Gefitinib Cytotoxicity in Non-small Cell Lung Cancer Cells is Enhanced by Low Dose Cisplatin Due to Ligand-independent EGFR Autophosphorylation

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Abstract: Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase overexpressed in non-small cell lung cancer (NSCLC) and many other solid tumors. EGFR is activated by specific ligands and various cell stresses, such as oxidative stress and UV irradiation. The present study investigates the effect of ligand-independent EGFR activation on gefitinib mediated cytotoxicity using the NSCLC cell line, PC-9. The induction of EGFR autophosphorylation by non-cytotoxic levels of hydrogen peroxide (H2O2) and cisplatin (CDDP) is completely inhibited by 100 nM gefitinib. Pretreatment of cells with both H2O2 and CDDP enhances gefitinib cytotoxicity in vitro. The growth inhibitory effect of gefitinib was examined in vivo using the xenografted severe combined immunodeficiency (SCID) mouse model. PC-9 cells were pretreated with/without a low dose of CDDP (1 μM) for 1h and injected subcutaneously into the right flank of SCID mice. Following the appearance of measurable tumors mice were treated by subcutaneous injection into the left flank with/without 10 mg/kg gefitinib for 4 days. Pretreatment with CDDP enhanced tumor growth by 20–30% compared to the control. Subsequent treatment with gefitinib resulted in disappearance of the tumor mass by day 10 in the CDDP-pretreated group and by day 16 in the control group. There was no reappearance of tumors in the CDDP-pretreated group. By comparison, tumors reappeared in the non-pretreated group by day 20 in 4/5 animals. These results suggest that chemotherapy may enhance tumor growth due to ligand-independent EGFR activation and that combination chemotherapy may result in enhanced sensitivity of tumors to the sequential administration of gefitinib.

Key words: EGFR, cisplatin, gefitinib, ligand independent activation

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Introduction

The epidermal growth factor receptor (EGFR/HER1/erbB1), a 170-kDa cell membrane glycoprotein with tyrosine kinase activity, plays a central role in cell proliferation, survival, migration, differentiation, and angiogenesis. Increased EGFR expression has been reported in a wide variety of human tumors and EGFR-mediated signaling is thought to play an important role in the progression of epithelial neoplasms. EGFR is dimerized and autophosphorylated by specific ligands, such as epidermal growth factor (EGF), transforming growth factor α (TGF-α), and HB-EGF. Several non-physiologic agents, such as radiation, oxidants and alkylating agents, induce ligand-independent activation of EGFR and numerous membrane receptor kinases. In vitro drug sensitivity tests frequently reveal that low doses of anti-cancer agents enhance cancer cell growth compared to non-treated controls (Fig. 1, gray area). This phenomenon is thought to relate to the ligand-independent activation of membrane receptor kinases.

Many small anti-cancer molecules targeting EGFR have been recently developed. Gefitinib (Iressa, [4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy)-quinazoline]) is an orally active EGFR-tyrosine kinase inhibitor (EGFR-TKI) that blocks signal transduction pathways implicated in cancer cell proliferation, survival, and other host-dependent processes that promote cancer growth. Anti-tumor efficacy has been demonstrated for gefitinib in patients with relapsed or recurrent non-small cell lung cancer (NSCLC) and it is now approved for the treatment of advanced NSCLC. A recent large phase III placebo-controlled clinical study failed to demonstrate a statistically significant increase in survival using gefitinib to treat patients with advanced NSCLC refractory to prior chemotherapy (IRESSA Survival Evaluation in Lung cancer (ISEL) trial). Despite these discouraging results, a remarkably rapid and often profound response to gefitinib was observed in a subgroup of patients.

![Figure 1](image-url)  
Fig. 1. Low dose CDDP exposure enhanced PC-9 cell growth. PC-9 cells were exposed to indicated concentrations of CDDP for 4 or 5 days. Cell viability was measured by the MTT assay. Low dose CDDP (less than 1 μM, gray area) exposure enhanced cell growth compared to the non-treated control.
patients with NSCLC\textsuperscript{11}). Previous clinical studies have demonstrated that a somatic mutation in EGFR identified in lung adenocarcinoma is associated with gefitinib sensitivity\textsuperscript{12}. Activation of this mutated EGFR is thought to occur in a ligand-independent manner\textsuperscript{13}. EGFR gene copy number is also associated with increased sensitivity to gefitinib\textsuperscript{14}. Based on these observations, Ono et al reported that EGFR activity and cellular dependency on EGFR determine sensitivity to gefitinib\textsuperscript{15}.

Since gefitinib cytotoxicity is thought to be enhanced by EGFR activation, we hypothesized that the ligand-independent EGFR activation by an alkylating agent would modulate gefitinib sensitivity. In this paper, we examined the effect of a non-cytotoxic dose of cisplatin (CDDP) on EGFR autophosphorylation and cell viability in the NSCLC cell line, PC-9. Moreover, we clarified the relationship between ligand-independent EGFR activation and gefitinib cytotoxicity \textit{in vitro} and \textit{in vivo}.

\section*{Materials and Methods}

\subsection*{Chemicals and antibodies}

Gefitinib (Iressa) was donated by AstraZeneca (Wilmington, DE). CDDP was donated by Bristol-Myers Squibb. K. K. Anti-EGFR antibody (1005) was purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-phosphotyrosine antibody (PY 20) was purchased from Transduction Lab. (Lexington, KY). Other antibodies were purchased from Santa Cruz Biotech. Chemicals were purchased from Sigma, unless otherwise mentioned.

\subsection*{Cell lines}

The PC-9 human NSCLC cell line, established from adenocarcinoma tissue from untreated patients, was kindly donated by Prof. K. Hayata (Tokyo Medical College, Tokyo, Japan). This cell line was cultured in RPMI 1640 media supplemented with 10\% fetal calf serum (FCS) and maintained in a 5\% CO\textsubscript{2} incubator at 37\textdegree C under humidified conditions.

\subsection*{In vitro growth-inhibition assay}

The cytotoxicity of anti-cancer drugs was assessed by the tetrazolium dye MTT assay using the Cell Titer 96 kit (Promega) according to manufacturer’s instructions. Cells were plated on 96-well micro culture dishes at $2 \times 10^3$ cells/well and were treated with drugs for 4 continuous days. Absorbance was measured at 570 nm using a Microplate reader Model 550 (BioRad).

\subsection*{Immunoblot analysis}

Cells were washed twice with ice-cold PBS then lysed in EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5\% Nonidet P-40, 100 \textmu M NaF, 200 \textmu M Na orthovanadate, and 10 \textmu g/ml of leupeptin, aprotinin and PMSF) using an ultrasonic disruptor (Tomy, Japan). The cell lysate was isolated after centrifugation. For the immunoprecipitation analysis, the supernatant was precleared with 20 \textmu l of Protein A-Sepharose 4B Fast Flow (Sigma) then incubated with anti-EGFR monoclonal antibody (1005) (Santa Cruz) for 1.5 h at 4\textdegree C. Immune complexes were precipitated by adding 25 \textmu l of Protein A-Sepharose 4B Fast Flow and incubating for 1.5 h and were then washed 4 times with EBC buffer.

The samples were resolved by 10\% SDS-PAGE, transferred to nitrocellulose membrane, and probed with respective antibodies. Bound antibodies were detected with horseradish peroxidase-linked Ig (Amersham) and ECL reagents (NEN Life Science).
In vivo growth-inhibition assay

Based on permission from the animal experiments committee of Showa University, the xenograft model experiments were performed in accordance with the Showa University guidelines for the welfare of animals. Six week old C.B-17 / IcrCrj-SCID mice were purchased from Oriental Kobo K. K. (Tokyo, Japan). Cultivated PC-9 cells were pretreated with/without a non-toxic dose (1 μM) of CDDP for 1 h at 37°C. After pretreatment, the cells were washed 3 times with PBS and the concentration of cells was adjusted to $1 \times 10^7$ cells/ml using saline. On day 0, $5 \times 10^6$ PC-9 cells were transferred by subcutaneous (s.c.) injection into the right flank of SCID mice. Five mice per group were injected with tumor cells. By day 4, all mice had developed measurable tumors. From day 4 to day 7, gefitinib was administered at 10 mg/kg/day by s.c. injection into the left flank (Fig. 6). The tumor volume was calculated using the following formula:

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\text{Tumor volume (mm}^3\text{)} = (\text{long axis}) \times (\text{short axis})^2 / 2
\]

All animals were sacrificed on day 60 following measurement of tumor volume.

Statistical analysis

Statistical analysis of tumor growth-inhibition was performed using Stat View II (Abacus Concepts, Berkeley, CA). Data was expressed as the mean±SE. Statistical differences were calculated by ANOVA using Fisher’s protected least significant difference test. A value of $p < 0.05$ was considered statistically significant.
Ligand-independent EGFR Activation Enhances Cytotoxic Effect of Gefitinib

Results

CDDP and H2O2 mediate ligand-independent EGFR autophosphorylation

The NSCLC cell line PC-9 was used to investigate the effect of ligand-independent EGFR autophosphorylation on gefitinib cytotoxicity. We previously identified a 15-bp in-frame deletion in exon 19 (2411 to 2425) of EGFR in PC-9 cells, a mutation that leads to a 1000-fold increase in gefitinib sensitivity compared to the NSCLC cell line, PC-14, which expresses wild type EGFR. The effect of cell stress on EGFR autophosphorylation was investigated by examining the sensitivity of PC-9 cells to hydrogen peroxide (H2O2) and CDDP using the MTT assay. Dose response analyses were performed to determine non-cytotoxic concentrations of H2O2 and CDDP for treatment of PC-9 cells to eliminate the adverse effect of these drugs on cell viability. The IC50 of CDDP was 38.4±0.4 μM (Fig. 1) and for H2O2 no cytotoxic effect was observed at levels less 1 μM (data not shown). PC-9 cells were exposed to 1 μM of H2O2 or 1 μM of CDDP for the indicated time periods. EGFR was precipitated from the cell lysate using EGFR specific antibodies and autophosphorylation of EGFR was detected by Western blotting using anti-phosphotyrosine antibody. H2O2 and CDDP induced significant autophosphorylation of EGFR (Fig. 2). H2O2 induced EGFR autophosphorylation after an exposure period of less than 15 min, which then decreased after exposure for 1 h (Fig. 2, 4). By comparison, EGFR autophosphorylation was induced gradually by CDDP and was maintained past 48 h of drug exposure. To confirm whether EGFR autophosphorylation was induced in a ligand-independent manner, we pretreated PC-9 cells with either actinomycin D (to inhibit mRNA synthesis) or cyclohexamide (to inhibit protein synthesis) and then assayed for EGFR autophosphorylation. Pretreatment with 5 μg/ml actinomycin D or 5 μg/ml of cyclohexamide did not inhibit EGFR autophosphorylation induced by H2O2 (data not shown) or CDDP (Fig. 3). This result suggests that EGFR autophosphorylation does not depend on ligand synthesis but is induced by autocrine or paracrine ligand activation.

Effect of gefitinib on ligand-independent EGFR autophosphorylation

Ligand-induced EGFR activation enhances the sensitivity of NSCLC cells to the EGFR specific inhibitor, gefitinib. It is possible that ligand-independent EGFR activation also
Fig. 3. Ligand synthesis did not contribute to CDDP-induced EGFR autophosphorylation. PC-9 cells were pretreated with 5 μg/ml of actinomycin D or 5 μg/ml of cyclohexamide for 30 min. Treated cells were then exposed to 1 μM CDDP for the indicated time periods. EGFR was immunoprecipitated (IP) with EGFR specific antibodies and autophosphorylation was determined by immunoblotting (IB) using anti-phosphotyrosine antibodies (PY). For EGFR detection, the membrane was stripped and rebotted using anti-EGFR antibodies.

Fig. 4. Effect of gefitinib on ligand-independent EGFR autophosphorylation. PC-9 cells were pretreated with 1 μM of H2O2 or 1 μM CDDP for 15 min. Cells were then treated with 100 nM gefitinib and incubated for the indicated time periods. Significant EGFR autophosphorylation was observed following pretreatment with both H2O2 and CDDP compared to the non-treated control (0 h).

sensitize cells to gefitinib. For this reason, we examined the effect of gefitinib on ligand-independent EGFR autophosphorylation. Cells were pretreated with 1 μM H2O2 or 1 μM CDDP for 15 min, prior to treatment with 100 nM gefitinib. As shown in Fig. 4, gefitinib completely inhibited ligand-independent EGFR autophosphorylation induced by H2O2 and CDDP within 15 min and continued to inhibit EGFR autophosphorylation over the 48 h time course of exposure.
Ligand-independent EGFR Activation Enhances Cytotoxic Effect of Gefitinib

Cytotoxic effect of gefitinib on EGFR preactivated PC-9 cells in vitro

The effect of ligand-independent EGFR activation on the sensitivity of PC-9 cells to gefitinib was investigated by pretreating cells with non-cytotoxic levels of H2O2 and CDDP then performing a dose response analysis with gefitinib (Fig. 5). PC-9 cells were pretreated with 1 μM H2O2 or 1 μM CDDP for 1 h and washed for 3 times with prewarmed PBS. Cells were then seeded into 96-well plates, exposed to gefitinib continuously for 4 or 5 days, then assayed for viability using the MTT assay. Pretreatment with both H2O2 and CDDP enhanced the sensitivity of PC-9 to gefitinib (Fig. 5). The IC50 for gefitinib with and without pretreatment with H2O2 was 29.1 ± 3.5 nM and 12.0 ± 0.9 nM, respectively (2.4-fold), and with and without pretreatment with CDDP was 24.9 ± 2.6 nM and 9.8 ± 0.4 nM, respectively (2.5-fold). These results show that pretreatment with H2O2 and CDDP enhanced the sensitivity of PC-9 cells to gefitinib by 2.4-fold and 2.5-fold, respectively. This result suggests that ligand-independent EGFR activation sensitizes cells to gefitinib.

Cytotoxic effect of gefitinib on EGFR preactivated PC-9 cells in vivo

To predict the clinical advantage of the sensitization effect to gefitinib by ligand-independent EGFR autophosphorylation, we performed a sensitivity test using the SCID mice xenograft model. PC-9 cells were pretreated with or without a non-toxic dose of CDDP (1 μM) for 1 h and washed 3 times with prewarmed PBS. The cells were then injected into the subcutaneous region of the right flank of SCID mice. Following the appearance of measurable tumors (day 4), the mice were administered 10 mg/kg of gefitinib from day 1 to day 4 by s.c. injection to the left flank. As shown in Fig. 5, the tumor growth rate was significantly higher in the CDDP-pretreated cell group than in the non-pretreated cell control group (p < 0.05). By day 21 the tumor volume increased by 362% and 269% in the CDDP-pretreated cell group and non-pretreated cell group, respectively. In the non-pretreated cell group, the xenografted tumor completely disappeared by day 14 following treatment with 100 μM gefitinib, and 4 of 5 mice had recurrence of the tumors after day 18.
Fig. 7. Effect of ligand-independent EGFR autophosphorylation on gefitinib sensitivity in vivo

PC-9 cells were pretreated with/without 1 μM of CDDP for 1 h. Cells were then washed 3 times with prewarmed PBS then injected into the right flank of SCID mice via s.c. lesions. Administration of gefitinib (10 mg/kg) by s.c. injection into the left flank commenced following the appearance of measurable tumors in all animals by day 4. Tumor size was measured 2 times/week and tumor volume was calculated using the formula described in Materials and Methods.

* significant difference (p < 0.05) compared to the non-treated control

By comparison, there was significant tumor reduction in the CDDP-pretreated cell group following treatment with gefitinib. In the CDDP-pretreated cell group the xenografted tumor completely disappeared by day 8, and there was no recurrence of the tumors during the 4 weeks of follow-up observation. These results strongly suggest that ligand-independent EGFR autophosphorylation enhances sensitivity to gefitinib in vivo.

Discussion

In this paper, non-cytotoxic doses of H2O2 and CDDP were used to elucidate the effect of ligand-independent EGFR activation on gefitinib cytotoxicity. Both H2O2 and CDDP induced EGFR autophosphorylation in a ligand-independent manner (Figs. 2, 4) and CDDP enhanced the rate of cell growth in vitro and in vivo (Figs. 1, 7). The effect of CDDP on EGFR autophosphorylation was maintained for more than 48 h (Fig. 4). This is a significantly greater activation period than that observed for EGFR specific ligands, which is usually less than 1 h. The activation period of autophosphorylation is significantly longer in mutant EGFR, which is hypersensitive to gefitinib, compared to wild type EGFR [12]. In the present study, the activation period of EGFR autophosphorylation induced by CDDP resembled that observed for mutant EGFR activation and enhanced sensitivity to gefitinib (Figs. 5, 7). The prolonged activation of EGFR is thought to be related to gefitinib sensitivity.
Tumor growth rate is occasionally enhanced following chemotherapy, which is thought to result from a decrease in the immune response caused by induction of myelosuppression by anti-cancer agents. In the present report, we demonstrate that non-toxic levels of CDDP enhance the growth rate of cancer cells via ligand-independent EGFR activation in vitro and in vivo (Figs. 1, 7). These results suggest that incomplete cancer chemotherapy may enhance tumor growth due to ligand-independent EGFR activation. Ligand-independent EGFR activation is also induced by irradiation\(^8\), alkylating agents\(^9\), and CPT-11\(^20\). Taken together, the phenomenon of ligand-independent EGFR activation may influence tumor behavior following chemotherapy and radiotherapy and as such impact on the prognosis of cancer patients.

Evidence suggests that drugs targeting EGFR are active against NSCLC. Several clinical trials assessing the combination of gefitinib with cytotoxic agents have been evaluated in patients with NSCLC\(^21-23\). There is no evidence that gefitinib in combination with cytotoxic agents improves the efficacy over cytotoxic agents alone. In the clinical trial evaluating gefitinib in combination with gemcitabine and CDDP in patients with advanced NSCLC, there was no significant difference in efficacy endpoints (including overall survival, time to progression, and response rates) with or without gefitinib. While an explanation for these negative results remains an issue of debate Normanno suggested that EGFR activation by EGF enhances the sensitivity to CDDP in patients with NSCLC\(^24\).

Our results indicate that the ideal combination therapy may be the sequential administration of gefitinib following a platinum containing regimen, for example, or alternative administration of both treatments. The rational is that the NSCLC cells remaining after standard chemotherapy would be effectively killed by gefitinib in a combination therapy regimen.

References


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