Enhancement of Sensitivity to Tumor Necrosis Factor α in Non–Small Cell Lung Cancer Cells with Acquired Resistance to Gefitinib

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Abstract

Tumor cells that have acquired resistance to gefitinib through continuous drug administration may complicate future treatment. To investigate the mechanisms of acquired resistance, we established PC-9/ZD2001, a non-small-cell lung cancer cell line resistant to gefitinib, by continuous exposure of the parental cell line PC-9 to gefitinib. After 6 months of culture in gefitinib-free conditions, PC-9/ZD2001 cells reacquired sensitivity to gefitinib and were established as a revertant cell line, PC-9/ZD2001R. PC-9/ZD2001 cells showed collateral sensitivity to several anticancer drugs (vinorelbine, paclitaxel, camptothecin, and 5-fluorouracil) and to tumor necrosis factor α (TNF-α). Compared with PC-9 cells, PC-9/ZD2001 cells were 67-fold more sensitive to TNF-α and PC-9/ZD2001R cells were 1.3-fold more sensitive. Therefore, collateral sensitivity to TNF-α was correlated with gefitinib resistance. PC-9/ZD2001 cells expressed a lower level of epidermal growth factor receptor (EGFR) than did PC-9 cells; this down-regulation was partially reversed in PC-9/ZD2001R cells. TNF-α-induced autophosphorylation of EGFR (cross-talk signaling) was detected in all three cell lines. However, TNF-α-induced Akt phosphorylation and IκB degradation were observed much less often in PC-9/ZD2001 cells than in PC-9 cells or PC-9/ZD2001R cells. Expression of the inhibitor of apoptosis proteins c-IAP1 and c-IAP2 was induced by TNF-α in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells. This weak effect of EGFR on Akt pathway might contribute to the TNF-α sensitivity of PC-9/ZD2001 cells. These results suggest that therapy with TNF-α would be effective in some cases of non-small-cell lung cancer that have acquired resistance to gefitinib.

Gefitinib (Iressa, ZD1839), a small-molecule epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, has been approved for the treatment of refractory and relapsed non-small-cell lung cancer (NSCLC) patients in a number of countries around the world. This drug, which is given continuously as a once-daily oral dose, showed antitumor activity in patients with relapsed or recurrent NSCLC; however, tumor responses were observed in 12% to 18% of patients with chemotherapy-refractory advanced NSCLC (1, 2). Even in cases sensitive to gefitinib, resistance might be acquired through continuous drug administration. Additional treatments for cases of NSCLC relapsing during treatment with gefitinib are urgently needed.

To investigate the mechanism of acquired resistance to gefitinib, we previously established gefitinib-acquired resistant cells, PC-9/ZD2001, from a NSCLC, PC-9, which is hypersensitive to gefitinib and has a 15-del mutation in exon 19 of EGFR (data not shown). After >6 months of culture in gefitinib-free conditions, the sensitivity of PC-9/ZD2001 cells to gefitinib was restored, and the cells were subsequently established as a revertant cell line, PC-9/ZD2001R. The active mutation of EGFR was sustained in both the resistant and the revertant cell lines and the existence of revertant cell line suggests the additional mutation of EGFR, such as a secondary mutation of T790M in EGFR that causes resistance to gefitinib (3, 4), is unlikely to be contribute to this gefitinib resistance. In the gefitinib-resistant cells, the expression levels of EGFR and mRNA decreased to 30% to 50% of those in parental cells. A ligand-induced EGFR activation minimally activated mitogen-activated protein kinase signaling pathways and the inhibitory effect of gefitinib on this...
pathway was significantly decreased in the resistant cells. To elucidate the cross-resistance to other anticancer agents, we examined the sensitivity to the conventional anticancer agents and tumor necrosis factor α (TNF-α). PC-9/ZD2001 showed cross-resistance to another EGFR inhibitor, AG1478. Interestingly, gefitinib-resistant cells were ~3-fold more sensitive than PC-9 cells to the cytotoxic effects of vinorelbine, paclitaxel, camptothecin, 5-fluorouracil, and a cytokine, TNF-α. The same tendency was confirmed in the other gefitinib-resistant clones established along with PC-9/ZD2001. The restoration of these collateral sensitivities (except 5-fluorouracil) in revertant PC-9/ZD2001R cells suggests that such sensitivities are correlated with the mechanism of gefitinib resistance.

TNF-α is the prototype of ~20 related cytokines that act through specific members of the TNF receptor (TNFR) super family (5–7). Several cancer therapies exploiting the cytotoxic effect of TNF-α on solid tumors and soft-tissue sarcomas have recently been examined in clinical trials (8, 9). The TNF-α stimulates inflammation by turning on gene transcription through signaling cascades such as the Akt/nuclear factor κB (NF-κB) pathway. This signaling subsequently serves as the primary mechanism to protect cells against apoptotic stimuli through several transcriptional genes, such as inhibitor of apoptosis proteins (IAP), the specific inhibitor of caspases (10, 11). In contrast, TNF-α-mediated signaling also triggers apoptosis through the activation of caspase-8 and the downstream caspase-3 or caspase-7 in a wide variety of cells (12). From these observations, it is possible to say that TNF-α has two different signaling pathways that contradict each other. The cytotoxic effect of TNF-α might be determined by ratios between the apoptosis-inducing and the apoptosis-inhibiting effects.

Akt/NF-κB signaling also occurs downstream of EGFR and this signaling mediates cell proliferation and anti-apoptotic signaling through this pathway (13). In the case of the anti-apoptotic signaling of TNF-α, TNFR is known to activate Akt/NFκB in three ways: directly through phosphatidylinositol 3-kinase activation, or indirectly through cross-talk signaling to EGFR, or both together (5–7, 12, 14, 15). Moreover, several recent articles report that the TNFR-mediated cross-talk signaling to EGFR occurs in a ligand-dependent and -independent manner (16–21). Therefore, to investigate the mechanisms of the collateral sensitivity to TNF-α in gefitinib-acquired resistant cells, we focused on TNF-α-induced cross-talk signaling to EGFR and analyzed the Akt/NFκB signaling pathway in response to TNF-α.

In this article, we show that a weakness of Akt/NFκB signaling from TNF-α-mediated cross-talk signaling via EGFR causes the collateral sensitivity to TNF-α in the gefitinib-acquired resistant cell line. Moreover, this cross-talk signaling is thought to be a dominant pathway of TNF-α-mediated Akt activation.

Materials and Methods

Chemicals and antibodies. Gefitinib was donated by AstraZeneca Pharmaceuticals (Wilmington, DE). An anti-phospho-EGFR antibody (Tyr1068) was purchased from Cell Signaling Technology (Beverly, MA). Other antibodies and chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), respectively, unless otherwise specified.

Cell lines and cultures. The PC-9 human NSCLC cell line, established from a previously untreated patient, was kindly donated by Prof. K. Hayata (Tokyo Medical College, Tokyo, Japan). The PC-9 cells were cultured with RPMI 1640 supplemented with 10% FCS and maintained in a 5% CO2 incubator at 37°C under humidified conditions.

Establishment of gefitinib-resistant cell lines. To establish gefitinib-resistant cell lines, PC-9 cells were continuously exposed to increasing dosages of gefitinib for 1 year. The surviving cells were cloned and three gefitinib-resistant cell lines, designated as PC-9/ZD2001, PC-9/ZD2002, and PC-9/ZD2003, were established. These cell lines can survive exposure to 200 nmol/L gefitinib. Sensitivity to gefitinib was restored by culture of PC-9/ZD2001 in gefitinib-free conditions for >6 months. The restored cells were cloned and subsequently established as a revertant cell line, PC-9/ZD2001R.

Established resistant cell lines were maintained by culture in a medium containing 200 nmol/L gefitinib. To eliminate the effects of gefitinib, the resistant cells were cultured in a drug-free medium for at least 2 weeks before all experiments. As the relative resistance values of these cell lines were stable for at least 3 months after culture under drug-free conditions (data not shown), we used the cells for experiments during this period.

Growth inhibition assay. To measure sensitivity to gefitinib, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done (Cell Titer 96 assay kit, Promega Corp., Madison, WI). In brief, PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells were seeded onto 96-well plates and preincubated overnight. The cells were continuously exposed to the indicated concentrations of gefitinib for 4 or 5 days. Absorbance was measured at 570 nm with a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA).

Analysis of tumor necrosis factor α–induced apoptotic cell death. The PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells were treated with 100 ng/mL TNF-α for the indicated time periods. They were then fixed with 4% paraformaldehyde at 4°C for 30 minutes. After 100 μL of 70% ethanol were added, the cells were permeabilized by incubation overnight at −20°C. Apoptotic DNA fragments were probed with the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling method (MEBSTAIN Apoptosis TUNEL Kit Direct, Medical & Biological Laboratories, Nagoya, Japan) and subpopulations of apoptotic cells were measured with a flow cytometer (FACScalibur, BD Biosciences Immunocytometry Systems, San Jose, CA).

Activity assays for CPP32/caspase-3 and FLICE/caspase-8. Activities of CPP32/caspase-3 and FLICE/caspase-8 were measured with caspase-3 and caspase-8 colorimetric assay kits (MRL Diagnostics, Cypress, CA) according to the instructions of the manufacturer. The PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells were incubated for 12 hours with 10 ng/mL TNF-α and then resuspended in 50 μL of chilled cell lysis buffer. The cells were incubated on ice for 10 minutes and the protein concentration of the supernatant was assayed with a bicinchoninic acid protein assay kit (Sigma-Aldrich). A certain amount of each sample was added to 50 μL of 2× reaction buffer containing the respective substrates DEVD-pNA and IETD-pNA, then incubated at 37°C for 1 hour. After incubation, absorbance was measured at 400 and 405 nm with a microtiter plate reader (Model 550, Bio-Rad Laboratories).

Immunoblot analysis. Cells were treated with 10 ng/mL of TNF-α for 30 minutes, then washed twice with ice-cold PBS and lysed in EBC buffer (50 mmol/L Tris-HCl (pH 8.0), 120 mmol/L NaCl, 0.5% NP40, 100 μmol/L NaF, 200 μmol/L Na orthovanadate, and 10 μg/mL of leupeptin, aprotinin, and phenylmethylsulfonyl fluoride) with an ultrasonic disruptor (Tomy Digital Biology Co., Ltd., Tokyo, Japan). The cell lysate was preclarified by centrifugation, resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies against EGFR, phospho-EGFR (Tyr1045), phosphatase and tensin homologue, Akt, phospho-Akt, trk, c-1AP1, and c-1AP2. Bound antibodies were detected with horseradish peroxidase-linked immunoglobulin (Amersham Biosciences, Buckinghamshire, United Kingdom).
and enhanced chemiluminescent reagents (Perkin-Elmer Life and Analytical Sciences, Boston, MA).

**Real-time reverse transcription-PCR method.** Total RNA was isolated with the guanidinium isothiocyanate method using an RNA purification kit (RNEasy Mini Kit, Qiagen, Venlo, the Netherlands) according to the instructions of the manufacturer. After RNA isolation, cDNA was prepared in the presence of random 9-mers with a reverse transcription-PCR (RT-PCR) kit (Takara Shuzo Co., Ltd., Kyoto, Japan). Expression levels of EGFR, c-IAP1, and c-IAP2 mRNA were quantified with a fluorescence-based real-time detection method (GeneAmp 5700 Sequence Detection System, Applied Biosystems, Foster City, CA). Cycling conditions were 40 cycles at 94 °C for 20 seconds, 55 °C (EGFR) and 64 °C (c-IAPs) for 20 seconds, and 72 °C for 30 seconds. Expression of the mRNA was measured with the following primer sets: EGFR, 5'-AGAGTTGCCGCTAGATC-3' and 5'-TCTCTGACCCATCTAGC-3'; c-IAP1, 5'-ATGTGGGTACAGTGATGATGTCA-3' and 5'-AAACCAC-TTGGCAGTGTGAAC-3'; and c-IAP2, 5'-CTAGTGTCACTGTGAAC-3' and 5'-CTTCAGGGCCACTCATCAAC-3'. The expression of β-actin mRNA was used as an internal control.

**Statistical analysis.** Statistical analysis was done with the StatView II software program (Abacus Concepts, Berkeley, CA). Activities of CPP32/caspase-3 and FLICE/caspase-8 were analyzed with paired Student’s t test. P < 0.05 was considered significant.

**Results**

**Establishment of acquired gefitinib-resistant cell lines.** To elucidate the mechanism of acquired resistance against gefitinib, we established gefitinib-resistant NSCLC cell lines through continuous exposure of this drug. Resistance against gefitinib developed quite slowly; the relative resistant values of 3- to 4-fold were reached after >1-year exposure to gefitinib. We picked the clones of gefitinib-resistant cell lines named PC-9/ ZD2001, PC-9/ZD2002, and PC-9/ZD2003. These cell lines can survive in 200 nmol/L gefitinib-contained medium. Sensitivities to gefitinib were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In the case of PC-9/ ZD2001 cells, the cell line was able to survive by >50% at the concentration of >500 nmol/L gefitinib. This concentration caused maximum inhibition in PC-9. The IC_{50} value of gefitinib in PC-9 cells was 53.0 ± 8.1 nmol/L. The gefitinib-resistant cell line PC-9/ZD2001 showed a 4-fold higher resistance to gefitinib than PC-9 cells (IC_{50} = 211.1 ± 32.4 nmol/L; Fig. 1). Culture of the cells in gefitinib-free conditions for 6 months restored sensitivity to gefitinib in PC-9/ZD2001 and subsequently established a revertant cell line, PC-9/ZD2001R, in which sensitivity to gefitinib was completely restored (IC_{50} = 46.3 ± 10.2 nmol/L).

**Analysis of tumor necrosis factor α–induced apoptotic cell death.** TNF-α-induced cytotoxic effect was measured by 3-(4,5- dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide assay. The IC_{50} values of TNF-α in PC-9, PC-9/ZD2001, and PC-9/ ZD2001R cell lines were 815.0 ± 44.8, 12.2 ± 1.4, and 626.2 ± 18.5 ng/mL, respectively. PC-9/ZD2001 R cells acquired new sensitivity to TNF-α. PC-9/ZD2001 was ~67-fold more sensitive to TNF-α as compared with PC-9, but this sensitization was restored to 1.3-fold in PC-9/ZD2001R (Fig. 2A). A collagen sensitivity to TNF-α was confirmed in the other gefitinib-resistant cell lines, PC-9/ZD2002 and PC-9/ZD2003 (data not shown).

Additionally, we measured TNF-α-induced apoptotic cell death by flow cytometry. The apoptotic cells were stained by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method. No significant apoptosis was observed in these three cell lines until 24 hours of exposure to TNF-α (10 ng/mL). Forty-eight hours of TNF-α exposure induced a 6-fold higher apoptotic cell death in PC-9/ZD2001 cells (70.3%) as compared with the parental PC-9 cells (11.8%). This enhancement was completely recovered in PC-9/ ZD2001R cells (16.6%; Fig. 2B; Table 1). These results suggest that the collateral sensitivity to TNF-α might be correlated with the resistance to gefitinib in these cell lines.

**Analysis of tumor necrosis factor α–mediated activations of CPP/caspase-3 and FLICE/caspase-8.** To clarify the difference of TNF-α-induced apoptotic cell death in these cell lines, we analyzed TNF-α-mediated CPP32/caspase-3 and its upstream FLICE/caspase-8 activations by caspase-8 and caspase-3 colorimetric protease assay kits (Medical and Biological Laboratories), respectively. PC-9, PC-9/ZD2001, and its revertant PC-9/ ZD2001R cells were incubated with the indicated concentrations of TNF-α for 12 hours. In the case of caspase-3, TNF-α did not cause any increases in the activity in PC-9 and PC-9/ZD2001R cells even at the highest concentration of 100 ng/mL. In contrast, TNF-α significantly enhanced caspase-3 activity in PC-9/ZD2001 cells even at the concentration of 1 ng/mL within this time course (Fig. 3A). In the case of caspase-8, TNF-α enhanced the activities in all three cell lines from 10 ng/mL (Fig. 3B). TNF-α at 100 ng/mL activated caspase-8 ~1.6-, 2.9-, and 1.9-fold higher in PC-9, PC-9/ZD2001, and PC-9/ZD2001R, as compared with the respective untreated cells. In PC-9/ZD2001 cells, TNF-α caused the highest relative induction of caspase-8 (Fig. 3B).

**Immunoblot analysis for the tumor necrosis factor α–induced cross-talk signaling to epidermal growth factor receptor and Akt/ nuclear factor κB pathway activation.** EGFR expression was significantly lower in PC-9/ZD2001 than in PC-9 cells (Fig. 4A). When measuring the expression of EGFR protein by a densitometer (calculated by the NIH image software), the expression was decreased to 52.4 ± 2.6% of that in parental cell line. Moreover, we measured the expression levels of EGFR mRNA by a real-time RT-PCR method. The expression level in PC-9/ZD2001 was decreased to 37.0 ± 3.2% of that in parental cell line (Fig. 4B).
cells. The same down-regulation of EGFR was seen in the other resistant cell lines (data not shown). In the case of PC-9/ZD2001R, expression levels of EGFR protein and mRNA were also decreased to 69.3 ± 1.1% and 56.8 ± 2.2%, respectively, as compared with PC-9. The expression of EGFR was restored, but not completely, in the revertant cell line.

In PC-9 cells, cross-talk signaling from TNFR to EGFR was observed and treatment with 10 ng/mL TNF-α for 30 minutes induced significant autophosphorylation of EGFR (Fig. 4A). According to the autophosphorylation of EGFR, definite phosphorylation of Akt and a decrease in IκB content were observed. The activation of Akt and down-regulation of IκB were inhibited by gefitinib at concentrations <10 nmol/L. Because gefitinib (100 nmol/L) mostly inhibited this signaling, we concluded that the cross-talk signaling from TNFR to EGFR might be the dominant pathway of TNF-α-mediated Akt/NF-κB activation in this cell line rather than the direct signaling from TNFR to Akt. In contrast, although EGFR autophosphorylation was observed, only partial phosphorylation of Akt and down-regulation of IκB, compared with those in PC-9, were observed after TNF-α exposure in PC-9/ZD2001 cells (Fig. 4A and B). Treatment with gefitinib inhibited this cross-talk signaling to EGFR but had no effect on downstream Akt phosphorylation.

These observations suggest that TNF-α-mediated EGFR signaling has less effect on the Akt/NF-κB pathway in the gefitinib-resistant PC-9/ZD2001 cell line. Other stimuli might activate Akt in an EGFR-independent manner. In the revertant PC-9/ZD2001R cell line, this weak effect of EGFR was largely reversed and TNF-α exposure induced autophosphorylation of EGFR and subsequent activation of the Akt/NF-κB pathway. The expression levels of phosphatase and tensin homologue, a suppressor of Akt signaling, did not differ significantly among PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells. This decreased effect of EGFR might be partially caused by the down-regulation of EGFR expression in PC-9/ZD2001. However, although the EGFR-mediated signaling and the resistance to gefitinib were mostly restored, EGFR expression remained only partially restored in PC-9/ZD2001R. For this reason, we speculated that the down-regulation of EGFR expression might not fully explain the weak EGFR signaling to Akt pathway in PC-9/ZD2001 cells.

To clarify the decreased EGFR signaling in PC-9/ZD2001, we examined the inhibitory effect of a phosphatidylinositol 3-kinase inhibitor, wortmannin, on the TNF-α-induced activation of this pathway (Fig. 4B). Interestingly, wortmannin inhibited the TNF-α-mediated phosphorylation of Akt in PC-9/ZD2001 cells at the same level as it did in PC-9 and PC-9/ZD2001R cells.

Expression of c-IAP1 and c-IAP2 on treatment with tumor necrosis factor α. After treatment with TNF-α (10 ng/mL) for 30 minutes, expression of c-IAP1 and c-IAP2 proteins was

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NOTE: After 72 hours of exposure to TNF-α, significant apoptotic cell death was observed in PC-9/ZD2001R cells not in PC-9 or PC-9/ZD2001R cells.
significantly increased in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells (Fig. 4A and B). According to the results of Akt phosphorylation, induction was inhibited by gefitinib in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells. Wortmannin could inhibit induction in all three cell lines. Consistent with the results of protein expression, treatment with TNF-α increased the expression level of c-IAP1 and c-IAP2 mRNAs in PC-9 and PC-9/ZD2001R cells in a dose-dependent manner (Fig. 5A and B). After treatment with 100 ng/mL TNF-α for 12 hours, the expression levels of both c-IAP1 and c-IAP2 mRNAs were significantly increased in PC-9 cells (c-IAP1, 7.05 ± 0.62; c-IAP2, 18.22 ± 0.25) and PC-9/ZD2001R cells (c-IAP1, 7.02 ± 0.54; c-IAP2, 11.56 ± 0.75) but not in PC-9/ZD2001 cells (c-IAP1, 2.60 ± 0.58; c-IAP2, 2.83 ± 0.66). These observations suggest that TNF-α-induced apoptotic signaling is not inhibited by its own antiapoptotic effects, such as IAPs induction, owing to the weak effect of TNF-α-mediated signaling and the Akt/NF-κB pathway via EGFR in this gefitinib-resistant cell line.

Discussion

We have shown that the gefitinib-acquired resistant NSCLC cell line PC-9/ZD2001 acquired collateral sensitivity to the apoptotic effect of TNF-α. Because this collateral sensitivity was significantly diminished in the revertant PC-9/ZD2001R, it might be correlated with gefitinib resistance. As described before, PC-9/ZD2001 also acquired collateral sensitivities to some anticancer drugs, such as vinorelbine, paclitaxel, camptothecin, and 5-fluorouracil. However, this cell line did not show the collateral sensitivities to cisplatin, etoposide, mitomycin C, and cyclophosphamide. Moreover, there was no difference of susceptibility to serum-starved condition between PC-9 and PC-9/ZD2001 (data not shown). From these observations, it can be concluded that the collateral sensitivities of the gefitinib-resistant cells are specific to some cell stresses and are not caused by the fragility of the cells. Because the same tendency of sensitivity was seen in the other resistant clones, PC-9/ZD2002 and PC-9/ZD2003, the acquired sensitivity to the anticancer drugs and TNF-α could be a general phenomenon even in the clinical gefitinib-resistant cells.

TNF-α activates not only apoptotic signaling but also antiapoptotic signaling via the Akt/NF-κB activation (22, 23). Activation of the downstream transcription factor NF-κB inhibits various types of apoptotic cell death by inducing apoptotic inhibitory proteins (22, 23), such as bcl-2 (24), bcl-xl (25), forkhead (26), and IAPs (10, 11, 27, 28). As described before, it is thought that the cytotoxic effect of TNF-α is determined by ratios between the apoptosis-inducing and the apoptosis-inhibiting effects (5–7, 12, 14, 15).

In parental PC-9 cells, TNF-α induced EGFR autophosphorylation and subsequent Akt/NF-κB pathway activation (Fig. 4A and B). This autophosphorylation was completely inhibited by a low concentration of gefitinib (10 nmol/L). From these observations, we think that TNF-α-induced Akt/NF-κB pathway activation occurs mainly through cross-talk from TNFR to EGFR in this cell line. Because the expression level of EGFR was partially diminished the TNF-α-induced activation of the Akt/NF-κB pathway. Our results are supported by those of an earlier study showing that resistance to the cytotoxic effect of TNF-α is associated with high expression of Her family receptors, such as EGFR (Her1), erbB2/Her2/neu, or Her3, in a panel of human tumor cell lines (29). However, the decreased EGFR signaling from the Akt/NF-κB pathway could not be fully explained by the lower EGFR expression in PC-9/ZD2001 because EGFR expression remained only partially restored in the revertant PC-9/ZD2001R cell line. In light of these observations, to clarify the mechanisms of collateral sensitivity to TNF-α in the gefitinib-resistant cells, we focused on the cross-talk signaling from TNFR to EGFR in PC-9, PC-9/ZD2001, and PC-9/ZD2001R cells.

Several recent articles have reported that TNFR mediates cross-talk signaling to EGFR through a ligand-dependent and -independent manner (16–19, 21, 23). Chan et al. (17) have reported that exposure of human mammary epithelial cells to TNF-α results in transactivation of EGFR through metallocroprotease-dependent shedding of EGFR ligand(s). Hirota et al. (18) reported that EGFR transactivation by TNF-α is...
regulated by means of redox-dependent mechanisms. The transactivation of EGFR was observed to occur quickly, after <30 minutes of exposure to TNF-α in PC-9 cells (Fig. 4A and B). No additional induction of ligands, EGF and transforming growth factor-α, were detected by ELISA in the culturing medium of the cells even after 6 hours of 100 ng/mL TNF-α exposure (data not shown). From these observations, we think that this activation could occur independently of ligands but not through TNF-α-mediated ligands synthesis or proteolytic releasing of preexisting ligands from the disrupted cells. Although TNF-α induced the same levels of EGFR autophosphorylation in all three cell lines, this EGFR activation is minimally transmitted to the downstream Akt/NF-κB pathway in the resistant PC-9/ZD2001 cells (Fig. 4A). Moreover, an inhibitory effect of gefitinib on TNF-α-induced Akt/NF-κB activation was not observed although wortmannin, a phosphatidylinositol 3-kinase inhibitor, completely inhibited this signaling in PC-9/ZD2001 cells (Fig. 4B). These results suggest that the weak effect of EGFR on Akt/NF-κB signaling could occur between EGFR and phosphatidylinositol 3-kinase in PC-9/ZD2001 cells.

Several articles reported that the sensitivity to gefitinib is regulated by active mutant EGFR (30, 31), by the expression level of phosphatase and tensin homologue/MMAC/TEP (32), and by levels of Akt phosphorylation (13, 33, 34). Because the gefitinib-hypersensitive PC-9 cells originally had 15-bp deletion mutation in exon 19 of EGFR, they were thought to have a gefitinib-sensitive active mutant EGFR (35); however, because we found no alteration of the EGFR mRNA sequence in PC-9/ZD2001 cells (data not shown), we conclude that this gefitinib-resistant cell line was a good model for acquired gefitinib resistance. In our previous study, EGFR signaling mediated by transforming growth factor-α, an EGFR ligand, could not activate the mitogen-activated protein signaling pathway but could partially activate the Akt signaling cascade in PC-9/ZD2001. In PC-9/ZD2001R cells, the association between EGFR and mitogen-activated protein kinase signaling was completely reconstituted. On the basis of this result, we conclude that the decrease of EGFR signaling to the mitogen-activated protein kinase signaling pathway might contribute to acquired gefitinib resistance.5 In this study, TNF-α significantly induced EGFR autophosphorylation but subsequent activation of the Akt signaling cascade was little observed in PC-9/ZD2001 (Fig. 4A and B). This decreased EGFR signaling on Akt could be partially caused by the decrease in EGFR expression but we have
no data to explain the discrepancy between transforming growth factor-α-mediated and TNF-α-mediated EGFR signaling in this cell line. Nevertheless, TNF-α-mediated cross-talk signaling to EGFR, although ligand independent, seems to cause downstream activation in a different way from that caused through ligand-mediated direct EGFR activation. Akt/NF-κB signaling is also known to be downstream of other receptors, such as other Her family receptors (36), platelet-derived growth factor receptor (37), and IFN receptor (38). We previously confirmed the expression of other Her family receptors, Her2 and Her3, in PC-9 cells. Possibly, signaling of these receptors may be able to modulate the TNF-α-mediated cross-talk signaling and Akt/NF-κB signaling. Various aspects of TNF-α-induced cross-talk signaling to EGFR, such as EGFR heterodimer formation with other Her family receptors and downstream signaling specificity, require further investigation.

Human IAPs, c-IAP1 and c-IAP2, have been reported to block the apoptotic events caused by caspase-8 activation by directly combining with caspase-3 and caspase-7 and restraining them (10, 27). IAPs play a key role in the resistance to apoptotic effect of TNF-α superfamily of proteins (39) and various anticancer drugs (40, 41); for this reason, IAPs are considered promising targets in anticancer therapy (42, 43). To evaluate TNF-α-mediated ant apoptotic signaling, we measured IAP induction in these cell lines by means of Western blotting analysis and real-time RT-PCR. As might be expected, IAPs and their mRNAs were markedly induced by TNF-α in PC-9 and PC-9/ZD2001R cells but not in PC-9/ZD2001 cells (Fig. 5A and B). TNF-α-induced activation of caspase-3, but rarely of caspase-8, was significantly lower in PC-9 and PC-9/ZD2001R as compared with PC-9/ZD2001 (Fig. 3A and B). These results suggest that TNF-α precisely activates apoptotic signaling through caspase-8 in all three cell lines and that induction of IAPs blocks downstream signaling by inhibiting caspase-3 in PC-9 and PC-9/ZD2001R. In this cell line, the induction of IAPs likely plays a key role in determining the sensitivity to TNF-α-mediated apoptosis among the ant apoptotic proteins that are induced by NF-κB-mediated transcription.

Several clinical studies of TNF-α as an anticancer treatment have been done, mainly in the 1970s; however, treatment with TNF-α was greatly limited by its side effects, particularly its toxicity to previously healthy organs (44–49). Recently, several new anticancer therapies using TNF-α have been developed, such as RGD-V29 (F4614) and TNF-erade (Biologic), in an attempt to reduce adverse effects (8, 9, 50, 51). We have shown that a NSCLC cell line with acquired resistance to gefitinib acquired collateral sensitivity to TNF-α. These data strongly suggest that treatment with TNF-α might be effective against tumors that have acquired resistance to gefitinib after long-term administration of this drug. Further analysis is required before clinical application.

In summary, the cross-talk signaling from TNFR to EGFR and subsequent IAP induction play important roles in the resistance to TNF-α-induced apoptosis in PC-9 cells. Because this signaling cascade is decreased in the gefitinib-resistant PC-9/ZD2001 cells, TNF-α did not activate the Akt/NF-κB cascade. This decrease of EGFR signaling to Akt/NF-κB pathway, which is related to gefitinib-acquired resistance, may contribute to the acquisition of hypersensitivity to TNF-α in this cell line.

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