Suppression of Epidermal Growth Factor Receptor, Mitogen-activated Protein Kinase, and Pak1 Pathways and Invasiveness of Human Cutaneous Squamous Cancer Cells by the Tyrosine Kinase Inhibitor ZD1839 (Iressa)

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Abstract
Abnormalities in the expression and signaling pathways downstream of the epidermal growth factor receptor (EGFR) contribute to malignant transformation in human cancers, including those of the cutaneous epithelium. Accordingly, novel agents such as the EGFR tyrosine kinase inhibitor ZD1839 (Iressa), are promising, biologically based treatments that are currently in preclinical and clinical development. The process of tumor progression requires, among other steps, increased transformation, directional migration, and enhanced cell survival. This study explored the effect of ZD1839 on the stimulation of p42/44 mitogen-activated protein kinase (MAPK) and p21-activated kinase 1 (Pak1), which are vital for transformation, directional motility, and cell survival, using immortalized keratinocytes (HaCaT cells) and cutaneous squamous cell carcinoma cells. The EGFR and a number of effector kinases (mitogen-activated protein extracellular signal-regulated kinase 1 and 2, MAPK, Pak1, p38, c-JunNH2-terminal kinase and extracellular signal-regulated kinase 1) and cell survival proteins (AKT, FKHR, and c-Src) showed constitutive pathway activation in HaCaT and cutaneous squamous cell carcinoma cells. ZD1839 effectively inhibited EGFR and MAPK activation and Pak1 activity in exponentially growing cancer cells. ZD1839 also suppressed EGF-induced autophosphorylation on Y1068 and Y1068, MAPK phosphorylation on T402 and Y404, and Pak1 activity in a dose-dependent manner.

In addition, ZD1839 blocked EGF-induced cytoskeleton remodeling, cell growth, and in vitro invasiveness of cancer cells and induced a differentiated squamous cell phenotype. These studies suggest that the EGFR-tyrosine kinase inhibitor ZD1839 may cause potent inhibition of the EGFR, MAPK, and Pak1 pathways, resulting in attenuation of transformed cell phenotypes and induced differentiation in human cancer cells deregulated in these growth factor receptor pathways.

Introduction
Despite the perceived relative innocuous biology of non-melanoma skin cancer, cuSCC3 continues to be the most aggressive and fatal form of skin cancer in the United States (1). Our limited understanding of the pathobiology of skin cancers has been a barrier to progress in treating cuSCC. Much of the biological research in cuSCC has focused on understanding the process of carcinogenesis. Although this research has been very fruitful, it is now clear that a more complete understanding of key regulatory signaling pathways is required for further significant gain in understanding biologic predictors and more effective therapy for those aggressive malignancies.

Growth factors and their receptors play an essential role in the regulation of epithelial cell proliferation, and abnormalities in their expression and signaling pathways contribute to progression and maintenance of the malignant phenotype in human cancers, e.g., deregulation of EGFR activation has been shown to be closely associated with the development and progression of cuSCC (2–4). The EGFR is one of a family of four closely related receptors: (a) EGFR (erbB1); (b) HER2/neu (erbB2); (c) HER3 (erbB3); and (d) HER4 (erbB4; Refs. 5, 6). The regulation of these family members is complex, as the receptors can be transactivated by heterodimeric interaction between two family members and thus use multiple pathways to execute their biological functions. EGFR signaling is also of physiological significance during normal epithelial development, and several EGFR ligands are secreted by normal keratinocytes (7). High expression of EGFR has been shown to induce transformed properties in recipient cells (8), possibly because of excessive

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3 The abbreviations used are: cuSCC, cutaneous squamous cell carcinoma; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; HER, human epidermal growth factor receptor; Pak1, p21-activated kinase 1; HRG, heregulin; MAPK, mitogen-activated protein kinase; RIPA, radioimmunoprecipitation assay; MEK, mitogen-activated protein extracellular signal-regulated kinase kinase.
activation of signal transduction pathways. Recent studies have shown the widespread presence of EGFR family members in keratinocytes and cuSCC (9).

On binding a ligand, EGFR dimerizes and becomes phosphorylated on multiple tyrosine residues and stimulation of signaling pathways, such as Ras/MAPK, Src kinase, and the Pak1 kinase (10–12). EGFR is reported as one of the major contributors to motility in keratinocytes (13–15). MAPK is one of the most well-characterized cell survival components of EGFR signaling; because MAPK is activated by MEK 1 and 2 in response to EGFR activation, it is possible that MAPK could serve an important biomarker of activated EGFR. The protein tyrosine kinase c-Src is also an important signal transduction element in many growth factor receptor signals for proliferation and transformation (16). Likewise, Pak1 is a critical component of many growth factor receptor-mediated signal transduction pathways, leading to directional cell motility, cell invasiveness, and angiogenesis (17–20). EGFR and signaling kinases are activated in cuSCC; thus, anticancer agents that could potentially inhibit these kinases need to be explored.

The exposure of cells to polypeptide growth factors causes reorganization of the cytoskeleton, formation of lamellipodia, membrane ruffling, and changes in cell morphology; accordingly, such exposure is implicated in stimulating cell migration and invasion (21). The motility function is normally repressed in many cells, but it can be activated by appropriate stimuli and/or oncogenic transformation. The small GTPases cdc42 and Rac1 regulate the formation of motile structures via Pak1, which is a serine/threonine kinase (17, 18). Recent studies have shown a mechanistic role for Pak1 activation in the increased invasiveness of breast cancer cells by the polypeptide growth factor heregulin (HRG) and Pak1-mediated promotion of cell migration and anchorage-independent growth (17–19). In addition to its effects on the cytoskeleton, Pak1 also activates c-JunNH2-terminal kinase and extracellular signal-regulated kinase kinases and thus influences nuclear signaling (19). Together, these findings suggest a central role of Pak1 in motility, invasion, and cell survival in human cancer.

Since EGFR pathways are commonly deregulated in human epithelial tumors, therapeutic agents directed against the EGFR represent a promising and important group of biologically based treatment strategies that are in various stages of preclinical and clinical development. One such selective inhibitor of the EGFR-TKI is ZD1839 [Iressa; 4-(3-chloro-4-fluorooanilino)-7-methoxy-6-(3-morpholinoproxy)quinazoline]. ZD1839 markedly inhibits the autophosphorylation of EGFR-stimulated EGFR in a broad range of EGFR-expressing human cancer cell lines and xenograft models and possesses 100-fold less activity against HER2 than EGFR (10, 22–24). In Phase I studies, encouraging antitumor activity has been observed in a selected range of tumor types, including squamous epithelial cancers (25). This study was undertaken to explore the effect of the EGFR-TKI ZD1839 on signaling pathways activated by EGFR that support cell proliferation, survival, and invasion using immortalized keratinocyte and cuSCC cell model systems.

### Material and Methods

#### Cell Cultures and Reagents.

The HaCaT, Colo16, SRB1, and SRB12 (provided by G. L. C.) cell lines were maintained in DMEM/F-12 medium supplemented with 10% FCS. The following antibodies were used: anti-HER1, -HER2, -HER3, and -HER4 (Neomarkers, Fremont, CA); antivinculin (Sigma, St. Louis, MO); phospho-EGFR and phospho-Src antibodies (Biosource International, Camarillo, CA); phospho-HER2 (NeoMarkers); phospho-MEK1, phospho-p42/44 MAPK, phospho-c-JunNH2-terminal kinase, phospho-p38 MAPK, phospho-AKT, phospho-FKHR, and total MAPK (Cell Signaling); and Pak1 (Santa Cruz Biotechnology, Santa Cruz, CA). ZD1839 was from AstraZeneca (Cheshire, United Kingdom). Recombinant human EGF was from Sigma.

#### Cell Extracts and Western Blotting.

Cells were lysed in radioimmunoprecipitation assay buffer supplemented with 100 μM NaF, 200 μM Na3VO4, and 1 × protease cocktail (Boehringer Mannheim, Indianapolis, IN) on ice for 15 min. Cell lysates containing equal amounts of protein were separated by SDS-PAGE and immunoblotted with the indicated antibodies as described previously (19). The protein vinculin was used routinely as a loading control.

Phospho-Pak1 Activity.

Lysate (200 μg) was immunoprecipitated with anti-Pak1, and kinase assays were performed in kinase buffer as described previously (19), using myelin basic protein as a substrate.

Immunofluorescence Studies.

For immunofluorescence studies, cells were plated on coverslips in regular DMEM/F-12 medium supplemented with 10% FCS. After 1 day, cells were shifted to serum-free medium for 48 h and treated with EGF (10 ng/ml; 1.6 nM) for 15 min with or without pretreatment with 1 μM ZD1839 for 15 min. Cells were fixed in 4% paraformaldehyde, blocked in 10% goat serum, and then processed for indirect immunofluorescence as described previously (18). Primary antibodies were visualized by using Alexa488- or Alexa546-conjugated goat second antibodies (Molecular Probes, Eugene, OR). Alexa488-labeled phalloidin was used to visualize F-actin, and nuclear staining used the far red wavelength, DNA-intercalating dye Topro-3 (Molecular Probes). Cells were analyzed by confocal microscopy using appropriate filters.

Cell Growth Assay.

Equal numbers of various cell lines were plated, grown in serum-free media for 48 h, and then treated with ZD1839 and/or EGF as indicated for 3 days, and cell numbers were counted in six fields per treatment group as described previously (26). Each experiment was repeated three times with data graphed as the mean ± SD.

Chemoimmunoassay.

To test the invasion behavior of treated cells, 8-μm filters were coated with Matrigel (20 μg/filter) and placed in Boyden chambers. Cells (105), suspended in DMEM containing 0.1% BSA and different ZD1839 and EGF treatments as indicated, were added to the top chamber. Conditioned medium from mouse fibroblast NIH3T3 cells was used as a source of chemoattractant and placed in the bottom compartment of the Boyden chambers. After 24 h of incubation at 37°C, noninvaded cells were scraped off, and the cells that had migrated to the lower surface of the filter inserts were fixed with 100% methanol for 10 min and stained with H&E (27).
Results and Discussion

To begin delineating the role of EGFR in cuSCC cells, we profiled the expression of HER family members. In A, Western blot analysis was used to evaluate the expression of HER family members in various cell lines grown in DMEM/F-12 (1:1) medium with 10% calf serum. In B, using phospho-specific antibodies in Western blot analysis, EGFR was differentially phosphorylated on tyrosine 1086 (autophosphorylation site), 1068 (Grb2-binding site), and 1173 (PTPase-binding site). In C, serum-starved HaCaT cells were treated with 1 nM EGF for 1 or 4 h. Top panel, autoradiogram of immunoprecipitated HER2 showed a time-dependent increase in receptor phosphorylation. Lower panel, Western blot for total HER2.

Extracellular signal-regulated kinase 1 and 2 (p42/44 MAPK or MAPK) is one of the most well-characterized cell survival components of EGFR signaling; because MAPK is activated by MEK 1 and 2 in response to EGFR activation, it is possible that MAPK could serve an important biomarker of activated EGFR. Next, we evaluated the status of MEK 1 and 2 phosphorylation (site) and MAPK phosphorylation as indicators of kinase activation by Western blot using phospho-specific antibodies. Data in Fig. 2A show the presence of activated MEK 1 and MAPK in immortalized keratinocytes and cuSCC cells. Interestingly, phosphorylation and activation of the MAPK activator MEK 1 were fairly consistent between cuSCC lines, but large differences were apparent in the phosphorylation states of MAPK, although total MAPK expression was fairly consistent. Importantly, the phosphorylation status (indicating activation) of the downstream ef-

Fig. 1. Differential expression and EGF-mediated activation of HERs in keratinocyte and squamous cell carcinoma cell lines. In A, Western blot analysis was used to evaluate the expression of HER family members in various cell lines grown in DMEM/F-12 (1:1) medium with 10% calf serum. In B, using phospho-specific antibodies in Western blot analysis, EGFR was differentially phosphorylated on tyrosine 1086 (autophosphorylation site), 1068 (Grb2-binding site), and 1173 (PTPase-binding site). In C, serum-starved HaCaT cells were treated with 1 nM EGF for 1 or 4 h. Top panel, autoradiogram of immunoprecipitated HER2 showed a time-dependent increase in receptor phosphorylation. Lower panel, Western blot for total HER2.

Fig. 2. Differential activation of signaling kinases in keratinocyte and cuSCC cell lines. Western blot analysis was used to evaluate the expression and phosphorylation states (as an indicator of activation) of kinases downstream of EGFR (A) and the cell survival proteins AKT, FKHR, and Src (B).
factors c-Jun NH2-terminal kinase and p38 was well correlated with MAPK phosphorylation. This was also true for the expression of two other established cell survival signaling molecules: (a) phospho-Akt, and (b) phospho-FKHR (Fig. 2B). Finally, we examined Src phosphorylation (Y215) as an indicator of an active Src kinase. Src was highly activated and fairly consistent among HaCaT and cuSCC cells. Interestingly, most the pathways analyzed above were activated in HaCaT cells and moderately stimulated in Colo16 cells. Therefore, we used HaCaT and Colo16 cells as model systems to further delineate the impact of EGFR activation and downstream signaling pathways on the biology of skin cells.

To further explore the role of MAPK in the action of EGFR in cuSCC cells, we evaluated the effect of EGF treatment on the phosphorylation of EGFR and MAPK in HaCaT cells. In addition, we measured the effects of the EGFR kinase inhibitor ZD1839 on EGF-stimulated phosphorylation of both EGFR and MAPK. As shown in Fig. 3A, EGF (1.6 nm, 15 min) induced heavy phosphorylation of EGFR on Tyr 1086 and 1068. MAPK phosphorylation was also induced by EGF (Fig. 3B). When cells were incubated with both EGF and increasing concentrations of ZD1839, EGFR and MAPK phosphorylation was reduced dramatically in a dose-dependent manner, with IC50s between 0.02 and 0.2 μM. These inhibitory ZD1839 concentrations are similar to those reported previously for EGFR phosphorylation in vitro and in breast cancer cells (22) and provide new data on the effectiveness of ZD1839 against EGFR activation in cuSCC. These data demonstrate that ZD1839 is a potent inhibitor of EGF-stimulated phosphorylation of both EGFR and the survival signal MAPK.
These findings underscore the significance of EGFR signaling in the activation of the MAPK pathway and suggest that MAPK may be an important mediator of EGFR action in cuSCC cells. Because EGF promotes cell motility and Pak1 activity, and EGFR is overexpressed in cuSCC cells, we evaluated the efficacy of ZD1839 in inhibiting Pak1 activity in cuSCC cells. We found that Pak1 activity was elevated in exponentially growing cells compared with serum-starved cells (Fig. 3). EGF treatment significantly increased Pak1 activity in both HaCaT and Colo16 cells (Fig. 3) and, ZD1839...
effectively reduced EGF-induced Pak1 activity to basal levels, albeit only at higher concentrations than those needed to inhibit activation of EGFR and MAPK. These data demonstrate that in this model system, EGF signaling regulates Pak1 kinase activity, and minimal phosphorylation of EGFR seen at higher ZD1839 doses may be sufficient to activate Pak1, or Pak1 may exhibit low-level constitutive activation in these cells through another mechanism. Given the established role of Pak1 activity in cytoskeletal reorganization and directional movement (17–19), these data reveal a putative mechanistic link between EGFR activation and regulation of cuSCC motility and invasiveness. Consistent with the inhibitory action of ZD1839 on the activation of EGFR and MAPK, suboptimal doses of ZD1839 also inhibited the growth of HaCaT and Colo16 cells cultured in 5% serum (Fig. 4). A similar level of inhibition was also achieved when cells were grown in serum-free conditions (data not shown). Exogenous EGF had a marginal effect on cell growth, suggesting that the cells may be constitutively activated by an autocrine mechanism. A low ZD1839 concentration of 0.02 μM did not block EGF-stimulated cell growth (Fig. 4), but higher concentrations were effective inhibitors, which may indicate a threshold for growth inhibitory effects of 0.2 μM in this model system. However, these data demonstrate effective cuSCC growth inhibition by the EGFR-TKI ZD1839.

Tumor cell migration is a critical factor in the formation of solid tumors and is necessary for their spread to distant organs. The process of metastasis requires changes in cell adhesion, increased cell migration, and angiogenesis. We have shown previously that Pak1 plays a role in leading-edge formation and invasiveness of noninvasive breast cancer cells (17) and maintenance of motile/invasive phenotypes of MDA-MB435 cells (18). Because Pak1 activation is also involved in directed migration and controlling downstream signaling (17), and HaCaT and Colo16 cells had significant Pak1 activity (Fig. 2), we investigated whether EGF also up-regulates invasiveness and motility of cuSCC cells and whether invasiveness was blocked by ZD1839. Using Boyden chambers coated with Matrigel and conditioned medium from cultured fibroblasts as a chemo-attractant, serum-starved HaCaT cells were treated with EGF (16 h) with and without ZD1839, and cells migrating to the bottom chamber were counted as invading cells. Results show that ZD1839 effectively blocked HaCaT motility in a dose-dependent manner (Fig. 5A). ZD1839 also induced a dose-dependent reduction in invasive Colo16 cells (Fig. 5B). Thus, the EGFR-TKI ZD1839 may be an effective tool in blocking cuSCC invasion and metastasis.

To validate EGF stimulation and ZD1839 inhibition of EGFR, MAPK, and Pak1 kinase activity, we examined in vivo protein phosphorylation in serum-starved HaCaT and Colo16 cells treated with EGF and/or ZD1839. In each case, cells grown in media supplemented with 10% serum was used as a positive control. Results show that for EGFR (Fig. 6A), as well as the effector kinases MAPK (Fig. 6B) and Pak1 (Fig. 6C), growth factor treatment dramatically increased protein phosphorylation in both cell lines. Induction of phosphorylation was blocked by ZD1839, suggesting that this EGFR effector pathways, resulting in growth inhibition (Fig. 4) and attenuated cell motility (Fig. 5).

The exposure of cells to growth factors causes cytoskeletal reorganization, the formation of lamellipodia, membrane ruffling, and changes in cell morphology; accordingly, such exposure is implicated in stimulating cell migration and invasification (29, 30). To understand the effect of EGFR activation on motile phenotypes, we used confocal microscopy to study cytoskeleton remodeling and subcellular distribution of EGFR in HaCaT and Colo16 cells. EGFR stimulation was associated with significant induction of membrane protrusions, such as pseudopodium and ruffles. EGFR staining was abundant at intercellular adhesive sites and on cellular projections (arrowheads) in cells grown in 10% serum (Fig. 6A). EGF stimulation (1.6 nm) of serum-starved cells was accompanied by distinct cytoplasmic internalization of activated EGFR (red staining, row 3) and drastic remodeling of F-actin containing membranes with formation of motile structures that contained EGFR (arrowheads). ZD1839 treatment alone (row 5) and EGF plus ZD1839 (row 4) blocked EGFR phosphorylation (red) lamellipodia formation and motile cell phenotypes. Importantly, ZD1839 treatment also induced the formation of thick actin cables (arrows) characteristic of nonmotile differentiated cells, independent of EGF treatment. These data support the hypothesis that ZD1839 suppresses cell migration and blocks aggressive cuSCC cell phenotypes.

In summary, we have shown that the EGFR-initiated signaling pathway is a potent inducer of MAPK and Pak1 pathways, as well as reorganization of the cytoskeleton, which allows increased tumor invasiveness. The observed EGF-mediated stimulation of MAPK and Pak1 was closely related to high EGFR expression in head and neck cancer cells, and inhibition of EGFR by ZD1839 was accompanied by inhibition of Pak1 activation and in vitro invasiveness of cancer cells. Because deregulation of EGFR expression, signaling, or both is commonly associated with stimulation of MAPK and Pak1 pathways, the results presented here suggest that the EGFR-TKI ZD1839 may lead to secondary inhibition of MAPK and Pak1 and invasiveness of human cancer cells. Together, these findings generate a testable hypothesis wherein the use of ZD1839 (Iressa) in cells with activated MAPK or Pak1 pathways may potentially lead to beneficial anticancer activity.

Acknowledgments

Iressa is a trademark of the AstraZeneca group of companies.

References

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