Antiproliferative Effects of Gefitinib are Associated with Suppression of E2F-1 Expression and Telomerase Activity

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Abstract. Background: Gefitinib (Iressa, ZD1839) is a selective epidermal growth factor receptor tyrosine kinase inhibitor. E2F-1 is a critical determinant in cell cycle. Growth signals up-regulate telomerase activity. The effects of gefitinib on E2F-1 and telomerase in A549, H23 and A431 cells were examined. Materials and Methods: Cell proliferation and cell cycle progression were measured by the WST-1 assay and flow cytometry. The expression of E2F-1 and cyclin-dependent kinase inhibitors was evaluated, and hTERT mRNA expression and telomerase activity were analyzed. Results: In the A431 and A549 cells, treatment with gefitinib inhibited cell proliferation and was associated with an increase in G1-phase. In both cell types, gefitinib decreased the expression of E2F-1 mRNA and protein, followed by the suppression of hTERT mRNA and telomerase activity. In the H23 cells, gefitinib did not affect cell proliferation. Conclusion: The antiproliferative effects of gefitinib may be, at least in part, due to the inhibition of E2F-1 expression and telomerase activity.

Gefitinib (Iressa, AstraZeneca Pharmaceuticals, Macclesfield, UK) is a selective inhibitor of the epidermal growth factor receptor (EGFR) - tyrosine kinase. Inhibition of EGFR activation blocks signal transduction pathways implicated in the proliferation and survival of cancer cells (1). Although gefitinib was approved for the treatment of non-small cell lung cancer in Japan and the United States, clinical trials have revealed significant variability in response to gefitinib (2). Recently, EGFR mutations in lung cancer appeared to correlate with clinical response to gefitinib therapy (3, 4). However, the mechanism involved in the antiproliferative effect of gefitinib has not been characterized completely. Gefitinib inhibits the tyrosine kinase activity of the EGFR by competing with adenosine triphosphate and by non-competitively inhibiting signaling by the EGFR peptide ligands (5). Gefitinib has been shown to inhibit the growth of EGFR-positive human cancer cell lines in vitro (5).

In some cell lines, the growth inhibitory effect of EGFR antagonists correlates with the arrest of cells in the G1-G0-phase of the cell cycle (6). During G1-phase progression, cyclin-dependent kinase (CDK)-mediated hyperphosphorylation of retinoblastoma protein (pRB) results in the dissociation of pRB and E2F-1 and entry into the S-phase. CDKs are negatively regulated by a group of functionally related proteins called CDK inhibitors (CDKIs) (7). E2F-1 mediates the transcription of genes required for DNA synthesis and thus binding of hypophosphorylated pRB to E2F-1 arrests cells in the G1-phase. In head and neck squamous carcinoma cells, p27KIP1 (p27) and p21WAF1/CIP1 (p21) are known to play critical roles in the antiproliferative effect of gefitinib (8). In the present study, the antiproliferative effect of gefitinib on two lung adenocarcinoma cell lines and on a human vulvar cancer cell line was examined. The effect of gefitinib on some regulatory proteins of the G1-phase of the cell cycle was also analyzed, with particular attention to E2F-1, p21, p27, p57KIP2 (p57) and p16INK4a (p16).

Growth signals and E2F-1 up-regulate telomerase activity, which is involved in the immortalization of cancer cells. Telomerase is reactivated in a large majority of cancer cells. Although human telomerase is a multicomponent complex, this catalytic holoenzyme may only consist of human telomerase
RNA and hTERT (9), hTERT was shown to be a determinant for telomerase activity control (9). A recent study documented the activation of the hTERT promoter by epidermal growth factor and the regulation of telomerase activity by various reagents including growth factors (10). Thus, it is hypothesized that gefitinib has a suppressive effect on telomerase activity.

Materials and Methods

Chemicals. Gefitinib was kindly provided by AstraZeneca.

Cell lines and conditions. The A431 human vulvar epidermoid carcinoma cells and A549 human lung adenocarcinoma cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). The H23 human lung adenocarcinoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco’s modified eagle medium (DMEM) was used as the culture medium and was supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Breda, The Netherlands) and 100 μg/ml kanamycin. All cell lines were cultured at 37°C in a humidified 5% CO2 atmosphere.

Cell growth and cell cycle analysis. Cell growth was determined by the WST-1 assay (Boehringer Mannheim, Mannheim, Germany). Briefly, the tetrazolium salt WST-1 was cleaved to form azan dye by mitochondrial dehydrogenase in viable cells and the dye was then quantified by spectrophotometry. The cells were plated into flat-bottomed 96-well plates. Twenty-four h after seeding, various concentrations of gefitinib were added to the medium and the cells were incubated for an additional 48 h. The WST-1 reagent was then added and was allowed to react for 1 h. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad Lab., Hercules, CA, USA).

For cell cycle analysis, 24 h-preincubated cells were cultured for 48 h with various concentrations of gefitinib and the cells were then washed with phosphate buffered saline (PBS) and harvested with trypsin-EDTA solution. Samples of 1x10⁶ cells were fixed in 70% ethanol on ice for 30 min, followed by washing with PBS. The cells were then treated with RNase A (100 μg/ml) for 30 min at 37°C and were stained for DNA with propidium iodide (10 μg/ml) for 15 min at room temperature. The fluorescence of stained cells was measured using a flow cytometer (Beckman Coulter, Inc.).

Western blot analysis. Whole cell lysates were prepared as follows. For the dose-dependent study, cells were harvested after 48-h treatment with various concentrations of gefitinib. For the time-dependent study, the A431 cells were treated with 1 μM gefitinib, while the A549 and H23 cells were treated with 20 μM gefitinib for 6, 12, 24 and 48 h. Harvested cells were lysed in buffer containing 20 mM of Tris (pH 8), 10 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The lysates were incubated on ice for 60 min, microcentrifuged at 10,000 xg for 15 min and the supernatant was then transferred to a fresh tube. Aliquots of 10 μg of protein were separated on a 12.5% SDS-polyacrylamide gel and were then electro-transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies against E2F-1 (Santa Cruz Biotechnology, CA, USA), p21 (Exalpha Biologicals, MA, USA), p27 (Santa Cruz Biotechnology), p57 (Santa Cruz Biotechnology) or p16 (BD PharMingen, CA, USA), followed by incubation with each of the respective horseradish peroxidase-conjugated secondary antibodies. The immunoreactive proteins were visualized using the enhanced chemiluminescence detection system (Amersham, Bucks, UK).

RT-PCR. For total RNA extraction, cells were plated onto 100 mm-diameter dishes. Following an overnight incubation, gefitinib was added to the cell culture medium. For the dose-dependent study, the cells were cultured in various concentrations of gefitinib and were harvested after 24-h exposure. For the time-dependent study, the A431 cells were treated with 1 μM gefitinib, while the A549 and H23 cells were treated with 20 μM gefitinib for 3, 6, 12 and 24 h. The cells were then harvested and total RNA was isolated using ISOGEN (Nippogene Co., Tokyo, Japan). A 2 μg amount of total RNA was reverse-transcribed with random hexamer and then the target sequence for each mRNA was separately amplified with gene-specific primers according to the protocol provided by the manufacturer (ThermoScript RT-PCR System; Invitrogen Corp., Carlsbad, CA, USA). The PCR primer sets were as follows: 5′-CCG AAG AGT GTC TGG AGG AA-3′ and 5′-GGA TGA AGC GGA GTG TGG A-3′ for hTERT mRNA (11), 5′-TGA TAC CCC AAC TCC CTC TA-3′ and 5′-AAA GCA GGA GGG AAC AGA GC-3′ for E2F-1 mRNA (12), 5′-CTGA AGA CAT GGG GAA GGT GA-3′ and 5′-ATG ATC TTG AGG CTA TCA TA-3′ for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (11). The reaction conditions for hTERT mRNA were: 36 cycles of 95°C for 30 sec, 56°C for 30 sec, and 72°C for 60 sec, for E2F-1 mRNA; 30 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 60 sec, and for GAPDH mRNA; 28 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec. The amplified products of hTERT mRNA were separated by 8% polyacrylamide gel electrophoresis and were then visualized by SYBR Gold staining (Molecular Probes, Inc., Eugene, OR, USA). The other products were electrophoresed through 2% agarose gels and stained with ethidium bromide.

Telomerase activity assay. Telomerase activity was evaluated using a telomeric repeat amplification protocol (TRAP) assay kit (Intergen Co., Purchase, NY, USA) as reported previously (13). Harvested cells were resuspended in the detergent-based lysis buffer and the lysate was then centrifuged for 30 min at 12,000 xg. The protein concentration of the supernatant was measured using a Bradford assay kit (Bio-Rad Lab.). A 2-μl aliquot of the extract (0.1 μg/μl of protein) was then incubated at 30°C for 30 min in 50 μl of reaction solution containing 0.1 μg TS primer [5′-AAT CCG TCG AGA GTT-3′] and 0.1 μg reverse primer. An internal control, together with a specific primer, was added to the reaction mixture to monitor the efficiency of PCR. The solution was subjected to 29 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 45 sec. The PCR products were electrophoresed through a 12.5% non-denaturing polyacrylamide gel and stained with SYBR Green 1 (Molecular Probes, Inc.). The presence of telomerase activity was demonstrated by 6-bp ladders starting from 50 bp. A 36-bp internal control band to monitor PCR efficiency was visible in every lane.

Results

Cell growth and cell cycle population. Gefitinib inhibited the growth of the A431 and A549 cells but not of the H23 cells...
(Figure 1). After the 48-h treatment, proliferation of the A431 cells was suppressed by clinically achievable serum levels of 0.25-1 μM gefitinib. The proliferation of A549 cells was not suppressed by the clinically achievable levels of gefitinib, but was inhibited by 20 μM gefitinib. In contrast, the growth of H23 cells was not altered by 48 h of treatment with 20 μM gefitinib.

To determine whether growth inhibition in the A431 and A549 cells by gefitinib was a consequence of cell cycle arrest, the cell cycle profile was examined after 48 h of treatment with gefitinib (Figure 2). In the A431 and A549 cells, treatment with gefitinib increased the G1 population and decreased the S and G2/M populations in a dose-dependent manner, with a corresponding suppression of cell growth (Figure 2). On the other hand, gefitinib did not affect either cell cycle population or cell growth in the H23 cells (Figure 2).

E2F-1 and CDKIs. The protein levels of E2F-1 were inhibited by 48 h-treatment with gefitinib in the A431 and A549 cells, whereas inhibition of E2F-1 was not observed in the H23 cells (Figure 3). In addition, gefitinib decreased p21 protein levels and increased p27 protein levels in the A431 cells in a dose-dependent manner. In the A549 cells, only a decrease in p21 protein levels was observed at 20 μM gefitinib. In contrast, gefitinib did not regulate CDKI proteins in the H23 cells. In addition, the time-dependent effects of gefitinib on E2F-1 in the A431 and A549 cells were examined. Suppression of E2F-1 by gefitinib was detected after 24-h treatment of the A431 and A549 cells (data not shown).

Whether the suppression of E2F-1 protein levels was associated with the inhibition of E2F-1 mRNA levels was subsequently analyzed. Gefitinib inhibited the expression of E2F-1 mRNA in a dose-dependent manner in the A431 and A549 cells, but not in the H23 cells (Figure 4). In addition, gefitinib suppressed the expression of E2F-1 mRNA in a time-dependent manner, and the suppression was evident from the 12-h treatment (data not shown).

HTERT mRNA expression and telomerase activity. In the A431 and A549 cells, the expression of hTERT mRNA
decreased after 24-h treatment with gefitinib in a dose-dependent manner, whereas the expression of hTERT mRNA was not influenced in the H23 cells (Figure 4). In the time-course experiment, the suppression of hTERT mRNA was seen in A431 and A549 cells from 12-h and 24-h treatment with gefitinib, respectively (data not shown). In addition, telomerase activity was reduced by 48-h treatment with gefitinib in the A431 and A549 cells, but not in H23 cells (Figure 5).

Discussion

The results of the present study demonstrated that gefitinib inhibited E2F-1 expression followed by telomerase activity in gefitinib-sensitive A431 cells and slightly-sensitive A549 cells, but not in gefitinib-resistant H23 cells. In addition, p27, which modulates E2F-1 expression, was increased in highly-sensitive A431 cells, but not in the slightly-sensitive A549 cells. Thus, the mechanisms of E2F-1 suppression differ between the A431 and A549 cells.

Gefitinib was shown to exert growth suppressive effects in various human cancer cell lines (14). These effects were associated with the ability of gefitinib to induce inhibition of cell growth and cell cycle arrest due to blocking of the signal-transduction pathway (6). The A431 and A549 cells used in the present study are sensitive to gefitinib, while the H23 cells are resistant. Clinically achievable levels of gefitinib induced growth inhibition in the A431 cells in the present study (1) and higher levels of gefitinib suppressed the cell growth of A549 cells. In the A431 and A549 cells, the growth inhibitory effect of gefitinib correlated with cell cycle arrest in the G1-phase, whereas the cell cycle was unaffected in the H23 gefitinib-resistant cell line.

Gefitinib inhibited the expression of E2F-1 mRNA and protein in the A431 and A549 cells. Since E2F-1 is a protein which regulates the G1/S transition in the cell cycle (15), cell cycle arrest in the G1-phase by gefitinib may, in part, be caused by the suppression of E2F-1 mRNA and protein expression. The CDKIs are known to regulate E2F-1 transcriptional activity and earlier reports showed that the up-regulation of p27 or p21 played an important role in the antiproliferative effects of gefitinib (8). In the present study, we showed that p27 expression was up-regulated in the A431 cells, but not in the A549 cells after treatment with gefitinib. In the A431 cells, acceleration of p27 expression by gefitinib may correlate with suppression of E2F-1 expression. In contrast, in the A549 cells, other mechanisms may be involved in the suppression of E2F-1. In both the A431 and A549 cells, p21 expression was suppressed by treatment with gefitinib. Several studies recently established anti-apoptotic roles for p21 (16, 17) and additional studies are required to clarify the relationship between the antiproliferative effects of gefitinib and the suppression of p21.

We also found that gefitinib suppressed hTERT mRNA expression and telomerase activity in A431 and A549 cells. A variety of transcription factors were shown to participate in hTERT gene expression (18). As the expression of hTERT is known to be influenced by E2F-1 expression (19), gefitinib may suppress telomerase activity via E2F-1 suppression in A431 and A549 cells. In human cutaneous squamous cell carcinoma cells, an earlier report indicated that the blockade of EGFR signaling induced antitumor effects by decreasing telomerase activity (20). The inhibition of telomerase induced telomere shortening and cellular senescence (9). In A549 and A431 cells, the suppression of telomerase activity may be another mechanism for the antiproliferative effects of gefitinib.

In conclusion, we demonstrated that gefitinib down-regulated E2F-1 expression followed by the suppression of
telomerase activity in the A431 and A549 cells. The antiproliferative effects of gefitinib may be, at least in part, due to the inhibition of E2F-1 expression and telomerase activity.

References


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