Erlotinib Induces Mitochondrial-Mediated Apoptosis in Human H3255 Non–Small-Cell Lung Cancer Cells with Epidermal Growth Factor Receptor (EGFR) L858R Mutation through Mitochondrial Oxidative Phosphorylation-Dependent Activation of BAX and BAK

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ABSTRACT

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, such as gefitinib and erlotinib, show promise in the treatment of non–small-cell lung cancer (NSCLC). Erlotinib is approved by the Food and Drug Administration as second and third line treatment for NSCLC. However, the molecular mechanisms by which erlotinib induces apoptosis remain to be elucidated. Here, we investigated the effect of erlotinib on apoptotic signal pathways in H3255 cells with the EGFR L858R mutation. Erlotinib induces apoptosis associated with the activation of caspases in a dose- and time-dependent manner. Erlotinib did not alter the expression of apoptotic receptors FAS and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), although it induced caspase activation and BAX translocation. In addition, cell death caused by erlotinib was not prevented by coincubation with FAS and TRAIL antagonists, ZB-4 monoclonal antibody, and TRAIL, suggesting that erlotinib-induced apoptosis is not associated with receptor-mediated pathways. Erlotinib activates BAX and BAK and induces mitochondrial membrane potential and release of cytochrome c and second mitochondrial-derived activator of caspases/direct IAP binding protein-3 (cIAP3).

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get the EGFR signaling pathway and have significant antitumor activity have been developed in the past few years (Noonberg and Benz, 2000). Erlotinib (Tarceva, OSI-774) is an orally bioavailable quinazoline derivative (Moyer et al., 1997). Preclinical studies demonstrated that erlotinib inhibits tumor cell growth coupled with inhibition of EGFR activation. Erlotinib as a single agent has shown significant clinical activity in previously treated patients with NSCLC (Pollack et al., 1999) and has received Food and Drug Administration-approval as second and third line treatment for NSCLC patients (Shepherd et al., 2005). Although erlotinib-induced antitumor activity is linked to the inhibition of EGFR and its related downstream pathways, the precise cellular and molecular mechanisms by which erlotinib induces cell death remain to be elucidated. Furthermore, several studies have clearly shown that human NSCLC cells with EGFR domain mutations are supersensitive to EGFR TKIs (Lynch et al., 2004; Paez et al., 2004) and that such sensitization may be involved by activation of apoptotic pathways (Tracy et al., 2004).

Apoptosis is a highly and genetically regulated cell suicide response to facilitate the correct development and homeostasis of multicellular organisms (Green, 2000). In mammals, apoptosis is an important mechanism of antimicrobial defense and induced cell killing and susceptibility to apoptosis among cells is an important determinant of chemotherapy efficacy (Kaufmann and Earnshaw, 2000). Two apoptotic pathways have been well characterized. The first is the cell death receptor-mediated (extrinsic) pathway involving activation of the death receptor such as FAS/APO-1/FADD/TRAIL/APO2L, the apoptotic caspase-8 cascade, and recruitment of adaptor molecule FADD and procaspase-8 forming the death-inducing signaling complex. Recruitment of caspase-8 to death-inducing signaling complex leads to downstream activation of effector caspases, 3 and 7 (Walczak and Krammer, 2001). Caspase-3 is involved in the activation of the mitochondrial pathway via the cleavage of the BCL-2 family member BID (Li et al., 1998). The second apoptotic signal pathway is the mitochondrial-mediated apoptotic pathway via the BCL-2 family proteins. Upon activation of the intrinsic pathways, mitochondrial integrity is disrupted and release of cytochrome c, subsequently results in the activation of caspase-9 (Reed et al., 2002). Caspase-9 directly cleaves and activates the effector caspase-3 and caspase-7, and the activity of caspase-9 and caspase-7 lead to the cleavage of several proteins, chromatin condensation, DNA ladder, and formation of apoptotic bodies. In both signal pathways, BCL-2 family proteins play a crucial role in the regulation of apoptotic events (Huang and Strasser, 2000). Overexpression of BCL-2 or BCL-xL results in prevention of apoptosis. In contrast, overexpression of BAX and BAK leads to an increase in cell susceptibility to apoptotic signals (Karbowski et al., 2006). Apart from alteration in gene expression of BCL-2 family, current studies show that BAX and BAK proteins undergo a set of activation steps in response to apoptotic stimuli (Desagher and Martinou, 2000). Upon initiation of the apoptotic cascade, BAX translocated from cytoplasm to mitochondria, both BAX and BAK proteins change their conformation and form homo-oligomers (Suzuki et al., 2000). On the outer membrane of the mitochondria, oligomerized BAX and BAK may form a channel or membrane pore, which allows the release of cytochrome c and Smac/DIABLO (Annis et al., 2005).

Several recent studies have reported that EGFR TKIs, including gefitinib and erlotinib, induce apoptosis in NSCLC cell lines through the activation of intrinsic pathways mediated by the induction of BH3-only BCL-2 proteins BIM and BAD playing key roles in inducibly killed cells with in Bcr/Abl leukemic cells. In this study, we examined human H3255 NSCLC harboring a mutant EGFR (L858R) mutation as a model to examine the molecular mechanism of erlotinib-induced apoptosis. We found that 0.1 μM erlotinib induces apoptosis early as 8 h after drug exposure. We also found that erlotinib-induced apoptosis is not dependent on the FAS/CD95/Apo1- and TRAIL/Apo2L-related pathways, although it caused BH3-only BCL-2 protein activation and cleavage of caspase-8. Erlotinib-induced apoptosis was dependent on activation of mitochondrial-mediated pathways, resulting in the loss of Apm and release of cytochrome c and Smac/DIABLO from mitochondria to cytoplasm. More importantly, we found that the induction of apoptosis by erlotinib involves the activation of BAX and BAK, including their conformational changes and oligomerization. Furthermore, we found that erlotinib-induced BAX and BAK activation and apoptosis are largely dependent on mitochondrial oxidant phosphorylation, but not dependent on intracellular oxalo. In addition, we tested the effects of gefitinib, both inhibition or overexpression of BCL-2 on BAX and BAK activation and apoptosis induced by erlotinib. Downregulation of BAX and BAK gene expression with siRNA resulted in an effective reduction of erlotinib-induced cell death. Overall, these observations provide a more detailed understanding of the mechanisms of action of the EGFR TKIs, and their role in erlotinib and should constitute more rational basis for the therapeutic use either alone or in combination with other chemotherapeutic agents.

Materials and Methods

Antibodies and Chemicals. Erlotinib was obtained from OSI Pharmaceuticals (Farmingdale, NY), dissolved in DMSO at a stock concentration of 10 mM, and diluted to the indicated concentration with RPMI 1640 medium. The polyclonal antibodies of caspase-3, caspase-8, caspase-9, FADD, BID, and BAX were purchased from Cell Signaling Technology Inc. (Danvers, MA); the antibodies of FAS, TRAIL, and cytochrome c were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and monoclonal antibodies of BCL-2, BAX, and polyclonal antibody of Smac/DIABLO were purchased from Calbiochem (San Diego, CA). Rotenone, antymycin A, oligomycin, and Z-VAD-fmk were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Lines. The human H3255 NSCLC cell line carrying an L858R EGFR mutation was a generous gift from Dr. Janne (Harvard Medical School, Boston, MA), and the H322 cell line with wild-type EGFR was purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in 75-cm² flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂.
Assay of Apoptosis and Cell Death. H3255 cells were treated with 0.1 μM erlotinib for the indicated times. After treatment, cells were fixed with 4% paraformaldehyde in PBS for 5 min and stained with 0.5 μg/ml DAPI in PBS for 15 min. Apoptotic cells (50 cells from three different fields) were counted with a Nikon E400 fluorescence microscopy. For sub-G0/G1 assay, cells were fixed with 75% cold (4°C) ethanol overnight and then incubated at room temperature for 3 h with 1 μg/ml propidium iodide and 5 μg/ml RNase I (Roche Molecular Biochemicals, Indianapolis, IN). The number of apoptotic cells (sub-G0/G1) was measured by FACScan flow cytometry (BD Biosciences, San Jose, CA). For annexin V assay, cells treated with erlotinib were double-stained with FITC-conjugated annexin V and propidium iodide (PI) using a kit according to the manufacturer’s instructions (Calbiochem). The percentage of annexin V-positive cells was measured by FACScan flow cytometry. For cell death assays, cells were treated with erlotinib for the indicated times, and cell survivals were determined by trypan blue exclusion.

Assay of Caspase Activity. H3255 cells were treated with 0.1 μM erlotinib for the indicated times. After treatment, cells were extracted with extraction buffer contained 20 mM HEPES, pH 7.2, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin at 4°C for 15 min. The soluble extracts were collected by centrifugation at 14,000g at 4°C for 15 min and stored at −80°C until assay. Ten microliters of cell extract (10 μg of protein) was added into a total 100-μl reaction mixture containing 12 μM Ac-DEVD-pNA, Ac-IETD-pNA, or Ac-Leu-Leu-Val-Ala-Asp-fluoromethyl ketone (BIOMOL Research Laboratories) as the substrates for caspase-8, and caspase-9, respectively, in reaction buffer for incubation at room temperature for 30 min. The absorbance of p-nitroaniline-derived substrate cleavage by caspases was determined in a microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm.

Measurement of Δψm and PARP. Cells were plated in a six-well plate and treated with 0.1 μM erlotinib for the same time of medium containing 0.01% DMSO as a control for the indicated time periods. After treatment, cells were incubated with 5 μM JC-1 (Invitrogen, Carlsbad, CA) at 37°C for 15 min for determination of Δψm, or with 10 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA; Invitrogen) at 37°C for 30 min to monitor the measurement of reactive oxygen species (ROS) activity. After incubation, cells were harvested and washed with PBS three times. JC-1 and ROS generation were analyzed by FACScan flow cytometry. In another assay for assay of release of cytochrome c, FITC-DIACA, and BAX translocation, cells were washed into cytosolic and mitochondrial fractions as described previously (Ling et al., 2003). In brief, cells were incubated in buffer containing 20 mM HEPES, pH 7.2, 10 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 20 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin at 4°C for 15 min. The supernatants were further centrifuged at 15,000g for 30 min at 4°C, and the pellets were then the supernatants. The pellet was further dissolved in lysis buffer containing 1% SDS as the mitochondrial fraction.

Immunoblot Analysis. Cells were scraped from culture dish, washed twice with ice-cold PBS, and then suspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1% Triton X-100, and 1% SDS at 0–4°C for 15 min. After centrifugation at 15,000g for 10 min at 0°C, the supernatants were collected, and the protein amount of cell lysate was measured with a DC Protein assay kit (Bio-Rad, Hercules, CA). An equal amount of cell lysate (30 μg of protein) was subject to 12 or 15% SDS-PAGE. After electrophoresis, protein blots were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST solution and incubated 4°C overnight with the corresponding primary antibodies in the blocking solution. After washing three times with TBST solution, the membrane was incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody diluted with TBST solution (1:500). The detected protein signals were visualized by an enhancement chemiluminescence detection system as recommended by the manufacturer (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Flow Cytometric Analysis of Annexin V and PARP. H3255 cells were treated with 0.1 μM erlotinib for the same volume of medium as control for the indicated times. After treatment, cells were fixed with 0.25% paraformaldehyde in PBS at room temperature for 5 min, washed three times with PBS, and then incubated with 6A7 monoclonal antibody against PARP (BD Biosciences, San Jose, CA) for 30 min at 4°C. The percentage of annexin V-positive cells was assayed by FACScan flow cytometry. For cell death assays, cells were stained with FITC-conjugated annexin V (BD Biosciences) containing 0.05% propidium iodide for 15 min. After washing three times with PBS, cells were incubated with FITC-conjugated Annexin V for 30 min at room temperature, and then 100,000/samples were analyzed by fluorescence-activated cell sorting (FACS) for validation of conformational change in BAX and BAK. We performed the assay by lysates with zwitterionic detergent CHAPs for detection of BAX (10 mM HEPES, pH 7.4, 150 mM NaCl, and 1% CHAPS) according to the methods of Tamaguchi and Wang (2001). The amounts of native BAX and BAK were detected by immunoblot using polyclonal anti-BAX and BAK antibodies.

In Vitro Cross-Linking for Detection of BAX and BAK Oligomers. In vitro cross-linking, the detection of BAX and BAK oligomers was performed according to the methods of Sundararajan et al. (2001). In brief, cells (1 × 106) were incubated in 0.5 ml of cross-linking buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM HEPES-KOH, pH 7.2, and 0.05% digitonin) containing 1 mM BMH (Pierce Chemical, Rockford, IL) or the same volume of DMSO as a vehicle in a 37°C temperature for 30 min. After incubation, cells were pelleted by centrifugation at 15,000g for 15 min at 4°C and suspended in 0.625 mM Tris-HCl, pH 6.8, 10% glycerol, and 5 μg/ml SDS. BAX and BAK oligomers were detected by immunoblot using polyclonal anti-BAX antibody or monoclonal anti-BAK antibody, respectively.

Transient Transfection of BCL-2. Vector and human wild-type BCL-2 cDNA was a gift from Dr. Xiaobo Cao (Texas A&M, College Station, TX). H3255 cells were plated in a six-well plate and grown to 80% confluence. Cells were transiently transfected with 4 μg/ml vector or BCL-2 cDNA by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After a 4-h transfection period, cells were reincubated in fresh medium with 10% fetal bovine serum at 37°C for 24 h. Then, 0.1 μM erlotinib or the same volume of medium containing 0.01% DMSO was added to the cell culture and incubated for an additional 24 h. After treatment, the transfected cells were harvested and divided into two cell aliquots for the determination of BCL-2 protein expression and apoptosis as described above. For determination of the effect of BCL-2 on BAX and BAK activation, transfected cells were immunoprecipitated by anti-BAX (6A7) and anti-BAK (Ab1) antibodies according to the methods of Tamaguchi and Wang (2001). The amounts of activated BAX and BAK were detected by immunoblot using polyclonal anti-BAX antibody or monoclonal anti-BAK antibody, respectively.

Knockdown of BAX and BAK Gene Expression with siRNA. BAX-siRNA, BAK-siRNA, and nonspecific control of siRNA were purchased from Dharmacon RNA Technologies (Lafayette, CO), and the corresponding antibodies. For validation of conformational change in BAX and BAK, we performed the assay by lysates with zwitterionic detergent CHAPs for detection of BAX (10 mM HEPES, pH 7.4, 150 mM NaCl, and 1% CHAPS) according to the methods of Tamaguchi and Wang (2001). The amounts of native BAX and BAK were detected by immunoblot using polyclonal anti-BAX and BAK antibodies.
Fig. 1. Erlotinib induces apoptosis and activation of caspases in H3255 cells. A, cells were treated with 0.1 μM erlotinib or with the same volume of medium as a control for 24 h. After treatment, cell morphological changes were visualized by phase-contrast microscopy, or the condensed and fragmented nuclei were visualized by fluorescent microscopy using DAPI staining. Apoptotic cells were determined by flow cytometric analysis after cell staining with PI (B) or staining with annexin V/PI (C). Cells were treated with varying concentrations of erlotinib at 37°C for 24 h (D) or with 0.1 μM erlotinib for the indicated time (E). After treatment, cells were stained with PI solution, and the apoptotic cells were determined by flow cytometric analysis. Data represent the mean ± S.D. of three independent experiments; ** p < 0.01 versus concentration at 0 μM or time at 0. F, cells were treated with 0.1 μM erlotinib for the indicated time. After treatment, cells were harvested and divided into two aliquots. One aliquot was used for determination of generation of active form of cleaved caspase-8, -9, and -3 by immunoblots using corresponding antibodies. G, the other cell aliquot was used for the determination of caspase activity by measurement of the release of pNA from substrates as described under Materials and Methods. Each point represents mean ± S.D. of three independent experiments.
erlotinib. After 24-h incubation, cells were harvested for the determination of BAX and BAK protein expression, and apoptosis as described above.

**Statistical Analysis.** Data are presented as mean ± S.D. of the number of experiments indicated. The comparisons were made with a t test, and the difference was considered to be statistically significant if the p value was <0.05.

**Results**

**Erlotinib Induces Apoptosis in H3255 Cells.** Because human H3255 NSCLC cells that carry an EGFR<sup>L858R</sup> mutation have shown to be very sensitive to gefitinib (Tracy et al., 2004), we selected this cell line as a model to determine the effect of erlotinib on triggering apoptotic pathways. At first, we treated H3255 cells with 0.1 μM erlotinib for 24 h to evaluate drug-induced apoptosis. As shown in Fig. 1A, erlotinib induces H3255 cell death with the characteristic apoptotic features, including cell detachment, shrinkage, and generation of apoptotic bodies as observed with a phase-contrast microscope, as well as the chromatin condensation and fragmentation in nucleus detected by cell staining with DAPI solution. Moreover, flow cytometric analysis revealed that erlotinib treatment led to an increase in apoptotic nuclei with a subdiploid DNA content (sub-G<sub>0</sub>/G<sub>1</sub>) (Fig. 1B), as well as an increase in the FITC-labeled annexin V-positive staining cells with exposure of phosphatidylserine on the outside of the plasma membrane (Fig. 1C). The kinetic studies showed that induction of apoptosis in H3255 cells by erlotinib occurred in a concentration- and time-dependent manner. It could be detected at a concentration as low as 0.05 μM for 24 h and as early as 8 h after exposure to 0.1 μM erlotinib (Fig. 1, D and E). We next explored whether erlotinib-induced apoptosis was associated with activation of caspases. After treatment with 0.1 μM erlotinib for the indicated times, cells were harvested and divided into two aliquots. One aliquot was used for determination of the active form of cleaved caspases by immunoblots. The other cell aliquot was used for determination of caspase activities measured by the colorimetric assay as described under Materials and Methods. The immunoblot analysis revealed that 0.1 μM erlotinib treatment resulted in the appearance of the active forms of cleaved caspase-8 (43 and 18 kDa) and caspase-9 (37 and 35 kDa) as early as 4 h after exposure, and the active forms of cleaved caspase-3 (26, 19, and 17 kDa) after 8 h.

![Fig. 2](image-url). Effect of erlotinib on extrinsic apoptotic pathway in H3255 cells. A, cells were treated with 0.1 μM erlotinib for the indicated time. After treatment, cells were harvested, and the total BID and t-BID were detected by immunobots using anti-BID antibody. β-Actin was used as a loading control. B, effect of erlotinib on expression of FAS, TRAIL, and FADD proteins. Cells were treated with 0.1 μM erlotinib for the indicated time, and the levels of FAS, TRAIL, and FADD were determined by immunoblot analysis using the corresponding antibodies. β-Actin was used as a loading control. C, effect of ZB-4 mAb on erlotinib- and FAS-induced cell death. Cells were treated with 0.1 μM erlotinib or with 0.5 μg/ml FAS alone or with plus 2 μg/ml ZB-4 mAb for 24 h. After treatment, cell death was determined by trypan blue exclusion. D, effect of TRAIL/Fc recombinant on erlotinib- and TRAIL-induced cell death. Cells were treated with 0.1 μM erlotinib or with 0.1 μg/ml TRAIL alone or with plus 2 μg/ml TRAIL/Fc recombinant for 24 h. After treatment, cell death was determined by trypan blue exclusion. Data represent the mean ± S.D. of three independent experiments; **, p < 0.001 and *, p < 0.05.
8 h of drug exposure (Fig. 1F). Quantitative determinations of caspase activities by colorimetric assays showed consistently that activities of caspase-8 and caspase-9 were markedly increased by approximately 2.8- and 2.4-fold at 4 h of erlotinib treatment, respectively, whereas caspase-3 activity was increased by approximately 2.9-fold after 8 h of drug treatment (Fig. 1G). These results indicate that erlotinib triggers the initiator caspase-8, and caspase-9 initially and subsequently activates the executioner caspase-3.

**Effect of Erlotinib on Extrinsic Apoptotic Pathways.** Because erlotinib treatment caused a remarkable activation caspase-8, which is an initiator of caspase in the extrinsic apoptosis pathways (Walczak and Krammer, 2000), we sought to determine whether the death receptor apoptotic signals were involved in erlotinib-induced apoptosis. Results as shown in Fig. 2A indicate that erlotinib at 0.1 \( \mu \)M caused a time-dependent caspase-8 induced cleavage of BH3-only BID protein and the formation of its truncated form of BID (t-BID), which could translocate to the mitochondria to enhance the mitochondrial-mediated apoptosis pathways (Li et al., 1998). Under the same experimental conditions, erlotinib did not markedly affect the expression of the death receptors FAS and TRAIL as well as FAS adaptor protein FADD over time (Fig. 2B). To further explore whether erlotinib-induced apoptosis is associated with the death receptor pathways, we treated H3255 cells with 0.1 \( \mu \)M erlotinib alone, or plus 2 \( \mu \)g/ml FAS-antagonistic mAb (ZB-4; Upstate, Billerica, MA), or 2 \( \mu \)g/ml TRAIL recombinant TRAIL/Fc recombinant (Apootech, San Diego, CA) for 24 h. Neither ZB-4 mAb nor TRAIL/Fc recombinant protected against erlotinib-induced cell death, whereas cotreatment with ZB-4 mAb resulted in a significant reduction of FAS-induced cell death (p < 0.01), and cotreatment with TRAIL/Fc attenuated TRAIL-induced cell death (p < 0.05) (Fig. 2C and D). These results suggest that erlotinib-induced apoptosis may be mediated with FAS/CD95/Apo1 and TRAIL/Apo2L apoptotic pathways, although erlotinib induced caspase activation and generation of t-BID.

**Erlotinib Induces Mitochondrial Dysfunctions.** Next, we examined whether erlotinib-induced apoptosis was mediated with the mitochondrial pathways. First, we treated H3255 cells with 0.1 \( \mu \)M erlotinib for the indicated time, and then we determined its effect on the \( \Delta \psi_m \) using a cationic JC-1 dye JC-1. As shown in Fig. 3A, the typical fluorescence histograms show that disruption of \( \Delta \psi_m \) determined by the ratio of JC-1 aggregates to monomers was observed in cells treated with 0.1 \( \mu \)M after 8 and 24 h, compared with control cells. Quantitative analysis of the loss measured by JC-1 aggregates of mitochondrial membrane potential in H3255 cells after treatment with 0.1 \( \mu \)M erlotinib for the indicated time indicated that erlotinib exposure increased by approximately 2.9-fold after 8 h of drug treatment (Fig. 1F). Quantitative determinations of \( \Delta \psi_m \) by flow cytometric analysis of mitochondrial membrane potential in H3255 cells after treatment with 0.1 \( \mu \)M erlotinib or with the same volume of medium as a control for the indicated time. After treatment, cells were stained with JC-1 solution at 37°C for 15 min, and the \( \Delta \psi_m \) was determined by flow cytometric analysis. A, histograms of flow cytometric analysis of mitochondrial membrane potential in H3255 cells after treatment with 0.1 \( \mu \)M erlotinib or with the same volume of medium as a control for the indicated time. B, quantitative analysis of \( \Delta \psi_m \) as detected by JC-1 at FL-2 in H3255 cells after treatment with 0.1 \( \mu \)M erlotinib for the indicated time compared with control. Data represent mean ± S.D. of three independent experiments; **, p < 0.01 compared with control. C, effect of erlotinib on cytochrome c release from mitochondria to cytoplasm. H3255 cells were treated with 0.1 \( \mu \)M erlotinib for the indicated time. After treatment, cytosol and mitochondrial fractions were prepared as described under Materials and Methods. The levels of cytochrome c and Smac/DIABLO in cytosol and mitochondria were detected by immunoblots using the corresponding antibodies. β-Actin and cytochrome c oxidase (Cox) IV were used as cytosol and mitochondrial sample loading control, respectively.
gates at FL-2 indicated that ~30% of loss of Δψm was observed after 8 h of erlotinib treatment, increasing over time compared at each time point with control cells (Fig. 3B). Second, we evaluated the effect of erlotinib on cytochrome c release from mitochondria to the cytoplasm by immunoblot analysis after cell fractionation. As shown in Fig. 3C, the signal of cytochrome c was barely detectable in cytosol at times 0 to 4 h and clearly detected at 8 h, reached the maximal level thereafter. Under the same experimental conditions, we also detected the release of Smac/DIABLO, another apoptotic regulator, from mitochondria to cytosol with a pattern similar to that of cytochrome c release.

Erlotinib Induces BAX Translocation to the Mitochondria and Conformational Changes of BAX and BAK. Previous reports have demonstrated that BAX translocation to the mitochondria and alteration in protein conformation of BAX and BAK are necessary steps for cells undergoing apoptosis (Desagher et al., 2000; Stankiewicz et al., 2000).

Fig. 4. Erlotinib induces BAX translocation to the mitochondria and BAX and BAK conformational changes. A, H3255 cells were treated with 0.1 μM erlotinib for the indicated time. After treatment, cytosolic and mitochondrial fractions were prepared as described above. The levels of BAX in cytosolic and mitochondrial fraction were detected by immunoblots. B and C, effect of erlotinib on BAX and BAK protein conformational changes. Cells were treated with 0.1 μM erlotinib for the indicated times. After treatment, cells were fixed with paraformaldehyde and then incubated with 6A7 monoclonal anti-BAX antibody or with AM03 monoclonal anti-BAK (Ab1) antibody for 60 min. After incubation with FITC-conjugated second antibody for 30 min, the signals of activated conformation of BAX and BAK proteins were measured by flow cytometric analysis as described under Materials and Methods. The typical histograms of BAX and BAK conformational changes in cells treated with erlotinib are presented in B and C, respectively. The increased folds of activities of BAX and BAK as measurement by flow cytometric analysis are presented in the bottom of B and C, respectively. Data represent the mean ± S.D. of three independent experiments. D and E, erlotinib-induced activation of BAX and BAK protein conformation was further validated by immunoprecipitation. H3255 cells were treated with 0.1 μM erlotinib for the indicated time. After treatment, cells were harvested, and cell pellets were divided into two aliquots. One aliquot was used for the preparation of immunoprecipitation with 6A7 anti-BAX or Ab1 anti-BAK antibodies. The other cell aliquot was lysed with nonionic detergent lysis buffer. The levels of BAX and BAK were detected by immunoblots using polyclonal anti-BAX and anti-BAK antibodies.
We thus examined whether erlotinib-induced apoptosis is associated with the activation of BAX and BAK. We investigated BAX localization in the cytosol and mitochondria after cell fractionation by immunoblots in H3255 cells after exposure to 0.1 μM erlotinib for the indicated times. As shown in Fig. 4A, BAX levels were high in the cytosol at 0 to 4 h of incubation, and then they declined at 8 to 24 h. In contrast, BAX levels in the mitochondrial fraction were low at 0 to 4 h postdrug exposure, and they increased thereafter, indicating that erlotinib treatment led to BAX translocation to the mitochondria. In addition, we found that erlotinib did not markedly alter the subcellular localization of BAK over time, remaining in the mitochondria all along (data not shown). Next, we analyzed the activity-related conformational changes in BAX and BAK by flow cytometric analysis using immunostaining with special anti-BAX (6A7) and BAK (AM03, Ab1) antibodies, which recognize the N-terminal epitopes of the active conformation of BAX and BAK (Papretakis et al., 2002). The typical fluorescence histograms shown in Fig. 4, B and C (top), demonstrate that exposure to 0.1 μM erlotinib induces a shift to the right of the BAX and BAK fluorescence curves compared with control, indicating that erlotinib leads to BAX and BAK activation. The quantitative analysis presented in Fig. 4, B and C, bottom, indicates that the increased BAX and BAK activities occurred as early as 4 h after erlotinib exposure, and gradually increased with time. To further validate these observations, we used the zwitterionic detergent CHAPS, which has been proven to retain the BAX active conformation (Yamaguchi and Wang, 2002), to prepare cell lysate and then to immunoprecipitate BAX and BAK proteins with anti-BAX 6A7 and anti-BAK Ab-1 antibodies. As shown in Fig. 4D, the signal of active BAX was barely detectable at time 0, was first detectable at 4 h after erlotinib exposure, and remained elevated thereafter, whereas the level of active BAK was low at time 0 and gradually increased with time (Fig. 4E). However, bands of BAX and BAK in the cell lysates extracted with nonionic detergent Nonidet P40 remained at almost identical points. These data indicated that erlotinib induces BAX translocation to mitochondria, and BAX and BAK conformational changes.

Erlotinib BAX and BAK Oligomerization.

Recent studies have demonstrated that oligomerization of BAX and BAK play a critical role in triggering mitochondrial-mediated apoptosis (Suzuki et al., 2000; Antignani and Youle, 2006). We explored whether erlotinib-induced apoptosis and activation of BAX and BAK could be tied to ROS generation. We first examined the intracellular ROS level by flow cytometric analysis after incubation with DCF-DA in cells treated with 0.1 μM erlotinib for 24 h. As shown in Fig. 5, A and B, erlotinib treatment not only did induce ROS but actually were slightly higher than control, as shown by the left shifting of the ROS fluorescence curve, whereas H2O2, as a positive control, led to ROS generation as showing by the right shift of the ROS fluorescence curve, compared with control cells (Fig. 5B). In parallel, we tested the effects of the antioxidants L-ascorbic acid, the (NAC), tiron, and dihydroxy-2,4-dihydroxynaphthalene, sodium thiosulfate, and sodium glutathione on erlotinib-induced activation of BAX and BAK and apoptosis, and we found that all tested antioxidants failed to alter drug-induced apoptosis, whereas Fig. 6A and to affect erlotinib-induced BAX and BAK conformational changes and oligomerization (Fig. 6B and E). These results suggest that erlotinib induces oligomerization of BAX and BAK proteins in H3255 cells. Cells were treated with varying concentrations of erlotinib for 24 h or with 0.1 μM erlotinib for the indicated time. After treatment, cells were harvested and incubated with 1 mM protein cross-linker BMH at room temperature for 30 min. After incubation, cells were lysed with lysis buffer, and an equal amount of lysates (30 μg of protein) was subjected to a 15% SDS-PAGE. After transferring to a membrane, the oligomers of BAX (A) and BAK (B) were detected by immunobots using the corresponding antibodies. The nonspecific bands (22 kDa) served as the sample loading control.

Fig. 5. Erlotinib induces oligomerization of BAX and BAK proteins in H3255 cells. Cells were treated with varying concentrations of erlotinib for 24 h or with 0.1 μM erlotinib for the indicated time. After treatment, cells were harvested and incubated with 1 mM protein cross-linker BMH at room temperature for 30 min. After incubation, cells were lysed with lysis buffer, and an equal amount of lysates (30 μg of protein) was subjected to a 15% SDS-PAGE. After transferring to a membrane, the oligomers of BAX (A) and BAK (B) were detected by immunobots using the corresponding antibodies. The nonspecific bands (22 kDa) served as the sample loading control.
that erlotinib-induced cell death is not dependent on ROS-mediated pathways.

**Effects of Inhibitors of Mitochondrial Oxidative Phosphorylation on Erlotinib-Induced Activation of BAX and BAK and Apoptosis.** Recent investigations have demonstrated that the activation of BAX and BAK may be controlled by either mitochondrial oxidative phosphorylation and/or by mitochondrial membrane permeabilization (Tomiyama et al., 2006). We thus tested the role of mitochondrial oxidative phosphorylation in the regulation of erlotinib-induced activation of BAX and BAK and cell death. We first determined the effects of 1 μM rotenone, 5 μM antimycin A, two inhibitors of mitochondrial electron transport chain complexes I and II (Okun et al., 1999), and 3 μM oligomycin, an inhibitor of mitochondrial ATPase (F1F0) (Linnett and Beechey, 1979), on the activation of BAX and BAK as measured by conformational change and oligomerization, and on the mitochondrial dysfunction as monitored by cytochrome c release in H3255 cells after exposure for 12 h with 0.1 μM erlotinib. We found that cotreatment with these inhibitors effectively blocked erlotinib-induced BAX and BAK activation including the reduction of the formation of active BAX and BAK, and decline in BAX and BAK oligomerization (Fig. 7, A and B), and protected cytochrome c release to cytosol compared with cells treated with erlotinib alone (Fig. 7C). Cotreatment with these inhibitors consistently caused a significant inhibition of erlotinib-induced apoptosis compared with cells treated with erlotinib alone (p < 0.01) (Fig. 7D). These data indicated that activation of BAX and BAK by erlotinib is clearly dependent on mitochondrial oxidative phosphorylation.

**Effects of Caspase Inhibitor on Erlotinib-Induced Activation of BAX and BAK and Apoptosis.** Because erlotinib-induced apoptosis correlates with the activation of BAX and BAK, we also tested the role of caspase inhibition in erlotinib-induced apoptosis. We found that treatment with a caspase inhibitor, z-VAD-FMK, effectively blocked erlotinib-induced BAX and BAK activation and apoptosis in H3255 cells (Fig. 7E).

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**Fig. 6.** Effect of erlotinib on ROS generation and effects of antioxidative agents on erlotinib-induced BAX and BAK activation and apoptosis in H3255 cells. A and B, histograms of erlotinib- and H2O2-induced ROS generation. Cells were treated with 0.1 μM erlotinib or with 1 mM H2O2 at 37°C for 6 h. After treatment, cells were incubated with 10 μM DCF-DA at 37°C for 15 min. The intracellular ROS levels were determined by flow cytometric analysis. C, effects of antioxidative agents on erlotinib-induced apoptosis. Cells were treated with 0.1 μM erlotinib or with 0.1 μM erlotinib plus 5 mM NAC, 1 mM tiron, and 2.5 mM reduced glutathione or with the same volume of medium as a control at 37°C for 24 h. After treatment, cells were stained with DAPI solution, and the percentage of apoptotic cells was determined by counting condensed and fragmented nuclei. Data represent the mean ± S.D. of three independent experiments. D, effect of caspase inhibitor on erlotinib-induced BAX and BAK conformational changes. Cells were treated with erlotinib alone or with erlotinib plus caspase inhibitor as described above. After treatment, the active BAX and BAK were immunoprecipitated with 6A7 anti-BAX or Ab1 anti-BAK antibodies as described above. The levels of active BAX and BAK were measured by immunoblots using the corresponding antibodies. E, effect of caspase inhibitor on erlotinib-induced BAX and BAK oligomerization. Cells were treated with erlotinib alone or with plus caspase inhibitor as described above. After treatment, cells were harvested and incubated with 1 mM protein cross-linker BMH at 37°C for 30 min. After subjecting to a 15% SDS-PAGE, the oligomerized BAX and BAK were detected by immunoblots using the corresponding antibodies. The nonspecific bands (22 kDa) served as the sample loading control.
caspases, we examined whether the activation of BAX and BAK by erlotinib could be linked to the caspase-dependent pathway. To this purpose, we cotreated H3255 cells with 0.1 μM erlotinib and 50 μM Z-VAD-fmk, a pan-caspase inhibitor for 24 h, and then examined the effect on induction of apoptosis. As shown in Fig. 8A, cotreatment with Z-VAD-fmk led to prevention of erlotinib-induced apoptosis. However, cotreatment with Z-VAD-fmk had no effect on erlotinib-induced BAX and BAK conformational changes and oligomerization (Fig. 8, B and C). These results suggest that BAX and BAK activation may not be through the caspase-dependent pathway, and caspase activation is downstream of BAX and BAK activation in erlotinib-induced apoptosis.

Effect of Overexpression of BCL-2 on Erlotinib-Induced BAX and BAK Activation and Apoptosis. It has been well established that BCL-2 plays a critical role in the regulation of the mitochondrial-mediated apoptotic pathway (Adams and Cory, 2001). We thus transiently transfected H3255 cells with BCL-2 cDNA, and we determined the effect of overexpression of BCL-2 on erlotinib-induced activation of BAX and BAK, and apoptosis. Immunoblot analysis revealed that endogenous BCL-2 was barely detectable in nontransfected and vector-transfected cells, and BCL-2 expression was clearly detected after a 24-h BCL-2 cDNA transfection (Fig. 9A). As expected, transient transfection with BCL-2 led to a significant inhibition of erlotinib-induced cell death; i.e., ~14% of cells were apoptotic in H3255/BCL-2 cells compared with ~27% in H3255 and H3255/vector cells after a 4-h treatment with 0.1 μM erlotinib (Fig. 9B). However, we also found that BCL-2 overexpression did not markedly alter erlotinib-induced BAK conformational change and oligomerization and slightly reduced the formation of active form of BAX.

Fig. 7. Effects of inhibitors of mitochondrial oxidative phosphorylation on erlotinib-induced apoptosis and activation of BAX and BAK in H3255 cells. A, cells were treated with 0.1 μM erlotinib alone or with 0.1 μM erlotinib plus 1 μM rotenone, 3 μM oligomycin, 5 μM antimycin A, or with the same volume of medium as a control at 37°C for 24 h. After treatment, cells were harvested, and the active BAX and BAK were immunoprecipitated with 6A7 anti-BAX or Ab1 anti-BAK antibodies as described above. The levels of active BAX and BAK were measured by immunoblots using the corresponding antibodies. B, for assay of BAX and BAK oligomerization, cells were treated with erlotinib alone or plus inhibitors as described above. After treatment, cells were harvested and incubated with 1 mM protein cross-linker BMH for 30 min. The oligomerized BAX and BAK were detected by immunoblots using the corresponding antibodies as described above. C, inhibitors of mitochondrial oxidative phosphorylation inhibit erlotinib-induced cytochrome c release to cytosol. Cells were treated with erlotinib alone or plus the inhibitors of mitochondrial oxidative phosphorylation as described above. After treatment, cells were harvested and the cytosolic fraction was prepared as described under Materials and Methods. The levels of cytochrome c in cytosol were detected by immunoblot analysis. β-Actin was used as a loading control. D, effects of inhibitors of mitochondrial oxidative phosphorylation on erlotinib-induced apoptosis. Cells were treated with erlotinib alone or plus inhibitors as described above. After treatment, cells were stained with DAPI solution, and the percentage of apoptotic cells was determined by counting the condensed and fragmented nuclei. Data represent mean ± S.D. of three independent experiments; **, p < 0.01 versus erlotinib alone.
BAX and dimer (Fig. 9, C and D). These data suggest that overexpression of BCL-2 may not be involved in the regulation of BAX and BAK activation, although it prevented erlotinib-induced cell death.

**Effect of Down-Regulation of BAX and BAK Gene Expression by siRNA on Erlotinib-Induced Apoptosis in H3255 Cells.** To further investigate the role of BAX and BAK expression on the regulation of cell death by erlotinib, we used BAX and BAK siRNA to down-regulate both proteins expression in H3255 cells, and to test the effect of down-regulation of BAX and BAK on erlotinib-induced apoptosis. As shown in Fig. 10A, immunoblot analysis demonstrated that transfection with BAX and BAK siRNA specifically down-regulated the expression of BAX and BAK protein in either control or erlotinib-treated cells compared with non-transfected cells or cells transfected with nonspecific siRNA. As expected, down-regulation of BAX or BAK protein expression by siRNA resulted in a significant attenuation of erlotinib-induced apoptosis compared with that of nontransfected cells (Fig. 10B). In addition, we found that the double gene silenced by transfection with both BAX and BAK siRNA caused a higher inhibitory effect on erlotinib-induced apoptosis than that observed with transfection with either BAX siRNA or BAK siRNA alone (p < 0.05). These findings suggest that the activation of either BAX or BAK gene may play an important role in the regulation of erlotinib-induced apoptosis.

**Discussion**

In previous work, we and others have demonstrated that erlotinib is a highly selective EGFR tyrosine kinase inhibitor with potent activity against human NSCLC cell lines in vitro and in vivo studies (Moyer et al., 1997; Dai et al., 2005). We also found that erlotinib exposure caused cell growth inhibition accompanied by cell cycle arrest at G1/S phase in human NSCLC cells. The G1/S phase arrest by erlotinib was shown to be due to induced p27KIP1 expression and Ser10 phosphorylation (Ling et al., 2007). In addition, recent studies have shown that EGFR gene mutations within the active loops of the tyrosine kinase domain confer very high induced susceptibility to the EGFR TKI gefitinib in NSCLC cells (Lynch et al., 2004; Paez et al., 2004). In the present work, we used human H3255 NSCLC cells which harbor an EGFR(L858R) mutation. We used this model to examine the effects of drug-induced apoptosis on the sequence of events involved in the inhibition of cell proliferation and cell death induced by erlotinib. Our results show that the exposure of H3255 cells to low concentrations of erlotinib results in apoptotic cell death, as verified from morphological observations and the assessment of sub-G0/G1 DNA content.
cells and annexin V-positive staining by fluorescence-activated cell sorter analysis, and it is associated with the activation of caspases at the initiation and execution stages. Furthermore, we found that erlotinib did not affect the expression of the apoptotic receptors FAS and TRAIL, although it induced activation of caspase-8 and cleavage of BH3-only BID protein. Coincubation with the FAS and TRAIL antagonists ZB-4 mAb and TRAIL/Fc recombinant did not prevent erlotinib-induced cell death, suggesting that apoptosis caused by erlotinib is not mediated by apoptotic receptor-related pathways. These results are consistent with the studies by Liu and Fan (2001), who showed that cetuximab, an anti-human EGFR monoclonal antibody, induces cell death in DiFi colon cancer cells, but it does not interact with or regulate the TRAIL- or FAS-related pathway, although it activates caspase-8 (Liu and Fan, 2001). Our studies demonstrated that erlotinib induces the disruption of mitochondrial function with loss of Δψm, as well as release of cytochrome c and Smac/DIABLO from mitochondria to the cytosol. We also observed that erlotinib-induced apoptosis is correlated with BAX translocation from the cytosol to the mitochondria, and BAX and BAK protein conformational changes as well as both proapoptotic protein oligomerization. To our knowledge, the observation that activation of BAX and BAK and their oligomerization are involved in erlotinib-induced apoptosis is new. The kinetic studies showed that activation of BAX and BAK, including their conformational changes and oligomerization, precede the release of cytochrome c and Smac/DIABLO and the activation of caspases and seem to be a major contributing factor in the induction of apoptotic signals by erlotinib. We also tested whether cetuximab-induced activation of BAX and BAK occurred in other human NSCLC cell lines. Preliminary results show that erlotinib treatment causes H322 cell death, accompanying the induction of BAX and BAK oligomerization, indicating that the activation of BAX and BAK protein is not restricted to H3255 cells (Supplemental Fig. S1). It has been suggested that BAX and BAK oligomerization can facilitate the formation of the megachannel in the outer mitochondrial membranes, allowing the release of cytochrome c. However, the precise molecular mechanisms of how the megachannel is formed and how cytochrome c is subsequently released remain unknown (Annis et al., 2005).

Fig. 9. Effect of transient transfection of BCL-2 cDNA on erlotinib-induced apoptosis and activation of BAX and BAK in H3255 cells. A, cells were transiently transfected with 2 μg BCL-2 cDNA or with the same amount of vector or with the same volume of medium as a control by Lipofectamine 2000 as described under Materials and Methods. After 24-h transfection, cells were treated with 0.1 μM erlotinib or with the same volume of medium as a control for an additional 24 h. Cells were harvested and divided into two aliquots. One aliquot was used for the determination of BCL-2 expression by immunobLOTS. β-Actin was used as a loading control. B, the other cell aliquot was used for examination of apoptotic cells after staining with DAPI and Actin. C, effect of BCL-2 c-DNA transfection on erlotinib-induced apoptosis and activation of BAX and BAK. Cells were treated with erlotinib as described above. After treatment, cells were harvested and the active BAX and BAK were immunoprecipitated with 6A7 anti-BAX and Ab1 anti-BAK antibodies as described above. The levels of active BAX and BAK were detected by immunoblots using corresponding antibodies. D, effect of BCL-2 cDNA transfection on erlotinib-induced BAX and BAK oligomerization. Cells were transiently transfected with BCL-2 cDNA and treated with erlotinib as described above. After treatment, cells were harvested and incubated with 1 mM protein cross-linker BMH for 30 min. The oligomerized BAX and BAK were detected by immunoblots using the corresponding antibodies. The nonspecific bands (22 kDa) served as the sample loading control.
Our results indicate that BAX and BAK oligomerization was an earlier event than BAX translocation, as well as cytochrome c release and caspase activation, suggesting that BAX and BAK oligomerization is the event triggering the apoptosis cascades. These results are consistent with the reports by Gross et al. (1998), who demonstrated that BAX oligomerization is a prerequisite for BAX translocation to the mitochondria. Although erlotinib induces apoptosis via the disruption of mitochondrial function and the activation of BAX and BAK, the precise mechanism by which the inhibition of EGFR and its related pathways could lead to the initiation of the apoptotic cascades remain to be further elucidated. One possible explanation for the erlotinib-induced activation of BAX and BAK may be a direct interaction with BAX and BAK, and then inducing protein conformational changes and oligomerization. However, preliminary results show that erlotinib has no effect on protein conformation by immunostaining using active anti-BAX and BAK antibodies, and does not induce oligomerization of BAX and BAK as measured by immunoblots in the presence of protein cross-linker BMH when it is added directly into the cell-free systems such as the cytosolic or mitochondrial fractions prepared from H3255 cells (data not shown), suggesting that erlotinib does not directly interact with BAX or BAK proteins.

Another possibility may be that erlotinib inhibits AKT/PKB and/or ERK/MAP kinase, and these events lead to the activation of BAX and BAK and the initiation of the apoptotic cascade. We thus compared the effects of erlotinib on the inhibition of AKT/PKB, LY-294002, and wortmannin, and the inhibition of ERK/MAP kinase, U-0126, and PD-98059 on the activation of BAX and BAK. We found that pharmacological concentrations, AKT/PKB inhibitors, but not ERK/MAP kinase inhibitors cause the activation of BAX and BAK. These results suggest that erlotinib-induced activation of BAX and BAK is associated with the inhibition of AKT/PKB rather than with the inhibition of ERK/MAP kinase pathway (Supplemental Fig. S2). Indeed, a recent study showed that the inhibition of AKT/PKB signal pathway by either growth factor deprivation or glucose deprivation resulted in the abrogation of erlotinib-induced BAX and BAK activation in mitochondria (Tajewski et al., 2004).

Tomiyama et al. (2006) have demonstrated that cotreatment with inhibitors of mitochondrial oxidative phosphorylation markedly prevented the oligomerization of BAX and BAK and cell death in rat-1 fibroblasts and human cancer cells subjected to apoptotic stimuli such as DNA damage, endoplasmic reticulum stress, and tumor necrosis factor-α (Tomiyama et al., 2006). These results also demonstrate consistently that treatment with a variety of inhibitors of mitochondrial oxidative phosphorylation, including rotenone, antimycin A, and dicyclohexyl carbodiimide, effectively blocked erlotinib-induced the formation of active BAX and BAK and oligomerization, and cytochrome c release as well as cell death. These data indicate that BAX and BAK activation by erlotinib may be regulated by mitochondrial oxidative phosphorylation and/ or with the modulation of mitochondrial membrane permeabilization. The other possibility may be that erlotinib treatment could alter the mitochondrial pathways and metabolites such as generation in the glycolysis for ATP generation and/or changes in NADPH oxidase in the membrane and the mitochondrial electron transport system (Harris and Dong, 1989).

In summary, our results demonstrate that erlotinib-induced apoptosis in H3255 cells is associated with activation of caspases at both an early and a late stage. Erlotinib-induced apoptosis is dependent on the mitochondrial-mediated path-

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**Fig. 10.** Down-regulation of BAX and BAK expression by siRNA attenuates erlotinib-induced apoptosis in H3255 cells. Cells with 75% confluency were transiently transfected with BAX and BAK siRNA or with nonspecific siRNA, or with the same volume of medium as a nontransfection as described under Materials and Methods. After transfection, cells were incubated in the presence of 0.1 μM erlotinib (E) or the same volume of medium as a control (C). After 24 h of incubation, cells were taken from culture, and cell pellets were divided into two aliquots. A, one of the aliquots was used for determination of BAX and BAK expression by immunoblots using anti-BAX and BAK antibodies. β-Actin was used as a loading control. B, the other aliquot was used for examination of apoptotic cells by counting the condensed and fragmented nuclei after staining with DAPI as described above. Data represent the mean ± S.D. of three independent experiments; ***, p < 0.01 compared with that in nontransfected cells, and **, p < 0.05 versus cells transfected with single siRNA.
way, but independent of extrinsic pathway. Furthermore, the activation of BAX and BAK, including BAX translocation, BAX and BAK conformational changes, and oligomerization, plays a crucial role in the initiation of erlotinib-induced apoptosis. In addition, the activation of BAX and BAK is dependent on the mitochondrial oxidative phosphorylation, but independent of ROS generation or redox signals. Overexpression of BCL-2 or inhibition of caspase activity by Z-VAD-fmk did not markedly affect the activation of BAX and BAK, but it inhibited erlotinib-induced apoptosis. Moreover, down-regulation of BAX and BAK protein expression by siRNA led to the attenuation of erlotinib-induced apoptosis. In addition, recent investigation by Gong et al. (2007) indicated that erlotinib-induced apoptosis through the alteration in the subcellular localization of BAX from nuclei to cytoplasm in PC-9 and H3255 cells. All findings suggest that the activation of proapoptotic BCL-2 proteins, including BAX, BAK, and BIM, is essential for the triggering of EGFR TKI-induced intrinsic apoptotic pathway. Overall, the characterization of the molecular sequences of events leading to erlotinib-induced apoptosis has yielded important information toward understanding the mechanisms of action of EGFR TKIs in lung cancer cells. These findings provide a better elucidation of the mechanisms involved in erlotinib-induced apoptosis and may help in optimizing the use of this compound in clinical combination with other agents to improve its efficacy.

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