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Toremifene, a Novel Antiestrogen, Can Overcome hsp27-Induced Drug Resistance in Human Breast Cancer Cells

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Human breast cancer cell lines derived from MDA-MB-231 were constructed to express hsp27 constitutively. The elevated presence of this protein resulted in an enhanced ability to survive a heat shock and exposure to doxorubicin, a chemotherapeutic agent. Hsp27 expression was unable to protect cells from doxorubicin if they were cultured in the presence of toremifene. Flow cytometry analysis indicated that cells exposed to both toremifene and doxorubicin accumulate at G2 + M. Protective effects of hsp27 were overcome by addition of an estrogen antagonist at clinically nontoxic levels. Addition of toremifene to chemotherapeutic regimes may enhance the sensitivity of breast cancer cells to doxorubicin.

Key Words: Toremifene; hsp27; heat shock protein; chemotherapy; breast cancer.

Introduction

Cells have the ability to survive and subsequently adapt to environmental stresses. The best-characterized adaptive response is the expression of the heat shock proteins (hsps) following exposure to elevated temperatures (reviewed in Carper et al., 1987; Welch, 1992; Craig et al., 1994). Most eukaryotic cell lines that express hsps express at least one small hsp in the mol-wt range of 20–30 kDa (Ciocca et al., 1993; Arrigo and Landry, 1994). In human cell lines, hsp 27 is expressed as four isoforms, all but one of which are phosphorylated. Following a heat shock, hsp27 is rapidly phosphorylated, forming large heat shock granules, which can migrate to the nucleus (Arrigo and Welch, 1987). The expression of the human hsp27 gene product can protect rodent cell lines from heat-induced cytotoxicity and from certain chemotherapeutic agents (Landry et al., 1989; Huot et al., 1991). The mechanism whereby hsp27 can increase survival against stresses is unclear at the present time. However, hsp27 has been reported to help stabilize actin filaments following a heat shock (Lavoie et al., 1993, 1995).

The role of hsp27 expression in breast cancer may have prognostic value. Overexpression of hsp27 by human primary breast cancers is independently associated with reduced disease-free survival (Thor et al., 1991; Love and King, 1994). Overexpression of hsp27 by human breast cancer could result from response to environmental stresses or from exposure to estrogen. Treatment of human breast cancer cell lines with 17 β-estradiol has been shown to elicit the expression of hsp27 (Fuqua et al., 1989). Human breast cancer cell lines that express hsp27 demonstrated an increase in cell survival when treated with doxorubicin or heat (Ciocca et al., 1992; Oesterreich et al., 1993). Therefore, expression of hsp27 may protect breast cancer cells from the effects of chemotherapy and may be one cause of tumor recurrence.

Toremifene is a triphenylethylene antiestrogen with a structure that is a modification of tamoxifen (Wiebe et al., 1992). It has a high affinity for estrogen receptors and has the ability to induce progesterone receptors (Kangas et al., 1986). Toremifene is capable of sensitizing both estrogen receptor-positive and estrogen receptor-negative human breast cancer cell lines to doxorubicin (DeGregorio et al., 1989). To characterize better the action of toremifene as a chemosensitizer, we investigated the ability of toremifene, in clinically tolerable doses, to overcome hsp27-induced doxorubicin resistance in an estrogen receptor-negative human breast cancer cell line. We demonstrate that the combination of toremifene plus doxorubicin is more effective in growth inhibition than either single agent in hsp27 expressing MDA-MB-231 derived cell lines.

Results

Control (pHβApr-1-neo) or the constitutive expression (pβ27) vectors (Fig. 1) were transfected into MDA-MB-231 cells, and single colonies were isolated following selection in G418 sulfate. The cell lines were maintained in the presence of G418 sulfate. All cells transfected with pβ27 demonstrated constitutive expression of hsp27 as determined by SDS-PAGE (data not shown). A control trans-

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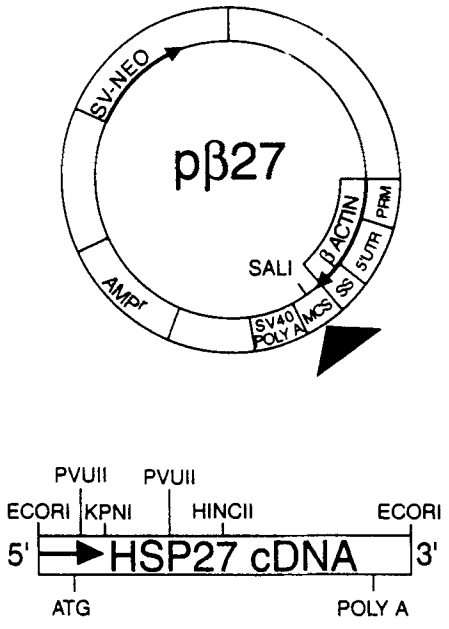

Fig. 1. Plasmid pβ27 used for the constitutive expression of hsp27 under control of the β-actin promoter. The gene for resistance to neomycin is under control of the simian virus early promoter.

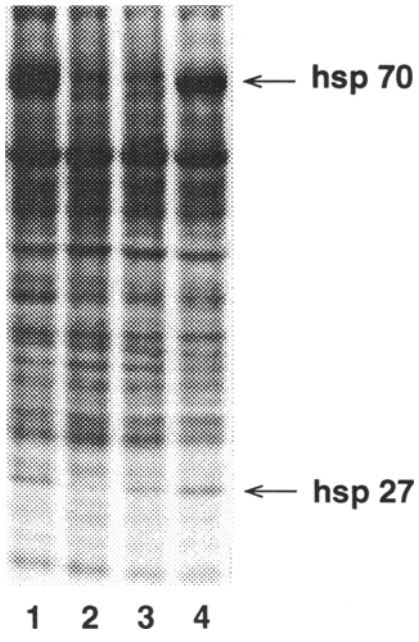

Fig. 2. SDS-PAGE of transfected MDA-MB231 cell lines. Lanes one and two are from the control transfected cell line DC4, whereas lanes three and four are proteins from the pβ27 transfected cell line DB46. Lanes one and four are from heat shocked cells, whereas lanes two and three are from nonheat shocked cells. The locations of hsp70 and hsp27 are indicated by arrows.

fected (DC4) and an hsp27-transfected (DB46) cell line were selected for further characterization.

Protein expression was characterized to demonstrate that the pβ27 plasmid resulted in the increased constitutive production of hsp27. Figure 2 contains proteins expressed prior to or following a heat shock in the DC4 and DB46 cell lines. DC4 expresses a low level of hsp27 prior to heating, but this expression is dramatically stimulated following a heat shock. DB46 expressed a high constitutive level of hsp27 owing to the presence of pβ27. The expression of hsp27 increased slightly in DB-46 cells following a heat shock.

The ability of this hsp to provide a survival advantage to a lethal heating was investigated (Fig. 3). Constitutive expression of hsp27 resulted in up to a 2-log increase in cell survival following heating at 45°C. The control transfected cell line displayed clonogenic survival values similar to that of the parental cell line (data not shown).

Constitutive expression of hsp27 resulted in an increased growth rate in the presence of doxorubicin. At all concentrations tested, the DB46 cell line displayed a statistically significant growth rate when compared to the DC4 cell line. Figure 4 shows the result from a single experiment in triplicate (mean ± SD). This experiment has been repeated three separate times with similar results. When transfected cell lines were grown in the presence of 6.6 μ*M* toremifene, subsequent treatment with doxorubicin produced an identical growth inhibition in the two cell lines. This demonstrates that hsp27-expressing cells were not resistant to doxorubicin when grown in toremifene. Figure 5 shows the results from a single experiment in triplicate (mean ± SD). This experiment has been repeated three separate times with similar results.

To evaluate the mechanism of this growth inhibition, MDA-MB-231 control transfected cells (DC4) were treated with 6.6 μ*M* toremifene, 1.0 μg/mL doxorubicin, or the combination, and evaluated by flow cytometry. Doxorubicin decreased the number of cells in G1/G0 and S phases, and results in accumulation in the G2 + M phase (Fig. 6). Toremifene decreased the number of cells in both the S and G2 + M fractions of the cell cycle. The combination of the two drugs resulted in an increased number of cells in the G2 + M phases of the cell cycle, as did doxorubicin alone, but after 24 h, the cells were not exclusively present in the G2 + M phase. This indicates that the two drugs are capable of blocking cell growth at different parts of the cell cycle.

When doxorubicin dosage is lowered to 0.1 μg/mL the doxorubicin-sensitive (DC4), but not the doxorubicin resistant (DB46) cell line is blocked at G2 + M. The DC4 control cell line was affected by 0.1 μg/mL doxorubicin as manifested by an increase in G2 + M (16% nontreated vs 41% of treated cells) (Fig. 7). This concentration of doxorubicin failed to cause an increase in the G2 + M fraction in the hsp27 transfected cell line (DB46) (13% nontreated vs 13% of treated cells). This result confirms that the DB46 cell line was resistant to doxorubicin. The combination of doxorubicin and toremifene resulted in accumulation of cells in G2 + M in both the DC4 and DB46 cell lines (27% of cells and 20% of cells, respectively). These data appear to confirm our growth inhibition experiments, and suggest that the combination of doxorubicin and toremifene is synergistic.

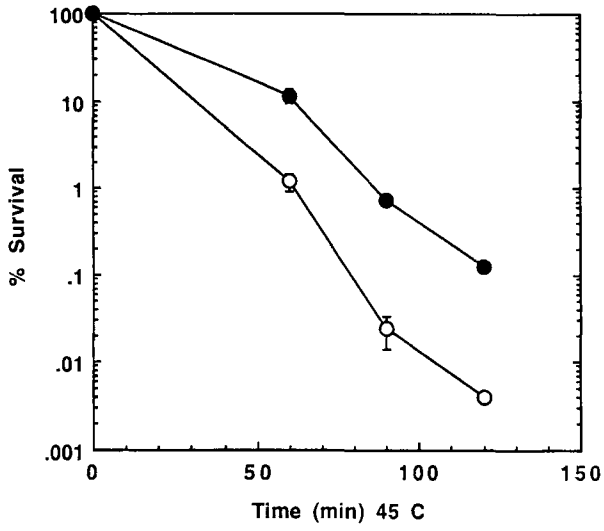

Fig. 3. Effect of hsp 27 expression on survival to a heat shock at 45 °C. The open circles (–○–) represent the DC4 control transfected cell line, whereas the filled circles (–●–) represent DB46, an hsp27 transfected cell line. The control plating efficiency for DC4 was 37% and for DB46, was 35%.

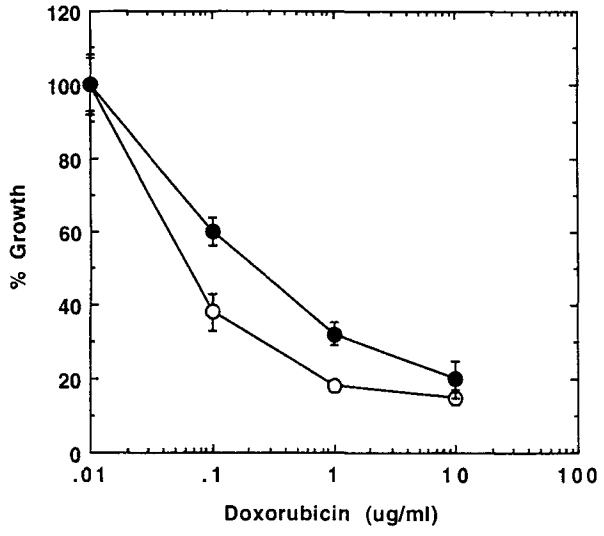

Fig. 4. Effect of hsp27 expression on growth in the presence of doxorubicin. The hsp27-transfected (–●–) and the control transfected (–○–) cell lines were treated with varying concentrations of doxorubicin for 2 h, and then grown for 48 h in medium and counted. Data presented as the mean ± SD of a single experiment in triplicate, and graphed as percent of DC4 or DB46 cell line growth in the absence of doxorubicin. The data were normalized to the control counts of DC4 (517) and DB46 (434). At all doxorubicin concentrations, a significant difference in growth inhibition was noted ($P < .05$).

Discussion

In these studies, we have shown that constitutive expression of hsp27 in the estrogen receptor-negative human breast cancer cell line MDA-MB-231 results in an increased ability to survive the cytotoxic stresses of heat (Fig. 3) or

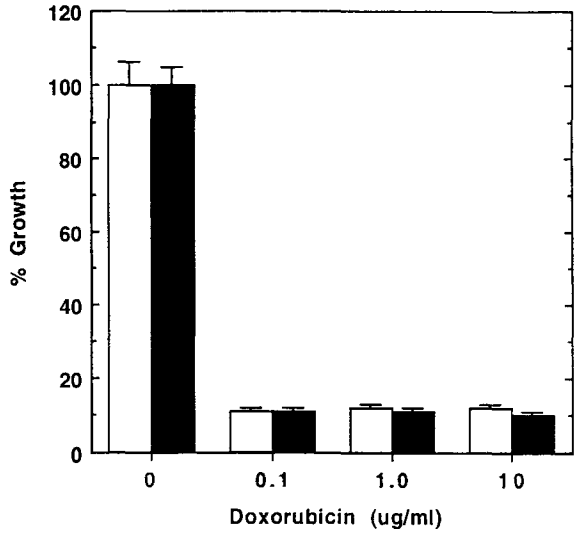

Fig. 5. Effect of toremifene on growth inhibition by doxorubicin. The hsp27-transfected (filled bars) and the control-transfected (open bars) were treated with varying concentrations of doxorubicin in the presence of chronic toremifene (6.6 μ*M*). Data presented as the mean ± SD of a single experiment in triplicate, and graphed as percent of DB46 or DC4 cell line growth in the absence of doxorubicin and toremifene. The data are expressed as the percent of control counts (DC4 [1121] and DB46 [1250]). □, DC4; ■, DB46.

exposure to the chemotherapeutic agent doxorubicin (Fig. 4). These results are consistent with those reported for cell lines that express hsp27 (Huot et al., 1991; Ciocca et al., 1992; Oesterreich et al., 1993). The ability of doxorubicin to inhibit cell growth was dose-dependent (Fig. 4). If cells were cultured in the presence of a clinically tolerable dose of toremifene (6.6 μ*M*), the cytotoxic action of doxorubicin was greatly enhanced (Fig. 5). This result is also consistent with previously published studies (DeGregorio et al., 1989; Baker et al., 1992). Toremifene exposure also resulted in overcoming the hsp27-mediated resistance to doxorubicin. Doxorubicin arrests cell growth by blocking at G2 + M phase of the cell cycle (Wiebe et al., 1992). We confirmed this observation with our parental cell line (Fig. 6) and the control transfected cell line (Fig. 7). However, constitutive expression of hsp27 protected cells from this block in the cell cycle (Fig. 7). If cells were cultured in the presence of clinically tolerable levels of toremifene (6.6 μ*M*), the cytotoxic action of doxorubicin was greatly enhanced. Addition of toremifene to parental cells resulted in a decrease in the number of cells in both S and G2 + M fractions of the cell cycle. The combination of toremifene and doxorubicin in the parental cell line resulted in an increased number of cells in the G2 + M phase of the cell cycle (Fig. 6). Similar results are seen in the control transfected cell line (Fig. 7). The time-course experiment depicted in Fig. 6 suggests that the two drugs are each blocking cell growth at different points in the cell cycle.

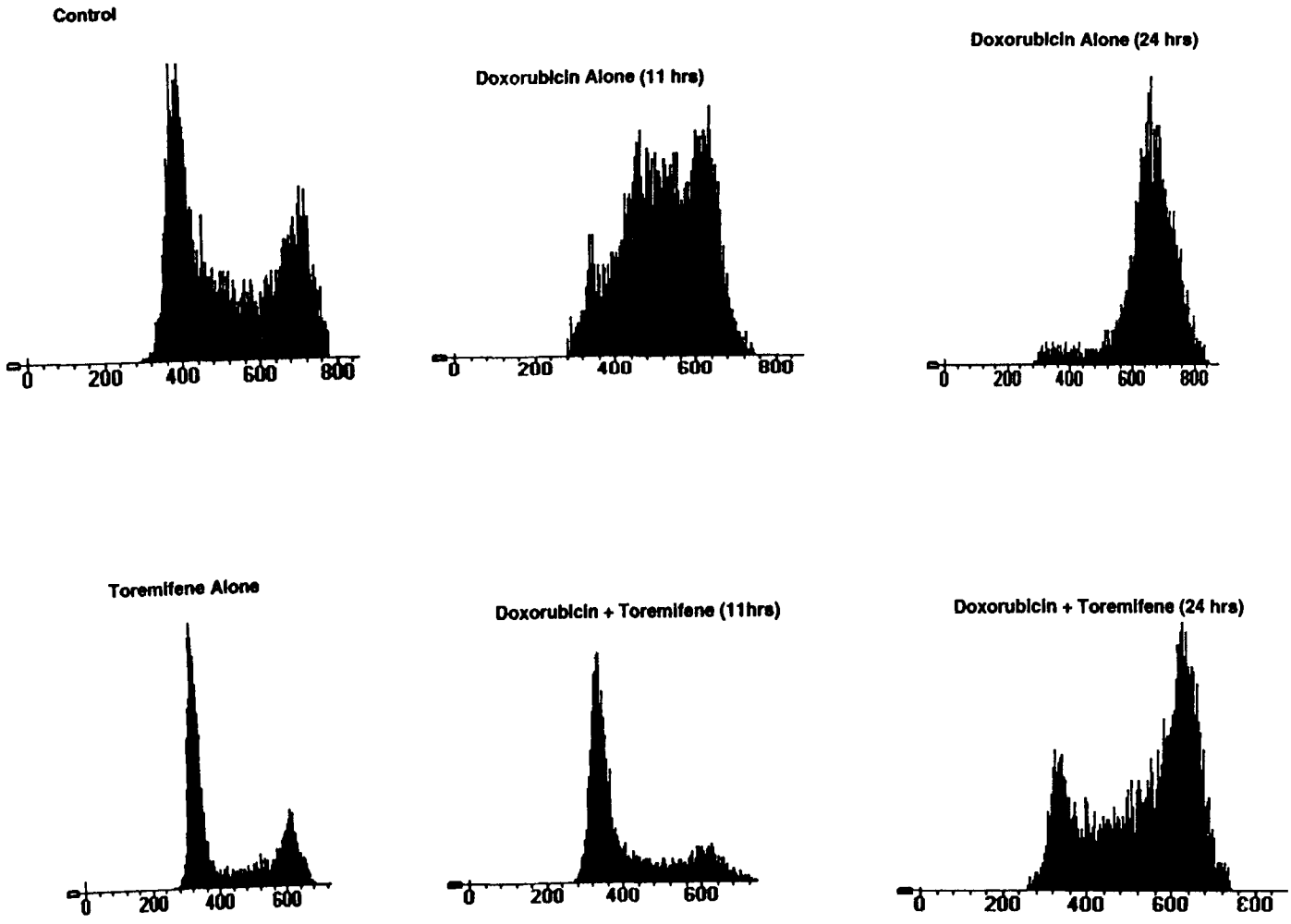

Fig. 6. Flow cytometric analysis of control-transfected (DC4) cells treated with doxorubicin (1.0 μg/mL) or doxorubicin (1.0 μg/mL) plus toremifene (6.6 μ*M*), and evaluated at 11 and 24 h after doxorubicin treatment. Control (untreated) and toremifene-only graphs were obtained at 24 h. A total of 10,000 cells were counted for each treatment. Two separate experiments yielded similar results. The *Y* axis represents the absolute number of nuclei, and the *X* axis represents DNA content analyzed over 256 channels.

The relative G0/G1 toremifene block shows accumulation of cells in the S_1 and G2 + M phases of the cell cycle, and doxorubicin prevents cells from leaving G2 + M. The hsp27 expressing cell line showed a dramatic accumulation of cells in the G2 + M phase following treatment with both toremifene and doxorubicin.

Toremifene has little antitumor effect, and has no effect on doxorubicin accumulation in wild-type MDA-MB-231 human breast cancer cells (Robinson et al., 1990; Wiebe et al., 1992). The synergy of toremifene and doxorubicin in doxorubicin-resistant MDA-MB-231 cells chronically cultured in doxorubicin has been reported (Baker et al., 1992). This combination of doxorubicin and toremifene resulted in greater doxorubicin accumulation in resistant cells (Wiebe et al., 1992). We have demonstrated that the combination of doxorubicin plus toremifene has superior cytotoxic effects compared to doxorubicin alone in cells resistant to doxorubicin owing to constitutive hsp27 expression.

Toremifene has two main biological actions. It is an effective antiestrogen, which can inhibit growth of ER-positive breast cancer cell lines (Kangas et al., 1986; Robinson et al., 1990; Warri et al., 1993; Iino et al., 1993), as well as a chemosensitizing agent (DeGregorio et al., 1989; Baker et al., 1992; Wiebe et al., 1992). In human breast cancer cells that express the estrogen receptor, toremifene has been reported to induce apoptosis (Warri et al., 1993). The mechanism whereby toremifene accentuates the growth-inhibiting action of doxorubicin is unclear. Toremifene's antiestrogen properties do not play a role in the mechanism of drug enhancement, since the MDA-MB-231 cells used in this study do not express an estrogen receptor (Thor et al., 1991). The mechanism used by hsp27 to provide resistance to doxorubicin is also unclear, but does not involve the MDR1 gene product or an enhanced efflux of drug (Huot et al., 1991). The novel finding presented in this study is that toremifene can overcome hsp27-induced drug resistance in human breast cancer cells.

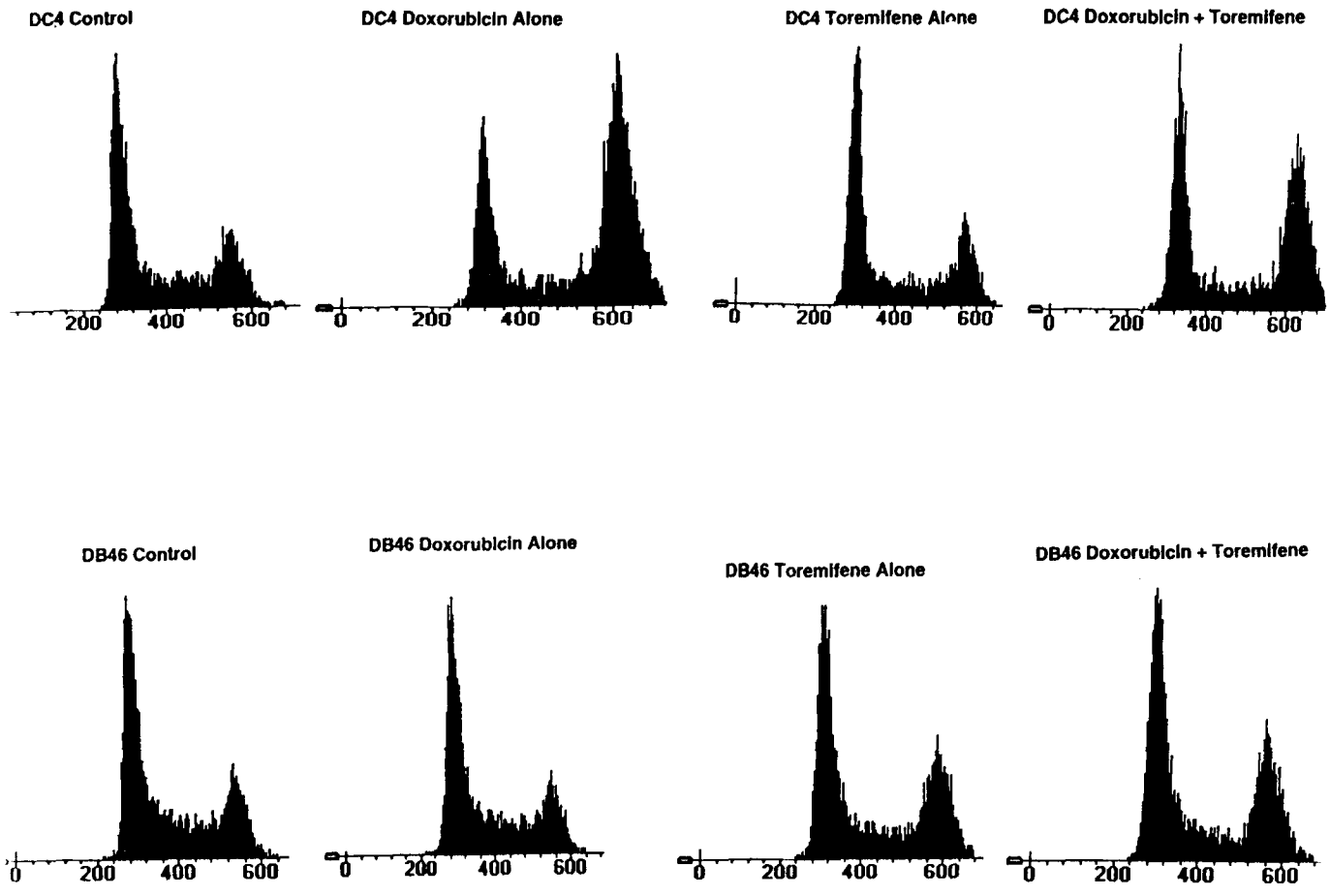

Fig. 7. Flow cytometric analysis of hsp27-transfected (DB46) and control-transfected (DC4) cells treated with 0.1 μg/mL doxorubicin and 6.6 μ*M* toremifene for 24 h. A total of 10,000 cells were counted for each treatment. Two separate experiments yielded similar results. The *Y* axis represents the absolute number of nuclei, and the *X* axis represents DNA content analyzed over 256 channels.

A significant clinical problem with breast cancer is *de novo* or acquired resistance to chemotherapy, which appears to be multifactorial. One factor may be expression of hsp27, which confers resistance to doxorubicin, an important clinical agent in breast cancer therapy. Several clinical trials have investigated potential modulators of drug resistance. Such modulators (e.g. cyclosporine, verapamil, trifluroperazine, quinine, tamoxifen) have been chosen owing to their ability to inhibit MDR1-related resistance (Ozols et al., 1987; Miller et al., 1988; Ford and Hait, 1990; Yahanda, et al., 1992). These drugs have not proven useful clinically because the doses required for modulation have been toxic to humans. However, recent reports have demonstrated that a new antiestrogen (toremifene) is capable of chemosensitization at nontoxic doses (Kangas et al., 1986; Baker et al., 1992; Wiebe et al., 1992).

The expression of the estrogen-responsive protein hsp27 can protect breast cancer cells from the potentially lethal effects of adjuvant chemotherapy, and thus, may be one cause of tumor recurrence and ultimately death in many patients with breast cancer. Breast cancer cell lines treated chronically with doxorubicin inevitably develop resistance to doxorubicin (Baker et al., 1992). The mechanism of this resistance is not well defined, but most probably is multifactorial. We and others have demonstrated that expression of hsp27 is an important factor in doxorubicin resistance. One strategy to overcoming this resistance is combining a drug known to reverse multidrug resistance with doxorubicin. The cytotoxic effect of toremifene plus doxorubicin is superior to doxorubicin alone in the treatment of doxorubicin-sensitive and doxorubicin-resistant breast cancer cell lines (DeGregorio et al., 1989; Baker et al., 1992; Wiebe et al., 1992). In cells resistant to doxorubicin owing to hsp27 overexpression, we have demonstrated that the combination of doxorubicin plus toremifene has superior cytotoxic effects compared to doxorubicin alone. Because the doses of toremifene employed in this study are nontoxic to humans, a clinical trial of these agents in doxorubicin-resistant tumors is warranted, with the expectation of improved outcome.

Materials and Methods

Cell Lines

Stable transfected MDA-MB231 cell lines were isolated and grown in Minimal Essential Media (MEM) (Gibco

BRL, Grand Island, NY), supplemented with 5% heat-inactivated fetal bovine serum, 25 m*M* HEPES buffer, 6 ng/mL bovine insulin (Sigma, St. Louis, MO), 100 IU penicillin/mL, 100 μg/mL streptomycin, 2 m*M* L-glutamine (Biowhittaker Inc., Walkersville, MD), and 600 μg/mL G418 sulfate (Gibco BRL). All Cultures were maintained at 37°C in humidified incubators with 5% CO_2:95% air. Eighteen hours prior to an experiment, cells were washed and resuspended in supplemented MEM, lacking G418.

Vector Construction

The constitutive expression vector (pβ27) utilizing the human full-length hsp27 cDNA (Carper et al., 1990) was constructed by cloning the *Eco*RI cDNA fragment into the *Sal*I site of pHβAPr-1-neo (Gunning et al., 1987) by a blunt-end ligation after filling in the fragments. This vector (Fig. 1) contains the β actin promoter (PRM), 5'-untranslated region (5'UTR), and an intron splice site (ss) followed by a multiple cloning site (MCS) and an SV40 polyadenylation signal (SV40 poly A).

Transfection

A calcium phosphate method was used for transfection (Bethesda Research Labs, Grand Island, NY). Cells (1×10^6) were fed with fresh serum-containing medium 2 h prior to the addition of 20–40 μg of DNA. Twenty-four hours following the addition of DNA, cells were washed, and serum-containing medium was added to the cells. Twenty-four hours after washing, cells were put under selection pressure by the addition of serum containing medium supplemented with 600 μg/mL of G-418 sulfate. Individual colonies were isolated 2 wk later and continuously cultured in the presence of 600 μg/mL of G418 sulfate.

Protein Analysis

Transfected cells were grown to a density of 40,000 cells/10×75 mm culture tube. Protein was labeled by the incorporation of [^{3}H] leucine (800 Ci/mmol, New England Nuclear, Boston, MA) at a concentration of 0.1 μCi/μL in a final volume of 50 μL of complete MEM medium containing fetal bovine serum. Cells were incubated under standard growth conditions following the heat shock. The total cellular protein was solubilized by the addition of SDS sample buffer (0.1*M* sodium phosphate buffer [pH 7.2], 1% SDS, 1% 2-mercaptoethanol, 0.25*M* sucrose), and the samples were then heated in a boiling water bath for 1 min. SDS-PAGE was performed (Laemmli, 1970) with 12% acrylamide gels. Equal amounts of radioactivity were added to each lane. Apparent molecular weights were determined by using molecular markers (Bethesda Research Labs). After electrophoresis, gels were fixed in: 10% (v/v) isopropanol; 5% (v/v) glacial acetic acid; 85% (v/v) water. The gels were then impregnated with En^{3}hance (New England Nuclear), dried at 60°C, and exposed to Kodak X-Omat XAR5 X-ray film at -80°C for fluorography.

Clonogenic Survival

Colony-forming assays were performed as previously described (Carper et al., 1991). Briefly, following a heat shock, all treated cells were harvested, counted with a Coulter counter, and then incubated at various cell densities in supplemented media lacking G418 sulfate for 9–12 d to allow for colony growth. Colonies that grew from the surviving cells were stained with crystal violet and counted (>50 cells /colony). The percentage survival was calculated as 100x (number of colonies formed/number of experimental cells plated)/(number of control colonies formed/number of control cells plated).

Growth Inhibition

Cell lines were incubated for 2 h with doxorubicin (Adria Laboratories, Columbus, OH), washed in PBS, and then grown in triplicate at 15,000 cells/well in T-35 plates for 48 h in fresh media. Cells were then trypsinized and counted with a Coulter counter. MDA-MB231 cell lines were grown continuously in 6.6 μ*M* toremifene for 72 h and then in doxorubicin for 2 h. Cells were washed, plated in triplicate at 10,000 cells/well, and grown in media lacking doxorubicin, but with toremifene still present. After 48 h, cells were counted. Cell growth data are presented as the percentage of cells present when counted 48 h after treatment with doxorubicin compared to cells grown in media alone. Data from assays were compared using the Wilcoxon two-sample rank test. P values < 0.05 were considered significant.

Flow Cytometry

Following a 72-h pretreatment with 6.6 μ*M* toremifene, 0.1 μg/mL or 1.0 μg/mL doxorubicin (Sigma) was added, and cells were incubated at 37°C for 1 h. The medium was removed, and cells were washed. Fresh medium containing 6.6 μ*M* toremifene was added to the flasks, and cells were incubated 24 h before harvesting. Cells were harvested with trypsin-EDTA and counted. Dilutions were made in sterile PBS to give 2×10^6 cells/tube. Cells were fixed by addition of an ethanol solution (95% ethanol and 0.5% Tween-20 in sterile PBS), stored at 4°C, and stained by a modified Krishan technique (Krishan, 1975). Following centrifugation for 5 min at 119*g*, the cell pellet was resuspended in staining solution (2 mg/mL RNase, 0.1 mg/mL propidium iodide, and 0.6% nonidet P-40 in sterile PBS). After incubating for 30 min in the dark at room temperature, cells were vortexed and filtered through a 37-μ nylon membrane (Tetko, Briar Cliff Manor, NY) into 12 x 75 mm tubes. Flow cytometric measurements were executed on a FACScan instrument (Becton Dickinson, Sparks, MD) with an argon-ion laser operating at 400 mW and 488 nm. Propidium iodide emission was collected through a 585-bandpass filter. Cell-cycle analysis was performed on 10,000 events using CellFit software (Becton Dickinson, Sparks, MD).

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