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Alteration of EGF-Receptor Binding in Human Breast Cancer Cells by Antineoplastic Agents

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Abstract—*Polypeptide growth factors bind to membrane receptors on human breast cancer cells and stimulate cell proliferation, suggesting that they may be important in growth regulation. Inhibition of the stimulatory effects of these factors might result in antineoplastic activity. Since cytotoxic drugs have been shown to alter cell membrane characteristics, we have examined the effects of a variety of antitumor drugs on the binding of epidermal growth factor (EGF) to the membrane receptor of human breast cancer cells. Twenty-four standard or investigational cytotoxic drugs were screened at a concentration of one-tenth the achievable peak plasma level for their ability to inhibit binding of ^{125}I-EGF to its receptor in MCF-7 human breast cancer cells. Although at this concentration statistically significant inhibition of binding was observed with 11 drugs, the maximum inhibition observed was only 27%. Five agents, representing classes of drugs with different modes of action, were then studied in more detail. Of these, preincubation with 5-fluorouracil, 4-hydroperoxy-cylophosphamide and doxorubicin inhibited MCF-7 colony formation in a dose-dependent manner, but these drugs had no effect on EGF-binding even at a concentration of 10 times the peak plasma level. Preincubation of cells with vinblastine and cisplatin, however, resulted in both reduced colony survival and a parallel reduction in EGF receptor binding. Membrane integrity, as measured by trypan blue exclusion, was not altered. Scatchard analysis of EGF binding demonstrated that the major effect of cisplatin was a reduction in binding affinity. We conclude that cisplatin and vinblastine at high concentrations can inhibit the binding of EGF to human breast cancer cells offering an additional possible mechanism for their antiproliferative activity.*

INTRODUCTION

GROWTH of human breast cancer cells is regulated by several growth factors *in vitro*. Among these, insulin, Insulin-like Growth Factor-I (IGF-I), Epidermal Growth Factor (EGF) [1, 2, 3], and alpha-Transforming Growth Factor (alpha-TGF) [4, 5] bind to membrane receptors and stimulate cell proliferation. Furthermore, IGF-I and alpha-TGF are secreted by human breast cancer cells and may act as autocrine growth factors [5–7]. Alpha-Transforming Growth Factor is a low mol. wt polypeptide which interacts with membrane receptors for EGF and, like EGF, activates a tyrosine kinase [8–10]. The reaction with the EGF receptor is thought to be a necessary event for all subsequent biological effects of alpha-TGF.

Alteration of the binding of growth factors to their receptors might lead to a disruption of growth stimulation. Binding could be altered either by stereo-specific antagonists or by non-specific disturbances of the receptor binding sites [11]. The purpose of the present study was to determine whether antineoplastic agents can alter growth factor binding to target cells. EGF receptor binding in MCF-7 human breast cancer cells was studied, since these cells are sensitive to EGF, and since they secrete alpha-TGF which may serve as an autocrine growth factor through the EGF receptor system [5, 6, 12].

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MATERIALS AND METHODS

Drugs

Actinomycin D (Cosmegen^{R}) was purchased from Merck, Sharp and Dohme. M-AMSA, 2-FLAMP, hexamethylmelamine, MGBG, and thioguanine sodium were provided by the National Cancer Institute. Cytosine arabinoside (Cytosar-U^{R}) and menogaril were obtained from Upjohn Co. Diaziquone (AZQ) was provided by Warner-Lam-

bert Co. Bisantrene-mitoxantrone, and methotrexate were obtained from Lederle. Bleomycin sulfate (Blenoxane^R), cisplatin (Platinol^R), mitomycin C (Mutamycin^R), and VP16 (VePesid^R) were purchased from Bristol Laboratories. Doxorubicin (Adriamycin^R) and 5-FU (Adrucil^R) were purchased from Adria Laboratories. Dacarbazine (DTIC^R) was obtained from Miles Pharmaceuticals, interferon A from the Schering Corporation, and melphalan (Alkeran^R) from Burroughs Wellcome Co. Vinblastine sulfate (Velban^R) and vincristine sulfate (Oncovin^R) were purchased from Eli Lilly and Co.

4-Hydroperoxy-cyclophosphamide was used as the biologically active form of cyclophosphamide and was a generous gift from Dr. David Alberts.

EGF binding studies

Epidermal Growth Factor was purchased from Collaborative Research. ^{125}I-EGF was obtained either by iodination using chloramine T [13] or purchased from Biomedical Technologies Inc. (specific radioactivity: 100–200 mCi/mg). MCF-7 cells were seeded in 6-well plates (Corning) at 4.5×10^5 cells/well and cultured for 2 days (nearly confluent monolayer) at 37°C, 5% CO_2 in a humidified atmosphere. In the initial screening experiments, drugs (final concentrations of one-tenth peak plasma level), unlabeled EGF, and ^{125}I-EGF (final concentration: 100 pg/ml) was added simultaneously to the wells. Binding was carried out for 30 min at 37°C, 5% CO_2. In subsequent experiments, MCF-7 cells were preincubated with drugs (30 min or 2 hr) and washed twice with phosphate-buffered saline to remove the drugs, and binding was performed for either 30 min at 37°C or 3 hr at 0°C (equilibrium conditions), 5% CO_2. The ^{125}I-EGF radioreceptor assay and Scatchard analysis were performed as described earlier [12].

Trypan blue exclusion

MCF-7 cells (2×10^6 cells/flask) were seeded in 25 cm^2 tissue culture flasks (Corning) and cultured for 2 days. The medium was discarded and 5 ml of fresh medium with the appropriate concentrations of drugs (0.01–10 fold peak plasma concentration achievable in man) were added. After various incubation times an aliquot of the supernatant was removed for cell counts and trypan blue (GIBCO) exclusion. The remaining medium was then discarded and the attached cells were harvested with 0.25% trypsin/1 mM EDTA (GIBCO) for cell counts and trypan blue exclusion. To assess membrane integrity, the percentage of trypan blue excluding cells was expressed in relation to the total number of cells per flask. The harvested cells were diluted to the appropriate concentration and were used for the determination of *in vitro* colony formation in the human tumor cloning assay.

Human tumor cloning assay

Cytotoxicity was assessed using a modification of the soft agar method of Hamburger and Salmon [14, 15]. Base and top layers consisted of improved MEM, 5% calf serum, penicillin (90 U/ml), streptomycin (90 μg/ml, all Irvine Scientific), glutamine (2 mM, GIBCO),and insulin (10^{-9} M, Iletin-100^R, Eli Lilly). The final agar concentration in base layers was 0.5%. Each top layer contained 3×10^3 MCF-7 cells in 0.3% agar. All determinations were done in triplicate. Cultures were incubated in a humidified atmosphere at 37°C, 5–7% CO_2 for 10–14 days. Colonies (cell clusters ≥ 70 μm in diameter) were counted with an inverted microscope. Colony counts from drug-pretreated cells were compared with colony counts from diluent-pretreated cells and expressed as percentage of the control. Chromomycin A3 (50 μg/ml, Sigma) served as positive control for cell kill to assure the presence of a good single cell suspension.

Statistical methods

Percentage survival and standard deviations of percentage survival were calculated as previously described [16]. The same method was used in the ^{125}I-EGF binding studies. The Student's *t*-test was used to calculate whether EGF binding by drug-pretreated cells differed significantly from binding by control cells.

RESULTS

Screening of antineoplastic agents

Twenty-four antineoplastic agents were tested in initial screening experiments in which the drugs, at a concentration of one-tenth the achievable peak plasma level, were added directly to the EGF radioreceptor assay (Table 1). Although a statistically significant ($P \leq 0.05$) inhibition of ^{125}I-EGF binding was observed under these conditions with 11 of the 24 drugs, the maximal inhibition was only 27%.

Effect of preincubation with selected drugs

Next we selected five antineoplastic agents and studied ^{125}I-EGF binding after the cells were preincubated with various concentrations of the drugs to determine if greater effects could be observed.

MCF-7 cells were preincubated for 30 min or 2 hr with cisplatin, 4-hydroperoxy-cyclophosphamide, doxorubicin, 5-fluorouracil, or vinblastine at concentrations ranging from 0.01 to 10-fold peak plasma level achievable in man. Identically treated cells were cloned in soft agar to assess the antitumor activity. Trypan blue exclusion was also performed

Table 1. Effect of 24 standard or investigational agents on ^{125}I-EGF binding

Drug	Concentration (μg/ml)	^{125}I-EGF-binding (% Control ± S.D.)	*P* value
Actinomycin D	0.01	78 ± 5	0.010
m-AMSA	0.50	73 ± 4	0.003
Ara C	10.00	116 ± 14	0.277
Bisantrene	0.50	87 ± 6	0.118
Bleomycin	0.20	88 ± 3	0.016
Cisplatin	0.20	80 ± 5	0.043
Dacarbazine (DTIC)	0.10	83 ± 3	0.006
Diaziquone (AZQ)	0.30	87 ± 6	0.101
Doxorubicin	0.04	87 ± 8	0.188
2-FLAMP	0.10	106 ± 6	0.326
5-Fluorouracil	6.00	74 ± 3	0.002
Hexamethylmelamine	1.00	111 ± 3	0.015
4-Hydroperoxy-cyclophosphamide	3.00	80 ± 5	0.015
Interferon A	1000.00*	104 ± 3	0.237
Melphalan	10.00	86 ± 5	0.053
Menogaril	0.02	87 ± 5	0.057
Methotrexate	10.00	116 ± 13	0.220
MGBG	10.00	75 ± 3	0.001
Mitomycin C	0.10	97 ± 4	0.545
Mitoxantrone	0.05	97 ± 5	0.631
Thioguanine	1.00	84 ± 2	0.004
Vinblastine	0.50	100 ± 15	0.991
Vincristine	0.01	84 ± 3	0.007
VP 16	3.0	87 ± 3	0.011

*International Units.

MCF-7 cells were cultured in six-well plates. When the cells were nearly confluent, EGF binding studies were performed in the presence of the specified concentrations of each drug. The results are expressed as percentage of untreated control. Numbers represent the mean and standard deviation of triplicate determinations.

as an index of membrane integrity.

After a 30 min preincubation, colony survival was reduced by each drug in a dose-dependent manner (Fig. 1). Trypan blue exclusion was not affected by drug treatment, indicating that cell membranes were not grossly altered. EGF binding was moderately increased by cisplatin at very low concentrations, but no consistent effect on EGF-binding was observed for the other four drugs. However, when the drug preincubation time was prolonged to 2 hr, a significant reduction in EGF receptor binding as well as colony survival was observed with two of the five drugs, vinblastine and cisplatin (Figs. 2D and 2E). At the highest concentrations tested, binding was reduced to 69% of control with vinblastine and to 32% of control with cisplatin. The reduction in binding with these two agents was reproducible in multiple experiments. Inhibition of binding required higher concentrations than inhibition of colony growth. 4-Hydroperoxy-cyclophosphamide and 5-fluorouracil inhibited colony survival, but had no effect on trypan blue exclusion or EGF binding (Figs. 2B, 2C). Doxorubicin led to moderate increase in EGF binding at the highest concentration (Fig. 2A).

Effect of cisplatin on receptor affinity and concentration

We next investigated the effects of cisplatin, the most potent drug, on EGF receptor binding in more detail. Binding studies were repeated at 0°C to prevent ligand degradation and hormone-receptor internalization. Scatchard analysis [34] of binding data obtained from cells incubated with constant tracer amounts of ^{125}I-EGF and increasing concentrations of unlabeled EGF revealed that the major effect of cisplatin was a 3-fold reduction in binding affinity (Fig. 3). Receptor concentration was not significantly altered. Identical data were obtained from Scatchard analyses of binding studies using increasing concentrations of ^{125}I-EGF (data not shown).

DISCUSSION

Our studies indicate that binding of EGF to target cells can be disturbed *in vitro* by pretreatment with high concentrations of vinblastine and cisplatin. The effect is time dependent and was observed after exposure to the drug for 2 hr; simultaneous incubation or preincubation with drug for 30 min did not result in consistent changes in EGF receptor binding. Scatchard analysis suggests that the affin-

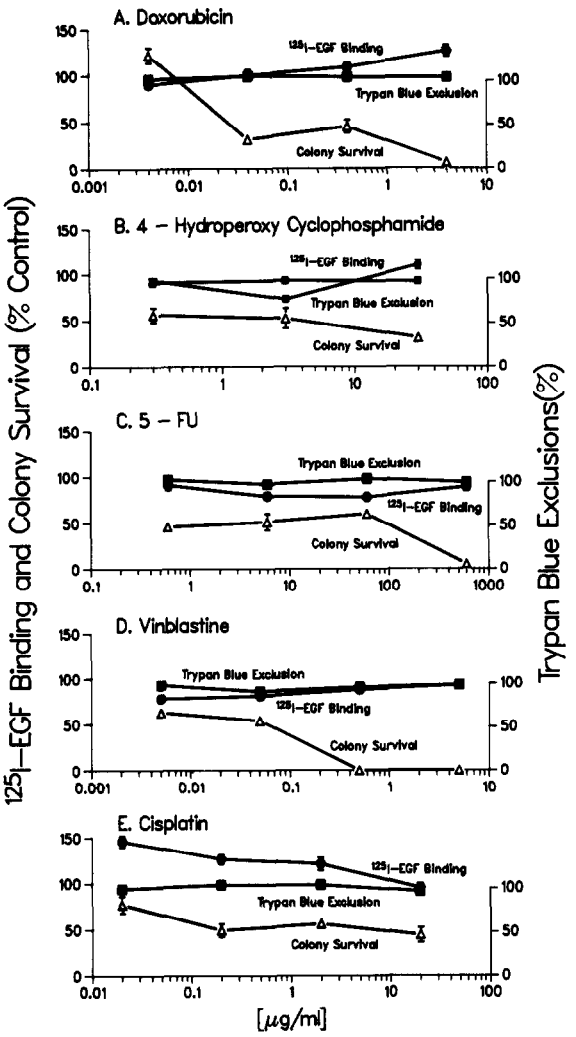

Fig. 1. ^{125}I-EGF binding, soft agar colony formation, and trypan blue exclusion for five antineoplastic agents. MCF-7 cells were grown in six-well plates. When subconfluent to confluent, the cells were preincubated with various drug concentrations for 30 min. *Binding of ^{125}I-EGF to its receptor was studied in a competitive assay. Binding conditions were* 30 min, 37°C, 5% CO_2. A: *doxorubicin*, B: *4-hydroperoxy-cyclophosphamide*, C: *5-fluorouracil*, D: *vinblastine*, E: *cisplatin. Note the absence of consistent effects on EGF binding except for a moderate increase by cisplatin at low concentrations.*

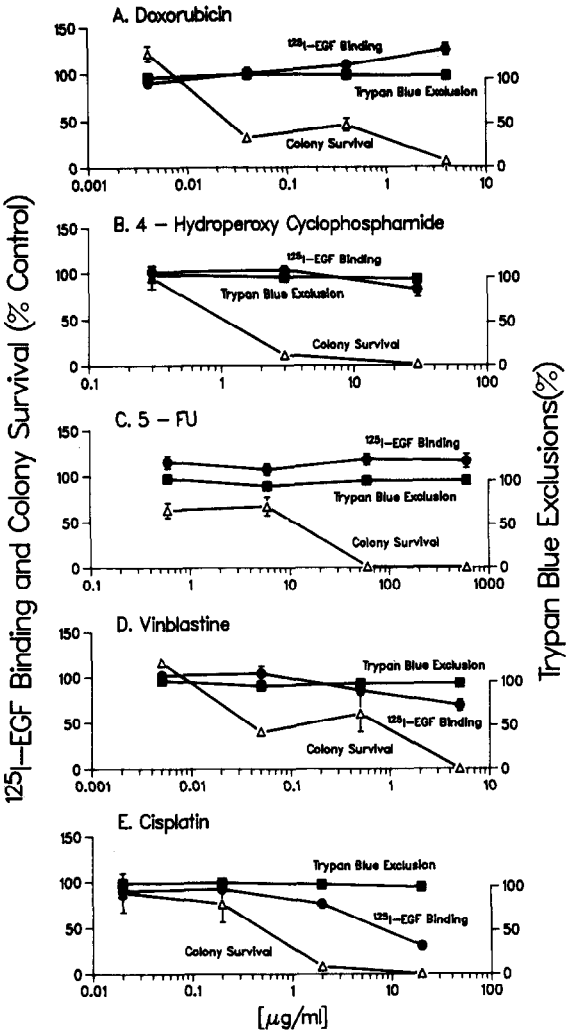

Fig. 2. ^{125}I-EGF binding, soft agar colony formation, and trypan blue exclusion after preincubation with five antineoplastic agents for 2 hr. *Binding conditions were as described in Fig.* 1. A: *doxorubicin*, B: *4-hydroperoxycyclophosphamide*, C: *5-fluorouracil*, D: *vinblastine*, E: *cisplatin. Note the dose-dependent decrease in ^{125}I-EGF binding after pretreatment with vinblastine and ciplatin.*

ity of the receptor for EGF is decreased by a factor of three after a 2-hr-preincubation with cisplatin at 20 μg/ml.

Drugs which did not interfere with ^{125}I-EGF binding under our experimental conditions have been associated with changes in cell membranes and altered EGF binding in other systems. Doxorubicin has been extensively studied and several reports provide evidence for a complex action on membranes. Like cisplatin, doxorubicin disturbs redox mechanisms by inhibition of enzymes [30, 31], and membrane fluidity may be altered [32]. Interestingly, incubation for 3 days at a concentration of 10 nM led to an upregulation of EGF receptors in HeLa and 3T3 cells [33]. In our experiments, no statistically significant change in EGF binding was observed with low concentrations of doxorubicin in

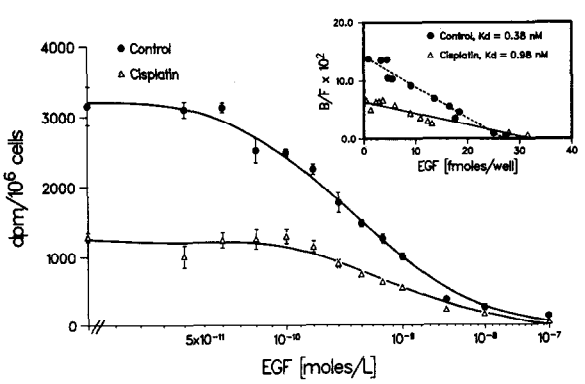

Fig. 3. Binding of ^{125}I-EGF to its receptor before and after pretreatment with cisplatin. MCF-7 cells were grown in six-well plates. When subconfluent to confluent, the cells to be treated were incubated with 20 μg/ml *cisplatin at* 37°C 5% CO_2 *for* 2 hr. *EGF binding was studied in a competitive assay after removal of the drug. Binding conditions were* 3 hr, 0° C, 5% CO_2. *Scatchard analysis is shown in the insert and indicates a change in receptor affinity but not receptor concentration.*

MCF-7 cells, but increased binding was observed at high concentrations perhaps indicating upregulation of receptors in these cells as well.

Vinblastine has been reported to act on cell membranes and to inhibit polypeptide hormone receptor binding. Binding of ^{125}I-insulin to its receptor on rat heart cells is inhibited by the drug (at 10–100 μM) due to a decrease in receptor concentration. This was interpreted to indicate a role for microtubules in the interaction of the hormone with its receptor [29]. Whether microtubule alterations are involved in the decreased EGF binding to MCF-7 cells after incubation with vinblastine in our experiments will require additional study.

Cisplatin has been reported to affect cell membranes of normal and malignant cells. Juckett and Rosenberg [17] reported that cisplatin can lead to the disappearance of cell surface nucleic acids in mouse tumor cells. Furthermore, cisplatin inhibits organic cation transport in brush border and basolateral membranes from the rat kidney cortex [18], selectively inhibits transplasmalemma dehydrogenase in HeLa cells as well as in isolated membranes from mouse erythrocytes and hepatocytes [19], suppresses capping of immunoglobulin and concanavalin A receptors on mouse spleen cells [20], and inhibits the activity of several membrane enzymes leading to increased membrane permeability in ascites sarcoma-180 cells [21]. Binding of the drug to erythrocyte membranes has been linked with the development of a hemolytic anemia occasionally seen during the clinical use of cisplatin [22]. Additional evidence for an action on cell membranes by cisplatin is provided by a report that the agglutinability of normal and transformed cells can be modulated by the drug [23].

Our results demonstrate that cisplatin also interacts with the cell membrane of MCF-7 and alters the binding of EGF, a potentially important growth factor. At least two classes of different polypeptides, EGF and alpha-transforming growth factors (alpha-TGFs) exert a mitogenic activity on MCF-7 cells by activating the EGF receptor system [12], and alpha-TGF-like factors might be involved as secondary messengers in the estrogen-mediated stimulation of breast cancer growth *in vitro* and *in vivo* [35]. Thus, disturbance of the EGF receptor might result in reduced tumor growth. However, it is unknown how much of an alteration of the EGF receptor binding is required for a specific reduction in tumor growth. It could be argued that, for cisplatin, colony formation of the MCF-7 cells in soft agar was decreased at concentrations at which EGF binding was apparently unaltered or slightly increased and that colony survival and EGF binding are thus unrelated. However, the current consensus for drug testing with the human tumor cloning assay—at least for primary tumors—is such that at least a 50–70% reduction in colony formation is required for biologically meaningful results [36, 37]. For cisplatin, decreased EGF binding was accompanied by such reductions in colony formation. Although this effect is observed most clearly at 20 μg/ml, 10 times higher than the usual peak plasma level, we suggest that this might still offer an additional mechanism for the antiproliferative effects of cisplatin. High doses of cisplatin have been used in regional intraperitoneal chemotherapy as well as in limb perfusions [24–28] and peak peritoneal concentrations may reach concentrations 20-fold higher than the peak plasma level (20.1 μg/ml–101.1 μg/ml) [24, 25]. Also, peak plasma concentrations well above 2 μg/ml have been reached during intraarterial infusion of cisplatin [26, 27].

The mechanisms leading to the observed affinity changes are currently poorly understood but comprise three possible events: direct competition with the natural ligand for binding to the receptor, binding to cell membrane compounds with secondary changes of receptor conformation, and secondary receptor alterations resulting from intracytoplasmatic drug action. As shown in Table 1, neither cisplatin nor vinblastine showed a statistically significant inhibition of ^{125}I-EGF receptor binding when incubated together with the ligand. This indicates that direct competition for receptor binding might not be a critical factor for the observed drug-related inhibition in EGF binding.

In summary, our studies provide evidence that vinblastine and cisplatin affect the plasma membrane of MCF-7 human breast cancer cells and reduce EGF binding; cisplatin is shown to alter the affinity of the EGF receptor for its ligand. Reduction in growth factor binding is a potential secondary mechanism for the antineoplastic activity of these drugs.

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