Effect of Stathmin on the Sensitivity to Antimicrotubule Drugs in Human Breast Cancer

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ABSTRACT

Stathmin is a p53-regulated protein known to influence microtubule dynamics. Because several chemotherapeutic agents used to treat breast cancer alter the dynamic equilibrium of tubulin polymerization, stathmin may play an important role in determining the sensitivity to these drugs. Therefore, we evaluated the effect of stathmin expression on the action of taxanes and Vinca alkaloids using a panel of human breast cancer cell lines. Cell lines harboring mutant p53 expressed high levels of stathmin. Two cell lines with different levels of endogenous stathmin expression and isogenic-paired cell lines transfected to overexpress stathmin were used to determine whether or not stathmin modulated the sensitivity to drugs. Overexpression of stathmin decreased polymerization of microtubules, markedly decreased binding of paclitaxel, and increased binding of vincristine. Stathmin overexpression decreased sensitivity to paclitaxel and, to a lesser extent, to vinblastine. In contrast, stathmin content had no significant effect on the sensitivity to chemotherapeutic drugs that do not target microtubules. Cell lines overexpressing stathmin were more likely to enter G2 but less likely to enter mitosis as determined by fluorescence-activated cell sorting and mitotic index. This effect was magnified when stathmin-overexpressing cells were treated with vinblastine as measured by the detection of proteins phosphorylated in early mitosis. These data suggest that the action of antimicrotubule drugs can be affected by stathmin in at least two ways: (a) altered drug binding; and (b) growth arrest at the G2 to M boundary. Mutant p53 breast cancers exhibiting high levels of stathmin may be resistant to antimicrotubule agents.

INTRODUCTION

Taxanes and Vinca alkaloids are antimicrotubule drugs that are commonly used chemotherapeutic drugs (1). Taxanes stabilize microtubules, and Vinca alkaloids destabilize microtubules (1, 2). Both classes of drugs promote cell death by affecting microtubule dynamics, thereby interfering with the function of the mitotic spindle and inducing apoptosis by promoting mitotic arrest (3, 4). Antimicrotubule drugs are among the most effective drugs in the treatment of breast cancer, but they produce remissions in no more than 50% of previously untreated patients (5). Therefore, many patients receive cytotoxic agents without receiving an anticancer benefit. The choice of most chemotherapeutic regimens is based on tumor origin without considering molecular determinants of drug sensitivity. Therefore, by identifying distinct tumor characteristics, it may be possible to more accurately predict the most effective drugs for an individual patient.

The functional status of p53 can regulate the sensitivity of cancer cells to chemotherapeutic drugs (6–8). p53 regulates the sensitivity to antimicrotubule drugs by controlling the expression of proteins that affect the dynamic equilibrium of microtubule assembly (6, 7). For example, Murphy and coworkers found that p53 transcriptionally repressed MAP-4 (9, 10) and stathmin (10, 11), two proteins that can affect the polymerization state of microtubules (12). We recently found that the regulation of MAP-4 by p53 markedly affected the sensitivity to taxanes and Vinca alkaloids (6, 7), and we now report the effects of stathmin.

Stathmin is an M, 18,000 cytosolic phosphoprotein (also known as oncoprotein 18 or Op18) shown to decrease microtubule polymer mass. The mechanism by which stathmin affects microtubules appears complex; data to support either tubulin sequestration or promotion of microtubule catastrophe exist (12–15). Stathmin is regulated during the cell cycle by transcriptional and posttranscriptional mechanisms. Decreased stathmin expression favors microtubule polymerization (15), whereas increased expression of stathmin reduces microtubule polymer mass (16, 17). During mitosis, stathmin is inactivated by phosphorylation (16–20), thus promoting polymerization and aiding in mitotic spindle assembly (21). Because of the effects of stathmin on tubulin dynamics, its transcriptional regulation by p53, and reports that stathmin is up-regulated in human breast cancers (22), we evaluated the effect of stathmin expression on the sensitivity of breast cancer cell lines to antimicrotubule drugs.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. MCF-10A immortalized human breast epithelial cells were cultured in 1:1 Ham’s F-12-DMEM with 2 mM L-glutamine and supplemented with 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone, and 5% horse serum. BT20 human breast epithelial cancer cells were cultured in Eagle’s MEM containing nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1.5 g/liter sodium bicarbonate (American Type Culture Collection, Manassas, VA) and supplemented with 10% fetal bovine serum. BT474 human breast cancer cells were cultured in RPMI 1640 with 2 mM L-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, and 1 mM sodium pyruvate and supplemented with 0.01 mg/ml bovine insulin and 10% fetal bovine serum. BT549 human breast cancer cells were cultured in RPMI 1640 with 0.023 IU/ml bovine insulin and 10% fetal bovine serum. MDA-MB-231 and MDA-MB-468 breast human breast cancer cells were cultured in Leibovitz’s L-15 medium supplemented with 10% fetal bovine serum. T47D human breast cancer cells were cultured in RPMI 1640 supplemented with 0.2 IU/ml bovine insulin and 10% fetal bovine serum. All cell lines were obtained from American Type Culture Collection, ATCC. BT20V1, BT20V3, BT20ST1, and BT20ST3 were stable clones generated from BT20 cells (described below). They were cultured as described above for the parental BT20 cell line with the addition of 200 μg/ml Genetin. All tissue culture media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin, and tissue culture reagents were obtained from Invitrogen (Carlsbad, CA) unless otherwise specified. All cell lines were maintained at 37°C and 5% CO2 with the exception of MDA-MB-231 and MDA-MB-468 cells, which were incubated without CO2.

Drugs and Reagents. Paclitaxel, vinblastine, camptothecin, and Colcemid were purchased from Sigma Chemical Co. (St. Louis, MO). Doxorubicin was obtained from Calbiochem (San Diego, CA). Additional reagents were purchased from the following sources: (a) fluorescent-conjugated paclitaxel (Oregon Green 488 paclitaxel) and fluorescent-conjugated vinblastine (BODIPY...
FL vinblastine), Molecular Probes (Eugene, OR); and (b) MTT, Sigma Chemical Co. Paclitaxel and fluorescence-conjugated drugs were dissolved in ethanol (final concentration of ≤5%). Camptothecin was dissolved in DMSO (final concentration of ≤0.25%). Vinblastine and doxorubicin were dissolved in distilled H2O and filter sterilized. All other reagents were from Sigma Chemical Co.

**Transfections.** Cells plated in 60-mm tissue culture plates were grown to 90% confluence and transfected with 9 μg of pcDNA3.1 vector (Invitrogen) containing the full-length human stathmin sequence (a generous gift from Dr. Maureen Murphy, Fox Chase Cancer Center, Philadelphia, PA) or empty vector using LipofectAMINE 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. For stable cell lines, colonies were selected with 400 μg/ml Geneticin (Invitrogen) from a single clone expansion 24 h after transfection. Positive clones were screened by Western blot for stathmin content.

**Western Blot Analysis.** Cells were harvested at 80% confluence in 50 mm Tris-HCl (pH 7.5), 1% sodium deoxycholate, 1% Triton X-100, 150 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 20 μg/ml aprotinin, 10 mM NaF, 50 mM sodium fluoride, and 500 μM sodium orthovanadate (23) and disrupted by pulsed four to six times with ~2.5 watts using a Virsonic 60 VirTis Sonicator. Protein concentrations were determined by the method of Bradford (24) using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Proteins were loaded onto 15% SDS-PAGE gels, followed by transfer to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Polyclonal stathmin (Calbiochem) and monoclonal β-actin (clone AC15; Sigma Chemical Co.) antibodies were used to detect protein content using enhanced chemiluminescence detection (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Protein expression was quantified by Molecular Analyst software (Bio-Rad Laboratories).

**Immunofluorescence Staining.** Cells were grown on glass coverslips until 80% confluent. After fixation with methanol, coverslips were blocked with 3% BSA and stained with α-stathmin and α-tubulin (clone D1A1; Sigma Chemical Co.) using Cy3- and FITC-conjugated secondary antibodies (Sigma Chemical Co.), respectively. Coverslips were mounted with ProLong Antifade Kit (Molecular Probes) according to the manufacturer’s instructions. Slides were analyzed using a Zeiss HBO 100 W2 Axioskop fluorescence microscope and ScionImage software (Scion Corp., Frederick, MD). Fluorescence intensity was evaluated per low-power field, and high-power images of representative fields are shown for better visualization of microtubules.

**Drug Binding.** Cells plated in tissue culture glass slide chambers were allowed to attach for 24 h and then treated with 20 μM fluorescein-conjugated paclitaxel or vinblastin for 1 h. Excess drug was removed by washing with 2% BSA in PBS, followed by a second wash with PBS. Live cells were observed by fluorescence microscopy as described above.

**MTT Assay.** Cells were plated in 96-well tissue culture plates, allowed to attach overnight, and then treated with drug for 2–3 times the cell doubling time determined for each line. Twenty μl of 5 mg/ml MTT in PBS were then added to each well, and cells were incubated for 4 h at 37°C. Formazan crystals were made soluble with DMSO (Fisher Scientific, Pittsburgh, PA), Optical densities were determined at 570 nm using a Dynatech MR5000 plate reader. Viability was expressed as a percentage of control by dividing the absorbance of each treated well by the average of the untreated or vehicle-treated controls. IC50 was defined as the concentration of drug that decreased cell viability by 50%.

**Cell Cycle Analysis.** Cells were plated and allowed to attach for 24 h and then incubated with 10 μM BrdUrd for 1 h (Roche Molecular Biochemicals, Indianapolis, IN). Adherent and supernatant cells were collected, generated into a single cell suspension, washed with PBS, and fixed with ice-cold 70% ethanol. Cells were then washed with PBS, made permeable with 2% HCl/0.5% Triton X-100, neutralized twice with 0.1 M borate buffer (pH 8.5), and washed once with 0.5% Tween 20/1% BSA in PBS. Cells were incubated with 20 μl of FITC-conjugated anti-BrdUrd (Becton Dickinson, San Jose, CA) for 30 min at room temperature, washed with PBS, and incubated with 50 μl of 1 mg/ml RNase A (Sigma Chemical Co.) and 5 μl of 1 mg/ml propidium iodide in 400 μl of PBS for ~30 min at room temperature. Propidium iodide incorporation and BrdUrd incorporation were measured on a Becton Dickinson FACScan analyzer using dual parameter flow cytometric analysis. Cells in G2-M were identified and quantified using quadrant statistics. Statistical significance was determined by Student’s t test.

**Mitotic Index.** Cells were collected and centrifuged onto glass slides and then fixed and stained with 10 μg/ml 4’,6-diamidino-2-phenylindole in PBS containing 5 μg/ml RNase A. For each sample, ~200 cells were randomly counted by fluorescence microscopy. Mitotic figures were scored blindly.

**Measurement of Phosphoproteins Detected by MMP2-** BT20V1 and BT20ST1 cells were plated in 60-mm dishes, allowed to adhere for 20 h, and treated for 6, 12, and 24 h with 2 nm vinblastine. HeLa cells were serum-starved for 24 h and either untreated or treated for 24 h with 50 ng/ml Colcemid in DMEM containing 10% fetal bovine serum. Adherent cells collected by trypsinization were pooled with supernant cells, washed with PBS, and extracted in cell lysis buffer (50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylnylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na3VO4, 1 μM NaF, and a 1:100 dilution of phosphatase inhibitor mixture I (Sigma Chemical Co.) by sonication using a Virsonic 60 VirTis Sonicator. Extracts were quantified as described above. Eighty μg of protein were loaded onto 9% SDS-PAGE gels and transferred to nitrocellulose membranes. Western blotting with MMP2-1, a monoclonal anti-phospho-Ser/Thr-Pro antibody, was carried out according to the manufacturer’s protocol (Upstate Biotechnology, Lake Placid, NY) and detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

**RESULTS**

**Stathmin Expression in Human Breast Cancer Cell Lines Harboring Mutant p53.** A panel of mutant p53-containing human breast cancer cell lines was examined for stathmin content by Western analysis. MCF-10A, a nontransformed breast cell line harboring wild-type p53, was used as a control. BT20, BT474, BT549, MDA-MB-231, MDA-MB-468, and T47D expressed 4-, 7-, 11-, and 10-, 7-, and 5-fold more stathmin normalized to that of the MCF-10A control (data not shown).

**Stathmin Expression and Microtubule Polymerization.** We investigated the functional significance of stathmin expression in breast cancer cell lines by comparing the expression of stathmin with the ability to visualize polymerized microtubules using immunofluorescence microscopy. BT20 cells exhibited less stathmin staining and a greater degree of microtubule polymerization (Fig. 1A, top panel) than BT549 cells (Fig. 1A, bottom panel).

We next produced transient stathmin transfectants to analyze the acute effects of forced stathmin expression on the same parameters. Fig. 1B demonstrates that BT20 transfectants had greater stathmin content and less microtubule polymerization than vector-transfected cell lines.

**Effects of Stathmin on Cellular Binding of Paclitaxel and Vinblastine.** We next generated stable isogenic cell lines overexpressing stathmin and used these cell lines to study the effects of stathmin on drug binding. BT20 cells were transfected with a stathmin expression vector; two clones transfected with empty vector (BT20V1 and BT20V3) and two clones transfected with stathmin-containing vector (BT20ST1 and BT20ST3) were isolated as described in “Materials and Methods.” Western analysis demonstrated that the stathmin transfectants had an 8-fold increase in expression of stathmin as compared with the parental line and empty vector-transfected controls (Fig. 2).

Live BT20V1, BT20V3, BT20ST1, and BT20ST3 cells were incubated with 20 μM fluorescein-labeled paclitaxel or vinblastine for 1 h and assayed for fluorescence intensity. Fig. 3 demonstrates that overexpression of stathmin (Fig. 3C, BT20ST1; Fig. 3D, BT20ST3) markedly decreased paclitaxel binding as compared with that seen in empty vector-transfected controls (Fig. 3A, BT20V1; Fig. 3B, BT20V3). BT20ST1 and BT20ST3 cells examined under visible light confirmed the presence of intact cells (data not shown) in the same fields as those shown in Fig. 3. Fig. 4 demonstrates that overexpression of stathmin in BT20ST1 (Fig. 4C) and BT20ST3 (Fig. 4D) cells increased vin-
Effect of Stathmin on Sensitivity of Breast Cancer Cell Lines to Antimicrotubule and Non-Antimicrotubule Drugs. To examine the effects of stathmin content on drug sensitivity, we first compared nonmanipulated cell lines that differed naturally in stathmin content with the MCF-10A control. BT20 cells (4-fold increase in stathmin) and BT549 cells (11-fold increase in stathmin) were treated with increasing concentrations of paclitaxel and vinblastine, and cell viability was measured by MTT. BT549 cells were 1000-fold less sensitive to paclitaxel ($IC_{50} \approx 2 \text{ nM}$) than BT20 cells ($IC_{50} \approx 2 \text{ pM}$). BT549 cells ($IC_{50} \approx 10 \text{ pM}$) were also 5-fold less sensitive to vinblastine than BT20 cells ($IC_{50} \approx 2 \text{ pM}$).

We next determined the effect of stathmin expression on drug sensitivity in isogenic cells. Stathmin transfectants (BT20ST1 and BT20ST3) were also less sensitive to paclitaxel (Fig. 5A) and vinblastine (Fig. 5B) than vector-transfected controls.

To determine whether or not the effects of stathmin expression on drug sensitivity were limited to antimicrotubule drugs, BT20V1, BT20V3, BT20ST1, and BT20ST3 cells were treated with increasing concentrations of doxorubicin or camptothecin, and cell viability was determined by MTT. Stathmin expression had no significant effect on the sensitivity to doxorubicin or camptothecin, drugs targeting topoisomerase II and I, respectively (Table 1).

Effect of Stathmin Content on Cell Cycle Progression. Stathmin affects microtubule polymerization, and its degree of expression could nonmanipulated cell lines that differed naturally in stathmin content with the MCF-10A control. BT20 cells (4-fold increase in stathmin) and BT549 cells (11-fold increase in stathmin) were treated with increasing concentrations of paclitaxel and vinblastine, and cell viability was measured by MTT. BT549 cells were 1000-fold less sensitive to paclitaxel ($IC_{50} \approx 2 \text{ nM}$) than BT20 cells ($IC_{50} \approx 2 \text{ pM}$). BT549 cells ($IC_{50} \approx 10 \text{ pM}$) were also 5-fold less sensitive to vinblastine than BT20 cells ($IC_{50} \approx 2 \text{ pM}$).

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affect the distribution of cells throughout the cell cycle and thereby affect drug sensitivity. Therefore, cell cycle distribution of nonsynchronized cultures of BT20V1, BT20V3, BT20ST1, and BT20ST3 cells were analyzed by flow cytometry. BT20ST1 (9.03\%/110060.65%) and BT20ST3 (9.21\%/110060.27%) had significantly more cells in G2-M than BT20V1 (6.47\%/110060.23%) and BT20V3 (5.70\%/110060.77%) cells (P < 0.003 and 0.00002, respectively).

To analyze the distribution of cells in mitosis, we determined the mitotic index in stathmin transfectants compared with that of empty vector-transfected controls. Fig. 6 demonstrates that stathmin transfectants had 2–3-fold fewer cells entering mitosis than the control cell lines.

To understand the relative resistance of stathmin transfectants to vinblastine in the face of increased drug binding, we compared the accumulation of cells in M phase after treatment with vinblastine in vector- and stathmin-transfected cells as determined by MPM-2 antibody staining of mitotic-specific phosphoproteins (25, 26). HeLa cells arrested in mitosis by treatment with Colcemid for 24 h were used as positive controls. As shown in Fig. 7, BT20V1 cells treated with vinblastine had a 5- and 9-fold increase in MPM-2 staining of high and low molecular weight proteins, respectively. In contrast, MPM-2 detection of the high and low molecular weight bands increased 2- and 4-fold, respectively in stathmin-transfected BT20ST1 cells treated with vinblastine.

**DISCUSSION**

The functional status of p53 affects sensitivity to chemotherapeutic drugs and radiation (6–8, 27, 28). Cells with intact p53 are often more sensitive to treatment due to their propensity to undergo apoptosis (8, 29, 30). In contrast, we previously found that cells harboring p53 mutations were more sensitive to taxanes but were resistant to *Vinca* alkaloids, and we attributed these changes to the regulation by p53 of MAP-4, a protein involved in the polymerization of microtubules (6, 7). We now report that stathmin, another p53-regulated gene product involved in microtubule dynamics, can affect the sensitivity to antimicrotubule drugs.

Expression of stathmin varied from 4–11-fold in human breast cancer cell lines harboring mutant p53 when normalized to MCF-10A cells, a nontransformed, immortalized breast epithelial cell line harb-
boring the wild-type protein. These results were anticipated from the work of others (10, 11), who demonstrated that stathmin was repressed by wild-type p53 protein through an interaction with mSin3a and histone deacetylase (10). Because stathmin participates in the control of microtubule polymerization, we determined its effect in breast cancer cells by comparing the content of polymerized microtubules as a function of stathmin expression. Fig. 1 demonstrates that in nonmanipulated cells (Fig. 1A) or in transfectants (Fig. 1B), stathmin expression correlates inversely with microtubule polymerization. Similar results were obtained by Gavet et al. (20), who found that overexpression of stathmin in HeLa cells decreased staining of the microtubule network. The precise mechanism by which stathmin decreases microtubule polymer mass is complex. Data to support a role of stathmin in promoting microtubule “catastrophe” as well as in sequestering tubulin dimers exist (12–15).

The polymerization state of microtubules can affect the binding of antimicrotubule drugs. Previously, we found that increased polymerization led to increased binding of paclitaxel and decreased binding of Vinca alkaloids (6, 7). Accordingly, decreased microtubule polymerization associated with overexpression of stathmin (Fig. 2) decreases the binding of paclitaxel and increases the binding of vinblastine to breast cancer cells (Figs. 3 and 4).

Overexpression of stathmin decreases the sensitivity of breast cancer cells to paclitaxel and to vinblastine. This effect on drug sensitivity was observed in both mutant p53 human breast cancer cell lines containing different levels of endogenous stathmin and in isogenic transfectants (Fig. 5). The effects of stathmin appear to be restricted to antimicrotubule drugs because stathmin transfectants do not demonstrate significant changes in sensitivity to doxorubicin or camptothecin (Table 1). Iancu et al. (31) recently found that inhibition of stathmin expression through antisense transfection increased the ratio of polymeric to depolymerized tubulin, increased the sensitivity to taxanes, and decreased the sensitivity to Vinca alkaloids. Thus, the results with the taxane were anticipated based on the alteration of tubulin dynamics and the decreased binding of paclitaxel in stathmin-overexpressing cell lines. These effects differ from the destabilization effects of tubulin mutations, which can be stabilized by paclitaxel (32).

The results with the Vinca alkaloid were unexpected because stathmin overexpression increased the binding of vinblastine (Fig. 4) but decreased drug sensitivity (Fig. 5). To understand the mechanism by which cells remain resistant to Vinca alkaloids despite increased drug binding, we focused on the effects of stathmin expression on the distribution of cells throughout the cell cycle. We found that stathmin increases the number of cells in G2-M as measured by fluorescence-activated cell-sorting analysis (see “Results”) but decreases the number of cells in mitosis as measured by counting mitotic figures (Fig. 6). When stathmin-overexpressing cells were treated with vinblastine, fewer cells entered mitosis compared with vector-transfected controls as measured by decreased expression of proteins detected by MPM-2, an antibody that recognizes several proteins that are selectively phosphorylated at the onset of mitosis (Refs. 25 and 26; Fig. 7). Therefore, overexpression of stathmin appears to diminish the cytotoxic effects of vinblastine by impeding progression of cells from G2 into mitosis. In support of this interpretation, Blagosklonny et al. (27) found that loss of p53-dependent G2-M checkpoint control increased the sensitivity to both Vinca alkaloids and taxanes. Thus, the mechanism of stathmin-induced resistance to paclitaxel can be explained by both decreased drug binding to a diminished microtubule polymer mass and decreased entry into mitosis. The effects of stathmin on vinblastine resistance occur despite increased drug binding and are attributable to decreased entry of treated cells into mitosis.

In summary, these data indicate that alterations in the functional status of p53 can produce significant and unexpected changes in drug sensitivity due to increased expression of stathmin. Because p53 mutations and stathmin overexpression are frequent accompaniments of breast epithelial transformation, and antimicrotubule drugs are standard treatments for breast cancer, understanding the effects of stathmin in the clinical setting may have important implications for the outcome of therapy.

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