Effects of c-erbB2 Overexpression on the Drug Sensitivities of Normal Human Mammary Epithelial Cells

Michael S. Orr, Patrick M. O’Connor, Kurt W. Kohn

Background: Overexpression of the gene c-erbB2, which encodes a receptor tyrosine kinase, in breast tumors has been linked with either increased or decreased response of breast cancer patients to various therapies. In breast cancer cell lines, overexpression of exogenous c-erbB2 sometimes alters drug sensitivities but sometimes has no effect. To avoid the genetic complexities associated with established cancer cell lines, normal human mammary epithelial cells (HMECs) were studied to determine whether c-erbB2 overexpression by itself would alter chemosensitivity. Methods: HMECs were designed to overexpress c-erbB2, and these cells were then evaluated for alterations in chemosensitivity. Results: HMECs overexpressing c-erbB2 failed to show any alterations in chemosensitivity to a panel of chemotherapeutic agents, as indicated by 95% confidence intervals on growth curves of cells treated with or without the agent of interest. With the use of fluorescence-activated cell sorting to enrich for HMECs overexpressing c-erbB2 on their surface, an 85% pure population of cells was isolated and their chemosensitivity was evaluated. Again, the cells failed to display any alterations in chemosensitivity. Conclusions: These results suggest that overexpression of c-erbB2 is not sufficient by itself to induce changes in chemosensitivity. Cellular studies using normal human cells in which the complexity of the system can be carefully controlled by the addition of one, two, or even more genes associated with cancer development may provide valuable information about how the products of the genes interact with each other and which combinations are critical in regulating chemosensitivity. [J Natl Cancer Inst 2000;92:987–94]

The c-erbB2 gene (also known as HER-2 or neu) encodes a 185-kd transmembrane glycoprotein receptor tyrosine kinase that contains sequence homology to members of the type-1 receptor tyrosine kinase family, which includes c-erbB1, c-erbB3, and c-erbB4 (1). Growth factors (such as epidermal growth factor [EGF] or Neu differentiation factor) interact with the extracellular domains of different type-1 tyrosine kinase family members, which facilitates the formation of homodimers and heterodimers of various receptor family members. The heterodimerization of c-erbB2 with other receptor family members leads to intracellular tyrosine autophosphorylation at multiple tyrosine residues on the carboxyl terminal region and provides docking (binding) sites for Src homology 2 adapter proteins (2–4). The docking proteins then relay the activation signal to proteins, such as ras, raf, mitogen-activated protein kinase (MAPK), c-Jun kinase, or ribosomal protein S6 kinase, which eventually leads to alterations in cell growth and transformation (1.5–10).

The association between c-erbB2 overexpression and clinical response is complex. C-erbB2-overexpressing tumors tend to be resistant to adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil (5-FU) chemotherapy (11,12). However, patients overexpressing c-erbB2 benefited from adjuvant therapy withCAF (cyclophosphamide, doxorubicin [Adriamycin], and 5-fluorouracil), while patients with low or undetectable levels of c-erbB2 did not (13). Two recent studies (14,15) provided further evidence that adjuvant therapy with CAF may be beneficial for breast cancer patients overexpressing c-erbB2; however, neither study was conclusive. Thus, the role of c-erbB2 in chemosensitivity of clinical tumors remains uncertain.

Cellular studies exploring the relationship between c-erbB2 overexpression and its effects on chemosensitivity have given conflicting results. A study of a panel of breast cancer cell lines designed to overexpress c-erbB2 indicated that two of four breast cancer cell lines tested were more resistant to cisplatin as compared with the empty-vector control cells, while there were no alterations in chemosensitivity in the other cell types (16). It appears that alterations in chemosensitivity are dependent on the combined effects of c-erbB2 overexpression and the intrinsic genetic alterations associated with a particular cell line studied.

Previously published in vitro studies suggest that a combination of genetic changes may work in concert with c-erbB2 overexpression, and alterations in chemosensitivity only become manifested when a particular set of genetic alterations has arisen in the breast cancer cell type studied. To avoid the genetic complexities associated with established cancer cell lines, normal human mammary epithelial cells (HMECs) were used in this study to determine whether c-erbB2 overexpression by itself would alter chemosensitivity.

Materials and Methods

Cell culture. HMECs cryopreserved in the seventh passage were purchased from Clonetics (San Diego, CA). HMECs from a 22-year-old woman were used for one, two, and four transductions. The HMECs for transduction 3 were from a 41-year-old woman (Clonetics). Both cell types had similar doubling times of approximately 24 hours. HMECs were grown at 37°C in 95% air–5% CO2, in mammary epithelial growth medium purchased from Clonetics. The cells were routinely seeded at 3500 cells/cm2 and then passaged when the cells were confluent. The medium was replaced every 2–3 days. The cells were maintained in culture for no more than 30 days because the doubling time increased to greater than 70 hours and phenotypic signs of senescence were displayed at subsequent passages. The doubling time between days 23 and 26 was approximately 24–27 hours for both cell types. PT67 cells were obtained from Clontech Laboratories, Inc. (Palo Alto, CA). These cells were grown in Dulbecco’s modified Eagle medium (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO).
St. Louis, MO), 4 mM l-glutamine (Life Technologies, Inc.), and 100 µg/mL penicillin-streptomycin (Life Technologies, Inc.). SK-OV-3 cancer cells obtained from the American Type Culture Collection (Manassas, VA) were grown in RPMI-1640 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co.).

Subcloning and transfection of PT67-packaging cells. The long terminal repeat-2/erbB2 plasmid containing the 3.8 kilobase (kb) complementary DNA (cDNA) of c-erbB2 was from the laboratory of Dr. J. Pierce (National Cancer Institute, Bethesda, MD) (8). The 3.8-kb fragment was digested at the XhoI site, and the fragment was isolated by use of a spin-X centrifuge tube filter (Corning Costar Corp., Cambridge, MA). The isolated c-erbB2 cDNA fragment was blunt ended and then ligated into the pLNCX retroviral vector in the HpaI site down stream of the human cytomegalovirus immediate early promoter. The retroviral viral vector is designed to overexpress a gene of interest as well as the neoymycin resistance gene for selection of cells that overexpress the gene of interest, c-erbB2 in this case. The pLNCX retroviral vector and PT67 packaging cell line were purchased from Clontech Laboratories, Inc. The PT67 packaging cells at a density of 6 × 10⁵ cells per 75-cm² flask were transfected with 10 µg of either the empty vector (pLNCX) that lacks the c-erbB2 cDNA or c-erbB2-overexpressing vector (pLNCX-erbB2) that contains the c-erbB2 cDNA by use of the lipofectamine reagent according to the manufacturer’s protocol (Life Technologies, Inc.). Following transfection, PT67 cells were selected for neomycin resistance for 2 weeks in 500 µg/mL of G418 medium (Life Technologies, Inc.), and the cells that survived the selection process were pooled and expanded, and aliquots were frozen for future use. Stably transfected PT67 cells capable of producing infectious but replication-incompetent virus of either the empty vector or c-erbB2 were used to transduce HMECs.

Transduction of HMECs. HMECs were plated at a density of 8000 cells/cm² 1 day before transduction. On the day of transduction, polybrene (Sigma Chemical Co.) was added to the viral supernatants at a final concentration of 4 µg/mL, and it was then filtered through a 0.45-µm cellulose acetate filter. The HMECs’ medium was removed, and virus-containing supernatants collected from the PT67 cells were layered over the cells. The HMECs were incubated with the viral supernatants for 8 hours. Stably transfectected, the medium was aspirated, the cells were rinsed once with sterile 1× PBS (pH 7.4) and then washed with 1× PBS (pH 7.4) and 10% fetal bovine serum (Sigma Chemical Co.) at 37 °C. The DNA content and c-erbB2 protein level analysis was performed by use of a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). For each sample, 40,000 cells were analyzed. HMECs containing the empty-vector or overexpressing c-erbB2 were labeled with anti-erbB2-FITC (Becton Dickinson Immunocytometry Systems), and enrichment by FACs was performed. The empty-vector control cells were also passed through the machine, and the total population of cells was isolated. The isolated cells were then expanded and analyzed for c-erbB2 protein levels and were also used in the MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] chemosensitivity assay.

FACS analysis of HER-2 protein levels by fluorescence intensity measurement. HMEC vector, c-erbB2-overexpressing protein, MCF-7, or SK-OV-3 cells were incubated with 40 µL of anti-erbB2-FITC (Becton Dickinson Immunocytometry Systems) for 30 minutes in the dark at room temperature. The cells were then washed with 1× PBS (pH 7.4), and analysis was performed by use of a FACScan flow cytometer. The mean fluorescence was determined for each sample in triplicate for each cell type.

MTS chemosensitivity assay. The empty-vector or c-erbB2-overexpressing cells were plated in a 96-well plate at a density of 500 cells per well in six replicate wells per concentration of a particular drug. The following day, the cells were exposed for 5 days to paclitaxel (Taxol, Dr. Jill Johnson, Drug Synthesis and Chemistry Branch, National Cancer Institute), 1 µg/mL of doxorubicin (Adriamycin) (NSC 123127), 5-fluorouracil (NSC 19893), methotrexate (NSC 740), cisplatin (Sigma Chemical Co.), or flavopiridol (from Dr. A. M. Senger, National Cancer Institute). The paclitaxel, doxorubicin, 5-fluorouracil, and methotrexate were obtained from Dr. Jill Johnson. After the 5-day exposure to one of the various drugs, the MTS tetrazolium salt reagent (inner salt; Promega Corp., Madison, WI) was added to each well. The plates were incubated for 6 hours, and the absorbance at 490 nm was measured. The tetrazolium salt reagent was prepared by dissolving 3 mg of MTS in 1.5 mL of dimethyl sulfoxide and 1 mL of water. The final concentration of MTS was 5 µg/mL.

Protein and lipid analysis. Cell surface protein levels of c-erbB2 and enrichment by FACS. HER-2 protein levels by fluorescence intensity measurement. HMEC vector, c-erbB2-overexpressing protein, MCF-7, or SK-OV-3 cells were incubated with 40 µL of anti-erbB2-FITC (Becton Dickinson Immunocytometry Systems) for 30 minutes in the dark at room temperature. The cells were then washed with 1× PBS (pH 7.4) and fixed in 70% ethanol. Samples were then washed with 1× PBS (pH 7.4) and stained with propidium iodide at a dose of 60 µg/mL (Sigma Chemical Co.) containing ribonuclease at a dose of 100 µg/mL (Sigma Chemical Co.) for 30 minutes at 37 °C. The DNA content and c-erbB2 protein level analysis was performed by use of a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). For each sample, 40,000 cells were analyzed. HMECs containing the empty-vector or overexpressing c-erbB2 were labeled with anti-erbB2-FITC (Becton Dickinson Immunocytometry Systems), and enrichment by FACs was performed. The empty-vector control cells were also passed through the machine, and the total population of cells was isolated. The isolated cells were then expanded and analyzed for c-erbB2 protein levels and were also used in the MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] chemosensitivity assay.
In the first transduction, the cells were used between days 20 and 26; in the second, between days 18 and 24; and in the third, between days 18 and 24. The FACS-enriched cells were used between days 23 and 29 in culture for their respective chemosensitivity assays.

**Statistical analysis.** Means and 95% confidence interval were calculated by use of the Microsoft Excel 97 SR-2 software package.

**RESULTS**

c-erbB2 Expression and Phosphorylation in Transfected HMECs

HMECs designed to overexpress c-erbB2 were evaluated for c-erbB2 protein levels by western blot analysis. As indicated in Fig. 1, A, HMECs expressed elevated levels of c-erbB2 protein as compared with the empty-vector-infected cells; subsequent infections produced cells that overexpressed similar levels of c-erbB2 protein (data not shown).

It was of interest to determine the tyrosine phosphorylation status of c-erbB2 protein, since tyrosine phosphorylation has been indicative of the presence of an activated receptor capable of initiating signals transmitted from the membrane to a variety of intracellular signaling proteins (1). As shown in Fig. 1, B, c-erbB2 was heavily tyrosine phosphorylated, while the empty-vector control cells displayed barely detectable levels of tyrosine phosphorylation. To confirm that the tyrosine phosphorylated protein was indeed c-erbB2, the same blot was stripped and probed again with the c-erbB2 antibody (lower panel, Fig. 1, B). C-erbB2 protein was detected at the same site at which the intense tyrosine phosphorylation was found.

C-erbB2 receptor signaling through the MAPK pathway was evaluated by the use of anti-active MAPK antibodies that recognize the dually phosphorylated, active form of MAPK. This method measured the levels of activated MAPK present in c-erbB2-overexpressing cells compared with the empty-vector control HMECs. PC12 cell extracts treated with NGF or untreated were used as controls for the anti-active MAPK antibody. As indicated in the upper panel of Fig. 1, C, PC12 cells treated with NGF had elevated levels of both active forms of MAPK (ERK1 [p44] and ERK2 [p42]); conversely, the activated forms of ERK1 and ERK2 in the untreated PC12 cells were undetectable. More important, HMECs overexpressing c-erbB2 contained elevated levels of the active form of ERK2 as compared with the empty-vector control cells. As shown in Fig. 1, C (lower panel), total levels of MAPK protein were relatively equivalent between the PC12-treated and untreated samples and also for the c-erbB2-overexpressing and empty-vector HMECs. This result indicates that c-erbB2-overexpressing HMECs contained enhanced signaling through activated MAPK (ERK2) as compared with the empty-vector control cells, although it appears that ERK1 is not activated in HMECs overexpressing c-erbB2. These results indicate that c-erbB2 overexpression is capable of inducing cellular signaling through the MAPK pathway in HMECs.

It was of interest to determine the levels of c-erbB2 protein in HMECs overexpressing c-erbB2 as compared with breast or ovarian cancer cells known to overexpress high levels of c-erbB2 protein on their surface. As indicated in Fig. 1, D,
the surface levels of c-erbB2 in c-erbB2-overexpressing HMECs were greater than those in SK-OV-3 cells as measured by fluorescence intensity by FACS on a single-cell basis. Furthermore, MCF-7 cells contained low levels of HER-2 protein per positive cell as compared with either the c-erbB2-overexpressing HMECs or the SK-OV-3 cells, and this result was consistent with previously published studies (16,18). A previous study (18) had indicated that SK-OV-3 c-erbB2 protein levels are greater than or equal to c-erbB2 expression levels in eight of 10 breast cancer cell types screened. The results provide strong evidence that the levels of c-erbB2 protein expression per cell are greater than the levels observed in SK-OV-3 cells and, therefore, indirectly indicating that the HMEC c-erbB2 expression levels are comparable to expression levels exhibited in most breast cancer cell lines that are known to overexpress c-erbB2.

**Protein Levels of c-erbB2 on the Surface of SK-OV-3 or HMECs**

SK-OV-3 ovarian cancer cells, which express c-erbB2 at elevated levels due to amplification of the c-erbB2 gene (19), were evaluated for the quantity of c-erbB2 present on their surface. Fig. 2, A, indicates that 79% of SK-OV-3 cells contain detectable levels of c-erbB2 on their surface as compared with the total population of propidium iodide-stained cells (Fig. 1, A). We wanted to determine if the quantities of c-erbB2 on the surface of c-erbB2-overexpressing HMECs were comparable to SK-OV-3 cells. Either the empty-vector or c-erbB2-expressing HMECs were analyzed for the percentage of cells with c-erbB2 protein levels on their surface as compared with the total population of cells analyzed. The empty-vector control cells had barely detectable levels of c-erbB2 on their surface, 0.03% (Fig. 2, B), while the c-erbB2-overexpressing cells were found to contain 11% c-erbB2 expression on their surface. As compared with the SK-OV-3 reference cells, c-erbB2-overexpressing HMECs contained lower levels of c-erbB2 on their surface.

Although these HMECs overexpressed high levels of c-erbB2 intracellularly, only a fraction of the cell population exhibited strong surface expression of c-erbB2.

To obtain an enriched population of cells expressing c-erbB2 on their surface (so as to determine—see below—whether this altered chemosensitivity), an aliquot of HMECs derived from the 22-year-old woman was transduced with c-erbB2 as described previously. The cells were then sorted by use of a FITC-conjugated monoclonal antibody to c-erbB2. The selected HMECs were expanded. Cell surface levels of c-erbB2 were determined on the same day that the cells were plated for chemosensitivity studies. The empty-
vector control cells had barely detectable surface levels of c-erbB2, while 85% of the c-erbB2-enriched cells exhibited c-erbB2 surface expression (Fig. 2, C). This technique increased the population of cells overexpressing c-erbB2 from approximately 11%–85%, which was comparable to the levels of c-erbB2 found on the surface of SK-OV-3 cells, 79% (Fig. 2, A). Next, we evaluated the chemosensitivity of cells that were or were not selected by FACS to a variety of agents commonly used in breast cancer therapy.

**Chemosensitivity of c-erbB2-Overexpressing HMECs**

Asynchronously growing isogenic pairs of cells that were not FACS selected were exposed to cisplatin, doxorubicin, 5-fluorouracil, paclitaxel, methotrexate, or flavopiridol for 5 days on plastic and then the MTS assay was performed. There was no difference in chemosensitivity to the various chemotherapeutic agents tested in three independent experiments (Fig. 3, A). In the case of paclitaxel, two of the experiments in which the cells were obtained from the 22-year-old woman had an IC_{50} (the concentration of drug that inhibited cell growth by 50% as measured by the MTS assay following a 5-day long-term exposure to the drug) of approximately 55 nM for both empty-vector and c-erbB2-overexpressing cells. In the third experiment, in which the cells were from the 41-year-old woman, the dose–response curve for paclitaxel was shifted to an IC_{50} of 5 nM. Even with the shift in the dose–response curve between the two different patient samples, there was still no difference in the chemosensitivity between the empty-vector and c-erbB2-overexpressing cells in each of the independent experiments (data not shown).

HMECs obtained from cell sorting and characterized to be 85% pure with respect to the detection of cell surface c-erbB2 protein were continuously exposed to five chemotherapeutic agents for 5 days before chemosensitivity was analyzed by the MTS assay. Insufficient cells were available at this stage to adequately test each of the chemotherapeutic agents, so flavopiridol was not tested. Again, we found no alterations in chemosensitivity in the c-erbB2-overexpressing cells as compared with the empty-vector control cells for any of the chemotherapeutic agents tested (Fig. 3, B). These results suggest that overexpression of c-erbB2 is not sufficient, by itself, to induce changes in chemosensitivity.

A compilation of the IC_{50}s derived from the dose–response curves in (Fig. 3, A and B) is shown in Table 1 (left side). Furthermore, there were no apparent differences between a relatively pure population of FACS-selected, c-erbB2-overexpressing cells as compared with the empty-vector control cells (Table 1, right side). These results suggest that overexpression of c-erbB2 fails to modulate chemosensitivity in normal HMECs.

**DISCUSSION**

Cell studies exploring the relationship between c-erbB2 overexpression and its effects on chemosensitivity have given seemingly conflicting results. A study utilizing a panel of breast cancer cell lines designed to overexpress c-erbB2 indicated that alterations in chemosensitivity may occur, in part, because of the combination of two parameters: the genetic background of the cell line studied and c-erbB2 overexpression (16). To eliminate genetic diversity in our cellular studies, we evaluated the role of c-erbB2 overexpression in the drug sensitivities of normal HMECs. The results suggest that overexpression of c-erbB2 is not sufficient, by itself, to induce changes in chemosensitivity as assayed by use of the MTS approach with cells cultured on plastic. Therefore, other as yet unidentified genetic changes within a cancer cell may work in combination with c-erbB2 to elicit alterations in chemosensitivity. These studies suggest that a combination of genetic changes may work in concert with c-erbB2 overexpression and that chemosensitivity alterations become manifested only when a particular set of genetic alterations has arisen.

Evidence indicating that multiple genetic alterations may be required to influence chemosensitivity by the overexpression of c-erbB2 was previously found in co-transfection studies performed in MCF-10A immortalized cells (20). Co-transfection of c-erbB2 and mutated c-H-ras induced the expression of mdr-1 and enhanced P-glycoprotein activity, resulting in resistance to doxorubicin in MCF-10A cells (20). In contrast, overexpression of either gene alone failed to induce resistance to doxorubicin (20). Even though the study using MCF-10A immortalized cells provides evidence that c-H-ras and c-erbB2 may cooperate, these findings may or may not be recapitulated in another immortalized breast cell line because of the genetic diversity sustained during immortalization.

Genetic alterations modifying the levels of the estrogen receptor, progesterone receptor, or EGF receptor by themselves or in various combinations have been identified in breast cancer, and several studies (21–23) have demonstrated associations between these alterations and changes in chemosensitivity and prognosis. HMECs exposed to tamoxifen at concentrations reaching 1 μM failed to display any alterations in growth, either indicating the absence of the estrogen receptor or, alternatively, a lack of growth dependence on the estrogen receptor in these cells (data not shown). Moreover, HMECs express only luminal cytokeratin 18 and not 19, information provided by Clonetics, while breast cancer cells express the luminal cytokeratins 8, 18, and 19. The role of c-erbB2 overexpression and chemosensitivity in a population of uniformly luminal cancer cells may be different from the overexpression of c-erbB2 in HMECs that lack the expression of cytokeratins 8 and 19. Furthermore, normal HMECs contain growth-inhibitory proteins necessary for senescence. The growth-regulatory proteins are perturbed in immortalized cell lines, allowing them to escape senescence. The presence of these regulatory proteins in normal HMECs may block the c-erbB2 signaling pathways that modulate chemosensitivity in the unsorted and/or sorted c-erbB2-overexpressing HMECs. Because only a small fraction of the cells overexpresses c-erbB2 on the surface in the unsorted HMECs, the mutations in chemosensitivity induced by c-erbB2 overexpression may not have been detected. However, a relatively pure population of HMECs overexpressing c-erbB2 on their surface (85%) still failed to display any alterations in chemosensitivity. This result indicates that c-erbB2 overexpression, by itself, does not modulate chemosensitivity in these cells.

To avoid evaluating just apoptosis that has been associated with the 2-day MTT viability assay, our studies consisted of using chronic drug exposures of 5 days. Many studies [reviewed in (24)] in the literature have indicated that longer drug exposures often discern between transient early apoptotic reductions in cell number and long-term growth inhibition. Clonogenic assays are an excellent platform for evaluating long-term alterations in che-
Fig. 3. Chemosensitivity of c-erbB2-overexpressing human mammary epithelial cells (HMECs). Panel A: Asynchronously growing HMEC-overexpressing c-erbB2 or the isogenic empty-vector control cells were continuously exposed to the various drugs for 5 days before growth was evaluated by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] thiazolium salt reagent assay. The chemosensitivity of HMECs overexpressing c-erbB2 was compared with that of the empty-vector control cells for each drug. The mean and 95% confidence interval for each point were derived from three independent experiments, except for paclitaxel. The paclitaxel data are the mean and range from the two replicate experiments, since the third experiment that used cells from a different human donor was much more sensitive to the paclitaxel. Panel B: chemosensitivity of HMECs sorted for the expression of c-erbB2 on their cellular surface. HMECs isolated by fluorescence-activated cell sorter in which 85% of the HMECs expressed c-erbB2 on their surface were continuously exposed to the various drugs for 5 days before growth was evaluated by the MTS assay. The chemosensitivity of the sorted HMECs was compared with that of the isogenic empty-vector control cells. CDDP = cisplatin; Adriamycin = doxorubicin.
mosensitivity in various immortalized cell types. However, clonogenic assays are very difficult to perform in normal HMECs engineered to overexpress c-erbB2, since half of their lifespan before senescence is lost during the transduction and selection process. Clonogenic assays routinely consist of plating cells previously exposed to chemotherapeutic agents for 14–21 days. Unfortunately, HMECs transduced to overexpress c-erbB2 have a limited period of time before their growth rate is reduced and the cells enter senescence. Clonogenic assays were not performed in this study because of the confounding effects of senescence at time points greater than 10 days.

Overexpression of EGF receptor (EGF-R), erbB3, or erbB4 in combination with c-erbB2 may lead to downstream alterations in gene expression and possibly changes in the sensitivity of these cells to chemotherapeutic agents. In breast cancer cells, antibodies specific to c-erbB2 or EGF-R have been shown to potentiate the cytotoxic effects of chemotherapeutic agents, indicating a link between growth factor receptors and sensitivities to cytotoxic agents (25–27). Another layer of complexity associated with cell signaling through erbB family members is dependent on the presence of EGF-related ligands. EGF-related ligands can induce different erbB receptor heterodimerizations, which are then capable of initiating distinct signaling pathways (28,29). Furthermore, elevated levels of a particular type of EGF-related ligand may be an essential component necessary to modulate chemosensitivity within the cell through erbB receptor signaling. Overexpression of heregulin β-2 in MCF-7 cells sensitized these cells to doxorubicin and etoposide, providing evidence that the presence of a particular ligand may be a key component in initiating the correct signaling cascade to induce modulations in chemosensitivity (30).

Cellular studies using normal human cells in which the complexity of the system can be carefully controlled by the addition of one, two, or even more genes may provide valuable information that could be used to determine the critical combinations of genes involved in chemosensitivity. Unfortunately, a cellular study cannot replicate the exact biologic conditions of one, two, or even more genes altering chemosensitivity in patients who have developed breast cancer. Nevertheless, evaluating which genes alter chemosensitivity in normal HMECs may provide the necessary insights to determine the key proteins that are involved in modulating chemosensitivity in breast cancer patients.

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**Table 1.** IC$_{50}$ of human mammary epithelial cells (HMECs) containing the empty-vector or overexpressing c-erbB2.$^*$

<table>
<thead>
<tr>
<th>Substrate</th>
<th>HMEC vector</th>
<th>HMEC c-erbB2</th>
<th>FACS selection of HMECs, IC$_{50}$, μM</th>
<th>FACS selection of HMECs, IC$_{50}$, μM derived from one experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HMEC vector</td>
<td>HMEC c-erbB2</td>
<td>HMEC vector sorted</td>
<td>HMEC c-erbB2 sorted</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2.60 (0.30–4.90)</td>
<td>2.10 (1.93–2.27)</td>
<td>3.13</td>
<td>2.81</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.18 (0.10–0.26)</td>
<td>0.17 (0.10–0.24)</td>
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<td>0.08</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>3.00 (2.70–3.30)</td>
<td>3.00 (2.71–3.29)</td>
<td>3.82</td>
<td>4.05</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.062 ± 0.047 ± 0.0048 $^a$</td>
<td>0.057 ± 0.054 ± 0.0045 $^a$</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.04 (0.03–0.05)</td>
<td>0.05 (0.04–0.06)</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>0.16 (0.14–0.18)</td>
<td>0.16 (0.12–0.20)</td>
<td>0.16</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*IC$_{50}$ was determined as the concentration of drug that inhibited cell growth by 50% as measured by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay following a 5-day long-term exposure to the drug. FACS = fluorescence-activated cell sorting.

†The mean and the 95% confidence intervals (CIs) were derived from three independent experiments.

‡Values obtained in two independent experiments.

§Values obtained in the third experiment. Results of the three experiments were not averaged together because these cells, from different individuals, were much more sensitive to paclitaxel than the first individual’s cells. For each individual, however, there was no difference in sensitivity between the c-erbB2-overexpressing cells and the empty-vector cells.


NOTES

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