Modulation of Radiation Response and Tumor-induced Angiogenesis after Epidermal Growth Factor Receptor Inhibition by ZD1839 (Iressa)

Shyh-Min Huang, Jing Li, Eric A. Armstrong, and Paul M. Harari

Department of Human Oncology, University of Wisconsin School of Medicine and Comprehensive Cancer Center, Madison, Wisconsin 53792

ABSTRACT

ZD1839 ("Iressa") is an orally-active, selective epidermal growth factor receptor-tyrosine kinase inhibitor. We evaluated the antitumor activity of ZD1839 in combination with radiation in human squamous cell carcinomas (SCCs) of the head and neck. ZD1839 produced a dose-dependent inhibition of cellular proliferation in human SCCs grown in culture. Flow cytometry analysis of cell cycle progression confirmed the accumulation of cells in G1 phase after exposure to ZD1839. Clonogenic analysis demonstrated that treatment of SCCs with ZD1839 reduced cell survival after radiation exposure. Flow cytometric analysis further demonstrated that treatment of SCCs with ZD1839 amplified radiation-induced apoptosis. Tumor xenograft studies confirmed that oral administration of ZD1839, or focal radiation, resulted in partial and transient tumor regression in both SCC-1 and SCC-6 xenografts. In contrast, profound tumor regression and regrowth delay was observed in mice treated with the combination of ZD1839 and radiation. To examine antiangiogenic effects, we studied the impact of ZD1839 on human umbilical vascular endothelial cells (HUVECs). In the presence of reconstituted Matrigel matrix, HUVECs established a capillary-like network structure (tube formation). Treatment with ZD1839 reduced the cell-to-cell interaction of HUVECs, resulting in disruption of tube formation. The effect of ZD1839 was further examined using an in vivo tumor xenograft model of angiogenesis (Matrigel plug) in athymic mice. Systemic treatment with ZD1839 significantly inhibited tumor-induced neovascularization across the Matrigel plug. Taken together, these results suggest that the antitumor activity of ZD1839 in combination with radiation appears to derive from not only proliferative growth inhibition (with associated cell cycle arrest and enhancement of radiation-induced apoptosis) but also from inhibition of tumor angiogenesis.

INTRODUCTION

SCC of the H&N represents a worldwide health care problem with ~750,000 new cases diagnosed annually. Radiation therapy plays a primary role in treatment for patients with SCC of the H&N. However, the rapid proliferation of surviving tumor clonogens during and after radiotherapy serves to compromise ultimate tumor control (1). Biological approaches that use growth factor inhibitors to modify tumor proliferation and/or radiosensitivity offer potential for improving tumor control. The EGFR represents a promising therapeutic target in epithelial tumors. The EGFR serves as an important regulator of cellular proliferation but also contributes to other processes that are central to cancer progression, including angiogenesis and metastatic spread (2). EGFR is overexpressed by a variety of human tumors, particularly SCC of the H&N. Several studies demonstrate a positive correlation between EGFR expression and cellular resistance to radiation (3-6). SCC cells derived from H&N cancer patients that express high levels of EGFR appear to be more radioresistant than those expressing low levels of EGFR (4). The degree of radioresistance correlates positively with the magnitude of EGFR overexpression (5). Additional studies suggest that cell survival and repopulation during a course of radiotherapy are influenced, in part, by activation of EGFR/TGF-α that is induced after exposure to radiation (7). These results suggest that modulation of EGFR signaling represents a promising strategy for improving tumor control with radiation (8).

ZD1839 ("Iressa") is an EGFR-selective tyrosine kinase inhibitor that blocks signal transduction pathways implicated in cancer cell proliferation, survival, and other host-dependent processes promoting cancer progression (9). ZD1839 inhibits EGFR in vitro with an IC50 of 23 nm (10) and demonstrates much lesser activity against HER2 (IC50 of 2 μM). ZD1839 possesses good oral bioavailability and demonstrates antitumor activity across a broad range of mouse models. A series of studies have evaluated the combined antiproliferative effect of treatment with ZD1839 and a variety of cytotoxic drugs with different mechanisms of action (11). A supra-additive growth-inhibitory effect was observed with ZD1839 and several cytotoxic drugs tested in vitro and in human xenograft tumor models. Phase I clinical trials with ZD1839 as monotherapy confirm that treatment is generally well tolerated and provide evidence of antitumor activity (12). These successes have led to additional clinical investigations regarding the effects of ZD1839 in combination with conventional cytotoxic drugs or radiation.

There are a series of recently published abstracts that describe the capacity of ZD1839 to modulate radiation response in human tumor cell lines and xenografts (13-19). The present study represents the first full publication to examine potential therapeutic advantage of combining ZD1839 with radiation in a series of in vitro and in vivo experimental systems. Our results confirm that treatment with ZD1839 reduces clonogenic survival of SCC cells after exposure to radiation. Moreover, coadministration of ZD1839 with focal radiation enhances the antitumor potency of radiation against human SCC xenografts in athymic mice. In addition, we offer experimental evidence suggesting that potentiation of the antitumor activity of ionizing radiation by ZD1839 is mediated in part by effects on cellular apoptosis and tumor angiogenesis.

MATERIALS AND METHODS

Chemicals and ZD1839. Cell culture media were obtained from Life Technologies, Inc. (Gaithersburg, MD). The Diff-Quik stain set was purchased from Dade International, Inc. (Miami, FL). Matrigel was obtained from Becton Dickinson (Bedford, MA). MCDB 131 medium without growth supplements was obtained from Life Technologies, Inc. (Grand Island, NY). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). ZD1839 was generously provided by AstraZeneca (Macclesfield, United Kingdom).

Cell Lines and Cell Culture. Human SCC cell lines were established from biopsies of H&N cancer patients. The SCC-13Y cell line was derived from the facial epidermis and was provided by Dr. B. Lynn Allen-Hoffmann (University of Wisconsin-Madison). The SCC-1 cell line (floor of mouth) and SCC-6 cell line (tongue) were provided by Dr. Thomas A. Carey (University of Michigan). SCC cells were cultured routinely in DMEM supplemented with 5% fetal bovine serum, 1 μg/ml hydrocortisone, and 1% penicillin and streptomycin.
HUVeCs were kindly provided by Dr. Deane F. Mosher (University of Wisconsin-Madison) and cultured in MCDB 131-complete medium purchased from VEC Technologies, Inc. (Rensselaer, NY).

**Growth Inhibition Assay.** The antiproliferative effect of ZD1839 on human SCC cell lines was evaluated using MTT assay as described previously (20). Briefly, exponentially growing SCC cells were seeded into 96-well plates and incubated in medium containing ZD1839 from 0 to 100 μM for 24 h at 37°C. Duplicate plates containing six replicate wells/assay condition were seeded at a density of 1500 cells in 0.1 ml of medium. ZD1839 was solubilized in DMSO. Final concentration of DMSO in all cultures, including controls, was 0.1%. After exposure of cells to ZD1839, 100 μl of MTT (1 mg/ml) were added to each well for 2 h at 37°C to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized overnight at 37°C in a solution containing 10% of SDS and 50% of N,N-dimethylformamide. The absorbance of each well was measured in a microplate reader at 600 nm. The percentage cell growth was calculated by comparison of the A570 reading from treated versus control cells.

**Flow Cytometric Analysis of Cell Cycle.** Control or ZD1839-treated cells were harvested by trypsinization, washed with PBS, then fixed in 95% ethanol, and stored at 4°C for up to 7 days before DNA analysis. After the removal of ethanol by centrifugation, cells were then incubated with phosphate-citric acid buffer (0.2 mM Na,HPO₄ (pH 7.8), and 4 mM citric acid) at room temperature for 45 min. After centrifugation, cells were then washed with a solution containing 33 μM/μl PI, 0.13 mg/ml RNase A, 10 μM EDTA, and 0.5% Triton X-100 at 4°C for 24 h. Stained nuclei were analyzed for DNA-PI fluorescence using a Becton Dickinson FACScan flow cytometer. Resulting DNA distributions were analyzed by ModFit (Verity Software House, Inc., Topsham, ME) for the proportion of cells in sub-G₀, G₁, S, and G₂-M phases of the cell cycle.

**Flow Cytometric Quantitation of Apoptosis.** Apoptosis was detected by examination of altered plasma membrane phospholipid packing, which was quantitated by the incorporation of the lipophilic dye MC540 and Ho342 dye (21). The validity of MC540 staining as a measure of entry into apoptosis has been established in previous experimental systems in which cell morphology, changes in light scatter parameter, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling DNA end-labeling were quantitated by the incorporation of the lipophilic dye MC540 and Hoechst 33342. MC540 bright cells in different cell cycle phases were marked by an increase in red fluorescence, collected at 575–590 nm, and further quantified by Adobe Photoshop software (Adobe Systems, Mountain View, CA).

**Statistical Analysis.** The effects of radiation and/or ZD1839 on growth inhibition, radiation survival, and apoptosis were assessed by multiple regression analysis using the PROC general linear model procedure in SAS (version 8; SAS Institute, Inc., Cary, NC). In experiments involving multiple radiation doses or ZD1839, the linear quadratic model with the following equation was used: log(surviving fraction) = αD + βD², where D represents the dose of radiation (Gy) or dose of ZD1839 (μM). For xenograft studies, data on tumor volume were transformed into ranks and analyzed by nonparametric procedures.

**RESULTS**

**ZD1839 Induces G₁ Cell Cycle Arrest.** The capacity of ZD1839 to inhibit cell cycle progression was evaluated via flow cytometry. The effect of ZD1839 treatment on cell cycle phase distribution in the
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SCC-1 and SCC-13Y cell lines is summarized in Fig. 2. Treatment with 1 μM ZD1839 for 48 h induced accumulation of cells in G1 phase with a significant decrease in the percentage of cells in S-phase relative to controls. No significant changes in the percentage of cells within G2-M phase were observed in these experiments. The ZD1839-induced G1 arrest was further confirmed by examining the effect of ZD1839 on the expression of several key regulators of the G1-S-phase transition. Western blot analysis confirmed that treatment with ZD1839 increased expression of the cyclin-dependent kinase inhibitor p27, hypophosphorylated retinoblastoma protein, and decreased expression of cyclin D1 and E (data not shown).

ZD1839 Enhances Radiosensitivity. To examine the potential usefulness of combining ZD1839 with radiation therapy for rapidly dividing human SCCs of the H&N, experiments were conducted to evaluate the effects of ZD1839 on clonogenic survival. Fig. 3 depicts radiation survival curves for SCC-1 and SCC-6 cells exposed to ZD1839 before radiation. Our results demonstrate that treatment with ZD1839 before radiation significantly reduced cell survival compared with control (P < 0.01) in both cell lines. When the data were analyzed according to the linear quadratic model in SCC-1 cells, the α:β ratios were 0.95 and 4.34 for the control and the ZD1839 groups, respectively. These data indicate that treatment with ZD1839 results in a steeper decline in cell survival with a higher α:β ratio and enhancement of radiosensitivity with the higher α coefficient. Similar results were observed in SCC-6 cells (control curve: α/β = 17.02; ZD1839-treated curve: α/β = 23.03).

ZD1839 Augments in Vivo Tumor Response to Radiation. Two human SCC cell lines (SCC-1 and SCC-6) were inoculated s.c. into female athymic mice and allowed to grow for 17 days before randomization into four groups. In an attempt to examine the in vivo interaction between ZD1839 and radiation, doses of both agents were specifically selected so that their independent effects on tumor growth inhibition would be modest. This was confirmed by our observation that treatment with radiation (3 Gy × 7) alone or with ZD1839 (0.5 mg × 12) alone produced a measurable but transient growth delay in both SCC-1 and SCC-6 xenografts. These tumors subsequently recapitulated their prior growth pattern upon withdrawal of treatment (Fig. 4). In contrast, combined treatment with radiation and ZD1839 significantly inhibited tumor growth and resulted in substantial growth delay in both SCC-1 and SCC-6 xenografts. For SCC-6 xenografts, statistical analysis indicated that combined treatment with radiation and ZD1839 was associated with an additive effect on tumor growth inhibition. The combined treatment with radiation and ZD1839 produced a synergistic tumor growth inhibition in SCC-1 xenografts beyond day 51 (P < 0.01). This synergistic interaction between ZD1839 and radiation increased over time, as indicated by diminishing P50 values from day 51 (P = 0.009) to day 68 (P = 0.008) and finally to day 75 (P = 0.005).

ZD1839 Enhances Radiation-induced Apoptosis. We further evaluated whether mechanisms of interaction between ZD1839 and radiation involve cell killing mediated by apoptosis. Enhancement of radiation-induced apoptosis by ZD1839 was first examined using MC540, which detects the early stages of apoptosis, i.e., conformational changes in the plasma membrane. As shown in Fig. 5, radiation alone (6 Gy) or ZD1839 alone induced early apoptosis to a similar degree (approximately 4–8%) compared with control. However, combined treatment with radiation and ZD1839 resulted in substantially
more potent induction of apoptosis (increased by 23% compared with control), which was greater than additive with P < 0.001. The enhancement of radiation-induced apoptosis by ZD1839 was further confirmed by a distinct assay approach (flow cytometry) using PI labeling of the DNA. This method examines the appearance of hypodiploid nuclei (sub-G0), which reflects the terminal stage of apoptosis. This approach also confirmed a potentiation of radiation-induced apoptosis in SCC cells by ZD1839 (data not shown).

**ZD1839 Inhibits Capillary-like Network Formation in HUVECs.** To investigate the effect of ZD1839 on angiogenesis, we first examined the impact of ZD1839 on human microvascular endothelial (HUVEC) cells. HUVECs were shown to attach to the reconstituted extracellular matrix (Matrigel) when plated and were capable of forming capillary-like structures. It is believed that these processes in extracellular matrix are representative of the latter stages in angiogenesis, during which vessels complete their differentiation and become stable (24). In this study, HUVECs were observed to spread out and generate lateral processes 2 h after plating onto Matrigel. By 6 h, the alignment of cells and the formation of intercellular tubular connections become evident (Fig. 6A). At 18 h, the inhibitory effect of ZD1839 was clearly noted. Conversely, the endotubes formed by control cells continued to differentiate and mature. As the incubation period lengthened, cells treated with ZD1839 gradually lost their cell-to-cell connections. This destabilizing effect became very obvious by 30 h, when the capillary-like network of HUVECs displayed substantial fragmentation and dissociation, resulting in the loss of most intercellular contacts. The number of endotubes was substantially reduced at 18 and 30 h (60 and 75%, respectively) in the ZD1839-treated group (Fig. 6B).

**Tumor-induced Vascularization of Matrigel Plugs.** To further investigate the influence of ZD1839 on tumor-induced angiogenesis in vivo, we adapted a tumor xenograft angiogenesis assay using Matrigel matrix. In this assay, Matrigel was injected s.c. into the abdominal wall of athymic mice, thereby forming semisolid plugs. Twenty-four h later, SCC-1 cells were introduced into a polyvinyl sponge that was then inserted into a surgically created pocket within the Matrigel plug. In each experimental group, at least three grafts were analyzed in two independent experiments. Plugs with Matrigel alone were pale in color and visibly clear, with no identifiable blood vessels migrating into the Matrigel. After implantation with tumor cells, blood capillaries were observed to grow from the edge of the Matrigel plug toward the implanted tumor as visualized by fluorescent microscopy (Fig. 7A). The control plugs containing SCC-1 cells showed extensive vascularization and growth of vessels toward the tumor core within 10 days. In contrast, plugs in those mice treated with ZD1839 showed markedly reduced vascularization (Fig. 7A), which was confirmed by the reduced intensity of fluorescence relative to untreated groups (Fig. 7B). These results suggest that ZD1839 can inhibit tumor-induced angiogenesis.
DISCUSSION

Blockade of EGFR signaling pathways represents a particularly promising new strategy for the development of novel and selective anticancer therapies. In this report, we demonstrate that ZD1839, a selective EGFR tyrosine kinase inhibitor, is capable of inhibiting cellular proliferation and augmenting radiation response in SCCs of the H&N. Treatment with ZD1839 enhances radiation-induced apoptosis and inhibits tumor angiogenesis. The apoptotic and antiangiogenic activity appears to facilitate the in vivo antiproliferative and antitumor effects of ZD1839 as observed in human xenograft studies.

Findings from our human xenograft studies demonstrate that ZD1839 treatment is associated with an additive (SCC-6) or synergistic (SCC-1) effect on tumor growth inhibition when combined with ionizing radiation. The success of radiation therapy to effect local tumor control is influenced by multiple factors including overall clonogenic burden, intrinsic radiosensitivity, oxygenation status, and cellular repopulation capacity and repair. It has been reported previously that SCC-1 and SCC-6 exhibit distinct radiosensitivities (SCC-6, radiosensitive; SCC-1, radioresistant; Ref. 25). Our current observation that radiation alone inhibited the growth of the SCC-6 tumor xenografts more significantly than for the SCC-1 tumor xenografts (Fig. 4) is consistent with these known radiosensitivity profiles. Therefore, differential effects observed with combined radiation and ZD1839 on tumor growth between SCC-1 and SCC-6 may be partially attributable to differences in their intrinsic radiosensitivity.

Inhibition of tumor angiogenesis may also contribute to the antitumor potency of ZD1839 plus radiation observed within the xenograft model systems because a mechanistic linkage between EGFR signaling and angiogenesis has been identified recently (26, 27). TGF-α can induce transcriptional activation of the angiogenic factor, VEGF (26).

Fig. 6. Effect of ZD1839 on capillary-like network (tube) formation in HUVECs. Tissue culture plates were coated with 250 μl of Matrigel for 1 h, and HUVECs (1000 cells/ml/well) were added with or without ZD1839 (1 μM). At 6, 18, and 30 h, plates were fixed and stained. A, photomicrographs of tube formation in the absence or presence of ZD1839. B, average number of tubes/ microscopical field from three separate experiments. For each time interval, data are expressed relative to the control group; bars, SE.

Fig. 7. Effect of ZD1839 on tumor xenograft angiogenesis. SCC-1 cells were implanted into dorsal Matrigel plugs prepared in athymic mice as described in “Materials and Methods.” A, photomicrographs of Matrigel plugs from control and ZD1839-treated mice. The Matrigel morphology is shown in the upper panels with the dark central images depicting location of tumor implantation. The distribution of blood vessels in the Matrigel plug was visualized (green) under fluorescence microscopy. Bottom panels, higher resolution photographs (×2) taken from the designated box within the middle panels. B, the intensity of fluorescence was further quantified as described in “Materials and Methods.” Results were obtained from six mice from two independent experiments; bars, SD.
Blockade of EGFR results in reduced expression of VEGF mRNA and protein, as well as interleukin 8 and basic fibroblast growth factor in tumor cells (27). Using the Matrigel plug neovascularization assay, we demonstrate that treatment of mice with ZD1839 significantly reduces vessel formation and inhibits the propensity of vessels to grow toward the central core of SCC-1 tumor implantation. We further demonstrate that ZD1839 inhibits the early angiogenic process by targeting endothelial cells, i.e., by decreasing cell-to-cell interaction and disrupting endothute formation (Fig. 6).

Endothelial cells from several neoplasms have been shown to express EGFR (28). Binding of TGF-α to EGFR on endothelial cells stimulates their proliferation (29). Conversely, blockade of the EGFR leads to apoptosis of endothelial cells and to a subsequent reduction in neovascularity (30). The respective roles of endothelial cells and tumor cells in the overall tissue response to radiotherapy remain controversial. Paris et al. (31) provide recent evidence that endothelial cells may represent principal targets for radiation, whereas tumor cell death may represent a secondary event after demise of endothelial cells on which tumor cells ultimately depend. The effect of ZD1839 on endothelial cells observed in our studies warrants further investigation regarding the capacity of ZD1839 to modify the radiation response profile of endothelial cells.

Cellular apoptosis represents another process that may influence the ultimate response of a particular tumor to ionizing radiation (32, 33). In a study comparing two murine tumors, one radiosensitive (ovarian carcinoma) and one radioresistant (hepatocellular carcinoma), the former was found to be much more susceptible to induction of apoptosis by radiation. A similar correlation was observed between the amount of apoptosis and the degree of radiosensitivity in two mouse lymphoma cell lines (34). These data suggest that apoptotic propensity may influence intrinsic tumor cell radiosensitivity. Recent studies in human SCCs (35) and colorectal cancers (36) indicate that EGFR signaling blockade (with the anti-EGFR monoclonal antibody C225) can itself induce apoptosis. The colorectal work further suggests that subcellular redistribution of Bax protein may influence this response. In the present study with SCCs, we demonstrate that molecular inhibition of EGFR tyrosine kinase with ZD1839 exerts a modest independent effect on apoptosis but effects a clear enhancement of radiation-induced apoptosis. It suggests that treatment with ZD1839 not only enhances cell death after radiation but also increases the fraction of tumor cells succumbing to radiation-induced apoptosis. ZD1839 inhibits cellular proliferation across a variety of SCC cell lines with various EGFR levels. Interestingly, the degree of proliferative inhibition may not correlate with the level of EGFR expression, as indicated by results in Fig. 1. Other studies report quite similar observations. A recent study suggests that the sensitivity of tumor cells to ZD1839 is not correlated with EGFR expression but rather with HER2 expression (37). In a panel of 10 human breast cancer and other epithelial tumor cell lines, Moasser et al. (37) found that HER2-overexpressing tumors were more sensitive to ZD1839 than others, with IC₅₀<sub>98</sub> < 10 µM. The highest sensitivity was observed among those tumors with the greatest HER2 expression, with IC₅₀<sub>98</sub> < 1 µM. No apparent correlation between the expression level of EGFR and tumor sensitivity to ZD1839 was identified in this study. In a study by Sirotnak et al. (38), tumor cell lines with various EGFR expression levels were used to examine the influence of EGFR expression on the cytotoxic effect of chemotherapy agents combined with ZD1839. The EGFR expression level did not substantially influence the cytotoxic antitumor activity by ZD1839 or the activity of ZD1839 alone. These results suggest that ZD1839 influences tumor response through inhibition of EGFR, HER2, and perhaps both. HER2 functions preferentially as a heterodimer with other HER family members including EGFR. ZD1839 may therefore also represent a valuable agent in the treatment of patients with HER2-overexpressing tumors, such as selected breast cancers (39, 40). Indeed, the clinical testing of ZD1839 need not necessarily be confined to patients with EGFR-overexpressing tumors.

In conclusion, ZD1839 offers promise in the treatment of epithelial tumors that rely on ErbB signaling for their growth advantage. The studies presented in this report indicate that human epithelial cancer cells (SCCs) are particularly sensitive to radiation when EGFR signaling is inhibited by ZD1839. This finding expands upon recent results with other EGFR inhibitory agents (i.e., C225), which act via blockade of the extracellular receptor domain to inhibit EGFR signaling (35, 41, 42). The fact that epithelial tumors comprise approximately two-thirds of human tumors, with many relying on EGFR signaling for cellular growth and differentiation, suggests a broad applicability of this molecular target in human cancer therapy. Clinical trials are now in progress to examine the impact of ZD1839 (and other EGFR-inhibitory agents) on radiation response and patient survival in a spectrum of human malignancies.

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