F16, a Mitochondriotoxic Compound, Triggers Apoptosis or Necrosis Depending on the Genetic Background of the Target Carcinoma Cell

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ABSTRACT

Mutations that lead to the emergence of resistance to apoptosis are commonly observed among tumor cells. Some of the proteins affected are integral parts of the apoptotic cascade such as pro- and antiapoptotic members of the Bcl-2 family. F16 is a small molecule that accumulates in mitochondria of a variety of tumor cells and interferes with their physiological function. Because this interference ultimately triggers apoptosis in many affected cell lines, we examined the effect of antiapoptotic Bcl-2 overexpression on the response of cells to F16. Our results showed that high levels of Bcl-2 did not block the ability of F16 to induce cell death. However, unlike the apoptotic response that followed F16 treatment of cells with moderate Bcl-2 levels, cells resistant to a variety of apoptotic stimuli by virtue of Bcl-2 overexpression succumbed to F16 by necrosis. Thus, this dual ability of the mitochondrial toxic compound F16 to induce apoptosis and necrosis may represent an added advantage by expanding its spectrum of action toward genetically altered tumor cells incapable of apoptosis.

INTRODUCTION

We have recently reported the identification of F16, a small molecule that exhibits selective cytotoxicity toward transformed cells (1). This compound is preferentially concentrated by mitochondria of several oncogene-initiated mouse mammary tumor cell lines and of human breast cancer cell lines. The basis for the selective accumulation of this delocalized lipophilic cation (DLC) lies in the higher membrane potential (\(\Delta \psi_m\)) and, to some extent, plasma membrane potential of the transformed versus nontransformed cells (2). Even though the accumulation of DLCs has been explained in terms of a general mechanism, the toxicity imposed by each on the mitochondria of carcinoma cells results from inhibition of different and specific essential molecular targets and processes (3). We have shown that F16 is capable of inhibiting oxidative phosphorylation and inducing mitochondrial transmembrane depolarization (1). This event is followed by an increase in the permeability of the inner mitochondrial membrane [mitochondrial permeability transition (PT)], cytochrome c release, and apoptotic cell death (4, 5).

Most anticancer drugs, regardless of their molecular target, rely on intact apoptotic cascades to effectively induce malignant cell death. However, impaired apoptosis is commonly observed in tumors. Alterations in the cell death program confer emerging neoplastic cells with selective advantages to withstand extreme environmental conditions (low \(pO_2\), low pH, and nutrient deprivation) in rapidly growing tumors and metastatic outgrowths. Many cancer cells that exhibit impaired apoptosis experience changes in the balance between pro- and antiapoptotic proteins (i.e., like those that belong to the Bcl-2 family) as well as down-regulation of members of the caspase family of proteases or mutations in tumor suppressors such as \(p53\) (6–8).

We have previously speculated that F16 might be capable of inducing cell death in cells resistant to apoptosis, and, thus, be a particularly effective antitumor agent. One distinctive feature of F16 is that its toxicity is a consequence of direct mitochondrial accumulation. F16 is capable of inducing mitochondrial PT in both cells and in isolated mitochondria. These effects are transiently blocked by cyclosporine A (1), an inhibitor of the voltage-dependent PT pore (4). Thus, we hypothesized that F16 cytotoxicity might not rely on the status of genes involved in the upstream apoptotic cascade. We focused our attention on alterations of proteins that participate directly in the mitochondrial apoptotic pathway. Of those, we decided to examine the role of proteins that, like members of the Bcl-2 family, have been implicated in the control of mitochondrial membrane permeability either by direct pore formation or by regulation of the PT pore opening (9–12). We have previously shown that Bax/Bak (DKO) cells, although resistant to apoptosis in response to various stimuli (13), were sensitive to F16 treatment (1). In the present study, we sought to determine whether overexpression of the Bcl-2 oncogene could induce resistance to F16.

Aberrant expression of antiapoptotic Bcl-2 has been identified in many hematological malignancies and solid tumors (14, 15). In transfection studies, high levels of Bcl-2 protect cells against a variety of apoptotic stimuli (16). In fact, overexpression of Bcl-2 has been linked to chemoresistance and radioresistance of cancer cells (17–20). How Bcl-2 protects cells from apoptosis is still the subject of intensive research and remains to be determined. The cytoprotective role of Bcl-2 has been associated with various mechanisms of action. For example, it has been reported that Bcl-2 is capable of blocking apoptosis by inhibiting the opening of the voltage-dependent PT pore and thus preventing mitochondrial membrane permeabilization (21). Bcl-2 has also been shown to prevent apoptosis by acting as an antioxidant and inhibiting cytochrome c release and downstream caspase activation (22–24).

Our results showed that overexpression of Bcl-2 in F16-sensitive cells did not prevent F16-induced cytotoxicity. Bcl-2 is capable of blocking cytochrome c release, caspase activation, and apoptosis induced by F16 and several other stimuli. However, the mitochondrial injury imposed by F16, due to its direct accumulation within the organelle matrix, leads to necrosis of the treated Bcl-2-overexpressing cells. Altogether, our data suggest that the death program triggered by F16 depends on the genetic background of the target cells. Cells capable of extensive mitochondrial accumulation of F16 succumb to apoptosis by activation of the mitochondrial pathway. Alternatively, cells harboring alterations that render them resistant to apoptosis may die from necrosis resulting from an energetic, ionic, and redox catastrophe.

MATERIALS AND METHODS

Reagents. Anti-Neu was purchased from Upstate Biotechnology Inc., anti-Bcl-2 from Transduction Laboratories, anti-caspase-3 from Cell Signaling, and anti-\(\beta\)-actin from Sigma. F16 was obtained from Asinex, and rhodamine 123 (Rh123) from Molecular Probes. Staurosporine (ICN), thapsigargin (Sigma), and etoposide (Sigma) were used where indicated. 4,5-Dihydroxy-1,3-benzene disulfonic acid (Tiron) was purchased from Sigma, and the broad spectrum caspase inhibitor zVAD-fmk from Calbiochem.

Cell Culture and Generation of Stable Cell Lines. Unless indicated, cells were grown in DMEM and 10% fetal bovine serum at 37°C/5% CO\(_2\). Mouse
mammary epithelial Eph4 cells stably expressing the neu<sup>T</sup> oncogene (Eph4-H6-A6) were established as described previously (1). Neu-initiated tumor cell lines SMF and NF324-1C have been established in our laboratory from tumors dissected from Neu-transgenic mice. pcDNA3 (Invitrogen) carrying the bcl-2 cDNA (25) was transfected into Eph4, Eph4-H6-A6, and SMF cells using Fugene reagent according to the manufacturer’s protocol (Roche). Selection of stable clones was done in medium containing 1.2 mg/ml G418 (Life Technologies, Inc.).

**Effect of F16 on Cell Proliferation.** Cells (1 x 10<sup>5</sup>) were seeded in each well of 6-well plates. On the following day, F16 or staurosporine was added to the medium (3 and 4 μM, respectively), whereas control cells were left untreated. Cells were harvested and counted whenever indicated. Counts were done in triplicate for untreated and treated cells, and the entire experiment was repeated three times.

**Cell Lysate Preparation and Immunoblotting.** Cells were washed with PBS and lysed in buffer [40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 10 mM sodium PP<i>i</i>, 2% NP40, 10 mM NaF, 2 mM EDTA, and 5 mM Na<sub>2</sub>VO<sub>4</sub> containing Complete protease inhibitor mixture (Roche). The insoluble material was separated by centrifugation at 4°C. Protein concentration in the supernatants was determined by Bradford assay (Bio-Rad). For each sample, aliquots containing equivalent amounts of protein were separated by SDS-PAGE and transferred to Polyvancrylon membranes (NEN) in Towbin’s buffer (25 mM Tris, 190 mM glycine, 20% methanol, and 0.005% SDS). Membranes were blocked with 1.5% BSA in Tris-Buffered Saline Tween-20 (TBST) [150 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.3% Tween 20]. The primary antibodies (1 μg/ml) and secondary antibodies were in TBST/0.2% BSA. Membranes were washed with TBST buffer. Detection was done using horseradish peroxidase conjugated to anti-rabbit IgG or to anti-mouse IgG (Amersham Biosciences) and the enhanced chemiluminescent substrate (Fierce).

**Electron Microscopy.** Cell were grown in 35-mm dishes and treated as indicated. Cells were formaldehyde fixed, and the fixed cells were stained with 1.25% glutaraldehyde (2.5%) fixation and treated as described previously (1). Ultrathin sections were analyzed using a JEOL 1200EX microscope.

**Oligonucleosomal DNA Fragmentation.** Cells were left untreated or treated for 48 h with 0.3, 1, and 3 μM F16 or with 4 μM staurosporine as a control. DNA was isolated in each case, from 1 x 10<sup>5</sup> cells using Suicide-Track (Oncogene) following recommended protocol. Samples were resolved in 1.5% agarose gel and ethidium bromide stained.

**Flow Cytometry.** For detection of apoptosis after various treatments, cells were stained for 15 min with annexin V-phycocerythrin conjugate and the vital dye 7-amino-actinomycin D (7-AAD) following manufacturer’s procedure (PharMingen)

For comparison of Δψ<sub>M</sub>, among various cell lines, the potentiometric probe tetramethylrhodamine methyl ester (TMRM; Molecular Probes) was used. To normalize the TMRM fluorescent signal according to mitochondrial mass, cells were incubated with nonyl-acridine orange (Molecular Probes). Cells were then stained with annexin V-FITC conjugate and the vital dye 7-AAD (Bio-Rad). The insoluble material was separated by centrifugation at 4°C.

**Oligonucleosomal DNA Fragmentation.** Cells were incubated for 72 h in medium containing F16 as a control. DHE was added to the cell suspension (10<sup>5</sup> cells/ml) in ice-cold mito buffer (0.25 M sucrose, 10 mM HEPES, 2 mM glucose, and 1 mM pyruvate (to sustain mitochondrial ATP synthesis). In addition, where indicated, the broad caspase inhibitor zVAD-fmk (50 μM), the superoxide scavenger Tiron (1 mM), and excess glucose (28 mM) were added alone or in combinations to the medium (26–28). Cells were then stained with annexin V-FITC conjugate and the vital dye 7-AAD (Bio-Rad). In all cases, the labeled cells were analyzed using the FACSCalibur (Becton-Dickinson). Each determination was done in duplicate, and the entire experiment was done in triplicate. The results were expressed as percentage of cell death (which includes apoptotic and/or necrotic cells). Annexin V positive/7-AAD negative were scored as apoptotic cells, annexin V negative/7-AAD positive were scored as necrotic cells, and annexin V positive/7-AAD positive were scored as apoptotic and/or necrotic cells.

**Detection of Cytochrome c Release by Immunofluorescence.** Untreated or cells treated with F16 or staurosporine were washed with PBS. Cells were then fixed and permeabilized for 20 min with ice-cold 3.7% paraformaldehyde and 0.01% (v/v) NP40, respectively. This step was followed by 15-min incubation in blocking solution (0.5% BSA/PBS). Immunodetection was performed with monoclonal anti-cytochrome c antibody (PharMingen) and tetramethylrhodamine isothiocyanate-conjugated antimouse secondary antibody (Jackson Immunoresearch) in PBS/0.3%M TWEEN 20/0.2% BSA. Hoechst stain was used to localize nuclear DNA. Cells were photographed with the Axioskop microscope using a Spot Camera (Diagnostic Instruments).

**Detection of Cytochrome c Release by Western Blot Analysis.** Mitochondria- and cytosol-containing fractions were obtained by differential centrifugation as described previously with some modifications (29). In brief, cells were subjected to various treatments as indicated. Adherent and floating cells were harvested, washed with PBS, and counted. Cells were pelleted and resuspended (5 x 10<sup>5</sup> cells in 2 ml) in ice-cold mito buffer (0.25 m sucrose, 10 mM HEPES, 1 mM EGTA). Cells were homogenized with Dounce homogenizer (10 strokes, tight pestle) on ice. The cell homogenates were centrifuged at 600 x g for 10 min at 4°C to pellet unbroken cells, nuclei, and cell debris. Next, the supernatants were centrifuged at 7,500 x g for 20 min at 4°C to pellet the mitochondria. The supernatants, containing the cytosolic extract, were carefully transferred. The fractions obtained were subjected to Western blot analysis as described previously. Immunodetection was performed with mouse monoclonal anti-cytochrome c antibody (Phar-Mingen) and anti-manganese superoxide dismutase (Stressgen).

** Determination of ATP.** Quantification of cellular ATP levels was done by the luciferin-luciferase method following manufacturer’s recommended protocol (Roche). Cells were incubated in medium containing 3 μM F16 for the periods of time indicated. Determinations were done in duplicate, using an equivalent number of cells in each case. The entire experiment was performed twice.

**RESULTS**

**Isolation and Characterization of Bcl-2-Overexpressing Clones.** To test whether Bcl-2 could protect cells from the cytotoxic effect of F16, the Bcl-2 cDNA was transfected into the previously described F16-sensitive Eph4-H6-A6 (transfomed by Neu<sup>T</sup> overexpression) and F16-insensitive Eph4-EV (transfected with the empty vector) cell lines (1). Clones stably overexpressing Bcl-2 were isolated (Fig. 1A). Eph4-H6-A6.C13, Eph4-H6-A6.C18 clones derived from Eph4-H6-A6 (A6) cells, and an Eph4-D6 clone derived from Eph4-EV cells exhibited between 8–10-fold higher Bcl-2 protein levels than the corresponding parental cells from which they were derived.

As previously mentioned, high levels of Bcl-2 have been shown to effectively protect cells against a variety of apoptotic stimuli (30, 31). To functionally evaluate Bcl-2 overexpression, we compared the effect of the potent protein kinase C inhibitor staurosporine, the endoplasmic reticulum Ca<sup>2+</sup> adenosine triphosphate pump inhibitor thapsigargin, and the topoisomerase II inhibitor etoposide on parental Eph4-H6-A6 cells and derived Bcl-2-overexpressing Eph4-H6-A6.C13 and Eph4-H6-A6.C18 cells. Phosphatidylserine is normally restricted to the inner leaflet of the plasma membrane. Its appearance on the outer side of the cell surface is an early event in the apoptotic response. To detect the externalization of phosphatidylserine, we monitored by flow cytometry cells stained with annexin V in conjunction with the vital dye 7-AAD (Fig. 1B). Treatment of Eph4-H6-A6 cells with the apoptosis-
As a control, cells were treated with the potent apoptosis inducer staurosporine for 48 h. Untreated or incubated for 24 h in medium containing F16 (3 μM) nor A6 nor H9262 M, a recently identified lipophilic cation with selective cytotoxicity against tumor cells. As a control, cells were left untreated or subjected to a time-course treatment with 3 μM staurosporine for 24 h and etoposide (ET, 3.4 μM in the same conditions, Bcl-2-overexpressing EpH4-A6 and EpH4-A6.C13 cells consistently showed that Bcl-2 overexpression could not prevent F16-induced cytotoxicity. However, Bcl-2-overexpressing cells experienced a delay in the onset of massive cell death upon F16 treatment, as observed in the course of the cell viability assay (Fig. 2). Bcl-2 family members have been shown to regulate PT pore opening and closing by interaction with the mitochondrial voltage-dependent anion channel and the adenine nucleotide translocator (9). In that regard, Bcl-2 might regulate Δψm (32). A differential response between Bcl-2-overexpressing and parental cell lines could result from the alteration of the Δψm in the inducing compounds for 24 h resulted in a detectable early apoptotic cell subpopulation (annexin V positive, 7-AAD negative cells). Under the same conditions, Bcl-2-overexpressing EpH4-A6.C13 cells appeared primarily viable, nonapoptotic (annexin V negative/-ADD negative). The Bcl-2-overexpressing clone EpH4-A6.C18 behaved in a similar way. These results confirmed that transfected Bcl-2 rendered cells resistant, capable of long-term survival to various apoptosis inducers. In contrast to the observed apoptosis in F16-treated EpH4-A6 cells (annexin V positive/-ADD negative), F16 treatment of Bcl-2-overexpressing cells resulted in the appearance of an annexin V negative/-ADD positive population characteristic of early necrotic cells. Thus, high levels of Bcl-2 are not capable of protecting cells from F16-induced cytotoxicity.

Bcl-2 Overexpression Is Not Capable of Protecting F16-Sensitive Cells from the Small Molecule-Induced Cell Death. The ability of F16 to affect cell growth of EpH4-A6 and Bcl-2-overexpressing EpH4-A6.C13 and EpH4-A6.C18 was evaluated by increase in cell number during a time course experiment in the presence of 3 μM F16. As a control, cells were treated with the potent apoptosis inducer staurosporine.

The three cell lines showed a progressive reduction in the number of cells upon treatment with F16 for 24 and 48 h when compared with untreated cells, and only Bcl-2-overexpressing cells remained resistant to staurosporine (Fig. 2). Even though the Bcl-2-overexpressing clones exhibited a slower response, these cells were largely affected by the small molecule as evidenced by the drastic decline in the number of living cells observed after 48 h of incubation in F16-containing medium. Thus, these results are in agreement with the appearance of annexin V negative/7-AAD positive subpopulation during the flow cytometric analysis of F16-treated EpH4-A6.C13 cells (Fig. 1B). Altogether, the data establish that antiapoptotic Bcl-2 is capable of blocking apoptosis in response to a variety of toxic agents but is unable to protect cells from F16-induced cell injury.

While performing the cell viability assays, we noticed that the parental cells and Bcl-2-overexpressing cells presented different morphological features upon F16 treatment. To examine in more detail these phenotypic differences, we examined untreated or F16-treated EpH4-A6 and Bcl-2-overexpressing EpH4-A6.C13 and EpH4-A6.C18 cells by electron microscopy. Upon F16 treatment, Bcl-2-overexpressing cells showed cellular swelling, characteristic of necrotic cells (Fig. 2B). In contrast, parental EpH4-A6 treated with F16 shrank and exhibited chromatin condensation, characteristic of apoptosis. These observations together with the results obtained by fluorescence-activated cell sorter analysis suggested that Bcl-2-overexpressing cells undergo necrosis in response to F16.

Bcl-2 neither Alters Δψm nor Affects F16 Uptake. The results obtained by cell counting and annexin V staining of F16-treated EpH4-A6 and EpH4-A6.C13 cells consistently showed that Bcl-2 overexpression could not prevent F16-induced cytotoxicity. However, Bcl-2-overexpressing cells experienced a delay in the onset of massive cell death upon F16 treatment, as observed in the course of the cell viability assay (Fig. 2). Bcl-2 family members have been shown to regulate PT pore opening and closing by interaction with the mitochondrial voltage-dependent anion channel and the adenine nucleotide translocator (9). In that regard, Bcl-2 might regulate Δψm (32). A differential response between Bcl-2-overexpressing and parental cell lines could result from the alteration of the Δψm in the

Fig. 1. Characterization of Bcl-2 overexpressing EpH-4 clones. A, Western blot analysis on whole-cell lysates from EpH4-EV and Bcl-2-overexpressing EpH4 clone D6 (EpH4-D6). Clones EpH4-A6.C14, EpH4-A6.C13, and EpH4-A6.C18 were derived by transfection of bcl-2 cDNA in the previously described Neu-overexpressing EpH4-A6 cells. Aliquots of lysates containing equivalent amount of protein were probed with antibodies against Bcl-2, HER-2/neu, and β-actin as described in “Materials and Methods.” B, Fluorescence-activated cell sorter analysis of cells subjected to annexin V-PE/7-AAD staining as detailed in “Materials and Methods.” EpH4-A6 (A6) cells and Bcl-2-overexpressing EpH4-A6.C13 (A6.C13) and EpH4-A6.C18 (A6.C18) cells were left untreated or incubated for 24 h in medium containing F16 (3 μM), a recently identified lipophilic cation with selective cytotoxicity against tumor cells. As a control, cells were incubated in the presence of the apoptosis-inducing agents staurosporine (ST, 4 μM) and thapsigargin (TH, 3 μM or 2 μg/ml) for 24 h and etoposide (ET, 3.4 μM or 2 μg/ml) for 48 h.

Fig. 2. Bcl-2 overexpression does not protect cells from F16-induced cell death. A, EpH4-A6 cells and two Bcl-2-overexpressing clones, EpH4-A6.C13 and EpH4-A6.C18, were left untreated or subjected to a time-course treatment with 3 μM F16 (■). Cells were counted after 24, 48, and 96 h of the initiation of the treatment as described in “Materials and Methods.” As a control, cells were treated with 4 μM staurosporine for 24 h and counted (○). The results are expressed as fold reduction in cell number with respect to untreated cells for each time point. They represent the average ± SE of three independent experiments. B, images obtained by electron microscopy of untreated or F16-treated (3 μM, 48 h) EpH4-A6 and EpH4-A6.C13 cells. Bar = 1 μm.
A6.C13 (A6.C13), and EpH4-A6.C18 (A6.C18) cells were stained with the overexpressing cell lines. We have previously shown that accumulation of enhanced production of ROI below and above physiological levels, mitochondrial respiration results in a decrease in cellular ATP levels and an increase in reactive oxygen intermediates (ROI). A blockade in mitochondrial mass between cell lines.

Mitochondria are a major source of cellular ATP and reactive oxygen intermediates (ROI). The accumulation of DLCs in transformed cells (1–3) recapitulated the positive correlation between Δψm and mitochondrial accumulation of DLCs in transformed cells (1–3). The accumulation of F16 as well as of Rh123, another DLC concentrated by cells with elevated Δψm, was monitored (Fig. 3B). We took advantage of the fact that both compounds are fluorescent and that their accumulation in cells can be followed by flow cytometry. No significant difference in the levels of fluorescent signal was detected between the parental and derived Bcl-2-overexpressing clones. These results ruled out a decrease in Δψm and/or an impairment in F16 uptake as factors that may contribute to the difference in the response of Bcl-2-overexpressing cells to F16.

**Bcl-2 Overexpression Does Not Protect Cells from F16-Induced Mitochondrial Dysfunction and the Concomitant Energetic and Redox Imbalance.** Mitochondria are a major source of cellular ATP and reactive oxygen intermediates (ROI). A blockade in mitochondrial respiration results in a decrease in cellular ATP levels and an enhanced production of ROI below and above physiological levels, respectively (34). We have previously shown that accumulation of F16 in mitochondria led to inhibition of oxidative phosphorylation. Treatment of cells with F16 resulted in the bionenergetic compromise of their mitochondria as a function of time.

Bcl-2 overexpression has been reported to prevent mitochondria depolarization during apoptosis (24, 35). Hence, we reasoned that because F16 inhibition of respiration and oxidative phosphorylation was related to its ability to depolarize mitochondria, the stabilizing effect of Bcl-2 on Δψm could partially compensate for F16-induced depolarization. This effect could in turn prevent or slow down F16-induced depletion of ATP levels and increase production of ROI and could explain the delayed onset of cell death observed in the Bcl-2-overexpressing cells. Cellular ATP levels in untreated or F16-treated EpH4-A6, EpH4-A6.C13, and EpH4-A6.C18 for 15, 24, and 48 h were assessed by detection of the chemiluminescent signal resulting from the luciferase-catalyzed, ATP-dependent oxidation of luciferin (Fig. 4A). Upon F16 incubation, a similar time-dependent decrease of the cellular ATP pool was observed in both parental EpH4-A6 and Bcl-2-overexpressing EpH4-A6.C13 and EpH4-A6.C18 cells. Superoxide anion production was followed in parallel by flow cytometric analysis of cells stained with DHE. The fluorescent DNA-ethidium complex formed as a result of the oxidation of DHE by superoxide is a measure of the anion levels. A comparable steady increase in the fluorescent signal was detected in Bcl-2-overexpressing and parental cell lines. Thus, overexpression of Bcl-2 cannot ameliorate the mitochondrial dysfunction imposed by F16 leading to the decline in ATP synthesis and overproduction of reactive oxygen species.
F16-Imposed Mitochondrial Injury Does Not Trigger Apoptosis in Bcl-2-Overexpressing Cells. As previously mentioned, an annexin V negative/7-AAD positive subpopulation, characteristic of necrotic cells, was detected among F16-treated EpH4-A6.C13 and EpH4-A6.C18 cells. These results along with the electron microscopy images and the decrease in the total number of cells in response to F16 treatment suggested that in the presence of the anti-apoptotic Bcl-2, F16 induced necrosis instead of apoptosis.

To assess this issue, we examined three additional apoptosis markers. First, we looked at cytochrome c release from mitochondria into the cytoplasm, an early event in the apoptotic cascade. Immunofluorescence detection of cytochrome c was performed on EpH4-A6 and Bcl-2-overexpressing EpH4-A6.C13 18 h after addition of F16 or staurosporine, as a control, to the medium. F16 treatment promoted early release of cytochrome c in transformed EpH4-A6 cells, whereas no release of cytochrome c was observed in the Bcl-2-overexpressing clones (Fig. 5A). Unlike staurosporine, after prolonged incubation (36 h and beyond) of EpH4-A6.C13 cells in F16-containing medium, diffuse cytochrome c staining was observed in about 15% of cells. For those cells in which release of cytochrome c was evident, localization of manganese superoxide dismutase to the mitochondria was also lost (data not shown). This result indicates that the relocation observed was due to loss of mitochondrial structural integrity, and the release of cytochrome c, a consequence rather than a cause of cell death. Likewise, cytochrome c release from the mitochondria to the cytoplasm was also detected by cell fractionation followed by Western blot analysis in F16-treated EpH4-A6 cells (3 μM for 18 h) but not in the Bcl-2-overexpressing clones (Fig. 5B).

Activation of the caspase family of cysteine aspartyl proteases is a common mechanism involved in the induction of apoptosis (11). Caspase activation has been associated with degradation of a variety of cellular components and cell death. This group of enzymes is synthesized as inactive pro-caspases that become enzymatically active upon proteolytic maturation. To monitor the processing of the effector caspase-3, Western blot analysis was performed on whole-cell lysates from untreated cells or cells treated for 24 h with F16 or staurosporine as a control. The appearance of the cleaved (M₄ 18,000) active form of caspase-3 was observed in EpH4-A6 cells in response to both F16 and staurosporine treatment, which suggested that in the presence of the antiapoptotic Bcl-2, the protective effect of caspase inhibition becomes less short period (24 h). After 48 h of incubation in F16-containing medium, the protective effect of caspase inhibition becomes less consistent with cell death.

In light of the inability of Bcl-2 to prevent cell death upon F16 treatment, we concluded that cell death in the absence of cytochrome c release, caspase-3 processing, or appearance of a DNA ladder was indeed consistent with necrosis of the treated cells.

F16-Induced Mitochondrial Dysfunction Triggers Apoptosis or Necrosis, Depending on the Genetic Background. Our experiments demonstrate that overexpression of Bcl-2 in EpH4-A6 cells does not prevent F16-induced cell death, but rather alters the way in which cells die. To further evaluate the role of caspase activation in death resulting from F16 treatment, EpH4-A6 and Bcl-2-overexpressing cells were left untreated or incubated in medium containing F16 in the presence of the pan-caspase inhibitor zVAD-fmk (Fig. 6). The results shown for clone EpH4-A6.C13 are representative of those for clones EpH4-A6.C18. Inhibition of caspase activity results in a reduction of the number of dead cells among EpH4-A6 cells exposed to F16 for a short period (24 h). After 48 h of incubation in F16-containing medium, the protective effect of caspase inhibition becomes less consistent with cell death.
Bcl-2 does not protect cells from F16-induced cytotoxicity

From our previous results, we speculated that the necrosis observed in F16-treated EpH4-A6 cells in response to staurosporine (data not shown). However, there is a significant percentage of cell death in the presence of the caspase inhibitor among F16-treated EpH4-A6 cells. No DNA ladder was detectable in these cells (data not shown). These results suggest that pharmacological inhibition of caspase activity in EpH4-A6 can recapitulate the effect of Bcl-2-overexpression in these cells. Thus, when apoptosis is impaired (e.g., by Bcl-2 overexpression) or blocked (e.g., by caspase inhibition) in EpH4-A6 cells, necrosis in response to F16 is uncovered. On the other hand, inhibition of caspase activity in EpH4-A6.C13 cells (already apoptosis resistant) does not affect the percentage of dead cells at any time point.

From our previous results, we speculated that the necrosis observed in F16-treated EpH4-A6.C13 and EpH4-A6.C18 cells could be the consequence of the energetic breakdown in these cells. Indeed, the lag phase observed in the response of the Bcl-2-overexpressing cells to F16 treatment correlated with the incubation time required for cells to show a drastic depletion of their ATP pool. It has been reported that manipulations aimed to maintain a high concentration of cellular ATP favored the onset of apoptosis over necrosis (28, 36). Conversely, conditions leading to a depletion of the ATP pool could shift the balance in the opposite direction. To test whether the depletion of ATP and the increase in ROI were direct triggers of the necrosis induced by F16 in the Bcl-2-overexpressing cells, we examined the effect of buffering ATP and superoxide levels in F16-treated cells (Fig. 6). For that purpose, the medium was supplemented with glucose (28 mm final concentration) to stimulate glycolysis and to maintain the ATP concentration at a high level. The cellular ATP concentration in F16-treated EpH4-A6.C13 cells was maintained at 11 ± 0.8 nmol/10⁶ cells in glucose-supplemented medium (in contrast to 2.8 ± 0.3 nmol/10⁶ cells in medium without glucose supplementation). In addition, we used the superoxide scavenger Tiron to protect against superoxide anion burst. Our results showed that caspase-independent cell death of Bcl-2- overexpressing EpH4-A6.C13 cells upon F16 treatment was largely prevented in high-glucose medium (1.7-fold; Fig. 6). This protective effect was potentiated by combination with Tiron (2.5-fold). Similar results were obtained with the Bcl-2-overexpressing clone EpH4-A6.C18. Interestingly, in the Bcl-2-overexpressing cells grown in high-glucose- and Tiron-containing medium, neither did F16 lead to the emergence of apoptotic phenotypes (data not shown), nor did addition of the caspase inhibitor zVAD-fmk exhibit any added beneficial effect.

These data show that the manipulation of ATP levels alone, even though sufficient to partially protect cells from necrotic death upon F16 treatment, is not enough to shift the Bcl-2-overexpressing cells into the apoptotic pathway. It is possible that, in addition to ATP concentration, alterations in mitochondrial Ca²⁺ levels play a key role in the commitment of cells to apoptosis or necrosis, as previously reported (37). Taken together, our results show that the limiting availability of ATP and the excess of ROI are important factors leading to necrotic death of Bcl-2 overexpressing cells in response to F16.

F16 induces necrosis in various cell lines resistant to apoptosis. Next, we examined in other F16-sensitive cell systems the ability of the small molecule to bypass Bcl-2-imposed blockade in the apoptotic cascade, by triggering a necrotic response. For that purpose, we examined the effect of F16 on the Neu-initiated adenocarcinoma cell line SMF and on SMF stably overexpressing Bcl-2 (Fig. 7A). We have previously reported that SMF cells are sensitive to F16 (Ref. 1; Fig. 7B). Similarly to Bcl-2-overexpressing EpH4-A6 cells, Bcl-2-overexpressing SMF cells were resistant to the apoptosis inducer staurosporine, albeit sensitive to F16 (Fig. 7B). Unlike the activation of caspase-3 observed in SMF cells after F16 or staurosporine treatment, no proteolytic processing of caspase-3 was detectable in the SMF cells overexpressing Bcl-2 (Fig. 7C). Moreover, the pan-caspase inhibitor zVAD-fmk could not prevent cell death of Bcl-2-overexpressing SMF cells in response to F16 (data not shown). F16-induced cell death in SMF cells was accompanied by the emergence of a DNA ladder, characteristic of apoptosis (Fig. 7D). In contrast, no oligonucleosomal DNA fragmentation was observed in Bcl-2-overexpressing SMF cells treated with F16. In addition, we included in this analysis a Neu-initiated tumor cell line that is inherently resistant to apoptosis although capable of F16 uptake into mitochondria. Bcl-2 protein levels in NF324-1C are comparable with those observed in other mammary epithelial cells (Fig. 7B). The underlying cause for their apoptosis resistance remains to be determined, however, these cells are sensitive to F16 (Fig. 7B). Like in Bcl-2-overexpressing SMF cells, cell death in NF324-1C in response to F16 occurred in the absence of apoptosis markers (Fig. 7, C and D). These cells experienced progressive cellular swelling (sign of necrosis) upon F16 treatment in a time-dependent manner (data not shown). In all of the cell lines tested, whether apoptosis sensitive or apoptosis resistant, F16 treatment resulted in mitochondrial dysfunction as evidenced by the decrease in the cellular ATP levels (Fig. 7E).

Thus, our data show that resistance to apoptosis achieved by Bcl-2 overexpression in transformed mammary epithelial EpH4-A6 and in adenocarcinoma SMF cells does not compromise the ability of F16 to trigger necrosis in these same cells. The analysis performed in NF324-1C additionally suggests that F16 is capable of triggering necrosis in cells with an impaired apoptosis response resulting from other genetic alterations in addition to Bcl-2 overexpression. Provided that F16 extensively accumulates in mitochondria of treated cells, the damage exerted to this organelle leads to loss of energetic and redox homeostasis and concomitant death in these cells.

Discussion

In the present study, we examined the effect of Bcl-2 overexpression on the response of cells to F16. Previous reports have looked at the cytoprotective role of Bcl-2 against an array of apoptotic inducers in a variety of cell systems. Even though Bcl-2 has been clearly shown...
to inhibit apoptosis, and in a few circumstances necrosis, this protein is not capable of universally preventing cell death (38). Our results showed that stable expression of antiapoptotic Bcl-2 in F16-sensitive cells did not induce resistance to the compound, despite rescuing several other forms of apoptotic cell death. However, unlike the apoptosis triggered by F16 in several transformed cell lines expressing moderate levels of Bcl-2, cells overexpressing the protein died of necrosis upon F16 incubation. This shift in the balance between apoptosis and necrosis by Bcl-2 overexpression upon toxic stimulation has been described previously (37). Indeed, in our case, death of Bcl-2-overexpressing cells was cytochrome c and caspase independent, and hallmarks of apoptosis such as phosphatidyl serine externalization and DNA laddering were absent.

Apoptosis and necrosis are two forms of cell death that exhibit very distinctive biochemical and morphological characteristics. However, these two modalities of cell death, once featured as opposed processes, are currently viewed as “two extremes of a continuum of possible types of cell death” (38, 39). In fact, apoptosis and necrosis can occur simultaneously in cells exposed to the same stimulus. Furthermore, the same stimulus at different concentrations can commit the same cells to either the apoptotic or the necrotic pathway.

During the apoptotic response, a highly orchestrated set of reactions is set into motion to efficiently execute the suicide of a mammalian cell. Several of these regulated cascades converge at the level of mitochondria, leading to mitochondria membrane permeabilization and the release into the cytoplasm of apoptogenetic factors that mediate cell death. Likewise, after toxin exposure, the poorly characterized necrotic response results in mitochondrial PT that translates into the breakdown of ΔΨm. It is now clear that mitochondria play a pivotal role in sensing and integrating signals during both apoptosis and necrosis.

F16, unlike other apoptosis inducers, acts exactly at the level of mitochondria, where both necrotic and apoptotic pathways intersect. F16 mitochondrial accumulation results in membrane depolarization and induction of PT, compromising the functional integrity of an organelle essential for cell survival. It has been proposed that, upon mitochondrial membrane permeabilization, the balance between the rate of the energetic depletion and oxidative burst, and the rate of caspase and nuclease activation determines the commitment of cells to one or the other form of cell death. High-energy equivalents are required for the complex set of catabolic reactions to proceed during the degradation phase of the apoptotic response. In contrast, conditions leading to a decrease in ATP levels result in cellular necrosis. Unlike the observed protective effect of caspase inhibition against F16 cytotoxicity in EpH4-A6 cells, our data indicate that cell death in F16-treated Bcl-2-overexpressing clones derived from these cells is prevented under conditions aimed to maintain a high concentration of ATP and to neutralize superoxide anion. Therefore, our results additionally show that the onset of necrotic death in Bcl-2-overexpressing cells coincides with F16-driven mitochondrial dysfunction.

Finally, it is important that an antiproliferative small molecule can still trigger cell death in a genetically altered environment that can resist apoptosis. Tumor cells are characterized by a high accumulation of genomic mutations (40). Alterations in proteins that are integral to the mitochondrial apoptotic pathway or tumor suppressors that affect the upstream apoptotic cascade are frequently observed in tumors. These types of mutations often render cells resistant to a wide range of antitumor drugs. In those cases, induction of necrosis might provide an alternative mechanism to stop tumor cell proliferation. Some authors have even argued that compounds capable of eliciting necrosis might have a wider spectrum of action because they would not rely on the actual status of genes that control the apoptotic response (41). Despite the crucial role of apoptosis in chemotherapy, necrotic elimination of cells significantly contributes to tumor clearance upon radiation treatment (42). It has also been noticed that in addition to apoptosis, commonly used anticancer drugs trigger necrosis in cancer cells (43).

In comparison with necrosis, apoptosis has been frequently regarded as a preferred and superior mechanism for tumor clearance. It has been argued that because apoptosis proceeds with an almost intact plasma membrane, it prevents the onset of an inflammatory response and favors phagocytic elimination of cells by macrophages. However, these ideas have been challenged by novel findings demonstrating that exposure of macrophages to necrotic dead cells resulted in pronounced stimulation of macrophage antitumor activity. This response might in fact positively contribute to tumor eradication (44). Furthermore, it has been proposed that the antitumor effect brought about by the nonspecific activation of the immune system by necrotic cells might lead to a longer lasting effect than that achieved by apoptotic cells (43–45). Compounds like the mitochondriotoxic molecule F16,
that possess dual ability to induce apoptosis as well as necrosis might present an added advantage for inducing cell death in a diverse array of malignant cells.

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F16, a Mitochondriotoxic Compound, Triggers Apoptosis or Necrosis Depending on the Genetic Background of the Target Carcinoma Cell

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