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, Vincenzo Damiano, Gabriella Fontanini, Sabina Cuccato, Sabino De Placido, A. Raffaele Bianco, and Giampaolo Tortora


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, 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.
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 quinoline-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 angiogenic 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 angiogenic 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% CO₂ at 37°C.

**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 (10⁴ cells/well) were suspended in 0.5 ml of 0.3% 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/10⁶ 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.
**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 125I-labeled EGFR 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/10⁶ cells/24 h.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGFR (relative expression)</th>
<th>TGF-α (pg/10⁶ cells/24 h)</th>
<th>bFGF (pg/10⁶ cells/24 h)</th>
<th>VEGF (pg/10⁶ cells/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR-75-1</td>
<td>0.6</td>
<td>285 ± 30</td>
<td>195 (±10)</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>MCF-7 ADR</td>
<td>6.2</td>
<td>580 ± 25</td>
<td>255 ± 35</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>3.7</td>
<td>680 ± 35</td>
<td>645 ± 55</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>GEO</td>
<td>1</td>
<td>405 ± 15</td>
<td>710 ± 55</td>
<td>150 ± 8</td>
</tr>
<tr>
<td>SW480</td>
<td>3.5</td>
<td>480 ± 20</td>
<td>600 ± 65</td>
<td>400 ± 12</td>
</tr>
<tr>
<td>CaCo2</td>
<td>0.6</td>
<td>255 ± 15</td>
<td>ND</td>
<td>110 ± 8</td>
</tr>
<tr>
<td>KATO III</td>
<td>1</td>
<td>340 ± 25</td>
<td>ND</td>
<td>180 (±8)</td>
</tr>
<tr>
<td>N87</td>
<td>0.4</td>
<td>360 ± 35</td>
<td>380 ± 40</td>
<td>145 (±20)</td>
</tr>
</tbody>
</table>

* ND, not detectable.

**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/10⁶ cells/24 h (Caco2 cells) to 680 pg/10⁶ 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 IC₅₀ 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. 2A, 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-
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.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ZD1839 IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR-75-1</td>
<td>0.2</td>
</tr>
<tr>
<td>MCF-7 ADR</td>
<td>0.15</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>0.25</td>
</tr>
<tr>
<td>GEO</td>
<td>0.4</td>
</tr>
<tr>
<td>SW480</td>
<td>0.3</td>
</tr>
<tr>
<td>CaCo2</td>
<td>0.6</td>
</tr>
<tr>
<td>KATO III</td>
<td>0.2</td>
</tr>
<tr>
<td>N87</td>
<td>0.9</td>
</tr>
</tbody>
</table>

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 cm³ 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).

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 mice bearing established GEO xenografts (0.25 cm³ 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).
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 IC₅₀ of ZD1839 for cancer cell growth inhibition is similar to the IC₅₀ for inhibition of growth factor production. Furthermore, in mice bearing established xenografts of the hu-

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/10⁶ 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/10⁶ cells/24 h.
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, 10^6 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 cm³. 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.

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, 10^6 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 cm³. 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 21 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.

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 EGFR and EGFR families. We have reported that treatment of GEO xenografts with the anti-EGFR blocking MAb C225 inhibited production of various paracrine growth factors that are necessary to the 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
PKI-166, a TKI that blocks the EGFR and, with lower potency, the VEGF-specific receptors KDR and Flt-1, significantly potentiates 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

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