Caspase Inhibition Blocks Cell Death and Results in Cell Cycle Arrest in Cytokine-deprived Hematopoietic Cells*

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Cytokine deprivation has been classically used to study molecular processes of apoptosis. Following interleukin (IL)-3 withdrawal in FL5.12 cells, Bax undergoes a conformational change that results in its mitochondria targeting, cytochrome c release, activation of caspase-9, and apoptosis. Cells overexpressing Casp9DN (dominant negative caspase-9) or treated with the caspase inhibitor Q-VD-OPh increased viability but failed to increase clonogenic survival. We find that caspase-inhibited cells had a significant fraction of viable cells (herein termed “rescued”) that failed to initiate cell division after IL-3 add back. The “rescued” cells had reduced mitochondrial potential, stained for active Bax, and had reduced staining with dihydroethidium, an agent sensitive to superoxide levels. Readdition of IL-3 after deprivation demonstrated that Bax activation was reversed, whereas altered 5,5‘,6,6‘-tetrachloro-1,1‘,3,3‘-tetraethylbenzimidazolylcarbocyanine iodide and dihydroethidium staining persisted for days. Furthermore, the “rescued” cells were resistant to rotenone, an inhibitor of mitochondrial respiration. The cells were highly sensitive to 2-deoxyglucose, an inhibitor of glycolysis and proposed anti-cancer agent. We conclude that the inhibition of caspase-9 allows cells to retain viability, but cells have prolonged mitochondrial dysfunction and enter a unique nondividing state that shares some properties with malignant cells.

Cytokine withdrawal has been classically used to study molecular processes of apoptosis. FL5.12 cells are a murine pro-B-cell line derived from fetal liver, which undergo apoptotic cell death following IL-3 deprivation (1). During apoptosis, the proapoptotic proteins Bax and Bak undergo a conformational change that ultimately results in outer mitochondria membrane permeabilization (2, 3). Mitochondria factors, such as cytochrome c, Smac, and ARTS, mediate the activation of proapoptotic proteases known as caspases. In this model, caspase-9 is the primary initiator caspase in a cascade that activates a number of effector caspases that cleave numerous downstream substrates that result in apoptosis.

The Bcl-2 family of proteins contain a number of both anti- and proapoptotic factors that can be subclassified based on the presence or absence of Bcl-2 homology domains (BH1 to -4). Antiapoptotic family members, such as Bcl-2 and Bcl-xL, contain all four homology domains, whereas proapoptotic Bax and Bak are multidomain proteins that contain BH domains 1, 2, and 3. Finally, a large number of family members contain limited homology to Bcl-2 (BH3 domain only) and function to promote apoptosis by facilitating the activity of Bax/Bak and/or inhibiting the function of Bcl-2 (4–7). It is well established that members of the proapoptotic, multidomain Bcl-2 family members directly participate in outer member permeabilization (8–10).

After mitochondrial outer membrane permeabilization, intermembrane space proteins, including cytochrome c, are released to activate caspases in the cytoplasm (11). Cell death initiated by caspase activation is considered to be the “classic” apoptotic pathway. In some cases, cell death is effectively blocked by caspase inhibition. However, in many cases, caspase inhibition fails to block cell death, and cells die by what has been called caspase-independent cell death (12, 13). The pathways involved in caspase independent death are not well described but may include apoptosis-inducing factor (AIF), reactive oxygen species, and mitochondria dysfunction. Moreover, the existence of caspase-independent cell death is postulated to explain why Bcl-2 is frequently oncogenic, whereas regulators of caspase activation are less commonly associated with tumor formation.

The activation of Bax through conformational change is one of the earliest steps in the apoptotic pathway in FL5.12 cells. Previous studies demonstrate that Bcl-2 expression acts upstream and prevents this conformational change in Bax (14). However, it is unclear at what point in the apoptotic pathway cells are fully committed to death. This paper addresses this issue by comparing cells where death was blocked at distinct steps in the apoptotic pathway. We found that molecular or chemical inhibition of caspase activity effectively blocks death but fails to enhance clonogenic survival. In this report, we have termed these cells that would otherwise have committed apoptosis “rescued” cells. Evidence is provided that these cells remain metabolically active, have altered mitochondrial properties, and remain in a distinctive, prolonged state of cell cycle arrest.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—FL5.12 cell lines were generated using the SFFV plasmids alone (Neo) or with cDNA from human Bcl-2, Casp9DN, and hemagglutinin-tagged Bax. Plasmids were made and cell lines generated as previously

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medium without IL-3 at 0.2–1 million cells/ml. Thymocytes from normal C57Bl/6 mice were isolated and cultured as previously described (16).

**Cell Cycle Analysis**—For DNA content analysis, cells were stained with Krishan propidium iodide buffer as previously described (16, 17). For BrdUrd uptake experiments, cells were deprived of IL-3 for 24 h; subsequently, IL-3 was added back, and cells were continuously exposed to BrdUrd for the next 48 h by adding 10 μM BrdUrd to culture medium at 0, 24, and 47.5 h after IL-3 readdition. Following 48 h of BrdUrd exposure, cells were pelleted and fixed with ice-cold 70% EtOH and stored at −20 °C prior to staining with anti-BrdUrd and PI as previously described (18). Viable/nondoublet cells were analyzed based on FL2 area versus FL2 width dot plots. For CFSE staining, cells were deprived of IL-3 for 24 h at 500,000 cells/ml, pelleted, and resuspended in PBS, 0.1% bovine serum albumin at 1 million cells/ml and exposed to 10 μM CFSE for 10 min at 37 °C. The reaction was then stopped by the addition of 5 volumes of ice cold culture medium. Cells were washed three times in culture medium and initially seeded at 250,000 cells/ml. Cell division was followed by visualizing relative CFSE staining in the FL1 channel of a flow cytometer. For simultaneous staining with DHE, compensation for CFSE detection in the FL2 channel was adjusted, utilizing cells that were stained with CFSE but not DHE.

**Cell Death Assays**—A Guava PCA cell analyzer (Guava Technologies Inc.) was used to assess cell viability. In brief, 10 μl of cells was added to 50 μl of Guava ViaCount reagent and allowed to stain for 5–20 min. Just prior to placement in the instrument, the cells were diluted with 40 μl of PBS and mixed, and the data were acquired. Experiments were performed in duplicate or triplicate, and the data represent the mean ± S.D. for each sample. For annexin V staining, cells were pelleted and resuspended in annexin V binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, pH 7.4) containing annexin V-fluorescein isothiocyanate (1:1000) and PI at 1 μg/ml. Cells were incubated for 5 min at room temperature and analyzed on a FACSCalibur flow cytometer. A DEVDase activity assay was performed as previously described (19).

**Flow Cytometry Assays**—For JC-1 staining, cells at 500,000–1,000,000 cells/ml were treated with 10 μg/ml JC-1 for 10 min at 37 °C in medium. The cells were then placed on ice until run on a flow cytometer. JC-1 staining was visualized simultaneously in the FL1 and FL2 channels of a flow cytometer. Compensation was adjusted so that the optimal separation of the viable and dead cells was obtained. To simultaneously stain for JC-1 and annexin V-Cy5, JC-1 stained samples were pelleted again and resuspended in annexin V binding buffer with annexin V-Cy5 (1:1000). Annexin V-negative (live) gated cells were then examined for JC-1 staining using a FACSCalibur flow cytometer. For DHE staining, cells were pelleted and resuspended in PBS containing 5 mM pyruvate and 10 μM DHE. Cells were then incubated for 15–20 min at 37 °C. DHE-stained cells were pelleted and stained with annexin V-Cy5 as described above. Cells were covered and kept on ice until analyzed on a FACSCalibur flow cytometer. Only viable cells (annexin V-negative) were analyzed for DHE fluorescence (FL2).
Caspase-independent Cell Cycle Arrest

**PROTOCOLS**

**RESULTS**

**DISCUSSION**

**FIGURE 1.** Protein levels, caspase activity, and cell death in FL5 cell lines. A, whole cell lysates were prepared from cells as described under “Experimental Procedures.” Equivalent protein levels were loaded (30 μg), and proteins were detected with a Western blot utilizing hemagglutinin (HA) epitope tag (12CA5), murine caspase-9, and human Bcl-2 (6C8) antibodies. Ponceau 5 staining indicated equivalent loading (data not shown). B, FL5.12 cells as indicated were deprived of IL-3, and cell death was measured at the indicated times utilizing Guava ViaCount reagent. FL5-Neo cells were treated with 50 μM Q-VD-OPh as indicated. C, FL5-Neo cells were deprived of IL-3 in the presence of 50 μM Q-VD-OPh or 5 mM 3-methyladenine (3-MA) as indicated. 48 h after deprivation, viability was determined in duplicate by Guava, and the means ± S.D. are shown. The experiment is representative of three independent experiments. D, FL5.12 cells of the indicated genotype were analyzed for annexin V viability (hatched bar) after 24-h IL-3 deprivation. In addition, clonogenic survival was measured as described under “Experimental Procedures” by the addition of IL-3 after 24 h of deprivation (filled bars). Clonogenic survival was normalized to control plates that had not been deprived of IL-3.

**Activated BAX Staining by Flow Cytometry**—Activated BAX staining was performed as previously described (15, 20–22). In brief, 1–2 × 10⁶ FL5.12 cells were pelleted and fixed in 0.25% paraformaldehyde for 5 min at room temperature. Fixed cells were washed with PBS and resuspended in 100 μl of blocking buffer (PBS with 0.01% Digitonin, 5 μg/ml anti-FC, and 10% normal rat serum). The sample was split in half and stained with anti-BAX antibody (clone 6A7; eBioscience) or isotype control (clone P3; eBioscience) at 2.5 μg/sample. Cells were stained for 30 min at room temperature, washed, and stained with 1 μg/100 μl fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody for 30 min. The cells were washed with PBS, 0.01% digitonin prior to staining for DNA content with PI and RNase as previously described (21). Cells were acquired and analyzed on a FACSCalibur flow cytometer. Doublets and subdiploid cells were excluded in analysis. Under all conditions, gates were adjusted so that less than 1% of the cells stained positive with the isotype control antibody.

**Clonogenic Survival Assay**—Clonogenic survival of FL5-Neo, FL5-Bcl-2, FL5-Bax, and FL5-Casp9DN cells following 24 h of IL-3 deprivation was performed by plating cells by limiting dilution into 96-well plates. Cells were spun down, washed with PBS, and resuspended in culture medium lacking IL-3. Cells were counted with the Guava PCA system and then plated out at the limiting dilution in 96-well round bottom plates. An equal volume of medium containing IL-3 was added either immediately at plating or 24 h later. The number of positive wells was determined after 10–14 days of growth. The number of colony-forming units/well was corrected using Poisson statistics with the following formula: colony-forming units/well = −ln (percentage of negative wells). For each experiment, samples were performed in triplicate, and the data were normalized to the clonogenic survival of the untreated cells.

**Oxygen Consumption Studies**—Oxygen consumption analysis was determined 20 h after IL-3 deprivation as previously described (23). In brief, cells were grown to a density of 600–800,000 cells/ml. Cells were counted and assayed for viability with ViaCount reagent and a Guava instrument. Approximately 10 × 10⁶ cells were harvested, spun down at −350 × g, and suspended in 3 ml of RPMI 1640 medium at 37 °C. Cells were then placed in a YSI oxygen chamber and stirred for 3 min at 37 °C to equilibrate oxygen pressure and temperature. Following the initial equilibration, a Clark-type YSI oxygen electrode, model 5331, was placed in the chamber and again equilibrated for 2 min. Oxygen consumption was followed for 15 min and was normalized to the number of viable cells in the chamber.

**RESULTS**

**Cell Death following IL-3 Deprivation**—Four cell lines (FL5-Bax, FL5-Neo, FL5-Bcl-2, and FL5-Casp9DN) were generated that express one of three proteins: Bax, Bcl-2, or caspase-9 dominant negative. The control cells (FL5-Neo) were transfected with a blank SFFV-Neo vector. Protein expression by Western blot confirmed expression of these proteins (Fig. 1A). Caspase activity levels in these cell lines following IL-3 deprivation were examined. As expected, cells overexpressing the antiapoptotic proteins Bcl-2 or Casp9DN showed reduced levels of caspase activity as compared with Neo cells or cells overexpressing the proapoptotic protein Bax (data not shown).
The pro- or ant apoptotic effects of the overexpressed proteins were observed by withdrawing IL-3 from the culture medium and observing cell viability by dye exclusion over time. Additionally, cell death following treatment with the caspase inhibitor Q-VD-OPh was also examined. As previously described, the antiapoptotic protein Bcl-2, which blocks apoptosis at a point upstream of Bax activation, provides long-term protection from cell death. FL5-Neo cells and FL5-Bax cells show rapid death by apoptosis, with FL5-Bax cells dying the quickest. Interestingly, cells that have the caspase cascade blocked through the addition of Q-VD-OPh or overexpression of Casp9DN show excellent protection from death at early time points, but viability significantly declines after about 24 h of IL-3 deprivation (Fig. 1B). During the course of the experiment, FL5-Bcl-2 cells retained very high viability, whereas FL5-Casp9DN or Q-VD-OPh-treated cell viability progressively decreased.

Autophagy Inhibitor Enhances Q-VD-OPh Protection—The progressive decrease in cell death in the FL5-Casp9DN cells with deprivation prompted us to examine alternative cell death pathways in these cells. Given that autophagy can be activated following IL-3 deprivation (24), the effect of the autophagy inhibitor 3-methyladenine was examined. In the absence of Q-VD-OPh, 3-methyladenine increased the level of apoptosis seen with IL-3 deprivation. In contrast, with Q-VD-OPh, there was a small but significant increase in viability of the IL-3 deprived cells (Fig. 1C). These data suggest that autophagy may mediate cell death of IL-3-deprived FL5 cells when caspases are blocked.

Caspase Inhibition Fails to Enhance Clonogenic Survival—One of the early changes during apoptosis is loss of plasma membrane asymmetry that exposes phosphatidylserine on the cell surface. This process precedes loss of plasma membrane integrity and can be detected by annexin V binding. Annexin V viability analysis after 24 h of IL-3 deprivation confirmed that caspase inhibition effectively blocked phosphatidylserine externalization (Fig. 1D). To determine if caspase inhibition acted upstream of the cell death commitment point, clonogenic survival was examined by limiting dilution. Replicate 96-well plates were prepared, and IL-3 was either added immediately or 24 h later. Clonogenic survival of cells that had been deprived of IL-3 for 24 h were normalized to undeprived cells. As expected, Bax decreased and Bcl-2 dramatically increased clonogenic survival relative to FL5-Neo cells (Fig. 1D). Surprisingly, caspase inhibition provided no significant increase in clonogenic survival relative to the control cells. Clearly, these results indicate a discrepancy in FL5-Casp9DN cells between early protection from apoptosis and observed ability to undergo cell division when IL-3 is added back following deprivation. One simple explanation for these results may be that the cells are committed to death by a caspase-independent pathway and proceed to die despite the addition of IL-3. If so, one would expect the FL5-Casp9DN cell viability to decrease despite the addition of IL-3. In contrast to this prediction, no significant decrease in cell viability is observed in FL5-Casp9DN cells following addition of IL-3 (data not shown).

Caspase Cascade Blockage Results in Cell Cycle Arrest—The discrepancy between 24-h viability and clonogenic capacity prompted us to look at cell cycle in FL5-Neo and FL5-Bcl-2 cells (with or without Q-VD-OPh) following 30 h of IL-3 deprivation and then 17 h after IL-3 had been added back. As expected, after 30 h of IL-3 deprivation, Q-VD-OPh treatment decreased the percentage of cells with subdiploid DNA content (data not shown). Q-VD-OPh had no effect on cell death or cell cycle arrest in FL5-Bcl-2 cells. 17 h after IL-3 was added back to these cultures, the fraction of viable cells that had entered cell division (S, G2, or M) was reduced in the FL5-Neo cells that had been deprived of IL-3 in the presence of Q-VD-OPh (35.6% versus 67.9%). In contrast, Q-VD-OPh had no effect on cell cycle in IL-3-deprived FL5-Bcl-2 cells (Fig. 2A). This finding suggested that FL5-neo but not FL5-Bcl-2 cells treated with Q-VD-OPh during IL-3 deprivation contained a population of viable cells that were arrested in G0/G1 phase of the cell cycle. Consistent with this interpretation, FL5-Neo but not FL5-Bcl-2 cells had a prolonged doubling time following IL-3 deprivation in the presence of Q-VD-OPh (data not shown). Herein, we will refer to the cells that are arrested by caspase inhibition as the “rescued” cells or population. In the experiments described here, they represent the fraction of cells (~20–40% after 24 h of IL-3 deprivation) that would have died if caspase activity were not inhibited.

The ability of these “rescued” cells to enter the S phase of the cell cycle was further tested by analyzing cell division with the BrdUrd uptake assay. Following IL-3 deprivation, IL-3 is added back in the continuous presence of BrdUrd. 48 h later, cells were harvested and stained for BrdUrd. In the cultures where caspase activity was not inhibited, nearly all of the viable cells (~2N DNA content) were BrdUrd-positive. However, in FL5-Casp9DN or Q-VD-OPh-treated FL5-Neo cells, a significant fraction of the viable cells were BrdUrd-negative at this time point (Fig. 2B). Of note, BrdUrd uptake was >99% in FL5-Bcl-2 cells, and therefore the results are not a byproduct of general apoptotic inhibition (data not shown). Of note, the percentage of “rescued” (BrdUrd-negative) cells decreased 48 h after IL-3 add back due to the high proliferation rate and rapid doubling time (~13 h) of the cells that are clonogenically competent.

Additional evidence that these “rescued” cells are nondividing was demonstrated with the CFSE assay. CFSE is a stable, cell-permeable dye that is a simple, effective indicator of cell division because it provides uniform fluorescence that is diminished as the cells undergo cell division. Cells were again deprived of IL-3 for 24 h and stained with CFSE prior to the readdition of IL-3. CFSE intensity of the viable cells was monitored over the next several days. Consistent with the BrdUrd results, a population of live, CFSE-bright cells was identified in the FL5-Casp9DN cells or FL5-Neo cells deprived with Q-VD-OPh but not in control cells (data not shown). The results from PI DNA content analysis, BrdUrd uptake, and CFSE intensity experiments all demonstrate that the “rescued” cells are unable to initiate cell division for at least 3–4 days following the readdition of IL-3.

“Rescued” Cells Have Altered Mitochondrial Membrane Potential—Since caspase-9 is activated downstream of outer mitochondria membrane permeabilization, the relative effect of Bcl-2 and caspase inhibition on mitochondria membrane potential was examined. Following IL-3 deprivation, the viable cells from FL5-Bcl-2, FL5-Neo, or FL5-Casp9DN cultures showed...
no differences in staining using 3,3'-dihexyloxacarbocyanine iodide (data not shown). However, distinct differences were observed when the cells were stained with JC-1. JC-1 is a lipophilic dye that is used to measure mitochondrial membrane potential. Recent studies suggest that JC-1 may be the most sensitive indicator of mitochondria membrane potential (25). At high concentrations within the mitochondria, JC-1 forms aggregates and fluoresces brightly in the FL2 channel (580 nm). Monomers of JC-1 are detected predominantly in the FL1 channel (530 nm). JC-1 uptake into mitochondria is dependent on potential, and with decreasing membrane potential the cells demonstrate decreased FL2 fluorescence and increased FL1 fluorescence (26, 27). Cells were stained simultaneously with annexin V-Cy5 and JC-1, and the JC-1 staining of viable (annexin V-negative) cells was examined. Following IL-3 deprivation, only FL5-Casp9DN cells had a viable population with significantly reduced FL2 fluorescence and increased FL1 fluorescence (Fig. 3A).

To demonstrate that the effects of caspase inhibition on viability and mitochondria potential was not unique to FL5.12 cells, Q-VD-OPh significantly protected primary thymocytes from both spontaneous cell death (78.5% versus 47.8%) and death following dexamethasone (33.5% versus <1%). In both spontaneous death and dexamethasone death, Q-VD-OPh significantly increased the number of viable cells with altered JC-1 staining (Fig. 3B).

Of note, in the dexamethasone-treated cells, 95% of the annexin V-negative cells had reduced membrane potential with JC-1. This demonstrates that in normal thymocytes, Q-VD-OPh maintains cell viability but does not block the change in JC-1 staining.

High Bax "Activation" in FL5-Casp9DN Cells Is Reversible—During apoptosis, Bax undergoes a conformational change that exposes the N terminus and results in the redistribution from the cytosol to the mitochondria (28). The conformation change in Bax is detected by antibodies that recognize the N-terminal domain (29, 30). Following IL-3 deprivation...
tion, immunoreactive, N-terminal Bax is detected in FL5-Neo and at even higher levels in FL5-Casp9DN cells (Fig. 4, A and B). Again, only viable cells (≥2N DNA content) were analyzed.

Negative controls included isotype control, cells not deprived of IL-3, and IL-3-deprived FL5-Bcl-2 cells that demonstrated little or no staining (Fig. 4A, data not shown). Similarly, examination of cytochrome c (31) release demonstrated a large fraction of cells with cytoplasmic cytochrome c (data not shown). Of note, the percentage of viable cells with active Bax appears to be higher than the percentage of cells with reduced membrane potential. This suggests that Bax activation precedes the loss of mitochondrial membrane potential (Fig. 4, B and C). Interestingly, 9 h following the readdition of IL-3, the percentage of cells that stained with the N-terminal antibody against Bax was dramatically reduced (Fig. 4B). During this time, only a small increase (3–5%) in total cell death was observed by DNA content analysis or dye exclusion analysis (data not shown). This demonstrates that a large fraction of the "rescued" cells remain metabolically active and able to either degrade or reverse the activation of Bax following the read- addition of IL-3.

Altered Mitochondria Potential Persists following IL-3 Readdition—Since N-terminal Bax staining was lost following the read addition of IL-3, the effect of IL-3 readdition on JC-1 staining was determined. Surprising-ly, 9 h after the addition of IL-3, the percentage of cells with altered JC-1 staining was not significantly affected (Fig. 4C). In fact, a viable population with altered membrane potential persisted up to 52 h after IL-3 was added back (Fig. 5). Of note, back-gating on the cells with reduced JC-1 staining demonstrates that this population of cells showed increased forward scatter following the readaddition of IL-3 (Fig. 5). This demonstrated that these "rescued" cells were metabolically active and persisted for at least 2 days following IL-3 addition.

Reduced DHE Staining of “Res- cued” Cells—Mitochondria are a major source of oxidants during normal respiration and cell death. Therefore, the oxidation status of the IL-3-deprived cells was examined by staining cells with either DHE or DCF. DCF is
relatively selective for peroxide, whereas DHE is oxidized by superoxide. As for the previous experiments, cells were simultaneously stained with annexin V-Cy5, and only viable cells were analyzed. No difference in DCF fluorescence was observed between FL5-Neo, FL5-Bax, FL5-Bcl-2, and FL5-Casp9DN cells following IL-3 deprivation (data not shown). Surprisingly, DHE staining of FL5-Casp9DN cells following IL-3 deprivation showed a population of viable cells with markedly reduced DHE fluorescence (Fig. 6A, bottom). DHE-low cells were not detected in the FL5-Neo, FL5-Bax, or FL5-Bcl-2 cells following IL-3 deprivation (Fig. 6A, bottom) or in any of the cells in the presence of continuous IL-3 (Fig. 6A, top). As a positive control, DHE fluorescence increased following treatment of the cells with antimycin A, an inhibitor of mitochondrial respiration (data not shown). For the FL5-Casp9DN cells, 20–30% of the viable cells demonstrated reduced DHE fluorescent staining. The fraction of DHE-low cells tightly correlated with the fraction of cells with altered JC-1 staining and high “active” Bax reactivity. We conclude that the “rescued” cells have markedly reduced DHE fluorescence.

**Nondividing Cells Are DHE-low**—The studies described so far demonstrate that inhibiting caspases during IL-3 deprivation results in altered mitochondrial membrane potential, DHE staining, and altered cell cycle properties. However, the spectral properties or staining requirements of these methods preclude simultaneous staining to demonstrate that these properties occur within the same cells. Since CFSE staining has minimal overlap with DHE, we examined the DHE staining in the “rescued” cells that had been stained with CFSE prior to the readdition of IL-3. Comparing DHE staining with CFSE 3 days after the addition of IL-3, it is clear that the CFSE-bright cells stain low for DHE (Fig. 6B). These results confirm that the DHE-low cells are the nondividing cells.

**“Rescued” Cells Survive for at Least 9 Days**—In order to study the “rescued” cells beyond 2–3 days, FL5-Neo cells were deprived of IL-3 in the presence of Q-VD-OPh for 40 h. Control cells were maintained in IL-3 medium in the presence or absence of Q-VD-OPh. All cells were washed to remove Q-VD-OPh and returned to fresh IL-3-containing medium. Cells were then treated with colcemid, a mitotic inhibitor that kills dividing cells. Under these conditions, the cells that had been deprived of IL-3 in the presence of Q-VD-OPh were remarkably resistant to colcemid, whereas the undeprived cells (with or without Q-VD-OPh) were very sensitive. Of note, a significant fraction of the “rescued” cells remained viable for at least 9 days following IL-3 add back (Fig. 7A). Given that >80% of the viable cells and ~60% of the total cells were DHE-low after 40 h of deprivation, it is clear that not all of the “rescued” cells survive for this duration, since overall survival is only ~20% after 9 days. Nonetheless, the ability of a fraction of the “rescued” cells to survive in
the presence of colcemid for 9 days even after the Q-VD-OPh has been effectively removed is remarkable. Washing effectively removed the Q-VD-OPh, since the undeprived cells maintained in Q-VD-OPh were not protected from colcemid. Strikingly, beyond 1 day in culture with colcemid, nearly all of the viable cells demonstrated reduced DHE staining, indicating survival of only the “rescued” cells (Fig. 7B). Of note, the addition of IL-3 to the deprived FL5 cells results in increased DHE staining of the DHE-high cells with no change in the staining of DHE-low cells (Fig. 7B, compare day 0 with day 1). Given the stimulation of metabolic activity by IL-3 (32), this suggests that DHE is a sensitive marker of mitochondria respiration.

Superoxide is normally produced as a result of mitochondria respiration. The reduced DHE staining in IL-3-deprived FL5-Casp9DN cells prompted us to examine oxygen consumption in FL5 cells following IL-3 deprivation. As expected, oxygen consumption was markedly reduced in all cells following IL-3 deprivation, with FL5-Bax cells showing the lowest oxygen consumption and FL5-Bcl-2 the highest (data not shown). However, despite near equivalent viability, the rate of oxygen consumption per cell in the FL5-Casp9DN cells (0.40 ± 0.072 fmol of O₂/min) was markedly lower than in FL5-Bcl-2 cells (0.65 ± 0.066 fmol of O₂/min).

Cell Death Sensitivity of “Rescued” Cells—The cellular ATP required for survival is derived from two major sources, aerobic mitochondria respiration and anaerobic glycolysis. Rotenone is an inhibitor of Complex I and therefore potently blocks mitochondria respiration. 2-Deoxyglucose (2-DG) is a glucose analog and competitive inhibitor of glucose uptake and utilization. It therefore acts as a potent competitive inhibitor of glycolysis. The sensitivity of the “rescued” cells to these two agents was determined. Following IL-3 deprivation (40 h) and add back (8 h), cells were treated with the drugs, and viability was measured 15 h after the initiation of drug treatment. Under these conditions, FL5-Neo cells in continuous IL-3 are sensitive to rotenone but relatively resistant to 2-DG. In contrast, the cells deprived of IL-3 in the presence of Q-VD-OPh were resistant to rotenone and sensitive to 2-DG (Fig. 8A).

DHE staining of these cultures was examined under each of these conditions. As expected, FL5-Neo cells that had not been deprived of IL-3 or had been deprived of IL-3 without Q-VD-OPh did not contain a significant fraction of viable (annexin V-negative) cells with DHE-low staining (see Fig. 6A and data not shown). However, the cells that had been deprived of IL-3 in the presence of Q-VD-OPh prior to the readdition of IL-3 contained a large fraction viable cells that stained DHE-low (Fig. 8B). With 40 h of IL-3 deprivation, the DHE-low population represents ~75% of the viable cells (Fig. 8B). Rotenone treatment for 15 h markedly reduced the proportion of DHE bright cells, whereas two different concentrations of 2-DG selectively reduced the DHE-low cells and therefore enriched for the DHE bright cells (Fig. 8, B and C).

**DISCUSSION**

FL5.12 cells are an immortalized cell line derived from normal mouse fetal liver that rapidly undergo apoptosis upon withdrawal of IL-3. These cells have been used extensively to study various apoptotic pathways and allow one to look at the reversibility of cell death by the readdition of IL-3. In this study, our major aim was to compare cell death blockade by Bcl-2 with either molecular (Casp9DN) or chemical (Q-VD-OPh) inhibition of caspase activation. Numerous other studies have examined the effects of caspase inhibition...
on cell death in response to other stimuli. Following Fas treatment, caspase inhibitors effectively and completely block cell death (33, 34). Other studies failed to demonstrate that caspase inhibitors block cell death and show that cell death occurs by a caspase-independent mechanisms (12, 13, 35). For example, caspase inhibitors effectively block DNA degradation but not cell death in a model of inducible Bax expression (36). In UVB-treated Jurkat cells, caspase independent cell death is proposed to involve AIF and/or reactive oxygen species (37). These studies suggest that cell death still proceeds in a caspase-independent manner but may involve distinct morphology and molecular pathways. The in vivo importance of the caspase-independent cell death pathway is not clear, but it has been suggested to occur during digit formation (38).

In the studies described here, we have demonstrated that caspase inhibition effectively blocks cell death in IL-3-deprived FL5.12 cells. However, caspase inhibition had little or no effect on clonogenic survival. Initially, we suspected a caspase-independent cell death pathway was engaged but was kinetically delayed relative to caspase-dependent cell death. However, upon the readdition of IL-3, we were unable to demonstrate an increase in cell death in the caspase-inhibited “rescued” cells. Instead, we provide evidence that caspase inhibition blocks death but results in cells with prolonged cell cycle arrest.

In these studies, 24 h after IL-3 deprivation, at least three populations of cells exist in the Q-VD-OPh-treated or the FL5-Casp9DN cells. Dead cells are positive for annexin V staining, whereas annexin V-negative “viable” cells can be further classified into two subsets. Cells with normal JC-1 staining retain the ability to proliferate and stain brightly for DHE. The unique population described here are the annexin V-negative cells that are unable to initiate proliferation. We have termed this third population the “rescued” cells, since they represent the cells that would normally have died without caspase inhibition. Culturing these cells with colcemid, we demonstrate that at least some of these “rescued” cells survive for up to 9 days following IL-3 add back.
Subsequent studies focused on the characterization of these “rescued” cells. Examination of these cells after staining with JC-1 demonstrated that these “rescued” cells have altered staining properties consistent with reduced mitochondrial membrane potential. The likely cause of this mitochondrial dysfunction is the high level of “active” Bax that was detected in these rescued cells with caspase inhibition. Therefore, although caspase cascade blockage prevents rapid cell death, it allows for substantial amounts of “active” Bax. We propose that the “active” Bax results in outer mitochondrial membrane permeabilization and altered mitochondrial membrane potential. This is in agreement with studies in actinomycin D-treated HeLa cells or mouse embryonic fibroblasts (39). In addition, DHE staining reveals that the “rescued” cells have markedly reduced fluorescence. This was only observed when cell death was blocked by caspase inhibition and not by Bcl-2. We propose that the reduced DHE staining is indicative of decreased formation of reactive oxygen species due to decreased mitochondrial respiration. In summary, “rescued” cells have high levels of “active” Bax, altered JC-1 staining indicative of decreased mitochondrial membrane potential, and reduced DHE staining consistent with altered respiration.

One of the striking findings in the current studies is the rapid “reversibility” of Bax activation upon IL-3 add back. We propose that whereas this apoptotic event is reversible, the effects on mitochondria function are long lasting and perhaps irreversible. As mentioned previously, the cells have a persistent, measurable alteration of JC-1 staining indicative of reduced mitochondrial membrane potential. Also, there is persistent reduction in DHE staining consistent with reduced superoxide generation. These two pieces of data prompted us to examine the effects of inhibitors of glycolysis and of mitochondria respiration on the “rescued” cells. The “rescued” cells were highly sensitive to 2-DG while remarkably resistant to rotenone, a potent inhibitor of mitochondrial respiration. We conclude that “rescued” cells have decreased mitochondrial respiration and are generating energy by glycolysis.

One recent report examined cell death in bone marrow-derived mast cells from Apaf1 or caspase-9-deficient mice (40). Consistent with our data, cell death following cytokine deprivation is dependent on both of these components of the apoptosome. Also, they find that cytokine-deprived Mast cells from Apaf1 or caspase-9-deficient mice are defective in cell proliferation when cytokines are added back to the culture medium. However, following readdition of cytokines, these cells were unable to express surface markers of activation. Marsden et al.
(40) propose that mitochondria dysfunction may explain the inability to proliferate but do not provide experimental evidence for this. The data described here extend these findings to FL5.12 cells and identify specific differences in mitochondria and in glycolytic metabolism that are tightly associated with the defect in proliferation. Furthermore, we find that “rescued” cells are metabolically active based on the loss of staining for “active” Bax and the increase in cell size following IL-3 readdition.

Through these studies, we have identified a unique, viable, nondividing population of cells that have altered proliferation and metabolism. This represents the first biochemical characterization of this unique form of cell cycle arrest. Recently, caspase inhibitors have been proposed as possible therapeutic agents. Proposals include prevention of cell death following ischemic injury and in neurodegenerative disorders. Identification of cells that remain viable but have altered mitochondrial function suggests that promotion of cell viability by caspase inhibition may not be sufficient to assure normal cellular function. Markers of cell or tissue function may be required to confirm the efficacy of caspase inhibitors in the treatment of disease.

FL5.12 cells are an immortalized but not transformed cell line. Caspase inhibition during IL-3 deprivation results in a marked alteration in cell metabolism. It has not escaped our attention that similar changes in metabolism are observed during transformation (41). In this old but persistent metabolic theory of cancer, transformation involves an increased dependence on glycolysis. In fact, recent studies demonstrate that transformed cells are particularly sensitive to glucose deprivation or to 2-DG (42). Further support for this theory comes from studies demonstrating that genetic defects in respiratory mitochondrial proteins cause cancer (43). The studies here do not directly address the impact of caspase inhibition on transformation. However, Casp9DN

FIGURE 8. “Rescued” cells are sensitive to 2-DG and resistant to rotenone treatment. A, FL5-Neo were prepared in three ways: 1) no IL-3 deprivation; 2) 40-h IL-3 deprivation without Q-Vd-Oph; 3) 40-h IL-3 deprivation with Q-Vd-Oph. IL-3 was added back to the culture for 8 h prior to the initiation of drug treatment. Cells were treated with either 50 μM rotenone (Rot) or 20 mM 2-DG. Viability was determined with Guava ViaCount reagent 16 h after treatment. The viability was normalized to the viability at the time of the initiation of drug treatment. For B and C, only the cells that had been deprived of IL-3 in the presence of Q-Vd-Oph are shown. A significant fraction of DHE-low cells was not observed in any of the other conditions. B, these samples were stained with DHE and annexin V-Cy5, and the DHE staining of the viable/annexin V-negative cells is shown. Data are representative of duplicate samples. C, the percentage of viable cells that stained DHE bright is shown (mean ± S.D.) for each treatment group.
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