Epidermal Growth Factor Regulates the In Vitro Sensitivity of Human Ovarian Carcinoma Cells to Cisplatin

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Abstract

Cisplatin (DDP) is the most effective drug for the treatment of human ovarian cancer, but the mechanisms that determine sensitivity to the cytotoxic action of DDP are not well understood. Treatment of two human ovarian carcinoma cell lines with epidermal growth factor (EGF) simultaneously increased sensitivity to DDP and caused a persistent change in morphology in the absence of any mitogenic effect. Sensitization to DDP was shown to be dependent on both EGF concentration and EGF receptor number in C127 mouse fibroblasts expressing the human EGF receptor after transfection with a pBPV plasmid construct containing the human EGF receptor gene under control of the transferrin receptor 3′-inducible regulator. Sensitization of human ovarian carcinoma cells to DDP was not blocked by inhibition of protein synthesis. EGF did not enhance sensitivity to DDP or alter morphology in DDP-resistant human ovarian carcinoma cells despite the presence of functional EGF receptors on these cells. These results showed that elements of the signal transduction pathway activated by EGF determined cellular sensitivity to DDP, and that a DDP-resistant phenotype is associated with a defect in this signal transduction pathway. (*J. Clin. Invest.* 1990. 86:1632–1640.) Key words: drug resistance • epidermal growth factor receptor • transferrin receptor3′-inducible regulator • pBPV plasmid construct • C127 mouse fibroblasts

Introduction

Platinum complexes, particularly cisplatin (DDP),[1] are drugs of major importance in cancer therapy. However, both intrinsic and acquired resistance to DDP occurs frequently. Cells selected for resistance to many antimetabolites (1), and to drugs participating in the multiple drug resistance phenotype (2), often exhibit very high levels of resistance. In contrast, both in vitro and in vivo selection with DDP at clinically relevant intensities usually results in low-level resistant cells which are only two- to fourfold resistant to this drug (3–6). Attempts to enhance the sensitivity to DDP have focused on strategies such as reduction of glutathione (7–10) and inhibition of DNA repair (11, 12).

Binding of epidermal growth factor (EGF) to its receptor induces tyrosine phosphorylation of various cellular proteins including the EGF receptor itself. It has been exceedingly difficult to identify the key substrates that become phosphorylated besides the receptor itself, so the exact role of tyrosine phosphorylation in signal transduction remains uncertain. EGF receptor activation induces a variety of changes in intracellular physiology including activation of the Na^+/H^+ transporter (13), increase in calcium influx (14), activation of glycolysis (15), enhanced prostaglandin biosynthesis (16), induction of ornithine decarboxylase and accumulation of putrescine (17), changes in membrane potential (18), inositol phosphate formation (19, 20), activation of protein kinase C (21), S 6 ribosome phosphorylation (22), oncogene expression (23–27), stimulation of DNA synthesis (28), and cell proliferation (28).

We report here that activation of the receptor signal transduction pathway of the naturally occurring ligand EGF, can both enhance sensitivity to DDP, and produce morphologic changes consistent with activation of a differentiation program in two human ovarian carcinoma cell lines. While stimulation of DNA synthesis or cell proliferation are not required for EGF-induced sensitization to DDP, which of the many EGF-induced changes in cellular physiology are involved is currently not understood.

Methods

Cell lines and cell culture. Two well-characterized human ovarian carcinoma cell lines, designated 2008 (29) and COLO 316 (30), and a DDP-resistant variant 2008/C13*, derived from the parent 2008 cell line (31) were used in these studies. The 10-fold DDP-resistant cell line 2008/C13* was generated by 13 monthly selections with 1 μM DDP (31), followed by chronic exposure to stepwise increasing concentrations of DDP from 0.25 to 5.0 μM. 2008 cells and COLO 316 cells were maintained in exponential growth in RPMI medium 1640 supplemented with 5% heat-inactivated bovine calf serum, 2 mM freshly added glutamine, and 1% penicillin-streptomycin solution (Irvine Scientific, Santa Ana, CA). The parent and transfected C127 mouse fibroblasts were maintained in exponential growth in DME containing 4.5 g glucose per liter, 10% heat-inactivated FCS, and 2 mM glutamine.

Clonogenic assay. Clonogenic assays were performed by seeding 300 cells per 60 mm tissue culture plastic dish (Corning Glass Works, Corning, NY). The cells were allowed to attach overnight. On the next day, cells treated with EGF (murine EGF, Sigma Chemical Co., St. Louis, MO) were exposed to 10 nM EGF for 1 h and then to both EGF

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1. *Abbreviations used in this paper:* DDP, cisplatin; EGF, epidermal growth factor.

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and DDP during the second hour. The media was changed, and the colonies that formed were counted after 10 d of incubation in humidified 5% CO_2 at 37°C. Cell clusters containing > 50 cells were scored as a colony.

Growth assay. COLO 316, 2008, and 2008/C13* were seeded in 24-well plates (Corning Glass Works) at a density of 50,000 cells per well. Cells were treated with 10 nM EGF either for 2 h or continuously. The cell numbers were determined after trypsinization using a hemocytometer.

Scatchard analysis of EGF binding. EGF binding assays were performed with some modifications as described by Kawamoto et al. (32). 3-(^{125}I) Iodotyrosyl EGF (human recombinant), specific activity 9,000 Ci/mmol, was purchased from Amersham Corp. (Arlington Heights, IL). Subconfluent 2008, 2008/C13*, and COLO 316 cells grown in 150 × 25 mm tissue culture plates were fixed with 0.2% paraformaldehyde for 10 min at room temperature to prevent receptor internalization during incubation with EGF (33). Cells were scraped off with a rubber policeman and washed three times with 0.2% BSA (Sigma Chemical Co.) in PBS and resuspended in the same buffer at a cell density of 5 × 10^6 per ml. For binding assays, 200 μl of cell suspension (~ 1 × 10^6 cells) were incubated with 1 μCi ^{125}I EGF dissolved in 200 μl 0.2% BSA in PBS. After a 2-h incubation, cells were quantitatively collected on low protein binding hydrophilic Durapore membranes (pore size 0.22 μm, purchased from Millipore Corp., Bedford, MA) by suction. To prevent nonspecific binding of EGF, the filters were pre-wet with 0.25% gelatin (J. T. Baker Chemical Co., Phillipsburg, NJ) and 1% BSA in PBS. After cell collection, the filters were washed five times with 0.2% BSA in PBS. The filters were dried and radioactivity was measured in a gamma counter. Calculation of receptor number and K_d was performed as described by Scatchard (34).

Assessment of the EGF receptor number with monoclonal antibodies (MAbs) directed toward the human EGF receptor. MAb 455 (35–37) was used to quantitate the EGF-induced down-regulation of the EGF receptor number in 2008 and 2008/C13* cells. MAb 455 is directed toward the carbohydrate moiety of the human EGF receptor, and does not competitively inhibit the binding of EGF to its receptor (35–37). After harvesting the cells by trypsinization, all the following steps were performed on ice. The cells were washed twice with wash buffer (0.5 mM EDTA, 2 mM sodium azide, and 1 mg/ml BSA in PBS). Then the cells were incubated with 100 nM 455 MAb in wash buffer for 45 min. After washing the cells were stained with a fluorescent goat anti-mouse IgG/IgM antibody from Caltag Laboratories (San Francisco, CA) for 45 min in the dark. The cells were analyzed on a CytoFluorograf (Ortho Diagnostics Systems, Raritan, NJ) with excitation and emission settings of 488 and 530 nm, respectively. Similarly, in transfected C127 cells, changes in expression of the human EGF receptor number were quantitated with MAb 528 (35–37) as primary antibody. This latter antibody is specific for the protein core of the human EGF receptor (35–37). To determine the change in EGF receptor number within a given cell line, the change in the peak of mean intensity of fluorescence was used.

Transfection of C127 mouse fibroblasts. C127 mouse fibroblasts were stably transfected with a pBPV plasmid construct containing the human EGF receptor gene under the control of the transferrin receptor 3′-inducible regulator (Porter, D. C., manuscript in preparation). In brief, the plasmid vectors pSV2B and pBPV were obtained from Dr. J. Rose (Yale University, New Haven, CT). Plasmid preparation and all molecular manipulations were carried out using standard molecular biological techniques (38). DNA inserts were isolated from low-melt agarose gels and fragments purified by absorption and elution from NACS-52 columns (Bethesda Research Laboratories, Gaithersburg, MD). The BPV vector expressing the transferrin receptor was constructed as follows. A full-length transferrin receptor cDNA was isolated as a 5-kb *Bam*H1 fragment from pCDTR1 (39). The fragment was cloned into the *Hind*III/*Bgl*II sites of pSV2B. pSV2B is a derivative of pSV2 in which the *Pvu*II site upstream of the SV40 promoter has been converted into a *Bam*H1 site. A *Bam*H1 fragment containing the SV40 promoter, transferrin receptor insert, SV40 polyadenylation sequences, and small *t* antigen splice site was then removed and cloned into the *Bam*H1 site of the transforming vector pBPV. The pBPV/transferrin receptor vector containing the coding and noncoding sequence of the transferrin receptor was used to construct a hybrid gene composed of the coding sequence of the EGF receptor gene in place of the transferrin receptor coding sequence. In this plasmid, the transferrin receptor noncoding sequence was left intact and was placed 3′ to the EGF receptor gene. The pBPV/transferrin receptor plasmid was cut with the restriction enzymes *Eco*RV and *Bgl*II which removed the entire transferrin receptor coding sequence while leaving the SV40 promoter and noncoding transferrin receptor sequence intact. The *Bgl*II sticky end was filled in with T, A, and G nucleotide triphosphates and DNA polymerase I (Klenow fragment). The final unpaired nucleotide was removed with mung bean nuclease. This created an *Eco*RV restriction site between the SV40 promoter and the transferrin receptor noncoding sequence upon self ligation. This plasmid was designated pBPV/transferrin receptor noncoding sequence. The entire EGF receptor cDNA coding sequence was removed from the vector pX-gen (kindly provided by Dr. Gordon Gill, University of California San Diego, La Jolla, CA) with restriction enzymes *Xba*I and *Hind*III. The 4.0-kb fragment containing the EGF receptor coding sequence was electroeluted from high-melt agarose, made blunt with mung bean nuclease, and ligated into the *Eco*RV site of the plasmid pBPV/transferrin receptor noncoding sequence just described. Finally, the plasmid pBPV/EGF receptor coding sequence/transferrin receptor noncoding sequence was successfully transfected into mouse C127 fibroblasts using calcium phosphate transfection method (38).

Expression of the transferrin receptor gene is normally under the control of the transferrin receptor 3′-inducible regulator, and is regulated by iron (40). Incubation with the iron chelator deferoxamine induces expression of the transferrin receptor gene (41). To induce the EGF receptor number expressed on transfected C127 cells, the cells were treated with 10 μM deferoxamine mesylate (Desferal mesylate, Ciba-Geigy, Basel, Switzerland) and 25 μg/ml transferrin (human transferrin iron saturated, ICN Immuno Biologicals, Lisle, IL) for 60 h. The total number of EGF receptors, including murine and human, was determined by Scatchard analysis in parent and transfected C127 cells. In addition to Scatchard analysis, expression of human EGF receptors in transfected C127 cells was quantitated by flowcytometry using as primary antibody MAb 528 specific for the human EGF receptor (35–37).

Tetrazolium/formazan growth rate assay. Because C127 mouse fibroblasts do not form uniform colonies on plastic dishes, the sensitivity of control and deferoxamine-induced transfected C127 cells to DDP was compared by using a growth rate assay in which the cell number was quantified by tetrazolium dye reduction (42). 1,500 cells were seeded in 180 μl of DME containing 4.5 g glucose per liter, 10% heat-inactivated FCS, 25 μg/ml human transferrin (holoform), and 10 nM EGF. DDP at different concentrations was added in 20 μl of saline to triplicate culture wells, and cultures were incubated for 6 d in humidified 5% CO_2 in air at 37°C. MTT (3-[4,5-dimethylthiazol-2-*yl*]-2,5-diphenyl tetrazolium bromide, Sigma Chemical Co.) was prepared at 1 mg/ml in DME medium and on day 7, 50 μl were added to the microculture wells. After a 4-h incubation at 37°C, all the media was removed from each well, and 150 μl of 100% DMSO was added to solubilize the MTT-formazan product. After thorough mixing absorbance at 540 nm was measured with a V_{max} Kinetic Microplate Reader (Molecular Devices, Inc., Palo Alto, CA).

Inhibition of protein synthesis. Cycloheximide was used to inhibit protein synthesis (43). In preliminary experiments we have shown that incubation of 2008 cells with 5 μg/ml cycloheximide reduced protein synthesis by 85% after 10 min, and by 90% after 2 h, respectively. To assess whether EGF-induced enhanced sensitivity to DDP was dependent on new protein synthesis, 2008 cells were preincubated with 5 μg/ml cycloheximide for 10 min, followed by exposure to cycloheximide and EGF for 1 h, and cycloheximide, EGF and DDP for another hour. Immediately after drug exposure, cells were exposed to 25 μCi/ml [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) for 3 h.

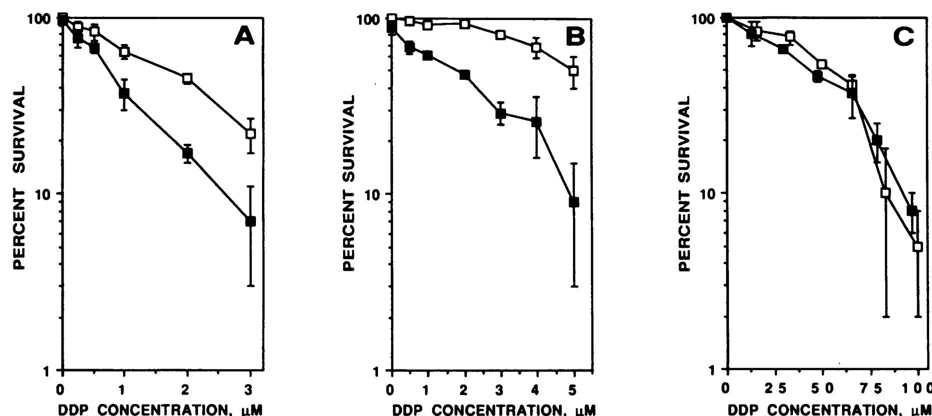

Figure 1. Effect of EGF on the sensitivity to DDP. COLO 316 (*A*), 2008 cells (*B*), 2008/C13* cells (*C*). Clonogenic assays were performed by seeding 300 cells per 60-mm plastic dish. Cells treated with EGF were exposed to 10 nM EGF for 1 h and then to both EGF and DDP during the second hour. The colonies that formed were counted after 10 d. Cell clusters containing > 50 cells were scored as a colony. Each data point represents the mean (±SD) of three separate experiments each performed with triplicate cultures (*Open squares*) DDP alone; (*solid squares*) DDP and EGF.

The incorporation of [^{35}S]methionine into protein was determined by precipitation of whole cell lysate proteins in 10% TCA (in the presence of 0.5% casamino acids) on glass-fiber filters; the precipitated material was counted in a β-scintillation counter.

Results

Fig. 1 shows the effect of EGF on the sensitivity to DDP in COLO 316, 2008, and 2008/C13* cells. Cells were exposed to 10 nM EGF for 1 h, and then to both EGF and DDP during a second hour. EGF increased the sensitivity of 2008 and COLO 316 cells by 3.1±0.9-fold, and 2.4±0.1-fold, respectively, as quantified by the ratio of the IC_{50} values. The EGF-induced decrease of the IC_{50} values were significant in both the 2008 and the COLO 316 cell lines ($P < 0.004$ and $P < 0.001$, respectively, by two-sided *t* test for the comparison with untreated cells). EGF did not alter the sensitivity of 2008/C13* cells to DDP.

The observed modulation of sensitivity to DDP by EGF was not due to an EGF-induced change in growth rate. The effect of both a 2-h exposure and continuous exposure to 10 nM EGF on growth rate was assayed using the same culture conditions under which EGF enhanced the sensitivity to DDP. During the first 5 d after cell seeding, cell growth was exponential. The doubling times of the 2008, 2008/C13*, and COLO 316 cells were 27.9, 28.2, and 29.2 h, respectively. Neither a 2-h nor a continuous exposure to 10 nM EGF had any demonstrable impact on the growth rate of these cell lines.

The effect of EGF on the sensitivity of 2008 cells to DDP was concentration dependent (Fig. 2). Control cells were exposed to increasing concentrations of EGF alone. EGF- and DDP-treated cells were exposed to EGF at increasing concentrations combined with DDP at a fixed concentration of 2 μM. First, the cells were exposed to EGF for 1 h, and then to both EGF and DDP concurrently during the second hour. A 2-h exposure to EGF (1 h before and 1 h concurrent with DDP) enhanced sensitivity to DDP at EGF concentrations as low as 0.4 nM, and the effect was maximal at concentrations of ~ 10 nM (approximately four times the K_d of the EGF receptor). As expected, a further increase in the EGF concentration up to 100 nM produced no additional change in sensitivity to DDP. The data presented in Fig. 2 is consistent with saturation of EGF binding at EGF concentrations over 10 nM, or with the presence of two distinct cell populations of which one is totally unresponsive to EGF. The latter possibility has not been formally excluded.

The time course of the EGF effect on the sensitivity of 2008 cells was determined by exposing cells to 10 nM EGF for 1 h, and then to DDP at a fixed concentration of 2 μM either concurrently or with an increasing delay between the 1 h EGF pretreatment and 1 h DDP exposure (Fig. 3). After the 1-h exposure to EGF the media was changed, and DDP at a fixed concentration of 2 μM was added for 1 h at the following time points: 0, 1, 2, 3, 4, 5, and 24 h after the start of the 1-h exposure to EGF. Thus, there was an increasing time period in which cells were incubated without EGF before DDP was added. When given concurrently with DDP, EGF significantly increased sensitivity to DDP as compared with control cells treated with DDP alone. Sensitivity to DDP increased even further when cells were exposed to EGF for 1 h before treatment with DDP. The EGF-mediated increase in sensitivity to DDP persisted for at least 5 h, but had largely disappeared by 24 h (Fig. 3).

In addition to enhancing sensitivity to DDP, and despite the lack of effect on growth, a 2-h exposure to EGF had marked effects on the morphology of the 2008 and COLO 316

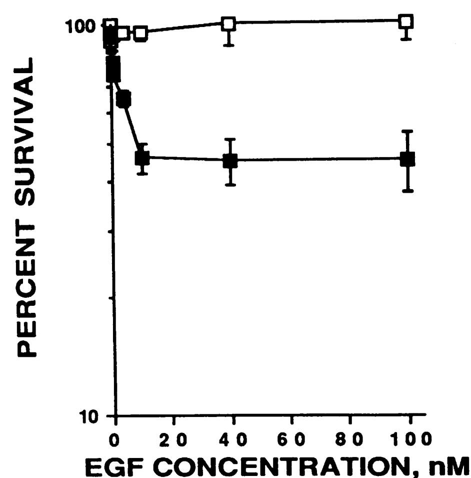

Figure 2. EGF concentration-dependence of DDP sensitization in 2008 cells. (*Open squares*) control cells, exposed to EGF alone for 2 h at increasing concentrations. (*Solid squares*) Cells exposed to EGF and DDP. Cells were exposed first to EGF for 1 h, and then to both varying concentrations of EGF and 2 μM DDP concurrently during the second hour. Colonies were counted after 10 d of incubation. Each data point represents the mean (±SD) of three different experiments.

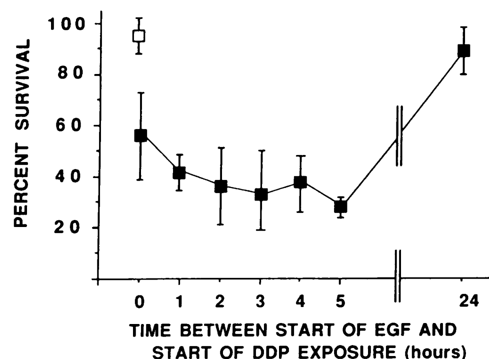

Figure 3. Time course of EGF-induced sensitization to DDP in 2008 cells. Cells were exposed to 10 nM EGF for 1 h. DDP at a fixed concentration of 2 μM was added to the media either concurrently with EGF or with increasing delay after the start of the 1 h EGF exposure. The abscissa represents the number of hours between the start of the 1-h EGF exposure and the start of the 1-h DDP exposure. Colonies were counted after 10 d of incubation. Each data point represents the mean (±SD) of three different experiments. (*Open square*) DDP alone; (*solid squares*) EGF and DDP.

cells (Fig. 4). At the macroscopic level, colonies formed by the 2008 and COLO 316 cells 10 d after a 2-h exposure to 10 nM EGF were much larger and stained less intensely with Giemsa. At the microscopic level, colonies formed from untreated cells consisted of tightly packed cells, whereas colonies arising after EGF treatment consisted of widely scattered cells of which 10–20% had formed prominent dendritic processes. The lower cell number displayed in the photographs taken from EGF-treated cells is due to the marked scattering of the cells, and does not reflect an EGF-induced change in growth rate. It was of particular interest that 2008/C13* cells, in which EGF failed to alter DDP responsiveness, showed no morphological response to a 2 h EGF exposure.

The receptor number per cell and the K_d of the EGF receptor were determined by Scatchard analysis (18) in the DDP-sensitive 2008 and COLO 316 cells, and the DDP-resistant 2008/C13* subline. The data is summarized in Table 1. The Scatchard plots were monophasic in all cell lines examined, suggesting the presence of a single class of EGF receptors with the same K_d. The K_d for 2008, 2008/C13*, and COLO 316 cells were of the same order of magnitude. 2008 cells expressed approximately threefold more EGF receptors as compared with 2008/C13* cells. Even though the EGF receptor number was similar in the COLO 316 and the 2008/C13* cells, the former were responsive to EGF and the latter nonresponsive to EGF in terms of sensitization to DDP and changes in morphology. Thus, no apparent correlation between EGF binding and its ability to sensitize cells to DDP and to induce morphological changes was found in different human ovarian carcinoma cell lines, suggesting that factors other than the EGF receptor number are determinants of the biological response to EGF in different cell lines.

The functionality of the EGF receptors was assayed in 2008 cells and 2008/C13* cells by demonstrating down-regulation of the receptor number upon binding to EGF. EGF receptors were quantitated by flowcytometry, using as primary antibody MAb 455 directed against the carbohydrate moiety of the human EGF receptor (35–37). A 1-h incubation of 2008 and 2008/C13* cells with 100 nM EGF at 37°C caused down-regulation of 60–70% of the EGF receptors on both DDP-sensitive and resistant cells (data not shown). Control cells were treated with 100 nM EGF for 1 h on ice, a condition known to prevent receptor down-regulation (37, 44). Using EGF receptor mutants lacking intrinsic protein tyrosine kinase activity, it has been convincingly shown that tyrosine kinase activity is essential for signal transduction and for receptor down-regulation upon binding to EGF (45, 46). Therefore, it can be concluded that both the 2008 and 2008/C13* cells have functional EGF receptors, which are capable of receptor autophosphorylation upon EGF binding.

To demonstrate that the modulating effect of EGF on DDP sensitivity was transduced by the EGF receptor, we investigated the effect of EGF on the sensitivity to DDP in C127 mouse fibroblasts stably transfected with a plasmid construct containing the human EGF receptor gene under the control of the transferrin receptor 3′-inducible regulator. The total number of EGF receptors including murine and human receptors was determined by Scatchard analysis in parent and transfected C127 cells. Parent C127 cells expressed ~ 3×10^3 receptors per cell (data not shown). Transfected C127 cells grown in media supplemented with FCS expressed 1.8×10^5 receptors per cell (including murine and human receptors, data not shown). After a 60-h incubation with 10 μM deferoxamine mesylate and 25 μg/ml transferrin, transfected C127 cells expressed 4.5×10^5 receptors per cell (including murine and human receptors, data not shown). In addition to Scatchard analysis, expression of human EGF receptors in transfected C127 cells was quantitated by flowcytometry, using as primary antibody MAb 528, specific for the protein core of the human EGF receptor (35–37). With this assay, deferoxamine treatment was shown to induce expression of the human EGF receptor to approximately twofold, as compared with untreated cells (data not shown). The same assay was used to demonstrate EGF-induced down-regulation of the human EGF receptor in transfected C127 cells. A 1-h incubation of transfected C127 cells with 100 nM EGF at 37°C caused down-regulation of 50–60% of the human EGF receptors (data not shown). Control cells were treated with 100 nM EGF for 1 h on ice, a condition known to prevent receptor down-regulation (37, 44). By demonstrating receptor down-regulation of the human EGF receptors expressed on transfected C127 cells upon binding of EGF, it can be inferred that these receptors are functional, i.e., capable of receptor autophosphorylation (*vide supra*). The sensitivity of control and deferoxamine-induced cells to DDP was compared by using a growth rate assay in which the cell number was quantitated by tetrazolium dye reduction (42). Fig. 5 shows that, in the presence of 10 nM EGF, the deferoxamine-treated cells were approximately twofold more sensitive to DDP than control cells. Deferoxamine at a concentration of 10 μM was nontoxic to transfected C127 mouse fibroblasts, even after prolonged incubation for 2 wk, and did not modulate the sensitivity of transfected C127 cells to DDP in the absence of EGF. Thus, within a given cell line, sensitivity to DDP could be regulated by both EGF concentration and EGF receptor number.

The EGF-induced increase in sensitivity of 2008 cells to DDP was not dependent on new protein synthesis. Under circumstances where DDP and EGF together reduced the survival of 2008 cells to 31±10% (SD) of control cells treated with DDP alone, pretreatment with cycloheximide sufficient to inhibit protein synthesis by 85–90% produced a survival of

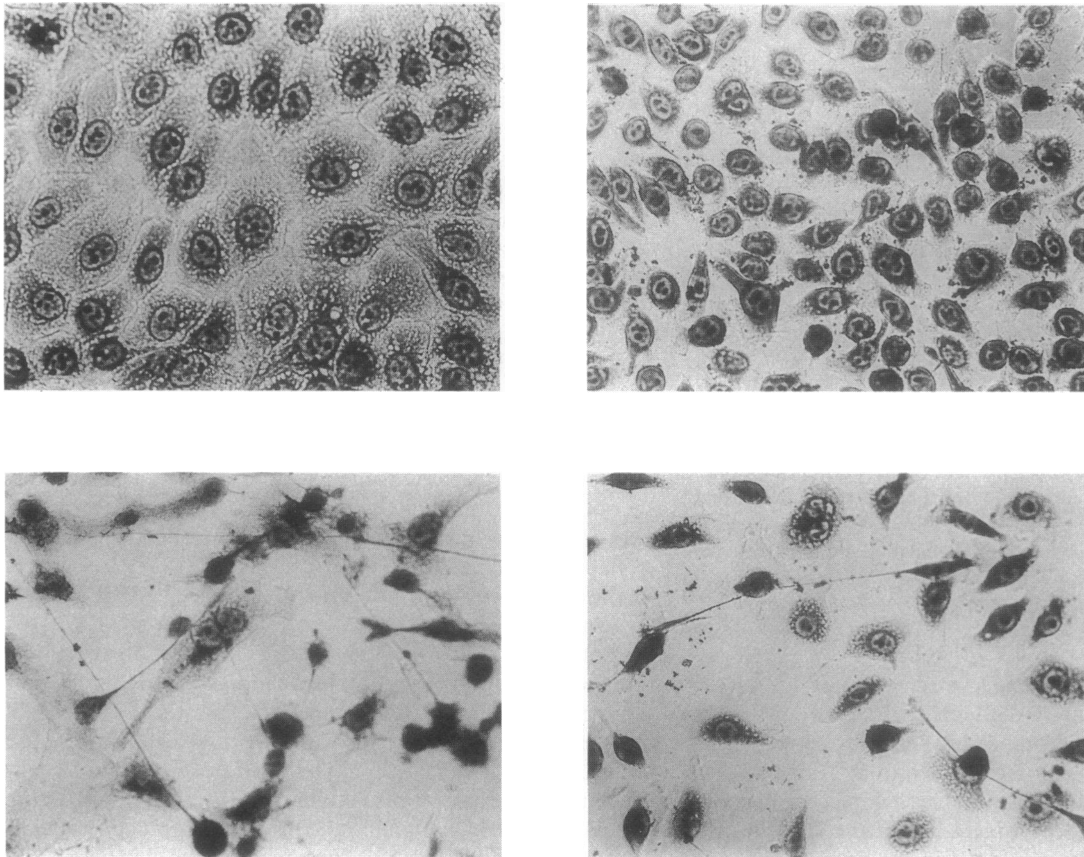

Figure 4. Morphological changes induced by a 2-h exposure to 10 nM EGF in 2008 and COLO 316 cells. Cells were seeded on 60-mm plastic dishes at a density of 300 cells per dish, allowed to attach overnight, and exposed to 10 nM EGF for 2 h on the following day. Colonies were inspected by light microscopy after 10 d of incubation. Untreated cells (COLO 316 cells, *top left;* 2008 cells, *top right*) showed a dense monolayer of ovoid cells with round nuclei and dense chromatin. The colonies formed by the COLO 316 cells appeared somewhat denser. Lower panels show cells exposed to EGF (COLO 316, *bottom left;* 2008 cells, *bottom right*). In both cell lines EGF induced a marked scattering of the cells and the formation of prominent dendritic processes.

47±8% (SD, data not shown). Thus, synthesis of new proteins is not required for EGF-induced sensitization to DDP in 2008 cells.

Discussion

We observed that EGF enhanced the sensitivity to DDP and induced marked changes in morphology in two human ovarian carcinoma cell lines. Interestingly, EGF failed to induce either effect in DDP-resistant 2008/C13* cells, suggesting a lesion in the EGF receptor signal transduction pathway associated with this DDP-resistant phenotype. The concept that activation of a growth factor signal transduction pathway can modulate the sensitivity of tumor cells to antineoplastic agents is novel, although the precise EGF-induced changes responsible for mediating the enhanced sensitivity to DDP are not yet known.

Our evidence suggests that binding of EGF to its receptor and activation of the EGF receptor tyrosine kinase activity are mandatory for EGF-induced sensitization to DDP. This evidence includes the observation that sensitization to DDP is dependent on both the EGF concentration and the EGF receptor number. Analysis of the EGF concentration dependence in 2008 cells shows that maximal sensitization to DDP occurred at an EGF concentration of ~ 10 nM. This is in agreement with a K_d of 2.4 nM found in this cell line, indicating that maximum sensitization is obtained as binding becomes saturated. Similarly, within a given cell line, the number of EGF receptors expressed per cell was a determinant of the degree of EGF-induced sensitization to DDP, as demonstrated in C127 mouse fibroblasts transfected with a pBPV plasmid construct containing the human EGF receptor gene under control of the transferrin receptor 3'-inducible regulator. In this model, sensitivity to DDP in the presence of EGF could be increased by approximately twofold by increasing the EGF receptor number by 2.5-fold. This is strong support for the contention that the effect of EGF on sensitivity to DDP is mediated by binding of EGF to its receptor.

Several lines of evidence indicate that EGF-induced enhancement of sensitivity to DDP is not dependent on new gene transcription. First, time course experiments showed that sensitization to DDP developed relatively rapidly. Almost maximal enhancement of DDP sensitivity was observed after just 1 h exposure to EGF. Second, blockade of new protein synthesis by preincubation with cycloheximide at a concentration sufficient to prevent 85–90% of protein synthesis did not prevent modulation of DDP sensitivity by EGF. Third, EGF-induced enhancement of sensitivity to DDP was independent of the ability of EGF to act as a mitogen, an effect that is related to

Table I. Scatchard Analysis of EGF Binding to Human Ovarian Carcinoma Cell Lines

Cell line	Fold increase in resistance to DDP	No. of EGF receptors per cell ($\times 10^4$)	K_d
			nM
2008	1	16.5±0.7	2.4±1.3
2008/C13*	8	5.8±0.9*	4.0±0.3
COLO 316	1	4.3±0.4	4.0±1.4

* $P < 0.001$ by two-sided *t* test for the comparison with 2008 cells. 200 μl of cell suspension ($\sim 1 \times 10^6$ cells) were incubated with 1 μCi ^{125}I-EGF. After a 2-h incubation, cells were quantitatively collected on membranes and radioactivity was measured in a gamma counter. Calculation of receptor number and K_d was performed as described by Scatchard. Mean and standard deviations of three different experiments are shown.

new gene transcription (47, 48). In the 2008 and the COLO 316 cell line, both of which demonstrated EGF-induced sensitization to DDP, the growth rate was not affected by addition of EGF to the media. Taken together, these three observations argue against a requirement for new gene transcription in mediating the modulation of DDP sensitivity by EGF.

We have demonstrated that exposure to EGF failed to stimulate the growth rate of human ovarian carcinoma cells under the experimental conditions in which EGF enhanced the sensitivity to DDP. In these experiments, control cells were maintained in RPMI medium supplemented with 5% bovine

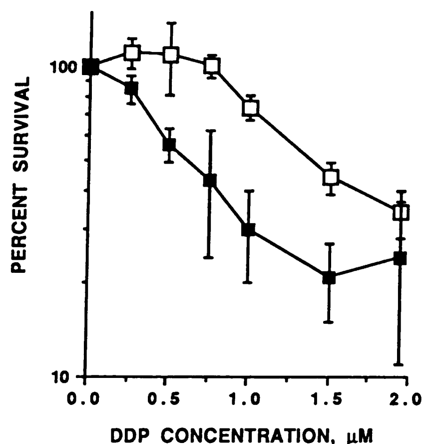

Figure 5. Effect of EGF receptor number on EGF-induced sensitization to DDP in transfected C127 mouse fibroblasts. C127 cells were transfected with a pBPV plasmid construct containing the human EGF receptor gene under control of the transferrin 3'-inducible regulator. (*Open squares*) Transfected C127 cells, expressing 1.8×10^5 EGF receptors per cell; (*solid squares*) transfected C127 cells expressing 4.5×10^5 EGF receptors per cell after pretreatment with 10 μM deferoxamine and 25 μg/ml transferrin for 60 h. The growth rate was determined by using a tetrazolium/formazan assay (16). 10 nM EGF and DDP at different concentrations were added to triplicate culture wells. The cultures were incubated for 6 d, and on day 7 MTT was added to the microculture wells. After a 4-h incubation, the MTT-formazan product was solubilized with DMSO and absorbance was measured at 540 nm. Each data point represents the mean (±SD) of three different experiments.

calf serum. It has been shown that maximal stimulation of DNA synthesis is achieved at EGF concentrations which are far below the concentration required for maximal binding (28). Thus, only a very small fraction of the available receptors need to be occupied by the growth factor to elicit maximal stimulation of the growth rate (28). Therefore, it is conceivable that in our system, control cells were maximally growth stimulated by the limited amount of EGF supplied by the bovine calf serum, which was added to the media. In this situation, addition of more EGF would not be expected to result in a further increase in the growth rate.

A 2-h exposure to EGF induced marked morphological changes in the 2008 and COLO 316 cell lines which were most marked at 10 d after a 2-h exposure to EGF. In contrast to untreated cells which formed tightly packed colonies, colonies developing after EGF exposure consisted of widely scattered cells, of which 10–20% formed long dendritic processes. It is conceivable that these changes in morphology were the result of an EGF-induced activation of a differentiation program. Because there are currently no widely recognized differentiation markers available for ovarian carcinoma cells, induction of differentiation by EGF cannot be proven at this point in time. Sensitization to DDP and induction of a differentiation program represent two distinct biological effects of EGF. The former appears very rapidly and is not dependent on new protein synthesis; in contrast, the morphological changes, indicating activation of a differentiation program, develop slowly and are dependent on new protein synthesis.

Interestingly, EGF failed to induce either sensitization to DDP or morphological changes in the DDP-resistant 2008/C13* cells, suggesting that this DDP-resistant phenotype is associated with one or more lesions in the EGF receptor signal transduction pathway. We have shown that EGF induces receptor down-regulation in DDP-sensitive and -resistant cells. This indicates that the initial steps in the signal transduction pathway, i.e., binding of EGF to its receptor and receptor autophosphorylation, are intact in both cell lines. This conclusion can be inferred from experiments with receptor mutants lacking intrinsic protein tyrosine kinase activity (45, 46). In this model it has been convincingly shown that receptor autophosphorylation, and thus protein tyrosine kinase activity, are essential for signal transduction and for receptor down-regulation secondary to binding of EGF to its receptor (45, 46). Therefore, the putative lesion in the EGF receptor signal transduction pathway in DDP-resistant 2008/C13* cells does not involve the protein tyrosine kinase activity of the receptor, but is located further downstream.

The DDP-resistant 2008/C13* cells, which were not responsive to DDP, had approximately threefold fewer EGF receptors compared with the parent 2008 cells. It seems unlikely that the lower receptor number alone can account for the complete lack of ability of EGF to sensitize to DDP or cause morphological changes. Rather, a weaker response would be expected if the decrease in receptor number were the only determinant of the response to EGF. The number of EGF receptors expressed on the EGF-responsive COLO 316 cells and EGF unresponsive 2008/C13* cells was similar. This indicates that despite the fact that EGF sensitization varies with receptor number within a cell line, the absolute number of receptors cannot predict the sensitivity to DDP in different cell lines. In an analogous situation, no correlation was found between EGF binding and its mitogenic activity in different

human mammary cell lines, suggesting that factors other than just the EGF receptor number are important determinants of the biological response to EGF in different cell lines (49).

We have recently shown that activation of two other signal transduction pathways involving protein kinase A and protein kinase C can modulate the sensitivity to DDP in human ovarian carcinoma cells (50, 51). Stimulation of protein kinase A by forskolin increased the sensitivity to DDP by increasing drug accumulation (50). Similar to EGF, forskolin failed to enhance sensitivity to DDP in DDP-resistant 2008/C13* cells. Stimulation of protein kinase C by the tumor promoter TPA increased the sensitivity of 2008 cells by 2.5-fold (51). Thus, activation of at least three different signal transduction pathways including protein kinase A, protein kinase C, and the EGF receptor can modulate sensitivity to DDP in human ovarian carcinoma cells. It is conceivable that the final target mediating sensitization to DDP is the same in all three pathways. Recently, exposure of CaSki human squamous carcinoma cells to EGF was shown to enhance radiosensitivity (52). It is currently not known whether there is a single element of the EGF signal transduction pathway capable of regulating both radiosensitivity and sensitivity to DDP, or whether different elements of the pathway are independently mediating these two effects.

Even though EGF induced only a two- to threefold increase in sensitivity to DDP, this change may be clinically significant. Several investigators have reported that patients pretreated with DDP had low-level resistance to DDP, and that human ovarian carcinoma cell lines derived from the patients' tumor at the time the tumor became unresponsive to DDP were only two- to fourfold resistant to DDP (3–6). In several in vivo murine models, DDP-resistant cell lines could be isolated from tumors treated with as few as two doses of DDP (53, 54). The level of resistance found in these animal studies was also low-level, in the range of two- to fourfold (53, 54). Taken together, these studies in the human and in animals indicate that in vivo low levels of resistance to DDP appear rapidly and are sufficient for treatment failure. Therefore, a two- to threefold increase in sensitivity to DDP, as observed in EGF-treated cells, is potentially clinically significant. Furthermore, several types of human tumors are known to express unusually large numbers of EGF receptors in vivo (55–59), and we speculate that EGF may be used to selectively enhance the DDP sensitivity of these tumors without increasing the toxicity of DDP to normal tissues. This may be facilitated by the fact that the number of EGF receptors can be increased by a variety of agents available for clinical use such as retinoic acid (60), estrogen (61), progesterone (62), triiodothyronine (63), and androgens (64). Furthermore, EGF may be used to stimulate differentiation of ovarian carcinomas, thus potentially reducing the degree of malignancy. To fully comprehend the biological significance of our findings, many cell lines from different tissues will have to be screened for EGF-induced sensitization to DDP, and the effect of EGF on sensitivity to DDP and potential induction of a differentiation program will need to be studied in the in vivo animal model.

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