HER2/neu Oncogene and Sensitivity to the DNA-Interactive Drug Doxorubicin

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ABSTRACT Breast tumor cells overexpressing the proto-oncogene HER2/neu are known to be less responsive to certain DNA-binding chemotherapeutic agents. The current study specifically investigates the correlation between chemosensitivity to the DNA-binding drug doxorubicin and cellular HER2/neu protein levels in a panel of eight breast cancer cell lines (HS-578, BT-474, MDA-MB-453, MDA-MB-231, MDA-MB-175, MCF-7, ZR-75-1 and T47D). The IC50 (the drug concentration required to inhibit cell growth by 50%) values for the cell lines were determined by the sulforhodamine B assay. IC50 values were correlated with HER2/neu protein levels determined by Western blotting. An almost linear relationship between IC50 and HER2/neu protein level for seven cell lines (p = 0.02, r² = 0.680) was found, with protein levels increasing as resistance increased. The findings suggest that overexpression of HER2/neu correlates with increased resistance to doxorubicin in seven of eight breast cancer cell lines studied. The observation that, in one cell line (MDA-MB-175), doxorubicin IC50 did not correlate with HER2/neu levels, suggests that in these cells, an as-of-yet unidentified factor contributes to resistance. If the observed correlation, which was present in seven of eight cell lines, is confirmed in a larger sample size, increased HER2/neu levels may be implemented as a predictor of breast tumor sensitivity to doxorubicin.

INTRODUCTION Breast tumors are characterized by a variety of cellular disorders, including mutations and overexpression of signal transduction proteins. Notably, the mutation and overexpression of the proto-oncogene HER2/neu has been observed in 15 to 35% of breast tumors (1-6). Many other malignancies, including ovarian, gastric and kidney tumors have also exhibited overexpression of the HER2/neu protein (1,7,8). These observations have generated attempts to use anti-HER2/neu antibodies to specifically deliver chemotherapeutic drugs to tumors (9).

The HER2/neu (c-erbB-2) proto-oncogene is located on chromosome 17q21 and encodes a 185 kD transmembrane phosphoglycoprotein, which consists of an intracellular kinase and a cysteine-rich extracellular receptor domain (10). HER2/neu belongs to a family of proteins, which, like the epidermal growth factor receptor, regulate cell growth as receptor tyrosine kinases (11-13).

The oncogenic potential of the HER2/neu protein can emerge in two ways. First, the proto-oncogene may be altered by a single point mutation that replaces valine 664 with a glutamic acid residue (12,14). A structurally altered gene product is generated that confers transforming potential, enhanced tyrosine kinase activity and ultimately enhanced cell proliferation (12). Second, gene transfer experiments have demonstrated that amplification of the normal HER2/neu gene product alone is sufficient to induce cell transformation and oncogenic potential (15), while antisense oligonucleotides specific for HER2/neu cause a reduction in cell growth (16).
The mechanism of enhanced proliferation is believed to be increased ligand-induced receptor-receptor interactions or overthreshold ligand-induced stimulation in cells overexpressing HER2/neu (12,17,18). Additionally, poor patient prognosis and aggressive tumor growth are associated with overexpression of HER2/neu in several tumor types (2,19-21). Further, studies have suggested that this oncogene may be involved in tumor resistance to chemotherapeutic drugs (20-22).

In the past 20 years, many antineoplastic agents have been isolated and synthesized. Of these, doxorubicin (DOX; Adriamycin) has emerged as the most utilized antitumor drug worldwide (23,24). Clinically, this anthracycline is used to treat many solid tumors, such as carcinomas of the breast, bladder, endometrium, lung, ovaries, stomach and thyroid, as well as sarcomas of bone and soft tissue, pediatric solid tumors and lymphoid tumors (24).

The cytotoxicity of DOX is mediated via its capacity to covalently bind double stranded DNA. This subsequently induces protein-linked double strand DNA breaks through the action of topoisomerase IIα (24). Topoisomerase IIα controls DNA supercoiling by cleaving and reannealing double stranded DNA. Intercalators such as DOX interfere with topoisomerase II by stabilizing the cleavable complex, which converts the enzyme into a cellular poison (25-27). The importance of topoisomerase II in the antineoplastic mechanism of DOX has been highlighted by studies demonstrating that decreases in topoisomerase II expression leads to resistance to DOX (28,29).

Other mechanisms of anthracyclines include inhibition of DNA synthesis and RNA polymerases, and formation of free radicals (24,30). Further, free radical formation has also been implicated as a mechanism by which DOX kills human breast cancer cell line MCF-7 in vitro (31,32).

Unfortunately, the use of DOX in cancer chemotherapy is frequently plagued by tumor resistance, which, in some cases, is mediated by the human multidrug resistance-associated protein Pgp (33,34). However, not all resistance can be explained by this mechanism. As previously mentioned, HER2/neu has also been implicated in DOX resistance (20-22). Evidence, such as increased sensitivity to DOX and other antineoplastic agents following reduction in HER2/neu activity by treatment with anti-HER2/neu antibody (35,36) and antisense oligonucleotides (37) and resistance to DOX correlating with HER2/neu expression in cancer cell lines (23,24,38,39), suggest that HER2/neu is related to DOX resistance. Furthermore, there is preliminary evidence that drug resistance linked to overexpression of the HER2/neu is a consequence of its role in DNA repair (40,41).

However, the relevant studies do not entirely agree with the hypothesis that HER2/neu expression correlates with chemoresistance. Namely, Pedram et al. demonstrated that HER2/neu overexpression was not sufficient to produce drug resistance in breast and ovarian cancer cell lines (42). Also, basic evidence suggests that increased HER2/neu activation leads to increases in topoisomerase II expression (43) and reduced gene copies of HER2/neu are linked to decreased gene copies of topoisomerase II (44). These studies suggest that increased HER2/neu would lead to increased topoisomerase IIα, which would increase the antineoplastic activity of DOX. Finally, clinical evidence from Muss et al. demonstrates that patients who overexpress HER2/neu are more likely to benefit from high doses of DOX (45). Due to the conflicting evidence suggesting that HER2/neu might not be involved in drug resistance, studies designed to further define the role of HER2/neu in tumor response to chemotherapeutic agents are in demand.

This work examines the correlation between the expression of HER2/neu and breast tumor cell response to DOX in a panel of eight breast cancer cell lines and finds that, in seven of the eight cell lines, there is a positive correlation of HER2/neu expression to DOX resistance.

**MATERIALS AND METHODS**

**Cell Culture**

Breast cancer cell lines (ATCC) (HS-578, BT-474, MDA-MB-453, MDA-MB-231, MDA-MB-175, MCF-7, ZR-75-1, T47D) were grown in RPMI 1640 (Gibco; Burlington, ON) complete medium containing penicillin streptomycin (2.5 mL/500mL RPMI), L-glutamine (5mL/500 mL RPMI) and fetal bovine serum (FBS) (10%) (Gibco). Incubation of cell lines was at 37°C in a 5% CO2 atmosphere. The growth medium was changed biweekly and plates approaching 100% confluence were split into new plates by trypsinization.

**Determination of the Sensitivity of the Breast Cell Lines to Doxorubicin**

Exponentially growing cell monolayers were incubated with serially diluted concentrations of DOX (Sigma, Oakville, ON) (0.1nM to 800nM in RPMI complete medium), and incubated for five days in 96-well plates. Cytotoxicity was evaluated by the sulforhodamine B assay. Briefly, the cells were fixed by the addition of 50μl of cold trichloroacetic acid (TCA) (50%) at 4°C for one hour. The wells were washed four times with water and stained with sulforhodamine B.
(0.4%) (Sigma) dissolved in 1% acetic acid. The plates were air-dried and the resulting colored residue dissolved in 200μl of Tris base (10mM). The optical density (OD) of each well was measured at 540nM with a microplate reader (Model 3550, BioRad; Mississauga, ON). The results were calculated from at least two independent experiments run in triplicate. Additionally, the IC_{50} value was calculated from each independent experiment. The graphs, therefore, represent the average IC_{50} for the indicated cell line.

**Determination of HER2/neu Levels in the Breast Tumor Cell Lines**

Exponentially growing cells were collected in cold PBS and centrifuged to separate a pellet that was then washed with hypotonic buffer. Cells were lysed by sonication (3 x 10 seconds) and the pellet (nuclei) separated from the supernatant (cytosol) by centrifugation (14,000 rpm). The supernatant was removed and the pellet was resuspended in hypotonic buffer containing 0.1% SDS which was kept on ice for 10 minutes. The protein concentrations of each cell line were measured against a prepared standard curve based on the absorbances of nine serial dilutions at 595 nM, using the method of Bradford (46).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as outlined (47). The samples were subjected to 8% SDS-PAGE gels, and finally transferred to nitrocellulose membranes. The membranes were rinsed in Tris buffered saline (TBS) and Tween 20 (T) and blocked to prevent antibody binding to non-specific sites with a solution of TBS+T+1% bovine serum albumin. To detect HER2/neu, the primary antibody (Cedar Lane; Hornby, ON) (mouse anti-human) was incubated with membranes overnight at 4°C (concentration 1:5000). To detect HER2/neu labeled proteins, an anti-mouse antibody (Cedar Lane) (concentration 1:3000) was incubated with membranes for 30 minutes at room temperature. Enhanced chemoluminescence (Amersham; Oakville, ON) was used to identify immuno-tagged proteins that were then visualized by exposure on Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY).

**Correlation Between Breast Tumor Cell Sensitivity to Doxorubicin with Levels of HER2/neu**

Levels of HER2/neu previously identified by a single Western blot were quantified by BioImage (Visage Electrophoresis Gel Analysis System; Ann Arbor, MI) densitometric scan and expressed in relative OD units. Statistical significance was assumed for \( p < 0.05 \) using Mann-Whitney rank sum test. All statistical analyses were performed with the GraphPad Prism (San Diego, CA) software package. OD units indicate relative levels of HER2/neu.

**RESULTS**

**Quantification of HER2/neu**

The relative levels of HER2/neu in the eight cell lines were quantified by Western blotting (Figure 1, Table 1). BT-474 and MDA-MB-453 expressed similar amounts of the protein. The levels of HER2/neu expressed in these two cell lines were approximately 2-fold greater than in MDA-MB-175, 1.7-fold greater in MCF-7, 5-fold greater than in both HS-578 and MDA-231, 10-fold greater than T47D, and 26.7-fold greater than ZR-75-1. This indicates that the levels of HER2/neu was quite variable within the cell population, a distribution pattern that is critical for the validity of the correlation.

**Chemosensitivity**

The IC_{50} for DOX was determined in each of the eight tumor cell lines (Table 1, Figure 2A and 2B). The

| Breast Cancer Cell Lines | IC_{50} DOX (nM) | HER2/neu Level (OD) |
|-------------------------|-----------------|---------------------|
| T47D                    | 2.706           | 0.154               |
| MDA-MB-231              | 3.560           | 0.357               |
| MCF-7                   | 4.160           | 0.978               |
| HS-578                  | 6.508           | 0.351               |
| ZR-75-1                 | 6.690           | 0.059               |
| MDA-MB-453              | 22.340          | 1.661               |
| BT-474                  | 64.600          | 1.691               |
| MDA-MB-175              | 121.100         | 0.850               |

IC_{50}: Inhibitory concentration; OD: Optical density
most sensitive cell line to DOX observed was T47D (IC50 = 2.706 nM) and the least chemosensitive cell line to DOX was MDA-MB-175 (IC50 = 121.1 nM). The IC50 for each of the other cell lines (MDA-MB-231, MCF-7, HS-578, ZR-75-1, MDA-MB-453, BT-474) was in the range of 3.56 nM to 64.6 nM.

Correlation Analysis
The DOX IC50 obtained for each of the cell lines was correlated with HER2/neu levels. When all eight possible points were displayed (Figure 3A) no significant correlation was observed ($p = 0.08$, $r^2 = 0.42$). The data point for MDA-MB-175 significantly deviated from linearity. Such a deviation may be due to additional mechanisms of resistance (please refer to discussion). Without this point (Figure 3B), a more linear correlation was observed between DOX chemosensitivity and HER2/neu levels ($p = 0.022$, $r^2 = 0.680$). High HER2/neu levels appeared to correlate with reduced cell sensitivity to doxorubicin.

DISCUSSION
This study was performed with eight breast tumor cell lines that presented diverse levels of expression of HER2/neu. MDA-MB-453 and BT-474 expressed the highest levels of the oncogene, while ZR-75-1 showed the lowest levels. It was demonstrated that DOX resistance may be positively related to the expression of the HER2/neu oncogene. Although the number of different cell lines used in this correlation was relatively low, a linear dependence of sensitivity to DOX on HER2/neu levels was apparent in seven cell lines. This, along with previous basic and clinical studies, form a strong basis of evidence suggesting that HER2/neu expression is linked to chemoresistance (23,24,35-39,45,48-50).
The exact mechanism that links overexpression of HER2/neu to DOX resistance is not clearly understood. It has been shown that altered drug accumulation or detoxification is not involved in HER2/neu mediated resistance (40). DNA repair, as well as dysregulation of cell cycle checkpoint and apoptotic mechanism seem to be responsible for the chemoresistance induced by HER2/neu overexpression, however, it remains to be determined which HER2/neu signaling cascade initiates these mechanisms (40).

In this study, one of the cell lines, MDA-MB-175, which expressed low levels of HER2/neu, showed resistance to DOX. The value for this cell line significantly deviated from the correlation established between the seven other cell lines. The current authors believe that this cell line may express another mechanism of resistance, most probably the P-glycoprotein-mediated mechanism (mdr-1). It has been shown that HER2/neu overexpression can lead to intrinsic drug resistance independent of mdr-1 in MDA-MB-435 cells (51). That is, it is possible that MDA-MB-175 is a case wherein the mdr-1 mechanism of chemoresistance dominates that of HER2/neu, and represents the central mode of resistance. The levels of P-glycoprotein (mdr-1) in MDA-MB-175 have not yet been reported. HER2/neu, however plays a prognostic role that is independent of its involvement in multi-drug resistance (mdr-1) (52).

The correlation reported in the current study establishes the value of HER2/neu levels in predicting chemosensitivity to doxorubicin. A firm correlation may then be applied in the clinic to design more effective chemotherapy regimens for solid breast tumors. The correlation may be further strengthened by extension to more cell lines. Carefully controlled experiments may begin to demonstrate whether overexpressed HER2/neu confers resistance alone or by a mechanism dependent on other factors, a finding that has applications to treatment and to patient prognosis.

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