bcl-2 Gene Transfer Increases Relative Resistance of S49.1 and WEHI7.2 Lymphoid Cells to Cell Death and DNA Fragmentation Induced by Glucocorticoids and Multiple Chemotherapeutic Drugs

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Introduction

The bcl-2 gene becomes transcriptionally deregulated in many non-Hodgkin’s B-cell lymphomas as a result of chromosomal translocations that bring this gene into juxtaposition with immunoglobulin genes (1). Previous studies have demonstrated that bcl-2 encodes an integral membrane protein that resides in mitochondria and possibly other intracellular compartments, and that appears to contribute to neoplastic cell expansion primarily by promoting cell survival, rather than by stimulating cellular proliferation (2–5). The mechanism by which bcl-2 enhances cell survival is probably through its ability to interfere with a process known as PCD or “apoptosis.” PCD is an active form of cellular suicide that typically requires ATP, involves new RNA and protein synthesis, and that culminates in the activation of endogenous endonucleases that degrade the DNA of the cell, thereby destroying the genetic template required for cellular homeostasis (6). Loss of plasma membrane integrity is a relatively late event in PCD, unlike the form of cell death termed “necrosis,” which can be caused by hypoxia and exposure to certain toxins and which typically is characterized early-on by increased membrane permeability, cell swelling, and rupture.

PCD is thought to play an important role in several physiological situations including the death of immature thymocytes during immune cell education (reviewed in Ref. 7). Previous studies have demonstrated that the cortical population of immature thymocytes is highly sensitive to induction of apoptosis by glucocorticoids, whereas the more mature medullary cells are resistant (8). Interestingly, immunohistochemical studies of the patterns of Bcl-2 protein production in the thymus have revealed bcl-2 protooncogene expression in the medulla but not the cortex of the thymus, consistent with the possibility that Bcl-2 might regulate the sensitivity of thymocytes to steroids (9, 10). Recently, we and others have created transgenic mice that produce high levels of human p26-Bcl-2 protein in the thymus, and have confirmed the ability of this protein to render immature thymocytes markedly resistant to glucocorticoids both in vivo and in vitro (11–13). Enforced expression of bcl-2 also increased resistance to other treatments that have been reported to induce apoptosis of thymocytes including γ-irradiation, and several reagents that either trigger the T-cell receptor complex on thymocytes or that emulate signals generated by T-cell receptor stimulation (anti-CD3 antibodies, IONO, TPA).

S49.1 and WEHI7.2 are T-lymphoid cell clones established from a murine lymphoma and thymoma, respectively, that are highly sensitive to killing by glucocorticosteroids (14, 15). Because these cells have been utilized extensively as clonal models for studies of steroid-induced apoptosis, they provided an opportunity to further explore the functions of p26-Bcl-2. Here we show that enforced expression of bcl-2 as a result of gene transfer manipulations renders S49.1 and WEHI7.2 cells markedly resistant to killing not only by the glucocorticoid DEX, but also to a wide variety of drugs that are known to induce apoptosis (16, 17) and that are commonly used in the treatment of cancer.

Materials and Methods

Cell Viability, Proliferation, and DNA Fragmentation Assays. For cell viability studies, S49.1 and WEHI7.2 cells were resuspended at 2.5 × 10^5/ml in RPMI or Dulbecco’s modified Eagle’s medium “complete medium,” respectively [RPMI-1640 or Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 0.1 mg/ml each of kanamycin, neomycin, and streptomycin], with various drugs. At various times after initiation of cultures in a humidified atmosphere of 5% CO₂/95% air at 37°C, cells were mixed with an equal volume of phosphate-buffered saline containing 0.4% trypan blue dye and were manually counted with the use of a hemocytometer.
For DNA fragmentation assays, cells were recovered from cultures after 1 day and 10⁶ cells (total live and dead) were analyzed by the gel electrophoresis method of Sorenson et al. (18).

Relative levels of DNA synthesis were measured in S49.1 and WEHI7.2 cells by culturing 6 × 10³ cells/well in 0.1 ml of complete medium with or without 10⁻⁷ M DEX in 96-well flat-bottomed plates (Falcon). After 16 h, 0.5 µCi of [³H]thymidine (6.7 Ci/mmol; New England Nuclear) was delivered to each microwell and the cells were lysed onto glass filters 8 h later for subsequent scintillation counting.

Recombinant Retrovirus Production and Infection of S49.1 and WEHI7.2 Cells. A bcl-2 expression plasmid was prepared by subcloning a 910-base pair EcoRI complementary DNA from the plasmid pB4 (19) into the retroviral expression plasmid pZip-NEO (20). The resulting recombinant plasmid pZip-BCL2 and the parental vector pZip-NEO were packaged as infectious amphotrophic retroviruses by the method of Miller et al. (21), using PA317 cells (22). Clones of PA317 cells exhibiting the highest titer virus production were then adapted to growth in RPMI complete medium and were subsequently used for infecting S49.1 and WEHI7.2 cells by coculturing for 2 weeks. Retrovirus-infected lymphoid cells were selected by growth for 3 weeks in medium containing ~750 µg/ml G418 (Gibco/BRL).

Immunoblot Assays. Expression of the bcl-2 virus in infected S49.1 and WEHI7.2 cells was confirmed by immunoblotting with the use of rabbit antisera specific for the human M, 26,000 bcl-2 protein, as described in detail elsewhere (23). Protein-containing cell lysates were normalized for protein content and 100 µg/lane were size fractionated in 12% sodium dodecyl sulfate-polyacrylamide gels prior to transfer to nitrocellulose filters. Rabbit antisera specific for the M, 95,000 glucocorticoid receptor (24) were obtained from Dr. J. Cidlowski (University of North Carolina) and were used at 1:250 (v/v) followed by 0.25 µCi/ml ¹²⁵I-protein A (30 mCi/mg; Amersham). Portions of the blots were stained with Ponceau-S to verify loading and transfer of equivalent amounts of intact protein for all samples.

Results

S49.1 and WEHI7.2 cells were stably infected with recombinant amphotrophic retroviruses carrying either a G418 antibiotic resistance gene (NEO) alone or in combination with a bcl-2 complementary DNA, thus establishing the cell lines S49.1-NEO, WEHI-NEO, S49.1-BCL2, and WEHI-BCL2. Immunoblot analysis of G418-resistant S49.1-BCL2, S49.1-NEO, WEHI-BCL2, and WEHI-NEO cells using highly specific anti-human Bcl-2 antibodies revealed levels of M, 26,000 Bcl-2 protein in S49.1-BCL2 and WEHI-BCL2 cells (Fig. 1, A and B) comparable to those found in a human lymphoma cell line (RSI1846) that contains a t(14;18) that deregulates bcl-2.

These immunoblot results were obtained with the polyclonal population of stably infected, G418-resistant S49.1-BCL2 and WEHI-BCL2 cells; hence, subcloning was unnecessary to achieve cells with high levels of bcl-2 retrovirus expression. The elevations in Bcl-2 protein were specific in that alterations were not observed in the relative levels of another mitochondrial protein (cytochrome C; not shown).

Overproduction of the Bcl-2 protein in S49.1 and WEHI7.2 cells rendered these thymus-derived cells highly resistant to killing by DEX. When assayed 2 days after addition of DEX to cultures, for example, S49.1-BCL2 and WEHI-BCL2 cells remained ≥90% viable despite exposure to concentrations of DEX as high 10⁻⁴ M, whereas ≥90% of S49.1-NEO and WEHI-NEO cells failed to exclude trypan blue dye when cultured in the presence of 10⁻⁵ M DEX (Fig. 2B; not shown). The survival of these Bcl-2-producing S49.1 and WEHI7.2 cells cultured in the presence of 10⁻⁷ M DEX was maintained at ≥90% for at least 3 days, in contrast to the NEO control cells which were nearly all dead by this time (Fig. 2B). The increased resistance of S49.1-BCL2 and WEHI7.2-BCL2 cells to DEX-mediated killing could not be attributed to a loss of glucocorticoid receptors in these cells. As shown in Fig. 1C, the relative levels of glucocorticoid receptors were not diminished by bcl-2 retrovirus expression, as assessed by immunoblotting, but rather appeared to be slightly increased in at least some experiments (Fig. 1C).

Although overproduction of Bcl-2 protein in S49.1 and WEHI7.2 cells interfered with the ability of DEX to cause cell death, it did not prevent DEX from suppressing the proliferation ([³H]thymidine incorporation) of these cells (Fig. 2C), consistent with previously published data (25). Enforced production of p26-Bcl-2 protein therefore markedly enhanced survival of DEX-treated S49.1 and WEHI7.2 cells without augmenting mitogenesis. In contrast to the striking resistance of bcl-2 retrovirus-infected S49.1 and WEHI7.2 cells to DEX, these cells displayed no enhanced survival when cultured in media containing various concentrations of H₂O₂ (Fig. 2D).

Because both DEX and H₂O₂ have been reported to induce apoptosis in some types of cells (26, 27) and because degradation of DNA into fragments that are integral multiples of the nucleosomal distance (=180 base pairs) is often considered a hallmark of this form of cell death (6, 7), we next compared S49.1-BCL2, S49.1-NEO, WEHI-BCL2, and WEHI-NEO
cells with regard to their relative levels of fragmented DNA by using a qualitative gel electrophoresis assay (18). Treatment for 1 day with $10^{-7}$ M DEX induced marked DNA fragmentation in the NEO control S49.1 and WEHI7.2 cells, but had little effect on S49.1-BCL2 and WEHI-BCL2 cells. We also explored the effects of a Ca$^{2+}$-ionophore (IONO) and a phorbol ester (TPA) on S49.1 and WEHI7.2 cells, since DEX has been reported to stimulate elevations in the concentrations of cytosolic Ca$^{2+}$ (28), which in turn can lead to activation of protein kinase C and other Ca$^{2+}$-dependent enzymes. Consistent with others' findings (28), 1 $\mu$M IONO and 10 ng/ml TPA induced cell death and DNA fragmentation in S49.1-NEO and WEHI-NEO cells. In contrast, S49.1-BCL2 and WEHI-BCL2 cells remained mostly viable and experienced much less DNA degradation (Fig. 3). High levels of p26-Bcl-2 production also protected S49.1 and WEHI7.2 cells from DNA fragmentation and cell death induced by cyclic AMP agonists (not shown). Unlike DEX, IONO, and TPA, H$_2$O$_2$ caused DNA degradation into oligonucleosomal-length fragments that were unaffected by overproduction of p26-Bcl-2 protein, even when used at concentrations as low as 2.5 $\mu$M (Fig. 3).

Many chemotherapeutic drugs are capable of initiating pathways leading to apoptosis (16, 17); we therefore determined whether high levels of p26-Bcl-2 protein could render S49.1 and WEHI7.2 cells resistant to several different types of antineoplastic reagents that were added to cultures at clinically relevant concentrations. When cultured for 1 day in the continuous presence of 10 $\mu$M methotrexate, 1.5 $\mu$M 1-$\beta$-d-arabinofuranosylcytosine, or 1 $\mu$M vincristine, S49.1-BCL2 and WEHI-BCL2 cells experienced much less DNA fragmentation and cell death than NEO-control cells (Fig. 3). Moreover, in contrast to S49.1-NEO and WEHI-NEO cells, drug-treated S49.1-BCL2 and WEHI-BCL2 cells did not appear to undergo the cell shrinkage which is characteristic of apoptotic cells, based on fluorescence-activated cell sorter-estimates of cell volume by forward light scatter (not shown). Treatment of S49.1 and WEHI7.2 cells with 35 $\mu$M concentrations of the DNA topoisomerase inhibitor etoposide (VP-16) produced comparable levels of DNA fragmentation and similar percentages of cell death in control and bcl-2 retrovirus-infected cells (Fig. 3), indicating that bcl-2 did not confer resistance to this drug at least at the concentrations used here. Similar to the situation with DEX, all of the chemotherapeutic drugs tested here were still capable of arresting the growth of S49.1 and WEHI7.2 cells, despite the enhanced survival of Bcl-2-producing cells.

Discussion

The bcl-2 gene is unique among cellular oncogenes described to date with regard to the mitochondrial location of its encoded
protein and its ability to enhance cell survival by interfering with pathways leading to apoptosis. The mechanism by which the M<sub>2</sub> 26,000 Bcl-2 protein regulates cell viability remains enigmatic, as the predicted amino acid sequence of this protein bears no significant homology with other known proteins and no biochemical activity has yet been ascribed to it. When high levels of p26-Bcl-2 protein were achieved in the murine T-cell lymphoma S49.1 and thymoma-derived WEHI7.2 cell lines through gene transfer methods, we found that these cells became markedly resistant to the acute cellular toxicity normally imparted by several chemotherapeutic drugs, as well as reagents that are known to induce apoptosis of immature thymocytes such as DEX, IONO, and TPA. The drugs to which Bcl-2-producing S49.1 and WEHI7.2 cells displayed increased resistance have diverse mechanisms of action, suggesting that p26-Bcl-2 functions in a final common pathway for PCD that can be activated by multiple mechanisms.

The relative resistance imparted by Bcl-2 however varied depending on the particular drug. Killing of S49.1 and WEHI7.2 cells by DEX, for example, was almost completely blocked by high levels of Bcl-2 protein production, when tested over a 6-log range of DEX concentrations (Fig. 2A). Some of the other drugs such as 1β-d-arabinofuranosycytosine, in contrast, killed 30–50% of S49-BCL2 and WEHI-BCL2 cells when used at concentrations just 2 times higher than those shown for Fig. 3 (data not presented), suggesting a much steeper dose-response curve. Although S49-BCL2 and WEHI-BCL2 cells were not resistant to VP-16, this finding may simply indicate that overproduction of p26-Bcl-2 is unable to provide protection against the particular concentration of etoposide (35 μM) chosen rather than a difference in the final pathway by which topoisomerase inhibitors kill cells, since we have observed relative resistance to this drug in another bcl-2 retrovirus-infected leukemic cell line. With regard to H<sub>2</sub>O<sub>2</sub>, however, treatment of the S49.1 and WEHI7.2 cells with a variety of concentrations of H<sub>2</sub>O<sub>2</sub> produced cell death and DNA fragmentation that was unaffected by the relative levels of p26-Bcl-2 present in these cells. Thus, Bcl-2 did not protect S49.1 and WEHI7.2 cells from all types of drugs that induced cell death accompanied by the DNA fragmentation pattern that is characteristic of apoptosis. Although some of the cell death produced by H<sub>2</sub>O<sub>2</sub> may occur by necrosis as a result of free radical generation, H<sub>2</sub>O<sub>2</sub> has been reported to induce apoptosis as defined by traditional morphological criteria in cultured embryonic cells and at least some neoplastic cell lines (26, 27). Furthermore, we did detect a ladder of DNA fragments during gel electrophoretic analysis of H<sub>2</sub>O<sub>2</sub>-treated S49.1 and WEHI7.2 cells consistent at least some of the death occurring via apoptotic mechanisms.

Regardless of the actual mechanism by which H<sub>2</sub>O<sub>2</sub> induces S49.1 and WEHI7.2 cell death, it is clear that high levels of Bcl-2 protein production cannot protect lymphoid cells from all pathways for cellular demise. In this regard, we have recently reported that Bcl-2 does not influence the sensitivity of a variety of types of hematopoietic and nonhematopoietic cells to death and DNA fragmentation induced by tumor necrosis factor (29). Like H<sub>2</sub>O<sub>2</sub>, tumor necrosis factor is thought to induce cell death at least in part by mechanisms involving hydroxyl radical production (30), and thus this cytokine may also activate pathways leading to both apoptotic and necrotic cell death. Our findings therefore provide additional evidence for the existence of both bcl-2-dependent and bcl-2-independent pathways for regulating cell survival, consistent with observations in bcl-2 transgenic mice, showing that negative antigenic selection in the thymus is relatively unperturbed by deregulated bcl-2 expression in contrast to thymocyte cell death induced by pharmacological reagents and X-irradiation (11–13). Taken together, the findings obtained previously in bcl-2 transgenic mice and those described here for S49.1 and WEHI7.2 cells suggest that a search for chemotherapeutic drugs that kill cells through bcl-2-independent mechanisms may be warranted for some types of lymphoid malignancies that involve the bcl-2 gene.

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