BAX Expression Is Associated with Enhanced Intracellular Accumulation of Paclitaxel: A Novel Role for BAX during Chemotherapy-induced Cell Death

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Abstract

SW626 cells that overexpress BAX are sensitized to the cytotoxic effects of paclitaxel and vincristine. It has been assumed that BAX mediates these effects through its ability to alter mitochondrial function, specifically by promoting the release of cytochrome c and facilitating the mitochondrial permeability transition. However, we have found that several early paclitaxel-mediated events are enhanced in SW626 transfectants that overexpress BAX, including G2-M-phase arrest, tubulin polymerization, and BCL-2 phosphorylation. We now demonstrate that these seemingly disparate effects are explained by an enhanced accumulation of paclitaxel in BAX-overexpressing cells, an effect due to diminished drug efflux. In contrast, drug efflux is increased in cells that do not overexpress BAX, resulting in low intracellular paclitaxel levels and relative resistance to the effects of this drug. Drug efflux in SW626 cells is mediated by a verapamil-inhibitable, non-MDR-1, non-MRP-1 transporter whose function or expression may be inhibited by BAX. These data suggest that stable transfectants that overexpress BAX may be sensitized to apoptotic cell death through a novel mechanism involving the enhancement of intracellular levels of naturally occurring toxins such as alkaloid derivatives.

Introduction

Apoptosis is the final common pathway through which most forms of chemotherapy mediate cell death. The most distal step in this process is the activation of a set of cysteine proteases known as caspases, which in turn degrade several protein substrates known to be involved in DNA repair and structural integrity (1). One of the best-studied members of this protease family is caspase-3, a cytoplasmic Mr 18,000 proenzyme that is activated by enzymatic cleavage into Mr 14,000 and Mr 3,500 fragments. Enzymatic cleavage of caspase-3 often requires the presence of cytochrome c, which is released from the inner mitochondrial membrane into the cytosol during apoptotic cell death (2). Mitochondrial cytochrome c release is now known to be a critical signaling event for many apoptotic stimuli and is modulated by members of the BCL-2 family of proteins, many of which are localized to the outer mitochondrial membrane. Specifically, BCL-2 proteins that protect against apoptosis, such as BCL-2 itself and BCL-xL, function partly through their ability to suppress the mitochondrial release of cytochrome c, whereas the proapoptotic BAX protein enhances cytochrome c release (3, 4). These observations have led to the hypothesis that many BCL-2 family members regulate the apoptotic threshold at a point upstream of caspase activation, largely through their ability to modulate the mitochondrial release of cytochrome c in response to cellular damage.

Overexpression of BAX is known to enhance many forms of apoptosis. For instance, BAX accelerates cell death after interleukin 3 withdrawal in the factor-dependent murine FL5.12 hematopoietic cell line, an event associated with the formation of BAX homodimers as well as heterodimers between BAX and either BCL-2 or BCL-xL (5). Also, SW626 ovarian cancer cells that stably overexpress BAX are 5- and 26-fold more sensitive to the cytotoxic effects of paclitaxel and vincristine, respectively, when compared to control transfectants (6, 7). Based on the central role that BCL-2 family members play in regulating apoptosis through the mitochondrial release of cytochrome c, it might be predicted that BAX overexpression would lead to a generalized enhancement of the apoptotic response to a variety of death stimuli. Surprisingly, however, BAX overexpression in SW626 cells does not enhance their sensitivity to DNA-damaging agents such as carboplatin or ionizing radiation, and cells from BAX-null mice do not display altered sensitivity to ionizing radiation in vitro (6–8). Thus, the ability of BAX to sensitize cells to apoptotic cell death appears to be stimulus-dependent, at least for some cell types.

Previous studies have focused on the ability of BAX to influence the most distal steps in the apoptotic pathway, especially those involving mitochondrial function (e.g., permeability transition and cytochrome c release; Refs. 3 and 9). However, the fact that BAX can sensitize ovarian cancer cells to paclitaxel and not to classical DNA-damaging agents suggests that this protein may function indirectly through a mechanism that is proximal to the mitochondria, specifically by affecting the early events that are associated with paclitaxel-mediated cytotoxicity. For instance, it is known that paclitaxel-induced killing is associated with multiple diverse molecular events, including tubulin polymerization, BCL-2 phosphorylation, RAF-1 phosphorylation, and p34<sup>cdc2</sup> activation, leading to the possibility that BAX might function to enhance one or more of these proximal pathways (10, 11). We now report that multiple, seemingly disparate proximal actions of paclitaxel are enhanced in the presence of BAX, and that this is mediated in large part by the presence of high intracellular levels of this drug in BAX-expressing clones. Furthermore, we demonstrate that paclitaxel efflux is impaired in cells that overexpress BAX and suggest that this may be a novel mechanism by which BAX predisposes cells to chemotherapy-induced cell death.

Materials and Methods

Source of Cells, Reagents, and Antibodies. The SW626 human ovarian epithelial carcinoma cell line used for transfection and drug sensitivity studies was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS (HyClone Laboratories, Logan, UT). Lipofection was used to generate stable SW626 clones expressing either pSV2-neo cDNA (a kind gift of Dr. Michel Streuli, Dana-Farber Cancer Institute, Boston, MA) or murine BAX DNA present in a HA<sup>1</sup>pSFFV expression vector (HA-BAX; a kind gift of Dr. Stanley Korsmeyer, Washington University, St. Louis, MO) as described previously (5, 6). Both neo-control and HA-BAX transfectants were selected for neomycin resistance in the continued presence of 500 µg/ml G418. The three separate SW626 HA-BAX transfectants used in these studies have...
been characterized previously and express a mean 10-fold higher level of BAX than neo-control cells (6, 7). Propidium iodide used for the assessment of DNA content was purchased from Sigma Chemical Co. Paclitaxel was purchased from Bristol-Myers Squibb (Princeton, NJ). Vincristine was purchased from Eli Lilly (Indianapolis, IN). [3H]Paclitaxel was a generous gift from Dr. Rudiger D. Haugwitz (National Cancer Institute, Bethesda, MD). [3H]Vincristine was purchased from Moravek Biochemicals, Inc. (Brea, CA). Fluorescein-labeled paclitaxel (BODIPY™FL Taxol) for use in confocal microscopy studies was purchased from Molecular Probes (Eugene, OR). Verapamil, vinblastine, and oligomycin were purchased from Sigma Chemical Co. The 4E3 murine antihuman MDR-1 antibody reactive with P-glycoprotein was used in flow cytometric analysis and was a kind gift of Dr. Robert Arceci (University of Cincinnati, Cincinnati, OH; Ref. 12). Antihuman MRPI-1 antibody (clone MRPI-1) was purchased from Kambay Biomedical Co. (Seattle, WA) and used in both flow cytometric analysis (after membrane permeabilization) as well as immunoblot studies. Antihuman LRP antibody (clone 42) was purchased from Transduction Laboratories (Lexington, KY) and was used in immunoblot studies. Antihuman BCL-2 antibody (murine clone 124, IgG1; DAKO Corp., Santa Barbara, CA) was used to assess the degree of paclitaxel-induced BCL-2 phosphorylation in immunoblot analysis as described previously (using the enhanced chemiluminescence detection system; Amersham; Ref. 6).

Detection of Sub-G₀ DNA Content by Propidium Iodide Staining. The fraction of cells containing a sub-G₀ DNA content has been shown to correlate with apoptotic cell death and was assessed by flow cytometry as described previously (6). The Multicycle software program (Multiple option cell cycle analysis; Phoenix Flow Systems, San Diego, CA) was used for curve fitting to quantitate the fraction of cells in the G₀-M phase of the cell cycle.

Tubulin Polymerization Assay. Tubulin polymerization was quantitated as described previously (10). Briefly, cells were lysed for 5 min at RT in 100 μM of hypotonic buffer (1 mM MgCl₂, 2 mM EGTA, 0.5% NP40, 1 mM phenylmethysulfonyl fluoride, 7.5 μg/ml aprotinin, 40 μg/ml leupeptin, 5 μg sodium vanadate, and 20 μM sodium fluoride), followed by centrifugation at 14,000 rpm for 10 min at RT. The supernatant containing soluble tubulin was then separated from the pellet containing insoluble, polymerized tubulin, followed by resuspension of the pellet in 200 μl of hypotonic buffer. Both the soluble and insoluble tubulin fractions were mixed with an equal volume of 2× sample buffer, heated for 5 min at 95°C, separated by SDS-PAGE, and subjected to immunoblot analysis using murine antihuman α-tubulin antibody as described (1:10,000 dilution; Sigma Chemical Co.). After quantitation of the tubulin signal by densitometry, the amount of polymerized tubulin was expressed as a percentage of the total tubulin at various times during the paclitaxel exposure.

Analysis of Drug Uptake and Efflux. Cells were incubated at 37°C for 2 h in 10% FCS/DMEM containing either 25 mM [3H]Hippoclash or 20 mM [3H]Vinblastine, followed by washing three times and lysis for 20 min at RT in radioimmunoprecipitation assay buffer (10 mM NaPO₄, pH 7.2, 150 mM NaCl, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethysulfonyl fluoride, 7.5 μg/ml aprotinin, 40 μg/ml leupeptin, 5 μg sodium vanadate, and 20 μM sodium fluoride). An incubation period of 2 h was based upon pilot studies determining maximum drug uptake over this time course. Intracellular [3H]-labeled drug was quantitated by liquid scintillation counting. In drug efflux studies, cells were incubated in [3H]-labeled drug for 2 h at 37°C, followed by washing and resuspension in drug-free medium for up to 2 additional hours, during which intracellular drug concentrations were serially determined as described above. In experiments using verapamil, cells were incubated with 100 μM verapamil or diluent alone (0.1% DMSO) for 1 h at 37°C before the addition of the [3H]-labeled drug for an additional 2 h at 37°C in the continued presence of verapamil.

Cytotoxicity Assessment. The relationship between drug dose and cytotoxicity was determined for each transfectant in suspension culture during continuous exposure to drug. Cells were seeded (2.5 × 10⁴ cells/well) in 24-well plates (Falcon, Oxnard, CA) in 10% FCS/DMEM in the presence or absence of paclitaxel over a broad concentration range, followed by incubation (37°C; 5% CO₂) and assessment of viable cell numbers by trypan blue exclusion at day 3. Cell counts were performed in duplicate for each drug dose and time point, with the SE typically being less than 20%. The percentage of survival was defined as 100 × [viable cell number (drug)/viable cell number (medium)] at the identical time point.

Statistical Analysis. Data are expressed as mean ± SE where appropriate. Significance levels for comparison of differences were determined using the two-sided Student's t test for unpaired samples.

Results

Several Early Effects of Paclitaxel Are Enhanced in the Presence of BAX. To determine whether BAX overexpression was associated with the enhancement of known proximal mechanisms of paclitaxel action, SW626 cells that were either stably transfected with the neomycin resistance gene alone (neo-control clones) or cotransfected with HA-BAX cDNA (BAX clones) were exposed to 0.2 μM paclitaxel for up to 60 h, during which the assessment of G₂-M-phase arrest, the degree of tubulin polymerization, and the amount of BCL-2 phosphorylation were assessed (Fig. 1). As shown previously, the three SW626 BAX clones used in these studies express a mean 10-fold higher level of BAX compared to their neo-control counterparts, grow at the same rate under baseline conditions, and are 10-fold more sensitive to the effects of paclitaxel in vitro (6, 7). As shown in Fig. 1A, G₂-M-phase arrest was induced by paclitaxel in both neo-control and HA-BAX-expressing cells, although the amount of G₂-M-phase accumulation was significantly enhanced in the presence of BAX. Specifically, at 24 h, the fraction of paclitaxel-treated neo-control cells (neo clone F8) in the G₂-M phase was 37.8% [compared to 77.2% for HA-BAX-expressing cells (BAX clone A9)] with both cell lines showing <5% of cells containing a sub-G₀ content of DNA (consistent with cells that have undergone apoptotic cell death). Thus, the enhancement of G₂-M-phase arrest in BAX clones (compared to neo-control cells) preceded the onset of significant cell death. At 36 h, the percentages of G₂-M-phase cells for neo-control and BAX clones were 34.8 and 94.1%, respectively, associated with 5 and 10% of the cells with a sub-G₀ DNA content, respectively. Consistent with the known ability of BAX to sensitize these cells to the effects of paclitaxel, the majority of HA-BAX-expressing cells exhibited a sub-G₀ DNA content by 60 h, as compared to only 5% of neo-control cells (Fig. 1A). The data shown in Fig. 1 are representative of the results obtained from three separate experiments and are consistent with the results obtained using neo-control clones A10 and B10 as well as BAX clones D7 and D8. To further evaluate the effects of BAX on paclitaxel action, we next assessed the degree of tubulin polymerization in neo-control versus BAX-expressing clones. As described previously, the method used to determined the fraction of polymerized α-tubulin in the presence of paclitaxel is based upon the fact that the polymerized species is insoluble in protein lysates prepared using low salt, hypotonic lysis buffer. The percentage of polymerized tubulin at 0, 2, 6, 14, and 24 h of paclitaxel exposure (0.2 μM) in neo-control A10 cells was 0, 25.9, 49.8, 31.1, and 19.1%, respectively, with a greater degree of polymerization occurring more rapidly and to a greater degree in HA-BAX cells as compared to their neo-control counterparts (Fig. 1B).

SW626 BAX Transfectants Accumulate High Intracellular Levels of Paclitaxel and Vinblastine in Association with Diminished Drug Efflux. The observation that several early, seemingly disparate paclitaxel actions were enhanced in BAX transfectants was unexpected in view of the known downstream function of BAX in modulating the release of mitochondrial cytochrome c. We therefore
considered that a unifying explanation for these pleiotropic effects might be enhanced intracellular levels of paclitaxel in BAX-expressing cells. To test this hypothesis, neo-control and BAX clones were exposed to 25 nM [3H]paclitaxel for 2 h at 37°C, followed by washing and determination of intracellular cpm. A 2-h incubation at 37°C was chosen based upon pilot studies that showed that maximum paclitaxel uptake occurred over this time period. As shown in Fig. 2A, BAX clones contained significantly higher steady-state levels of paclitaxel compared to neo-control clones (mean, 11.7-fold higher; data derived from three separate BAX clones and three separate neo clones; P = 0.002). Intracellular levels of paclitaxel in neo clones were similar to those observed in the original SW626 parental line (data not shown). Likewise, identical experiments performed with [3H]vinblastine (20 nM; 2 h at 37°C) revealed a mean 22.6-fold higher drug concentration in BAX versus neo clones (P = 0.02). The increased drug accumulation observed in HA-BAX clones was not due to an artifact related to the HA tag, because three other SW626 clonal transfectants expressing HA-BAD as opposed to HA-BAX failed to demonstrate this effect (data not shown). To determine whether enhanced intracellular levels of paclitaxel might be due to diminished drug efflux in BAX clones, cells were loaded with paclitaxel (25 nM; 2 h at 37°C),
followed by washing and incubation for another 2 h in paclitaxel-free medium, during which intracellular drug concentrations were serially determined. As shown in Fig. 2B, SW626 BAX clones exhibited a diminished rate of paclitaxel efflux compared to neo-control cells. Specifically, the efflux rate during first 60 min for BAX clones was 0.53% specific cpm/min/10^5 cells, compared to a value of 1.09 for neo-control clones (P = 0.027; derived from the slopes of the curves shown in Fig. 2B). Finally, to independently validate the impression that BAX clones contain higher levels of intracellular paclitaxel than neo-control clones, we incubated cells with fluorescein-labeled paclitaxel for 2 h, followed by confocal microscopy (Fig. 3). BAX cells contained significantly greater amounts of intracellular paclitaxel compared to neo-control clones (Fig. 3, a versus b), which on high power appeared to be partly concentrated in a honeycombed pattern suggestive of endoplasmic reticulum localization (Fig. 3, c and d; Ref. 13).

Existence of a Verapamil-inhibitable Drug Efflux Pump in SW626 Cells. The data in Fig. 2B suggest that paclitaxel efflux is either inhibited in BAX clones or, conversely, accelerated in neo-
In this regard, multidrug resistance is known to be partly mediated by energy-dependent, membrane-bound drug transporters such as MDR-1 (p170; Ref. 14) and MRP-1 (15), and these pumps are inhibited by drugs such as verapamil and cyclosporin A. However, neither MDR-1 nor MRP-1 protein was significantly expressed by any SW626 clones used in these studies (as determined through the use of anti-MDR-1 antibody 4E3 and anti-MRP-1 antibody MRPm6; data not shown). Furthermore, the LRP-1 multidrug resistance protein (which is not known to be a membrane-bound paclitaxel efflux pump; Ref. 16) was expressed at similar levels in all clones (as assessed by immunoblotting with anti-LRP antibody clone 42; data not shown).

To investigate the possibility that a MDR-1-independent, MRP-1-independent drug efflux mechanism was partly responsible for the low intracellular paclitaxel levels in the neo-control clones shown in Fig. 2A, we pretreated SW626 neo-control cells with 100 μM verapamil for 1 h before incubation in 25 nM [3H]paclitaxel for 2 h at 37°C in the continued presence of verapamil. Verapamil was chosen because of its known ability to block drug efflux mediated by ATP-dependent transporters such as MDR-1 and MRP-1, and a dose of 100 μM was used because it has been previously shown to inhibit MDR-1 without affecting cell viability in short-term culture (17). Verapamil significantly enhanced the intracellular levels of paclitaxel in each neo-control clone tested (Fig. 4A, mean 20.9-fold increase in paclitaxel; P = 0.001; n = 3 clones), an effect that was associated with diminished drug efflux and verified by confocal microscopy (data not shown).

To determine whether the drug efflux was ATP dependent, cells were pretreated with 20 μg/ml oligomycin for 1 h before the determination of intracellular paclitaxel levels as described. This oligomycin dose is similar to that previously shown to result in a significant depletion of ATP pools through the inhibition of mitochondrial respiration (18). Oligomycin pretreatment of SW626 neo clones increased the intracellular paclitaxel concentration by >10-fold (the mean of two separate experiments), consistent with the presence of an energy-dependent drug efflux pump. Finally, the incubation of SW626 neo clones with verapamil (10 μM continuous exposure over 3 days) was shown to significantly sensitize these cells to paclitaxel (Fig. 4B). A lower dose of 10 μM verapamil was chosen in these experiments because we determined that this was the maximum concentration compatible with normal cell viability over a 3-day incubation period. Remarkably, the paclitaxel sensitivity of neo-control cells coincubated with verapamil was similar to that observed for their BAX-expressing counterparts. Specifically, the mean paclitaxel LD_{50} value for verapamil-treated SW626 neo clones was 0.018 ± 0.009 μM, as compared to a value of 0.046 ± 0.031 μM for SW626 BAX clones (the mean of the LD_{50} values from three separate clones in each group).

**Discussion**

The well-known localization of many BCL-2 family members to the outer mitochondrial membrane is consistent with the hypothesis that a major role of these molecules is to regulate mitochondrial function during the apoptotic response (2, 9). The addition of purified BAX protein to isolated mitochondria has been recently shown to directly induce the mitochondrial release of cytochrome c, a critical step in the subsequent activation of caspase-3 during many forms of apoptotic death (3). Conversely, mitochondrial cytochrome c release is inhibited by the BCL-x, antiapoptotic protein (4). In addition to its ability to promote caspase activation through mitochondrial cytochrome c release, BAX is also capable of predisposing to nonapoptotic, caspase-independent cell death through a mechanism that involves the disruption of mitochondrial membrane potential, the generation of reactive oxygen species, and the subsequent ATP depletion through loss of oxidative phosphorylation (9). These observations suggest that the ability of BAX to exert its prodeath function appears to be directly related to its mitochondrial localization and its subsequent effects on mitochondrial function.

We became interested in exploring alternative mechanisms of BAX-mediated cell death after observing that SW626 cells that overexpress this protein were sensitized to the effects of tubulin-binding drugs such as paclitaxel and vincristine, but not to DNA-damaging agents such as carboplatin and ionizing radiation. The stimulus-specific nature of this effect in SW626 cells prompted us to investigate whether BAX affects the proximal action of drugs such as paclitaxel and, if so, to determine the mechanism by which this effect occurs. Our studies show for the first time that the enhancement of paclitaxel-mediated apoptosis in stable BAX-expressing transfectants is at least partly mediated by the diminished drug efflux by these cells, resulting in enhanced intracellular paclitaxel levels. This effect was documented by both [3H]paclitaxel uptake studies and independently validated through the use of confocal microscopy. Moreover, the use of six independent clonal transfectants (three neo-control and three

**Fig. 4. Effects of verapamil on paclitaxel accumulation and cytotoxicity in SW626 neo-control transfectants. A. intracellular paclitaxel was determined after the pretreatment of cells with either diluent alone (0.1% DMSO) or 100 μM verapamil for 1 h, followed by incubation in 25 μM [3H]paclitaxel for 2 h in the continued presence of verapamil. A mean 20.9-fold increase in paclitaxel levels was observed in verapamil-treated cells. B. neo clones were exposed to either diluent alone (0.01% DMSO) or verapamil (10 μM) during a 3-day incubation in paclitaxel over a broad dose range as indicated. A lower dose of verapamil was used in these studies to maintain normal cellular viability, as indicated in the text. Viability was assessed at day 3 by trypan blue exclusion, and the results are expressed as the mean percentage of specific survival from three separate neo clones (A10, B10, and F8) at each paclitaxel concentration tested (diluent exerted no effect on viability over this time period). The dose-response curve using mean data from three separate BAX clones (A9, D7, and D8) is shown for comparison. SEs are less than 10% (data not shown for clarity).**
BAX-overexpressing clones) reduces the possibility that the observed results are due to clonal selection, as opposed to a BAX-mediated effect. The ability of BAX-overexpressing cells to accumulate higher intracellular levels of paclitaxel explains the observed enhancement of multiple seemingly diverse, early paclitaxel-mediated events such as G2/M-phase arrest, tubulin polymerization, and BCL-2 phosphorylation.

These observations led to the discovery of a drug efflux mechanism in SW626 cells, which, when inhibited by verapamil, resulted in elevated intracellular paclitaxel levels and the restoration of drug sensitivity in neo-control clones (Fig. 4). The precise nature of this pump is uncertain at the present time, although known candidate molecules may be excluded based upon several lines of evidence. One such candidate is the MDR-1 protein, a member of the ATP binding cassette family of surface membrane-localized, drug efflux pumps that is capable of transporting a variety of structurally distinct, hydrophobic proteins including paclitaxel and Vinca alkaloids (14). However, MDR-1 protein is not expressed by any of the SW626 clones used in this study, as determined through the use of the 4E3 antibody, which recognizes a surface membrane epitope of MDR-1 on flow cytometric analysis (12). The verapamil-inhibitable MRP-1 protein, which is not known to be an efficient transporter of paclitaxel, is also not significantly expressed in these clones. Other members of the MRP family have been recently identified as possible drug transporters, including MRPs 2-5, although their association with the drug resistance phenotype in vitro is not clear (19). Finally, although the LRP-1 protein is expressed in SW626 cells and is associated with a multidrug resistance phenotype, this protein is not known to function as a verapamil-inhibitable paclitaxel efflux pump and has instead been correlated with resistance to DNA-damaging agents such as platinum analogues (16). Therefore, based upon the available evidence, it is likely that SW626 neo-control cells express a potentially novel, verapamil-inhibitable paclitaxel efflux pump that is responsible for an important component of paclitaxel resistance in vitro.

It is not possible at present to determine the mechanism by which paclitaxel efflux is inhibited in SW626 BAX transfectants. The question of whether BAX serves to inhibit the function of a putative paclitaxel efflux pump in SW626 cells or whether BAX-overexpressing cells are deficient in pump expression can only be addressed once precise molecular identification of the paclitaxel efflux pump is made. It is also important to point out that these observations have been made using one model system, and it is not possible at present to determine whether these results will be generalizable to other cell types. Nevertheless, these data suggest that BAX overexpression may predispose cells to apoptotic cell death under some circumstances through a novel mechanism involving the enhancement of intracellular levels of naturally occurring toxins such as alkald derivatives.

References

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