Reconstitution of Caspase 3 Sensitizes MCF-7 Breast Cancer Cells to Doxorubicin- and Etoposide-induced Apoptosis

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INTRODUCTION

Chemotherapeutic resistance is a major problem in human oncology. Mechanisms of chemotherapeutic resistance are diverse and poorly defined for most cancer subtypes. Recent studies suggest that aberrant apoptosis (programmed cell death) likely contributes to this process (1). Apoptosis is a genetically controlled process that can be triggered by different extracellular and intracellular stimuli (2). Apoptotic execution requires coordinated activation of a special group of proteases, known as caspases (3, 4). The activation of caspases is a signaling cascade mediated by proteolysis (5). Activated caspases subsequently cleave cellular death substrates and cause biochemical and morphological changes, leading to apoptosis (6). Fourteen mammalian caspases have been cloned (4, 7). Caspases 2, 8, 9, and 10 (apical caspases) initiate apoptosis and activate downstream caspases. Caspases 3, 6, and 7 (effector caspases) are activated by apical caspases and further cleave cellular death substrates (4).

Caspase 3 (also known as cpp32, yama, and apopain) is a key caspase in this signaling cascade (8–12). Caspase 3 activity has been detected in apoptosis induced by a variety of apoptotic signals, including death receptor activation (13), growth factor deprivation (14), ionizing radiation (15), and treatment with granzyme B (16) or different chemotherapeutic agents (17). Caspase 3 knockout mice displayed abnormal brain tissue development due to lack of apoptosis (18). A growing number of substrates cleaved by caspase 3 have been identified, such as PARP (10), sterol-regulatory element-binding protein (19), gelsolin (20), the U1-associated M17 70,000 protein (21), D4-GDI (22), DFF (23), DNA-dependent protein kinase and θ (24, 25), α-fodrin (26), and huntingtin (27). Caspase 3 is believed to play a pivotal role in apoptotic execution.

Alterations in apoptosis-associated genes are often observed in cancers. The p53 tumor suppressor gene, a key regulator in DNA damage-induced apoptosis, is frequently mutated in human tumors (28). Overexpression of apoptotic inhibitors, such as bcl-2 and bcl-xL (29, 30), and down-regulated apoptosis-promoting factors, such as Bax-α and Fas (31, 32), has been detected in primary tumors and tumor cell lines. These alterations have been linked to chemotherapeutic resistance (31, 33). Correction of these alterations has resulted in sensitization of the defective cells to chemotherapeutic agents (34).

Caspase 3 deficiency was recently detected in MCF-7 breast cancer cells. It is due to a deletion mutation in exon 3 of the gene (35). Overexpression of caspase 3 in MCF-7 cells indicates that caspase 3 plays a critical role in both death receptor- and mitochondria-mediated apoptotic pathways (35–38). Given the important role of caspase 3 in apoptotic execution and the correlation between the alterations of other apoptotic regulators and chemotherapeutic resistance, we postulated that caspase 3 deficiency might also significantly contribute to chemotherapeutic resistance. Although caspase 3-like activity has been detected in the apoptosis induced by various chemotherapeutic drugs (17), the specific role of caspase 3 in this process warrants further investigation due to the overlapping activities among effector caspases (18, 39). To evaluate the role of caspase 3 in chemotherapy-induced apoptosis, we reconstituted caspase 3 in MCF-7 cells and characterized the apoptotic responses of the MCF-7 cells to doxorubicin and etoposide in comparison with control cells. We found that reconstitution of caspase 3 significantly sensitized MCF-7 cells to doxorubicin- and etoposide-induced apoptosis.

MATERIALS AND METHODS

Cell Culture, Plasmid Construction, and Transfection. MCF-7 cells were maintained in Iscove’s modified Dulbecco’s medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and penicillin/streptomycin. The pBabe/caspase 3 plasmid was constructed by treating the BamHI/PvuI caspase 3 cDNA insert from pBS/caspase 3 plasmid (a gift from Drs. David Boothman and John Pink) with T4 DNA polymerase and then subcloning it into the blunt-ended pBabe/puro retroviral vector (40). MCF-7 cells were placed into 60-mm dishes at 3 × 10^4 cells/dish and allowed to grow overnight. Two µg of caspase 3 encoding pBabePuro plasmid were mixed with 10 µl of LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) and transfected into the cells according to the manufacturer’s instructions. Empty pBabePuro vector was also transfected as a control. Twenty-four h after transfection, the cells were trypsinized, diluted, and placed into 96-well plates. Transfected cells were then selected with 2 µg/ml puromycin. Individual puromycin-resistant clones were screened for caspase 3 by Western blot. Five caspase 3-positive clones were pooled for further characterization. Morpho-

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logical changes were observed and photographed with a phase-contrast microscope.

**Drug Treatment and Sample Collection.** For doxorubicin (Bedford Laboratories, Bedford, OH) and etoposide (Bristol-Myers Squibb Co., Princeton, NJ) treatments in studies other than the MTT assay (see below), 1 x 10^6 cells were seeded into 60-mm dishes 24 h before drug treatment. Various doses were added to the dishes 18 h before cell collection. Treated cells to be analyzed by flow cytometry and DAPI staining were trypsinized. Cells to be analyzed by DEVD (Asp-Glu-Val-Asp) cleavage assay and Western blot were scraped off the dish. In all cases, medium from individual dishes, which might contain floating dead cells, was collected and mixed with the cell pellet from the same dish.

**MTT Survival Assay.** Three hundred cells were placed into each well of 96-well plates. Twenty-four h later, the medium was replaced with new medium containing defined doses of doxorubicin or etoposide. Six days after treatment, the medium was changed with phenol red-free medium containing 500 µg/ml MTT (Sigma). Three h after incubation, MTT-containing medium was removed. The incorporated dye was dissolved in 100 µl/well DMSO, and the plates were read at the wavelength of 570 nm using an ELISA reader.

Absorbance in the treated cells was expressed as a percentage of control. Eight parallel samples were treated in each concentration point. Five separate experiments were performed.

**DEVD Cleavage Assay.** Drug-treated cells were washed with PBS and resuspended in lysis buffer [50 mM Tris-HCl (pH 8.0), 130 mM KCl, 1 mM EDTA, 10 mM EGTA, and 10 µM digitonin] at 320 µl/60-mm dish. After incubation at 37°C for 10 min, the samples were spun for 3 min (5000 rpm), and the supernatant was collected. After adding 100 µl of lysis to each well of a fluorometer plate, 100 µl of substrate solution, 2 µM DEVD-AMC (PharMingen, San Diego, CA) in lysis buffer was added right before the reading. Fluorescence was measured in a microplate fluorometer (Cambridge Technology, Cambridge, MA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Results are reported as the fluorogenic activity over 1 h (T₇₀ to T₀). Samples were prepared in triplicate.

**Western Blot.** PBS-washed cells were treated with lysis buffer (41) on ice for 30 min. Lysed cells were centrifuged at 14,000 rpm for 10 min to remove cellular debris. Protein concentrations of the supernatant were determined using BCA Protein Assay (Pierce, Rockford, IL). Fifty µg of cell lysate were loaded onto each lane of a gel. Protein was separated by either 10% or 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with TBS-T (5% milk in Tris-buffered saline-Tween 20) washing buffer (41) and probed with specific primary antibodies. Concentrations of the primary antibodies used ranged from a 1:500 dilution to a 1:2,000 dilution. Antibodies against caspases 3 and 7 were purchased from Transduction Laboratories (Lexington, KY). Antibodies against caspase 6 and DFF were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-lamin B antibody was from PharMingen. The anti-PARP antibody was from Boehringer Mannheim (Indianapolis, IN). Washed membranes were then probed with horseradish peroxide-labeled antimouse, antirabbit, or antigoat secondary antibodies (Amersham Pharmacia, Arlington Heights, IL), respectively. The membranes were washed again and treated with enhanced chemiluminescence reagents (Amersham Pharmacia). The specific protein bands were visualized by autoradiography (41).

**Flow Cytometry.** Drug-treated cells were trypsinized and washed with PBS. The cells were then fixed in 50 µl of 0.125% paraformaldehyde in PBS at 37°C for 5 min, followed by the addition of 450 µl of ice-cold methanol to each sample. After being washed three times with PBS containing 0.1% Triton X-100 and treated with RNase A (0.04 µg/ml) for 30 min, the cells were stained with 50 µg/ml propidium iodide. Cell analysis was performed using a Coulter Epics 751 flow cytometer. The fraction of the total cell population was removed. The incorporated dye was dissolved in 100 µl/well DMSO, and the plates were read at the wavelength of 570 nm using an ELISA reader.

Absorbance in the treated cells was expressed as a percentage of control. Eight parallel samples were treated in each concentration point. Five separate experiments were performed.

**RESULTS**

**Stable Reconstitution of Caspase 3 in MCF-7 Cells to Doxorubicin- and Etoposide-mediated Killing.** To compare the sensitivity of MCF-7/c3 cells and MCF-7/pv cells to doxorubicin and etoposide, we studied the viability and morphological changes of treated cells. The IC₅₀ values were determined using MTT assays, in which MCF-7/pv and MCF-7/c3 cells were exposed to 0.5–37 nM doxorubicin or 0.05–1 µM etoposide for 6 days. The sensitivities of MCF-7/pv and MCF-7/c3 cells to each drug are shown in Table 1. The results indicated that MCF-7/c3 cells were significantly sensitized to both drugs (P < 0.01 for doxorubicin; P < 0.05 for etoposide). This suggests that caspase 3 reconstitution sensitized MCF-7 cells to doxorubicin and etoposide treatments.

Morphological changes commensurate with striking cytopathic differences in chemotherapeutic sensitization were observed in the caspase 3-reconstituted cells. To reflect in situ cell death in the original culture plates, the cells were treated at higher concentrations for a shorter period. When MCF-7/pv and MCF-7/c3 cells were treated with doxorubicin at concentrations of 0, 2.5, 5, and 10 µM or with etoposide at concentrations of 0, 100, 200, and 400 µM for 18 h, the differences between the two cell lines were evident at all doses. This effect was magnified at increased concentrations. Cellular alterations included shrinkage, rounding, detachment, membrane blebbing, and segregation of cellular structure. In the 10 µM doxorubicin (Fig. 2A)-treated group or 400 µM etoposide (Fig. 2B)-treated group, MCF-7/c3 cells displayed diffused apoptosis as compared with MCF-7/pv cells, which showed only sporadic islands of cell death.

![Fig. 1. Reconstitution of caspase 3 in MCF-7 cells. Protein levels of caspase 3 in Jurkat, MCF-7/pv, and MCF-7/c3 cells were detected using Western blot. MCF-7/pv and MCF-7/c3 cells were MCF-7 cells transfected with pBabe/puro vector and the vector encoding caspase 3 cDNA, respectively.](image-url)
CASPASE 3 RECONSTITUTION-MEDIATED SENSITIZATION TO DOXORUBICIN AND ETOPOSIDE

Table 1  Effects of doxorubicin and etoposide on survival fractions of MCF-7/pv and MCF-7/c3 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC₅₀a (µM)</th>
<th>95% CI</th>
<th>IC₅₀b (µM)</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>8.44</td>
<td>7.30-9.58</td>
<td>4.23</td>
<td>3.31-5.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.36</td>
<td>0.19-0.53</td>
<td>0.09</td>
<td>0.08-0.10</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

a Mean of IC₅₀a, IC₅₀b were determined as described in “Materials and Methods.” Five data sets were used for analysis.

b CI, confidence interval.

Fig. 2. Enhanced killing in MCF-7/c3 cells treated with doxorubicin (A) or etoposide (B). MCF-7/pv (A and B, a and b) and MCF-7/c3 (A and B, c and d) cells were treated with 10 µM doxorubicin (A, b and d) or 400 µM etoposide (B, b and d) for 18 h, as compared with untreated cells (A and B, a and c) cells. Photographs were taken under a phase-contrast microscope (10 × 20).

Activation of Effector Caspases in Caspase 3-reconstituted Cells. Activation of effector caspases is a biochemical hallmark of apoptosis. To verify that the above-described sensitization to the chemotherapeutic drugs occurred through caspase 3-mediated apoptosis, we analyzed the activation of effector caspases in MCF-7/c3 and MCF-7/pv cells. DEVD cleavage assay is a quantitative method that detects caspase 3-like activity (39). As shown in Fig. 3, DEVD cleavage activity in drug-treated MCF-7/pv cells was very limited, even in the cells treated with 5 µM doxorubicin or 200 µM etoposide. However, DEVD cleavage activity in MCF-7/c3 cells increased over 10–20-fold when the cells were treated with 200 µM etoposide or 2.5 µM doxorubicin. The strong caspase 3-like activity in drug-treated MCF-7/c3 cells indicates that caspase 3 expressed in MCF-7/c3 cells was functional and that activation of reconstituted caspase 3 contributed to the sensitization.

To detect the activation of specific effector caspases, Western blots were performed. As indicated by decreased proform and subunit generation, activation of caspase 3 was detected in MCF-7/c3 cells treated with both drugs (Fig. 4, A and B). Although Western blotting was less sensitive than DEVD cleavage assay, the results obtained using either method were consistent with each other. Because caspases 6 and 7 are commonly activated in different apoptosis, we compared the extent and pattern of their activation between MCF-7/pv and MCF-7/c3 cells. In drug-treated MCF-7/pv cells, which were deficient of caspase 3, caspase 7 processing/activation was minimal (Fig. 4, A and B). In contrast, activation of caspase 7 in MCF-7/c3 cells was remarkably increased when the cells were treated with 10 and 50 µM doxorubicin or 200 and 400 µM etoposide, as indicated by the formation of p32 and p20 fragments. These results indicate that caspase 7 activation in doxorubicin- and etoposide-treated cells was primarily caspase 3 dependent. These observations were consistent with our reported finding of granzyme B-induced apoptosis (16).

Analysis of caspase 6 activation in the two cell lines revealed a more specific action of caspase 3. As shown in Fig. 4A, a p32 band product was identified in doxorubicin-treated MCF-7/pv cells but not in MCF-7/c3 cells, consistent with caspase 6 activation at low levels in the absence of caspase 3. Reconstitution of caspase 3, however, significantly enhanced caspase 6 activation at both 10 and 50 µM doxorubicin. Because the combined size of pLarge and pSmall subunits of caspase 6 is about Mₛ 32,000 (42), the appearance of a p32 band in MCF-7/pv cells suggests that caspase 6 was processed by a caspase other than caspase 3 between the propeptide and pLarge subunit. The disappearance of the p32 band and an increase in the pLarge subunit (p20) in treated MCF-7/c3 cells suggests that caspase 3 processes caspase 6 between the pLarge and the pSmall subunits. In etoposide-treated MCF-7/c3 cells, the extent of caspase 6 activation was not as great as that observed in doxorubicin-treated MCF-7/c3 cells (Fig. 4B). However, a cleavage product with a size around Mₛ 30,000 appeared specifically in etoposide-treated MCF-7/c3 cells. Disappearance of the p32 band in MCF-7/c3 cells treated with 800 µM etoposide also suggests the cleavage between pLarge and pSmall subunits. Taken together, these results support that activation of caspase 3, as well as the subsequent activation of caspases 6 and 7, contributed to the sensitization in MCF-7/c3 cells.

Cleavage of Cellular Death Substrates in MCF-7/c3 Cells. Because proteolytic cleavage of cellular death substrates by activated...
Caspases is responsible for the cellular dysfunction and structural destruction of apoptosis (6), we studied the cleavage of PARP, lamin B, and DFF as representative substrates in the control and reconstituted cells. As shown in Fig. 5, there was only limited cleavage of all three substrates in MCF-7/pv cells treated with either drug (even when the doxorubicin concentration was as high as 50 μM, and the etoposide concentration was as high as 400 μM). In contrast, all three substrates were significantly or even completely cleaved in the drug-treated MCF-7/c3 cells. These results suggest that caspase 3 was also required for DNA and nuclear fragmentation in chemotherapy-induced apoptosis.

DISCUSSION

In this report, we describe the establishment of a stable MCF-7 cell line reconstituted with caspase 3. This line was useful for studying the specific role of caspase 3 and caspase 3-dependent signaling in response to doxorubicin and etoposide. As demonstrated by IC₅₀ determination and morphological data, caspase 3 reconstitution sensitized MCF-7 cells to doxorubicin- and etoposide-induced apoptosis. Increased DEVD cleavage and amplified activation of caspases 6 and 7 were also observed after treatment in caspase 3-reconstituted cells. Significant increases in the proteolysis of cell death substrates and DNA fragmentation further verified a caspase 3-mediated sensitization in doxorubicin- and etoposide-induced apoptosis.

Doxorubicin and etoposide are active chemotherapeutic agents used in clinical oncology. Doxorubicin is a key adjuvant drug for breast cancer treatment. It triggers apoptosis through several mechanisms. As with many chemotherapeutic agents, it induces DNA damage by interacting with topoisomerase II, leading to DNA breakage (43). It can also induce membrane alterations and the generation of ceramide at higher concentrations (44). Recently, it has been reported that up-regulation of the Fas/Fas ligand system may also be involved in doxorubicin-mediated killing (45). For etoposide-induced apoptosis, DNA damage secondary to topoisomerase II inhibition appears to be...
a major mechanism (46). Despite the variance in the chemotherapeutic initiation process, the resulting release of cytochrome c from mitochondria followed by activation of caspase 9 and the effector caspases is believed to be the final common pathway in chemotherapy-induced cell death (38, 47, 48). Microinjection of cytochrome c induced apoptosis in 293 cells with functional caspase 3 or caspase 3-transfected MCF-7 cells but not in caspase 3-deficient MCF-7 cells (36), indicating that caspase 3 was required for cytochrome c-mediated apoptosis. Abrogation of mitochondrial cytochrome c release and caspase 3 activation have been associated with acquired multidrug resistance (49). As shown in this presentation, caspase 3 reconstitution restored the integrity of the doxorubicin- and etoposide-induced killing mechanism. This direct evidence links caspase 3 deficiency and chemotherapeutic efficacy, suggesting caspase 3 defects as one mechanism for chemoresistance.

Activation of caspase 3 in chemotherapy-induced apoptosis has been reported by many groups (17, 38, 47, 50, 51). Involvement of caspase 3 in this process was shown either by detecting its activation as a representative of effector caspases (38, 50, 52) or by using synthetic inhibitors, such as DEVD-CHO (51, 53), to block caspase 3-like activities. Nevertheless, little work has been done to differentiate the role of caspase 3 from that of other effector caspases in this process. In our experiments, comparison between caspase 3-deficient and -reconstituted cell lines more specifically defined the specific role of caspase 3 in doxorubicin- or etoposide-induced apoptosis and in the activation of other effector caspases. Although caspases 3, 6, and 7 are all categorized as effector caspases (4, 5), our results demonstrated an additional apical-like nature of caspase 3. These data, derived from a whole cell system (in contrast to a cell-free system), show that activation of caspase 6 and especially caspase 7 was largely dependent on caspase 3 activation (Fig. 4). Although caspase 6 activation was detected in caspase 3-deficient cells, efficient activation of caspase 6 required caspase 3 activity (Fig. 4). As a result, by direct cleavage and amplification through the activation of other effector caspases, caspase 3 reconstitution led to a striking increase in death substrate cleavage and DNA fragmentation (Figs. 5 and 6). Our preliminary results showed that reconstituted caspase 3 also had feedback effects on its upstream factors.4

Although our experiments were based on an in vitro cell line model, our data are consistent with a recent report that was based on an in vivo model. Using a rat AH130 liver tumor model, Yamabe et al. (54) found that transduction of human caspase 3 in combination with etoposide administration induced extensive apoptosis and significantly reduced tumor volume, as compared with the group with caspase 3 transduction or etoposide treatment alone. Although our focus was on reconstitution of caspase 3, and theirs was on caspase 3 overexpression-mediated therapy, both reports demonstrate that

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4 Unpublished observations.
Caspase 3 is critical in chemotherapy-induced apoptosis and that caspase 3 reconstitution overexpression and chemotherapy have synergistic effects.

In our experiments, two drug treatment conditions were used. For IC₅₀ determination using MTT assays, the cells were exposed to the drugs for 6 days. Significant sensitization was detected when drug concentrations were between 0.5 and 37 μM for doxorubicin and 0.05 and 1 μM for etoposide, respectively. This compares favorably with the plasma concentrations of the two drugs in clinical application, which could reach up to 2 μM for doxorubicin (55) and 170 μM (100 μg/ml) for etoposide (56), respectively. To evaluate early biochemical changes, the cells were also treated for shorter periods (18 h) at much higher concentrations, although this very high dose and short duration approach is not currently clinically feasible.

One noteworthy finding was that when MCF-7/c3 cells were treated with doxorubicin for 18 h, caspase activation and death substrate cleavage displayed a sharp increase when the drug concentration was increased from 2 to 10 μM (Figs. 4 and 5). This suggests that there may be a concentration threshold for doxorubicin to induce maximal caspase 3-mediated apoptosis under a given treatment condition. This is consistent with the clinical benefit observed with dose intensification of doxorubicin, as shown for node-positive breast cancer patients (57) and topical administration of doxorubicin for ovarian cancer (58).

Doxorubicin appears to induce two types of cellular response, i.e., slow cell death at low concentrations and rapid cell death at high concentrations. This may be due to an increased number of activated mechanisms at higher drug concentrations. This may be a concentration threshold for doxorubicin to induce maximal caspase 3-mediated apoptosis under a given treatment condition. This is consistent with the clinical benefit observed with dose intensification of doxorubicin, as shown for node-positive breast cancer patients (57) and topical administration of doxorubicin for ovarian cancer (58). Because significant sensitization to doxorubicin was also detected in MTT assays, we agree with Han et al. (59) on the action model of doxorubicin. Doxorubicin appears to induce two types of cellular response, i.e., slow cell death at low concentrations and rapid cell death at high concentrations. This may be due to an increased number of activated mechanisms at higher drug concentrations.

Distinct differences in apoptotic activities between MCF-7/pv and MCF-7/c3 cell lines in response to doxorubicin or etoposide treatment underscore the possible significance of caspase 3 deficiency in cancer resistance. Caspase 3 reconstitution sensitized MCF-7 breast cancer cells to commonly applied chemotherapeutic agents, suggesting that caspase 3 deficiency may contribute to chemotherapeutic resistance. Caspase 3 reconstitution also sensitized MCF-7 cells to radiotherapy and granzyme B (16). Reconstitution of caspase 3 in MCF-7 cells may also enhance apoptosis in response to Fas ligand and tumor necrosis factor α treatment, as shown by others (35, 37). Therefore, it appears that caspase 3 deficiency may have a broad clinical relevance, including both chemotherapeutic and radiotherapeutic resistance and immune-associated antimurder mechanisms. Our preliminary results show down-regulation or deficiency of caspase 3 in many breast cancer specimens, which supports the non-breast cancer findings of others (60). We therefore speculate that caspase alterations may be linked to poorer prognosis and therapeutic resistance in human breast cancer.

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