Inhibition of the epidermal growth factor receptor increases expression of genes that stimulate inflammation, apoptosis, and cell attachment

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Introduction

The epidermal growth factor receptor (EGFR) belongs to a family of related cell surface receptors, including ErbB1 (EGFR), ErbB2/Her-2, ErbB3, and ErbB4 (1). These receptors contain an extracellular domain for ligand binding, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. Most epithelial cells express the EGFR and secrete several EGF-like growth factors. Ligand binding induces homodimerization of the EGFR, or heterodimerization with other EGFR family members, causing activation of the tyrosine kinase domain on the cytoplasmic terminus. The resulting autophosphorylation on tyrosine promotes interaction of the receptor with adaptor molecules in the cytoplasm and activates multiple signal pathways, including ras/raf/mitogen-activated protein kinase, phospholipase C, and phosphatidylinositol-3 kinase (2). The EGFR is critical for promoting growth, survival, and differentiation of epithelial cells. Mice with a targeted disruption of the EGFR gene display strain-dependent embryonic lethality due to multiple epithelial abnormalities (3). The EGFR is also critical for wound healing due to its ability to stimulate cell proliferation (4), cell migration (5), and angiogenesis (6).

The EGFR is overexpressed or constitutively activated in many types of human cancer, including cervical carcinoma (7, 8), and overexpression has been associated with a poor prognosis (7). EGFR activation can be inhibited using antibodies (9, 10) or small molecule tyrosine kinase inhibitors (11), and inhibition of EGFR function has been shown to decrease growth of several types of human cancer in preclinical studies (12, 13). Gefitinib (Iressa, ZD1839), a kinase inhibitor, was recently approved for treatment of non–small cell lung cancer, and additional drugs are being tested in phase II or III clinical trials. The clinical response to EGFR inhibition varies in different patients; however, factors that influence this response have recently been identified (14–16). EGFR inhibitors have low toxicity, and the most common side effect in patients is an inflammatory acne-like skin rash (17). PD153035 is a specific and reversible inhibitor of the EGFR tyrosine kinase (18). It blocks binding of ATP to the tyrosine kinase domain on the EGFR and suppresses tyrosine phosphorylation and receptor function. Nanomolar concentrations block EGF-mediated cellular activities, including mitogenesis, gene expression, and transformation (19).

Inhibition of the EGFR diminishes growth and survival of cancer cells by several mechanisms. These include arresting cell cycle progression in G1 due to increased expression of the cyclin-dependent kinase inhibitor p27kip (20), increasing the susceptibility of tumor cells to apoptosis by altering expression of multiple proapoptotic and...
antiapoptotic genes (21), and altering attachment of tumor cells to the extracellular matrix and blocking invasion (22). EGFR inhibitors decrease expression of vascular endothelial growth factor and interleukin-8 and inhibit angiogenesis in experimental tumor models (23), and they can stimulate epithelial differentiation (24). Because inhibition of EGFR function has multiple effects on malignant development, it is important to understand how EGFR inhibition alters gene expression in human carcinoma cells.

Most in vitro studies of EGFR inhibitors have been done on carcinoma cells in monolayer culture (19, 20). However, tumors are three-dimensional and individual cells form cell-cell and cell-stromal interactions that strongly influence their biological properties. These interactions are particularly important for growth factor signaling, cell survival, and regulation of gene expression (25). Organotypic culture is an alternative to monolayer culture that allows cells to grow in a three-dimensional arrangement that preserves cell-cell and cell-matrix interactions. Cultures are formed by layering epithelial cells on collagen rafts containing stromal fibroblasts and maintaining rafts at the interface between the culture medium and the air (26). Cultures are nourished by diffusion of nutrients from below, as occurs in vivo. When normal cervical epithelial cells are placed in organotypic culture, they form a well-differentiated stratified squamous epithelium. In contrast, carcinoma cells form an undifferentiated epithelium composed of rapidly growing cells.

The objective of this study was to examine changes in gene expression of carcinoma cells that result from inhibition of the EGFR. Three cervical carcinoma cell lines that are sensitive to EGFR inhibition were grown in organotypic culture and treated with PD153035. Inhibition of EGFR function decreased proliferation of carcinoma cells and blocked invasion into the collagen substrate. Microarray analyses showed that PD153035 altered expression of multiple genes involved in inflammation, cell attachment and migration, apoptosis, and cell cycle regulation.

Materials and Methods

Cell Culture

Human cervical epithelial cells were cultured from biopsies of patients with nonmalignant disease (usually fibroids or endometriosis) as described (27), and carcinoma cell lines were derived from primary cervical cancers (28). Cells were maintained in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA). Organotypic cultures were prepared as described (29) and maintained in raft medium composed of Ham’s F12 and DMEM (50:50 mixture) containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT) plus growth factors, including EGF (5 ng/mL), insulin, transferrin, triiodothyronine, hydrocortisone, and cholera toxin (26). After 10 days, organotypic cultures were used for isolation of RNA or they were fixed with 3% buffered formalin for histology.

PD153035 (Calbiochem, San Diego, CA) was dissolved in DMSO to make a 1.0 mmol/L stock and aliquots were stored at −20°C in the dark. Cultures were treated with fresh medium containing different concentrations of PD153035 (0.03, 0.1, 0.3, 1.0, and 3.0 µmol/L) every 2 days for a total of 10 days (chronic treatment) or for 12 hours (acute treatment). Controls were treated with medium containing 0.1% DMSO.

Immunoprecipitation and Western Blot Analyses

Monolayer cultures of carcinoma cells were treated with PD153035 for 2 hours and 100 ng/mL of EGF were added 5 minutes before cells were lysed in radioimmunoprecipitation assay buffer. Lysates were processed for immunoprecipitation and Western blotting as described (30). Cell lysates (500 µg) were immunoprecipitated with 1 µg of anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), the proteins were fractionated in 7.5% polyacrylamide gels and then transferred to nitrocellulose membranes, and the membranes were blocked overnight in 20% nonfat dry milk at 4°C. Membranes were incubated with anti-phosphotyrosine primary antibody (Upstate Biotechnology, Waltham, MA; 1:750) for 1 hour at room temperature and incubated in secondary antibody for 30 minutes. The chemiluminescent signal was visualized using the SuperSignal Western Detection Kit (Pierce, Rockford, IL). Membranes were treated with antibody stripping buffer (Chemicon, Temecula, CA) and reprobed with anti-EGFR primary antibody diluted 1:500 (Santa Cruz Biotechnology) to ensure that equal levels of EGFR were present in each immunoprecipitation.

DNA Synthesis and Apoptosis Assays

Organotypic cultures were pulsed with bromodeoxyuridine (BrdUrd) for 2 hours immediately before fixation (31). Slides were stained with antibody to BrdUrd, counterstained with hematoxylin, and the percentage of BrdUrd-positive cells (per 300 cells in each of six specimens) was counted as described (31). The percentage of apoptotic cells in organotypic cultures was determined using the Apoptag Plus Kit (Intergen Co., Purchase, NY) as described (31). Differences between the means of each experimental group were compared by the Student’s t test at P < 0.05.

Invasion into the Collagen Raft

Histologic sections of rafts were stained with hematoxylin, and three microscopic fields from four different cultures were randomly chosen and photographed at low power. The number of epithelial cells that penetrated into the collagen raft was counted directly from the digital image. Invading tumor cells were distinguished from NIH 3T3 fibroblasts within the raft because the tumor cells were larger in size, they stained more darkly, and they invaded as aggregates of cells. The mean number of invading cells was compared in different groups by the Student’s t test at P < 0.05.

RNA Isolation

Total RNA was isolated from the epithelial layer of organotypic cultures by gently scraping the carcinoma cells from the collagen raft and lysing cells with Trizol (Invitrogen) as described (30). Carcinoma cells were
recovered from rafts as an intact sheet without noticeable contamination by fibroblasts or collagen. RNA was stored at −80°C and was intact and of good quality as judged by gel electrophoresis and observation of 28S and 18S rRNA bands (data not shown).

**cDNA Synthesis and cDNA Array Hybridization**

The National Cancer Institute oncochip glass arrays were manufactured and printed at the National Cancer Institute microarray core facility (Advanced Technology Center, Gaithersburg, MD). The oncochip contains 2,208 named cDNAs that are immobilized on poly-L-lysine–coated glass slides. The procedures for cDNA synthesis and cDNA array hybridization have been described in detail previously (30).

**Scanning and Analysis of cDNA Array Data**

Detection of the fluorescent hybridization signals was done using a GenePix 4000A laser scanner. Arrays were scanned at 5-μm resolution with variable photomultiplier tube voltage and laser intensity to obtain maximal signal intensities with <1% saturated spots. Images for both the Cy3 (control) and the Cy5 channels (treated cell cultures) were merged and analyzed using the Gene Pix 4.0 software package. The intensity of array elements was measured as medians of all pixels covered by an individual spot diameter, and local background was subtracted. For each set of experiments, at least three replicate experiments were done (Table 1). The Gene-Spring 6.0 software package (Silicon Genetics, Redwood City, CA) was used for normalization and scaling of all genes and arrays. Using the 50% percentile of each dye channel’s intensity range, relative expression values were normalized per chip by scaling the average of the intensities of all genes to a constant target intensity. All 20 microarrays within the experiment set were scaled to a constant average intensity. Each gene’s measured intensity was divided by its control channel value in each sample (relative Cy3/Cy5 ratio, raw expression values/ratios available as supplementary data file). Spots with low normalized intensity (<300 units), a spot size of <60 μm, or high background (ratio of signal to background <2.5 or <2 SDs of signal intensity) were excluded from analysis unless the other channel was >2,000. The Student’s t test/ANOVA on the normalized expression ratios was used to identify those genes with normalized expression ratios that are statistically different from 1.0 (95% confidence interval; significance level for a two-sided test, P < 0.05). This resulted in 312 genes that were significantly changed by a factor of 2-fold in at least one subset of experiments. We used Onto-Express to automatically translate the lists of genes into functional profiles using information available from the GeneOntology database. Of 312 significant genes, sufficient functional information was available for 186. Relative expression ratios were log transformed (2), sorted by expression similarity (self-organizing map), and displayed using the TreeView program package (32).

**Nuclear Factor κB Reporter Gene Assay**

Monolayer cultures of carcinoma cell lines in 12-well dishes were treated with PD153035 (0.1, 0.3, and 1.0 μM/L) for 24 hours. Triplicate wells were then cotransfected with 1.0 μg per well of pLucNF-κB [four nuclear factor κB (NF-κB) binding sites joined to a firefly luciferase reporter gene; Promega, Madison, WI] and 0.1 μg per well of pTKLuc (weak thymidine kinase promoter joined to the Renilla luciferase gene; Promega) for 3 hours using LipofectAMINE reagent (Invitrogen). Cell lysates were prepared after 24 hours and analyzed using the dual luciferase assay (Promega). The data were normalized for transfection efficiency by taking the ratio of NF-κB firefly luciferase activity to the corresponding Renilla luciferase activity. In control experiments, cells were cotransfected with an expression vector for p65 (RelA) or a dominant-negative mutant of IκBα to confirm that the reporter assay could measure increases or decreases in NF-κB activation. All experiments were repeated thrice and statistically significant values were determined by the Student’s t test at P < 0.05.

**PCR**

Real-time PCR with the LightCycler system (Roche Diagnostics, Mannheim, Germany) was used to quantitatively assess gene expression as described (33). The DNA sequences of all primers used for amplification are available on request. Glyceraldehyde-3-phosphate dehydrogenase was used to control for equal RNA loading and to normalize all other genes tested in identical cDNA samples. The ratio of each analyzed cDNA was determined as the mean of two experiments. Melting curves of the PCR products were routinely done for quality control, and primer pairs resulting in multiple products were excluded from further analyses. Semiquantitative reverse transcription-PCR was done as described (31).

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**Table 1. Summary of array hybridizations**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cells</th>
<th>Treatment</th>
<th>No. arrays</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CXT1</td>
<td>Chronic exposure</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>CXT2</td>
<td>(10 d) on rafts</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>CXT3</td>
<td>Chronic exposure</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>CXT1, CXT2, CXT3</td>
<td>Acute exposure (12 h) on rafts</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>CXT1, CXT2, CXT3</td>
<td>Exposure in monolayer culture (5 d)</td>
<td>3</td>
</tr>
</tbody>
</table>

**NOTE:** A total of 20 microarray hybridization experiments were performed using three different cervical carcinoma cell lines. Experiments were divided into five groups based on cell line, length of exposure to PD153035, or culture conditions.

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4 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org).
5 http://vortex.cs.wayne.edu
6 http://www.godatabase.org
To exclude the saturation or plateau effect of amplification and to optimize PCR conditions for each gene individually, PCR was repeated using a total number of 20, 25, and 30 cycles. Each reaction was done at least twice using independent reverse transcription reactions to confirm reproducibility.

Results

Cervical Carcinoma Cells Express High Levels of the EGFR in Organotypic Culture

Organotypic cultures of normal cervical cells formed a well-differentiated stratified squamous epithelium similar to normal cervix (Fig. 1A). In contrast, carcinoma cells formed thick undifferentiated epithelia (Fig. 1A). The carcinoma lines expressed higher levels of EGFR RNA than normal cervical cells (Fig. 1B). However, expression of ErbB2, ErbB3, and ErbB4 mRNAs and EGF-like growth factors (transforming growth factor-α, amphiregulin, β-cellulin, epiregulin, and heparin-binding EGF) was variable, and no consistent differences were observed between normal and carcinoma cells (data not shown). Thus, carcinoma cells expressed EGFR mRNA at increased levels relative to normal cells, as occurs in many cervical cancers (34).

PD153035 Inhibits EGFR Tyrosine Phosphorylation in a Dose-Dependent Manner

We did dose-response experiments with PD153035 to determine the minimal dose required to completely inhibit EGFR activity in two different carcinoma cell lines. PD153035 strongly inhibited phosphorylation of the EGFR at concentrations above 0.1 μmol/L (Fig. 2A). However, similar levels of PD153035 did not inhibit tyrosine phosphorylation of platelet-derived growth factor receptor (data not shown). We used PD153035 at concentrations between 0.1 and 3.0 μmol/L to treat cells in organotypic culture.

Biological Effects of PD153035 in Organotypic Culture

Three cervical carcinoma cell lines (CXT1, CXT2, and CXT3) previously determined to be sensitive to EGFR inhibition were maintained in organotypic culture for 10 days and treated every other day with PD153035. Controls consisted of cultures treated with 0.1% DMSO (the vehicle used to dissolve PD153035). The untreated cultures formed thick undifferentiated epithelia that invaded into the underlying collagen raft (Fig. 2B). The number of invading cells varied for each carcinoma cell line. However, treatment with PD153035 strongly blocked invasion of each cell line in a dose-dependent manner (Fig. 2C). To measure the effect of PD153035 on DNA synthesis, cultures were pulsed with BrdUrd for 2 hours and processed for immunohistochemistry using an anti-BrdUrd antibody. PD153035 significantly decreased the percentage of BrdUrd-positive cells in each cell line (Fig. 2D). Experiments also examined whether PD153035-induced apoptosis as measured by the terminal deoxynucleotidyl transferase–mediated nick-end labeling assay. In untreated cultures, the number of apoptotic cells varied in each carcinoma cell line, but treatment with PD153035 did not significantly increase the number of apoptotic cells in any cell line (data not shown).

Microarray Analyses

Microarray analyses were done to examine changes in gene expression that resulted from inhibition of the EGFR. The experiments were organized into five different groups (Table 1). The majority of experiments (groups 1–3) examined carcinoma cell lines CXT1, CXT2, and CXT3 treated with PD153035 chronically (10 days) in organotypic culture. However, a small number of experiments explored the response of cells after acute exposure to PD153035 (12 hours) in organotypic culture, or after chronic treatment in conventional monolayer culture. The overall analysis of microarray data identified 312 genes that were significantly up-regulated or down-regulated in at least one
Many of these genes could be classified into one of the four largest functional clusters of genes, including genes that regulate (a) inflammation and immunity, (b) cell attachment and motility, (c) apoptosis, and (d) cell cycle progression (Fig. 3). The response to PD153035 varied in each carcinoma cell line and was significantly different between CXT2 and the other carcinoma cell lines, CXT1 and CXT3 (one-way ANOVA, $P < 0.05$). Chronic exposure of cells in organotypic culture induced a different pattern of gene expression than acute exposure in organotypic culture or chronic exposure in monolayer culture (Fig. 3; Supplementary Data). Quantitative real-time reverse transcription-PCR was done to determine whether changes in gene expression observed with microarrays could be confirmed by an alternate method. Primers were constructed for selected genes in each of the four functional groups. As shown in Fig. 4, genes whose expression was altered in microarray analyses were also confirmed to be up-regulated or down-regulated by real-time reverse transcription-PCR.

**Functional Classes of Genes Perturbed by EGFR Inhibition**

Alterations in expression of cell cycle and growth regulatory genes were expected because PD153035 inhibited DNA synthesis (Fig. 2D). PD153035 decreased expression of genes that are required for growth such as cell cycle kinases (cdc6 and cdc2L5), transcription factors (c-myc and E2F4), and mitogens such as insulin-like growth factor 1 (IGF-1), insulin receptor (INSR), and fibroblast growth factor 9 (FGF9). Many of these genes were down-regulated in each of the five experimental groups (Fig. 3, cluster IV). Although treatment with PD153035 did not directly stimulate apoptosis in organotypic culture, the drug inhibited expression of several antiapoptotic genes (Fig. 3, cluster III). These included survivin (BIRC5) an apoptotic inhibitor that is expressed during the G2-M phase of the cell cycle; BCL2-associated athanogene (BAG1) that enhances the antiapoptotic effects of BCL2; and RELA, which is the p65 subunit of NF-$\kappa$B. PD153035 also stimulated expression of death associated protein kinase 1 (DAPK1) and the proapoptotic transcription factor requiem (REQ).

The most obvious effect of EGFR inhibition was decreased invasion of carcinoma cells into the collagen substrate. In this regard, PD153035 decreased expression of genes encoding cytoskeletal proteins including vinculin (VCL) which anchors $\alpha$-actin to the inner surface of the plasma membrane and the actin-binding protein $\alpha$ 1 actinin (ACTN1; Fig. 3, cluster II). PD153035 stimulated expression of cell adhesion molecules, including integrin $\alpha$ 8 (ITGA8), a component of the receptor for fibronectin, and transforming growth factor $\beta$-induced protein (TGFBI) that mediates cell-collagen interaction.
A most novel finding was that PD153035 altered expression of multiple genes that can stimulate inflammation and the innate immune response (Fig. 3, cluster I). These included chemokines that attract T lymphocytes, such as CCL18, XCL1, CXCL9, and CX3CL1; chemokines for inflammatory cells, such as macrophage inflammatory protein-1α (CCL3); and the chemokine receptor CXCR6. PD153035 induced expression of cytokines that stimulate immune function, including interleukin-6 (IL6), IL-7, and tumor necrosis factor ligand superfamily member 4 (TNFSF4). Inhibition of EGFR function also increased genes that regulate IFN expression or signaling, including IFN regulatory factor 5 (IRF-5) and IFN consensus sequence-binding protein 1 (ICSBP1) that control expression of IFN-α and IFN-β–regulated genes that are induced by viral infection.

Activation of NF-κB by EGFR Inhibition

The transcription factor NF-κB is known to stimulate expression of proinflammatory genes (35); therefore, we examined whether PD153035 activated NF-κB using a reporter gene assay. EGFR inhibition induced a dose-dependent activation of NF-κB in two of three carcinoma.

**Figure 3.** Cluster analysis of gene expression in response to PD153035. Treatment groups (top) and differentially expressed genes (right). Lanes 1-6, median changes in gene expression (left); lanes 7-26, results of each individual experiment (right). The concentration of PD153035 was 1.0 μmol/L in most experiments, although selected experiments were done with 0.1 or 0.3 μmol/L drug. Cluster I, inflammation and immune response; cluster II, cell attachment; cluster III, apoptosis; cluster IV, cell cycle.
cell lines used in this study and two of three additional carcinoma cell lines (Fig. 5). PD153035 also activated NF-κB in two independently isolated cultures of normal cervical epithelial cells that were infected with retroviruses containing the HPV-16 E6/E7 genes. These cultures were examined shortly after infection with HPV-16, before they had undergone immortalization or transformation. Thus, this response was not unique to carcinoma cells. The CXT1 and CXT6 carcinoma cell lines that exhibited the largest induction of NF-κB also had a strong induction of proinflammatory gene expression. These results show that inhibition of the EGFR activates NF-κB, and they suggest that NF-κB activation might contribute to the induction of cytokine gene expression by PD153035.

Discussion

The EGFR is overexpressed in several types of human cancer (36, 37), and overexpression is frequently associated with a poor prognosis. Inhibition of EGFR function is a potential strategy for therapy or chemoprevention of several types of human cancer (10–13). Thus, it is important to understand how inhibition of EGFR function alters gene expression in carcinoma cells. We used cDNA microarrays to examine this question in cells grown in three-dimensional organotypic culture. Our experiments identified 312 genes that were increased or decreased after treatment with the EGFR inhibitor, PD153035. These genes were classified into four major functional groups, including genes associated with (a) inflammation, (b) cell attachment, (c), apoptosis, and (d) cell cycle regulation. Our results also showed that the effects of PD153035 on gene expression varied in each carcinoma cell line. Similar variability has been reported after treatment of human tumor xenografts in nude mice with the EGFR inhibitor, gefitinib (15).

Our most novel observation was that EGFR inhibition altered expression of many genes that regulate the inflammatory or innate immune response. PD153035 stimulated expression of multiple chemokine genes including chemokine genes ligand 1 (XCL1), fractalkine (CXCL1), and macrophage inflammatory protein 4 (CCL18) that are chemotactic for T lymphocytes (38). Other proinflammatory or immunoregulatory cytokines (IL-6, IL-7, and TNFSF4), cytokine receptors (IL-13RA1 and CXCR6), and signaling molecules (IRF3 and ICSBP1) were also stimulated. Our results are consistent with a recent report that blockade of EGFR function induces a deranged chemokine expression in keratinocytes leading to enhanced skin inflammation (39). In this study, inhibition of EGFR activity in skin before antigen challenge resulted in a markedly enhanced immune response with increased chemokine expression and heavier inflammatory cell infiltrate. The ability of EGFR inhibitors to activate genes associated with inflammation is consistent with their common side effect of producing an inflammatory, acne-like skin rash (17). Interestingly, patients who develop a skin rash often respond to anti-EGFR therapy better than patients who do not (40).

In the cervix, epithelial cells secrete multiple cytokines that activate inflammation and regulate the immune response to human papillomavirus (HPV) infection (41, 42), which is the major risk factor for cervical cancer. However, the innate and adaptive immune responses to HPV are frequently delayed or ineffective (43, 44). In animal models, HPV-associated carcinogenesis is often accompanied by an absence of inflammation, tolerance to HPV antigens, and immunosuppression (45). The ability of EGFR inhibitors to stimulate NF-κB activity and expression of proinflammatory or immunoregulatory cytokines could provide important “danger signals” that facilitate immune recognition and destruction of HPV-containing premalignant cells or tumor cells. Thus, activation of inflammation and innate immunity might represent a novel mechanism by which EGFR inhibitors could help to prevent persistent HPV infection or progression to cervical cancer.

The transcription factor NF-κB is an important activator of the inflammatory and immune responses (35). NF-κB protects against apoptosis, it stimulates cell proliferation, and it is often activated during the process of carcinogenesis (46). Signaling through the EGFR has been shown

Figure 4. Confirmation of array results for a subset of genes by quantitative real-time reverse transcription-PCR. Raft cultures of CXT3 carcinoma cells were treated with PD153035 for 10 d and expression of specific genes was assessed by real-time reverse transcription-PCR. Normalized to expression of glyceraldehyde phosphate dehydrogenase.

Figure 5. PD153035 activates NF-κB in a dose-dependent manner. Cervical carcinoma cell lines (CXT1, CXT2, CXT5, and CXT6) or normal cervical epithelial cells infected with HPV-16 E6 and E7 (HCX1 and HCX2) were treated with various doses of PD153035 for 24 h and lysates were examined in a NF-κB reporter gene assay. Columns, mean of three experimental wells; bars, ±SE. Experiments were repeated three times. *, P < 0.05, statistically different from untreated cultures (t test).
to activate NF-κB (47); however, we observed that inhibition of EGFR signaling caused a 2- to 10-fold activation of NF-κB. PD153035 stimulated a dose-dependent increase in activation of NF-κB in four of six carcinoma cell lines examined. This response was not limited to cancer cells, because PD153035 also stimulated NF-κB activation of normal cervical epithelial cells (the progenitor cells of cervical carcinoma) shortly after they were infected with HPV-16 E6 and HPV-16 E7 genes (<1 week). E6 and E7 functionally inactivate the p53 and retinoblastoma tumor suppressor proteins and are sufficient to induce immortalization of normal keratinocytes. These infected cervical cells are not transformed, but they are also not normal because the cell cycle is deregulated and they are more sensitive to apoptosis. Interestingly, the carcinoma cell line that exhibited the greatest induction of proinflammatory gene expression (CXT1) also exhibited strong activation of NF-κB (Fig. 5). The pathway by which PD153035 activates NF-κB reporter gene activity in cultured carcinoma cells is unclear, but it may be important for understanding the role of EGFR inhibitors in cancer therapy.

Inhibition of EGFR function increases the susceptibility of carcinoma cells to a variety of apoptosis-inducing agents including radiation (48) and chemotherapeutic drugs (49, 50), and this may be an important mechanism for antitumor activity (21). Our results showed that PD153035 decreased expression of several antiapoptotic genes including secreted frizzled-related protein 1 (SFRP1), the apoptosis inhibitor survivin (BIRC5), and BCL2-associated athanogene (BAG1). PD153035 up-regulated two proapoptotic genes proteins, death-associated protein kinase-1 (DAPK) and apoptosis response zinc finger protein requiem (REQ). These results are consistent with previous work showing that EGFR inhibitors stimulate important proapoptotic genes and down-regulate antiapoptotic genes (21, 51). However, our terminal deoxynucleotidyl transferase–mediated nick-end labeling assays showed that PD153035 did not significantly stimulate apoptosis of cervical carcinoma cells in organotypic culture. This apparent contradiction might be explained by that fact that cells treated with PD153035 were not directly exposed to a strong apoptosis-inducing agent, and cells in organotypic culture establish cell-cell and cell-matrix interactions that protect against programmed cell death (52). Furthermore, our observation that EGFR inhibition activates NF-κB, a potent antiapoptotic factor, suggests that EGFR inhibition might exert both positive and negative effects on survival of cervical cancer cells.

Inhibition of EGFR function prevented invasion of cancer cells into the collagen substrate of organotypic cultures. Inhibition was dose-dependent and observed for each carcinoma cell line. Our results support previous observations that EGFR inhibition stimulates cell-cell attachment (55) and that the EGFR is critical for invasion and metastasis of cancer cells (56, 57).

The response to PD153035 varied in each carcinoma cell line. Some of this variation was due to the microarray procedure. However, the K-means clustering algorithm showed that the CXT2 carcinoma cell line was intrinsically different than the others. In particular, many genes were less strongly changed in CXT2 in response to the drug than in CXT1 or CXT3 cell lines.

In summary, our results identify multiple genes whose expression is altered by the EGFR inhibitor, PD153035. The ability of EGFR inhibition to activate NF-κB and stimulate expression of multiple genes involved in inflammation and innate immunity defines a novel pathway that might contribute to recognition and elimination of premalignant or malignant cervical cells.

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