Prevention of Bronchial Hyperplasia by EGFR Pathway Inhibitors in an Organotypic Culture Model

Jangsoon Lee1, Seung-Hee Ryu1, Shin Myung Kang1, Wen-Cheng Chung1, Kathryn Ann Gold2, Edward S. Kim1, Walter N. Hittelman3, Waun Ki Hong1, and Ja Seok Koo1

Abstract

Lung cancer is the leading cause of cancer-related mortality worldwide. Early detection or prevention strategies are urgently needed to increase survival. Hyperplasia is the first morphologic change that occurs in the bronchial epithelium during lung cancer development, followed by squamous metaplasia, carcinoma in situ, and invasive tumor. This study was designed to determine the molecular mechanisms that control bronchial epithelium hyperplasia. Using primary normal human tracheobronchial epithelial (NHTBE) cells cultured by using the 3-dimensional (3D) organotypic method, we found that the epidermal growth factor receptor (EGFR) ligands, EGF, TGF-α, and amphiregulin induced hyperplasia, as determined by cell proliferation and multilayered epithelium formation. We also found that EGF induced increased cyclin D1 expression, which plays a critical role in bronchial hyperplasia; this overexpression was mediated by activating the mitogen-activated protein kinase pathway but not the phosphoinositi de 3-kinase/Akt signaling pathway. Erlotinib, an EGFR tyrosine kinase inhibitor, and U0126, a MAP/ERK kinase (MEK) inhibitor, completely inhibited EGF-induced hyperplasia. Furthermore, a promoter analysis revealed that the activator protein-1 transcription factor regulates EGF-induced cyclin D1 overexpression. Activator protein-1 depletion by using siRNA targeting its c-Jun component completely abrogated EGF-induced cyclin D1 expression. In conclusion, we showed that bronchial hyperplasia can be modeled in vitro by using primary NHTBE cells maintained in a 3D organotypic culture. EGFR and MEK inhibitors completely blocked EGF-induced bronchial hyperplasia, suggesting that they have a chemopreventive role.

Cancer Prev Res; 4(8); 1306–15. ©2011 AACR.

Introduction

Hyperplasia in the bronchial epithelium, as evidenced by increased cell proliferation, is associated with conditions such as trauma, smoking, chronic cough, chronic inflammatory airway disease, and cancer. It is the first of several progressive, cumulative, genetic, and morphologic changes associated with lung squamous cell carcinoma, followed by squamous metaplasia, dysplasia, and carcinoma in situ (1–3). These extensive, multifocal changes occur throughout the respiratory tree when the lungs are chronically exposed to common carcinogens, a phenomenon known as field cancerization (4).

In the developed lung, growth factors and their signaling receptors support cellular activities in equilibrium, preserving normal lung structure, and function (5). However, this homeostatic control can be compromised during the accumulation of genetic and molecular alterations that lead to lung cancer. Several decades of research have revealed that the ErbB system is a critical growth factor system in normal and abnormal epithelial cell proliferation (6, 7). The ErbB family, ErbB1-4, plays an important role in lung cancer development. In addition, several ErbB ligands are aberrantly regulated in cancer cells. Therefore, we hypothesized that bronchial hyperplasia results from ErbB hyperactivation in bronchial epithelial cells. To test this hypothesis, we evaluated ErbB ligands for their ability to induce bronchial hyperplasia by using a 3-dimensional (3D) organotypic air–liquid interface primary bronchial epithelial...
cell culture system (8–10). We then determined which downstream signaling pathways and genes were involved in bronchial hyperplasia development. Epidermal growth factor receptor (EGFR) ligands induce bronchial hyperplasia via the MAP/ERK kinase (MEK)/extracellular signal regulated kinase (ERK) signaling pathway. EGF-induced cyclin D1 overexpression plays a critical role in the development of bronchial hyperplasia. EGFR and MEK inhibitors completely blocked EGF-induced bronchial hyperplasia.

As monotherapy, erlotinib, a small molecular inhibitor targeting the intracellular tyrosine kinase domain of EGFR, significantly prolonged survival in previously treated advanced non–small cell lung cancer (NSCLC) patients compared with placebo (13) and was recently approved by the Food and Drug Administration. Erlotinib has antiproliferative effects arising from G1 arrest and proapoptotic effects on cancer cells (14). However, its effects on normal and hyperplastic bronchial epithelial cells are unknown. Our results showed that erlotinib blocks EGF-induced bronchial hyperplasia and can reverse hyperplasia, restoring normal bronchial epithelial morphologic characteristics. We identified several mechanisms involved in the onset of changes leading to lung cancer, such as abnormal cell proliferation, which may be targets for preventing malignant progression.

Materials and Methods

Chemicals

Erlotinib (LKT Laboratories, Inc.), U0126, LY294002, and Akt inhibitor VIII (Calbiochem) were dissolved in dimethylsulfoxide.

Cells

Normal human tracheobronchial epithelial (NHTBE) cells were obtained from the Lonza Walkersville, Inc. A549 lung cancer cells were obtained from the American Type Culture Collection. We authenticated A549 cell by genotyping through MD Anderson Cancer Center DNA analysis core facility.

3D organotypic air–liquid interface cell culture and treatment

We cultured NHTBE, or A549 cells by using the 3D organotypic air–liquid interface method described previously (9, 11). The medium in the bottom chamber was changed every 24 hours. We treated 7-day-old confluent NHTBE cells grown on a porous membrane of a culture plates (Corning) overnight and cotransfected with several siRNAs by using the Dharmafect 1 transfection reagent (Dharmacon), according to the manufacturer’s instructions. A mixture of several siRNAs ensured that the targeted gene product was effectively deleted. Cells at 60% to 70% confluency were treated with EGF (5 ng/mL) for 24 hours, when target protein levels had been reduced more than 70%, as assessed by Western blot analysis.

Western blot analysis

We prepared total protein extracts by using cold radioimmunoprecipitation assay lysis buffer (50 mmol/L HEPES, pH 7.4; 1% NP-40, 150 mmol/L NaCl, 1 mmol/L EDTA, phosphates inhibitors, and protease inhibitors). Protein (15 μg) was resolved by 10% SDS-PAGE gel. Membranes were incubated with rabbit polyclonal antibodies against cAMP responsive element binding protein (CREB), phospho-CREB-133 (Upstate Biototechnology), ERK, phospho-ERK-202/204, cyclin A1, cyclin B1, cyclin D1, cyclin E2, Akt, phospho-Akt-473, c-Jun, phospho-c-Jun-73, p-EGFR-1068, and EGFR (Cell Signaling Technology) overnight. β-Actin (clone AC-15; Sigma-Aldrich) was used as a loading control. The reaction of proteins with primary antibodies was visualized with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents (GE Healthcare).

siRNA

Human c-Jun (accession no. NM_002228) siRNAs were used to knock down c-Jun expression (Sigma-Aldrich), according to the manufacturer’s instructions. A mixture of several siRNAs ensured that the targeted gene product was effectively deleted. Cells at 60% to 70% confluency were transfected for 48 hours with a final concentration of 100 nmol/L c-Jun siRNA or nonspecific control pooled siRNAs by using the Dharmafect 1 transfection reagent (Dharmacon), according to the manufacturer’s instructions. The cells were treated with EGF (5 ng/mL) for 24 hours, when target protein levels had been reduced more than 70%, as assessed by Western blot analysis.

Immunohistochemistry and immunofluorescence

The NHTBE cells were fixed in neutral-buffered formalin and embedded in paraffin. Sections (5 μm each) were prepared by using a microtome, mounted on slides, deparaffinized in xylene, rehydrated in graded alcohols, and washed in distilled water. Endogenous peroxidases were quenched by incubation in 3% H2O2. Antigens were retrieved by microwaving the sections in 10 mmol/L citric acid (pH 6.0) for 5 minutes. The slides were washed 3 times with PBS and blocked for 30 minutes with 10% normal goat serum in 1% bovine serum albumin (BSA)/PBS. Immunohistochemical staining was visualized by using the Histostain-Bulk–SP and the AEC red substrate kits (Zymed Laboratories). Immunohistochemical staining without a primary antibody was done as a negative control. Immunofluorescence staining was visualized by using Cy-3 (Jackson ImmunoResearch) and 4',6-diamidino-2-phenylindole (Invitrogen). Stained slides were visualized with an Axioskop 40 fluorescence microscope (Carl Zeiss); the images were captured at a magnification of 200× and stored by using Axiovision LE software v4.5 (Carl Zeiss) according to the manufacturer’s instructions.

Luciferase reporter assay

NHTBE cells (5 × 104) were cultured in 12-well tissue culture plates (Corning) overnight and cotransfected with
cyclin D1 promoter-luciferase constructs (wild type, AP-1 site mutant, or CRE site mutant; kindly provided by Dr. Richard Pestell at Thomas Jefferson University) and Renilla luciferase control vector by using Lipofectamin 2000 (Invitrogen). After 24 hours, the culture medium was changed to 0.1% BSA in bronchial epithelial basal medium (BEBM; Lonza Walkersville, Inc.), and cells were treated with or without EGF (5 ng/mL) for 24 hours. Luciferase activity was detected by using the dual-luciferase reporter assay (Promega) and measured by using a Lumat LB 9507 tube luminometer (Berthold). All assays were done in triplicate and repeated at least 3 times. Figures show representative results.

Hyperplasia evaluation via cell layer thickness and cell number

To determine ErbB receptors ligands’ effects on the histomorphologic characteristics of NHTBE cells, we incubated NHTBE cells with EGF (10 ng/mL), TGF-α (10 ng/mL), AR (50 ng/mL), or HR (100 ng/mL) for 4 days. After making paraffin-embedded blocks, we captured 3 images from each block within a 10-mm area from the center of the Transwell membrane with an Axioskop 40 microscope (Carl Zeiss) under light microscopy (200×). To evaluate hyperplasia, we measured thickness by using Axiosvision LE software v4.5 (Carl Zeiss) according to the manufacturer’s instructions. Total cell numbers in the captured area were counted manually under light microscopy (200×). Bars, SE; **, P < 0.01 and ***, P < 0.001.

Statistical analysis

For each experimental outcome, descriptive statistics (mean, SD, median, and range) were summarized for each group. An ANOVA model was used to detect any differences between treatment and control groups.

Results

EGFR ligands induce hyperplasia in bronchial epithelial cells grown in 3D organotypic culture

To determine whether ErbB receptors are involved in bronchial hyperplasia morphologic changes, we cultured NHTBE cells by using the organotypic air–liquid interface method with various ErbB receptor ligands, including EGF (10 ng/mL), TGF-α (10 ng/mL), AR (50 ng/mL), and HR (100 ng/mL), for 4 days. We histochemically evaluated ErbB ligands’ effects on morphologic changes in NHTBE cells and found that the NHTBE cell layer was statistically significantly thicker after treatment with EGFR ligands, EGF, TGF-α, and AR but not with HR (Fig. 1A). Quantitative changes are shown as mean thicknesses: control = 13 (± 2) μm, EGF treated = 42 (± 6) μm, TGF-α treated = 31 (± 4) μm, AR treated = 28 (± 3) μm, and HR treated = 15 (± 2) μm cells. These data clearly show that high EGFR ligand concentrations induce bronchial epithelial cell hyperplasia. The most prominent hyperplastic morphologic changes in NHTBE cell culture histologic patterns were induced by EGF; therefore, we selected EGF for subsequent study.

To determine dose-dependent effects of EGF on NHTBE cell hyperplasia, we incubated NHTBE cells with EGF for 4 days. Immunohistochemical analysis and cell quantitation clearly indicated that EGF-induced hyperplasia in a dose-dependent manner. NHTBE cell layers were significantly thicker after treatment with 5 ng/mL of EGF, and cell quantitation showed a similar pattern (Fig. 1B). To determine time-dependent effects of EGF, we incubated NHTBE cells with 5 ng/mL of EGF for 1 to 4 days. The NHTBE cell layer expanded in a time-dependent manner. Four days of treatment resulted in an approximately 2.5-fold increase in cell number and cell layer thickness (Fig. 1C).

EGF induces cell proliferation at only the basal layer of NHTBE cell cultures

Multilayered hyperplasia is believed to result from uncontrolled bronchial epithelial cell proliferation. To identify the specific NHTBE cell population after EGFR ligand exposure, we conducted Ki-67 immunostaining in NHTBE cells grown in a 3D organotypic culture system. We used A549 cells as a positive control. As shown in Figure 2, immunohistochemical (Fig. 2A) and immunofluorescence (Fig. 2B) analyses of Ki-67 as a cell proliferation indicator revealed positive staining in cells in the basal layer of NHTBE cell cultures. In contrast, Ki-67-positive cells were detected randomly in both basal and parabasal layers in A549 cells. These results indicate that only NHTBE cells in the basal layer divide and grow in response to EGF; cancer cells are not limited to the basal layer.

MEK/ERK pathway is a critical signaling pathway for EGF-induced NHTBE cell hyperplasia

The MEK/ERK and PI3-K/Akt pathways are well-established downstream signaling pathways of the EGF–EGFR pathway (12). To determine the relative importance of these pathways for transmitting EGF-induced hyperplasia signals in NHTBE cells, we treated fully confluent 7-day-old NHTBE cells with 5 ng/mL of EGF for 2 hours. EGF induced Akt, ERK, and CREB phosphorylation in NHTBE cells (Fig. 3A), showing that the Akt and ERK pathways may participate in induction of cell proliferation of EGF and layer thickening. To further understand the relative role of these pathways, we treated 7-day-old NHTBE cells with EGF (5 ng/mL) alone or in combination with pharmacologic inhibitors targeting select molecules in the PI3-K/Akt and ERK signaling pathways: erlotinib (EGFR-TKI, 1 μmol/L), U0126 (MEK inhibitor, 5 μmol/L), LY294002 (PI3-K inhibitor, 10 μmol/L), and Akt inhibitor VIII (10 μmol/L) for 15 minutes. Inhibitors were pretreated for 30 minutes before EGF treatment. Erlotinib completely inhibited EGFR, Akt, and ERK phosphorylation (Fig. 3B). U0126 inhibited ERK and CREB phosphorylation. However, LY294002 and Akt inhibitor VIII had no effect on EGF-induced ERK and CREB phosphorylation (Fig. 3B).
To further determine the morphologic consequences of inhibiting critical molecules in signaling pathways, we cultured NHTBE cells for 7 days until they became confluent and then treated with EGF (5 ng/mL) alone or in the presence of various inhibitors for 4 days. NHTBE cells treated with EGF alone experienced hyperplastic changes and increased thickness, whereas those treated in the presence of erlotinib or U0126 underwent no such changes. However, LY294002- and Akt inhibitor VIII-treated NHTBE cells still showed hyperplasia and increased thickness (Fig. 3C). These results suggest that the MEK/ERK pathway is the main pathway by which EGF induces hyperplasia in NHTBE cells. We next determined whether inhibitors reverse EGF-induced hyperplasia such that normal morphologic characteristics were restored (Fig. 3D).

**Cyclin D1 is increased during EGF-induced NHTBE cell hyperplasia**

Increased cell proliferation is partly responsible for hyperplasia induction. Because elevated cyclin levels play a role in enhancing cell proliferation, we determined cyclin levels in NHTBE cells after EGF treatment. EGF robustly increased cyclin D1 and slightly increased cyclin E2 expression (Fig. 4A). However, A1 and B1 were not significantly increased. The EGFR and MEK inhibitors erlotinib and U0126, respectively, markedly blocked EGF-induced expression of cyclin D1, but LY294002 and Akt inhibitor VIII did not (Fig. 4B). To determine which transcription factors are involved in EGF-induced cyclin D1 gene expression, we conducted a cyclin D1 promoter-luciferase activity assay (Fig. 4C).
We transfected NHTBE cells with various cyclin D1 promoter-luciferase reporters (wild type, AP-1 site mutant, and CRE sites mutant in the cyclin D1 promoter region) and treated the transfected cells with or without EGF. EGF increased luciferase activity more than 20 times when wild-type or mutated CRE cyclin D1 promoter reporters were introduced (Fig. 4C). However, when the AP-1 recognition sequence was mutated or removed, the EGF response was dramatically lower than that in the wild-type promoter. This result shows the importance of the AP-1 transcription factor in EGF-induced cyclin D1 overexpression.

Next, we investigated the activation status of AP-1 components c-Jun and c-Fos. EGF induced c-Jun expression and phosphorylation (Fig. 4D) but not c-Fos (data not shown). In addition, erlotinib and U0126 markedly blocked EGF-induced c-Jun phosphorylation (Fig. 4E). c-Jun knockdown with c-Jun siRNA prevented EGF-induced cyclin D1 expression, suggesting that EGF-induced cyclin D1 expression is mediated by c-Jun (Fig. 4F). Thus, we concluded that EGF induces cyclin D1 overexpression and that this overexpression is mediated by AP-1 (c-Jun) transcription factor. In addition, EGF-induced cyclin D1 overexpression is blocked by EGFR and MEK inhibitors.

**Discussion**

We showed that bronchial hyperplasia can be modeled and manipulated in vitro by using primary NHTBE cells maintained in a 3D organotypic air–liquid interface culture. The EGFR ligands EGF, TGF-α, and AR induce hyperplasia in NHTBE cells. This histomorphologic change is regulated by the MEK/ERK signaling pathway but not the PI3-K/Akt signaling pathway. The MEK/ERK signaling pathway induces cyclin D1 expression by activating AP-1 transcription factor. The EGFR and MEK inhibitors erlotinib and U0126 completely blocked EGF-induced hyperplasia.

In view of multistep lung carcinogenesis and field carcinization, our results suggest that erlotinib may be useful as a chemopreventive agent as such agents inhibit, delay, or reverse carcinogenesis. First, erlotinib may be beneficial for high-risk patients, such as those with a strong smoking history. Erlotinib is currently being studied in the adjuvant setting after surgery and chemotherapy in NSCLC. Lung cancer develops in a field with extensive and multifocal hot spots throughout the respiratory trees, which are consistently exposed to common carcinogens. Even after tumor resection, these hot spots can develop into lung cancer. After resection, the risk of a second primary lung cancer is...
approximately 1% to 2% per patient per year, with a cumulative risk of up to 20% at 6 to 8 years after resection (15). Erlotinib would target 2 cell populations at once: micrometastatic NSCLC cells and evolving bronchial epithelial cells. Inhibition of erlotinib and reversal of the first step in lung carcinogenesis in NHTBE cells warrant further investigation. We are conducting a clinical study and have enrolled 50 patients with early-stage lung cancer who have undergone neoadjuvant chemotherapy with cisplatin and docetaxel, followed by surgical resection and 1 year of adjuvant erlotinib. Patients will undergo bronchoscopy at 6 months and 1 year to assess possible changes in the bronchial epithelium after erlotinib treatment (16).
We found that EGF robustly increased cyclin D1 in primary NHTBE cells grown in organotypic culture. The malignant transformation of bronchial epithelial cells is driven by the dysregulation of oncogenes, growth factors, or tumor suppressor genes. Cyclin D1 is strongly implicated as an oncogene in lung cancer and several other...
human cancers, including B-cell lymphomas, head and neck squamous cell carcinomas, esophageal cancer, and breast cancer (17). Cyclin D1 is part of the cyclin-dependent kinase (CDK)-cyclin complex that increases retinoblastoma (Rb) protein phosphorylation at the G1-S transition and may play a role in transcriptional regulation. Cyclin D deregulation, by amplification or transcriptional upregulation, has been found in many tumor types (18–22). The p53-Rb pathway that mediates G1 arrest is the most commonly affected pathway in lung cancer. Defects in G1-regulatory proteins, especially p53-p21WAF1, p16-Rb-cyclin D1, and cyclin E-p16 pathway deregulation, seem to be essential to lung cancer development (23, 24). An immunohistochemical analysis showed cyclin D1 and E overexpression in bronchial preneoplasia that precedes the development of squamous cell carcinoma (25). These data imply that increased cyclin levels play a critical role in preneoplastic bronchial lesion progression. This conclusion was confirmed in bronchial epithelial cellular models (14) and carcinogen-induced lung tumors in animal models (26, 27).

Cyclin D1 overexpression may portend a worse prognosis in patients with resected lung cancer (28), although results have not been consistent (29). Cyclin D1 seems to be regulated by EGFR in gefitinib-resistant EGFR mutant cell lines, and these cell lines are sensitive to flavopiridol, a CDK inhibitor (30). Cyclin D1 repression is an indirect marker of erlotinib treatment response in aerodigestive tract cancers (31). Chemoprevention trials have found that rexinoid, a selective retinoid X receptor agonist, suppresses cyclin D1 expression in NSCLC (32), and low cyclin D1 expression predicts longer cancer-free survival in laryngeal premalignancy patients (33). Thus, cyclin D1 levels have been studied as markers for abnormal cell growth in chemoprevention trials (34). Cyclin D1 gene expression regulation has been reported to include the ras/raf/mitogen-activated protein kinase (MAPK) cascade in fibroblast cells (35, 36), p60Scrc pathways through CREB/activating transcription factor 2 activation in breast cancer cells (37), and PI3-K/Akt/NF-kB pathway-involved prooncogenic effects in human bronchial epithelial cells (38). Our data showed that EGF activates the PI3-K/Akt and MEK/ERK pathways in a time-dependent manner. However, only the MEK/ERK pathway was involved in EGF-induced cyclin D1 expression, suggesting that it is involved in early-stage lung carcinogenesis.

As shown in Figure 5, we found that bronchial hyperplasia can be modeled in vitro by using a 3D organotypic culture method and prevented by blocking the EGFR/MEK signaling pathway. We further found that bronchial hyperplasia is dependent on cyclin D1, which is regulated by AP-1 activation through the MEK/ERK pathway rather than the PI3-K/Akt pathway. Our model system and results will help elucidate the molecular mechanisms of lung carcinogenesis at its early stages and may support the prophylactic use of EGFR-targeting agents in patients at high risk of tumor development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

We thank Dr. Richard Pestell (Thomas Jefferson University, Philadelphia, PA) for the gift of cyclin D1 luciferase vectors.
Grant Support

Department of Defense VITAL grant W81XW-04-1-0142 (J.S. Koo, E.S. Kim, and W.K. Hong), National Heart, Lung and Blood Institute grant R01-HL-077556 (I.S. Koo), and National Cancer Institute Cancer Center Support grant CA-16672 (The University of Texas MD Anderson Cancer Center).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 8, 2010; revised March 23, 2011; accepted April 3, 2011; published OnlineFirst April 19, 2011.

References

12. Loupakis F, Vasilie E, Santini D, Masi G, Falcone A, Graziano F. EGF-receptor targeting with monoclonal antibodies in colorectal carcino-
35. Takuwa N, Takuwa Y. Ras activity late in G1 phase required for p27kip1 downregulation, passage through the restriction point, and entry into S phase in growth factor-stimulated NIH 3T3 fibroblasts. Mol Cell Biol 1997;17:5348–58.


Prevention of Bronchial Hyperplasia by EGFR Pathway Inhibitors in an Organotypic Culture Model


Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-10-0364

This article cites 37 articles, 19 of which you can access for free at: http://cancerpreventionresearch.aacrjournals.org/content/4/8/1306.full#ref-list-1

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

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

To request permission to re-use all or part of this article, use this link http://cancerpreventionresearch.aacrjournals.org/content/4/8/1306. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.