Relationship between Antiapoptotic Molecules and Metastatic Potency and the Involvement of DNA-Dependent Protein Kinase in the Chemosensitization of Metastatic Human Cancer Cells by Epidermal Growth Factor Receptor Blockade

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Received May 3, 2004; accepted July 22, 2004

ABSTRACT

The failure to treat metastatic cancer with multidrug resistance is a major problem for successful cancer therapy, and the molecular basis for the association of metastatic phenotype with resistance to therapy is still unclear. In this study, we revealed that various metastatic cancer cells showed consistently higher levels of antiapoptotic proteins, including Bcl-2, nuclear factor-κB, MDM2, DNA-dependent protein kinase (DNA-PK), and epidermal growth factor receptor (EGFR), and lower levels of proapoptotic proteins, including Bax and p53 than low metastatic parental cells. This was followed by chemo- and radioresistance in metastatic cancer cells compared with their parental cells. EGFR and DNA-PK activity, which are known to be associated with chemo- and radioresistance, were demonstrated to be mutually regulated by each other. Treatment with PKI166, an EGFR inhibitor, suppressed etoposide-induced activation of DNA-PK in A375SM metastatic melanoma cells. In addition, PKI166 enhanced markedly the chemosensitivities of metastatic cancer cell sublines to various anticancer drugs in comparison with those of low metastatic cancer cells. These results suggest that the activities of DNA-PK and EGFR, which is positively correlated with each other, may contribute to metastatic phenotype as well as therapy resistance, and the EGFR inhibitor enhances the effect of anticancer drugs against therapy-resistant metastatic cancer cells via suppression of stress responses, including activation of DNA-PK.

Despite the improvement of strategies against cancer therapy, the metastasis and development of multidrug resistance of tumor cells are the critical problems for successful cancer therapy, because recurrent or metastatic cancers after initial treatment with radiotherapy and chemotherapy are generally refractory to second treatments with these anticancer therapies (Yanagisawa et al., 1998). Therefore, it is necessary to elucidate the therapy-resistant mechanism of metastatic cancer cells for development of effective therapeutic modalities against metastatic cancers.

Recently, the interest has been focused on the possible relationship between cancer metastasis and therapy resistance, because the molecular basis for the association of aggressive metastatic phenotype with resistance toward apoptosis remains to be elucidated (Eckhardt, 2002). Some metastatic cancers are more resistant to chemotherapeutic drugs than their poorly metastatic counterparts (Molinari et al., 2002). Some cancer cells selected for resistance to drugs are more invasive/metastatic relative to nonresistant parental cells (Liang et al., 2001). In other instances reported in the literature, no correlation is seen between drug resistance and cancer invasion/metastasis (Liang et al., 2002). On the other hand, there are some correlations between the metastatic capacity of tumor cells and the radiation resistance (Lewis et al., 1996; Haffty and Glazer, 2003).

Several lines of evidence have identified the epidermal growth factor receptor (EGFR) as a rational target for anti-

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ABBREVIATIONS: EGFR, epidermal growth factor receptor; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; VBL, vinblastine; VCR, vincristine; CPT, camptothecin; DOX, doxorubicin; BLM, bleomycin; VP-16, etoposide; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; MEF, murine embryonic fibroblast; NF-κB, nuclear factor-κB; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide; EMSA, electrophoretic mobility shift analysis; MDR, multidrug resistance.
cancer therapy (Ciardiello et al., 2003; Jannata and Giaccone, 2003). The blockade of EGFR leads to inhibition of cell cycle progression, induction of apoptosis, enhanced chemosensitivity, and inhibition of the repair efficiency of the damaged DNA. The receptor is highly expressed in a range of solid tumors, including breast, head and neck, nonsmall cell lung, and prostate cancer (Salomon et al., 1995). The EGFR is not only involved in survival signaling, cell migration, metastasis formation, and angiogenesis but also confers reduced responses of cancer cells toward drug or radiation (Klijn et al., 1992; Salomon et al., 1995; Sartor, 2000; Brandeker et al., 2001; Baselga, 2002; Kopp et al., 2003).

In addition, there are some evidences showing that bel-2 family proteins and DNA-dependent protein kinase (DNA-PK) can be modulated by EGFR (Huang et al., 1999; Wang et al., 1999; Magne et al., 2003). The increase of Bax and decrease of Bel-2 and DNA-PK were found after inhibition of EGFR with various methods. Bel-2 family proteins play a pivotal role in the regulation of the mitochondrial pathway of apoptosis. They comprise both antiapoptotic members such as Bel-2, Bel-XL, and Mcl-1 as well as proapoptotic molecules such as Bax, Bad, and Bek, and BH3 domain-only molecules that link the death receptor pathway to the mitochondrial pathway such as Bid, Bim, Puma, and Noxa (Debatin, 2004). DNA-PK is a nuclear serine/threonine protein kinase, which composed of 460-kDa catalytic subunit (DNA-PKcs) and a heterodimer of Ku70 and Ku80 regulatory subunits, and it plays role in repairing double strand breaks as well as sensing and transmitting a damage signal to downstream targets leading to cell cycle arrest. The activity of DNA-PK is known to be associated with both radioresistance and chemoresistance in various cancers (Kim et al., 2000; Shintani et al., 2003).

In this study, we determined the correlation between antiapoptotic molecules such as EGFR and DNA-PK and metastatic potency to understand drug resistance mechanisms of metastatic cancer cells and tried to find out strategies improving the effect of chemo-and radiotherapy against metastatic cancer cells.

Materials and Methods

Reagents. The following reagents were obtained from the listed sources and used at the concentrations indicated in the text. PKI166 was kindly donated by Dr. I. J. Fidler (University of Texas MD Anderson Cancer Center, Houston, TX). Vinblastine (VBL), vincristine, doxorubicin (DOX), bleomycin (BLM), camptothecin (CPT), and etoposide (VP-16) were obtained from Sigma-Aldrich (St. Louis, MO). All other materials were purchased in the highest grade.

Cell Lines and Culture Conditions. The poorly metastatic A375 human melanoma cell line was originally established in culture from a lymph node metastasis of melanoma. The highly metastatic A375SM line was established from lung metastases produced by the A375 cells growing subcutaneously in nude mice, and another highly metastatic A375-C28 cell line was derived from A375 cells by a limited dilution technique. The poorly metastatic KM12 cell line was established from a primary colorectal carcinoma classified as Dukes B2. The highly metastatic cell line, KM12L4A, was derived from KM12. A highly metastatic LNcAP-LN3 variant was isolated by intraprostatic injection of human androgen-dependent LNcAP prostate cancer cells. Human androgen-independent PC3 prostate adenocarcinoma cells and PC3-MM2 cells, variant of PC3 selected for their highly metastatic potential were also used in this study. All these cells (provided by Dr. I. J. Fidler) were maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, l-glutamine, and vitamin solution.

The multidrug-resistant (MDR) variants, CEM/MDR cells isolated from the human lymphoblastic leukemia CCRF-CEM (CEM) cells and MCF7/MDR cells isolated from human breast carcinoma MCF-7 cells (provided by Dr. I. J. Fidler), were maintained in minimal essential medium supplemented with 10% FBS. Murine embryonic fibroblast MEF and its Ku-deficient Ku80−/− cells immortalized by SV40 transfection, and Rat fibroblast Rat-1 cells were maintained in DMEM supplemented with 10% FBS and antibiotics. R7080-6 cells, which constitutively overexpress both human Ku70 and Ku80 in Rat-1 cells, were maintained in the medium containing hygromycin (100 μg/ml) and G418 (200 μg/ml) (Kim et al., 2000). Murine SCID (DNA-PKcs deficient) and isogenic wild-type murine embryonic fibroblast CB-17 cells were maintained in DMEM supplemented with 10% FBS and antibiotics (Um et al., 2003).

Western Blot Analysis. Whole cell lysates or nuclear extract containing an equal amount of protein was resolved on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were blotted to nitrocellulose membrane. The membrane was incubated with antibody as each specific antibody for anti-EGFR, MM2, Bel-2, Ku70/80, DNA-PKcs, EGFR, Bax, p53, and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively, followed by secondary antibody conjugated with horseradish peroxidase. The blots were visualized by the Amersham Biosciences Inc. (Freiburg, Germany) enhanced chemiluminescence system. Secondary antibodies were obtained from Amersham Biosciences Inc.

Radiation and Drug Sensitivity Assay. For radiation sensitivity profiles of metastatic and its parental cells, cells (5 × 10^6 cells/well) were seeded 16 h before irradiation and were irradiated at various doses. After irradiation, the cells were incubated at 37°C for 96 h. It was followed by the addition of 100 μl of MTT solution (5 mg/ml), and incubation for 4 h in the dark room. The water-insoluble MTT-formazan crystals were dissolved in dimethyl sulfoxide, and reduction of MTT was determined at 570 nm using ELISA reader (Bio-Tec Instruments, Winooski, VT). The experiment was carried out at least twice in each cell line. The 50% inhibitory dose (ID_{50}) was calculated as the dose of radiation, which caused a 50% reduction in cell viability.

In case of drug sensitivity, exponentially growing cells (5 × 10^5 cells/well) were plated in 96 well and incubated in growth medium at 37°C for 96 h the culture medium with or without anticancer drugs at various concentrations, and MTT assay was used to assess drug sensitivity. The concentration of each anticancer drug that reduced cell growth by 50% after 96 h treatment (IC_{50}) was determined from the growth inhibition plots. The treatment period of 96 h was determined empirically not to allow outgrowth of untreated cells.

Electrophoretic Mobility Shift Analysis (EMSA). Preparation of nuclear extracts from metastatic and its parental cells and the gel mobility-shift assays were performed as described previously (Um et al., 2001). In brief, 3 × 10^6 cells were washed with cold phosphate-buffered saline and harvested quickly and resuspended in 300 μl of lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The cells allowed swelling in ice for 10 min. After 0.05% Nonidet P-40 was added, the tube was vigorously mixed 3 times for 3 s on a vortex, and centrifuged at 250g for 10 min to pellet the nuclei. The nuclear pellet was resuspended in 30 μl of ice-cold nuclear extraction buffer (5 mM HEPES, pH 7.9, 26% glycerol (v/v), 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and then incubated on ice for 30 min with intermittent mixing, and centrifuged at 24,000g for 20 min at 4°C. The nuclear extract was washed with prechilled alcohol and centrifuged at 10,000g for 10 min. The antibodies included Ku consensus oligonucleotide (AGTTGAGG GGACTTTCCC). The protein-bound and free oligonucleotides were electrophoretically separated on 4.5% native polyacrylamide gels in 0.5× TBE buffer.
DNA-PK Kinase Assay. The kinase activity of whole DNA-PK complex was determined using the Sigma TECT DNA-dependent protein kinase assay system (Promega, Madison, WI). DNA-PK was assayed by measuring phosphorylation of a p53 peptide substrate (EPLLQEQAFADLWKKR). In brief, 10 μg of nuclear extract was incubated with activator DNA, a biotinylated p53-derived peptide substrate, and [γ-32P]ATP at 30°C for 5 min. The reaction was terminated by adding termination buffer. The terminated reaction sample was spotted onto SAM2TM biotin capture membrane and then washed with 2 M NaCl and 2 M NaCl in 1% H$_3$PO$_4$. The SAM2TM membrane squares were analyzed using Molecular Imager system (model GS 525; Bio-Rad, Hercules, CA).

Statistical Analysis. All experiments were performed in triplicates at least twice, and significance was determined using unpaired Student's t test (Tables 1 and 2) or analysis of variance (Table 3).

Results

Increase of Antiapoptotic Proteins and Decrease of Proapoptotic Proteins in Metastatic Cancer Cells. The molecular basis for the association of metastatic phenotype with resistance to therapy is not clear. Because changes that inhibit the apoptotic machinery might play a role in resistance to therapy, we determined the expression level of antiapoptotic proteins in various human cancer cells with different metastatic capacity to evaluate the relationship between antiapoptotic molecules and metastatic potency. As shown in Fig. IA, the highly metastatic A375SM cells showed higher levels of antiapoptotic proteins, including Bcl-2, NF-κB, and MDM2 than those of the poorly metastatic parental A375 cells, whereas levels of proapoptotic proteins, including Bax and p53 in A375SM cells, were significantly decreased, compared with those of A375 cells.

To confirm this result, other cell lines with different metastatic potency and tissue origin were tested. Similar results were obtained with various metastatic sublines, including KM12L4A cells, LNCaP-LN3 cells, and PC3M-MM2 cells compared with their parental cancer cells as shown in Fig. 1, B to D. These results suggest that elevated expression of antiapoptotic proteins, including Bcl-2, MDM2, and NF-κB, and concurrently decreased expression of proapoptotic proteins, including Bax and p53, may be common characteristics of highly metastatic cancer cells.

Table 2 shows the comparison of radiosensitivity in metastatic and parental cells.

Elevated Expression of DNA-PK and EGFR in Metastatic- and Drug-Resistant Cancer Cells, and the Correlation of DNA-PK with EGFR. Because there is some evidence that metastatic cancer cells might be associated with acquisition of drug resistant phenotype. The expression of DNA-PK components, Ku-DNA binding activity, DNA-PK kinase activity, and EGFR expression were compared between each paired poorly and highly metastatic cell lines from different tissue origins (Fig. 2). The A375SM cells showed remarkable increase of DNA-PKcs, Ku80 levels, and DNA-PK kinase activity (2.7-fold) as well as Ku-DNA binding activity in comparison with A375 cells. Concurrently, increase of EGFR expression was observed in A375SM cells (Fig. 2A, left). Similar results were obtained in other highly metastatic cells, including the PC3-MM2 and KM12L4A cells that showed 3.4- and 2.3-fold increased DNA-PK activities compared with each parental cell line, respectively (Fig. 2A, middle and right). These results suggest that the increased expression of DNA-PK and EGFR may be associated with the metastatic potency of cancer cells.

Because there is some evidence that metastatic cancer cells may be drug-resistant cells, we examined whether the DNA-PK components and EGFR are also increased in drug-resistant cancer cells (Fig. 2B). The MDR variants CEM/MDR and MCF7/MDR cells showed increased levels of DNA-PKcs, Ku70/80, and EGFR compared with their parental cells, suggesting that the increase of EGFR and DNA-PK might be associated with acquisition of drug resistant phenotype as well as metastatic phenotype.

To examine whether the expression of EGFR can be affected by DNA-PK, the EGFR level was determined in Ku-deficient and Ku-overexpressed cells. The EGFR level of R7080-6 cells was higher than that of parental Rat-1 cells, but conversely the EGFR level of Ku80−/− cells was lower than that in parental MEF cells (Fig. 3A). In addition, the EGFR level of DNA-PKcs-deficient SCID cells was much lower than that of wild-type CB-17 cells (Fig. 3B). These results suggest that the expression of EGFR may be positively regulated by DNA-PK activity.

Influence of Metastatic Capacity on Chemo- and Radiosensitivities. Because the correlation between cancer metastasis and therapy resistance is not clear (Liang et al.,...
2001, 2002; Molinari et al., 2002), the drug sensitivities between cancer cells with different metastatic capacity and tissue origin and their parental counterparts were compared by MTT assay (Table 1). The highly metastatic KM12L4A and LNCaP-LN3 cells were 8.5- and 4.8-fold resistant to VP-16 and 3.2- and 3.8-fold resistant to BLM than each parental KM12 and LNCaP cells, respectively. The resistances to DOX of KM12L4A, LNCaP-LN3, and A375SM cells were increased about 2-fold compared with those of their parental cells. In the case of VCR and VBL, the highly metastatic A375SM cells were 3.8- and 2.0-fold more resistant than their parental A375 cells, respectively. These results indicate that metastatic capacity could be positively correlated with drug resistance.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Drugs (nM)</th>
<th>PKI166 Free IC50</th>
<th>± 0.5 µM PKI166 IC50</th>
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<tr>
<td>KM12</td>
<td>VP-16</td>
<td>36 ± 2.4</td>
<td>20 ± 1.1 (1.8)</td>
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<tr>
<td>KM12SM</td>
<td>VP-16</td>
<td>350 ± 12</td>
<td>80.4 ± 7.5 (4.4)</td>
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<tr>
<td>KM12L4A</td>
<td>VP-16</td>
<td>310 ± 5</td>
<td>73.8 ± 5.0 (4.2)</td>
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<td>A375</td>
<td>VP-16</td>
<td>15 ± 1.8</td>
<td>4.7 ± 0.3 (3.2)</td>
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<tr>
<td>A375SM</td>
<td>CPT (ng/ml)</td>
<td>26 ± 1.5</td>
<td>2.0 ± 0.1 (1.3)</td>
</tr>
<tr>
<td>PC3</td>
<td>CPT (ng/ml)</td>
<td>2.1 ± 0.3</td>
<td>1.7 ± 0.03 (1.2)</td>
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<tr>
<td>PC3-M-M2</td>
<td>VP-16</td>
<td>3.0 ± 0.2</td>
<td>0.6 ± 0.01 (5.0)</td>
</tr>
<tr>
<td>CEM</td>
<td>VP-16</td>
<td>255 ± 18</td>
<td>230 ± 12 (1.1)</td>
</tr>
<tr>
<td></td>
<td>CPT</td>
<td>2.5 ± 0.2</td>
<td>0.4 ± 0.02 (6.3)</td>
</tr>
<tr>
<td></td>
<td>VBL (nM)</td>
<td>12 ± 1.7</td>
<td>10 ± 0.7 (1.2)</td>
</tr>
<tr>
<td>CEM/MDR</td>
<td>VP-16</td>
<td>1300 ± 76</td>
<td>170 ± 10 (7.6)</td>
</tr>
<tr>
<td></td>
<td>CPT</td>
<td>1.6 ± 0.1</td>
<td>0.12 ± 0.01 (13.3)</td>
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<tr>
<td></td>
<td>VBL</td>
<td>210 ± 10.1</td>
<td>58 ± 3.2 (3.6)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>VP-16</td>
<td>54 ± 1.8</td>
<td>50 ± 2.3 (1.1)</td>
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<tr>
<td></td>
<td>DOX (ng/ml)</td>
<td>3.2 ± 0.3</td>
<td>3.1 ± 0.1 (1.0)</td>
</tr>
<tr>
<td>MCF7/MDR</td>
<td>VP-16</td>
<td>1400 ± 90</td>
<td>370 ± 22 (3.8)</td>
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<tr>
<td></td>
<td>DOX</td>
<td>35 ± 1.2</td>
<td>11 ± 0.8 (3.2)</td>
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</table>

Table 3: Potentiation of chemosensitivity by PKI166 in metastatic and MDR cells.

Each cell line (4 × 10^4 cells/well) was treated with each anticancer drug for 96 h in the presence or absence of 0.5 µM PKI166. Growth inhibition assay was performed by MTT method. Values in parentheses indicate the ratio of IC50 for drug alone to the IC50 for drug in the presence of PKI166. Data shown are the mean ± S.D. of two experiments carried out in triplicates. Results from a completely randomized design were analyzed with analysis of variance. The chemosensitizing effects of PKI166 were significantly higher in metastatic and MDR cells than in their respective drug-sensitive parental cells (P < 0.001).
Because DNA-PK plays a role in the acquisition of a resistant phenotype of human tumors to radiotherapy as well as chemotherapy (Kim et al., 1999, 2000; Tai et al., 2000), we compared the modulation of the components of DNA-PK between KM12L4a and KM12 cells after treatment with γ-ray irradiation (Fig. 4A). The DNA-PKcs and Ku70 expressions of KM12L4A cells were increased and maintained by treatment with γ-ray irradiation, respectively, whereas those of KM12 cells were gradually decreased by treatment with γ-ray irradiation. Similar results were obtained between PC3 and its metastatic PC3M-MM2 cells after treatment with γ-ray and UV irradiation (Fig. 4B). These results were followed by increased radioresistance in metastatic cancer cells compared with their parental cells (Table 2). The highly metastatic KM12L4A, LNCaP-LN3, A375-C28, and PC3M-MM2 cells showed increased resistance (about 2-fold) to irradiation compared with their each parental counterpart. These results suggest that metastatic capacity can be associated with the induction of radioresistance, at least due to increased expression of DNA-PK as a stress response.

Suppression of DNA-PK and Chemosensitization by Inhibition of EGFR. Because DNA-PK and EGFR were consistently increased in metastatic cancer cells and DNA-PK was inhibited by ZD1839 (Magne et al., 2003), it was examined whether the expression of DNA-PK could be modulated by the treatment of metastatic cancer cells with PKI166, an EGFR tyrosine kinase inhibitor. Because it has been well known that EGFR tyrosine phosphorylation was significantly inhibited at dose of 0.1 to 1 μM PKI166 in various cancer cell lines (Bruns et al., 2000; Solorzano et al., 2001; Bonomi, 2003; Holsinger et al., 2003), the effect of PKI166 on the expressions EGFR and DNA-PK of the metastatic cancer cells was examined after exposure to increasing concentration of PKI166 (0.25–1 μM) for 90 min. Treatment of A375SM with PKI166 resulted in the decreased levels of Ku80 and DNA-PKcs as well as EGFR in dose-dependent manner. DNA-PK kinase activity was also inhibited 1.7-, 2.5-, and 3.3-fold by treatment with 0.25, 0.5, and 1 μM PKI166, respectively (Fig. 5, left). When A375SM cells were exposed to PKI166 (0.5 μM) for various periods, the inhibition of Ku70/80 and DNA-PKcs expression was occurred in time-dependent manner (Fig. 5, right). Similar results were observed in PKI166-treated MCF7/MDR cells, showing decreased levels of EGFR and Ku70/80. These re-
results strongly suggest that EGER and DNA-PK activity can be mutually regulated by each other, and DNA-PK and EGFR may be potential molecular targets for therapy against metastatic and/or drug-resistant cancer cells.

Because PKI166 suppressed the activities of DNA-PK and EGFR, the combination effect of PKI166 on drug sensitivity of metastatic and MDR cells, both of which have increased level of DNA-PK and EGFR, was determined (Table 3). PKI166 treatment strongly potentiated the chemosensitivity of various anticancer drugs in metastatic and MDR cells. In the case of VP-16, the drug sensitivity of metastatic KM12SM and KM12LA4 cells were increased about 4-fold by cotreatment with PKI166 (0.5 μM), whereas the drug sensitivity of the parental KM12 cells showed only 1.8-fold increase by cotreatment with PKI166. In A375SM cells, treatment with PKI166 caused a 13-fold increase in the sensitivity to VP-16, whereas parental A375 cells showed 3.2-fold increase in drug sensitivity by cotreatment with PKI166. PC3 and PC3M-MM2 cells showed 1.2- and 5.0-fold increase in drug sensitivity for CPT by cotreatment with PKI166, respectively. Similar results also were observed in MDR cells. Treatment of CEM cells with PKI166 caused 1.1-, 6.3-, and 1.2-fold increase in the sensitivity to VP-16, CPT, and VBL, respectively, whereas treatment of CEM/MDR cells with PKI166 caused 7.6-, 13.3-, and 3.6-fold increase in the sensitivity to VP-16, CPT, and VBL, respectively. Treatment of MCF-7 cells with PKI166 did not increase the sensitivity to VP-16 and DOX, whereas treatment of MCF7/MDR cells with PKI166 caused 3.8- and 3.2-fold increase in the sensitivity to VP-16 and DOX, respectively. These results suggest that chemosensitizing effect of PKI166 seems to be higher in metastatic and MDR cells than drug-sensitive parental cells, and thereby PKI166 can be an effective chemosensitizer for MDR cells as well as the highly metastatic cells.

Because PKI166 could inhibit the activity of DNA-PK, we investigated whether PKI166 could suppress the induction of DNA-PK by VP-16 in A375SM cells. When the A375SM cells were treated with VP-16 in the presence or absence of 0.1 μM PKI166 for 6 h, VP-16-induced DNA-PK activation was
markedly inhibited by PKI166 treatment (Fig. 6). These results demonstrate that anticancer drug-induced DNA-PK activation in metastatic cells can be prevented by treatment with EGFR inhibitors, and this leads to enhancement of cytotoxic effect of anticancer drugs.

**Discussion**

In this study, we compared the levels of antiapoptotic molecules between metastatic cancer cells and their parental cancer cells to understand the mechanism of therapy resistance in metastatic cancer cells and thereby to determine the targets for treatment of metastatic and therapy-resistant cancers. Using the human cancer cells with different metastatic potential and tissue origin, we revealed that in various metastatic cancer cell lines, the antiapoptotic proteins such as Bcl-2, MDM2, NF-κB, DNA-PK, and EGFR were increased, whereas proapoptotic proteins such as Bax and p53
were decreased, compared with their parental cancer cell lines, and an inhibitor of EGFR improved chemosensitivity of metastatic cancer cells at least via suppression of DNA-PK.

Because the increase of antiapoptotic proteins and decrease of proapoptotic proteins are often associated with therapy resistance (Mow et al., 2001; Debatin, 2004), and our results showed that both metastatic cells and drug-resistant cells have similar expression patterns of apoptosis-controlling molecules, metastatic cells seemed to be associated with apoptotic resistance, suggesting that it may be difficult to treat metastatic cancer due to resistance to anticancer drugs and ionizing radiation. Although we do not understand the correlation between the molecules studied here, there is some evidence showing that Bcl-2 family proteins and DNA-PK can be modulated by EGFR. For example, the increase of Bax and decrease of Bcl-2 and DNA-PK were observed after EGFR-antisense transfection and treatment with C225, an anti-EGFR antibody or ZD1839 (Iressa) (Huang et al., 1999; Wang et al., 1999; Magne et al., 2003).

Many cancer therapeutics target and disrupt DNA function through adduct formation or by causing single- and double-chain scissions. DNA-PK participates in the repair of DNA double strand breaks by activating the nonhomologous end-joining pathway and could play an important role in conferring cells resistant to ionizing radiation (Sirzen et al., 1999) or DNA-damaging anticancer drugs (Muller and Salles, 1997; Shen et al., 1998; Kim et al., 1999, 2000). Although the expression of DNA-PK was shown to be suppressed by treatment with ZD1839 (Magne et al., 2003) and increased concomitantly with EGFR in metastatic cancer cells, the correlation between DNA-PK and EGFR is not clear. However, the expression of EGFR was increased by overexpression of Ku and decreased by deficiency of Ku and DNA-PKcs, and the expression of DNA-PK was suppressed by treatment with PKI166. Therefore, it seemed that the expression of DNA-PK and EGFR was positively correlated.

There has been a growing body of opinion that cancer cells progress from a less malignant to a more malignant (metastatic) phenotype due to genetic instability (Usmani and Sherbet, 1996; Webb and Vande Woude, 2000; Zhang et al., 2004), which could be resulted from inappropriately activated DNA repair pathways stimulated by growth factors (Harris, 1985). It was reported that overactivity of the Ku-mediated repair, which resulted in repair infidelity, is a candidate mechanism for chromosomal instability (Gaymes et al., 2002). Therefore, it seems likely that the enhanced DNA-PK activity could be associated with aberrant use of DNA repair, which may contribute to cancer progression and metastatic potency as well as induction of therapy resistance. Because both DNA-PK and EGFR are associated with therapy resistance and positively correlated, blocking one of them would lead to recovery of therapy resistance in metastatic cancers. With the activation of growth factor receptors represents a promising strategy for the development of novel and selective anticancer therapies (Zwick et al., 2002), and small molecule EGFR tyrosine kinase inhibitors represent one of the most promising classes of anticancer drugs (Jannaat and Giacone, 2003). Therefore, we have used PKI166 to target EGFR. In the present study, treatment with PKI166 resulted in decrease of DNA-PK components as well as EGFR in metastatic cancer cells. This was followed by potentiation of cytotoxicity induced by various DNA-damaging agents, especially in various metastatic and drug-resistant cancer cells compared with parental cancer cells. Probably, these results may be due to the suppression of strong stress responses including activation of DNA-PK by PKI166 in metastatic cancer cells. Potentiation of chemosensitivity by Iressa and C225 was observed in various cancer cell lines, irrespectively to basal EGFR levels in some experiments (Ciardiello et al., 1999, 2000; Sirotnak et al., 2000). Although we could not exclude the possibility of dual or nonspecific effects of PKI166, which has been known to inhibit both the intracellular domain of the EGFR and the HER2 tyrosine kinases, EGFR inhibitor, including PKI166, may be more effective to advanced cancer than to early cancer, because there was a tendency that the level of EGFR was higher in highly metastatic sublines than in low metastatic sublines.

In conclusion, we suggest that the DNA-PK and EGFR-mediated signaling pathway, which seemed to be correlated with each other, might contribute to acquirement of metastatic phenotype as well as therapy resistance, and the inhibition of EGFR may be helpful to enhance the effect of anticancer drugs against therapy-resistant metastatic cancer cells via suppression of strong stress responses, including activation of DNA-PK.
autoantigen affects the susceptibility to anticancer drugs. Cancer Res 53:4012–4017.


