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Biochemistry

Bcl-2 interrupts the ceramide-mediated pathway of cell death

(*Rb*/apoptosis/chemotherapy/cell cycle/signal transduction)

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ABSTRACT **Ceramide, a product of sphingomyelin turnover, has been proposed as a novel lipid second messenger with specific roles in mediating antiproliferative responses including apoptosis and cell cycle arrest. In this study, we examine the relationship between the ceramide-mediated pathway of growth suppression and the *bcl-2* protooncogene. In ALL-697 leukemia cells, the addition of the chemotherapeutic agent vincristine resulted in a time-dependent growth suppression characterized by marked apoptosis. The effects of vincristine on cell death were preceded by a prolonged and sustained accumulation of endogenous ceramide levels reaching ≈10.4 pmol ceramide/nmol phospholipids at 12 hr following the addition of vincristine—an increase of 220% over vehicle-treated cells. Overexpression of *bcl-2* resulted in near total protection of cell death in response to vincristine. However, the ceramide response to vincristine was not modulated by overexpression of *bcl-2*, indicating that *bcl-2* does not interfere with ceramide formation. Overexpression of *bcl-2* prevented apoptosis in response to ceramide, suggesting that *bcl-2* acts at a point downstream of ceramide. On the other hand, *bcl-2* did not interfere with the ability of ceramide to activate the retinoblastoma gene product or to induce cell cycle arrest, suggesting that the effects of ceramide on cell cycle arrest can be dissociated from the effects on apoptosis. These studies suggest that ceramide and *bcl-2* partake in a common pathway of cell regulation. The results also cast ceramide as a gauge of cell injury rather than an "executor" of cell death with clearly dissociable biological outcomes of its action depending on downstream factors.**

The novel lipid mediator ceramide is emerging as a key regulator of various antiproliferative responses (1, 2). The action of a number of extracellular agents such as 1,25-dihydroxyvitamin D_3, tumor necrosis factor α, nerve growth factor, interleukin 1, dexamethasone, and activators of the Fas receptor cause sphingomyelin hydrolysis and the generation of intracellular ceramide (3–11). In turn, the addition of exogenous ceramide (or the induction of ceramide accumulation through metabolic manipulation) has been associated with several antiproliferative responses including cell differentiation, apoptosis, and cell cycle arrest (1, 2). Although in several cell systems the kinetics of ceramide formation are somewhat rapid, occurring over several minutes (3–7), in most systems associated with apoptosis such as growth factor withdrawal (12), dexamethasone (8), Ara-C (11), and Fas activation (9) the accumulation of ceramide has been observed to be delayed and persistent. These results suggest that ceramide may act as a common and downstream mediator of apoptotic mechanisms.

The mechanisms by which ceramide induces apoptosis remain poorly understood. A major regulator of apoptosis is the protooncogene *bcl-2* (13, 14). *bcl-2* has been shown to inhibit apoptosis in response to a number of extracellular agents and stress responses including dexamethasone, chemotherapeutic agents, Fas activation, tumor necrosis factor α, and irradiation (13–15). These results suggest that ceramide and *bcl-2* may participate in either a common pathway of cell death or in redundant mechanisms activated by similar agents leading to cell death. We therefore undertook this study to determine the relationship of *bcl-2* and ceramide.

In this study we demonstrate that *bcl-2* acts downstream of ceramide preventing ceramide-induced cell death but not ceramide accumulation in a model of chemotherapy-induced cell death. However, *bcl-2* does not modulate ceramide-induced cell cycle arrest through activation of the retinoblastoma gene product (*Rb*). These results raise the possibility that ceramide may function as a critical endogenous activator of a *bcl-2* inhibitable pathway leading to cell death.

METHODS

Cell Culture. ALL-697 human pre-B leukemia cells were grown in RPMI 1640 medium and 10% fetal bovine serum in 2.5% Hepes buffer. Cell death was evaluated by trypan blue exclusion studies as well as by propidium iodide flow cytometry (12, 16).

Ceramide Measurements. At the time of treatment, cells were seeded at 5×10^5 cells/ml. Ceramide levels were measured using a modification of the diacylglycerol kinase assay (17, 18). Lipids were collected according to the method of Bligh and Dyer (19), and lipid phosphate was measured as described (20). Ceramide phosphorylated by diacylglycerol kinase was normalized to total cellular phospholipids.

***Rb* Phosphorylation.** *Rb* dephosphorylation was evaluated by Western blot analysis of *Rb* migration with the faster migrating forms indicating progressive dephosphorylation of *Rb* as described (21, 22) using a monoclonal mouse anti-human antibody (PharMingen).

Transfection of *bcl-2* and Selection of Cells. Molt-4 cells were transfected with pMEP4 vector with and without full length murine *bcl-2* gene using electroporation. Cells were seeded at 1×10^7/ml in serum free RPMI 1640 medium. Cells (300 μl) were electroporated (capacitance of 960 μF; 210 V for 28 sec) with 10 μg DNA. Treated cells were resuspended in 10 ml RPMI 1640 medium plus 10% fetal bovine serum and incubated for 48 hr. Hygromycin B1 (from Sigma) (400 μg/ml) was used for clonal selection of cells by performing 1:10 dilutions for 10 passages. Whole cell extracts were prepared from hygromycin B1-resistant cells from both cell lines for Western blot analysis to verify Bcl-2 protein expression.

Statistical Analysis. All experiments were performed at least three to five times. Results are shown as means ± SEM.

RESULTS

To gain understanding into the role of ceramide in cell death and its relationship to *bcl-2*, we used the chemotherapeutic

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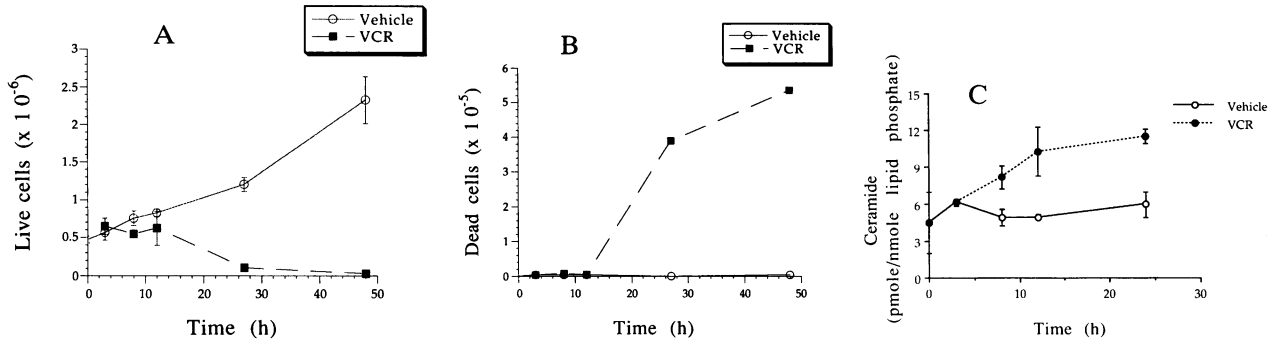

FIG. 1. Effects of vincristine (VCR) on cell death and ceramide levels in ALL-697 cells. ALL-697 cells were treated with 1 μg (1.08 μM) of vincristine per ml. At the indicated times, cells were collected and evaluated for viability (*A*) and cell death (*B*) by trypan blue exclusion analysis. Simultaneously, cells were analyzed for their content of total lipid and ceramide levels (*C*).

agent vincristine (23). In ALL-697 cells, vincristine induced significant growth suppression by 24–48 hr following addition of 1 μg of vincristine per ml (Fig. 1*A*). This growth suppression was primarily a result of cytotoxicity with a time-dependent accumulation of dead cells in response to vincristine (Fig. 1*B*). Propidium iodide flow cytometry demonstrated a pre-2N peak (data not shown) confirming death by apoptosis, as previously reported (23). We next evaluated the effects of vincristine on intracellular ceramide levels. The addition of vincristine resulted in a time-dependent accumulation of ceramide detected as early as 8 hr following treatment and peaking at 12 hr (Fig. 1*C*). These changes were specific to ceramide in that the levels of other membrane phospholipids at 8–12 hr remained unchanged. Also, the levels of diglycerides, which were measured simultaneously, did not show significant differences in response to vincristine (data not shown). Comparison of Figs. 1 *B* and *C* discloses that the accumulation of ceramide preceded the onset of cell death by several hours. Notably, these kinetics of ceramide accumulation differ significantly from the previously documented changes in ceramide in response to extracellular agents such as tumor necrosis factor α, interleukin 1, and nerve growth factor (3–7) in at least two aspects. First, there was a significant delay of at least 4 hr before any detectable increase in intracellular ceramide levels following vincristine treatment. Second, ceramide accumulation was persistent and prolonged such that the intracellular levels of ceramide remained at around two fold elevated until the onset of cell death at 12–24 hr. Indeed, by 12 hr, the levels of ceramide had increased to 10.4 pmol/nmol phospholipid, corresponding to a 2.2-fold increase over untreated control cells.

We next evaluated the effects of *bcl-2* on vincristine-induced death and vincristine-induced accumulation of ceramide. This was particularly important in view of the delayed kinetics of ceramide accumulation which raised the possibility that ceramide may function as an effector of cell death acting "downstream" of *bcl-2*, especially since *bcl-2* has been shown to interfere with the formation of lipid peroxides (15). ALL-697 cells overexpressing *bcl-2* demonstrated a survival advantage over ALL-697 cells transfected with control expression vector (neo) when challenged with vincristine (Fig. 2*A*). Indeed, vincristine-treated *bcl-2* cells demonstrated a marked cell cycle arrest primarily at G_2/M with a small component at G_0/G_1 (data not shown); the latter is consistent with the primary effect of vincristine on spindle formation. A more profound effect of *bcl-2* was observed when cell death in response to vincristine was examined where *bcl-2* afforded near total protection from cell death in response to vincristine (Fig. 2*B*).

Because cell death with vincristine was an all-or-none response depending on *bcl-2* expression, it became important to determine the effects of *bcl-2* on ceramide accumulation. In simultaneous experiments using either neo or Bcl-2 cells, the addition of vincristine (1 μg/ml) resulted in a nearly identical profile for ceramide accumulation with peak levels obtained ≈12 hr following addition of vincristine (Fig. 2*C*). Therefore, *bcl-2* did not interfere with ceramide accumulation, demonstrating that *bcl-2* does not act upstream of ceramide generation. These results also demonstrate that this delayed accumulation of ceramide is not a result of cell death because, in the presence of *bcl-2*, cell death is totally inhibited whereas ceramide accumulation is not.

These results suggest that either *bcl-2* acts at a point down stream of ceramide accumulation or that ceramide accumulation is an independent event related to signaling damage through vincristine. Therefore, we pursued these possibilities by asking whether *bcl-2* can prevent ceramide-induced apoptosis. The addition of 3 μM C_6-ceramide resulted in a time-dependent

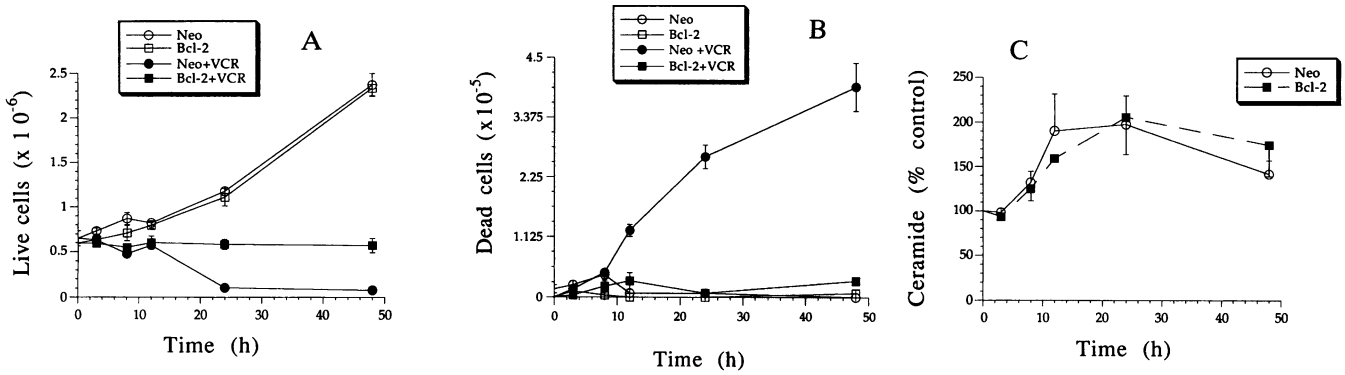

FIG. 2. Bcl-2 prevents vincristine-induced apoptosis but not the elevations in ceramide levels. ALL-697 cells transfected with either vector alone (neo) or with vector expressing human *bcl-2* cDNA (Bcl-2) were treated with 1 μg of vincristine (VCR) per ml. At the indicated time points, cells were evaluated for viability (*A*), death (*B*), or ceramide levels (*C*). Ceramide levels are shown as percent of time-matched controls. The basal level of ceramide was 4.9 pmol/nmol phospholipid.

cytotoxicity that was nearly completely abrogated by the expression of *bcl-2* (Fig. 3*A*). Also, the ability of *bcl-2* to inhibit C_6-ceramide-induced death was dose-dependent such that death in response to either 3 or 5 μM C_6-ceramide was totally inhibited by *bcl-2* (Fig. 3*B*). These results are consistent with the ability of *bcl-2* to interrupt the signaling pathways mediating apoptosis downstream of ceramide.

In other studies, we have shown that ceramide induces cell cycle arrest (12) through activation of the retinoblastoma gene product (*Rb*) in the Molt-4 T-leukemia cells (24). These observations suggest that ceramide's effects on cells are either cell-specific or that ceramide is capable of simultaneously coupling to more than one cellular response. Given the ability of *bcl-2* to interrupt ceramide-mediated apoptosis, we investigated the effects of *bcl-2* on ceramide regulation of *Rb* function. To this end, Molt-4 cells were transfected with either *bcl-2* or with vector alone (Fig. 4*A*). As in ALL-697 cells, vincristine resulted in time-dependent cell death that was inhibited by *bcl-2* [3×10^5 versus $<0.1 \times 10^5$ dead cells per ml (at 48 hr)]. In this cell line vincristine resulted in an even more pronounced accumulation of ceramide that was not inhibited by *bcl-2* (Fig. 4*B*). C_6-ceramide caused time-dependent cell death that was nearly totally inhibited by *bcl-2* (Fig. 4*C*). Consistent with previous results (24), C_6-ceramide induced dephosphorylation of *Rb* in a concentration-dependent manner (Fig. 4*D*). However, unlike the effects on death, *bcl-2* was unable to interrupt ceramide-induced *Rb* dephosphorylation (Fig. 4*D*). Phenotypically, C_6-ceramide induced a G_0/G_1 cell cycle arrest phenotype in this cell line which was not prevented by *bcl-2*. Thus, cells exposed to C_6-ceramide demonstrated a significant increase in the G_0/G_1 population (from 62% in untreated vector or Bcl-2 cells to 75% in cells treated with 5 μM C_6-ceramide) with a corresponding decrease in S and G_2/M cells (from 29% to 19% and from 9% to 6%, respectively). These results demonstrate that ceramide is capable of inducing both cell cycle arrest and apoptosis, but *bcl-2* interrupts selectively the apoptotic downstream pathway.

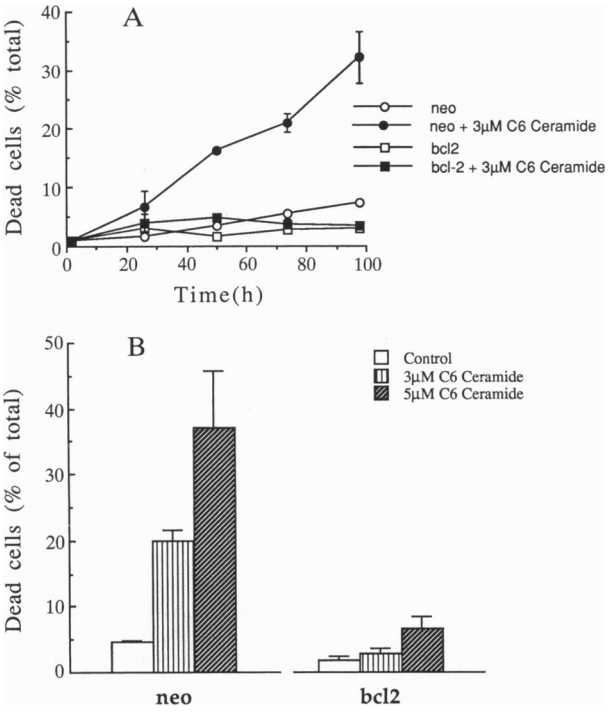

FIG. 3. Bcl-2 prevents ceramide-induced cell death. ALL-697 cells were seeded at 5×10^5 cell/ml in 2% fetal bovine serum and then treated with 3 μM C_6-ceramide and evaluated for ceramide-induced cell death (*A*). (*B*) Cells were treated with 3 or 5 μM C_6-ceramide, and cell death was evaluated at 72 hr.

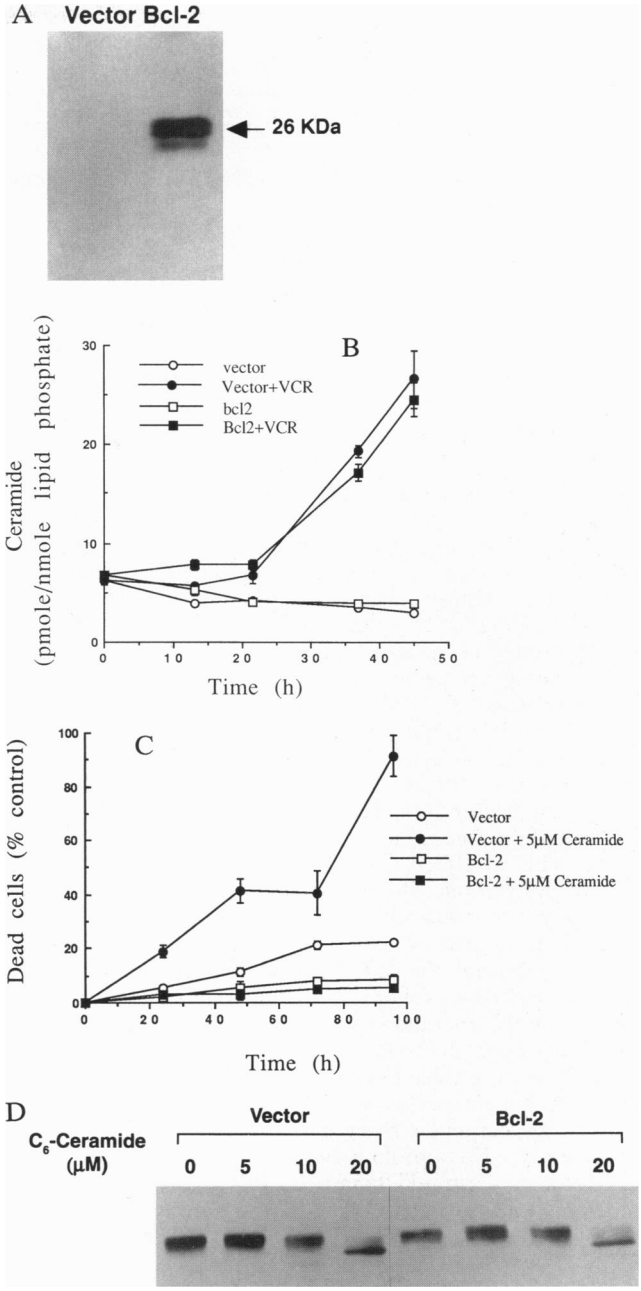

FIG. 4. Bcl-2 prevents ceramide-induced cell death but not ceramide induced activation of *Rb*. (*A*) Expression of *bcl-2* in Molt-4 cells transfected with mouse *bcl-2* cDNA as detected by an antibody specific to mouse *bcl-2*. (*B*) Effects of vincristine (1 μg/ml) on ceramide levels in vector and Bcl-2 cells. (*C*) Effects of *bcl-2* on C_6-ceramide-induced cell death in Molt-4 cells. (*D*) Effects of ceramide on dephosphorylation of *Rb* in vector and Bcl-2 cells 6 hr following addition of ceramide.

DISCUSSION

The results from this study support a ceramide-dependent mechanism of programmed cell death that acts upstream of the "commitment" point defined by *bcl-2* action. The ability of *bcl-2* to inhibit ceramide-induced death suggests that ceramide does not act at the "execution" stage of apoptosis but rather in the "sensing" phase of this pathway. While this manuscript was being prepared, another study with *bcl-x*, a member of the *bcl-2* family, showed that ceramide-induced death was attenuated by *bcl-x* (25). In our hands, *bcl-x* does not protect from ceramide-induced death under conditions where *bcl-2* is active, raising the possibility that these two related genes may have different molecular mecha-

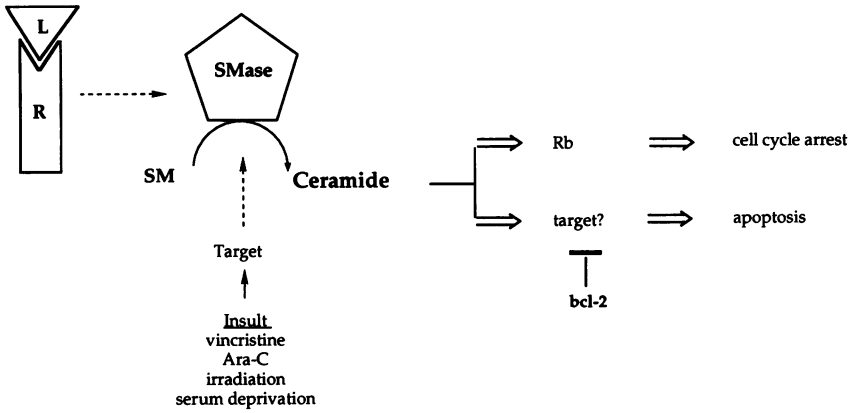

FIG. 5. Scheme for proposed role of ceramide in sensing and signaling cell injury.

nisms of action (G. Dbaibo and Y.A.H., unpublished observations).

These studies suggest that ceramide may emerge as a final common sensor for many extracellular agents of death or of intracellular damage. This contention is supported by the ever-expanding list of inducers of ceramide formation which includes tumor necrosis factor α (4), the Fas antigen (9, 10), the chemotherapeutic agent Ara-C (11), withdrawal of serum (12), dexamethasone (8), HIV (26), and ionizing irradiation (8, 27). Indeed, the degree and persistence of ceramide elevation may gauge the extent of cell damage, thus leading the cell to execute programs of cell death.

Consistent with a role for ceramide in sensing or gauging cell injury and damage rather than in executing apoptosis are the results with the effects of ceramide on cell cycle arrest and *Rb* dephosphorylation. These studies demonstrate clearly that ceramide can induce *Rb* dephosphorylation and cell cycle arrest independent of *bcl-2* action. Therefore, ceramide appears to "inform" the cell of the occurrence of cell damage. How the cell reacts (apoptosis or cell cycle arrest) appears to depend on the operation of subsequent downstream modulators (such as *bcl-2* and *Rb*).

The precise mechanism by which ceramide acts to launch apoptosis remains poorly defined, with several candidate direct targets for ceramide being proposed, including a protein phosphatase (28), a proline-directed protein kinase (29), and an isoform of protein kinase C, PKCζ (30) Nevertheless, positioning ceramide upstream of the "presumed" protease target for *bcl-2* action will allow further mechanistic development of this question.

In conclusion, an apoptosis-mediating pathway is emerging (Fig. 5) that is activated by either extracellular agents (e.g., tumor necrosis factor α, Fas-ligand) or intracellular insults (e.g., as a consequence of action of chemotherapeutic agents) converging at a point that results in accumulation of cellular ceramide. Ceramide then launches antimitogenic responses that primarily include an *Rb*-dependent mechanism of cell cycle arrest and a *bcl-2*-inhibited mechanism of cell death.

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