Interaction of the epidermal growth factor receptor and the DNA-dependent protein kinase pathway following gefitinib treatment

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Abstract

The epidermal growth factor receptor (EGFR) is an important target for cancer therapy. We previously showed that the EGFR inhibitor gefitinib modulated repair of DNA damage following exposure to cisplatin and etoposide involving the DNA-dependent protein kinase (DNA-PK) pathway. In this study, we specifically investigated the effect of EGFR inhibition by gefitinib on functional activity of DNA-PK in cancer cell lines and the interaction between EGFR and DNA-PK. The effects of DNA-PK inhibition by wortmannin and small interfering RNA to the catalytic subunit of DNA-PK (DNA-PKcs) on cell proliferation and DNA interstrand cross-link repair were investigated in the human MCF-7 breast cancer cell line and compared with the effects of gefitinib. DNA-PK activity was quantitated and expression measured by immunoblotting following gefitinib treatment. Immunoprecipitation experiments were done with and without gefitinib in MCF-7 cells, the AR42J pancreas cell line with high EGFR, and the human MDA-453 breast cancer cell line expressing low EGFR. Nuclear and cytoplasmic extracts were immunoblotted with antibody to DNA-PKcs to determine if gefitinib treatment altered cellular expression. Reduction of DNA-PK activity by wortmannin and expression by small interfering RNA to DNA-PKcs sensitized cells to cisplatin and inhibited repair of cisplatin-induced interstrand cross-links. Gefitinib treatment reduced DNA-PK activity in MCF-7 and AR42J but not MDA-453 cells. Immunoprecipitation experiments showed interaction between EGFR and DNA-PKcs in a dose-dependent and time-dependent manner following gefitinib treatment in MCF-7 and AR42J but not MDA-453 cells. Gefitinib treatment reduced nuclear expression and increased cytosolic expression of DNA-PKcs in MCF-7 and AR42J but not MDA-453 cells. Treatment with gefitinib modulates association of EGFR and DNA-PKcs. This is correlated with decreased function of DNA-PKcs. Inhibition of DNA-PKcs may be an important factor in sensitization to chemotherapy and radiation following treatment with inhibitors of the EGFR pathway. [Mol Cancer Ther 2006;5(2):209–18]

Introduction

The epidermal growth factor receptor (EGFR) is involved in many cellular processes, including growth proliferation, angiogenesis, and metastasis. EGFR is expressed at high levels in a variety of tumors including breast, colon, and lung cancers (1). Inhibition of EGFR in vitro by antisense RNA has been shown to inhibit proliferation and induce apoptosis (2). Several classes of EGFR-inhibitory agents have undergone preclinical and clinical assessment, notably small molecules, which inhibit (block) receptor tyrosine kinase activity and antibodies blocking interaction of receptor and ligand (3, 4).

There has been increasing clinical interest in the use of compounds targeting EGFR in the treatment of solid tumors (5). Gefitinib (Iressa, ZD1839) is an orally active quinazoline derivative that selectively inhibits EGFR tyrosine kinase activity by competitively inhibiting the ATP-binding domain and is active against several cancers, including non–small cell lung cancer (6, 7). The factors that predict clinical response following treatment with EGFR inhibitors remain unclear; EGFR expression per se does not correlate with efficacy of the EGFR antibody cetuximab (8). There is recent evidence that mutations in the catalytic kinase domain of EGFR result in a receptor that is more sensitive to gefitinib than the wild-type receptor (9, 10). In transfection experiments, these mutant EGFRs could activate signaling pathways (signal transducers and activators of transcription and AKT), which are antiapoptotic, but had no effect on extracellular signal-regulated kinase signaling pathways that induce proliferation (11).

Experiments with a variety of cell lines and xenografts indicate superadditive or synergistic effects of EGFR
inhibition and chemotherapy (2, 12, 13). Although effects of EGFR inhibition on the apoptotic threshold have been shown, there is less understanding of the mechanisms by which the anticancer activity of specific chemotherapeutic agents could be increased by concurrent EGFR inhibition (14). The results of clinical trials involving combinations of EGFR inhibitors and chemotherapy have been mixed. Studies combining gefitinib with a variety of agents in non–small cell lung cancer were negative (15, 16). However, a pivotal study in patients with advanced colon cancer refractory to irinotecan showed that addition of cetuximab could resensitize a proportion of cancers to irinotecan (17). Although gefitinib and erlotinib monotherapy have shown significant anticancer efficacy in vivo, the overall response rates are below 20%. It is therefore important to determine schedules and combinations of EGFR inhibitors with chemotherapeutic agents to improve therapeutic benefit.

We previously showed a synergistic effect on inhibition of proliferation when gefitinib was combined with cisplatin in the MCF-7 human breast cancer cell line (18). The repair of DNA interstrand cross-links, which contribute to cisplatin cytotoxicity, was delayed when gefitinib and cisplatin were combined. Immunoprecipitation experiments showed an association between EGFR and DNA-dependent protein kinase (DNA-PK), which was increased following gefitinib treatment.

In view of the potential role of the DNA-PK pathway in modulating the effects of gefitinib and cisplatin treatment, the aim of this study is to investigate the role of the DNA-PK pathway in EGFR inhibitor–mediated sensitization to cisplatin in more detail. Using wortmannin and small interfering RNA directed against DNA-PK, the effects of EGFR inhibition by gefitinib were mimicked in increasing sensitivity to cisplatin and inhibiting repair of interstrand cross-links. Additionally, these experiments show increased association of DNA-PK and EGFR in the cytosol following gefitinib treatment.

Understanding the relationship between EGFR inhibition and DNA repair is critical in the design of regimens integrating signal transduction inhibitors and chemotherapeutic agents for treatment of cancers.

Materials and Methods

Materials

Clinical-grade gefitinib was kindly provided by AstraZeneca (Macclesfield, United Kingdom). Wortmannin, cisplatin, and melphalan were purchased from Sigma-Aldrich (Dorset, United Kingdom).

Cell Lines and Culture Conditions

Human breast cancer cell line, MCF-7 cells (obtained from Cancer Research UK London Research Institute), were grown in Earle’s MEM (Autogen Bioclear, Wiltshire, United Kingdom). Rat pancreatic cell line, AR42J cells (European Collection of Animal Cell Cultures), was grown in RPMI (Autogen Bioclear). Human breast cancer cell line, MDA-453 cells (obtained from CR-UK London Research Laboratories), were grown in DMEM (Autogen Bioclear). All cells were supplemented with 10% FCS and 1% glutamine and incubated at 37°C in 5% CO₂.

Growth Inhibition Assay

Cells (5 × 10⁵) were seeded into 96-well microtiters plates and left for 48 hours. For combination studies, drugs were either added concomitantly for 5 days or sequentially by adding each drug in turn for 24 hours followed by 48 hours in drug-free growth medium.

Control wells were treated in the same way with aspiration at each 24-hour period. Two concentrations of gefitinib were used, which inhibited proliferation by 10% and 20% as a single agent, and the chemotherapeutic agents were assessed at a range of concentrations. Cytotoxicity was assessed using the sulforhodamine B assay (19). Briefly, at the end of the incubation period, plates were stained with sulforhodamine B, and the mean absorbance at 540 nm for each drug concentration was expressed as a percentage of the control (untreated) well absorbance.

Alkaline Single-Cell Gel Electrophoresis (Comet) Assay

MCF-7 cells were treated with appropriate agents and assessed using the single-cell gel electrophoresis (comet) assay as described previously (20). All procedures were carried out on ice and in subdued lighting. All chemicals used were obtained from Sigma Chemical (Poole, United Kingdom) unless otherwise stated. Briefly, cells were embedded in 1% agarose on a precoated microscope slide and lysed for 1 hour in lysis buffer [100 mmol/L disodium EDTA, 2.5 mol/L NaCl, 10 mmol/L Tris-HCl (pH 10.5)] containing 1% Triton X-100 (added immediately before analysis) and washed every 15 minutes in distilled water for 1 hour. Slides were then incubated in alkali buffer [50 mmol/L NaOH, 1 mmol/L disodium EDTA (pH 12.5)] for 45 minutes followed by electrophoresis in the same buffer for 25 minutes at 18 V (0.6 V/cm), 250 mA. The slides were finally rinsed in neutralizing buffer [0.5 mol/L Tris-HCl (pH 7.5)] saline. After drying, the slides were stained with propidium iodide (2.5 μg/mL) for 30 minutes then rinsed in distilled water. Images were visualized with the use of a NIKON inverted microscope with high-pressure mercury light source (NIKON UK Ltd., Kingston upon Thames, United Kingdom), 510- to 560-nm excitation filter, and 590-nm barrier filter at ×20 magnification. Images were captured by using an on-line charge-couple device camera and analyzed with Komet Analysis software (Kinetic Imaging, Liverpool, United Kingdom). For each duplicate slide, 25 cells were analyzed. DNA damage was measured by the increase in the tail moment, a function of the amount of DNA in the tail and the length of the tail (20). Detection and measurement of DNA interstrand cross-links was achieved using a modification of the comet assay (21). Immediately before analysis, cells were irradiated (12 Gy) to deliver a fixed number of random DNA strand breaks. The tail moment for each image was calculated by using the Komet Analysis software (Kinetic Imaging) as the product of the percentage DNA in the comet tail and the distance between the means of the head and tail distributions, based
on the definition of Olive et al. (22). Cross-linking was expressed as the percentage decrease in tail moment compared with irradiated controls, calculated by the following formula: percentage of decrease in tail moment = [1 – (TMdi – TMcu/TMci – TMcu)] × 100, where TMdi = tail moment of drug-treated irradiated sample, TMcu = tail moment of untreated unirradiated control, and TMci = tail moment of untreated irradiated control.

**Immunoblotting**

Cells were washed twice in PBS and scraped into lysis buffer [radioimmunoprecipitation assay buffer: 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 250 mMol/L NaCl, 50 mMol/L Tris (pH 7.5), 100 µg/mL 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 17 µg/mL aprotnin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 5 µmol/L fenvalerate, 5 µmol/L potassium bisperoxo (1,10-phenanthroline) oxovanadate, and 1 µmol/L okadaic acid]. Lysates were refined by centrifugation at 14,000 g for 10 minutes at 4°C, and protein concentrations were determined using a protein assay kit (Bio-Rad, Hertfordshire, United Kingdom). Protein cell lysates (250 µg) were immunoprecipitated using 2 to 4 µg of antibody (anti-EGFR or anti-DNA-PK CS) overnight at 4°C and then bound to protein G-Sepharose. Immune complexes were washed thrice in lysis buffer before further analysis. Cell lysates (15–50 µg) and immunoprecipitates were separated by SDS-PAGE, electrophoretically transferred to Immobilon P membrane (Millipore, Gloucestershire, United Kingdom), and probed with appropriate primary antibodies (anti-EGFR, anti-DNA-PK CS, anti-Ku70, or anti-HER-2) and then horseradish peroxidase–conjugated secondary polyclonal antibody. Immunoreactive bands were visualized with the enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, United Kingdom). For nuclear and cytosolic preparations, the Clontech Tranfactor Extraction kit was used. Immunoblotting antibodies were horseradish peroxidase–labeled secondary antibody (BD Biosciences, Oxford, United Kingdom), anti-EGFR and anti-EGFR (phosphate–labeled secondary antibody (BD Biosciences, Oxford, United Kingdom), anti-DNA-PK CS, and anti-tubulin (Sigma, Dorset, United Kingdom).

**Ethidium Bromide Treatment of Cleared Lysates**

Ethidium bromide was added (400 µg/mL) as previously described, and the lysates were incubated on ice for 30 minutes (23). Precipitates were removed by centrifugation for 5 minutes at 4°C in a microcentrifuge, and the supernatant was transferred to a fresh tube. The resulting lysate was then ready for immunoprecipitation. The original concentration of ethidium bromide was maintained during the washing steps.

**RNA Interference Transfection and DNA-PK Deletion**

Gene-specific pSuper constructs were generated expressing the following RNA interference target sequences (24): (a) human DNA-PK Cs5'-AGTATATGAGCTCTAGGA-3', (b) scrambled control 5'-CAUGCAGAUCCGCUAGUC-3'. A 1.6-kb fragment containing the CMV IE promoter and eCFP (enhanced cyan fluorescent protein) was subcloned from pECFP-Mito (Invitrogen, Paisley, United Kingdom) into the Sap1 site of the resultant pSUPER constructs, generating pSUPER-eCFP-Parp-1 and pSUPER-eCFP-control. Transfection was carried out using the Fugene (Roche, Hertfordshire, United Kingdom) according to the manufacturers instructions. Transient transfection occurred after ~ 40 hours and persisted for ~ 18 hours. All experiments were done within this timeframe.

**DNA-PK Functional Assay**

DNA-PK activity was detected using the Promega SignaTect DNA-PK assay system, according to the manufacturer’s protocol. Briefly, 20 µg of whole-cell extract was incubated with DNA-PK, biotinylated peptide substrate, [γ-32p]ATP, and either DNA-PK activation buffer or DNA-PK control buffer for 5 minutes at 30°C. Termination buffer was added, and 10 µL of each reaction sample were spotted onto a SAM2Tm-biotin capture membrane. The SAM2Tm membrane squares were washed and dried before analysis by scintillation counting. The enzymatic activity of DNA-PK was expressed as a percentage decrease of control DNA-PK activity.

**Immunofluorescent Staining**

Cells were seeded at 2 × 105 per well on circular glass slides in 12-well plates, treated as required, and subsequently washed twice with cold PBS. Cells were then fixed using 500 µL/well of 50% methanol/50% acetone mix at 4°C for 8 minutes. Following this, the slides were then permeabilized using 500 µL/well of 0.5% Triton X-100 in PBS. Slides were then blocked in 3% casein blocking buffer (3% skimmed milk powder (Marvel, London, United Kingdom) in TBS-Tween: 20 mMol/L Tris-Base, 0.15 mol/L NaCl (pH 7.5) in Elga H2O with 0.1% Tween 20) overnight at 4°C.

Slides were then washed thrice in cold PBS, following which the cells were incubated with anti–DNA-PK Cs antibody for 1 hour. Slides were then washed thrice with washing buffer (0.1% TritonX-100 in PBS) and then incubated for 1 hour at room temperature with FITC-labeled secondary antibody: Alexa Fluoro 488 goat anti-mouse IgG (green). Nuclear counterstaining was done using 2 µg/mL propidium iodide (blue) for 3 minutes followed by destaining with distilled water for 20 minutes. The slides were viewed and photographed using a confocal microscope.

**Results**

**Inhibition of the DNA-PK Pathway Mimics Effects of Gefitinib in Combination with Cisplatin**

Our previous experiments showed an interaction between the DNA-PK pathway and EGFR, which was modulated by gefitinib and could contribute to sensitization by gefitinib of cisplatin treatment (18). To investigate further the effects of inhibiting the DNA-PK pathway on chemosensitization to cisplatin compared with gefitinib treatment, we used wortmannin, an inhibitor of DNA-PK. The dose of wortmannin used (100 nmol/L) has been shown to be inhibitory to phosphatidylinositol 3-kinase related kinases (25). Following exposure of MCF-7 cells to 100 nmol/L wortmannin there was a 47% decrease in DNA-PK activity (data not
shown). MCF-7 cells were incubated in the presence of cisplatin in combination with gefitinib, wortmannin, or both agents (Fig. 1A). As previously shown, there was a synergistic effect of the combination of cisplatin and gefitinib. There was a similar effect observed when wortmannin was added to cisplatin (IC$_{50}$ = 3 ± 0.8 μmol/L). The addition of all three agents in combination resulted in no greater effect than that seen with gefitinib and cisplatin. These results suggest that the effects of gefitinib on inhibition of DNA repair may be modulated through the DNA-PK pathway.

We previously showed inhibition of repair of cisplatin-induced interstrand cross-links following exposure of MCF-7 cells to the phosphatidylinositol 3-kinase inhibitor LY294002. To confirm the role of DNA-PK in this effect, we incubated cells with cisplatin, in combination with wortmannin and/or gefitinib, and measured the production and rate of repair of interstrand cross-links using a modification of the comet assay (Fig. 1B). There was a delay in interstrand cross-link repair in cells treated with the combination of cisplatin and wortmannin. By 16 hours, 47% of interstrand cross-links were repaired in

**Figure 1.** Effect of wortmannin and DNA-PK-specific RNA interference in combination with cisplatin on MCF-7 Cells. A, growth-inhibitory effects of cisplatin treatment with gefitinib and/or wortmannin. B, formation of cisplatin-induced DNA interstrand cross-links and repair alone or in the presence of gefitinib and/or wortmannin. C, transient inhibition of DNA-PK by RNA interference as shown by immunoblotting. D, growth-inhibitory effects of cisplatin treatment with gefitinib and/or DNA-PK-specific RNA interference. E, formation of cisplatin-induced DNA interstrand cross-links and repair alone or in the presence of gefitinib and/or DNA-PK-specific RNA interference. For proliferation studies, cells were treated with the indicated concentrations, and surviving cells were fixed and stained using sulforhodamine B as before. Cisplatin (●), cisplatin with 10 μmol/L gefitinib (■), cisplatin with 100 nmol/L wortmannin (○), cisplatin with RNA interference (○), cisplatin with 10 μmol/L gefitinib and 100 nmol/L wortmannin (▲), cisplatin with 10 μmol/L gefitinib and RNA interference (▲). Points, averages of three different experiments, each done in triplicate; bars, SD. For interstrand cross-link studies, cells were treated with cisplatin and gefitinib and/or wortmannin or RNA interference for 1 h and then replaced with gefitinib and/or wortmannin or RNA interference alone. Cells were irradiated (12 Gy), lysed, and electrophoresed (comet assay). Interstrand cross-link formation, as represented as percentage decrease in tail moment, peaks at 6 h (cisplatin) after 1 h of drug treatment; 200 μmol/L cisplatin (●), cisplatin with 100 nmol/L wortmannin or RNA interference (○), cisplatin with 10 μmol/L gefitinib (■), cisplatin with 10 μmol/L gefitinib and 10 μmol/L wortmannin or RNA interference (▲, cisplatin and Scrambled RNA interference (▲). Points, averages; bars, SE.
PKCS does not entirely account for the cellular sensitization to DNA-PK CS, and cisplatin resulted in additional effects when combined with gefitinib, pSUPER-CFP-117 in MDA-453 cells (Fig. 2A). Cells were treated with gefitinib (10 μmol/L) and lysed. DNA-PK activity was measured using the Promega SignaTECT DNA-PK assay system. The enzymatic activity of DNA-PK was expressed as a percentage decrease of control DNA-PK activity. Columns, mean of three independent experiments; bars, SD. D, inhibition of DNA-PK expression in MCF-7 cells using different doses and time points following treatment with gefitinib.

Although wortmannin has inhibitory effects on the DNA-PK pathway, it has limited specificity and also has inhibitory effects on other members of the phosphatidylinositol 3-kinase family. To examine more precisely the interaction between inhibition of DNA-PK and treatment with gefitinib, we used plasmid-directed RNA interference to reduce expression of DNA-PK CS. Transfection of MCF-7 cells with a pSUPER plasmid expressing small interfering RNA directed against DNA-PK (pSUPER-CFP-117) achieved a 57% reduction in expression of DNA-PK CS. Transfection of MCF-7 cells with a pSUPER plasmid expressing small interfering RNA directed against DNA-PK (pSUPER-CFP-117) achieved a 57% reduction in expression of DNA-PK levels 40 hours following transfection. This persisted for ~18 hours as measured by immunoblotting (Fig. 1C). No effects were seen with a pSUPER plasmid expressing a scrambled small interfering RNA (pSUPER-CFP-Scrambled). All experiments were therefore carried out within this timeframe. To investigate the effects of reduction in DNA-PK CS levels on sensitization to cisplatin, a sulfonamide B assay was done on MCF-7 cells transfected with pSUPER-CFP-117 or pSUPER-CFP-Scrambled (Fig. 1D). The IC50 for cells treated with cisplatin and pSUPER-CFP-117 to DNA-PK CS was 2.5 ± 0.1 μmol/L, comparable with the combination of cisplatin and wortmannin (3 ± 0.8 μmol/L). Experiments in which cells were exposed to the combination of gefitinib, pSUPER-CFP-117 to DNA-PK CS, and cisplatin resulted in additional inhibition of cellular proliferation with an IC50 of 0.18 ± 0.04 μmol/L, indicating that the inhibition of DNA-PK CS does not entirely account for the cellular sensitization by gefitinib of cisplatin. However, as noted above pSUPER-CFP-117 achieved only partial inhibition of DNA-PK CS expression. Inhibition of repair of interstrand cross-links was shown in cells transfected with pSUPER-CFP-117 to DNA-PK CS and treated with cisplatin (Fig. 1E). There was 22% repair of interstrand cross-links in cells transfected with pSUPER-CFP-117 to DNA-PK CS at 16 hours; no effect was seen following transfection of pSUPER-CFP-Scrambled. Addition of gefitinib to pSUPER-CFP-117 to DNA-PK CS and cisplatin did not result in inhibition of repair greater than that seen with gefitinib and cisplatin.

Inhibition of DNA-PK Activity by Gefitinib

These experiments suggested the involvement of the DNA-PK pathway in gefitinib-induced sensitization to cisplatin. To investigate whether gefitinib directly affected the activity of DNA-PK, functional assays were carried out on cellular extracts following treatment with gefitinib. Following exposure of MCF-7 cells to 10 μmol/L gefitinib, there was a 31 ± 5.5% decrease in DNA-PK activity at 24 hours and 49 ± 8.5% decrease in DNA-PK activity after 48 hours (Fig. 2A). We previously found the IC50 for inhibition of MCF-7 cell proliferation by gefitinib after 72 hours exposure to be 17 ± 0.02 μmol/L. Therefore, a decrease in DNA-PK activity could be shown following exposure to concentrations of gefitinib, which result in inhibition of EGFR activation. To determine if the inhibitory effects of gefitinib on DNA-PK activity could be found with other cell lines, we analyzed extracts from the rat pancreatic cell line AR42J, which expresses high levels of EGFR. This showed comparable results with 48 ± 1.6% decrease in activity after 24 hours (Fig. 2B). We also investigated the effects of gefitinib treatment on the MDA-453 breast cancer cell line in which low levels of EGFR expression occur and which is resistant to gefitinib (26). Interestingly, the MDA-453 cell line showed minimal reduction in DNA-PK activity following prolonged exposure to gefitinib (Fig. 2C). These results show that treatment with gefitinib results in reduction of DNA-PK activity.

Treatment with gefitinib also results in reduced cellular expression of DNA-PK CS. There is no evidence of apoptosis following 24 hours of exposure as assessed by fluorescence-activated cell sorting analysis (18). Incubation of cells with the broad-spectrum caspase inhibitor, benzoyl-VAD-fluoromethyl ketone (zVAD-fmk) had no effect on reduction of DNA-PK CS expression (data not shown). We measured levels of DNA-PK CS using different...
doses and time points following treatment with gefitinib (Fig. 2D). This showed that inhibition of DNA-PK expression occurs at doses of gefitinib, which inhibit EGFR phosphorylation.

**Altered Association of EGFR and DNA-PKCS following Treatment with Gefitinib**

To further examine the interaction of EGFR and DNA-PKCS, we did immunoprecipitations on cell extracts following incubation with gefitinib. As previously shown, increased association of EGFR and DNA-PKCS was found after a 24-hour treatment with gefitinib in MCF-7 cells (Fig. 3A; ref. 18). To investigate whether the EGFR/DNA-PKCS complex contains other components of the DNA-PK pathway, we also did immunoblotting with antibody to Ku70. This indicated that the Ku70 complex is also associated with EGFR after treatment with gefitinib (Fig. 3A).

We further investigated the association of EGFR and DNA-PKCS by immunoprecipitation in other cell lines (Fig. 3A). The AR42J line, which expresses high levels of EGFR, showed an increase in association of EGFR and DNA-PKCS following treatment with gefitinib. However, the MDA-453 cell line, in which inhibition of DNA-PK activity following gefitinib treatment was not shown, showed no alteration in EGFR/DNA-PK association following gefitinib treatment. Experiments with MCF-7 cell extracts revealed that following immunoprecipitation with EGFR, HER-2 protein was detectable by immunoblotting as expected. However, following immunoprecipitation with DNA-PKCS, HER-2 was not detectable by immunoblotting (Fig. 3B). This indicates that the association between EGFR and DNA-PKCS does not additionally involve an interaction with HER-2.

**Association of EGFR and DNA-PKCS following Gefitinib Treatment Is DNA Independent**

The main functions of DNA-PKCS involve association with nuclear DNA. Additionally, although EGFR is a membrane-associated glycoprotein, there are reports that EGFR is detectable in the nucleus and binds to DNA acting as a transcription factor (27, 28). The immunoprecipitation results could have been due to contaminating DNA in protein preparations, which have been shown to stabilize DNA-dependent associations, which seem DNA independent (23). To determine if the association between EGFR and DNA-PKCS was direct or mediated by DNA, we did immunoprecipitations with ethidium bromide treated extracts. There was no alteration in the association of EGFR and DNA-PKCS in immunoprecipitation experiments following ethidium bromide treatment (Fig. 4). This confirms that the association of DNA-PKCS and EGFR following gefitinib treatment is independent of DNA.

**Gefitinib-Induced Association of EGFR and DNA-PKCS Is Unaffected by Treatment with Cisplatin and Etoposide**

Treatment with both etoposide and cisplatin result in DNA damage, which activates the DNA-PK pathway. To investigate whether preexisting DNA damage could influence the association of EGFR and DNA-PKCS, MCF-7 cells were incubated with cisplatin (to induce cross-link formation) and etoposide (to induce DNA double-strand breaks) before the addition of gefitinib. As shown in Fig. 5, there was no effect on the association of EGFR and DNA-PKCS following treatment with these agents. Simultaneous exposure of MCF-7 cells to gefitinib and either cisplatin or etoposide did not affect the increased association of EGFR and DNA-PKCS found following gefitinib treatment alone.

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**Figure 3.** A, gefitinib-induced EGFR/DNA-PKCS association. Cells were treated with gefitinib (10 μmol/L) for 24 h, and lysates were immunoprecipitated (IP) and immunoblotted (IB) as indicated. Lysates immunoprecipitated with anti-EGFR were then reprobed with anti-Ku70 antibody. B, HER-2 association with EGFR. Lysates immunoprecipitated with anti-DNA-PKCS or anti-EGFR and immunoblotted with anti-HER-2 antibody. A431 cell lysate loaded as a positive control for HER-2.

**Figure 4.** Gefitinib-induced DNA-independent association of EGFR with DNA-PKCS in MCF-7 cells. Cells were treated with gefitinib (10 μmol/L, 24 h) and ethidium bromide (400 μg/mL, 30 min), and lysates were immunoprecipitated (IP) and immunoblotted (IB) as indicated.
Subcellular Distribution of DNA-PKCS following Treatment with Gefitinib

DNA-PK CS is a nuclear enzyme involved in the nonhomologous end-joining pathway (29, 30). The association with EGFR following gefitinib treatment suggested an alteration in cellular localization as the majority of EGFR is extranuclear. Cells were treated with gefitinib, and nuclear and cytosolic fractions were isolated and immunoblotted for DNA-PKCS. As shown in Fig. 6A, basal expression of DNA-PKCS was detectable in the cytosol of MCF-7 cells, although expression was predominantly nuclear. Following treatment with gefitinib, increased DNA-PKCS was detectable in the cytosol. Cellular extracts from gefitinib-treated AR42J cells, which showed increased EGFR/DNA-PK CS association and inhibition of DNA-PK activity, also showed increased cytosolic DNA-PK CS following gefitinib treatment. In contrast, the MDA-453 cell line, in which neither inhibition of DNA-PK CS activity or association of EGFR and DNA-PK CS occurred following gefitinib treatment, showed no alteration in cytosolic expression (Fig. 6A).

Additionally, experiments were done using different time points and doses of gefitinib treatments (Fig. 6B) in MCF-7 cells. Following treatment with gefitinib, there was decreased nuclear and increased cytosolic DNA-PK CS detectable at 6 hours following gefitinib treatment. An effect on cellular distribution of DNA-PK CS was found following treatment with 5 μmol/L gefitinib. To confirm that the increased DNA-PK CS expression in the cytoplasm was not due to a leak from the nuclear fraction into the cytoplasm during the fractionation process, confocal microscopy was carried out using antibodies to DNA-PK CS. This showed cytoplasmic expression of DNA-PK CS following exposure to gefitinib (Fig. 6C).

Discussion

This study shows that interactions between EGFR and the DNA-PK pathway contribute towards cellular sensitization to cisplatin following gefitinib treatment. This is shown by expression of DNA-PK CS was detectable in the cytosol of MCF-7 cells, although expression was predominantly nuclear. Following treatment with gefitinib, increased DNA-PK CS was detectable in the cytosol. Cellular extracts from gefitinib-treated AR42J cells, which showed increased EGFR/DNA-PK CS association and inhibition of DNA-PK activity, also showed increased cytosolic DNA-PK CS following gefitinib treatment. In contrast, the MDA-453 cell line, in which neither inhibition of DNA-PK CS activity or association of EGFR and DNA-PK CS occurred following gefitinib treatment, showed no alteration in cytosolic expression (Fig. 6A).

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Additionally, experiments were done using different time points and doses of gefitinib treatments (Fig. 6B) in MCF-7 cells. Following treatment with gefitinib, there was decreased nuclear and increased cytosolic DNA-PK CS detectable at 6 hours following gefitinib treatment. An effect on cellular distribution of DNA-PK CS was found following treatment with 5 μmol/L gefitinib. To confirm that the increased DNA-PK CS expression in the cytoplasm was not due to a leak from the nuclear fraction into the cytoplasm during the fractionation process, confocal microscopy was carried out using antibodies to DNA-PK CS. This showed cytoplasmic expression of DNA-PK CS following exposure to gefitinib (Fig. 6C).

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the similar magnitude of chemosensitization to cisplatin shown by gefitinib and small interfering RNA directed against DNA-PKCS, migration of DNA-PKCS to the cytosol following gefitinib treatment, coimmunoprecipitation of EGFR and DNA-PK induced by gefitinib treatment, and reduction of functional DNA-PK activity by gefitinib.

Although many studies have used cell lines with high EGFR levels and increased sensitivity to gefitinib, we tested effects on MCF-7 cells, a widely used breast cancer cell line. The MCF-7 line is relatively resistant to gefitinib treatment with an IC$_{50}$ of 17 µmol/L in proliferation assays (18, 31). Although these levels are high, they are of clinical relevance. It has become clear that effective tumor concentrations in both xenografts and clinical studies are considerably higher than previously estimated. A recent study showed that the effective concentration of gefitinib in breast tumor tissue is around 17 µmol/L (32). These gefitinib tumor concentrations are considerably higher than those reportedly required in vitro to achieve complete inhibition of EGFR autophosphorylation. Additionally, recent work in xenografts suggests that pulsatile administration of gefitinib at high concentrations is significantly more effective at chemosensitization (33). We have shown that ≥10 µmol/L gefitinib has no effect on DNA cross-link repair in EGFR-negative cell lines, but repair inhibition can be shown following transfection of EGFR.5 Thus, it is unlikely that the effects on DNA repair of 10 µmol/L gefitinib treatment in this study are due to inhibition of pathways other than EGFR, although it remains a possibility that some of the effects described here could be due to inhibition of other pathways.

There are clearly general pathways by which inhibition of EGFR results in reduction in cell proliferation and apoptosis. Inhibition of EGFR by gefitinib may directly sensitize cells to chemotherapy and radiation through down-regulation of genes involved in the apoptotic pathway, including bcl2 and Bax (34). However, the generalized reduction in apoptotic threshold cannot explain sensitization to specific agents. For example, in our previous study, we showed that although gefitinib had a synergistic effect with cisplatin and etoposide, there was no additive effect with melphalan (18). The factors determining response to EGFR inhibitors remained unclear until recently. Mutations in the EGFR intracellular ATP-binding domain in some lung cancers have been found to be associated with sensitization to gefitinib and erlotinib in several studies (9, 10, 35). Additionally, in vitro studies suggested that the mutations result in resistance to several chemotherapeutic agents, including cisplatin and doxorubicin (11). Whether individual mutations affect responses to specific chemotherapeutic agents remains unclear. Although clinical studies with gefitinib and erlotinib have not shown benefit with the addition of chemotherapy, the antibody cetuximab has shown super-additive effects in chemotherapy-refractory colon cancer (17). This study indicates that an important factor in the sensitization of EGFR inhibitors to chemotherapeutic agents may be through effects on the DNA-PK pathway. The DNA-PK complex consists of three proteins involved in DNA repair, V(D)J recombination, and transcription. DNA-PKCS is a serine/threonine kinase regulated in a heterodimeric complex with Ku70/Ku80 and is believed to be involved in the repair of DNA double-strand breaks (29, 30). Activated DNA-PKCS has several substrates, including p53, c-abl, and cdc2; the src-like protein-tyrosine kinase Lyn interacts directly with DNA-PKCS near a leucine zipper homology domain (36). The major functions of DNA-PKCS have been localized to the nucleus. However, immunohistochemistry has shown the presence of DNA-PK in the cytosol in cancer cells (37). A study of squamous cancer cell lines suggested that there was an altered distribution of DNA-PKCS following radiation and gefitinib treatment (38). However, the effects of gefitinib alone were not included and, unusually, DNA-PKCS was not detectable in the nucleus in untreated cells. Another study on radiation response following treatment of squamous cancer cells to the anti-EGFR antibody C225 showed inhibition of DNA repair and a redistribution of DNA-PKCS from nucleus to cytosol. The effect of C225 alone was not included (39).

The functional significance of cytosolic DNA-PKCS remains unclear. However, recent studies have identified DNA-PK as a kinase activating Akt (40, 41). DNA-PKCS was shown to be colocalized with Akt at the plasma membrane and phosphorylated Akt on Ser$^{473}$ resulting in a ~10-fold enhancement of activity. This provides important evidence that DNA-PKCS can be functionally active within the cytosol. These studies indicate the importance of DNA-PK not only in the response to DNA repair but also in modulation of the phosphatidylinositol 3-kinase pathway. Studies with the anti-EGFR antibody cetuximab (C225) similarly showed coimmunoprecipitation of EGFR and DNA-PKCS (32). Our studies suggest that there is a strong effect of gefitinib on DNA-PKCS cellular localization, and this is likely to be a factor in the inhibition of cellular DNA-PK activity. The results from the MDA-453 cells differed from MCF-7 and AR42J in that reduction in DNA-PK activity, increased association of EGFR and DNA-PKCS, and increased cytosolic DNA-PKCS did not occur following gefitinib treatment. It will clearly be important to define the molecular basis of the interaction between EGFR and DNA-PKCS to investigate reasons for the differences between cell lines. It has recently been shown that treatment of cells with ionizing radiation and cisplatin triggers EGFR translocation into the nucleus and is accompanied by an increase in DNA-PK activity (42). Furthermore, inhibition of EGFR with cetuximab was shown to block nuclear EGFR import and radiation-induced activation of DNA-PK and localize the EGFR/DNA-PK complex in the cytoplasm (43). These studies confirm the importance of interactions between the EGFR and DNA repair pathways by novel therapeutic agents targeting EGFR.

5 Friedmann et al., in preparation.
Thus far, despite the abundant evidence in vitro that combining EGFR inhibition and chemotherapy is synergistic, this has not been borne out in clinical studies. However, the combination of cetuximab with chemotherapy was shown to resensitize cells to irinotecan (17). Additionally, preliminary results in head and neck cancer strongly suggest the value of combining irradiation with cetuximab (44). Understanding the effects of EGFR inhibition on DNA repair pathways will allow design of novel studies with schedules and agents that could provide major therapeutic benefit.

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References


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