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Prolactin-Regulated Apoptosis of Nb2 Lymphoma Cells

pim-1, bcl-2, *and* bax *Expression*

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Lactogen-dependent Nb2 lymphoma cells, widely employed for studying prolactin (PRL) mitogenic mechanisms, are also useful for investigations of apoptosis in T-lineage lymphocytes. Utilizing PRL-dependent Nb2-11 cultures, apoptosis-regulatory genes were evaluated for participation in dexamethasone- (DEX) provoked cell death or its inhibition by PRL. Treatment of lactogen-starved, G_1-arrested Nb2-11 cells with DEX (100 n*M*) activated apoptosis within 12 h evaluated by flow cytometric analysis of fragmented DNA. This effect was not associated with altered expression of *bcl-2*, *bax*, or *pim-1*. PRL (10 ng/mL), coincubated with DEX-treated cells, completely blocked DEX-induced apoptosis. This inhibition was associated with increased expression of *bcl-2* and *pim-1* mRNAs, genes reported to suppress apoptosis, within 2-6 h after addition of the hormone. Moreover, the increased transcription of *bcl-2* and *pim-1* was coupled to increases in their protein levels. The results suggest that *bcl-2*, *bax*, and *pim-1* do not play a critical role in DEX-induced apoptosis in Nb2 cells. However, expression of *bcl-2*, together with *pim-1*, may have a role in mediating the antiapoptotic actions of PRL.

Key Words: Apoptosis; dexamethasone; prolactin; Nb2 cells.

Introduction

Cultured rat pre-T Nb2 lymphoma cell lines have been widely employed to investigate molecular mechanisms mediating mitogenic stimulation by the anterior pituitary hormone, prolactin (PRL). Originally derived from a malignant lymphoma, which developed in an estrogen-treated Noble rat *(1)*, Nb2 cells resemble T lymphocytes at an intermediate stage of differentiation, as indicated by specific enzymatic and antigenic properties *(2)*. As previously demonstrated, the parental Nb2 cell line is critically dependent on PRL (or other lactogens) for maintenance of growth and viability *(1,3)*. Culturing the cells in lactogen-deficient medium for only a limited time (e.g., 18 h) results in their arrest, as a viable population, in the early G_1-phase of the cell cycle. The sole addition of PRL (picomolar concentrations) to such "stationary" cultures reinitiates cell-cycle progression in a partially synchronized fashion *(4)*. As a result, the PRL-dependent Nb2 cell cultures have been extensively utilized for studying mitogenic signaling by PRL coupled to expression of growth-associated genes *(5–11)*.

In addition to the use of Nb2 cells as a model for investigating PRL-dependent cell proliferation, recent evidence by Witorsch et al. *(12–14)* has extended the usefulness of these cells to include studies of apoptosis (programmed cell death). It was demonstrated that, as reported for thymocytes, treatment of lactogen-starved Nb2 lymphoma cells with glucocorticoids, such as dexamethasone (DEX), led to highly increased cell death associated with genomic DNA fragmentation, a hallmark of apoptosis. Moreover, the cytolytic effect of the glucocorticoids was abrogated by the addition of PRL, suggesting a role for growth factor action in the regulation of apoptosis in this model.

Apoptosis, a form of programmed, cellular self-destruction, is distinct from accidental, necrotic cell death, and characterized by a number of specific morphological and biochemical changes, including nuclear condensation and fragmentation, apoptotic body formation, protease (caspase) activation, as well as cleavage of genomic DNA *(15–17)*. Hydrolysis of DNA reflects activation of endogenous endonucleases presumably by apoptotic stimuli-increased caspase activity *(18)*. Apoptosis is considered instrumental in the elimination of undesirable cells produced during normal development of multicellular organisms; it can also occur in cells during inflammatory and other

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physiological or pathological responses *(15,16)*. Importantly, apoptosis has been demonstrated to be a gene-regulated process, and a number of genes have been identified as molecular mediators of either activation or inhibition of the cell death program.

A key, apoptosis-associated protooncogene, *bcl-2*, was originally identified in lymphoid neoplasia *(19–21)* and is considered to be the founding member of a family of homologous, apoptosis-regulating proteins *(22,23)*. *Bcl-2* has been well characterized as a suppressor of apoptosis in hematopoietic and other systems. Importantly, overexpression of *bcl-2* prolongs cell survival, but apparently does not lead to increased cell division *(24–26)*. It is notable that the mitogenic and viability-supporting action of PRL in Nb2 lymphoma cells *(1,3)* is associated with rapid induction of *bcl-2* expression *(27)*.

The protein product of the *bcl-2* gene, a 26-kDa membrane-associated protein, can interact with itself, or with other homologous protein family members via dimerization involving three highly conserved regions of the protein, i.e., Bcl-2 homology domains BH1, BH2, and BH3 *(28,29)*. The interaction of the Bcl-2 protein with Bax, a 21-kDa homologous protein family member thought to promote apoptosis, appears to be an important factor governing the apoptotic process. Accumulated evidence to date suggests that the survival of cells, receiving an apoptotic stimulus, is determined by the relative amounts of Bcl-2 and Bax proteins present in the cells: relatively high concentrations of Bax promote cell death, whereas elevated levels of Bcl-2 favor cell survival *(22)*.

Pim-1 is a protooncogene that encodes a conserved serine/threonine kinase that can be induced in myeloid and lymphoid cells, including the Nb2-11 line, by cytokines, such as interleukin 2 *(30–32)*, and PRL, a cytokine-like factor *(11)*. Whereas *pim-1* may have a role in the progression of cells through the cell cycle *(33)*, various studies have indicated that it may also have a function in the regulation of apoptosis. Thus, overexpression of this kinase in transgenic *lpr*/*lpr* (lymphoproliferation locus) mice has been reported to inhibit DEX-induced apoptosis of thymocytes *(34)*. However, other studies have shown that overexpression of *pim-1* leads to increased DNA fragmentation in mouse NS-1 myeloma cells, suggesting that in these cells, *pim-1* may act as an activator of apoptosis *(35)*.

In the present study, the apoptosis-enhancing effect of glucocorticoids in lactogen-starved Nb2 lymphoma cells, and the antiapoptotic effect of PRL, have been further evaluated. Results are presented suggesting that *pim-1* and *bcl-2*, but not *bax* may mediate the antiapoptotic effect of the hormone.

Results

DEX-Enhanced Apoptosis: Antiapoptotic Effect of PRL

Nb2-11 cells were lactogen-starved by an 18-h incubation in lactogen-free medium and then incubated for a further 12 h with DEX in the range 10–1000 n*M*. The apoptotic effect of the DEX treatment on the stationary cells is presented in Figs. 1B and 2A. DEX caused a concentration-dependent increase in the percentage of apoptotic cells, with the effect leveling off at 100 n*M* DEX and a total increase of about 100% relative to the control (no DEX). Notably, 13% of the control cells, incubated in lactogen-free medium for a total of 30 h, exhibited fragmented DNA. This finding is consistent with the induction of apoptosis observed in other growth factor-dependent T-cell systems following growth factor (cytokine) starvation *(39)*.

To evaluate the antiapoptotic effect of PRL, the hormone (0.01–10 ng/mL) was added to lactogen-starved Nb2

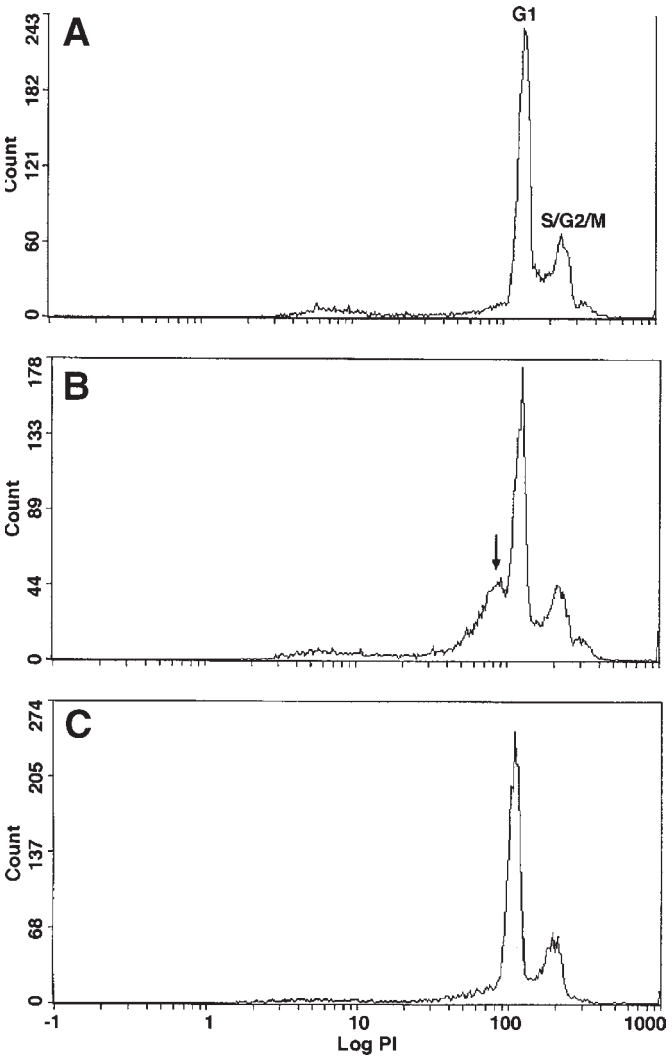

Fig. 1. Flow cytometric analysis of Nb2 lymphoma cells. Stationary Nb2-11 cells (5×10^5 cells/mL) were incubated for 12 h with DEX or DEX (100 n*M*) plus PRL (10 ng/mL) and then evaluated for the presence of apoptotic cells by flow cytometry (*see* Materials and Methods). **(A)** Control cultures; **(B)** cells incubated with DEX, apoptotic cell population indicated (arrow); and **(C)** cells treated with DEX and PRL. Note the relative absence of apoptotic cells. Representative DNA histograms from an experiment replicated at least five times are presented.

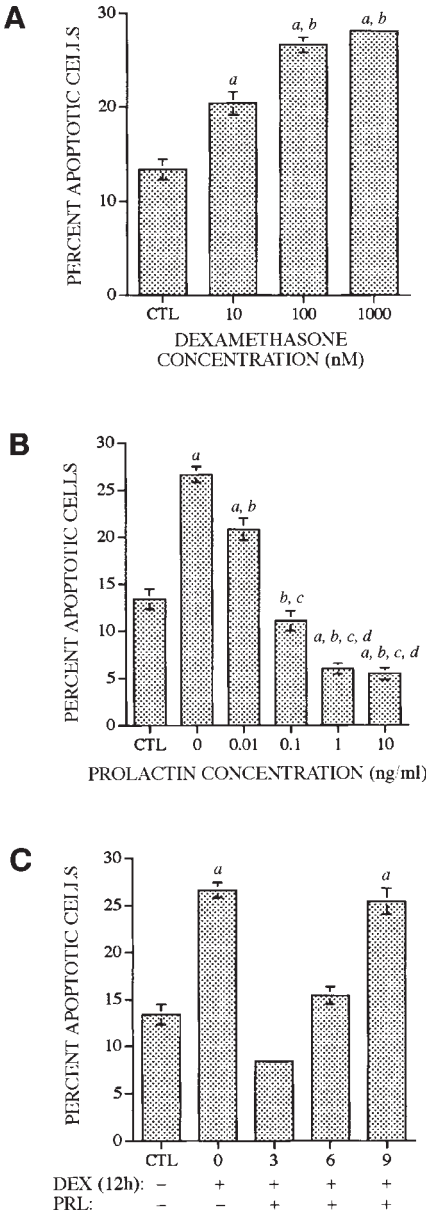

Fig. 2. DEX-induced apoptosis in lactogen-starved Nb2 lymphoma cell cultures: antiapoptotic effect of PRL. Stationary Nb2-11 cells (5×10^5 cells/mL) were incubated for 12 h with DEX (100 n*M*) and then analyzed for apoptosis by flow cytometry (*see* Materials and Methods). **(A)** Effects of DEX at increasing concentrations (a: $p < 0.001$ vs CTL; b: $p < 0.001$ vs 10 n*M* DEX); **(B)** effects of increasing concentrations of PRL added to cultures at the same time as DEX (a: $p < 0.001$ vs CTL; b: $p < 0.001$ vs 0; c: $p < 0.01$ vs 0.01 ng/mL PRL; d: $p < 0.01$ vs 0.1 ng/mL PRL); **(C)** effect of PRL (10 ng/mL) added to cultures at various hours after addition of DEX (a: $p < 0.001$ vs CTL). Data are presented as means ± SE from three separate experiments. CTL = controls (no DEX, no PRL).

cells at the same time as DEX (100 n*M*) for a 12-h incubation and subsequent flow cytometric DNA fragmentation analysis. As shown in Figs. 1C and 2B, the addition of PRL led to a concentration-dependent decrease in the amount of apoptosis induced by DEX. Even relatively low concentrations of PRL (e.g., 0.1 ng/mL), which led to only a modest proliferative response in Nb2 cells *(40)*, significantly ($p < 0.001$) reduced the DEX-induced apoptosis. Maximally mitogenic concentrations of PRL (1 and 10 ng/mL) completely blocked the cytolytic action of DEX. In addition, these two PRL concentrations reduced the apoptosis to levels that were significantly ($p < 0.001$) lower than those found in control cultures (no Dex, no PRL). This indicates that there was an increase in apoptosis in the control cultures from 18 to 30 h resulting from the absence of lactogen, which, as expected, was blocked by the addition of PRL. It appears from these results that after 12 h of DEX treatment, the apoptosis in the lactogen-starved Nb2 cell cultures was mainly owing to glucocorticoid action and to a minor extent to lactogen starvation. Notably, both effects were blocked by PRL.

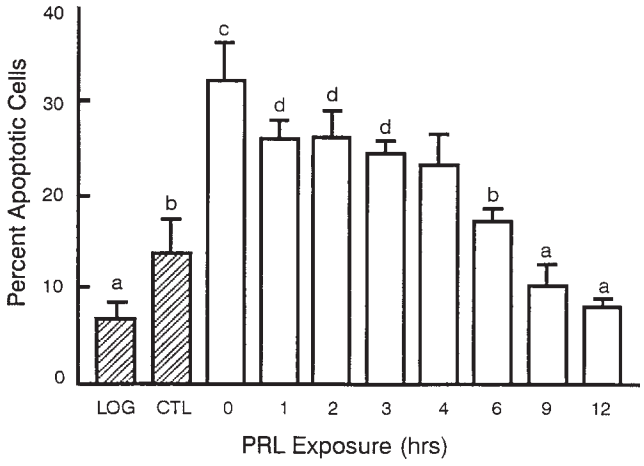

Fig. 3. Temporal relationship between PRL exposure and its suppression of DEX-induced apoptosis. Nb2-11 cultures (1×10^6 cells/mL) were treated with DEX (100 n*M*) or DEX + PRL (10 ng/mL). At the times indicated, cells were washed and resuspended in PRL-free, DEX-containing medium. The percentage of cells undergoing apoptosis was determined by flow cytometry at 12 h as described in Materials and Methods. Open bars represent DEX-only (time 0) and DEX + PRL (1–12 h). Crosshatched bars represent exponentially proliferating (LOG) and untreated (CTL) cells also evaluated at 12 h. Results obtained from an experiment replicated three times are presented as means ± SE. a: $p < 0.001$ vs DEX-only (time 0); b: $p < 0.01$ vs DEX-only (time 0); c: $p < 0.01$ vs CTL; d: $p < 0.05$ vs CTL.

In a subsequent experiment, PRL (10 ng/mL) was added to lactogen-starved Nb2 cell cultures at 3, 6, or 9 h following addition of DEX (100 n*M*). As shown in Fig. 2C, addition of PRL 9 h after the addition of DEX did not change the percentage of apoptotic cells found at 12 h. In contrast, addition of the hormone at 3 or 6 h after DEX administration led to a significant ($p < 0.001$) decrease in apoptosis. In a companion experiment, cultures were continuously exposed to DEX for 12 h in the presence of PRL for 1–12 h (Fig. 3). At the times indicated, cultures were washed free of PRL and resuspended in DEX-containing medium; the level of apoptosis was determined after 12 h by flow

cytometry. As shown, exposure to PRL for 6–12 h after the addition of DEX significantly reduced the percentage of cells undergoing apoptosis. However, PRL stimulation for <6 h failed to suppress DEX-induced cell death. These results suggest that the protection conferred by PRL most likely reflects its alteration of biochemical/molecular events that are stimulated by DEX within 6 h of exposure to the steroid.

Effects of DEX and PRL on pim-1, bcl-2, *and* bax *Gene Expression*

In previous studies we demonstrated that the proto-oncogenes, *pim-1* and *bcl-2*, represent immediate early genes, the expression of which was markedly increased within 1 h of PRL addition to stationary Nb2-11 cells *(11,27)*. In contrast, PRL provoked a gradual and only moderate increase in *bax* mRNA accumulation, followed by a significant reduction in the expression of the Bax protein within 2-6 h *(27)*. Since the expression of these apoptosis-associated genes in Nb2 cells had been shown to be sensitive to PRL, the effects of DEX alone and DEX + PRL were evaluated in Figs. 4 and 5.

As shown in Figs. 4A, B, and C, incubating stationary Nb2-11 cultures with DEX (100 n*M*) for up to 12 h did not lead to significant alterations in the relative amounts of *bcl-2*, *bax*, or *pim-1* mRNA expressed by the cells in the absence of DEX (at 0 h). This suggests that the DEX-mediated apoptosis is not dependent on altered transcription of these genes. In contrast, addition to the lactogen-starved cultures of both DEX and PRL (at an antiapoptotic concentration of 10 ng/ml; *see* Figs. 1C and 2B) led to substantial accumulations of *pim-1* and *bcl-2* transcripts within 2 h compared to the 0-h values. Maximal levels of the transcripts were attained by 3 h, showing fivefold elevated levels for *bcl-2* transcripts ($p < 0.05$) and 15-fold elevated levels for *pim-1* transcripts ($p < 0.05$). Moreover, the combination of DEX + PRL also led to a significant ($p < 0.05$), but only slight increase in *bax* mRNA accumulation from 6–12 h (Fig. 4B).

Shown in Fig. 5 are autoradiographs demonstrating the effect of PRL + DEX to augment *bcl-2* (5A), *bax* (5B), and *pim-1* (5C) mRNA expression. Ethidium bromide staining of 28 and 18S rRNA, to verify equal total RNA loading per lane, is presented in Fig. 5D. The results presented in Figs. 4 and 5 demonstrate that whereas treatment of the lactogen-starved Nb2 cultures with DEX alone had no effect on *pim-1*, *bcl-2*, or *bax* gene expression, addition of antiapoptotic (mitogenic) concentrations of PRL to the DEX-treated cultures markedly elevated *bcl-2* and *pim-1* mRNAs.

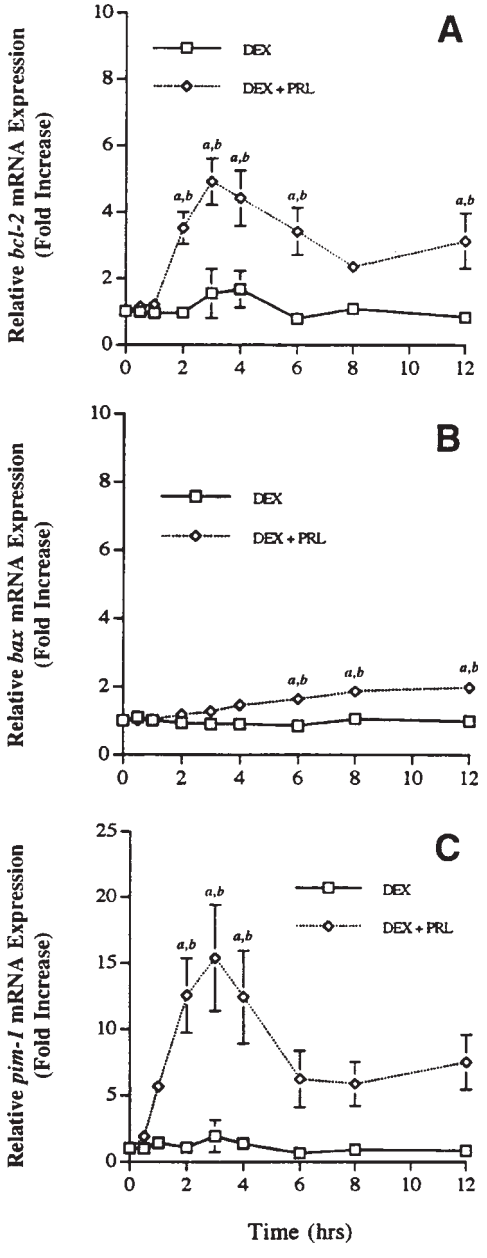

Fig. 4. Relative levels of *bcl-2*, *bax*, and *pim-1* mRNA in Nb2-11 cells as a function of time after addition (at 0 h) of DEX (100 n*M*), or DEX + PRL (10 ng/mL), to stationary cultures (2×10^7 cells/time-point). Northern blotting of total RNA (15 μg/lane) was conducted as described in Materials and Methods, using ^{32}P-labeled complementary DNA (cDNA) probes, which recognize rat *bcl-2* **(A)**, *bax* **(B)**, and *pim-1* **(C)** transcripts followed by densitometric analysis. Data are presented as the means ± SE from three separate experiments (a: $p < 0.05$ vs control, b: $p < 0.05$ vs DEX treatment at the same time-point). When not visible, error bars are obscured by the symbols.

Effects of PRL on Expression of Bax, Bcl-2, and Pim-1 Proteins

Since antiapoptotic PRL concentrations in the DEX-treated Nb2 cell cultures led to marked increases in the accumulations of the *bcl-2* and *pim-1* transcripts (Figs. 4 and 5), it was important to determine whether those increases were coupled to enhanced levels of the translated proteins. The time-course (0–12 h) for the effect of PRL (10 ng/mL) on Bcl-2, Bax, and Pim-1 protein expression in Nb2-11 cultures treated with DEX (100 n*M*) is shown in Fig. 6. The addition of both PRL and DEX to the lactogen-

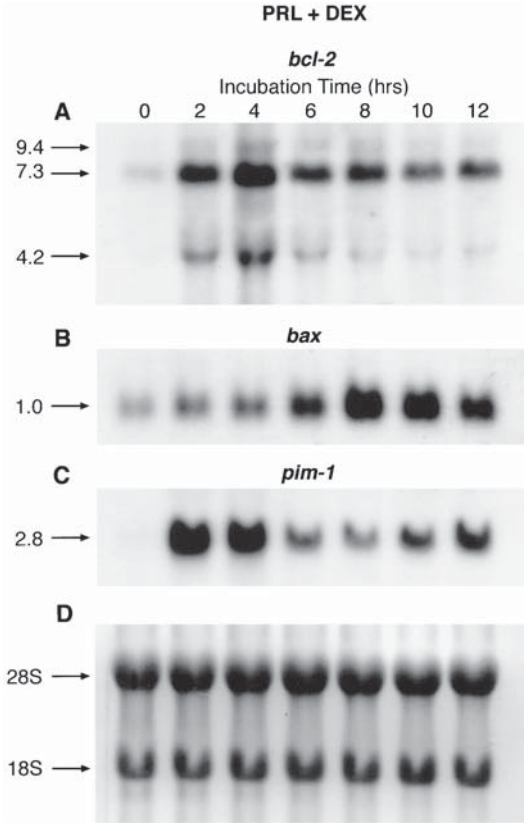

Fig. 5. Effect of DEX + PRL on *bcl-2*, *bax*, and *pim-1* mRNA accumulation in Nb2-11 cells. Stationary cultures (2×10^7 cells/time-point) were treated with DEX (100 n*M*) + PRL (10 ng/mL) and harvested at the times indicated. Northern blot analysis of total RNA (15 μg/lane) was conducted as described in Materials and Methods, using ^{32}P-labeled complementary DNA (cDNA) probes that recognize rat *bcl-2* **(A)**, *bax* **(B)**, and *pim-1* **(C)** transcripts. Equal loading of total RNA **(D)** was verified by ethidium bromide staining of the gels followed by densitometric analysis. A representative result of an experiment performed at least three times is presented.

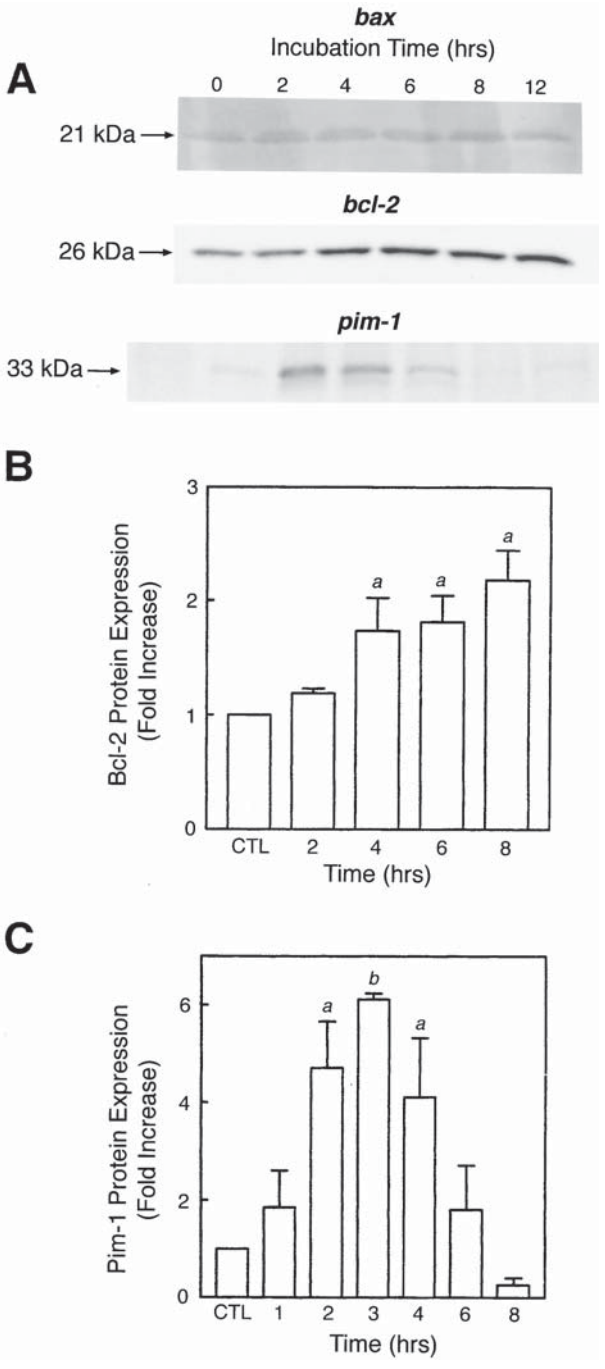

Fig. 6. Relative levels of Bax, Bcl-2, and Pim-1 protein **(A)** in Nb2-11 cells as a function of time after addition (at 0 h) of DEX (100 n*M*) plus PRL (10 ng/mL) to stationary cultures (2.5×10^7 cells/time-point). Graphic representation of Bcl-2 and Pim-1 protein expression is presented (**B** and **C**, respectively). Levels of Bcl-2 and Bax proteins were assessed by immunoblot analysis using cell lysates (100 μg/lane) and of Pim-1 protein by immunoprecipitation, as described in Materials and Methods. A representative autoradiograph obtained from an experiment repeated three times is presented.

starved cultures did not alter the level of Bax (Fig. 6A). However, increased expression of Bcl-2 and Pim-1 proteins was provoked by the combined treatment (Fig. 6B and C). The level of Bcl-2 was enhanced twofold ($p < 0.05$) within 4 h, whereas significantly ($p < 0.05$) increased Pim-1 was observed by 2 h. Thus, the significant elevation of *bcl-2* and *pim-1* mRNA accumulation, but not that of *bax* was coupled to rapid increases of their protein products. These results suggest that the two protooncogenes previously linked to suppression of apoptosis, i.e., *bcl-2* and *pim-1*, both may have a role in the mediation of the antiapoptotic action of PRL in glucocorticoid-treated Nb2 cell cultures.

Discussion

Results from the present study confirm and extend the initial observations by Witorsch et al. *(12,13)* that DEX (and other glucocorticoids) markedly enhance apoptosis in lactogen-starved, PRL-dependent Nb2 lymphoma cells and that addition of PRL can protect the cells from the cytolytic action of the steroid. Moreover, studies of the temporal relationships among DEX-provoked cytolysis and PRL-induced expression of positive and negative regulators of

the cell death program indicate that growth factor stimulation leads to enhanced mRNA accumulation and translation of proteins that suppress apoptosis.

To clarify mechanisms that underlie DEX-activated cell death and the protective effect of PRL, we evaluated the expression of genes associated with apoptosis in lymphocytes and other systems at the mRNA and protein levels. In previous studies, we demonstrated that expression in Nb2-11 cells of both *bcl-2* and *bax* mRNAs, and their encoded proteins, is regulated by PRL *(27)*. Addition of PRL to stationary cultures markedly induced *bcl-2* mRNA expression within 3 h, an effect that was independent of protein synthesis *(27)*. Moreover, mitogenic stimulation also led to a twofold elevation in the Bcl-2 protein by 6 h. Importantly, coincident with increased Bcl-2 protein levels, PRL provoked a 50% decrease in the expression of Bax protein. Together, these observations suggest that the mitogenic and viability-supporting action of PRL in the Nb2-11 cells is accompanied by an overall increase in the Bcl-2/Bax protein ratio *(27)*, which is likely critical for cell survival *(22)*.

In the present study, we analyzed the expression of *bcl-2* and *bax* mRNA and protein in DEX-treated, lactogen-starved Nb2-11 cultures to determine whether the glucocorticoid caused a decrease in the Bcl-2/Bax protein ratio favoring induction of apoptosis. Interestingly, there were no changes in *bcl-2* or *bax* mRNA, or protein, in the cultures exposed to the glucocorticoid for 12 h in the absence of lactogen, suggesting that DEX-induced apoptosis in Nb2 cells is independent of these genes. In examining the antiapoptotic action of PRL, it was observed that addition of PRL to DEX-treated cultures evoked a rapid, fivefold increase in the level of *bcl-2* mRNA and only a modest elevation in *bax* transcripts (Figs. 4B and 5B). Moreover, the increase in the levels of *bcl-2* mRNA was found to be coupled to increased protein levels (Fig. 6). Therefore, these results suggest that the antiapoptotic action of PRL in DEX-treated Nb2 cells is mediated by a mechanism that reflects augmented *bcl-2*, but is independent of altered *bax*. In this setting, the relative Bcl-2:Bax protein ratio would favor suppression of apoptosis.

The observed lack of change in intracellular Bcl-2 and Bax protein levels, following induction of apoptosis, has also been demonstrated in other systems. Broome and coworkers *(39)* analyzed the relative levels of Bcl-2 and Bax proteins at various times after withdrawal of IL-2 from activated splenic T lymphocytes, an event leading to apoptosis. After withdrawal of the cytokine, the level of Bcl-2 did not decrease before the T cells underwent apoptosis. In contrast, the level of Bcl-x protein, a Bcl-2 homolog and inhibitor of apoptosis, decreased by about 50% within 12 h of IL-2 deprivation *(39)*. These observations indicate that the *bcl-2* gene does not appear to play a central role in glucocorticoid-induced apoptosis in Nb2 cells. However, it is possible that other *bcl-2* homologs may be involved.

Previous studies have also shown that PRL regulates expression in Nb2-11 cells of *pim-1*, another apoptosis-associated protooncogene *(11)*. Administration of PRL to stationary Nb2-11 cells produced a rapid, 40-fold increase in *pim-1* mRNA levels, an effect found to be independent of protein synthesis. Since *pim-1* has previously been reported to suppress *(34)* and in another study to activate apoptosis paradoxically *(35)*, we assessed whether DEX-induced apoptosis in Nb2 cells was associated with an alteration in the levels of *pim-1* mRNA or protein. Similar to the findings with *bcl-2* and *bax* expression, DEX had no detectable effect on the *pim-1* mRNA and protein levels in the lactogen-starved Nb2-11 cultures. However, addition of PRL to the DEX-treated cultures led to a 15-fold increase in the level of the *pim-1* transcript (Fig. 4C). Most importantly, the increased *pim-1* mRNA accumulation was accompanied by significantly increased levels of Pim-1 protein (Fig. 6). Although correlative, these results suggest that *pim-1*, together with *bcl-2*, may mediate the antiapoptotic action of PRL in Nb2 cells. Furthermore, the close association of both oncoproteins suggests that they may function in concert as general inhibitors of hematopoietic cell apoptosis.

In this article, we demonstrate that addition of PRL to DEX-treated, lactogen-starved Nb2-11 cell cultures rapidly induced *pim-1* and *bcl-2* mRNA as well as protein expression, which was temporally associated with inhibition of apoptosis in this paradigm. The results also suggest that *bax* may not function as a critical regulator of DEX-induced apoptosis of the Nb2 cells. In addition, alterations in this apoptosis-associated gene may not mediate the protective effect of PRL in DEX-treated Nb2 cells. Further studies are required to define fully whether the Pim-1 kinase plays a functional role in the antiapoptotic effect of PRL in Nb2 lymphocytes.

Materials and Methods

Hormones, Antibodies, and Other Supplies

Ovine PRL (NIDDK oPRL-20, AFP10677C) was obtained through the NIH Pituitary Hormone and Antisera Program (Rockville, MD). A mouse (600 bp *Xho*I/*Hin*dIII probe) *bcl-2* complementary DNA (cDNA) was generously provided by T. J. McDonnell (Houston, TX). The mouse (600 bp *Eco*RI probe) *bax* cDNA and Bax antisera utilized have been previously described *(27)*. Antiserum to Pim-1 protein was generated against a recombinant glutathione *S*-transferase-Pim-1 fusion protein as described *(11)*. Bcl-2 antiserum (catalog no. sc-492), which specifically recognizes mouse, rat, and human Bcl-2 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Unless otherwise specified, all other reagents were of molecular biology grade and obtained from Sigma (St. Louis, MO).

Nb2 Lymphoma Cell Cultures

The PRL-dependent cell line, Nb2-11, originally cloned in H. G. Friesen's laboratory (Winnipeg, MB, Canada) was utilized. Nb2-11 cultures were maintained at 37°C in Fischer's medium containing 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD) as a source of lactogen, 10% horse serum (BioWhittaker), 2-mercaptoethanol (2-ME, 10^{-4} *M*), penicillin (50 U/mL), and streptomycin (50 μg/mL), as described previously *(1,3)*. For various experiments, stationary cell cultures were obtained by an 18-h preincubation of the cells in lactogen-free medium, i.e., Fischer's medium supplemented with 2-ME, antibiotics, and 10% nonmitogenic gelding serum (ICN, Irvine, CA).

Flow Cytometric Analysis of Apoptosis

The percentage of apoptotic cells in a population was assessed using flow cytometric analysis of propidium iodide-stained cell nuclei for the detection of DNA fragmentation, as described by Nicoletti et al. *(36)* and shown in Fig. 1. Briefly, aliquots of 1–2 × 10^6 cells were centrifuged at 200*g* and fixed by resuspension in 2 mL cold 70% ethanol. The cells were then washed by centrifugation, resuspension in phosphate-buffered saline (PBS), and then resuspended in 1.0 mg/mL RNase. After 1 h at 37°C, the cells were washed twice in PBS and finally resuspended in a hypotonic propidium iodide solution (50 μg/mL). The stained cells were analyzed using an Elite flow cytometer (Coulter Electronics, Hialeah, FL) with the 488-nm line of an enterprise laser (Coherent, Palo Alto, CA). Red fluorescence of the stained cells was monitored through a 600-nm dichroic and a 610 LP filter and collected in a three-decade log histogram. Forward scatter and side scatter were simultaneously measured. Time-of-flight measurement was used to exclude cellular debris and clumps. All samples were evaluated using the same instrument settings. Cells undergoing apoptosis represent a hypodiploid population as shown in Fig. 1B (arrow).

Northern Blotting Procedures

As previously described *(27)*, total RNA was isolated from 2 × 10^7 Nb2-11 cells cultured in 25-cm^2 flasks using RNAzol-B (Tel-Test, Friendswood, TX). The RNA was quantitated spectrophotometrically. For Northern analysis, RNA was denatured in formaldehyde and fractionated on 1% agarose gels, and then transferred to GeneScreen Plus (DuPont, Wilmington, DE). Equal loading per lane was verified by ethidium bromide staining of 18S and 28S ribosomal RNA, which was visualized and photographed under UV illumination. For evaluation of *pim-1*, *bcl-2*, and *bax* mRNA, isolated inserts were labeled with [^{32}P]deoxy-CTP (New England Nuclear, Boston, MA) using the random primer method of Feinberg and Vogelstein *(37)*. Hybridization and wash procedures were conducted using the methods of Church and Gilbert *(38)*.

Immunoprecipitation of [^{35}S]Pim-1 Protein

Prior to experiments, stationary Nb2-11 cells were washed free of extracellular methionine (MET) using MET-free RPMI 1640 (Gibco-BRL, Gaithersburg, MD), containing 2% gelding serum (dialyzed to remove MET), antibiotics, and 2-ME, and further incubated at a density of 5 × 10^6 cells/mL. Following addition of DEX, or DEX + PRL, and further incubation for selected time periods, the cultures were pulse-labeled for 2 h with 100 μCi/mL [^{35}S]MET prior to harvesting, then disrupted in radioimmunoprecipitation assay (RIPA) buffer containing 50 m*M* Tris (pH 8.0), 250 m*M* NaCl, 2 m*M* EDTA, 1% Nonidet P-40, 0.25% deoxycholate, 1 m*M* phenylmethylsulfonylfluoride (PMSF), and 10 μg/mL each of chymostatin, leupeptin, and aprotinin. The lysates were centrifuged for 20 min at 14,000*g* at 4°C. Sodium dodecyl sulfate (SDS) was added to the resulting supernatants to a final concentration of 0.1%. The samples were precleared by the addition of 6 μL normal rabbit serum (Sigma) and 25 μL recombinant protein G-agarose (Gibco-BRL) followed by incubation for 2 h at 4°C. After centrifugation, supernatant aliquots were precipitated with trichloroacetic acid, and [^{35}S]Met incorporation was determined by scintillation counting. Lysates (1–1.5 × 10^8 cpm) were incubated overnight at 4°C in the presence of 6 μL anti-Pim-1 antiserum and 25 μL protein G–agarose with continuous mixing. The immunoprecipitates were washed five times with RIPA buffer, resuspended in SDS-sample buffer (containing 10% 2-ME), and boiled for 10 min. Immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using 10% gels. The gels were stained, incubated with Enhance (DuPont), dried, and exposed to X-ray film for 1–3 d *(27)*.

Immunoblot Analysis of Bcl-2 and Bax Protein

Nb2-11 cells (2.5 × 10^7) were incubated for various time periods after addition of DEX and DEX + PRL. The cells were rapidly cooled in an ice bath and then resuspended in 4°C lysis buffer containing 10 m*M* Tris-HCl (pH 7.4), 0.15 *M* NaCl, 5 m*M* EDTA, 1% Triton X-100, 1 m*M* PMSF, and 25 μg/mL each of leupeptin and aprotinin. The lysates were centrifuged for 10 min at 14,000*g* at 4°C. Total protein was determined using the Bradford reagent (Bio-Rad, Hercules, CA). Lysates (100 μg total protein) were fractionated by SDS-PAGE using 10% gels and electrophoretically transferred to Immunolite blotting membrane (Bio-Rad). Membranes were blocked overnight at 4°C in 5% nonfat dried milk in Tris-buffered saline. Bcl-2 and Bax proteins were visualized by first incubating the membranes in the presence of antibodies (1/100 and 1/1500, respectively), followed by chemiluminescence detection using a secondary antibody (1/3000) coupled to alkaline phosphatase and a chemiluminescence substrate (Bio-Rad). The membranes were exposed to X-ray film for 5–15 min *(27)*.

Data Analysis

Data are presented from experiments repeated at least three times. For Northern blotting procedures, equal loading per lane was verified by densitometric analysis of ethidium bromide-stained 18S and 28S ribosomal RNA. Statistical analysis was performed by ANOVA followed by the Student Newman-Kuel's post-test for multiple comparisons. Where applicable, data are presented as means ± SE.

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