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ORIGINAL ARTICLE

Yi-He Ling · Nicholas J. Donato · Roman Perez-Soler

Sensitivity to topoisomerase I inhibitors and cisplatin is associated with epidermal growth factor receptor expression in human cervical squamous carcinoma ME180 sublines

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Abstract The relationship between expression and function of the epidermal growth factor (EGF) family of receptors and chemosensitivity remains controversial. We studied the chemosensitivity to various anticancer agents of human cervical squamous carcinoma ME 180 cells, and two resistant subclones, ME180/TNF and ME180/Pt, which also differ in their EGF receptor (EGFR) expression. Compared with ME180 cells, EGFR is overexpressed sixfold in ME180/TNF cells and is barely detectable in ME180/Pt cells. Cell cycle analysis by flow cytometry and BrdU incorporation into DNA showed a correlation between EGFR expression and percentage of cells in S phase and active DNA replication (35% in high EGFR-expressing ME180/TNF cells, 19% in non-EGFR-expressing ME180/Pt cells and 23% in parental, intermediate-level EGFR-expressing ME180 cells). By MTT assay and compared with parental, intermediate-level EGFR-expressing ME180 cells, high EGFR-expressing ME180/TNF cells had a three- to fourfold increased sensitivity to cisplatin, camptothecin (CPT), and topotecan, and low EGFR-expressing ME180/Pt cells had a five- to ninefold reduced sensitivity to the same agents. In contrast, the degree of cross-resistance with the topoisomerase II inhibitors doxorubicin and etoposide was minimal and the pattern of sensitivity to the anti-microtubulin agents vinblastine and paclitaxel was different, with a two- to fourfold decreased sensitivity in the high EGFR-expressing ME180/TNF cells and only a 1.5-fold decreased sensitivity in the low EGFR-expressing ME180/Pt cells. Neither alterations in intracellular CPT levels nor changes in topoisomerase I expression or activity, measured as ability to form DNA-protein complexes, were found to explain the differences in sensitivity to CPT among the three cell lines. Co-treatment with CP358774, a specific EGFR tyrosine kinase inhibitor, reduced the enhanced sensitivity of high EGFR-expressing ME180/TNF cells to the values observed in intermediate EGFR-expressing ME180 cells, but only reduced modestly the sensitivity of intermediate expressing ME180 cells. As a result, the resistance index of low EGFR-expressing ME180/Pt cells compared with intermediate EGFR-expressing ME180 cells was reduced only from five- to fourfold for cisplatin and from seven- to fourfold for CPT when ME180 cells were exposed to CP358774. CP358774 did not affect the sensitivity to either agent in low EGFR-expressing ME180/Pt cells. These results provide evidence that changes in EGFR expression or function may play a role in determining chemosensitivity to platinum and topoisomerase I poisons in some human tumor systems, and that the EGFR-related changes in chemosensitivity may vary depending on the level of EGFR expression and/or function.

Keywords Topoisomerase I inhibitors · Cisplatin · Epidermal growth factor receptor · ME180 sublines

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Y.-H. Ling (✉) · R. Perez-Soler
Kaplan Comprehensive Cancer Center,
New York University School of Medicine,
550 First Avenue, New York, NY 10016, USA
E-mail: lingy01@gcrc.med.nyu.edu
Tel.: +1-212-2638487
Fax: +1-212-2630715

N.J. Donato
Department of Bioimmunotherapy,
M.D. Anderson Cancer Center, Houston, TX 77030, USA

Introduction

The epidermal growth factor receptor (EGFR) is a 170-kilodalton transmembrane glycoprotein with specific tyrosine kinase activity. EGFR plays an important role in the regulation of the normal proliferation and differentiation, survival after stress, and transformation of epithelial cells [1, 2, 3]. Binding of EGF and transforming growth factor-alpha to EGFR originates a cell proliferation signal that leads to entry of cells in S phase,

in part through downregulation of the cyclin inhibitor p27 [4, 5, 6]. EGFR is commonly overexpressed in many epithelial cancers, including breast, ovarian, brain, bladder, head and neck, pancreas, and lung cancer [7, 8]. High EGFR expression has also been associated with a poor prognosis in some cases [9]. In tumor cells, EGFR blockade with monoclonal antibodies or specific EGFR tyrosine kinase inhibitors produces in general a cytostatic effect. However, in some tumor cell systems that depend on the EGFR pathway for cell survival, such blockade can induce apoptotic death [10].

The role of EGFR expression and other receptors from the same family in determining sensitivity to cytotoxic agents remains controversial. Some studies indicate that EGFR and Her-2 expression can be associated with relative chemoresistance, since these pathways can initiate a survival response for cells subjected to a cytotoxic insult [11, 12]. In contrast, other studies have demonstrated that overexpression or activation of EGFR can sensitize cells to the effects of chemotherapy, probably by enhancing cellular proliferation, which increases susceptibility to S and M phase-specific agents [13]. Combinations of cytotoxic agents and agents that disturb EGFR tyrosine kinase activity have also been shown to be synergistic, and surprisingly the synergism has been observed both with inhibitors and activators of the pathway [13, 14, 15].

The camptothecin (CPT) analogues topotecan and irinotecan have been approved for the treatment of relapsing ovarian carcinoma, small-cell lung cancer, and colorectal carcinoma. CPT and its analogues form a covalent complex with DNA and topoisomerase I (topo I), thus causing DNA single-strand breaks, inhibition of RNA transcription [16, 17], and apoptosis. Because of their selective topo I poisoning effect, the CPTs exert their cytotoxic effect predominantly in cells in S phase [18].

Since the EGFR pathway plays an important role in cell proliferation by promoting entry of cells in S phase [19] and topo I poisons are quite selectively toxic to cells in S phase [20], it is reasonable to hypothesize that cells with a higher proliferation rate, regulated by EGFR expression, might be more sensitive to topo I poisons. Under different selective pressures we have cloned variants of the human cervical squamous carcinoma ME180 cells that present distinct response patterns to various apoptotic triggers in the absence of changes in p53 function. The subline resistant to tumor necrosis factor (TNF), ME180/TNF, has a two- to threefold enhanced sensitivity to cisplatin and displays a six- to sevenfold EGFR overexpression, whereas the subclone resistant to cisplatin (ME180/Pt) has very low EGFR expression and displays p-53-independent overexpression of p21 [21]. We present here the cytotoxicity of these three cell lines to a variety of cytotoxic agents. Interestingly, we observed that CPT is cross-resistant with cisplatin in these cell lines, suggesting a similar correlation between decreased EGFR expression and resistance to topo I poisons. Neither changes in drug uptake nor in formation of topo I-DNA complexes were found to be associated with topo I poison sensitivity in these cell lines. In addition, co-treatment with the specific EGFR tyrosine kinase inhibitor CP358774 partially reversed the EGFR-associated enhanced chemosensitivity to topo I poisons. No cross-resistance with other agents was observed. These results suggest that, at least in some human tumor systems, expression and/or activation of EGFR within a certain range may confer an enhanced sensitivity to some anticancer agents.

Materials and methods

Chemicals and drugs

CPT was purchased from Sigma (St. Louis, Mo., USA) and the stock solution (1 mg/ml) was dissolved in dimethyl sulfoxide (DMSO) and stored at –20 C until use. Topotecan was a gift from SmithKline Beecham Pharmaceutical and was dissolved in phosphate-buffered saline (PBS). CP-358774, a specific EGFR tyrosine kinase inhibitor, was kindly provided by Pfizer and was dissolved in DMSO as a stock solution (1 mg/ml). Doxorubicin was obtained from Cetus (Emeryville, Calif., USA). Other anticancer agents were purchased from Sigma. Monoclonal anti-topo I antibody was a kind gift from Dr. Y.-C. Cheng (Yale University, New Haven, Conn., USA). Monoclonal anti-MDR and anti-EGFR (Ab-2) antibodies were purchased from Oncogene Sciences (Cambridge, Mass., USA). [^{3}H]-thymidine was purchased from Amersham (Arlington Heights, Ill., USA).

Cell culture and treatment

Wild type ME180, ME180/TNF, and ME180/Pt cells were maintained as a monolayer culture as described previously [22]. In brief, cells were incubated in DMEM medium with 10% fetal bovine serum and 100 mM glutamine at 37 C in a humidified incubator with 5% CO_2 and 95% air. Exponentially growing cells (0.1×10^5 cells/ml) were plated in 96-well microplates overnight and continuously exposed to different concentrations of anticancer agents for 72 h. Cell survival was determined by MTT dye reaction as described previously [23]. The ID_{50} value resulting in 50% cell killing was calculated from the graphical evaluation of the results.

Determination of cell growth characteristics

For cell growth rate analysis, cells (5×10^5 cells/ml) were plated in 12-well plates and incubated in DMEM medium with 10% fetal bovine serum and 100 mM glutamine at 37 C. At indicated time points, cells were harvested and the viable cell numbers were counted by trypan blue exclusion, and the doubling time determined graphically. For assay of DNA replication, cells were labelled with 20 μM BrdU (Baton Dickinson Immunocytometry System, Mountain View, Calif., USA) at 37 C for 1 h and detached with 0.25% trypsin. After fixing with 70% cold ethanol at 4 C for 4 h, cells were incubated with 0.1 HCl, 0.5% Triton X-100 at 4 C for 10 min, washed twice with water, and cells were incubated with 100 μl of PBS containing 0.1% bovine serum albumin, 0.5 μg fluorescein isothiocyanate-conjugated BrdU antibody at room temperature for 30 min in a dark room. The labelled cells were counted in a Becton Dickinson flow cytometer. For cell cycle analysis, cells were fixed with ethanol at 4 C overnight, treated with 500 units of RNase at 37 C for 1 h, and cellular DNA was stained with 10 μg/ml propidium iodide for 2 h. Cells were analyzed with a Becton Dickinson flow cytometer.

CPT uptake measurement

Exponentially growing cells were exposed to different concentrations of CPT at 37 C for 30 min. After removal of medium, cells were washed three times with cold Ca^{2+}-, Mg^{2+}-free PBS three times and detached with 0.5 ml of 0.25% trypsin. CPT was extracted by chloroform and measured in a Perkin Elmer MPF-44 A spectrofluorometer (Perkin-Elmer, Norwalk, Conn., USA). Excitation and emission wavelengths for CPT assay were 370 nm and 480 nm, respectively.

DNA-protein complex assay

DNA-protein complexes were determined as described by Trask et al. [24]. In brief, cells were labelled with 1 μCi of [^{3}H]-thymidine at 37 C for 24 h and then labelled cells were treated with different concentrations of CPT at 37 C for 1 h. Cells were lyzed with 1 ml of lysis buffer containing 1.25% sodium dodecyl sulfate (SDS), 5 mM EGTA (pH 8.0), 0.4 mg/ml of thymus DNA, and 130 mM KCl at 65 C for 10 min. Following incubation in an ice bath for 5 min, DNA-protein complexes were precipitated by centrifugation at 3,000 rpm at 4 C and washed three times with washing buffer containing 10 mM TRIS-HCl (pH 8.0), 100 mM KCl, 1 mM EGTA, and 0.1 mg/ml of thymus DNA. The labelled DNA-protein complex was determined by liquid scintillation counting.

Western blot analysis

Cells were lyzed with lysis buffer containing 50 mM TRIS-HCl (pH 7.4), 0.1% Triton X-100, 1% SDS, 250 mM NaCl, 1 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, and 10 μg/ml of aprotinin. The protein content of each sample was determined using a DC protein assay kit (Bio-Rad, Hercules, Calif., USA). An equal amount of lysate from each sample was separated by SDS-polyacrylamide gel electrophoresis. The proteins were then transferred onto a nitrocellulose membrane. After blocking with 5% of nonfat milk in TRIS-buffered saline at room temperature for 1 h, the corresponding antibodies were used to probe for EGFR, topo I, and MDR gene products. The resulting immunoblots were analyzed using an ECL detection system according to the manufacturer's protocol (Amersham).

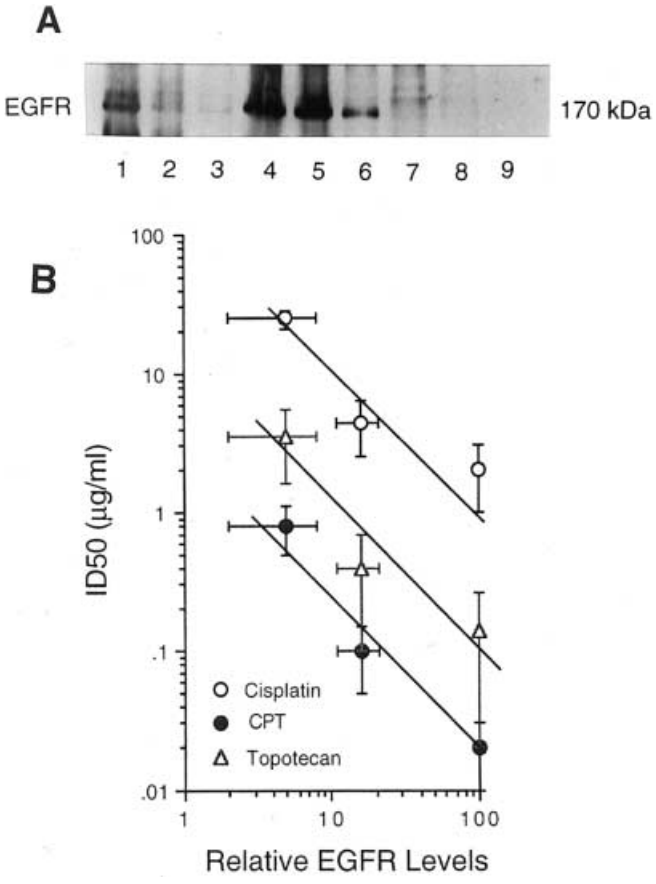

Fig. 1A, B Relationship between drug-induced cytotoxicity and relative levels of epidermal growth factor receptor (*EGFR*) in ME180, ME180/TNF, and ME180/Pt cells. **A** Expression of EGFR in different cell lines was determined by western blot analysis. Lanes1, 4, 7, loading 50 μg; 2, 5, 8, loading 25 μg; and 3, 6, 9 loading 10 μg of lysates from ME180, ME180/TNF, and ME180/Pt cells, respectively. **B** The relationship between ID_{50} values from Table 1 and relative levels of EGFR from quantification of EGFR by a laser densitometer. Each point is mean ± 3 independent experiments (*CPT* camptothecin, *kDa* kilodaltons)

Results

EGFR expression

We have previously cloned TNF- and cisplatin-resistant sublines of the human cervical squamous carcinoma ME180 and determined the EGFR expression of these cell lines by western blot analysis. Compared with parental ME 180 cells, the relative EGFR expression in ME180/TNF cells is increased by about sixfold, whereas EGFR expression is barely detectable in ME180/Pt cells (Fig. 1A).

Cytotoxicity

We determined the cytotoxic effects of different anticancer agents in these cell lines using a 72-h continuous drug exposure. As shown in Table 1, ME180/TNF cells are two- to threefold more sensitive than the parental ME 180 cells to the DNA-damaging agent cisplatin and the topo I poisons CPT and topotecan, and slightly more sensitive to the topo II poisons doxorubicin and etoposide. The ID_{50} values are about 2.3-, 3.0-, 2.8-fold ($P < 0.05$) lower for cisplatin, CPT, and topotecan, and about 1.6-fold lower for doxorubicin and etoposide in ME180/TNF cells compared with those in parental ME180 cells, respectively. In contrast, ME180/TNF cells are more resistant to the anti-microtubulin agents paclitaxel and vinblastine. The ID_{50} values for paclitaxel and vinblastine in ME180/TNF cells are 4.2- ($P < 0.01$) and 1.9-fold higher than those in the parental ME180 cells. ME180/Pt cells display a fivefold resistance to the DNA-damaging agent cisplatin, complete cross-resistance to the topo I poisons CPT and topotecan, and a much lower level of cross-resistance to topo II poisons and anti-microtubulin agents. The ID_{50} values are 5.5-, 6.6-, and 9.8-fold ($P < 0.01$) higher for cisplatin, CPT, and topotecan, 2.6-fold ($P < 0.01$) higher for etoposide, and only about 1.5-fold higher for doxorubicin, paclitaxel, and vinblastine in ME180/Pt cells compared

Table 1 Cytotoxicity of different anticancer drugs against human cervical carcinoma ME180, ME180/TNF, and ME180/Pt cells (*EGFR* epidermal growth factor receptor, CPT camptothecin)

Drugs	ID_{50} (μg/ml)				
	ME180 (intermediate EGFR)	ME180/TNF (high EGFR)	RI[a]	ME180/Pt (low EGFR)	RI[b]
Cisplatin	4.36 ± 1.99	1.88 ± 1.13*	0.4	23.75 ± 3.8**	5.5
CPT	0.12 ± 0.08	0.03 ± 0.01*	0.3	0.79 ± 0.39**	6.6
Topotecan	0.36 ± 0.28	0.14 ± 0.12*	0.4	3.54 ± 2.29**	9.8
Doxorubicin	0.25 ± 0.06	0.15 ± 0.11	0.6	0.38 ± 0.04	1.5
Etoposide	3.50 ± 0.53	3.03 ± 1.01	0.9	9.00 ± 0.71**	2.6
Paclitaxel	0.62 ± 0.18	2.60 ± 0.21**	4.2	1.00 ± 0.03**	1.6
Vinblastine	0.61 ± 0.19	1.17 ± 1.59	1.9	0.94 ± 0.39	1.5

Cells were exposed to various concentrations of drugs at 37 C for 72 h. Cytotoxicity was determined by MTT assay. Data represent the mean ± SD of 4 independent experiments
*$P < 0.05$, **$P < 0.01$ compared with ME180 cells
[a]RI: the ratio ID_{50}(ME180/TNF)/ID_{50}(ME180)
[b]RI: the ratio ID_{50}(ME180/Pt)/ID_{50}(ME180)

with those in ME180 cells, respectively. Figure 1B is a plot of the relationship between cytotoxicity and the relative expression of EGFR in these cell lines. The cytotoxicity induced by either the DNA-damaging agent cisplatin or by the topo I poisons CPT and topotecan is directly correlated with EGFR expression.

CPT cellular accumulation

We investigated whether the differences in CPT sensitivity in these cell lines were related to changes in intracellular CPT accumulation and P-glycoprotein (PGP) expression. We exposed cells to different concentrations of CPT at 37 C and determined the intracellular CPT accumulation at 30 min. As shown in Fig. 2B, the intracellular accumulation of CPT was directly related to the CPT concentration used, but no significant differences in intracellular CPT accumulation were observed among the three cell lines. None of the cell lines was found to express PGP as assessed by western blot analysis (Fig. 2A).

Topo I expression and function

Because topo I is the major target molecule for CPT, we next determined whether changes in topo I expression and/or activity could explain the differences in sensitivity to CPT. First, we used a monoclonal anti-topo I antibody to determine topo I expression and found that the three cell lines expressed similar amounts of topo I (Fig. 3A). Subsequently, we utilized the KCl-SDS assay to determine the formation of DNA-topo I complexes in cells treated with different concentrations of CPT. As shown in Fig. 3B, the formation of DNA-protein complex was increased as the CPT concentration increased, but DNA-protein complex formation in ME180/TNF cells was similar to that in ME180 cells, and only slightly reduced in ME180/Pt cells. All data indicate that the different sensitivity of these cell lines to CPT is not associated with alterations in topo I expression or its ability to form cleavable complexes.

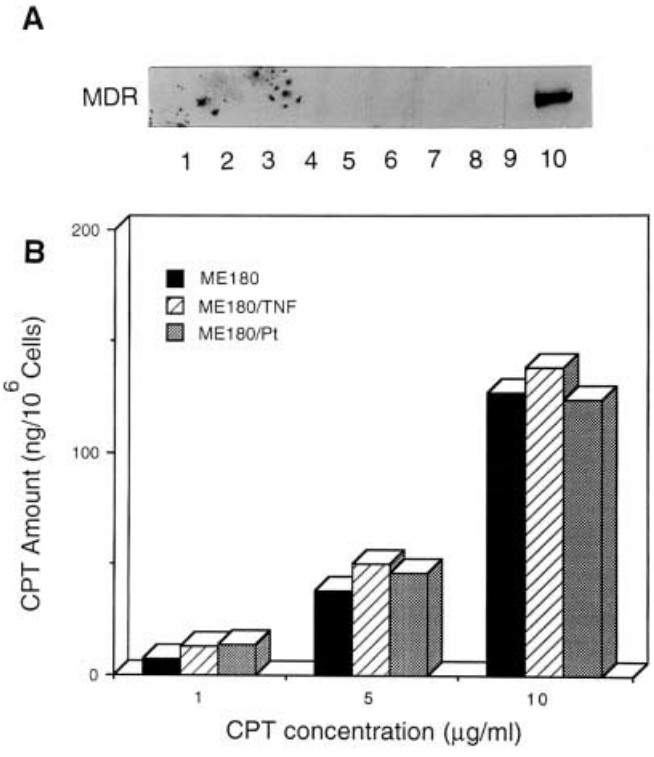

Fig. 2A, B Expression of MDR protein and intracellular concentrations of CPT in ME180, ME180/TNF, and ME180/Pt cells. **A** Expression of MDR was determined by monoclonal anti-MDR antibody. Lanes 1, 4, 7, loading 50 μg; 2, 5, 8, loading 25 μg; and 3, 6, 9, loading 10 μg of lysates from ME180, ME180/TNF, and ME180/Ptcells, respectively. Lane 10, loading 10 μg of lysate from MCF-7/Dox cells as positive control. **B** Cells were exposed to different concentrations of CPT at 37 C for 30 min and then the intracellular amount of CPT was determined. Each point is mean of two independent experiments

Correlation between chemosensitivity and cell proliferation

Because the EGFR pathway plays an important role in cell proliferation and the cytotoxic effects of many anticancer agents are highly dependent on cell prolifer-

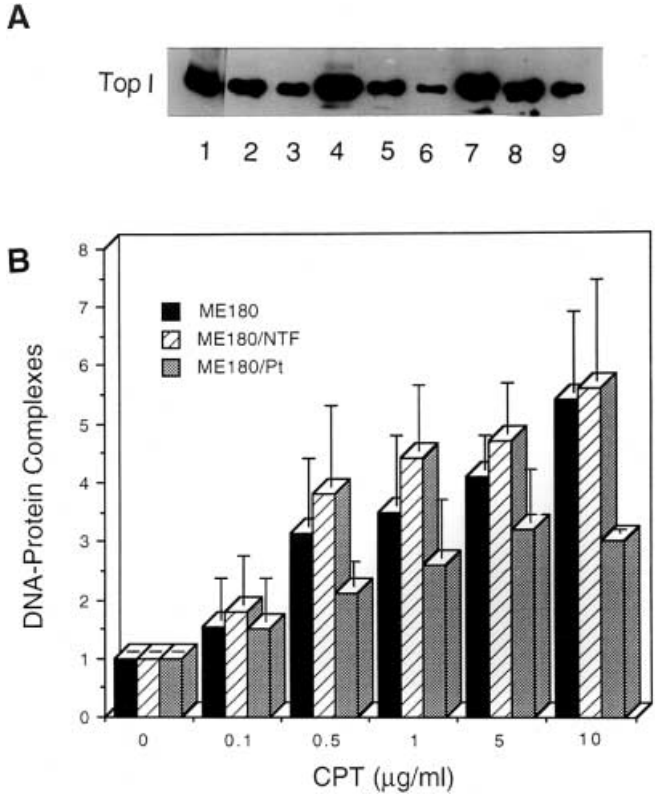

Fig. 3A, B Expression of topoisomerase I (*Topo I*) and the formation of DNA-protein complex caused by CPT in ME180, ME180/TNF, and ME180/Pt cells. **A** Topo I protein was determined by western blot analysis. Lanes 1, 4, 7, loading 50 μg; 2, 5, 8, loading 25 μg; and 3, 6, 9, loading 10 μg of lysates from ME180, ME180/TNF, and ME180/Pt cells, respectively. **B** [^{3}H]-thymidine-labelled cells were exposed to different concentrations of CPT at 37 C for 1 h and then the formation of DNA-protein complex was determined by cell lysis, followed by precipitation and washing of complexes, and liquid scintillation counting. Each point is mean ± 3 independent experiments

ation [25], we next investigated whether the observed differences in cellular sensitivity could be explained by EGFR pathway-related changes in cell proliferation. We assessed the cellular growth rate, cell cycle distribution, and DNA replication rate of the three cell lines by determining their doubling time, propidium iodide staining, and BrdU incorporation into DNA, respectively. As shown in Table 2, the doubling time was about 1.8-fold shorter in ME180/TNF cells (19 h vs. 33.7 h) and about 1.5-fold longer in ME180/Pt cells (49.7 h vs. 33.7 h) compared with that in ME180 cells, respectively. The percentage of cells in S phase and incorporating BrdU was highest in EGFR-expressing ME180/TNF cells (32%), lowest in non-EGFR-expressing ME180/Pt cells (19%), and intermediate in parental ME180 cells (23%). These results indicate that EGFR overexpression is associated with increased cellular proliferation. However, the differences in proliferation appear to be disproportionately smaller than the differences in cytotoxicity, particularly between ME180/Pt and parental ME180 cells.

Table 2 Growth characteristics of cervical carcinoma ME-180, ME-180/TNF, and ME-180/Pt cell lines (*DT* doubling time)

Cells	DT (h)	% Cells			
		G1	S	G2/M	BrdU
ME-180	33.7 ± 3.3	64 ± 6	23 ± 4	13 ± 2	23 ± 2
ME-180/TNF	19.0 ± 2.6	58 ± 12	32 ± 5	11 ± 8	36 ± 7
ME-180/Pt	49.7 ± 4.0	61 ± 2	19 ± 2	18 ± 2	19 ± 6

Cells were incubated in the presence of 1 μM BrdU at 37°C for 1 h, fixed with 75% cold ethanol for 5 min, and reacted with fluorescein isothiocyanate-labelled anti-BrdU antibody. DNA was stained with propidium iodide. The BrdU incorporation into DNA and cell cycle. distribution were determined by flow cytometry. Each data point represents the mean ± SD of three independent experiments

Effect of specific EGFR tyrosine kinase inhibitor CP358774

EGFR tyrosine kinase activity is triggered by binding of EGF to the EGFR ectodomain, thus inducing EGFR autophosphorylation, and initiation of intracellular signalling [26]. We investigated whether the inhibition of EGFR tyrosine kinase activity could affect the cytotoxic effects of cisplatin and CPT in these three cell lines. Cells were continuously exposed to different concentrations of cisplatin and CPT in the absence or the presence of 0.1 μM CP358774, a specific EGFR tyrosine kinase inhibitor [27] for 72 h, and then the cytotoxicity was determined by MTT assay. As shown in Table 3, co-treatment with CP358774 decreased the sensitivity of ME180/TNF cells (high EGFR expressors) to cisplatin and CPT to the level of sensitivity of parental ME180 cells (intermediate EGFR expressors) and decreased the cellular sensitivity of parental ME180 cells by 1.5- to 2-fold ($P < 0.05$). However, the changes in sensitivity in intermediate EGFR-expressing ME180 cells were only modest. As a result, the resistance index of low EGFR-expressing ME180/Pt cells compared with intermediate EGFR-expressing ME180 cells was only reduced from five- to fourfold for cisplatin and from seven- to fourfold for CPT when ME180 cells were exposed to the inhibitor. In non-EGFR-expressing ME180/Pt cells, cellular sensitivity to cisplatin and CPT was unchanged by CP358774.

Discussion

The results of this study indicate that: (1) acquired resistance to cisplatin in cervical carcinoma ME 180/Pt cells confers cross-resistance to topo I poisons but not to topo II poisons or antitubulin agents and is associated with EGFR downregulation and a decreased cellular proliferation rate; (2) resistance to topo I poisons in ME180/Pt cells is not mediated by changes in cellular drug accumulation nor alterations in the expression or function of topo I; and (3) sensitivity to topo I poisons in high EGFR-expressing ME 180/TNF cells is restored to

Table 3 Effect of EGFR tyrosine kinase inhibitor, CP358774, on cisplatin- and CPT-induced cytotoxicity in cervical carcinoma ME180, ME180/TNF, and ME180/Pt cell lines

Treatment	ID_{50} (μg/ml)		
	ME180/TNF (high EGFR)	ME180 (intermediate EGFR)	ME180/Pt (low EGFR)
Cisplatin	3.12 ± 1.07	4.16 ± 0.77	20.23 ± 5.36
+ CP358774	4.15 ± 1.28	5.66 ± 1.43	19.36 + 7.18
CPT	0.03 ± 0.01	0.07 ± 0.02	0.51 ± 0.18
+ CP378774	0.06 ± 0.02*	0.13 ± 0.09	0.57 ± 0.24

Cells were exposed to various concentrations of cisplatin and CPT in the absence or presence of CP358774 at 0.1 μM at 37 C for 72 h. Cytotoxicity was determined by MTT assay. Each data point represents the mean ± SD of 4 independent experiments. *$P < 0.05$ compared with CPT alone

that of intermediate EGFR-expressing ME 180 cells by EGFR tyrosine kinase inhibition, but only modestly affected in intermediate EGFR-expressing ME180 cells. Overall, these studies suggest that, at least in some tumor systems, a certain level of EGFR expression may be a requirement and a determinant for sensitivity to platinum and topo I poisons.

The relationship between the expression and function of the EGFR family of cellular receptors and chemosensitivity is highly complex, because disruption of the EGFR pathway may result in potentially opposite effects and such effects vary from tumor to tumor. One aspect of this complex relationship is whether baseline EGFR expression is a determinant of intrinsic chemoresistance or chemosensitivity and whether such effects are proportional to EGFR expression across all or broad ranges of expression. EGFR expression may be a determinant of chemoresistance in a particular tumor cell system if activation of the EGFR pathway is required for appropriate repair of cell damage caused by the cytotoxic agents, as has been previously suggested [19]. EGFR expression may at the same time and in the same system be a determinant of chemosensitivity if the cellular proliferation rate is EGFR expression dependent, since the ability of specific cytotoxic agents to inflict cellular damage is highly dependent on active cell proliferation. The interplay of these opposite effects in each particular tumor is what is likely to determine whether EGFR expression or activation confers chemoresistance or chemosensitivity.

A second aspect of this complex relationship is whether EGFR expression modulation is involved or mediates acquired resistance of tumor cells to different cytotoxic agents. Most cytotoxic agents are more effective in cells that are actively cycling because they affect functions that are essential for progression through S or M phases. Therefore, cells could protect themselves from the cytotoxic effects of chemotherapy by decreasing their cellular proliferation by downregulating EGFR and/or other cell growth regulatory pathways. Our results and those of other studies support this hypothesis but suggest that downregulation of the EGFR pathway is only partially responsible for such mechanism, thus suggesting that other growth regulatory pathways may also be involved [28].

Finally, a third aspect is how disturbance of EGFR function, either inhibition or stimulation, affects chemosensitivity, and whether such effects are uniform or variable across different ranges of EGFR expression. Since tumors vary in the intactness of the G1/S checkpoint, disruption of EGFR function may produce opposite effects in different tumors. In cells highly EGFR dependent for growth and survival, inhibition of EGFR function may enhance the chemotherapeutic effect by inhibiting damage repair. However, a net synergistic effect prevails only when inhibition of damage repair offsets the protection from cytotoxicity conferred by the resulting decreased cell proliferation. The opposite may occur in cells that are not solely dependent on the EGFR pathway for survival. Similarly, EGFR stimulation above a certain threshold may sensitize cells to chemotherapy by stimulating cell proliferation, provided that this effect offsets the putative enhanced damage repair.

Our study clearly suggests that in some human cancer systems, EGFR downregulation may be a contributory mechanism to the acquired resistance to cisplatin and topo I poisons. It is tempting to speculate that a reduced cell proliferation secondary to downregulation of the EGFR and other cell regulatory pathways might be an adaptation mechanism that develops in cells exposed to these chemotherapeutic agents. It is surprising that such reduced cellular proliferation does not alter the sensitivity to other cytotoxic agents. However, a likely explanation is that the impact of changes in cellular proliferation on the processing of cell damage and repair produced by different agent may vary among the agents.

Some clinical observations indirectly support a relationship between EGFR overexpression and chemosensitivity to topo I poisons, but there are no clinical data available supporting a relationship between acquired resistance to topo I poisons and EGFR downregulation. Squamous cell carcinoma of the lung expresses a higher level of EGFR receptors and displays a higher proliferation rate than adenocarcinoma [29]. Topotecan has been found to be more active against squamous cell carcinoma than adenocarcinoma of the lung [30]. In contrast, regarding the putative cross-resistance between cisplatin and topo I poisons suggested by our study, clinical studies with topo I poison in ovarian carcinoma do not support this, since topotecan has definite activity in cisplatin-resistant tumors [31].

Intrinsic expression of Her-2 has been directly correlated with chemoresistance in a panel of human lung cancer cell lines [11]. No similar correlation with EGFR

expression has been reported to date. However, data suggesting the opposite and supporting our results have been reported by Dixit et al. [32]. Treatment of MDA-468 breast cancer cells with EGFR antisense RNA was found to abrogate cisplatin-induced apoptosis, thus indicating that a critical threshold of EGFR expression appears to be required for cisplatin cytotoxicity. We have previously reported that stimulation of the EGFR pathway with EGF in these cell lines partially restores the sensitivity of the low EGFR-expressing ME180/Pt cells to cisplatin but does not enhance the sensitivity of the other subvariants [21]. In this study, we found that inhibition of EGFR tyrosine kinase reduced the sensitivity of the high EGFR-expressing cells to that of the intermediate EGFR-expressing cells, but only modestly decreased the sensitivity of the latter and did not affect the sensitivity of the low EGFR-expressing cells. All these results together suggest that changes in sensitivity as a result of EGFR activation or inhibition may only occur within a certain range of expression or activation.

The role of Her-2 in mediating chemosensitivity in breast cancer is better known. Her-2 is overexpressed as a result of gene amplification in about 20% of breast cancers [33]. If anything, a critical analysis of the clinical experience in breast cancer supports a dose- response to doxorubicin-containing regimens in Her-2-positive tumors, which is not observed in Her-2-negative tumors, suggesting a stronger relationship between overexpression and sensitivity rather than downregulation and resistance. In a retrospective analysis of adjuvant therapy trials, Her-2-negative tumors appear to do as well with CMF (Cytoxan, methotrexate, 5-fluorouracil) as with a doxorubicin-containing regimen. Her-2-positive tumors do better with a doxorubicin-containing regimen than with CMF, thus indicating that Her-2 expression mediates a certain chemosensitivity to doxorubicin [34, 35, 36]. In addition, Her-2-positive tumors do as well as Her-2-negative tumors with CMF, thus indicating that Her-2 does not mediate resistance to CMF. There are no clinical data in breast cancer on the potential relationship between Her-2 expression and clinical sensitivity to cisplatin or topo I inhibitors, since these agents are not commonly used in the treatment of breast cancer.

In addition to changes in cell proliferation, there are several possible mechanisms of cell resistance to CPT, including alterations in topo I levels [37], decreased topo I activity secondary to gene mutation or loss of some cofactor [38], increased repair of topo I-induced DNA breaks [39], and alterations in apoptotic pathways. We did not find any differences in topo I expression and activity in ME180/Pt cells, but we did not investigate the possibility of a gene mutation, increased DNA repair activity in the resistant cells, or alterations in apoptotic pathways shared by topo I poisons and cisplatin. In the case of cisplatin, ME180 cells were found to be less efficient in repairing DNA-Pt adducts, but the difference did not appear to account for the fivefold resistance observed [21].

In summary, this study sheds some new light on the role of the EGFR signal transduction pathway in mediating the response of tumor cells to cytotoxic agents. Our results using variants of a single tumor cell system indicate that EGFR overexpression above a certain level of expression may be associated with enhanced chemosensitivity, whereas downregulation below a certain level of expression may be associated but not solely responsible for acquired resistance to topo I poisons and cisplatin. However, these results may not be applicable to all tumor cell systems. Since the EGFR pathway is involved in regulating cellular functions related both to susceptibility to cytotoxic damage and damage repair, its disruption may cause opposite effects in cell systems that differ in their levels of EGFR expression, activation, dependence on the EGFR pathway for proliferation and survival, and intactness of the G1/S checkpoint.

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