Abrogation of Mitochondrial Cytochrome c Release and Caspase-3 Activation in Acquired Multidrug Resistance*

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Acquired multidrug resistance to anti-cancer agents has been associated with overexpression of the P-glycoprotein and other members of the ATP-binding cassette superfamily. The present studies demonstrate that SCC-25 cells selected for resistance to the alkylating agent cisplatin (CDDP) overexpress the anti-apoptotic Bcl-xL protein. In contrast to parental cells, the SCC-25/CDDP-resistant variant failed to exhibit activation of caspase-3, cleavage of protein kinase C δ, and other characteristics of apoptosis in response to CDDP. Similar results were obtained when SCC-25/CDDP cells were exposed to the structurally and functionally unrelated antitumor metabolite 1-β-D-arabinofuranosylcytosine (ara-C). Other cells selected for resistance to doxorubicin or vincristine also exhibited overexpression of Bcl-xL and failed to respond to CDDP and ara-C with activation of caspase-3. The results further demonstrate that multidrug-resistant cells exhibit a block in the release of mitochondrial cytochrome c into the cytosol and that this effect is dependent on overexpression of Bcl-xL. The demonstration that lysates from the resistant cells respond to the addition of cytochrome c with activation of caspase-3 confirms that the block in apoptosis is because of inhibition of mitochondrial cytochrome c release. These findings demonstrate that cells respond to diverse classes of anti-cancer drugs with overexpression of Bcl-xL and that this response represents another mechanism of acquired multidrug resistance.

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Acquired multidrug resistance to chemotherapeutic agents is an obstacle to the therapy of human malignancies. The development of resistance to certain cytotoxic drugs has been associated with insensitivity to structurally and functionally unrelated classes of anticancer agents. One type of acquired multidrug resistance is attributable to overexpression of the P-glycoprotein (Pgp) (1, 2). As a member of the ATP-binding cassette (ABC) superfamily of membrane transport proteins and an energy-dependent efflux pump, Pgp confers resistance to the anthracyclines, vinca alkaloids, and epipodophyllotoxins. The multidrug resistance-associated protein is another member of the ABC superfamily that when overexpressed also results in resistant phenotypes (3, 4). The finding that acquired multidrug resistance occurs in the absence of ABC protein overexpression has provided support for the existence of other mechanisms (5, 6).

The cellular response to the effects of diverse anticancer agents includes the induction of apoptosis (7). Whereas the precise signals responsible for the induction of apoptotic cell death remain unclear, recent studies have demonstrated that cytochrome c release from mitochondria contributes to this response (8). In the cytosol, cytochrome c associates with a complex of Apaf-1 and caspase-9 and thereby induces the activation of caspase-3 (9, 10). The induction of apoptosis is associated with caspase-3-mediated cleavage of poly(ADP-ribose) polymerase (PARP), protein kinase C δ (PKCδ), and other proteins (11–13). Importantly, overexpression of the anti-apoptotic Bcl-2 and Bcl-xL proteins blocks cytochrome c release and the activation of caspase-3 (14–16). In addition to effects on cytochrome c release and in concert with the finding that Bcl-2 is not restricted to mitochondria (17), Bcl-2 exhibits a downstream caspase-inhibiting activity (18, 19).

Treatment of cells with DNA damaging agents and staurosponine has been associated with release of cytochrome c (8, 16). By contrast, cytochrome c release was not observed in tumor necrosis factor-, Fas-, or dexamethasone-induced apoptosis (20). Thus, release of cytochrome c appears to be induced by only certain forms of cellular stress. Distinct mechanisms responsible for inhibition of cytochrome c release may also be restricted to certain apoptotic stimuli. In this context, selection of cells for resistance to vincristine and doxorubicin is associated with induction of Bcl-xL, and not Bcl-2, expression (21). These findings have suggested that overexpression of Bcl-xL may contribute to inhibition of cytochrome c release in acquired multidrug resistance.

The present studies demonstrate that acquired resistance to cytotoxic drugs is associated with inhibition of cytochrome c release. The findings support a Bcl-xL-dependent mechanism that is induced during selection to cytotoxic agents and thereby confers resistance to apoptosis.

MATERIALS AND METHODS

Cell Culture and Transfection—Human SCC-25 squamous carcinoma cells were grown as monolayers in Dulbecco’s modified Eagle’s minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 0.4 μg/ml hydrocortisone. SCC-25 cells were selected for resistance to cisplatin (CDDP) (22, 23). The cloning of resis-
ant cells designated SCC-25/CDDP was accomplished by isolating a single large colony in a cloning chamber. SCC-25 cells were transfected with either the pSFFV-Neo plasmid (SCC-25.neo) or pSFFV-Neo containing bcl-xL (SCC-25/bcl-xL) (21). Transfectants were selected in the presence of 400 μg/ml geneticin sulfate. U-937 cells and drug-resistant variants U-A20 and U-V20 were grown as described (21). Cells were treated with 100 μM CDDP or 10 μM ara-C (Sigma).

**Immunoblot Analysis—**Preparation of cell lysates and immunoblot analysis were performed as described using C-2-10 anti-PARP (24), anti-PKCα, anti-caspase-3 (25, 26), anti-Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Pgp antibodies (Oncogene Research Products, Cambridge, MA). Antigen-antibody complexes were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech).

**Cytochrome c Release—**Cells were washed twice with phosphate-buffered saline, and the pellet was suspended in 5 ml of ice-cold buffer A (20 mM HEPES, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin, apro tinin, and pepstatin A) containing 250 mM sucrose. The cells were homogenized by being run through a Dounce homogenizer 14 times with a sandpaper-polished pestle. After centrifuga tion for 5 min at 4 °C, the supernatants were centrifuged at 105,000 x g for 30 min at 4 °C. The resulting supernatant was used as the soluble cytosolic fraction. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-cytochrome c (Pharmingen, San Diego, CA).

**Analysis of DNA Fragmentation—**Cells (5 x 10⁶) were harvested, washed, and suspended in 50 μl of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% SDS, and 0.5 mg/ml protease K (Sigma). After incubation at 50 °C for 6 h, the samples were mixed with 50 μl of 10 mM EDTA (pH 8.0) containing 1% (v/v) low melting point agarose and 40% sucrose for 10 min at 70 °C. The DNA was separated in 2% agarose gels. After electrophoresis for 30 min at 30 V, the resulting bands were stained with 1 μg/ml ethidium bromide (Sigma) and visualized by UV illumination.

**In Vitro Assay for Cytochrome c-dependent Activation of Caspase-3—** S-100 cytosolic fractions from untreated cells were immunodepleted of cytochrome c by incubation with anti-cytochrome c (Pharmingen) and protein A-Sepharose beads for 2 h at 4 °C (8). The immunodepletion procedure was repeated twice with centrifugation to remove the beads. [35S]Methionine-labeled caspase-3 was synthesized by the coupled transcription and translation method (Promega, Madison, WI). Caspase-3 was incubated with 10 μl of cytochrome c-free lysate, 1 mM dATP, 1 μM cytochrome c (Sigma), and 1 mM MgCl2 at 30 °C for 1 h in a final volume of 25 μl of buffer A. The reaction products were analyzed by 15% SDS-PAGE and then autoradiography.

**RESULTS AND DISCUSSION**

SCC-25 cells, designated SCC-25/CDDP, were selected for growth in the presence of 17 μM CDDP (23). The SCC-25 and SCC-25/CDDP cells express low to undetectable levels of Pgp (data not shown). Parental SCC-25 cells responded to CDDP exposure with activation of caspase-3 and cleavage of PARP and PKCα (Fig. 1A). The parental cells also responded to CDDP with internucleosomal DNA fragmentation (Fig. 1B). By contrast, there was no detectable activation of caspase-3, cleavage of PKCα, or DNA fragmentation following exposure of SCC-25/CDDP cells to 100 μM CDDP (Fig. 1, C and D). These findings indicated that SCC-25/CDDP cells are resistant to CDDP-induced apoptosis.

Previous studies have demonstrated that cells selected for resistance to anthracyclines or vinca alkaloids overexpress the Bcl-xL protein (21). Analysis of SCC-25 cell lysates by immunoblotting with anti-Bcl-xL demonstrated that the CDDP-resistant line overexpresses this anti-apoptotic protein compared with the parental cells (Fig. 2A). The finding that Bcl-2 is not increased in the SCC-25/CDDP cells indicates that resistance to CDDP results in the selective induction of Bcl-xL expression (data not shown). To determine whether overexpression of Bcl-xL in the SCC-25 cells contributes to the apoptosis-resistant phenotype, we stably transfected SCC-25 cells to express Bcl-xL (Fig. 2A). CDDP treatment of SCC-25/neo cells that express the empty vector was associated with activation of caspase-3 (Fig. 2B). By contrast, there was no apparent cleavage of caspase-3 to the active fragment in SCC-25/bcl-xL cells treated with CDDP (Fig. 2B). The SCC-25/bcl-xL cells also failed to respond to CDDP with cleavage of PARP or PKCα, whereas this response was detectable in the SCC-25/neo cells (data not shown). These results suggest that overexpression of Bcl-xL in drug-resistant cells or in stably Bcl-xL transfectants blocks CDDP-induced activation of caspase-3.

To extend these findings to an agent that is structurally and mechanistically distinct from CDDP, we treated SCC-25/CDDP cells with the antimitabolite 1-β-D-arabinofuranosylcytosine (ara-C). In contrast to CDDP, ara-C incorporates into DNA and functions as a relative chain terminator (27). Treatment of
parental SCC-25 cells with ara-C was associated with activation of caspase-3 and cleavage of PARP (Fig. 3A). Similar results were obtained in the SCC-25/neo transfectants. By contrast, the SCC-25/CDDP and SCC-25/Bcl-xL cells failed to respond to ara-C with caspase-3 activation or substrate cleavage (Fig. 3A). U-937 cell lines selected for resistance to doxorubicin (U-A20) and vincristine (U-V20) overexpress both Pgp and Bcl-xL (21). Treatment of wild-type U-937, but not U-A20 or U-V20, cells with CDDP resulted in caspase-3 activation (Fig. 3B). Similar results were obtained when the U-A20 and U-V20 cells were treated with ara-C (Fig. 3C). Thus, resistance to activation of caspase-3 and apoptosis occurs in cells selected for growth in the presence of diverse agents (CDDP, doxorubicin, or vinca alkaloids).

Overexpression of Bcl-xL blocks release of mitochondrial cytochrome c to the cytosol and thereby activation of caspase-3 (16). To assess whether similar mechanisms are involved in drug-resistant cells, we assayed SCC-25 cells for increases in cytosolic cytochrome c following CDDP treatment. Whereas SCC-25 cells responded to CDDP with release of cytochrome c, CDDP had little if any effect on this response in SCC-25/CDDP or SCC-25/bcl-xL cells (Fig. 4A). Similar findings were obtained in the drug-resistant U-A20 and U-V20 cells (data not shown). These results indicate that cells selected for resistance to cytotoxic agents exhibit defects in cytochrome c release and thereby activation of caspase-3. To determine whether the SCC-25/CDDP cells acquire other mechanisms of resistance to cytochrome c that are downstream from mitochondrial release, we prepared cell lysates and asked whether caspase-3 is activated upon addition of cytochrome c. The results demonstrate that addition of cytochrome c to SCC-25 cell lysates is associated with activation of caspase-3 (Fig. 4B). Similar findings were obtained with lysates from the SCC-25/CDDP and SCC-25/bcl-xL cells (Fig. 4B).

The development of resistance to cytotoxic agents is often multifactorial but is for the most part selective for particular classes of drugs. To our knowledge, there are no known mechanisms that are applicable to all classes of cytotoxic agents. The present data suggest that selection for resistance to CDDP is associated with inhibition of cytochrome c release and caspase-3 activation. The finding that the CDDP-resistant cells are also unresponsive in terms of caspase-3 activation to other drug-resistant cells, we assayed SCC-25 cells for increases in cytosolic cytochrome c following CDDP treatment. Whereas SCC-25 cells responded to CDDP with release of cytochrome c, CDDP had little if any effect on this response in SCC-25/CDDP or SCC-25/bcl-xL cells (Fig. 4A). Similar findings were obtained in the drug-resistant U-A20 and U-V20 cells (data not shown). These results indicate that cells selected for resistance to cytotoxic agents exhibit defects in cytochrome c release and thereby activation of caspase-3. To determine whether the SCC-25/CDDP cells acquire other mechanisms of resistance to cytochrome c that are downstream from mitochondrial release, we prepared cell lysates and asked whether caspase-3 is activated upon addition of cytochrome c. The results demonstrate that addition of cytochrome c to SCC-25 cell lysates is associated with activation of caspase-3 (Fig. 4B). Similar findings were obtained with lysates from the SCC-25/CDDP and SCC-25/bcl-xL cells (Fig. 4B).

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**Fig. 3.** Resistance to caspase-3 activation in drug-selected cells. A, lysates from SCC-25, SCC-25/CDDP, and SCC-25/bcl-xL cells treated with 10 μM ara-C for 24 h were immunoblotted with anti-caspase-3 or anti-PARP. U-937 cells and the U-A20 and U-V20 resistant variants were treated with 100 μM CDDP (B) or 10 μM ara-C (C) for 6 h. Immunoblot analysis of the lysates was performed with anti-caspase-3. Cleavage products are indicated by small arrow(s). FL, full length.

**Fig. 4.** Accumulation of cytochrome c in response to CDDP and its role in drug resistance. A, the indicated cell lines were treated with CDDP and harvested at 6 h. Cytosolic proteins were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with anti-cytochrome c. To monitor for equal loading of proteins, the same filters were also stained with Ponceau S. B, lysates from the indicated cells were subjected to immunodepletion with anti-cytochrome c. After addition of dATP, [35S]methionine-labeled caspase-3 was incubated for 15 min with the lysates in the presence and absence of purified cytochrome c. Cleavage products of caspase-3 are indicated by two arrows.
structurally and mechanistically distinct agents, such as ara-C, supports a phenotype that applies broadly to the response of cells to cytotoxic agents. Indeed, the U-937 cells selected for resistance to doxorubicin or vincristine failed to respond to CDDP with activation of caspase-3. Also, in contrast to wild-type U-937 cells, the U-A20 and U-V20 lines failed to respond to cytotoxic agents. Indeed, the U-937 cells selected for resistance to CDDP also overexpress Bcl-xL (38, 22). These findings confirm that induction of Bcl-xL expression in drug-resistant cells is associated with abrogation of cytochrome c release and thereby caspase-3 activation. The present data confirm that induction of Bcl-xL expression in drug-resistant cells is associated with abrogation of cytochrome c release and thereby caspase-3 activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation. The finding that lysates from the drug-resistant cells respond to cytochrome c activation.
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