Advances in Brief

Overexpression of Bcl-xS Sensitizes MCF-7 Cells to Chemotherapy-induced Apoptosis

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Abstract

Resistance to apoptosis plays an important role in tumors that are refractory to chemotherapy. We report that Bcl-xS, which functions like Bcl-2 to inhibit apoptosis, is highly expressed in MCF-7 human breast carcinoma cells. We used Bcl-xS, a dominant negative inhibitor of Bcl-2 and Bcl-xL, to demonstrate the role of these genes in modulating chemotherapy-induced apoptosis. Bcl-xS overexpressed in MCF-7 cells by stable transfection does not affect viability by itself but induces a marked increase in chemosensitivity to VP-16 or taxol. Using an ELISA assay which quantitates DNA damage, we demonstrate that this sensitization is due to apoptosis, suggesting the therapeutic utility of targeting this pathway.

Introduction

An understanding of the molecular events responsible for the development of breast cancer may identify novel targets for therapeutic intervention. There is evidence that several chemotherapy agents used to treat cancers act through induction of apoptosis. The p53 and bcl-2 genes regulate apoptosis (1-4) and have been shown to directly affect resistance to chemotherapy (3, 4). In other systems, Bcl-2 has been shown to block both p53-dependent and p53-independent apoptosis mediated by chemotherapy (3, 4). Other systems, Bcl-2 has been shown to block both p53-dependent and p53-independent cell death pathways (2-4). These studies suggest that strategies designed to block Bcl-2 might prove useful in sensitizing tumor cells to chemotherapy-induced apoptosis.

bcl-2 belongs to a family of genes involved in modulating apoptosis. The bcl-x gene is transcribed into two forms by alternate splicing (8). The product of the long form, Bcl-xL, like Bcl-2, functions as an inhibitor of apoptosis (8). In contrast, the short form, Bcl-xS, serves as a dominant negative inhibitor of both Bcl-xL and Bcl-2 (8). We postulated that overexpression of Bcl-xS in cells that express high levels of Bcl-2 or bcl-xL would cause sensitization to chemotherapy-induced apoptosis. We report that Bcl-xS-overexpressing MCF-7 cells are 5-10-fold more sensitive to apoptosis induced by the chemotherapy agents VP-16 or taxol than are MCF-7-neo-transfected control cells treated with these agents.

Materials and Methods

Transfections. The MCF-7 human breast carcinoma cells were stably transfected with an expression plasmid encoding bcl-xS (pSFFVneo-bcl-xS) or a control plasmid (pSFFVneo; SFFV, spleen focus-forming virus). Transfections were performed using lipofectamine (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's protocol. The bcl-xS or neo-transfected cells were subcultured in selection medium [containing MEM, 10% FCS, insulin (10 mg/liter or 10 μg/ml), and 1 mg/ml geneticin (G418)] for 4 weeks. The MCF-7 clone with the highest Bcl-xS expression, as determined by Western analysis, was used. Alternately, transient overexpression of Bcl-xS was achieved by infecting MCF-7 cells with a replication-deficient adenovirus vector carrying the bcl-xS minigene.3

Western Blot Analysis. Protein samples were prepared and resolved by denaturing SDS-PAGE using standard methods. The proteins were transferred to nitrocellulose and Western blotted with a mouse monoclonal antibody specific to human Bcl-x (a kind gift of Dr. Craig B. Thompson, Howard Hughes Medical Institute and Department of Medicine, Molecular Genetics & Cell Biology, University of Chicago, Chicago, IL). The specificity of this antibody has been demonstrated (9). A goat anti-mouse antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and ECL (Amersham, Arlington Heights, IL) were used to visualize protein bands.

Growth Studies. MCF-7-neo and bcl-xS transfectants were cultured in medium lacking G418. Cells were plated in 12-well plates (10⁴/well) and treated with/without varying doses of VP-16 (Sigma Chemical Co., St. Louis, MO) dissolved in DMSO (Sigma). Final DMSO concentrations (0.01-0.1%) were included in controls. Cells were treated with taxol (Paclitaxel; Bristol Myers Squib Corp., Princeton, NJ) or cremophor (the carrier for taxol; Sigma). Cell viability was measured by hemocytometry using trypan blue exclusion.

Quantitation of Apoptosis by ELISA. To provide quantitative evidence in support of an apoptotic cell death mechanism, we used a "cell death" ELISA (Boehringer-Mannheim, Indianapolis, IN) that measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation. An antihistone first antibody is coated on wells, which are then loaded with the cytoplasmic fraction of lysates from 2-5 × 10⁶ cells/well. Cytoplasmic extracts from control and drug-treated cells were equalized on the basis of total cell number. The second antibody is an anti-DNA antibody conjugated to peroxidase. The ELISA was developed with peroxidase substrate, and the absorbance at 405 nm was measured using a Microplate autoreader (EL311; Bio-tek Instruments, Winooski, VT).

This ELISA measures cytoplasmic, DNA-bound histone as opposed to free histone or free DNA that may be non-specifically released into the cytoplasm during nonapoptotic cell death (10). As controls, cytoplasmic extracts from HL-60 cells treated with/without the topoisomerase I inhibitor, camptothecin, were used. Camptothecin-treated HL-60 cells demonstrated a DNA ladder (180 bp) when compared with untreated HL-60 cells (data not shown).

Statistical Analysis. Statistical significance was measured by Student's paired t test. P for each data set is shown in each figure legend.

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Bcl-x<sub>S</sub> INCREASES CHEMOTHERAPY-INDUCED APOPTOSIS

Results

Overexpression of Bcl-x<sub>S</sub> in MCF-7 Cells. The expression of Bcl-x<sub>S</sub> and Bcl-x<sub>L</sub> in MCF-7-neo- and bcl-x<sub>S</sub>-transfected cells was assessed by Western blotting using a bcl-x monoclonal antibody specific for bcl-x proteins. As shown in Fig. 1, MCF-7-neo-transfected cells express large amounts, approximately 25-fold greater levels of Bcl-x<sub>S</sub> compared to Bcl-x<sub>L</sub>. Stable transfection of bcl-x<sub>S</sub> into these cells results in approximately a 3.0-fold increased expression of Bcl-x<sub>S</sub> compared to MCF-7-neo-transfected cells (Fig. 1, Lanes 3 and 4 versus Lanes 1 and 2). As positive and negative controls, protein samples were prepared from MCF-7 cells infected with an adenovirus vector expressing Bcl-x<sub>S</sub> (Lane 5) or lac Z (Lane 6).

**Effect of Bcl-x<sub>S</sub> Overexpression on Cell Viability.** MCF-7 cells transfected with bcl-x<sub>S</sub> showed approximately a 20% (± 6%; n = 4) decrease in growth rate compared to MCF-7-neo-transfected cells. However, in the absence of chemotherapy, the bcl-x<sub>S</sub> transfecants maintained viability and did not undergo apoptosis (see below).

**Effect of Bcl-x<sub>S</sub> Overexpression on the Chemosensitivity of MCF-7 Cells to VP-16 and Taxol.** We examined the sensitivity of MCF-7-neo- and bcl-x<sub>S</sub>-transfected cells to the cytotoxic effects of VP-16. At concentrations of VP-16 from 0.5–2 µM, Bcl-x<sub>S</sub>-overexpressing cells showed markedly increased cytotoxic effects compared to MCF-7-neo-transfected cells treated with equivalent concentrations of VP-16 (Fig. 2). The most striking decrease in viable cell number in cultures of Bcl-x<sub>S</sub>-overexpressing cells relative to MCF-7-neo control cells was observed at 2 µM VP-16 (Fig. 2, D and H). When the viability of cells shown in Fig. 2 was quantitated using trypan blue exclusion, we found that Bcl-x<sub>S</sub>-overexpressing cells showed a 4.0- and 9.0-fold decrease in viability at 1 and 2 µM VP-16, respectively, compared to MCF-7-neo transfectants (Fig. 3A). As an additional method of measuring viability of VP-16-treated cells, we measured the replating efficiency of MCF-7-neo versus Bcl-x<sub>S</sub>-overexpressing MCF-7 cells after VP-16 treatment.

Fig. 3B demonstrates that after VP-16 (2 µM) treatment for 6 days, Bcl-x<sub>S</sub>-overexpressing MCF-7 cells showed a 2.5-fold decrease in replating efficiency when compared with VP-16-treated, MCF-7-neo-transfected cells. The untreated MCF-7-neo and bcl-x<sub>S</sub> transfectants formed similar numbers of colonies after 6 days of treatment with DMSO alone (VP-16 solvent).

These studies indicate that as determined by morphology, replating efficiency, and viability (trypan blue exclusion), overexpression of Bcl-x<sub>S</sub> sensitizes MCF-7 cells to cell death induced by low doses of VP-16.

Since overexpression of Bcl-x<sub>S</sub> increased the sensitivity of MCF-7 cells to VP-16-induced death, it was important to determine if Bcl-x<sub>S</sub> overexpression sensitized these cells to the apoptotic effects of other chemotherapy agents with different mechanisms of cytotoxicity. Taxol, whose mechanism of action differs from VP-16, has been shown to induce apoptosis less effectively in Bcl-2-overexpressing cells (11). Therefore, we measured viability of MCF-7-neo-transfected versus Bcl-x<sub>S</sub>-overexpressing MCF-7 cells in the presence/absence of taxol (0.10 µM for 2 days). Fig. 3C shows that taxol caused a 15% decrease in the viability of the neo transfectants and a 60% decrease in the viability of Bcl-x<sub>S</sub>-overexpressing MCF-7 cells. Thus, Bcl-x<sub>S</sub> overexpression increased the chemosensitivity of MCF-7 cells to taxol and VP-16 by 4- and 9-fold, respectively.

As an alternate method of increasing Bcl-x<sub>S</sub> expression, MCF-7 cells were infected with the adenovirus bcl-x<sub>S</sub> or lacZ vectors (2000 particle-forming units/cell) and then treated with taxol (0.01 µM for 6 days). Viability studies showed that the bcl-x<sub>S</sub>-infected cells were 3.25 ± 0.30-fold more sensitive to taxol-induced cell death than were uninfected cells or lacZ-infected cells (n = 3).

**Effect of Bcl-x<sub>S</sub> Overexpression on VP-16- and Taxol-Induced Apoptosis.** In mammary epithelial cells, demonstration of apoptosis has been difficult, since classical apoptotic morphology and DNA laddering have not been detected in cells undergoing death (12, 13). In order to provide evidence in support of an apoptotic cell death mechanism, we used a quantitative ELISA that measures cytoplasmic DNA histone complexes generated during apoptotic DNA fragmentation.

As measured by ELISA, MCF-7-bcl-x<sub>S</sub> transfectants showed a 13- and 5-fold increase in apoptosis at 0.50 and 2 µM VP-16, respectively,
when compared to neo transfectants treated similarly (Table 1A). Due to a high percentage of dead cells and fragile live cells in bcl-xS transfectants treated with 2 μM VP-16, some cell loss may have occurred during preparation of the ELISA sample. This may account for the decreased level of apoptosis measured in this sample compared to the sample from bcl-xS transfectants treated with 0.50 μM VP-16. Table 1A also shows that taxol induced a 5-fold higher level of apoptosis in MCF-7-bcl-xS transfectants than in MCF-7-neo control cells. Thus, the ELISA measured 5–10-fold increased cytoplasmic DNA in VP-16- or taxol-treated MCF-7-bcl-xS transfectants compared to MCF-7-neo-transfected cells treated similarly.

Taxol-induced cell death in MCF-7 cells infected with the bcl-xS or lacZ adenoviral vectors was measured by ELISA. We observed a significant level of apoptosis in bcl-xS-infected cells relative to uninfected or lacZ-infected cells in the absence of taxol, suggesting that transient overexpression of Bcl-xS itself can induce apoptosis (Table 1B). However, taxol induced a 24-fold increase in apoptosis in bcl-xS-infected cells compared to a 5-fold higher level of apoptosis in the uninfected or lacZ-infected cells treated with the same dose of taxol (Table 1B). Thus, transient overexpression of Bcl-xS sensitizes MCF-7 cells to taxol-induced apoptosis, a result that is consistent with that observed in MCF-7 cells stably transfected with bcl-xS (Table 1).

These results are indicative of increased DNA fragmentation in Bcl-xS-overexpressing MCF-7 cells undergoing VP-16- or taxol-induced apoptosis, suggesting that the increased chemosensitivity of these cells to VP-16 or taxol is in part due to a marked increase in chemotherapy-induced apoptosis.

Discussion

To examine the role of the bcl-2 family of genes in mediating chemotherapy-induced apoptosis in breast cancer, we used MCF-7 human mammary carcinoma cells. These cells have been reported to express wild-type p53 (14) and high levels of Bcl-2 (15). In addition, we found that these cells express high levels of Bcl-xS compared to Bcl-xS (Fig. 1).

The finding that MCF-7 cells express predominantly inhibitors of apoptosis, Bcl-2 and Bcl-xS, allowed us to determine the effect of stable transfection of the dominant negative inhibitor of this pathway, Bcl-xS, on apoptosis in these cells. Stable transfection of bcl-xS resulted in an approximately 3-fold increase in Bcl-xS expression in MCF-7 cells compared to neo transfectants. However, the bcl-xS transfectants still expressed approximately 8-fold greater levels of Bcl-xS compared to Bcl-xS (Fig. 1). The inability to produce stable transfectants that expressed higher levels of Bcl-xS suggested that high levels of this protein might prove lethal. Indeed, we have found that higher levels of Bcl-xS expression produced by bcl-xS adenovirus

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**Table 1A**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control</th>
<th>0.5 μM VP-16</th>
<th>2 μM VP-16</th>
<th>0.10 μM taxol</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7-neo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7-bcl-xS</td>
<td>1.00</td>
<td>2.22 (0.62)</td>
<td>6.68 (1.11)</td>
<td>2.67 (1.09)</td>
</tr>
<tr>
<td>MCF-7-lacZ</td>
<td>1.00</td>
<td>29.05 (9.00)</td>
<td>37.32 (9.96)</td>
<td>13.65 (0.95)</td>
</tr>
</tbody>
</table>

**Fold increase in apoptosis**

Mean units of cytoplasmic DNA-histone (± SEM)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Uninfected</th>
<th>bcl-xS infected</th>
<th>lacZ-infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>6.07 (1.94)</td>
<td>1.32 (0.25)</td>
</tr>
<tr>
<td>Taxol (0.01 μM)</td>
<td>5.01 (1.30)</td>
<td>23.80 (0.60)</td>
<td>5.68 (2.25)</td>
</tr>
</tbody>
</table>

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**Table 1B**

Quantitative ELISA for DNA fragmentation in adenovirus-infected cells.

MCF-7 cells were infected with the bcl-xS or lacZ adenoviral vectors (2000 particle-forming units/cell). Cells were treated with or without taxol for 6 days. ELISA samples were prepared as described (see Table 1A) (n = 3; P ≤ 0.01).

Mean units of cytoplasmic DNA-histone (± SEM)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Uninfected</th>
<th>bcl-xS infected</th>
<th>lacZ-infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Taxol</td>
<td></td>
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</tr>
</tbody>
</table>

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Fig. 3. Effect of VP-16 and taxol on viability of Bcl-xS-overexpressing MCF-7 cells. MCF-7-bcl-xS (C) or neo (B)-transfected cells were treated with or without the indicated concentrations of VP-16 or taxol. A, percentage viability of both cell lines treated with or without VP-16 (0–2.0 μM) for 6 days (bars, SE; n = 3; P ≤ 0.01). B, percentage replating efficiency of VP-16-treated cells. MCF-7-bcl-xS-transfected cells (C) and MCF-7-neo cells (D) were treated with or without VP-16 (2 μM for 6 days). On day 6, cells from each treatment were harvested and replated at a density of 1000 or 2000 cells/well in triplicate wells. After 10 days, colonies that formed from each sample were counted. Replating efficiency of VP-16-treated bcl-xS cells is expressed as the percentage of colonies formed relative to that of the untreated bcl-xS transfectants. Similarly, the replating efficiency of VP-16-treated MCF-7-neo transfectants is expressed relative to that of untreated MCF-7-neo-transfected cells (bars, SE; n = 3; P ≤ 0.001). C, the percentage viability of MCF-7-bcl-xS (C) and MCF-7-neo cells (D) treated with or without 0.10 μM taxol for 2 days (bars, SE; n = 3; P ≤ 0.01).
infection of MCF-7 cells compared to stable transfection with plasmid-encoded Bcl-xS decreased cell viability, even in the absence of chemotherapy. Furthermore, the increased levels of Bcl-xS expression obtained by infection with the Bcl-xS adenoviral vector sensitized these cells to taxol-induced apoptosis (Table 1B). This observation suggests that the effects of overexpression of Bcl-xS on chemosensitivity in the stably transfected cells are not simply an artifact of transfection or clonal selection.

In this report, we demonstrate that overexpression of Bcl-xS in MCF-7 cells sensitizes these cells to apoptosis induced by low concentrations of VP-16 or taxol. Both of these agents have clinical utility in treating breast cancer (16, 17). VP-16, a topoisomerase II inhibitor, induces DNA damage and subsequent apoptosis. Taxol is cytotoxic because it inhibits mitotic spindle function. The observation that overexpression of Bcl-xS causes sensitization to apoptosis by two chemotherapy agents with different mechanisms of action suggests that these agents induce cell death by a common pathway inhibited by Bcl-2 and/or Bcl-xL.

Recently, proteins that modulate apoptosis have been described. BAG-1 is a Bcl-2 binding protein that inhibits apoptosis (18), whereas the Bad and Bax proteins promote apoptosis (19, 20). Thus, the apoptotic threshold of a cell may be the result of a dynamic balance between the positive and negative regulators of apoptosis.

The molecular mechanism by which Bcl-xS overexpression increases chemosensitivity of MCF-7 cells remains to be determined. Interactions between Bcl-2, Bcl-xL, and Bcl-xS proteins have been analyzed using the yeast two-hybrid system. These studies suggest that the Bcl-xS protein can heterodimerize with Bcl-2 and Bcl-xL (21). Thus, loss of Bcl-xL and/or Bcl-2 function upon Bcl-xS binding is a plausible mechanism by which Bcl-xS overexpression in MCF-7 cells become sensitized to chemotherapy-induced apoptosis. The relative overabundance of Bcl-xL over Bcl-xS, even in the stably transfected cells, suggests that other factors may be involved. For example, the Bax and Bad proteins may also play important roles in modulating the chemosensitivity of MCF-7 cells.

Our demonstration that overexpression of Bcl-xS in MCF-7 human breast carcinoma cells induces sensitization to apoptosis induced by low concentrations of VP-16 and taxol provides evidence that Bcl-xL and Bcl-2 play a role in modulating apoptosis in these cells. Furthermore, these studies suggest that development of pharmacological agents that target this pathway represents a rational therapeutic strategy.

Acknowledgments

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References


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