Bcl-2 protects lymphoma cells from apoptosis but not growth arrest promoted by cAMP and dexamethasone

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Zhang, Lingzhi, and Paul A. Insel. Bcl-2 protects lymphoma cells from apoptosis but not growth arrest promoted by cAMP and dexamethasone. Am J Physiol Cell Physiol 281: C1642–C1647, 2001.—Glucocorticoids or increases in cellular cAMP promote apoptosis in many cell types, including murine S49 cells. We examined the impact of Bcl-2, an antiapoptotic protein, on S49 cell growth and death promoted by the glucocorticoid dexamethasone or agents that increase cAMP: isoproterenol (a β-adrenergic agonist) + 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) and forskolin (diterpene). These agents promoted apoptosis (i.e., increased expression of annexin V) of wild-type (WT) S49 cells, but Bcl-2-overexpressing S49 cells were protected from this response. Bcl-2 overexpression did not protect cells from G1 growth arrest but did allow cells to grow longer in culture and protected cells from culture-dependent necrosis. Commitment to and reversal from apoptosis vs. G1 growth arrest by isoproterenol + 3-isobutyl-1-methylxanthine showed different kinetics. Although both processes required several hours to develop, removal of agonists readily reversed growth arrest, but not apoptosis. Thus commitment to apoptosis is less reversible than G1 growth arrest. The findings also indicate that glucocorticoid- and cAMP-mediated G1 growth arrest is unaffected by Bcl-2 overexpression, even though increased Bcl-2 allows these lymphoma cells to resist necrosis and apoptosis.

glucocorticoids; S49 cells; isoproterenol; 3-isobutyl-1-methylxanthine; forskolin; annexin V

HORMONES ARE WELL KNOWN to have an impact on pathways involved in cell growth and cell death. Although previously the focus was on mitogenesis and stimulation of cell proliferation, in recent years the ability of hormones to regulate programmed cell death (apoptosis) has attracted considerable attention. Steroid hormones, such as glucocorticoids, which act via cytosolic receptors that translocate to the nucleus to regulate gene transcription, can decrease cell proliferation in a variety of cell types, including lymphoid cell lines of the T cell lineage (recently reviewed in Refs. 1 and 13). In such cells, the apoptotic pathway is accompanied by changes in gene expression (1, 13, 19). Many G protein-coupled receptors (GPCRs) are also able to regulate cell growth and death through their ability to alter protein phosphorylation. GPCRs that act via Gα to increase adenylyl cyclase activity, cAMP levels, and the activity of protein kinase A (PKA) have cell type-dependent pro- or antiapoptotic actions (see references cited in Refs. 4 and 23). Promotion of apoptosis by the cAMP-PKA pathway has been proposed as a potentially useful therapeutic approach in certain forms of cancer and in other settings characterized by cellular proliferation (9,12). The precise determinants of pro- vs. antiapoptotic responses by cAMP/PKA remain obscure but may relate to expression and regulation of members of the Bcl-2 family, which play a key role in apoptotic pathways, perhaps via effects on mitochondrial physiology (recently reviewed in Refs. 5, 10, 20, and 22).

Murine S49 cells, a T cell lymphoma cell line, have provided a useful model system to explore the action of glucocorticoids and Gs-linked GPCRs, especially as related to cell proliferation (7, 18, 23). Originally isolated 30 years ago, wild-type (WT) S49 cells undergo a patterned response to such hormones: cell division with ~20 h of doubling time, growth arrest in the G1 phase of the cell cycle, and apoptosis. A number of S49 variants have been isolated by virtue of their resistance to this cell death, while other S49 cells have been generated with altered expression of particular proteins (21). For example, Caron-Leslie et al. (3) transfected S49 cells with a construct that gave overexpression of Bcl-2; such cells were shown to be resistant to dexamethasone-promoted apoptosis and somewhat less resistant to cell killing promoted by calcium ionophore or cycloheximide. In recent studies, we found that these Bcl-2-overexpressing S49 cells are also resistant to apoptosis promoted by agents that increased cellular cAMP levels (23). In the present studies, we sought to define further aspects of the role of Bcl-2 in the growth and death of S49 cells, in particular, to ascertain the ability of Bcl-2 to affect G1 growth arrest promoted by glucocorticoids or increases in cAMP.

MATERIALS AND METHODS

Materials. All reagents were from standard sources: (−)-isoproterenol, 3-isobutyl-1-methylxanthine (IBMX), and 1% IGEPAL CA-630 were obtained from Sigma; FITC-annexin V, propidium iodide, dexamethasone, and forskolin from Calbiochem; [3H]cAMP from New England Nuclear; and Bcl-2 antibody from Santa Cruz Biotechnology.

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Fig. 1. Effect of isoproterenol (Iso, 100 μM), forskolin (Frsk, 50 μM) + IBMX (100 μM), or dexamethasone (Dex, 10 μM) on apoptosis of wild-type (WT) and Bcl-2-overexpressing S49 cells. Cell viability was determined 48 h after drug treatment. A: cell viability as assessed by trypan blue (0.1%) exclusion. Each bar represents mean ± SD of 3 separate experiments, each conducted in duplicate. B: flow cytometric analysis of S49 cells as assessed by FITC-annexin V staining. Data are presented as fluorescent intensity units of annexin V (abscissa) vs. number of counted cells (ordinate). M1, defined as high intensity (≥ 70 units intensity), was the same in each data set and reflects apoptotic cells. Percent apoptotic cells is shown in each panel.
Cell culture. S49 [WT and Bcl-2 (overexpressing Bcl-2)] were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 10 μM HEPES in a humidified atmosphere containing 10% CO₂ at 37°C. For most experiments, cultures were initiated at 2–3 × 10⁵ cells/ml.

cAMP determination. Cells were centrifuged for 3 min at 1,000 rpm at room temperature. The medium was discarded, and the cells were resuspended in Dulbecco’s modified Eagle’s medium in which 20 mM Na⁺-HEPES replaced the NaHCO₃ (DMEH), centrifuged again, and resuspended in DMEH at a cell density of 5 × 10⁶–1 × 10⁷ cells/ml. Aliquots (50 ml) of the cell suspension were incubated for 5 min in the presence of the cyclic nucleotide phosphodiesterase inhibitor IBMX (100 or 300 μM), the β-adrenergic agonist isoproterenol, or isoproterenol + IBMX at 37°C for 5 min. Incubations were terminated by addition of TCA to a final concentration of 7.5% and then assayed for cAMP by radioimmunoassay, as previously described (11).

Measurement of cell number and cell viability. Cell number was determined by using a hemocytometer or Coulter counter. Cell viability was determined by trypan blue exclusion or flow cytometry. Trypan blue was added to cell suspensions (final concentration 0.1%), which were incubated at room temperature for 1 min, and the cells were counted with a hemocytometer. At least 100 cells were counted per sample. Cell viability was represented as the percentage of cells excluding trypan blue divided by the total number of cells. For flow cytometric analysis, cells were pelleted, washed, and resuspended in phosphate-buffered saline (PBS). Cells were examined on a FACScan using CELLQuest software (Becton-Dickinson Immunocytometry System, San Jose, CA). Individual populations of cells (5,000–10,000 cells per experimental sample) were selected by gating with a forward light-scattering vs. side light-scattering dot plot as a measure of cell viability, as previously described (23).

Annexin V binding assay by flow cytometry. Apoptosis was evaluated by assessment of annexin V binding. We previously showed that this method yields results similar to other
means to define apoptosis of S49 cells (23). Cells were treated for the designated time, and then 0.5–1.0 × 10^6 cells were pelleted and washed with PBS containing 1% FCS. Cells were then washed with Hanks’ balanced salt solution (HBSS) containing 1 mM Ca²⁺. FITC-conjugated annexin V (annexin V-FITC, 0.2 μM) was added to the cells resuspended in 100 μl of HBSS and incubated at 37°C for 15 min. The cells were washed and resuspended in 0.5 ml of HBSS and analyzed as described elsewhere (23) for cell number and viability. 

Analysis of DNA content and cell cycle by flow cytometry. Cells were pelleted from the culture medium, washed once with PBS, fixed by addition of cold 75% ethanol to a volume of 2 ml with agitation, and incubated for 30 min at room temperature or stored at 4°C for up to 1 wk. Fixed cells were pelleted from the ethanol, washed once in PBS, and then stained for DNA content by use of 0.5 ml of 50 μg/ml propidium iodide and 100 μg/ml preboiled RNase A (GIBCO) in PBS. The suspension was incubated at room temperature for 30 min, protected from light, and then subjected to flow cytometric analysis with excitation at 488 nm and emission at 560–640 nm (FL2 mode).

Western blot analysis. At appropriate time points after various treatments, cells were pelleted from the culture medium, resuspended, and washed once with PBS. Cell pellets were lysed in 0.2 ml of RIPA buffer (PBS containing 1% IGEPAL CA-630, 0.5% sodium deoxycholate, and 0.1% SDS with freshly added proteolysis inhibitors), incubated for 30 min on ice, and centrifuged (30 min at 4°C, 11,000 rpm); the resulting supernatants were used as cell lysates. Protein concentration in the supernatant was measured using a Bio-Rad protein assay with bovine serum albumin as standard. Samples were denatured with SDS loading buffer at 100°C for 3 min and then separated on an SDS-10% polyacrylamide gel with a 5% stacking gel in SDS-triglycine running buffer. The protein was electroblotted to a polyvinylidene difluoride membrane. The filter was blocked with 3% nonfat dry milk in PBS overnight at 4°C, probed with Bcl-2 antibody for 2 h at room temperature or overnight at 4°C, visualized with enhanced chemiluminescence detection system, and exposed to X-ray film (Pierce, Rockford, IL).

Statistical analyses. All determinations were performed in duplicate or triplicate for each group, and each experiment was repeated at least three times. Values are means ± SD. Representative results from Western blot and flow cytometry analysis from a single experiment are presented. Statistical analyses were performed by ANOVA. Differences were considered to be statistically significant at P < 0.05.

RESULTS AND DISCUSSION

Treatment of WT S49 cells with agents that increase cAMP alters cell growth and promotes cell death (21, 23). As shown in Figs. 1 and 2 and Table 1, incubation of WT S49 cells with isoproterenol + IBMX or with forskolin, as a means to increase cAMP levels, arrests cell growth in the G1 phase of the cell cycle and decreases cell viability and promotes apoptotic cell death, as reflected by increased expression of annexin V. Other studies, which included assessment of morphological appearance and DNA laddering, provided further evidence that these agents kill S49 cells by apoptosis (23). As a further reflection of apoptotic cell death, WT cells incubated with isoproterenol show a depletion in G1/G0 cells between 24 and 48 h (Fig. 3B vs. 3C). The glucocorticoid dexamethasone also kills S49 cells via apoptotic mechanisms (Fig. 1) (3, 7).

In contrast to results obtained with WT S49 cells, S49 cells that overexpress Bcl-2 were protected from apoptosis promoted by isoproterenol + IBMX, forskolin, and dexamethasone but still responded to those agents with arrest of cells in G1 (Figs. 2B and 4A). Because the Bcl-2-overexpressing cells undergo G1 growth arrest, when these cells were grown for 48 h in the presence of isoproterenol + IBMX, we observed a decrease in cell number (Fig. 2A) without a substantial decrease in viability (Fig. 1A) or increase in apoptotic cell death (Fig. 1B). Moreover, expression of Bcl-2, as assessed by immunoblotting (Fig. 4B), was not altered by isoproterenol + IBMX or dexamethasone in the Bcl-2-overexpressing cells, even though growth of the cells was arrested in G1 (Figs. 3, B and C, and 4A).

Table 1 summarizes data for viable cell number for control WT S49 cells and Bcl-2-overexpressing cells incubated with isoproterenol + IBMX or dexamethasone for 24 and 48 h. WT cells incubated for 24 h with dexamethasone have a much lower viability than do cells incubated with isoproterenol + IBMX, while both treatments markedly decrease viability at 48 h. The decrease in viable cell number for Bcl-2-overexpressing cells after 24 and 48 h of treatment with isoproterenol + IBMX or dexamethasone is totally accounted for by G1 growth arrest, in that viability was 94–97% under all conditions at both time points.

In addition to their resistance to cell killing by several exogenous agents, the Bcl-2-overexpressing cells showed a pattern of cell growth that was different from that of WT S49 cells (Fig. 5). WT S49 cells grew logarithmically in suspension culture for several days before growth arrest and then death by necrosis; i.e.,
there was no increase in expression of annexin V in the dying cells. By contrast, Bcl-2-overexpressing cells, although they also stopped dividing, were able to stay alive for many days longer than were the WT cells, thus providing evidence for protection by Bcl-2 of cell culture- or density-dependent necrosis of the cells.

We undertook additional studies to assess the reversibility of response to isoproterenol + IBMX as well as the time required to "commit" cells to G₁ growth arrest and apoptosis. Cells were incubated with the agonists for variable times, washed to remove the drugs, and then returned to culture for up to 48 h. A: apoptosis was determined by annexin V staining, as described in Fig. 1 legend, and expressed as increase relative to control cells (cells not incubated with drugs). B and C: cellular growth arrest, defined as percentage of cells stained with PI that sized as G₀/G₁ cells, was determined for WT and Bcl-2 S49 cells, as described in Fig. 2 legend, at 24 h (B) or 48 h (C).

this rapid and sustained increase in cAMP, the cells appeared to require ≥6 h to commit to G₁ growth arrest, such that removal of the agonists at that time or earlier allowed cells to reverse cycle arrest, as assessed at 24 or 48 h. By contrast, cells were not able to reverse their commitment to apoptosis: cells incubated with the agonists for 6 h showed substantial cell death, even when assessed up to 42 h later after removal of the agonists (Fig. 3A). Thus the commitment to apoptosis

Fig. 3. Time course of apoptosis and G₁ growth arrest of S49 cells promoted by Iso and IBMX on apoptosis as assessed by flow cytometry. Cells were treated with Iso (200 μM) + IBMX (200 μM) for the indicated time, washed to remove the drugs, and then incubated in fresh medium for up to 48 h. A: apoptosis was determined by annexin V staining, as described in Fig. 1 legend, and expressed as increase relative to control cells (cells not incubated with drugs). B and C: cellular growth arrest, defined as percentage of cells stained with PI that sized as G₀/G₁ cells, was determined for WT and Bcl-2 S49 cells, as described in Fig. 2 legend, at 24 h (B) or 48 h (C).
appears to be more irreversible than is the commitment to G₁ arrest.

The present data show that overexpression of Bcl-2 protects S49 cells from apoptosis, but not from G₁ growth arrest promoted by several types of agents, including a β-adrenergic agonist/phosphodiesterase inhibitor, forskolin, and the glucocorticoid dexamethasone. Such results imply that Bcl-2 must regulate one or more events that occur subsequent to cell cycle arrest of these cells by cAMP (or glucocorticoids) and that G₁ growth arrest and apoptosis in response to these agents are independent events. Because Bcl-2 can protect cells from changes in mitochondrial membrane permeability and probably other events implicated in cell death pathways (5, 6, 10, 20, 22), prolonged G₁ growth arrest is insufficient to cause killing of S49 lymphoma cells and, by inference, other cell types that show cell cycle growth arrest as well as apoptosis. In addition, the data show that Bcl-2 overexpression does not seem to prevent cell culture-dependent growth arrest of S49 cells but can substantially blunt the necrosis that such cells undergo after prolonged culture. Data from other cell systems have suggested that Bcl-2 can protect from necrosis, which may occur by pathways that are different from those mediating apoptosis (15, 16).

The time dependence and reversibility of G₁ growth arrest and apoptosis of S49 cells treated with isoproterenol + IBMX imply that these events are not likely to be the direct result of PKA-mediated phosphorylation of ambiently expressed proteins in the cells. Perhaps more long-term transcriptional events, posttranscriptional events, and/or altered turnover of key proteins are involved. Killing of cells by dexamethasone appears to be somewhat more rapid than that occurring in response to isoproterenol + IBMX. Although these different treatments almost certainly activate different upstream components, it is conceivable that convergence of signaling and/or involvement of “downstream” response pathways are involved in cell death and execution. Future studies are needed to address if and how such different signals converge in the apoptotic responses in S49 cells.

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