EGF Receptor and p21WAF1 Expression are Reciprocally Altered as ME-180 Cervical Carcinoma Cells Progress from High to Low Cisplatin Sensitivity

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ABSTRACT

Cell cycle regulators and signal transduction pathways can influence apoptotic sensitivity of tumor cells, and we previously described an association between EGFr overexpression, reduced DNA repair activity, and increased apoptotic sensitivity of ME-180 cervical carcinoma cells toward cis-diammedichloroplatinum (cDDP; K. Nishikawa, et al., Cancer Res., 52: 4758–4765, 1992). In the present study, the characteristics of ME-180 cells selected for high or low apoptotic sensitivity to cDDP (or camptothecin) were examined and compared to determine whether signal transduction components and cell cycle regulation were distinct in these isogenic drug response variant populations. As ME-180 cells progressed from high to low cDDP sensitivity [IC50 ~80 ng/ml in cDDP sensitive (PT-S) to ~2000 ng/ml in cDDP-resistant (PT-R) cells], there was a significant decrease in EGFr expression that paralleled the relative reduction in cDDP apoptotic responsiveness (~30-fold). cDDP-resistant cells had the slowest rate of growth and more effectively reduced DNA adduct levels following cDDP exposure than parental cells. Cellular levels of the cell cycle inhibitor p21WAF1 inversely correlated with cDDP responsiveness with high levels of p21WAF1 expressed in drug-resistant Pt-R cells in the absence of elevated p53. cDDP stimulated a 2-fold increase in p53 levels in both drug-sensitive and drug-resistant cells but caused a delayed reduction in p21WAF1 levels, suggesting p53-independent regulation of p21WAF1 in ME-180 cells. Activation of EGFr in Pt-R cells stimulated cell cycle progression (2-fold), reduced p21WAF1 levels (>2-fold), and increased sensitivity to cDDP (3-fold), suggesting that receptor signaling enhanced the efficacy of cDDP to induce cell death by relieving cell cycle restriction. These results demonstrate that the transition of ME-180 cells from a drug-sensitive to drug-resistant phenotype correlates with reciprocal changes in EGFr and p21WAF1 expression and provides additional evidence that the pathways controlled by these proteins may contribute to some forms of drug resistance.

INTRODUCTION

Signal transduction elements are frequent targets of mutation, alteration, and amplification during oncogenic transformation. Both receptors for growth factors and intermediate mediators of signaling pathways can therefore generate inappropriate or constitutive signals that activate several processes, including those promoting cell cycle progression, induction of metastasis, and increased cell survival (1, 2). However, although these processes encourage the outgrowth and development of tumor foci, they may also expose the transformed cell to possible elimination by host defenses or agents that target cells with high proliferative potential or aberrant cell cycle control (3, 4). In this regard, the selective toxicity of many chemotherapeutic agents may require a degree of cellular dysfunction to mediate their apoptotic effects on tumor cells. However, the role of specific signaling entities and the cellular context, which contributes to chemosensitivity of transformed cells, is only poorly understood.

EGFr4 and other proteins with structural and functional homology (HER2, HER3, and HER4) are frequently amplified or overexpressed in neoplastic cells (5, 6). Overexpression of the EGFr gene results in its increased transmembrane protein expression and activation of its tyrosine kinase activity through ligand-induced formation of homodimers. The activated receptor transduces signals through tyrosine phosphorylation of itself and other adaptor proteins that mediate the activation of proximal cascades, including phosphatidylinositol-3′ and mitogen-activated protein kinase (7, 8). These cascades can then regulate nuclear and cytoplasmic events controlling transformation, mitogenesis, and cell survival (9). Thus, the level of EGFr or homologous proteins and autocrine or paracrine growth factors that stimulate their activation are important elements in the transformation process, and increasing evidence suggests that

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4 The abbreviations used are: EGFr, epidermal growth factor receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; EGF, epidermal growth factor; FAAS, flame-atomic absorption spectrophotometry.
they may also influence cellular sensitivity to several antitumor agents.

Recent reports have confirmed that EGFr and related proteins influence chemotherapeutic and radiation sensitivity. Selection of cervical carcinoma cells for high levels of EGFr correlated with their increased sensitivity to cDDP (10). Conversely, antisense-mediated reduction of EGFr expression decreased breast carcinoma cell apoptotic sensitivity to cDDP (11). Ovarian cancer cells resistant to cDDP expressed reduced levels of HER2, thereby providing evidence that changes in cDDP responsiveness correlate with modulation of receptor kinases in several tumor cell types (12). However, overexpression of EGFr or HER2 proteins alone does not account for chemosensitivity in all tumors (13), and attempts to modulate EGFr or HER2 levels (or their state of activation) to amplify chemotherapeutic efficacy have met with some success, but no clear mechanism of action has emerged. EGF and anti-EGFr antibodies have been shown to induce both receptor activation and inhibition but have similar effects on cDDP chemosensitization (14, 15). Similar divergent influences of anti-HER2 agents and activating ligands have been associated with increased drug sensitivity in breast and other cancers (16–18). These studies suggest that expression of these proteins may provide opportunities for modifying chemotherapeutic responses, but the overall influence of these molecules on drug-induced apoptosis may be controlled by other elements or downstream cascades controlled by these receptors.

The stress and cell cycle regulatory protein, p53, is able to regulate apoptotic responses following a number of stress-inducing episodes, including radiation, chemotherapy, and cytokines (19). However, as described above, other genes or cascades may be critical to the overall influence of p53 on apoptosis. Studies have shown that both chemoprotection and sensitization can be promoted by wild-type p53, and differential effects of this protein on chemoresponsiveness may be related to its transactivation of downstream regulators of apoptosis or cell cycle control (20, 21). In this regard, p21WAF1 may play an important regulatory role because its cell cycle inhibitory activity can be regulated by p53 and can influence the apoptotic response to DNA damaging agents (22, 23). Studies of p21WAF1 in clinical specimens have shown that high levels of this protein are associated with chemoresistance and poor patient prognosis (24, 25), and cells derived from p21WAF1 knockout mice have greater sensitivity to DNA damaging drugs and radiation (26). Through cell cycle checkpoint restriction, p21WAF1 can reduce the toxicity of DNA damaging agents by increasing repair intervals or by directly coordinating DNA repair activity through interaction with the proliferating cell nuclear antigen (22, 23, 26). The former action has also been proposed as a mechanism of chemoresistance induced by other cell cycle inhibitors (p27Kip1; Ref. 27). Therefore, control of cell cycle checkpoints by receptors capable of transmitting external signals to the nucleus (EGFr) and those endogenous regulators of cyclin-dependent kinases (p21WAF1) can play a significant and important role in determining apoptotic responsiveness to DNA damaging agents or conditions.

As previously described, the characteristics of a clonal variant ME-180 cell line selected for high sensitivity to cDDP suggested a role for EGFr overexpression in chemosensitivity (10). In this report, additional cell cycle studies of this cell line are described and compared to parental cells. In addition, to determine the role of EGFr and other proteins in drug resistance, a cDDP-resistant ME-180 cell variant was isolated from the ME-180 cell line and compared to both parental and highly cDDP-sensitive cells. This selection allows examination of characteristics associated with the cellular transition from a drug-sensitive to a drug-resistant phenotype from an isogenic background and supports a role for both EGFr and p21WAF1 as modulators of drug sensitivity.

**MATERIALS AND METHODS**

**Cell Lines.** ME-180 cervical carcinoma cells were originally purchased from American Type Culture Collection (Rockville, MD) and cultured in minimal essential media containing 10% FBS (Hyclone, Logan, UT). The highly cDDP-responsive ME-180 cell variant (Pt-S) was previously isolated and characterized (denoted as ME-180R in earlier publications; Ref. 10). By a similar strategy, a cDDP-resistant clone (Pt-R) was isolated from the parental population by culturing cells in increasing concentrations of cDDP (beginning at 0.1 μg/ml or 0.33 μM). Surviving cells were recovered and stabilized (by trypsinizing and transferring to drug-free media for 7 days) before additional selection with 2-fold greater concentrations of cDDP. This procedure was repeated until a population was selected that survived exposure to 2 μg/ml cDDP. This population was cloned by limiting dilution in culture media containing 1 μg/ml cDDP. Of the clones recovered following selection, one stable colony was obtained (Pt-R) that grew slowly in the absence of cDDP and was not apoptotic in the presence of cDDP (up to 1 μg/ml). This cell line maintained resistance to cDDP even after 2 months of passage in the absence of drug. When compared to the parental population, the clonal Pt-R population expressed ~8-fold resistance to cDDP and cross-resistance to camptothecin and topotecan, but near equivalent sensitivity to doxorubicin and paclitaxel.

**Cytotoxic and Apoptotic Measurements in Drug-Treated Cells.** Variant and parental cell sensitivity to cDDP (and other agents) was determined by crystal violet staining or MTT assay following drug exposure (at indicated concentrations) for 48–72 h (as noted) as previously described (10, 28). Drug-induced DNA fragmentation was also used to detect apoptotic cell death in parental and variant cell populations as previously described (28).

**Preparation of Cell Lysates and Immunoblotting.** Cell lysates were prepared in lysis buffer as previously described (29). Protein was quantitated (bicinchoninic acid reagent; Pierce Chemical), and equal protein aliquots were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies against p53 (Ab-6; Oncogene Sciences, Cambridge, MA) or p21WAF1 (Pharmingen, San Diego, CA or Transduction Labs, Lexington, KY). Primary antibody binding was detected with horseradish peroxidase-labeled secondary antibody (Bio-Rad, Richmond, CA) and enhanced chemiluminescence reagent (Amersham).

**Cell Cycle Analysis.** Cell cycle distribution was examined in confluent and subconfluent cultures of variant ME-180 cells. Briefly, 0.5 × 10⁶ cells were plated into 60-mm culture
plates, and cells were harvested by trypsinization at ∼30% (24–48 h) or near complete confluence (72–144 h). Harvested cells were fixed with 70% ethanol, stained with propidium iodide, sorted on a Becton Dickinson FACScan, and quantitated as previously described (28).

To determine the effect of EGF on cell cycle distribution, cDDP-response variant ME-180 cells were plated at a density of 10^6 cells (60 mm) in complete growth medium (minimal essential media + 10% FBS) and incubated 18 h to allow adherence. Complete growth medium was removed, and cells were rinsed in serum-free medium and incubated 24 h in medium containing 0.2% FBS. Under these conditions, serum-mediated cell cycle progression is restricted, thereby providing a means to measure EGF-stimulated cell cycle alterations in synchronous populations. Cells were treated with 10 nM EGF and harvested by trypsinization after 0, 8, 16, and 24 h. Cells were ethanol-fixed, stained with propidium iodide, and sorted by FACScan as described above.

**EGFr Expression and Tyrosine Kinase Activity.** Equal protein cell lysates were analyzed for EGFr expression by direct immunoblotting with anti-EGFr (Transduction Laboratories, Lexington, KY) and detected with secondary antibody as described above. EGFr was also immunoprecipitated (A108; Rorer Rhone-Poulenc; King of Prussia, PA) from cell lysates, washed extensively, and incubated in kinase buffer containing 32P-ATP (10 μCi) to measure EGFr tyrosine kinase activity as previously described (30).

**Measurement of Platinated-DNA Content in cDDP-Treated Cells.** Subconfluent parental or Pt-R cells (~1.5 × 10^6 cells in 60-mm culture plates) were treated with 30 μg/ml (100 μM) cDDP for 2 h at 37°C, washed free of drug, and directly harvested by trypsinization for an additional 14 h in normal growth media before harvesting. DNA was extracted from treated and control cells by chloroform/phenol extraction and precipitated with ethanol. The DNA pellets were air-dried and resuspended in Tris-EDTA, and A260/280 ratios were determined to estimate DNA content. Pt content was then determined by subjecting DNA aliquots from control and treated cells to FAAS as previously described (31). Pt standard curves were derived by “spiking” DNA samples (from untreated ME-180 cells) with known quantities of cDDP before FAAS analysis.

**Cell Growth Measurements.** Population doubling times were measured by direct cell counts of log phase cultures monitored over a 96-h period as previously described (30). Pt-R cell proliferation was also measured in the presence or absence of EGF with a commercially available proliferation assay. Five-thousand Pt-R cells were plated into individual wells of a 96-well plate and stimulated with 10 nM EGF for 72 h before quantitation by staining cells using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Absorbance in EGFr-treated cells (four replicates) was compared and reported as a fold increase over control cell values.

**Drugs and Growth Factor.** cDDP (cis-diaminedichloroplatinum) was kindly provided by Dr. Kenji Nishikawa (Nippon-Kayaku, Tokyo, Japan). Camptothecin was purchased from Sigma Chemical Co. (St. Louis, MO), and recombinant human EGF was purchased from R&D Systems (Minneapolis, MN).

**RESULTS**

Earlier studies of ME-180 cells provided evidence for EGFr overexpression in cells with increased sensitivity to select chemotherapeutic agents, including cDDP (10). More recent studies of breast carcinoma cells have suggested that specific reduction of EGFr expression reduces their sensitivity to cDDP-mediated apoptosis (11). To determine whether ME-180 cell survival following cDDP exposure results in an altered expression of EGFr, a clonal cDDP-resistant ME-180 cell line was established (Pt-R). As shown in Fig. 1, Pt-R cells were compared to highly cDDP-responsive (Pt-S) and parental cells for drug-induced DNA fragmentation and concentration-dependent cytotoxicity. Pt-R cells were 8-fold and 30-fold resistant to cDDP when compared to parental and Pt-S cells, respectively. Similar distinctions in camptothecin sensitivity were measurable in these cells, but no significant change in paclitaxel or doxorubicin sensitivity between parental and Pt-R cells was detected (data not shown). DNA fragmentation in cDDP- or camptothecin-treated cells also illustrated distinctions in apoptotic responsiveness between cell lines.
Signaling in cDDP-mediated Apoptosis

There was a parallel increase in p21WAF1 expression, and high levels in Pt-S, parental cells, and Pt-R cells, distinguishing basal p21WAF1 expression with low, intermediate, and high drug sensitivity (Pt-S). Untreated cells also expressed wide variation in p21WAF1 expression levels between Pt-R and Pt-S cells correlated with the parental cell line. Kinase activity measurements from Pt-R cells estimated an 8-fold or 30-fold reduction in EGFr kinase activity when compared to parental or Pt-S cells, respectively.

Because previous studies had suggested an important role for EGFr in drug-responsiveness (10, 11, 13–15), receptor expression was examined and compared between parental and variant cells. Measurement of receptor tyrosine kinase activity and receptor protein levels demonstrated that EGFr expression and tyrosine kinase activity were distinct in parental and variant cells, which leads to an examination of other cellular and biochemical characteristics that distinguish these cell lines.

The significant change in both EGFr and p21WAF1 levels as ME-180 cells express greater levels of drug resistance suggested that cell cycle control may also be affected. As previously reported (10), we were unable to detect major distinctions in the population doubling times for parental and cDDP-sensitive cells (26.3 ± 3.2 versus 28.1 ± 4.1 h). However, drug-resistant Pt-R cell growth was significantly reduced when compared to the parental population (63.5 ± 6.4 h), and cell cycle analysis suggested that increased accumulation of cDDP-resistant cells in both G1 and G2 correlated with their reduced cell growth (Fig. 4). Distinctions in cell cycle control were noted between sparsely seeded log phase cells and those approaching confluence, with drug-resistant Pt-R cells exhibiting greater G2 arrest under subconfluent conditions than cDDP-sensitive Pt-S cells (Table 1). However, as cells approached confluence, increased G1 arrest and a 3-fold reduction in S-phase fraction were noted in Pt-R cells. Therefore, selection for cDDP-resistance in ME-180 cells correlated with alterations in cell cycle control and expression of both positive and negative signal transduction molecules.

The efficacy of cDDP to induce apoptosis is dependent on several cellular properties, including the level of drug uptake, its stability in the cell, and its ability to form stable DNA adducts. The stability of the Pt-DNA adduct appears to be regulated by DNA repair processes that may be directly or indirectly regulated by cell cycle checkpoint proteins. To investigate the role of potential changes in DNA platination and adduct removal in cDDP-resistant Pt-R cells, platinated DNA levels were compared in Pt-R and parental cells after a 2-h incubation with and as shown in Fig. 2, a decrease in EGFr levels. Interestingly, high expression of p21WAF1 in Pt-R cells was not directly correlated with increased p53 expression, providing evidence for p53-independent regulation of p21WAF1 in Pt-R cells. Further, p53 mutations (exons 4–9) as determined by sequence analysis of RT-PCR products (32), were not detected in either Pt-S, Pt-R, or parental cells (data not shown), suggesting that p53 mutations alone do not account for changes in cDDP sensitivity or differential p21WAF1 expression in this cell model.
cDDP (Fig. 5). DNA platinum content was also measured 14 h after drug removal (before the onset of DNA fragmentation). The initial level of DNA platination was slightly lower in cDDP-resistant Pt-R cells than the parental population (53.6 versus 76.7 ng Pt/mg DNA, respectively), and following a 14-h incubation in the absence of drug, Pt-DNA levels were more effectively reduced in Pt-R cells (61.1% versus 39.8% for parental cells). Thus, reduced cDDP sensitivity in Pt-R cells may at least be partially controlled by their distinct capacity to minimize platinated DNA adduct levels. However, this small change in platination levels between cell lines does not account for their 8-fold difference in cDDP sensitivity or their parallel changes in sensitivity to other drugs with distinct mechanisms of action (camptothecin). This suggests the possibility that downstream elements are involved that reduce the lethality of specific drugs in Pt-R cells.

Wild-type p53 and p21WAF1 are important determinants of chemosensitivity, and their induction in response to cytotoxic agents may be important in triggering apoptosis. The induction of these proteins was therefore examined in cDDP-treated cells and, as shown in Fig. 6, p53 levels were increased in all cell lines after 6 h of cDDP exposure. Conversely, p21WAF1 levels (examined by immunoblotting the same cell lysates) were unchanged or partially reduced following cDDP incubation. A second experiment examining extracts prepared from cells treated for longer intervals (12 h) confirmed a reduction in p21WAF1 levels following cDDP treatment. Further examination of the early affects of cDDP treatment on p21WAF1 levels in Pt-S and Pt-R cells demonstrated that alterations in p21WAF1 levels were detected early after cDDP treatment (~60% of

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Fig. 4 Cell cycle analysis of clonal drug-response variant ME-180 cells. Pt-R or Pt-S cells were plated into 60-mm culture dishes and harvested by trypsinization at 30% (log-phase) or 90% confluence (near-confluent). Cells were collected by centrifugation, washed, fixed, and stained with propidium iodide as described in “Materials and Methods.” Stained cells were sorted by flow cytometry (above), and the results are tabulated below.

Fig. 5 Platinated DNA content in cDDP-treated ME-180 parental and Pt-R cells. ME-180 parental and Pt-R variant cells were treated with 100 μM cDDP (33 μg/ml) for 2 h, washed free of drug with media, and harvested immediately (0 h) or incubated for an additional 14 h before DNA extraction and quantitation. DNA samples from these cell lines were subjected to FAAS to determine Pt content. Values represent the average ± SE of three experiments and are reported as nanograms of Pt per mg of DNA. The values above the solid bars represent the percent decrease in Pt content after 14 h in the absence of drug.
Signaling in cDDP-mediated Apoptosis

Highly cDDP-sensitive clones of ME-180 cells were previously shown to have high level expression of EGFr (10), in agreement with recent studies of MDA-468 cells (11), which both express high sensitivity to cDDP and overexpress EGFr. Targeted reduction of EGFr expression in these cells resulted in a diminished sensitivity to cDDP and, as demonstrated in the

**DISCUSSION**

Tumor cell sensitivity to chemotherapeutic agents is controlled by several factors, including those regulating cellular drug levels and intracellular metabolism (4). However, it is also clear that specific biochemical processes, such as growth factor signal transduction and cell cycle progression, have a major impact on the cellular response to insult, including chemotherapy (3). Although the mechanisms are not fully characterized, an opportunity exists for exploitation or disruption of these pathways to allow greater therapeutic efficacy of existing anti-tumor agents and to help guide the development of novel therapies. In an effort to more closely define the role of signaling proteins in drug responses, a cDDP-resistant clonal variant was isolated from the ME-180 cell line and compared to a previously characterized cell variant expressing high sensitivity to cDDP (10). By comparing these two populations to the parental cell line, we were able to follow the signaling events that accompany the phenotypic transition of a tumor population from clinically relevant levels of drug sensitivity to resistance. The studies suggest that a reciprocal change in expression of EGFr and p21WAF1 underlie the molecular events that regulate cDDP sensitivity in some tumors.

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**Fig. 7** Effect of EGF on Pt-R cell growth and tyrosine kinase activity. Pt-R cells were treated with 10 nM EGF and assayed for cell growth after 72 h (Promega proliferation assay; left) or EGFr tyrosine kinase activity by immune-complex kinase assay after 15 min (right). Control cells received buffer alone. EGF consistently stimulated Pt-R cell growth by 175–220% when compared to untreated cells, and the results depicted represent the average ± SE of three independent measurements. EGF significantly increased tyrosine kinase activity (by 11- to 24-fold) in three independent experiments.

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Control levels after 4 h) in Pt-R cells but were not detectable in Pt-S cells that express low basal levels of this protein. These results support earlier observations of p53-independent regulation of p21WAF1 in ME-180 cells and the possible activation of p21WAF1 degradation (by apoptotic proteases), as previously noted in ME-180 clonal cells (29). Interestingly, CDDP-stimulated reduction of p21WAF1 levels occurs in both drug-sensitive and drug-resistant clones, suggesting that differences in intrinsic p21WAF1 expression, but not metabolism of this protein, correlate with distinctions in cDDP sensitivity.

Previous reports from this and other laboratories provided evidence for EGF-driven sensitization to cDDP and other agents (3, 10, 11, 13). To determine whether EGF altered sensitivity to cDDP in Pt-R cells, EGF receptor activation and mitogenic signaling were evaluated in EGF-treated cells. As shown in Fig. 7, EGF both activated receptor kinase activity and induced a mitogenic response in Pt-R cells, thereby increasing the growth rate by ~2-fold. Cell cycle analysis of EGF-treated Pt-R and Pt-S cells demonstrated that EGF induced a 2-fold increase in the % S-phase cells, with a concomitant reduction in both the G1 and G2 fraction of Pt-R cells (Fig. 8; Table 2). As described in cDDP-treated Pt-R cells (Fig. 6), EGF caused a time-dependent reduction in p21WAF1 levels and increased sensitivity to cDDP in Pt-R cells (~3-fold; Fig. 9). Conversely, although EGF stimulated receptor kinase activity, it did not significantly increase cell growth, alter cell cycle regulation (Fig. 8; Table 2), or affect cDDP sensitivity in Pt-S cells (10, 30). Therefore, although EGFr was down-regulated in Pt-R cells, stimulation with ligand reduced p21WAF1 levels, increased cell cycle progression, and partially restored cDDP sensitivity in Pt-R cells.

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**Fig. 6** Top, effect of CDDP on p53 and p21WAF1 induction in ME-180 parental and drug response variant cells. Parental, Pt-S, or Pt-R cells in 60-mm culture plates (1.5 × 10⁶ cells) were treated with CDDP (5 μg/ml) or PBS for 6 h, and equal protein cell lysates (50 μg) were immunoblotted with anti-p53 or p21WAF1 (Exp. 1). In subsequent studies, cell lysates were prepared from Pt-S and Pt-R cells treated with drug for 12 h (Exp. 2) and were subjected to p21WAF1 immunoblotting. It should be noted that a long exposure was required to detect p21WAF1 in drug-treated Pt-S cells (Exp. 2). Bottom, time-dependent effects of CDDP on p21WAF1 levels in Pt-R and Pt-S cells. Pt-R or Pt-S cells (as described above) were treated with CDDP (5 μg/ml) for 0, 4, or 8 h before cell lysates were prepared, and equal protein aliquots were analyzed for p21WAF1 expression by immunoblotting. The membrane was stripped and reprobed with an antibody against β-actin (Sigma) to demonstrate the equivalent protein amount in each lane.
present study, selection for cDDP-resistance results in the recovery of variant cells with low levels of EGFr. Therefore, transition from a drug responsive state to one of limited sensitivity resulted in a 30-fold change in response to both cDDP and EGFr. The independent selection procedures (selection for drug resistance and disruption of EGFr) in two unrelated cell types support a role for EGFr pathways in the regulation of drug sensitivity. However, EGFr overexpression alone does not universally correlate with increased cDDP sensitivity and does not appear to be coincident with clinical observations of limited sensitivity to therapies in patients with EGFr-positive tumors (5, 6, 13, 33). Therefore, other independent cellular components are likely to influence the role of receptor expression in tumor sensitivity (13). The results provided in the ME-180 cell model described in this report support a role for p53-independent regulation of p21WAF1 in EGFr-expressing tumors cells as a co-contributor to the drug-resistant phenotype.

p21WAF1 expression has been associated with drug resistance in glioma and leukemic patients and in cell lines derived from these diseases (24, 25). These cell lines may also be deficient or low in EGFr expression, thereby reflecting the biochemical properties described in the drug-resistant Pt-R cell line. Therefore, by clonal selection, targeted reduction of EGFr (11), and clinical observations (24, 25), a role for both receptor proteins and cell cycle regulators in drug responsiveness emerges (13). However, the mechanism and scope of drug resistance associated with EGFr/p21WAF1 changes are not completely understood. For example, cross-resistance to camptothecin (and analogues) but not doxorubicin or paclitaxel was detected in cDDP-resistant ME-180 cells. The distinct mecha-

Fig. 8 Effect of EGF on cell cycle progression in Pt-R and Pt-S cells. Subconfluent Pt-R or Pt-S cells were plated in the presence of serum (10%) and, after incubation overnight, were shifted to media containing low serum (0.2%). After 24 h, cells were treated with EGF (10 nM) and harvested at the indicated interval. Cells were collected, prepared, stained, and analyzed as described above (Table 1). Cytometric profiles are shown and tabulated below in Table 2.
nism of action and susceptibility of these drugs to multidrug resistance proteins suggests that common downstream processes, but not drug metabolism, regulate the toxicity of both camptothecin and cDDP. Further, these processes may be influenced by EGFr and p21WAF1 expression. One common activity that may regulate both cDDP and camptothecin cytotoxicity is the repair of DNA cross-links or adducts. Signaling proteins, such as EGFr, HER2, and p21WAF1, which influence cell cycle progression and consequently control the repair interval, may indirectly regulate the repair of these lesions (3, 10, 11, 17, 18, 21, 26). However, p21WAF1, through its association with the proliferating cell nuclear antigen, can directly affect the DNA repair process (34, 35), and EGF has been noted to block DNA repair activity without an overall effect on cell growth (3, 11, 13). In the drug-resistant Pt-R cell line, although EGFr had been down-regulated, EGF reduced p21WAF1 levels, activated cell growth, and increased cDDP toxicity, in contrast with previous studies in which EGF enhanced cDDP responses in the absence of cell cycle progression (3, 11). The distinctions may be related to the high level expression of p21WAF1 in Pt-R cells, which correlates with an overall reduction in their S-phase fraction (35). In the absence of p21WAF1 expression, as frequently occurs in cells with inactive or mutant p53 (19, 22), cell cycle restriction at the G1 and G2 checkpoints may be difficult to maintain and easily overcome in cells overexpressing EGFr. Further activation of receptor signaling in these cells may modify cell cycle intervals without influencing cell growth patterns in rapidly proliferating cultures. However, in cells with checkpoint restrictions and low levels of EGFr (as described in Pt-R cells), EGF can overcome checkpoint restriction, possibly through a reduction in p21WAF1, and increase cytotoxic activity of specific agents. In contrast, in cells with overexpressed receptors, activation may have a negative impact on cell growth and reduce drug activity through sustained induction of p21WAF1 (36). In EGFr overexpressing Pt-S cells, EGF had no significant effect on cell cycle control and, as previously described, did not further increase sensitivity to cDDP (10, 30).

Therefore, the intrinsic expression levels of both EGFr (and other tyrosine kinase receptor proteins) as well as cell cycle regulators, such as p21WAF1, appear to be important in determining direct cellular responses to some apoptotic agents in this and other cell models (10, 11, 16). These same molecules may determine whether growth factors enhance or disrupt the apoptotic response.

Elevated expression and drug- or growth factor-induced reduction of p21WAF1 in Pt-R cells appear to be independent of p53 regulation. Neither the intrinsic level of p53 expression nor its drug-induced accumulation correlated with the cellular levels of p21WAF1 in Pt-R cells. Other proteins may regulate p21WAF1 expression without altering the cellular levels of p53. Posttranslational modification of p53 (37) or altered expression of p53 modulatory proteins, such as mdm-2 (38), can affect p21WAF1 levels without a change in p53 content and may be associated with elevated p21WAF1 levels. However, we have been unable to detect evidence of p53 phosphorylation or altered mdm2 expression in parental or Pt-R cells (data not shown).

### Table 2: Effect of EGF on cell cycle distribution of Pt-R and Pt-S cells

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![Fig. 9](image_url) Effect of EGF on p21WAF1 levels and cellular response to cDDP in Pt-R cells. Top, Pt-R cells were treated with EGF (10 nM) for the indicated interval before cell lysates (50 μg) were prepared, resolved by SDS-PAGE, and immunoblotted for p21WAF1 or β-actin (as control). Bottom, Pt-R cells were treated with 10 nM EGF for 4 h (top) or 1 h (bottom) before incubation with cDDP at the concentrations indicated. After 72 h, cell survival was examined by MTT assay and compared to untreated cells or EGF-treated cells. The EGF results are based on EGF-treated cells as a control, and increased cDDP responses are not simply related to increased proliferation of EGF-treated cells. Each data point represents the average ± SE of four determinations. Three-fold increased sensitivity to cDDP (based on IC₅₀ measurements) was consistently measured in EGF-treated cells in several additional experiments.
Therefore, other p21WAF1 regulatory elements, such as IFN (39), extracellular signal-regulated kinase, or c-Jun-NH$_2$-kinase cascades (40, 41) and activated transcription complexes (42, 43), may be driving p21WAF1 expression in Pt-R cells. These possibilities are presently being examined.

In Pt-R cells, cDDP and EGF cause a similar reduction in p21WAF1 levels, which may occur through distinct mechanisms. Apoptotic protease cascades, as previously described in other ME-180 clonal cells (29), may mediate the reduction in p21WAF1 levels in drug-treated but not EGF-stimulated cells. Activation of a caspase cascade by tumor necrosis factor resulted in the time-dependent proteolysis of p21WAF1 to a 16-kDa proteolytic fragment that was co-incident with the degradation of other caspase substrates (poly ADP-ribose polymerase). Other studies have shown similar effects on p21WAF1 following radiation (44), although other proteolytic processes may also play a role (45). Growth factor-induced p21WAF1 reduction does not appear to be mediated by caspasas because these cascades are not engaged in EGF-treated Pt-R cells, and we have been unable to demonstrate specific proteolytic fragmentation of p21WAF1, which is characteristic of caspase activation (data not shown), in EGF-treated Pt-R cells. Other studies have demonstrated the involvement of the proteosomal complex in the regulation of cell cycle components, and some evidence suggests that growth factor signaling influences this process (46, 47). The mechanistic distinctions between EGF- and cDDP-mediated regulation of p21WAF1 are presently being investigated.

The characteristics of tumor cell clones isolated from an isogenic background but expressing high or low sensitivity to cDDP and camptothecin were described in this report. The results reveal that reciprocal changes in EGF and p21WAF1 may underlie a novel mechanism of drug resistance in which cell cycle control is modified as a consequence of altered signal transduction. The in vitro clonal selection procedure may mimic the clinical course of therapy in which drug-resistant clonal variants are likely to emerge after chemotherapeutic intervention. Understanding the characteristics of these variants and directing more effective therapies to eliminate residual disease are certain to impact the clinical outcome in cancer chemotherapy. The results described in this report suggest that disruption or exploitation of specific molecules, such as EGF and p21WAF1, are likely to increase chemosensitivity and overcome drug resistance in some patients.

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EGF Receptor and p21WAF1 Expression are Reciprocally Altered as ME-180 Cervical Carcinoma Cells Progress from High to Low Cisplatin Sensitivity

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