations of ZD1839 for 5 days. Data are the average of two different experiments, each performed in triplicate; *bars*, SD. *B*, time-dependent inhibition by ZD1839 treatment of TGF-α secretion in the CM collected from GEO and OVCAR-3 cancer cell lines. Both cancer cell lines were treated with 1 μM ZD1839 for 1 (*Lane 1*), 3 (*Lane 2*), and 5 (*Lane 3*) days. *C*, untreated control cells. Data are the average of two different experiments, each performed in triplicate; *bars*, SD. Results were normalized for the number of producing cells and reported as pg of growth factor/10^6 cells/24 h.

mice bearing established GEO xenografts (0.25 cm^3 in volume) were treated with 400 μg/dose paclitaxel on day 1 of each week for 3 weeks and/or with 3.75 mg/dose ZD1839 on days 1–5 of each week for 3 weeks. As shown in Table 4, the combined treatment was highly effective, with no histological evidence of GEO tumors in 6 of 16 mice at the end of the 3 weeks. Paclitaxel treatment only slightly affected the expression of TGF-α, bFGF, and VEGF and determined a reduction in MVC from 21 to 15 microvessels/field as compared with control mice, whereas an almost complete suppression in TGF-α, bFGF, and VEGF expression and of tumor microvessel development was observed after combined treatment with ZD1839 and paclitaxel (Table 4).

Downloaded from clincancerres.aacrjournals.org on February 25, 2021. © 2001 American Association for Cancer Research.

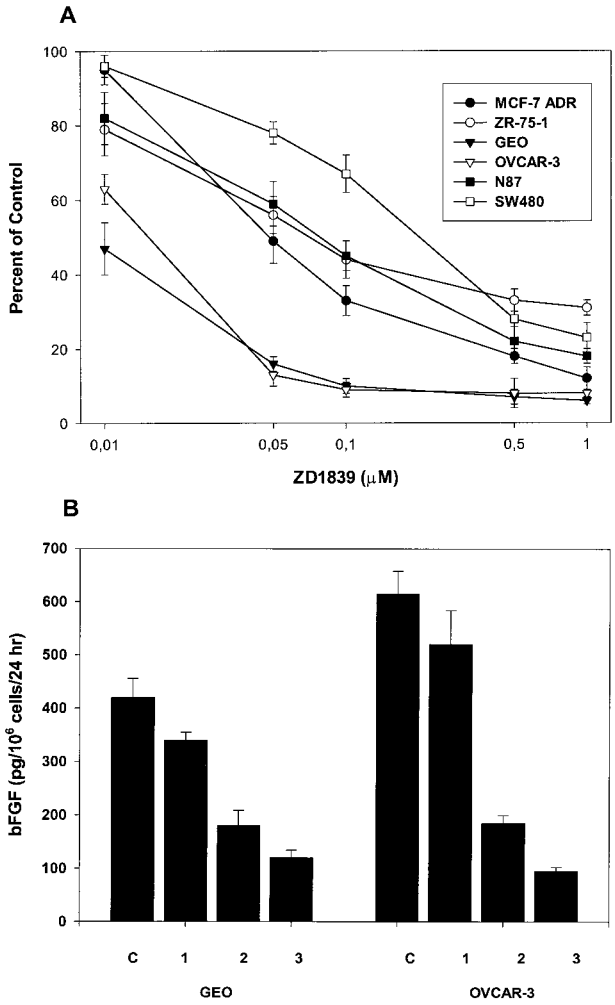

Fig. 3 *A*, dose-dependent inhibition by ZD1839 treatment of bFGF secretion in the CM collected from the indicated human cancer cell lines. Cancer cells were treated with the indicated concentrations of ZD1839 for 5 days. Data are the average of two different experiments, each performed in triplicate; *bars*, SD. *B*, time-dependent inhibition by ZD1839 treatment of bFGF secretion in the CM collected from GEO and OVCAR-3 cancer cell lines. Both cancer cell lines were treated with 1 μM ZD1839 for 1 (*Lane 1*), 3 (*Lane 2*), and 5 (*Lane 3*) days. *C*, untreated control cells. Data are the average of two different experiments, each performed in triplicate; *bars*, SD. Results were normalized for the number of producing cells and reported as pg of growth factor/10^6 cells/24 h.

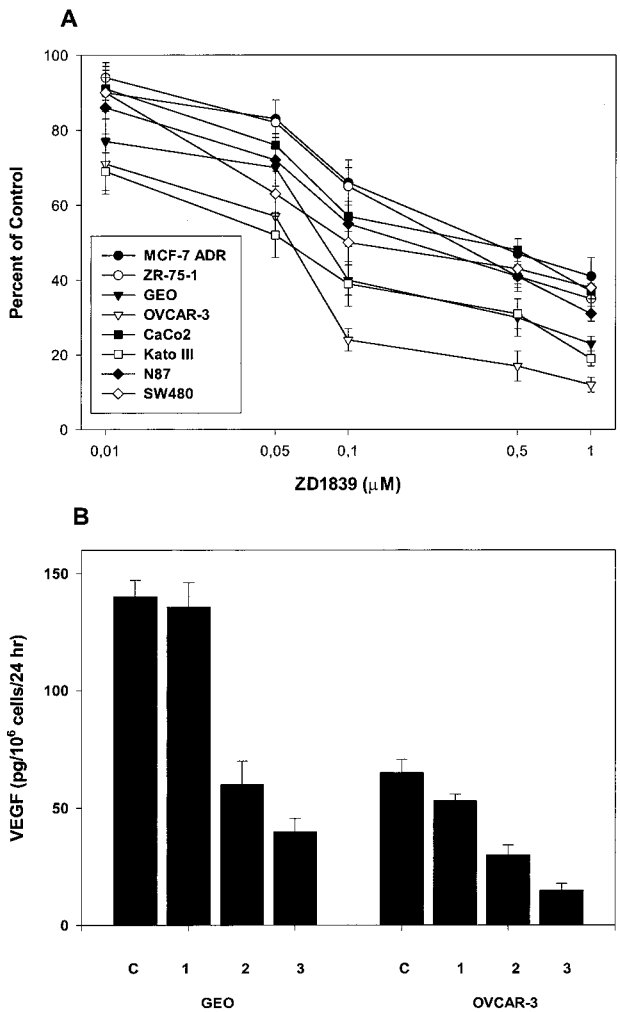

Fig. 4 *A*, dose-dependent inhibition by ZD1839 treatment of VEGF secretion in the CM collected from the indicated human cancer cell lines. Cancer cells were treated with the indicated concentrations of ZD1839 for 5 days. Data are the average of two different experiments, each performed in triplicate; *bars*, SD. *B*, time-dependent inhibition by ZD1839 treatment of VEGF secretion in the CM collected from GEO and OVCAR-3 cancer cell lines. Both cancer cell lines were treated with 1 μM ZD1839 for 1 (*Lane 1*), 3 (*Lane 2*), and 5 (*Lane 3*) days. *C*, untreated control cells. Data are the average of two different experiments, each performed in triplicate; *bars*, SD. Results were normalized for the number of producing cells and reported as pg of growth factor/10^6 cells/24 h.

DISCUSSION

Interference with the activation of growth factor receptors and/or with the intracellular growth factor-activated signal transduction pathways represents a promising strategy for the development of novel and selective anticancer therapies (19). A large body of experimental evidence has been provided for a key role of EGFR activation in a wide variety of human epithelial cancers (12), and blockade of EGFR is one of the most promising approaches in this area (26, 27). It has been suggested that EGFR-driven intracellular signaling controls not only cancer cell proliferation but also several processes that are important for tumor progression, including invasion, angiogenesis, and metastasis (13). In this respect, EGF and TGF-α can up-regulate the production of VEGF in human cancer cells (14, 15).

In this study, we have demonstrated that treatment of several different human epithelial cancer cell lines that express functional EGFR with ZD1839, a selective EGFR-TKI, inhibits in a dose-dependent manner the production of TGF-α, bFGF, and VEGF, which are angiogenic growth factors for endothelial cells. This antiangiogenic activity may contribute to the antiproliferative and antitumor effect *in vivo* of ZD1839 treatment. In fact, the IC_{50} of ZD1839 for cancer cell growth inhibition is similar to the IC_{50} for inhibition of growth factor production. Furthermore, in mice bearing established xenografts of the hu-

Downloaded from clincancerres.aacrjournals.org on February 25, 2021. © 2001 American Association for Cancer Research.

Table 3 Immunohistochemical analysis of GEO colon cancer xenografts after treatment with ZD1839

Mice bearing GEO tumor xenografts were treated as described in "Materials and Methods." Briefly, 10^7 cells were injected, after being suspended in 200 μl of Matrigel, into the dorsal flank of 4–5-week-old nude mice on day 0. Treatment was started on day 7 after tumor cell injection, when tumor volume was ~0.25 cm^3. Mice were treated i.p. daily from day 1 to day 5 with the indicated doses of ZD1839 for 2 weeks. Analysis was performed on day 21 after tumor cell injection. Each group consisted of six mice. The percentage (± SD) of specifically stained GEO cancer cells for Ki67, TGF-α, bFGF, and VEGF was recorded. To determine the percentage of positive cells, at least 1000 cancer cells/slide were counted and scored. The number of microvessels for field (± SD) was measured using a monoclonal antibody raised against the human factor VIII-related antigen and was scored by averaging five field counts of three individual tumors for each group.

Dose of ZD1839 (mg/day)	Tumor volume (cm^3 ± SD)	% positive cells (± SD)				Factor VIII-related antigen[a]
		Ki67	TGF-α	bFGF	VEGF	
0	0.92 ± 0.15	75 ± 10	55 ± 8	45 ± 10	50 ± 4	19 ± 3
1.25	0.68 ± 0.08	50 ± 7	40 ± 6	25 ± 3	35 ± 2	8 ± 2
2.5	0.44 ± 0.05	40 ± 5	25 ± 2	10 ± 2	15 ± 4	5 ± 1
3.75	0.31 ± 0.07	35 ± 5	20 ± 2	5 ± 3	5 ± 1	4 ± 2
5	0.16 ± 0.04	25 ± 2	5 ± 1	5 ± 1	3 ± 2	4 ± 1

[a] Figures for factor VII-related antigen are number of positively staining microvessels.

Table 4 Immunohistochemical analysis of GEO colon cancer xenografts after treatment with ZD1839 and paclitaxel

Mice bearing GEO tumor xenografts were treated as described in "Materials and Methods." Briefly, 10^7 cells were injected, after being suspended in 200 μl Matrigel, into the dorsal flank of 4–5-week-old nude mice on day 0. Treatment was started on day 7 after tumor cell injection, when tumor volume was ~0.25 cm^3. Mice were treated i.p. daily from day 1 to day 5 with the indicated doses of ZD1839 for 3 weeks or with the indicated dose of paclitaxel on day 1 of each week for 3 weeks. Mice were sacrificed, and histological and immunohistochemical analyses were performed on day 28 after tumor cell injection. The data presented are pooled data from two experiments in which each group consisted of eight mice. No histological evidence of GEO tumor was detected in 6 of 16 mice treated with the combination of paclitaxel and ZD1839. The percentage (± SD) of specifically stained GEO cancer cells for Ki67, TGF-α, bFGF, and VEGF was recorded. To determine the percentage of positive cells, at least 1000 cancer cells/slide were counted and scored. The number of microvessels for field (± SD) was measured using a monoclonal antibody raised against the human factor VIII-related antigen and was scored by averaging five field counts of three individual tumors for each group.

Treatment	Tumor volume (cm^3 ± SD)	Mice tumor-free	% positive cells				Factor VIII-related antigen[a]
			Ki67	TGF-α	bFGF	VEGF	
Control	1.45 ± 0.2	0/16	70 ± 5	65 ± 5	55 ± 7	55 ± 5	21 ± 2
Paclitaxel 400 μg	0.56 ± 0.1	0/16	40 ± 10	50 ± 5	35 ± 6	40 ± 5	15 ± 3
ZD1839 3.75 mg	0.35 ± 0.05	0/16	40 ± 5	20 ± 4	7 ± 3	5 ± 1	5 ± 2
Paclitaxel 400 μg + ZD1839 3.75 mg	0.06 ± 0.01	6/16	20 ± 5	2 ± 2	1 ± 1	1 ± 1	1 ± 1

[a] Values for factor VIII-related antigen are number of positively staining microvessels.

man GEO colon cancer, treatment with ZD1839 determines a dose-dependent growth inhibition that is accompanied by a reduction in TGF-α, bFGF, and VEGF production by cancer cells and a suppression of angiogenesis. These results extend, and are in agreement with, those obtained previously by our group and by other investigators on the antiangiogenic effect of blocking the activity of members of the EGF and EGFR families. We have reported that treatment of GEO xenografts with the anti-EGFR blocking MAb C225 inhibited production of various endogenous growth factors of the EGF family, such as TGF-α, amphiregulin, and CRIPTO, and of bFGF and VEGF (16). This effect was accompanied by a reduction in angiogenesis in GEO tumor xenografts in nude mice as assessed by MVC. Similarly, it has been shown that MAb C225 treatment inhibits the production of VEGF and neovascularization in A431 human epidermoid carcinoma xenografts (17). Recently, it has been demonstrated that MAb C225 treatment inhibited angiogenesis in a model of human transitional cell carcinoma grown orthotopically in nude mice by blocking the secretion of various angiogenic factors, including VEGF, bFGF, and interleukin 8 (18). Taken together, these data suggest that treatment with selective anti-EGFR agents has an antitumor effect *in vivo* that is attributable to direct blockade of the EGFR-dependent mitogenic pathway and, at least in part, to inhibition of the secretion of various paracrine growth factors that are necessary to sustain the proliferation and the functional differentiation of tumor blood vessels.

We have demonstrated previously that ZD1839 treatment potentiates the antitumor activity of several conventional cytotoxic drugs including paclitaxel (21). Because there is evidence that paclitaxel has an antiangiogenic effect (25), in this study we also investigated whether the antitumor activity of combined treatment with paclitaxel and ZD1839 could be attributable to inhibition of neovascularization *in vivo*. Treatment with the two drugs produced a complete regression of established palpable GEO tumors in mice; no histological evidence of GEO tumors was found in 6 of 16 mice at the end of 3 weeks of treatment. Furthermore, in the remaining mice, combined treatment with ZD1839 and paclitaxel resulted in almost complete suppression of tumor growth, of TGF-α, bFGF, and VEGF expression, and of MVC. In this respect, two recent reports have also suggested that EGFR-TKI inhibition in combination with cytotoxic drugs may inhibit angiogenesis. In a model of human pancreatic carcinoma, treatment with the anti-EGFR MAb C225 or with the

Downloaded from clincancerres.aacrjournals.org on February 25, 2021. © 2001 American Association for Cancer Research.

PKI-166, a TKI that blocks the EGFR and, with lower potency, the VEGF-specific receptors KDR and Flt-1, significantly potentiated the antitumor activity of gemcitabine, at least in part by antiangiogenic mechanisms (28, 29).

In summary, the results of the present study demonstrate that the antitumor activity of ZD1839 is accompanied by significant antiangiogenic activity and that these effects can be greatly enhanced by combined treatment with ZD1839 and paclitaxel. The suppression of synthesis of endogenous growth factors by cancer cells by ZD1839 treatment may have important therapeutic relevance; chronic treatment with such a selective EGFR-TKI, which could be p.o. administered for prolonged periods, could produce long-term control of local cancer cell growth and metastasis. These data provide a scientific basis for the clinical development of therapeutic approaches, based on the combination of this EGFR-selective TKI with cytotoxic drugs in cancer patients with epithelial cancers that express EGFR.

ACKNOWLEDGMENTS

We thank Gaetano Borriello for excellent technical assistance. ZD1839 was kindly provided by AstraZeneca, Macclesfield, United Kingdom.

REFERENCES

1. Sporn, M. B., and Roberts, A. B. Autocrine secretion: 10 years later. Ann. Intern. Med., *117:* 408–414, 1992.

2. Folkman, J. Tumor angiogenesis. *In:* J. Mendelsohn, P. Howley, L. A. Liotta, and M. Israel (eds.), The Molecular Basis of Cancer, pp. 206–232. Philadelphia: W. B. Saunders, 1995.

3. Klagsbrun, M., and D'Amore, P. A. Regulation of angiogenesis. Annu. Rev. Physiol., *53:* 217–239, 1991.

4. Goldfarb, M. The fibroblast growth factor family. Cell Growth Differ., *1:* 439–445, 1990.

5. Ferrara, N., Houck, K., Jakeman, L., and Leung, D. W. Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocr. Rev., *13:* 18–32, 1992.

6. Ferrara, N. The role of vascular endothelial growth factor in pathological angiogenesis. Breast Cancer Res. Treat., *36:* 127–137, 1995.

7. Folkman, J. Clinical applications of research on angiogenesis. N. Engl. J. Med., *333:* 1757–1763, 1995.

8. Gasparini, G., and Harris, A. L. Clinical implications of the determination of tumor angiogenesis in breast carcinoma: much more than a new prognostic tool. J. Clin. Oncol., *13:* 765–782, 1995.

9. Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis correlation in invasive breast carcinoma. N. Engl. J. Med., *324:* 1–8, 1991.

10. Horak, E. R., Leek, R., Klenk, N., LeJeune, S., Smith, K., Stuart, N., Greenall, M., Stepniewska, K., and Harris, A. L. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastasis and survival in breast cancer. Lancet, *340:* 1120–1124, 1992.

11. Macchiarini, P., Fontanini, G., Hardin, J. M., Squartini, F., and Angeletti, C. A. Relation of neovascularization to metastasis of non-small-cell lung cancer. Lancet, *340:* 45–46, 1992.

12. Salomon, D. S., Brandt, R., Ciardiello, F., and Normanno, N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit. Rev. Oncol. Hematol., *19:* 183–232, 1995.

13. Woodburn, J. R. The epidermal growth factor receptor and its inhibition in cancer therapy. Pharmacol. Ther., *82:* 241–250, 1999.

14. Goldman, C. K., Kim, J., Wong, W. L., King, V., Brock, T., and Gillespie, G. Y. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. Mol. Biol. Cell, *4:* 121–133, 1993.

15. Gille, J., Swerlick, R. A., and Caughman, S. W. Transforming growth factor α-induced transcriptional activation of the vascular permeability factor (*VPF/VEGF*) gene requires AP2-dependent DNA binding and transactivation. EMBO J., *16:* 750–759, 1997.

16. Ciardiello, F., Damiano, V., Bianco, R., Bianco, C., Fontanini, G., De Laurentiis, M., De Placido, S., Mendelsohn, J., Bianco, A. R., and Tortora, G. Antitumor activity of combined blockade of epidermal growth factor receptor and protein kinase A. J. Natl. Cancer Inst., *88:* 1770–1776, 1996.

17. Petit, A. M. V., Rak, J., Hung, M. C., Rockwell, P., Goldstein, N., Fendly, B., and Kerbel, R. S. Neutralizing antibodies against epidermal growth factor and erbB-2/*neu* receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*. Am. J. Pathol., *151:* 1523–1530, 1997.

18. Perrotte, P., Matsumoto, T., Inoue, K., Kuniyasu, H., Eve, B. Y., Hicklin, D. J., Radinsky, R., and Dinney, C. P. N. Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. Clin. Cancer Res., *5:* 257–264, 1999.

19. Gibbs, J. B. Anticancer drug targets: growth factors and growth factor signaling. J. Clin. Investig., *105:* 9–13, 2000.

20. Noonberg, S. B., and Benz, C. C. Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily. Drugs, *59:* 753–767, 2000.

21. Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Pomatico, G., De Placido, S., Bianco, A. R., and Tortora, G. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. Clin. Cancer Res., *6:* 2053–2063, 2000.

22. Fontanini, G., De Laurentiis, M., Vignati, S., Chinè, S., Lucchi, M., Silvestri, V., Mussi, A., De Placido, S., Tortora, G., Bianco, A. R., Gullick, W., Angeletti, C. A., Bevilacqua, G., and Ciardiello, F. Evaluation of epidermal growth factor-related growth factors and receptors and of neoangiogenesis in completely resected stage I-IIIA non-small-cell lung cancer: amphiregulin and microvessel count are independent prognostic indicators of survival. Clin. Cancer Res., *4:* 241–249, 1998.

23. Normanno, N., Bianco, C., Damiano, V., de Angelis, E., Selvam, M. P., Grassi, M., Magliulo, G., Tortora, G., Bianco, A. R., Mendelsohn, J., Salomon, D. S., and Ciardiello, F. Growth inhibition of human colon carcinoma cells by combinations of anti-EGF-related growth factor antisense oligonucleotides. Clin. Cancer Res., *2:* 601–609, 1996.

24. Ciardiello, F., Bianco, R., Damiano, V., Fontanini, G., Caputo, R., Pomatico, G., De Placido, S., Bianco, A. R., Mendelsohn, J., and Tortora, G. Anti-angiogenic and antitumor activity of anti-epidermal growth factor receptor C225 monoclonal antibody in combination with vascular endothelial growth factor antisense oligonucleotide in human GEO colon cancer cells. Clin. Cancer Res., *6:* 3739–3747, 2000.

25. Belotti, D., Vergani, V., Drudis, T., Borsotti, P., Pitelli, M. R., Viale, G., Giavazzi, R., and Taraboletti, G. The microtubule-affecting drug paclitaxel has antiangiogenic activity. Clin. Cancer Res., *2:* 1843–1849, 1996.

26. Baselga, J., and Mendelsohn, J. Type I receptor tyrosine kinases as targets for therapy in breast cancer. J. Mammary Gland Biol. Neopl., *2:* 165–174, 1997.

27. Fan, Z., and Mendelsohn, J. Therapeutic application of antigrowth factor receptor antibodies. Curr. Opin. Oncol., *10:* 67–73, 1998.

28. Bruns, C. J., Harbison, M. T., Davis, D. W., Portera, C. A., Tsan, R., McConkey, D. J., Evans, D. B., Abbruzzese, J. L., Hicklin, D. J., and Radinsky, R. Epidermal growth factor receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by antiangiogenic mechanisms. Clin. Cancer Res., *6:* 1936–1948, 2000.

29. Bruns, C. J., Solorzano, C. C., Harbison, M. T., Ozawa, S., Tsan, R., Fan, D., Abbruzzese, J., Traxler, P., Buchdunger, E., Radinsky, R., and Fidler, I. J. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. Cancer Res., *60:* 2926–2935, 2000.

Downloaded from clincancerres.aacrjournals.org on February 25, 2021. © 2001 American Association for Cancer Research.

AACR American Association for Cancer Research

Clinical Cancer Research

Inhibition of Growth Factor Production and Angiogenesis in Human Cancer Cells by ZD1839 (Iressa), a Selective Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor,

Fortunato Ciardiello, Rosa Caputo, Roberto Bianco, et al.

Clin Cancer Res 2001;7:1459-1465.

Updated version Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/7/5/1459

Cited articles This article cites 27 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/7/5/1459.full#ref-list-1

Citing articles This article has been cited by 88 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/7/5/1459.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/7/5/1459.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.

Downloaded from clincancerres.aacrjournals.org on February 25, 2021. © 2001 American Association for Cancer Research.