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Gefitinib, a Selective EGFR Tyrosine Kinase Inhibitor, Induces Apoptosis Through Activation of Bax in Human Gallbladder Adenocarcinoma Cells

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Abstract Although gefitinib, a selective inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase, has been clinically demonstrated to be effective for certain cancer cell types, the molecular mechanisms of the anti-tumor activity have not been fully elucidated. In this study, we investigated the mechanism of gefitinib-induced growth inhibition and apoptosis in HAG-1 human gallbladder adenocarcinoma cells. Treatment of gefitinib at a dose of 1 μM resulted in a significant growth inhibition, and the cell number irreversibly declined after 72-h incubation, with a progressive expansion of apoptotic cell population over 120-h. Following 2-h treatment, gefitinib significantly inhibited EGFR autophosphorylation and subsequent downstream signaling pathway through Erk and Akt, and induced accumulation of cells in the G0/G1 phase of the cell cycle at 24-h, accompanied by a concomitant increase in p21 transcript and increased expression of p27. Gefitinib did not affect the amount of total and phosphorylated p53 at serine 15, but upregulated the expression of total Bax, with subsequent increase in p18 Bax, an active form of Bax. The expression of Bcl-2 and Bad was unchanged. An increase in gefitinib-induced expression of total Bax might be due to the decreased degradation of Bax, because the level of Bax mRNA has not been altered by gefitinib treatment. Gefitinib promoted the cleavage of full-length p21 Bax into p18 Bax in mitochondrial-enriched fraction, a characteristic feature of Bax activation toward apoptosis. Moreover, blockade of Bax by using anti-Bax small interfering double stranded RNA (siRNA) significantly reduced gefitinib-induced apoptosis. Taken together, these data suggest a critical role of p18 Bax in gefitinib-induced apoptosis. J. Cell. Biochem. 97: 724–734, 2006.

Key words: gefitinib; EGFR; Bax; apoptosis; Akt; Erk

The EGFR, a receptor tyrosine kinase, is overexpressed in a wide variety of epithelial malignancies including non-small cell lung, head, neck, colon, and breast cancers [Salomon et al., 1995; Shirai et al., 1995; Grandis et al., 1998; Brabender et al., 2001] and enhanced expression of epidermal growth factor receptor (EGFR) is associated with more aggressive disease and a poor patient prognosis [Fox et al., 1994; Rusch et al., 1997]. Upon ligand binding, EGFR is activated through autophosphorylation by forming homodimerization or heterodimerization with other members of the HER family tyrosine kinases [Olayioye et al., 1998; Muthuswamy et al., 1999], and transduces a variety of signals to downstream signal transduction cascades that lead to cellular proliferation and survival [Alroy and Yarden, 1997; Schlessinger, 2000].

Gefitinib, a quinazoline derivative that inhibits EGFR tyrosine kinase activity, has been shown to be effective in preclinical studies and in late stages of clinical trials for non-small cell lung cancer [Fukuoka et al., 2003; Sirotnak, 2003], although the activity is associated with

Abbreviations used: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; RTK, receptor tyrosine kinase; MAPK, mitogen activated protein kinase; Erk, extracellular signal-regulated kinase; PI-3K, phosphatidylinositol 3′-kinase.

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the certain background of population, specific types of histology, and activating somatic mutations in the tyrosine kinase domain of EGFR [Lynch et al., 2004; Paez et al., 2004; Sordella et al., 2004]. This drug has been shown to inhibit major cell survival and growth signaling pathways such as Ras-Raf-MAP kinase pathway and phosphatidylinositol-3 kinase (PI-3K)-Akt pathway, as a consequence of inactivation of EGFR [Anderson et al., 2001; Moasser et al., 2001; Moulder et al., 2001; Janmaat et al., 2003]. Although induction of apoptosis has been considered as a major mechanism for gefitinib-mediated anti-cancer effects [Gilmore et al., 2002; Janmaat et al., 2003], the molecular mechanism for gefitinib-induced apoptosis has not been fully elucidated. Pro-apoptotic Bad, a BH3 only member of the Bcl-2 family, and anti-apoptotic Bcl-2 have been shown to be respectively involved in sensitivity and resistance to gefitinib-induced apoptosis [Gilmore et al., 2002; Janmaat et al., 2003], while the role of Bax, a multi-BH domain pro-apoptotic protein which appears to act downstream of Bad, in the gefitinib-induced apoptosis has yet to be clarified. Bax appears to have a more direct role than Bad in the regulation of pore formation in the outer membrane of the mitochondrion [Epand et al., 2002]. Bax protein undergoes conformational changes that expose membrane-targeting domains, resulting in its translocation from cytosol to mitochondrial membranes where Bax inserts and causes release of cytochrome C, followed by caspase activation and DNA degradation [Wolter et al., 1997; Pastorino et al., 1998]. Bax has been shown to undergo post-translational modification during apoptosis. For example, p18 Bax generation through wild type Bax cleavage has been observed in response to various stimuli such as Interferon-alfa [Yanase et al., 1998] and chemotherapeutic agents [Wood et al., 1998]. This p18 Bax fragment has been shown to be as efficient as full-length Bax in promoting cytochrome C release [Wood et al., 1998; Gao and Dou, 2000] or more potent than full-length Bax in inducing apoptotic cell death [Toyota et al., 2003].

In this report, we have investigated the molecular mechanism of gefitinib-induced growth inhibition and apoptosis using EGFR-expressing HAG-1 human gallbladder adenocarcinoma cells. We present evidence that blockade of the EGFR activity with gefitinib causes suppression of downstream signaling pathway through Erk and Akt, and induces apoptosis through activation of p18 Bax.

MATERIALS AND METHODS

Cell Culture and Chemicals

HAG-1 is a human cell line derived from a moderately differentiated adenocarcimona of the gallbladder and its cellular and molecular features were well characterized [Nakano et al., 1994]. The cells was cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Gefitinib was kindly provided by AstraZeneca (Macclesfield, United Kingdom). Stock solutions were prepared in dimethyl sulfoxide (DMSO, Wako, Osaka, Japan) and stored at −20°C. The final concentration of DMSO for all experiments and treatments (including controls, where no drug was added) was maintained at less than 0.02%. These conditions were found to be non-cytotoxic. Anti-EGF receptor, anti-Bax, anti-Bad, anti-Bcl-2, anti-p27, anti-p53 antibodies, and Protein A agarose were purchased from BD Biosciences (San Jose, CA). Anti-phospho-p53 (Ser15) antibody was purchased from Cell Signaling Technology Inc. (MA).

The siRNA (sense and anti-sense strands) against Bax gene was purchased from Qiagen (Germantown, MD). The sense and anti-sense strands sequences of Bax were 5′-GATGATTGCCGCCGTGGACA-TT and 5′-AAAGTAGGAGAGGAGGCCGT-TT, respectively. In vitro transfections were performed using the Transit-TKO polymer/lipid from Mirus (Madison, WI) as recommended. For 6×10^6 cells in 10 ml of medium, 2 μg of siRNA were used. Cells were washed 24 h after transfection.

Determination of Growth and Growth Inhibition

To determine the effect of gefitinib on cellular growth, replicate dishes (Falcon 3001) inoculated with $1 \sim 2 \times 10^4$ HAG-1 cells were incubated with or without gefitinib. Cell number was determined every day by Coulter counter after removal of the cells from plates with 0.05% trypsin and 0.02% EDTA in Ca- and Mg-free phosphate-buffered saline. The anti-proliferative effect of gefitinib on HAG-1 cells was assessed by WST assay, using manufacturer's

instructions (DOJIN, Kumamoto, Japan). The WST assay is a colorimetric method in which the intensity of the dye is proportional to the number of the viable cells. Briefly, 100 μl cell suspension of HAG-1 cells was seeded into a 96-well plate at a density of 1,000 cells/well. After overnight incubation, 100 μl drug solution at various concentrations were added. After incubation for 69 h at 37°C, 10 μl of solution A and solution B mixture was added to each well, and the plates were incubated for a further 3 h at 37°C. Then the optical density was measured at 450 and 620 nm using an IMMUNO-MINI NJ-2300 spectrophotometer (Nalge Nunc International, Chester, NY). Each experiment was performed using six replicate wells for each drug concentration and was carried out independently three times. The IC_{50} value was defined as the concentration needed for a 50% reduction in the absorbance.

Detection of EGFR by Flow Cytometry

Cells were harvested using trypsin and incubated for 1 h at 4°C with 1 μg of the anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA). As a control for non-specific binding, 1 μg of protein of human IgG1lamda (Sigma) was used as isotype-matched non-binding antibody for the EGFR. Subsequently, cells were washed twice with ice-cold PBS containing 0.5% BSA and incubated at 4°C in the dark for 1 h with FITC-conjugated goat anti-human IgG antibody, diluted 1:50 in PBS/BSA. After two washing steps with ice-cold PBS/BSA, cells were resuspended in 0.5 ml of ice-cold PBS/BSA and analyzed on a FACS/Calibur Flow Cytometer using CELLQuest software. Relative expression levels were calculated as the ratio between the mean fluorescence intensity of cells stained with the specific antibody and the mean fluorescence intensity of cells stained with the control antibody.

Cell Cycle Analysis and Apoptosis Measurement

Control or gefitinib-treated cells were harvested by trypsinization, washed with PBS, and then fixed in 100% ethanol and stored at 4°C for up to 3 days prior to cell cycle analysis. After the removal of ethanol by centrifugation, cells were then washed with PBS and stained with a solution containing PI and RNase A on ice for 30 min. Cell cycle analysis was performed on a Becton Dickinson FACS/Calibur Flow Cytometer using the CELLQuest or ModFit 3.0 software packages (Becton Dickinson, San Jose, CA), and the extent of apoptosis was determined by measuring the sub-G1 population.

Reverse Transcriptase Polymerase Chain Reaction

mRNA was extracted from HAG-1 cells using the Trizol Reagent (Life Technologies, Grand Island, NY). cDNA first-strand synthesis was performed by incubating 250 ng RNA in 20 μl RT reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol, and 500 μM dNTP containing 20 pmol of random primers) with 200 U avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI) at 42°C for 1 h. The cDNA was amplified in 50 μl PCR buffer containing 50 pmols if each primer, 200 μM dNTP, and one unit of Taq polymerase (Promega). The primer pairs for cyclin D1, p21, p27, and Bax were: cyclin D1: forward, 5′-TGCATCTACACCGACAACTC-3′, reverse, 5′-CAATGAAATCGTGCGGGGTC-3′, p21: forward, 5′-GAAGTAAACAGATGGCACTT-3′, reverse, 5′-TATCAAGAGCCAGGAGGGTA-3′, p27: forward, 5′-TCTGAGGACACGCATTTGGT-3′, reverse, 5′-TGAGTAGAAGAATCGTCGGT-3′, Bax: forward, 5′-TGGTTGCCCTTTTCTACTTTG-3′, reverse, 5′-GAAGTAGGAAAGGAGGCCATC-3′. After a first denaturation step (5 min at 97°C), samples were subjected to 30 cycles consisting of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C, with a final extension step of 10 min. PCR products were resolved by a 1.2% agarose gel electrophoresis and bands were visualized by ethidium bromide staining.

Immunoprecipitation and Western Blot Analysis

The cells were washed twice with ice-cold PBS and scraped into 1 ml of radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 0.4% (v/v)TritonX-100, 400 μM EDTA.2Na, 400 μM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7 \cdot 10H_2O$, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin). After removal of cell debris by centrifugation, protein concentrations of the supernatants were determined by using Bradford method or a BCA protein assay kit (Pierce, Rockford, IL). For immunoprecipitation, equal amounts of protein were incubated for 1 h at 4°C with specific antibodies against p53, phosphorylated p53 (pS^{15}). Immune complexes were precipitated with protein A agarose beads, washed with radioimmunoprecipitation assay lysis buffer and then boiled in electrophoresis sample buffer

(250 mM Tris pH6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol). For Western blot, equal amounts of proteins or immunoprecipitated target proteins were resolved by 5–20% SDS–PAGE (polyacrylamide gel electrophoresis) and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Non-specific binding sites were blocked by incubating the membranes in blocking buffer (5% nonfat milk in 1× TBS with 0.1% Tween-20) at room temperature for 1 h. The membranes were then incubated with primary antibodies against either phospho-EGFR (Tyr1068, Cell Signaling Technology), phospho-p44/42 MAPK (Thr202/Tyr204, Cell Signaling Technology), phospho-Akt (Ser473, Cell Signaling Technology), p27 (Transduction Laboratories), Bax, Bad, or Bcl-2 (Cell Signaling Technology). The membranes were hybridized with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Immunoblots were developed with the enhanced chemiluminescence (ECL) system from Amersham Biosciences (Buckinghamshire, UK) and then were exposed to ECL hyperfilm according to the manufacture's instructions (Amersham Biosciences). The blots were striped and reprobed with primary antibodies against EGFR (2232; Cell Signaling Technology) and MAPK (9102; Cell Signaling Technology) and Akt (9272; Cell Signaling Technology). For reblotting, membranes were incubated in stripping buffer (62.5 mM Tris/HCl, pH 6.8/2% (w/v) SDS/100 mM 2-mercaptoethanol) for 30 min at 50°C before washing, blocking, and incubating with antibody. Triplicate determinations were made in separate experiments.

Isolation of Mitochondrial Fraction

Cells were lysed in 1 ml of 20 mM HEPES–KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 250 mM sucrose. The cells were broken open with 6 passages through a 26-gauge needle applied to a 1 ml syringe. The homogenate was centrifuged at 800*g* for 10 min at 4°C to remove nuclei and unbroken cells. The supernatant was transferred to a 1.5 ml centrifuge tube. Centrifugation was conducted at 10,000*g* for 15 min at 4°C. The supernatant contained the cytosolic fraction. The resulting mitochondrial pellet was lysed in 50 μl of 20 mM Tris (pH 7.4), 100 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% Triton X-100. Then the lysate was centrifuged at 15,000*g* for 5 min at 4°C, and the resultant supernatant was kept as the solubilized enriched mitochondria fraction. Cell fractions were assayed for protein concentration using the Bio-Rad Dye Binding protein assay (Bio-Rad Laboratories), then equivalent amounts of protein were analyzed for Bax expression by Western/ECL analysis.

Statistical Analysis

The data were analyzed by the Mann–Whitney *U*-test for statistical significance of the difference between groups. A P value of <0.01 was considered to indicate statistical significance.

RESULTS

Effect of Gefitinib on Proliferation and Survival in HAG-1 Cells

The EGFR expression was examined in HAG-1 cells by flow cytometry. As shown in Figure 1A, EGFR was detected in HAG-1 cells, with approximately 10-fold relative EGFR expression. The IC_{50} of the gefitinib against HAG-1 cells was 0.12 μM for 72 h exposure (Fig. 1B). The population doubling times of HAG-1 cells was 26.4 h (Fig. 1C), but was prolonged to 104 h when the cells were treated with 1 μM gefitinib, indicating that gefitinib depressed the growth of HAG-1 cells by approximately fourfold (Fig. 1D). When the treatment exceeded 72 h, the cell number abruptly decreased, and the decline of the growth appeared to be irreversible, because the cell number still decreased upon removal of gefitinib at 72 h (Fig. 1D). These data indicate that gefitinib delays the growth of the cells initially, but leads to cell death when treated over 72 h.

Time-Course Analysis of the Effect of Gefitinib on Cell Cycle Progression and Apoptosis

To examine whether the inhibitory effect observed in growth assays reflects a delay or arrest of cells in the G0/G1 phase, cells were treated with gefitinib for indicated times, and the cell cycle progression was evaluated after PI staining by fluorescence-activated cell sorting analysis (Fig. 2). Upon treatment with gefitinib at a dose of 1 μM, the proportion of cells in a G0/G1 phase increased from 60 to 87% at 24 h from the beginning of the treatment, with

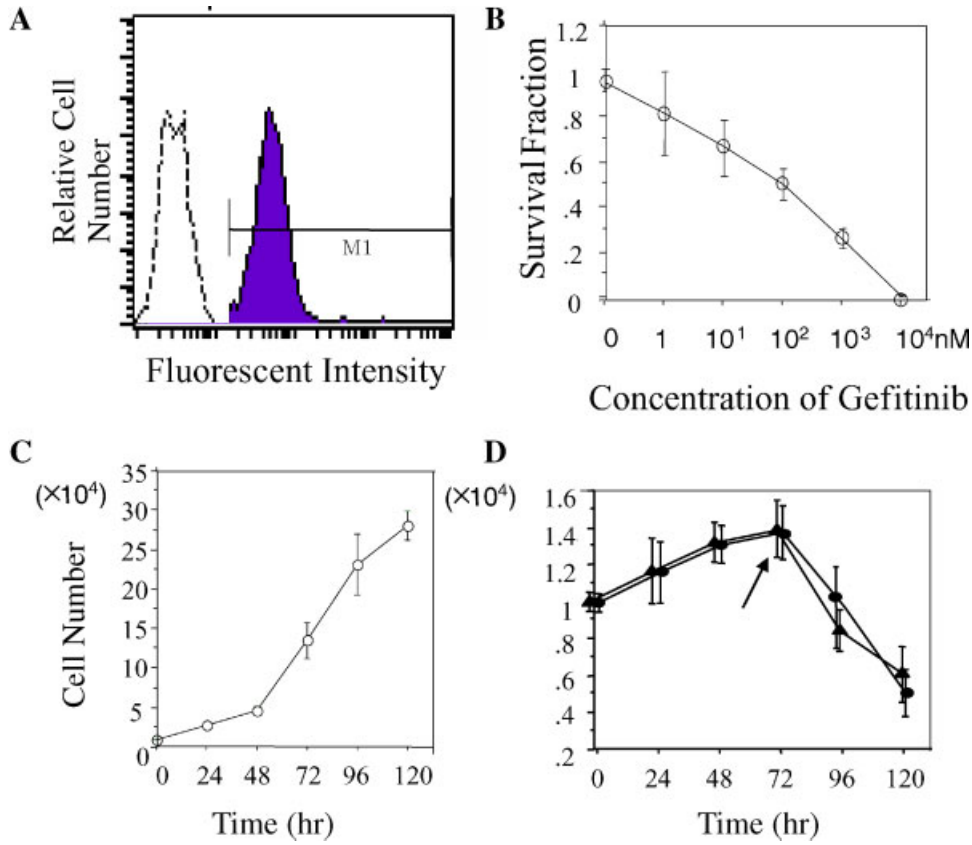

Fig. 1. Expression of EGFR, cytotoxicity of gefitinib, and effects of gefitinib on the growth of HAG-1 cells. **A**: Expression of EGFR was analyzed by FACS after treatment of cells for 1 h at 4°C with 1 μg of the anti-EGFR antibody. **B**: Cytotoxicity was determined by using WST-1 assay. Cells were seeded into a 96-well microplate, and treated with gefitinib at various concentrations of gefitinib for 72 h. **C**: The proliferation of HAG-1 cells without gefitinib treatment (○). **D**: Effect of gefitinib on cell growth. Cells were seeded and treated with gefitinib at 1.0 μM for 120 h (●), or treated with gefitinib at 1.0 μM for 72 h, followed by incubation with normal medium (▲). Arrow indicates removal of gefitinib. Values represent the means of three experiments; *bars*, SE. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

corresponding decrease in cells in S and G2–M phase, and reached almost a plateau afterwards. By contrast, the sub-G0/G1 cell population became evident (72 h, 20%) 72 h post-treatment, and progressively increased upon further treatment (96 h, 34% and 120 h, 50%). Because cells in the sub-G0/G1 population represent apoptotic cells [Janmaat et al., 2003], the irreversible growth decline appeared to be due to progressive expansion of apoptotic cell population.

Effects of Gefitinib on Autophosphorylation of EGFR, Akt, and Erk

To assess the effect of gefitinib on the EGFR activation and subsequent downstream activation, we examined the expression and activation of EGFR, Akt, and Erk. As shown in Figure 3, phosphorylated EGFR was detected without EGF stimulation. Upon treatment with 1 μM gefitinib, tyrosine phosphorylation of EGFR was significantly inhibited with incubation for 2 h, and continued to be suppressed over 24 h, without changing the relative amount of EGFR. In parallel, Erk was also phosphorylated without EGF, and significantly suppressed upon treatment with gefitinib. Unlike EGFR and Erk, autophosphorylation of Akt was modest, but subsequent suppression of Akt was also observed.

Gefitinib Induces Growth Inhibition and Apoptosis Through G1 Arrest and p18 Bax Expression

To identify the molecular basis for gefitinib-induced G0/G1 arrest, we examined the effects of gefitinib on the mRNA expression level of the cyclin D1 and p21 by using a

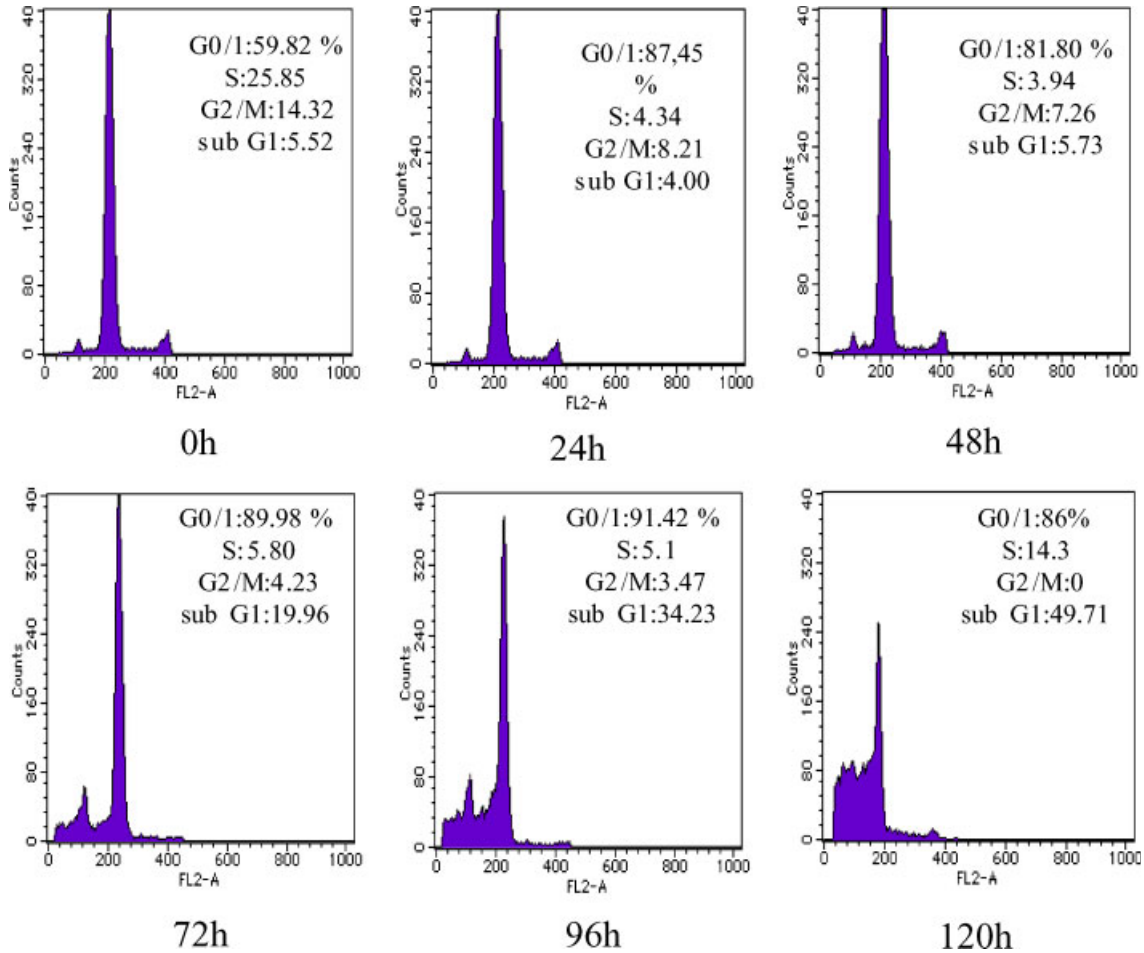

Fig. 2. Time course analysis of the effect of gefitinib on cell cycle progression and apoptosis. HAG-1 cells were stained with propidium iodide after exposure to gefitinib (1.0 μM) for 0, 24, 48, 72, 96, and 120 h, and analyzed by flow cytometry. Percentages of the total cell population in the different phases of cell cycle were determined with curve fitting using the ModFit 3.0 software. The mean values for each phase of the cell cycle are shown on the top right of each panel. Representative results of at least three experiments are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

semi-quantitative RT-PCR method. As shown in Figure 4A, mRNA expression level of cyclin D1 is decreased significantly at 24 h from the beginning of the treatment, and remained low during the entire period of experiments. In contrast, mRNA expression of p21 was upregulated. It has been demonstrated that a blockade of the EGFR-mediated pathway induced upregulation of p27 [Busse et al., 2000], we next examined the effect of gefitinib on the expression of p27. Although mRNA expression of p27 was not affected by the treatment of gefitinib throughout the experiments (Fig. 4A), gefitinib increased p27 protein by fivefold at 24 h from the beginning of the treatment, and levels remained high up to 120 h (Fig. 4B). These results, together with the cell cycle analysis, indicate that accumulation of p27 might be responsible for gefitinib-induced growth arrest at the G0/G1 phase. To investigate the apoptotic mechanism, pro-apoptotic p53, Bad, and Bax and anti-apoptotic Bcl-2 were evaluated following gefitinib treatment. As shown in Figure 4B, total p53 protein level, which acts upstream of p27, and phosphorylated p53 at serine 15, which stabilizes and enhances accumulation of p53, both were not altered after treatment with gefitinib (Fig. 4B). As shown in Figure 4C, gefitinib substantially increased the expression of p18 Bax, an active subtype of Bax protein, 72 h post-treatment, with maximal expression at 120 h. By contrast, Bcl-2 and Bad expressions were unchanged during the incubation period. Since gefitinib did not affect Bax mRNA levels, an

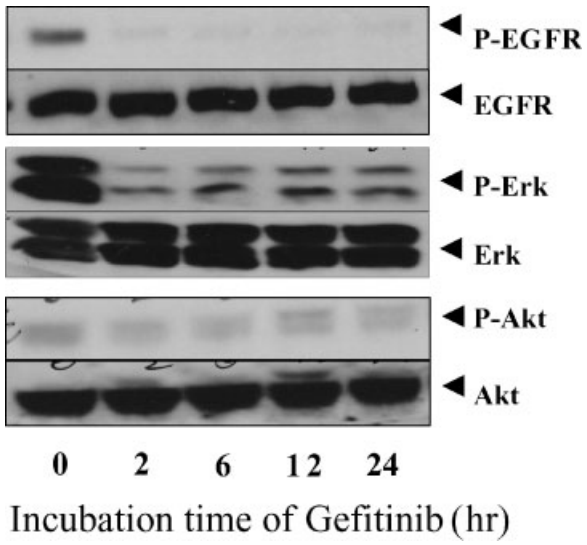

Fig. 3. Effects of gefinitib on the phosphorylation of EGFR and downstream Akt and Erk 1/2. Western blots are shown for phospho- and total EGFR, Erk 1/2, and Akt.

increase in gefitinib-induced expression of total Bax (p21 Bax and p18 Bax) could not be explained on a transcription level.

Attenuation of Apoptosis by Blocking Bax Activity With RNA Interference and Translocation of Bax to Mitochondria

To investigate the direct role of Bax in gefitinib-induced apoptosis, HAG-1 cells were transfected with anti-Bax siRNA, and gefitinib-induced apoptosis was evaluated. Anti-Bax siRNA significantly prevented the cells from gefitinib-induced apoptosis from 45 to 25% (45% reduction in apoptosis) (Fig. 5A,B) after incubation with gefitinib for 120 h. In parallel with the inhibition of apoptosis, anti-Bax siRNA was shown to significantly inhibit the amount of gefitinib-induced p18 Bax and p21 Bax protein, as compared to control siRNA that was constructed based on no significant homology with Bax RNA. Densitometric analyses showed approximately 70% reduction in Bax protein level (Fig. 5C). Western immunoblot analysis of mitochondrial-enriched fractions, obtained after cells were treated with 1 μM of gefitinib for indicated times, showed a time-dependent increase of p18 Bax, accompanied by time-dependent decrease of p21 Bax (Fig. 5D). Since wild-type p21 Bax has been shown to be cleaved into p18 Bax in the mitochondria [Wood et al., 1998], these data indicate that gefitinib

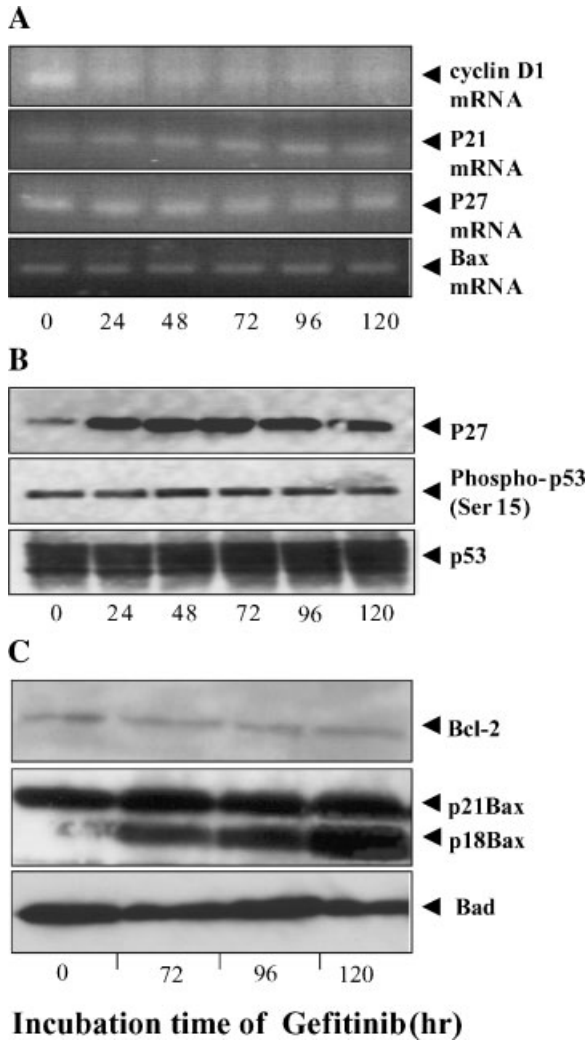

Fig. 4. Quantitative evaluation of apoptosis-associated proteins and RNA transcript in HAG-1 cells treated with gefitinib. The cells were exposed to 1.0 μM gefitinib for indicated times, and processed for RT-PCR and immunoblot analyses as described in Materials and Methods. **A**: Quantitative analysis of transcripts by RT-PCR of cyclin D1, p21, p27 and Bax. **B**: Western blot analyses of p27, p53, and phosphorylated p53 at serine 15. Equivalent amounts of immunoprecipitates were subjected to 12% SDS–PAGE, followed by transfer to nitrocellulose, and then blotting by respective antibodies. **C**: Western blot analyses of Bcl-2, Bax, and Bad.

activates Bax through translocation of Bax from the cytosol to the mitochondria, thereby inducing apoptosis.

DISCUSSION

The most frequent molecular abnormalities associated with pathogenesis of gallbladder cancer are overexpression of EGFR [Yukawa

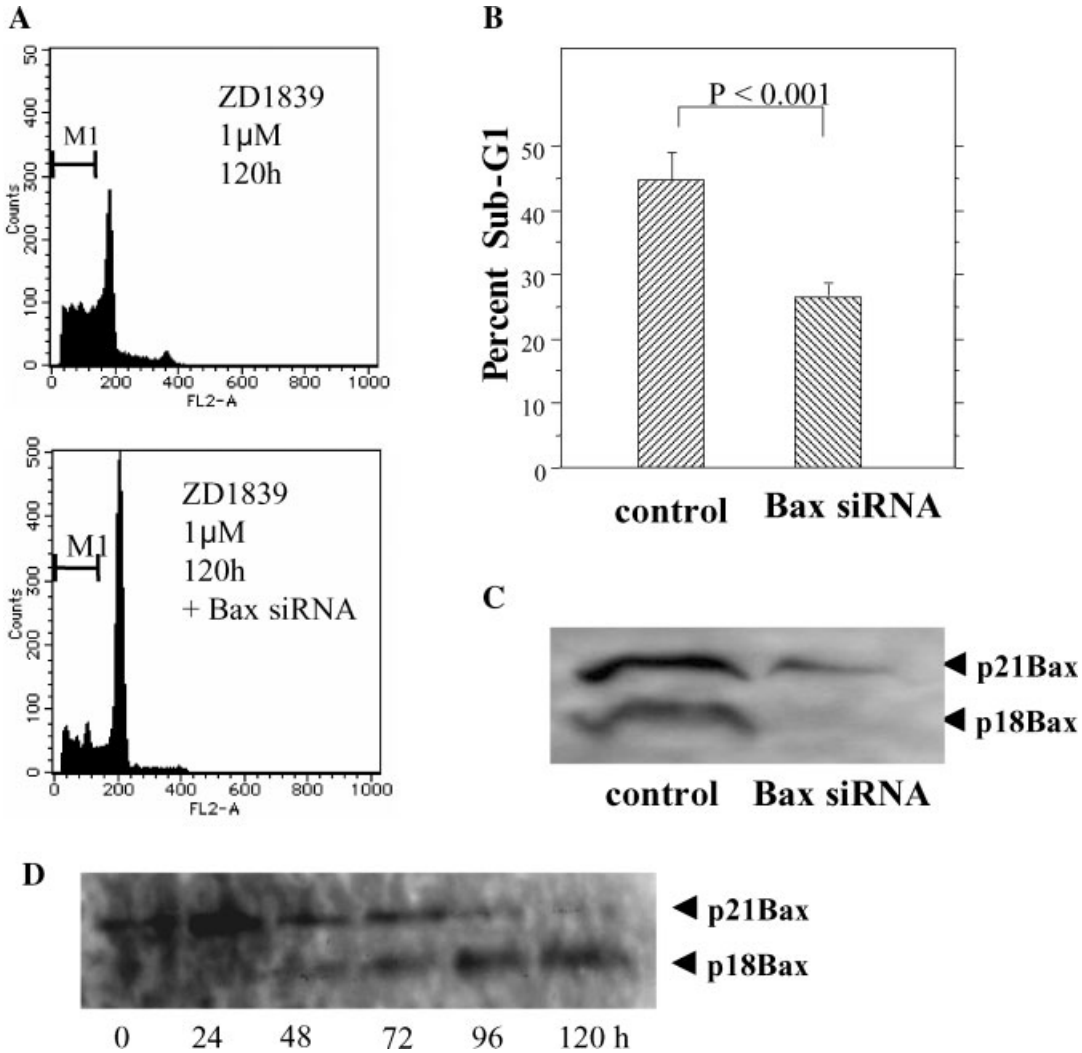

Fig. 5. Attenuation of apoptosis by Bax siRNA and translocation of Bax to mitochondria. HAG-1 cells were transfected with anti-Bax siRNA and processed for FACS analyses as described in Materials and Methods. **A**: Flow cytometric analysis of cell cycle progression at 120 h following treatment with gefitinib. **B**: Levels of gefitinib-induced apoptosis as measured by the percentage of sub-G1 phase cell population at 120 h post-treatment. **C**: Amount of Bax protein at 120 h post treatment, measured by Western blot. **D**: Amount of Bax protein in mitochondrial-enriched fraction.

et al., 1993; Valerdiz-Casasola, 1994; Lee and Pirdas, 1995]. Thus, we investigated here the possibility of EGFR signaling as a potential therapeutic target for gallbladder cancer by studying in vitro effects of the orally active EGFR inhibitor, gefitinib, against an EGFR-expressing HAG-1 gallbladder adenocarcinoma cell line. We have found that the IC_{50} of gefitinib against HAG-1 cells was 0.12 μM for 72 h exposure, a comparable IC_{50} concentration exhibited by highly sensitive A431 squamous carcinoma cell line [Janmaat et al., 2003]. Using this cell line, we showed that gefitinib inhibited the cell growth by arresting the cells in G0/G1 phase, followed by the increase in apoptotic cell population (sub-G0/G1 phase). The arrest of the cell cycle at the G0/G1 phase was accompanied by depression of cyclin D1 mRNA as well as accumulation of p27 protein, a critical negative regulator of the cell cycle, that inhibits the activity of cyclin/cdk complexes during G0 and G1 [Slingerland and Pagano, 2000], being consistent with a previous report showing a critical role of p27 in the anti-proliferating activity of gefitinib on tumor cells using p27 anti-sense construct [Di Gennaro et al., 2003]. Moreover, gefitinib upregulated p27 protein levels without affecting p27 mRNA expression.

Degradation of p27 has been shown to be a critical event for the G1/S transition occurring through ubiquitination and subsequent degradation by the 26S-proteasome [Slingerland and Pagano, 2000; Masuda et al., 2002]. Therefore, it is suggested that gefitinib may affect the ubiquitin–proteasome pathway of p27 degradation.

When the treatment of HAG-1 cells with gefitinib exceeded 72 h, cell death became evident with a progressive expansion of apoptotic population with incubation time until 120 h. Correspondingly, gefitinib upregulated the expression of total Bax, with subsequent increase in p18 Bax that has been shown to be generated through cleavage of full-length Bax during apoptosis [Wood et al., 1998] and regarded as a more potent inducer of apoptotic cell death than full-length Bax [Toyota et al., 2003]. The observed expression of p18 Bax appears to be a cause of gefitinib-induced apoptosis, not only because the amount of p18 Bax increased in the mitochondria, a characteristic feature of Bax activation toward apoptosis [Gross et al., 1999], but also because the blockade of Bax using anti-Bax siRNA significantly reduced gefitinib-induced apoptosis. This is the first report demonstrating the direct role of Bax in gefitinib-induced apoptosis. With regard to the mechanism of gefitinib-induced Bax upregulation, it has been reported in colorectal cancer that inhibition of EGFR by anti-EGFR monoclonal antibody C225 induces apoptosis by enhanced expression of newly synthesized Bax protein [Mandal et al., 1998]. However, in the present study, an increase in the gefitinib-induced Bax protein might be due to the decreased degradation of Bax, because levels of Bax mRNA expression and levels of total and phosphorylated p53 that regulates Bax [Zhan et al., 1994] were not altered following treatment with gefitinib. Recently, it has been demonstrated that Bax is degraded by the ubiquitin-proteasome pathway [Chang et al., 1998; Li and Dou, 2000]. Moreover, inhibition of proteasome function has been shown to increase levels of ubiquitinated forms of Bax protein, without any effects on Bax mRNA expression, thereby inducing apoptosis as a consequence of upregulation of Bax [Fan et al., 2001; Nam et al., 2001]. We are currently investigating the mechanism of gefitinib-induced accumulation and activation of Bax through the ubiquitin-proteasome pathway as well as cleavage pathway of wild-type Bax into p18 Bax.

There are two major cell survival and growth signaling pathways downstream of EGFR, i.e., the Ras-Raf-MAPK and PI-3K-Akt pathways. Recently, it has been reported that simultaneous inhibition of both the MAP kinase and PI-3K Akt pathways is important for the execution of gefitinib-induced anti-proliferative effect and apoptosis, and that persistent activity of either of these signaling pathways is involved in the decreased or lack of sensitivity to EGFR inhibitors [Janmaat et al., 2003; Li et al., 2003]. The inactivation of Bad through activation of these pathways has been demonstrated to be involved in gefitinib-induced apoptosis, since activation of either of MAP kinase or Akt pathway has been shown to abrogate the pro-apoptotic function of Bad by phosphorylating its specific serine residues [Datta et al., 1997; Fang et al., 1999; Shimamura et al., 2000; Zhou et al., 2000]. In the present study, however, Bad appears not to be involved in the gefitinib-induced apoptotic events, because Bad is unchanged during the treatment despite inactivation of Akt and Erk. With regard to Bax, there is only a report that inhibition of Akt led to an increased protein level of Bax in a pancreas cancer cell line [Fahy et al., 2003]. In this study, we have found that activation of MAP kinase and Akt is significantly inhibited by gefitinib, suggesting that simultaneous inhibition of these pathways by gefitinib may lead to Bax accumulation and subsequent apoptosis.

Although the observations were obtained on a single human gallbladder cancer cell line, the present data suggest the possibility of EGFR signaling as a potential therapeutic target for gallbladder carcinoma and may serve as a rational basis for a therapeutic approach to this incurable disease with EGFR tyrosine kinase inhibitors.

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