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Prolactin-Stimulated X-Linked Inhibitor of Apoptosis Protein Expression During S Phase Cell Cycle Progression in Rat Nb2 Lymphoma Cells

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The rat Nb2 lymphoma is useful for studying prolactin (PRL) receptor signaling to mitogenesis and apoptosis suppression. Previous results showed that PRL rapidly induced expression of several apoptosis suppressor genes during the G1 phase of cell cycle in this model. The X-linked inhibitor of apoptosis protein (XIAP) gene product acts to suppress apoptosis by direct inhibition of caspases. The present study was conducted to determine whether PRL alters *XIAP* expression in lactogen-dependent Nb2-11 or -independent Nb2-SFJCD1 cells. Stationary Nb2-11 cultures expressed detectable levels of an 8.9-kb *XIAP* transcript. PRL (20 ng/mL) stimulated its expression, reaching maximal levels within 12 h. Expression of *XIAP* was also evaluated in Nb2-SFJCD1 cells subsequent to treatment with differentiating agents (sodium butyrate [2 m*M*, 72 h], all *trans*-retinoic acid [10 µ*M*, 72 h], or 1,25-dihydroxycholecalciferol [100 n*M*, 24 h]). PRL significantly increased XIAP expression in cells previously treated with these compounds. Further analysis revealed that PRL stimulated XIAP expression during S phase of the cell cycle. To determine whether XIAP suppressed apoptosis, its cDNA was stably transfected into Nb2-11 cells. Compared to controls, cells overexpressing XIAP exhibited substantially less DNA fragmentation when apoptosis was induced by PRL deprivation or glucocorticoids. We conclude that PRL-stimulated *XIAP* expression likely serves to suppress apoptosis as cells progress through the later phases of the cell cycle.

Key Words: Prolactin; X-linked inhibitor of apoptosis; cell cycle; Nb2 lymphoma cells; apoptosis.

Introduction

Rat Nb2 lymphoma cell lines are widely used to study mechanisms underlying PRL receptor signaling in T-lymphocytes. Originally derived from a malignant lymph node that arose in an estrogen-treated Noble rat *(1)*, these cells morphologically and biochemically resemble T-lymphocytes in an intermediate stage of differentiation *(2,3)*. Early studies indicated that the parental Nb2 cell line was highly dependent on PRL for stimulation of growth and survival *(1)*.

Culturing PRL-dependent Nb2 cells in lactogen-deficient medium for 18–24 h leads to cell-cycle arrest in the early G1 phase of the cell cycle *(4)*. However, prolonged culturing in the absence of hormone induces apoptosis, a process that is reversed by readdition of PRL *(5–7)*. This characteristic of PRL-dependent Nb2 cells has led to their use as a model to investigate mechanisms underlying PRL suppression of apoptosis *(1,8)*.

In addition to PRL-dependent cell lines, several autonomous Nb2 sublines have been developed by culturing in lactogen-deficient medium and cloning of surviving cells *(9)*. The Nb2-SFJCD1 cell line produced in this manner grows in the complete absence of PRL *(9)* and resists activation of apoptosis *(6)*. However, treatment with differentiating agents such as sodium butyrate (NaBT) transiently reverts the cells to a lactogen-dependent phenotype *(9)*. Thus, NaBT-treated Nb2-SFJCD1 cells transiently require PRL for proliferation, and in its presence, resist apoptosis *(9)*.

A potential role for PRL as a physiologic suppressor of apoptosis in immune cells represents an emerging avenue of investigation. In Nb2 T-cells, PRL suppresses cell death provoked by glucocorticoids *(5–7)*, anticancer drugs, and heat shock *(10)*. However, the underlying mechanism of hormonal action is not fully understood.

Previously, we demonstrated that PRL induces several apoptosis-associated genes in lactogen-dependent Nb2-11 cells. Expression of several members of the *bcl-2* family of apoptosis regulatory genes including *bcl-2* and *bax* were markedly altered by the addition of PRL *(11)*. Moreover, PRL induced the expression of *pim-1*, a ser/thr kinase that, like *bcl-2*, inhibits apoptosis *(12–14)*. Therefore, we hypothesized that the antiapoptotic effects of PRL in proliferating cells and in those exposed to cytotoxic agents reflected the combined actions of *bcl-2* and *pim-1 (8)*.

X-linked inhibitor of apoptosis protein (XIAP) is a recently identified member of the highly conserved inhibitor of apoptosis (*IAP*) gene family *(15)*. Originally identified

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in *baculovirus*, IAPs were found to suppress host-cell responses, which allowed cell survival and viral propagation *(16,17)*. The antiapoptotic action of XIAP was shown to reflect its inhibition of at least two caspase family members *(18)*. Moreover, it has been demonstrated that during Fas (CD95)-induced apoptosis, XIAP was cleaved into two fragments, each with distinct caspase inhibitory specificities *(19)*. In addition, it was observed that human malignant glioma cell lines express XIAP and IAPs *(20)*, suggesting that owing to their capacity to inhibit cell death by interfering with caspase catalysis, these proteins may facilitate the development of certain malignancies.

Since PRL suppresses apoptosis induced by disparate activators in Nb2 lymphoma cells, in part, by augmenting the G1 expression of genes that attenuate this process, the present study was conducted to determine whether it similarly affected XIAP levels. The results indicate that PRL increases *XIAP* mRNA accumulation in Nb2 cells coincident with their entry into the S phase of the cell cycle.

Results

PRL-Stimulated XIAP Expression in Nb2-11 Cells

Previously, we demonstrated that PRL stimulated rapid expression of several antiapoptotic genes including *pim-1* and *bcl-2* during the G1 phase in the lactogen-dependent cells *(11,14)*. Since XIAP was recently found to suppress apoptosis in a variety of cell systems, it was of interest to determine whether its expression was also regulated by PRL in Nb2-11 cells. Quiescent cultures were treated with PRL (20 ng/mL) and harvested at various times spanning a 24-h time course. Expression of *XIAP* mRNA was evaluated by Northern blot analysis of total RNA. As shown in Fig. 1A, expression of an 8.9-kb *XIAP* transcript was readily detected in exponentially proliferating cultures (log); its expression was reduced in quiescent cells. The addition of PRL significantly increased *XIAP* by 12 h ($p < 0.05$), reaching maximal levels two- to threefold above those observed in untreated cells, an effect that was sustained through 24 h (Fig. 1A,C). Results from other experiments indicated that the effect of PRL to increase *XIAP* expression was concentration dependent (not shown).

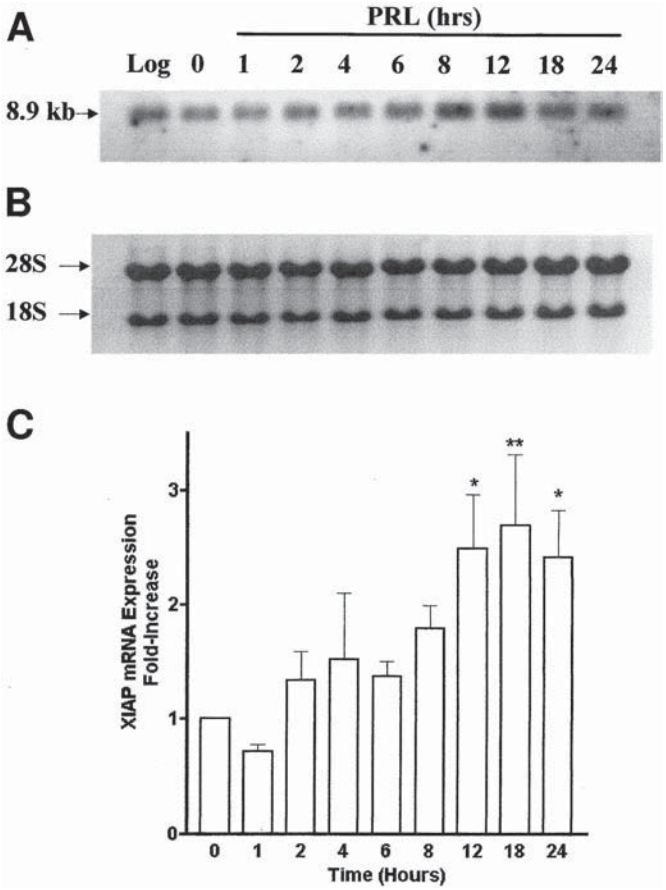

Fig. 1. Expression of *XIAP* in PRL-dependent Nb2-11 cells. Log represents exponentially proliferating cells. Quiescent cultures (0 h) (2×10^7 cells/time point) were stimulated with PRL (20 ng/mL) and harvested at the times indicated. Northern blot analysis of total RNA (20 µg/lane) was performed as described in Materials and Methods using a ^{32}P-labeled cDNA that recognizes the 8.9-kb *XIAP* transcript. (**A**). Representative autoradiograph from an experiment replicated five times; (**B**) ethidium bromide staining of 18S and 28S RNA; (**C**) densitometric analysis. Results are presented as the mean ± SEM of four independent experiments. *$p < 0.05$ vs unstimulated cells (0 h); **$p < 0.01$ vs unstimulated cells (0 h).

PRL-Stimulated XIAP Expression in Differentiating Agent-Exposed Nb2-SFJCD1 Cells

Since PRL had been previously shown to regulate expression of apoptosis suppressor genes during the G1 phase of cell cycle in Nb2-SFJCD1 cultures following their exposure to NaBT *(21)*, the effect of several differentiating agents on hormone-induced *XIAP* expression was investigated in this cell line. In initial experiments, exponentially proliferating Nb2-SFJCD1 cells were exposed to NaBT (2 m*M*). After 72 h, the fatty acid was removed by washing; the cells were further incubated with PRL (20 ng/mL) for an additional 24 h. As shown in Fig. 2, exposure of proliferating cells to NaBT did not appear to affect the constitutive level of the *XIAP* transcript (log vs 0 h; Fig. 2A). However, the addition of PRL increased the level of *XIAP*, which was detectable within 6–8 h with its maximal mRNA expression observed from 6 to 12 h at levels that were sustained through 24 h. Notably, this kinetic pattern for PRL-stimulated *XIAP* expression in Nb2-SFJCD1 cells was similar to that observed in the hormone-dependent line (Fig. 1).

Earlier reports revealed a beneficial effect of the dietary substance retinoic acid (RA) as an adjunct agent in the therapy of certain hematopoietic cancers in combination with cytotoxic drugs owing to its ability to induce differentiation and suppress proliferation *(22,23)*. Since PRL significantly increased *XIAP* expression in the autonomous subline following NaBT-mediated differentiation, the effect

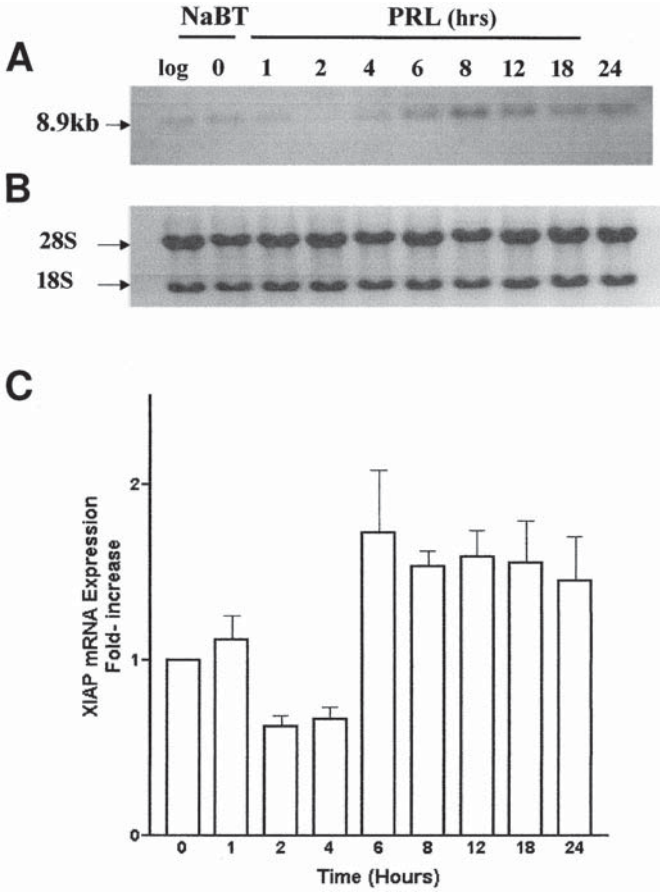

Fig. 2. Effect of PRL on *XIAP* mRNA expression in NaBT–pretreated Nb2-SFJCD1 cells. Exponentially proliferating Nb2-SFJCD1 cells (Log) were cultured in the presence of 2 m*M* NaBT for 72 h. After removal of NaBT, cultures were treated with PRL (20 ng/mL) and harvested at the time points indicated (2×10^7 cells/time point). Northern blot analysis of total RNA (20 µg/lane) was conducted as described in Materials and Methods. (**A**) Representative autoradiograph from an experiment performed five times; (**B**) ethidium bromide staining of 18S and 28S rRNA; (**C**) densitometric analysis. Linear trend analysis indicated that PRL significantly ($p < 0.0001$) altered *XIAP* expression. Zero hours represents cells treated with NaBT alone.

of PRL on its expression was determined in Nb2-SFJCD1 cells subsequent to exposure to RA (10 µ*M*, 72 h). However, prior to determining the effect of the hormone on *XIAP* expression, it was important to determine the PRL responsiveness of this cell line following RA treatment. Cells were treated with the vitamin for 72 h; it was then removed by washing. The cultures were subsequently incubated with increasing concentrations of PRL, and population density was determined, after 48 h, by electronic cell counting. As shown in Fig. 3, PRL stimulated a concentration-dependent increase in proliferation in the cells pretreated with RA. These results demonstrate that pretreatment with RA increases the responsiveness of the lactogen-independent Nb2-SFJCD1 cells to PRL, an effect similar to that observed following NaBT *(21)*.

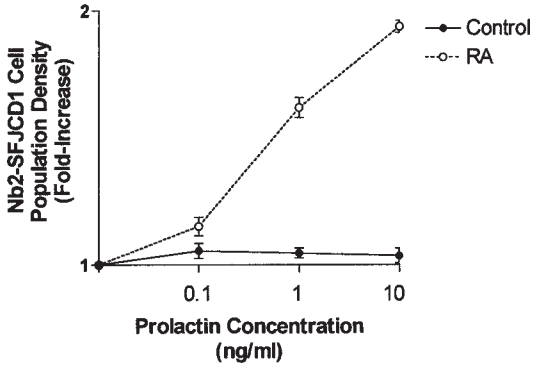

Fig. 3. Effect of PRL on cell population density in Nb2-SFJCD1 cells pretreated with RA. Exponentially proliferating Nb2-SFJCD1 cells were cultured in the presence of RA (10 µ*M*, 72 h). After removal of RA by washing, cells (1.2×10^5 cells/well) were treated with increasing concentrations of PRL. After 48 h, population density was determined by electronic cell counting. Control represents exponentially proliferating Nb2-SFJCD1 cultures without RA treatment. Results are presented as the means ± SEM of triplicate samples from an experiment replicated three times.

Figure 4 shows the effect of PRL on *XIAP* expression in Nb2-SFJCD1 cells previously exposed to RA. As before, RA was removed after 72 h by washing prior to the addition of PRL (20 ng/mL). Exposure to RA significantly ($p < 0.05$) reduced the constitutive expression of XIAP to a level about 50% of that observed in untreated controls. The addition of PRL to these cultures significantly ($p < 0.01$) increased accumulation of the transcript from 12 to 24 h (Fig. 4A,C), which reached maximal levels of fourfold above that observed in control cultures. Thus, just as with NaBT, PRL treatment of Nb2-SFJCD1 cells increased *XIAP* expression coincident with its effect to stimulate proliferation following RA-provoked differentiation.

In other experiments, the effect of PRL was evaluated on *XIAP* mRNA expression in Nb2-SFJCD1 cells that had been previously treated with vitamin D, a third dietary factor that induces differentiation in some types of hematopoietic cells *(24)*. Here, cells were exposed to 100 n*M* of vitamin D for 24 h prior to hormone treatment. Following its removal, the addition of PRL again significantly ($p < 0.0001$) increased *XIAP* mRNA levels following addition of the hormone (Fig. 4D). The maximum expression of *XIAP* mRNA was observed at 12–24 h. However, pretreatment with vitamin D was less efficacious regarding enhancement of the responsiveness of the Nb2-SFJCD1 line to PRL stimulation compared to either NaBT or RA. Nevertheless, each of the differentiating agents rendered the lactogen-independent cultures sensitive to PRL with respect to XIAP expression.

Effect of Dexamethasone + PRL on XIAP Gene Expression

We reported previously that PRL-mediated suppression of glucocorticoid-induced cell death positively correlated

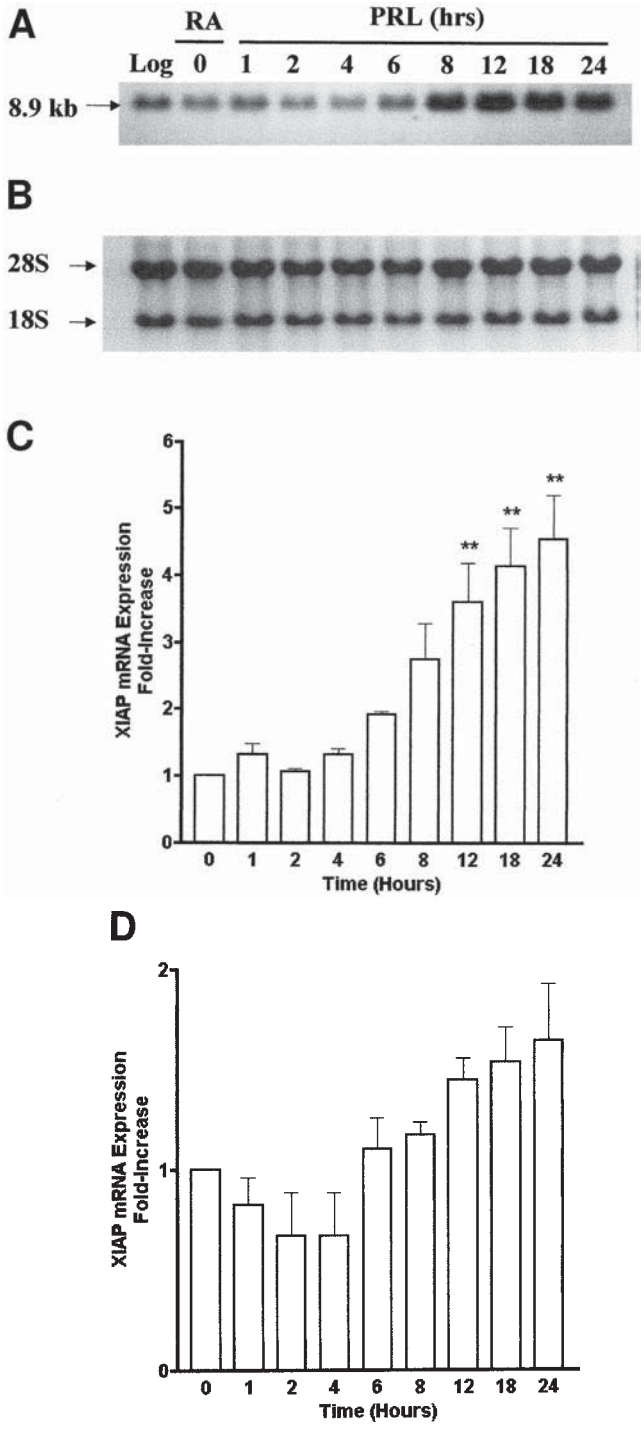

Fig. 4. Effect of PRL on *XIAP* mRNA expression in Nb2-SFJCD1 cells pretreated with RA and vitamin D. **(A–C)** Exponentially proliferating Nb2-SFJCD1 cells (Log) were cultured in the presence of 10 μ*M* all *trans*-RA for 72 h. After removal of RA, cultures were treated with PRL (20 ng/mL) and harvested at the time points indicated (2×10^7 cells/time point). Northern blot analysis of total RNA (20 μg/lane) was conducted as described in Materials and Methods. (**A**) Representative autoradiograph from an experiment performed three times; (**B**) ethidium bromide staining of 18S and 28S rRNA; (**C**) densitometric analysis. $^{**}p < 0.01$ vs 0 h (cells treated with all *trans*-RA alone). (**D**) PRL-stimulated *XIAP* mRNA expression in Nb2-SFJCD1 cells pretreated with vitamin D. Exponentially proliferating Nb2-SFJCD1 cells were cultured in the presence of 100 n*M* of vitamin D for 24 h. After removal of vitamin D, the cultures were treated with PRL (20 ng/mL) and harvested at the time points indicated (2×10^7 cells/time point). Results represent densitometric analysis of four independent experiments. PRL stimulation of *XIAP* mRNA exhibited a significant linear trend with time ($p < 0.0001$). Zero hours represents cells treated with vitamin D.

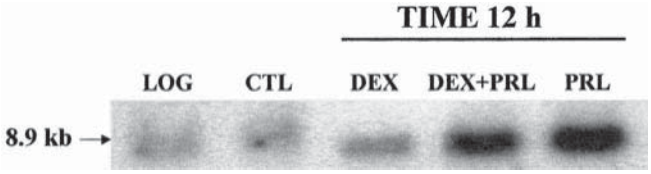

Fig. 5. Effect of DEX on PRL-stimulated *XIAP* mRNA expression. Quiescent cultures (0 h) (2×10^7 cells/time point) were treated with DEX (100 n*M*), PRL (20ng/mL), or DEX + PRL and harvested at the times indicated. Exponentially proliferating cells (Log) were included for comparison. Northern blot analysis of total RNA (20 μg/lane) was performed as described in Materials and Methods using a ^{32}P-labeled cDNA that recognizes the 8.9-kb XIAP transcript. A representative autoradiograph from an experiment replicated four times is shown.

with expression of *bcl-2* and *pim-1 (8)*, suggesting that these oncoproteins may underlie the antiapoptotic actions of the hormone. To determine whether XIAP may also participate in this process, the effect of dexamethasone (DEX) in combination with PRL was assessed on its expression. Stationary Nb2-11 cells were treated with DEX (100 n*M*) or DEX + PRL (20 ng/mL) for 12 h. As shown in Fig. 5, the level of *XIAP* was unaltered by the steroid compared to untreated control cells. However, addition of PRL to the DEX-treated cultures significantly increased its expression. Therefore, together with *bcl-2* and *pim-1*, XIAP may protect Nb2 cells from the cytotoxic effects of glucocorticoids.

PRL-Stimulated XIAP Expression During S Phase of Cell Cycle

In previous studies, we showed that PRL stimulated the entry of Nb2 cells into S phase within 12 h *(14)*. Since hormone-stimulated *XIAP* mRNA accumulation occurred within this time in PRL-treated Nb2-11 cells and in differentiating agent-pretreated Nb2-SFJCD1 cultures, it was of interest to evaluate whether this effect of the hormone was associated with cell-cycle progression. To determine whether increased XIAP expression occurred during S phase, it was necessary to synchronize the cells at the G1/S interface. Aphidicolin (APC), a compound previously shown to arrest cycling cells at G1/S *(25,26)* by inhibition of DNA polymerase α *(27)*, was chosen for these experiments. Initial studies were conducted to determine an efficacious and nontoxic concentration of APC that would block entry of PRL-stimulated Nb2-11 cells into S phase.

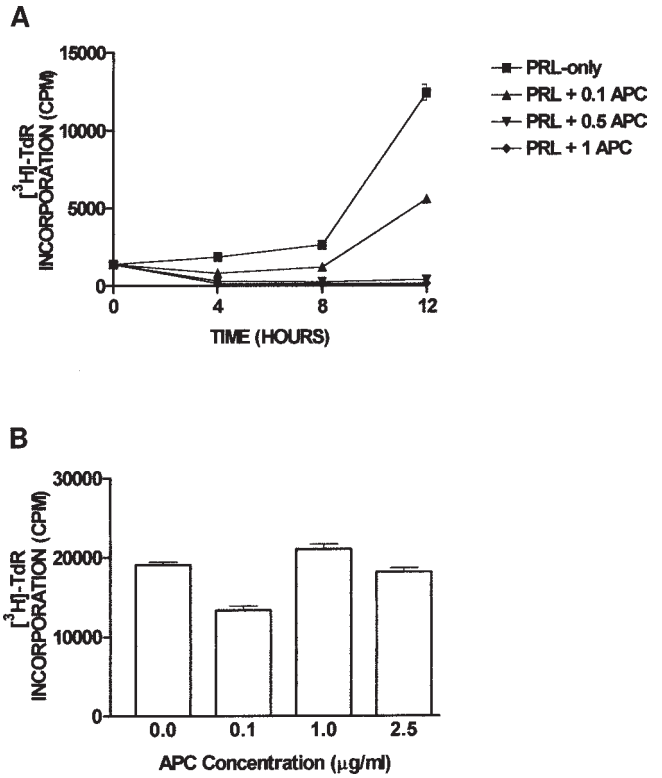

Fig. 6. Effect of APC on [^{3}H]-thymidine incorporation in Nb2 lymphoma cells. (**A**) Quiescent Nb2-11 cultures (1.1×10^5 cells/mL) were treated with varying concentrations of APC + PRL (20 ng/mL). Cells were harvested at the times indicated after pulse labeling at 4-h intervals with 0.5 μCi of [^{3}H]-thymidine. Results are represented as the mean ± SEM of sextuplicate values from an experiment performed three times. (**B**) Quiescent Nb2-11 cultures were incubated in the presence of increasing concentrations of APC + PRL (20 ng/mL). After 12 h, the APC was removed and the cells were cultured with PRL (20 ng/mL). At 20 h, [^{3}H]-thymidine was added and cells were harvested at 24 h. Results are presented as the mean ± SEM of sextuplicate values from an experiment performed twice.

Figure 6A shows the effect of increasing concentrations of APC on [^{3}H]-thymidine incorporation pulsed at 4-h intervals in PRL-stimulated Nb2-11 cells. Quiescent Nb2-11 cells were treated with PRL and varying concentrations of APC, and cells were harvested at the times indicated. Consistent with our earlier observations *(14)*, PRL stimulated entry of cells into S phase within 12 h. A concentration of 0.1 μg/mL of APC was ineffective at blocking entry into S phase whereas concentrations of 0.5 μg/mL and above completely inhibited [^{3}H]-thymidine incorporation.

The reversibility of APC blockade was also evaluated as an index of viability of the arrested cells. Quiescent Nb2-11 cells were treated with APC (0.1–2.5 μg/mL) and PRL (20 ng/mL). After 12 h, the compound was removed by washing, and fresh medium containing only PRL was added. The cells were cultured for an additional 12 h and [^{3}H]-thymidine incorporation was determined. As shown in Fig. 6B, cells treated with APC > 0.5 μg/mL (1 and 2.5 μg/mL) exhibited equivalent [^{3}H]-thymidine incorporation as those treated only with PRL. These results indicated that 0.5 μg/mL of APC was nontoxic to Nb2-11 cells and effectively blocked [^{3}H]-thymidine incorporation prior to their entry into S phase.

Table 1
Cell-Cycle Analysis of APC-Treated Nb2 Lymphoma Cells[a]

	Phase of cell cycle		
Treatment	G1	S	G2/M
Quiescent	77.4 ± 2.6	2.5 ± 0.2	8.6 ± 1.3
12-h PRL	42.5 ± 4.7	43.5 ± 4.8	6.6 ± 0.5
15-h PRL	34.5 ± 1.3	32.1 ± 4.7	26.4 ± 3.3
18-h PRL	62.7 ± 1.6	13.0 ± 1.4	19.9 ± 0.7
APC + PRL-12 h	86.5 ± 1.0	1.4 ± 0.3	9.9 ± 0.9
15-h PRL	25.4 ± 3.5[a]	65.5 ± 4.1[a]	1.9 ± 0.1
18-h PRL	18.6 ± 0.9	27.8 ± 5.7[a]	49 ± 5.8[a]

[a] Quiescent Nb2-11 cells (1×10^7 cells) were incubated with PRL (20 ng/mL) for 12, 15, or 18 h. Cells were harvested, fixed, and cell-cycle analysis was performed by flow cytometry as described in Materials and Methods. Quiescent Nb2-11 cells (1×10^7 cells) were incubated with APC (0.5 μ/mL) and PRL (20 ng/mL) for 12 h. Cells were harvested and fixed after 12 h (APC + PRL-12 h). APC was removed and PRL (20 ng/mL) was readded to other cultures (15- and 18-h PRL). Cells were harvested at the times indicated. Results represent the mean ± SEM of four separate experiments. $p < 0.001$ for G1 (15-h PRL) vs G1 (APC + PRL-12 h); S (15-h PRL) vs S (APC + PRL-12 h); S (18-h PRL) vs S (15-h PRL); G2/M (18-h PRL) vs G2/M (15-h PRL).

To determine more precisely at which point in the cell cycle APC-treated Nb2-11 cells accumulated, quiescent cells were treated with 0.5 μg/mL of APC and PRL (20 ng/mL). After 12 h, the inhibitor was replaced with fresh medium containing only PRL. The cells were harvested at 15 and 18 h, 3 and 6 h, respectively, after the addition of PRL. Cell-cycle progression was evaluated by flow cytometric analysis of propidium iodide–stained cells. Control cultures were treated identically but without the addition of APC. The results presented in Table 1 show the percentage of cells in each phase of the cell cycle following PRL alone, APC, or PRL subsequent to APC removal. In quiescent cultures, 77% of the cells were found to reside in G1 with <3% in S phase, demonstrating that withdrawal of lactogen arrested the cells in the initial phase. The addition of PRL significantly ($p < 0.001$) stimulated entry into S phase, as shown by a nearly 20-fold increase in the percentage of cells in this phase by 12 h (Table 1). By 15 h, a significant ($p < 0.001$) proportion of the cells had progressed to G2/M in control cultures. By contrast, approx 86% of the cells remained in the G1 phase while <2% were synthesizing DNA after 12 h in the presence of APC and PRL. These results were strikingly similar to those observed in quiescent cultures. However, subsequent to the removal of APC, 3 h in the presence

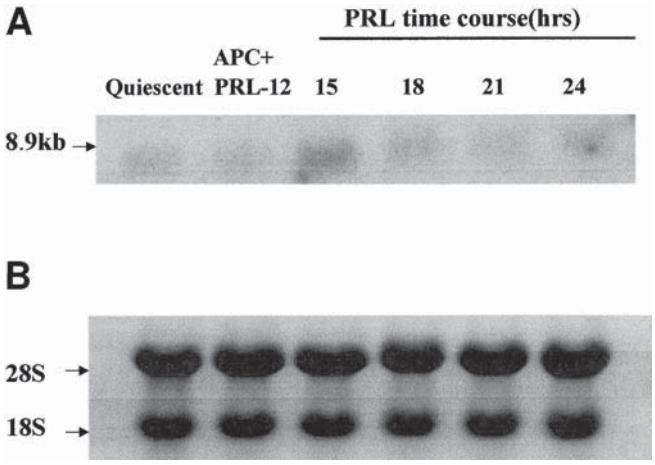

Fig. 7. Effect of PRL on *XIAP* mRNA expression in APC pretreated Nb2-11 cells. Quiescent Nb2-11 cultures were incubated in the presence of 0.5 μg/mL of APC and PRL (20 ng/mL) for 12 h. APC was removed and PRL was readded to the medium. Cells were harvested at the times indicated. (**A**) Representative autoradiograph from an experiment performed four times; (**B**) ethidium bromide staining of 18S and 28S rRNA.

of PRL stimulated nearly a 60-fold increase ($p < 0.001$) in the percentage of cells in S phase, indicating that the APC-treated cells were blocked near the G1/S boundary. Culturing the cells with PRL for an additional 3 h resulted in their movement from S phase to G2/M in a partially synchronous manner observed as a 25-fold increase ($p < 0.001$) in the percentage of cells in the latter phase. These results indicate that APC produced a G1/S blockade; on removal of the inhibitor, PRL stimulated a partially synchronous entry into S phase.

Having demonstrated that APC arrested Nb2-11 cells near the G1/S transition point, the expression of XIAP was evaluated in PRL-treated cultures previously synchronized with the inhibitor. Quiescent cultures were treated with 0.5 μg/mL of APC and 20 ng/mL of PRL. After 12 h, the APC-containing medium was replaced with fresh medium to which PRL had been added; cells were harvested at the times indicated in Fig. 7. In cultures treated with APC + PRL for 12 h, lower levels of *XIAP* mRNA transcript were observed compared with quiescent cultures. The addition of PRL caused a 5- to 10-fold increase in the level of the transcript within 3 h subsequent to the readdition of hormone (Fig. 7). However, its expression was markedly reduced 6–12 h subsequent to the addition of PRL. These results suggest that PRL-stimulated expression of *XIAP* mRNA occurred as Nb2-11 cells entered S phase of the cell cycle.

Suppression of Apoptosis in Nb2-11 Cells Overexpressing XIAP

Since we observed that XIAP expression is regulated by PRL in the Nb2 line, it was important to determine whether it suppressed apoptosis in these cells. The *XIAP* cDNA or empty vector was stably transfected into Nb2-11 cells. Overexpression was verified by Northern blot analysis (data not shown). Apoptosis was induced by PRL deprivation in proliferating *XIAP* transfectants, cells in which the empty pcDNA 3.1 vector was introduced, and in non-transfected Nb2-11 cells. Cells were harvested after 24 and 48 h and fragmentation of DNA was assessed by agarose gel electrophoresis. As shown in Fig. 8A, PRL starvation caused marked DNA fragmentation in nontransfected and empty vector–transfected control cultures within 24–48 h. However, cells in which XIAP was overexpressed exhibited substantially less fragment staining.

Agarose gel electrophoresis of DNA fragmentation is, at best, semiquantitative. Therefore, additional experiments were conducted to evaluate more precisely the antiapoptotic effects of XIAP. Exponentially proliferating XIAP or vector-only transfectants were deprived of lactogens and then were subjected to DEX (100 n*M*) or PRL deprivation for an additional 12 h. Following each apoptotic stimulus, the percentage of cells undergoing active cell death was determined by flow cytometric analysis of annexin V binding. As shown in Fig. 8B, PRL deprivation or DEX caused 55 and 65% apoptosis, respectively, in the empty vector–transfected cells. However, *XIAP* overexpression significantly ($p < 0.001$) blocked the effects of each apoptotic stimulus. Together, these results demonstrate that increased levels of XIAP suppress apoptosis induced by glucocorticoids or PRL deprivation.

Discussion

XIAP, a recently identified antiapoptotic protein, was assessed to determine whether it might participate in PRL-regulated suppression of apoptosis. Previous studies indicated that XIAP inhibited apoptosis by blocking cell death proteases, caspase-3 and -7 *(18)*. Most recently, XIAP has been reported to function as an inhibitor of caspase-9 in addition to caspase-3 *(28)*. Structurally, XIAP possesses a baculovirus inhibitory domain (BIR) *(29)*. The conserved amino acids within the linker region between the BIRs were shown to be critical for inhibiting caspase-3 *(30)*. *XIAP*, thought to be a nuclear factor-κB (NF-κB)-regulated gene, counteracted apoptotic signals triggered by tumor necrosis factor-α to prevent apoptosis in endothelial cells *(31)*. XIAP increased nuclear translocation of NF-κB via a signaling pathway involving the mitogen-activated protein kinase TAK1 *(32)*, which inhibited the caspase cascade *(33)*. Zhang et al. *(34)* showed that inhibition of nuclear translocation of NF-κB resulted in apoptosis. Therefore, NF-κB may be an important component in XIAP signaling to apoptosis inhibition.

Results from other studies have also suggested that XIAP may play an antiapoptotic role subsequent to activation of focal adhesion kinase, which stimulates phosphatidylinositide 3'-OH-kinase (PI3K) and downstream Akt result-

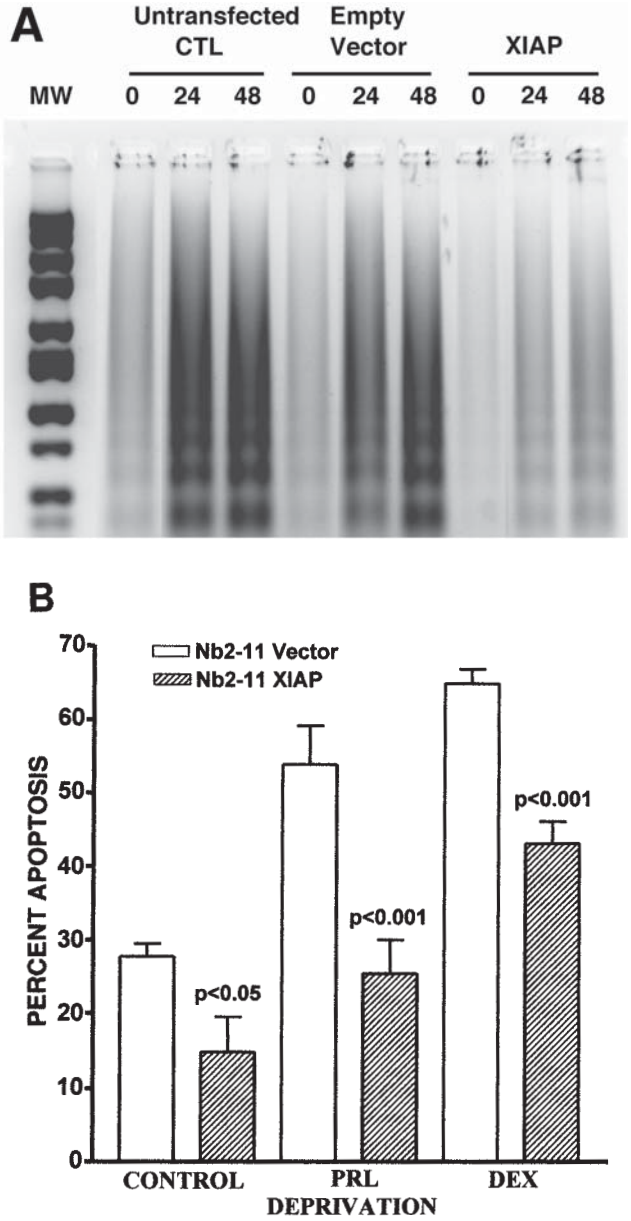

Fig. 8. Effect of XIAP overexpression on apoptosis suppression in PRL-deprived Nb2-11 cells. Exponentially proliferating Nb2-11 cells were transfected with XIAP and subjected to antibiotic selection. (**A**) The cells (0 h) were incubated in a medium lacking PRL, and samples were collected at the times indicated. Cells were lysed using phosphate-citrate buffer; treated with NP-40, RNase A, and proteinase K; incubated at 37ºC; and resolved on 1.8% agarose gels. Presented is an ethidium bromide–stained gel from an experiment performed twice with similar results. Untransfected CTL represents untransfected Nb2-11 cells. Empty vector represents cells transfected with pcDNA 3.1 alone, and XIAP represents cells transfected with pcDNA 3.1-XIAP. (**B**) Exponentially proliferating Nb2-11 cells overexpressing XIAP and vector only (control) were incubated in PRL-deprived medium for 12 h. Some cells were incubated for an additional 12 h in the absence of PRL (PRL deprivation), whereas others were treated with DEX (100 n*M*). Cells were harvested and incubated with FITC-conjugated annexin V in PBS-BSA for 30 min at 4ºC, washed, resuspended in PBS, and subjected to flow cytometric analysis.

Table 2
Apoptosis Suppressor Genes Regulated by PRL in Nb2-T Cells

Gene	Family/ characterization	Cell-cycle association	Reference
XIAP	IAP-family	S phase	—
bcl-2	Bcl-2 family	G1 phase	*11*
pim-1	Serine/threonine kinase	G1 phase	*14*
bag-1	Bcl-2 binding protein	—	*44*
bcl-X_L	Bcl-2 family	G1 phase	Kochendoerfer and Buckley, unpublished

ing in the marked induction of XIAP together with other IAPs *(35)*. Asselin et al. *(36)* showed that increased XIAP protein levels resulted in activation of Akt and reduced apoptosis in human ovarian epithelial cancer cells. These observations suggest that the antiapoptotic actions of XIAP may involve the PI3K-Akt signaling cascade. More important, we showed that PRL signaling to *pim-1* expression, and presumably apoptosis suppression, requires Akt activation *(37)*. Since PRL, XIAP, and Akt are each implicated in apoptosis suppression and cell survival, it is possible that PRL regulation of XIAP may also be a consequence of activation of this pathway.

In epithelial cells, a role for XIAP in angiogenesis was suggested based on its induction by vascular endothelial growth factor, suggesting it may represent a potential target for antiangiogenic therapy *(38)*. Notably, in this setting, as in Nb2 cells, growth factor stimulation increased XIAP while simultaneously provoking a proliferative response. Previous studies have shown that PRL regulates the expression of apoptosis-associated genes in Nb2 cells exposed to DEX (Table 2). Thus, it was important to evaluate the expression of XIAP in cells treated with DEX + PRL. The addition of PRL to DEX-treated cells increased the level of XIAP. Thus, we conclude that XIAP represents a candidate gene, together with *bcl-2* and *pim-1*, that likely plays an important role in PRL antagonism of apoptosis provoked by glucocorticoids.

PRL-mediated XIAP expression was also observed in the lactogen-independent cell line, Nb2-SFJCD1. Here, the effect of the differentiating agents NaBT, RA, and vitamin D as well as the effect of PRL in cells pretreated with these substances was evaluated on its expression. Butyrate has been shown to inhibit cell proliferation and induce differentiation in colonic epithelial cells *(39)* as well as to reduce the expression of protooncogenes such as c-*myc* and *pim-1* in Nb2 cultures *(21)*. Treatment with butyrate did not alter *XIAP* levels compared with those of the untreated cells. Nevertheless, PRL increased *XIAP* expression within 12 h

in the butyrate pretreated cells, an effect similar to that observed in the Nb2-11 line. Similar effects were observed in vitamin D–treated cultures. RA significantly reduced *XIAP* compared with that observed in untreated cells, whereas PRL increased its levels within 12 h. It is notable that previous reports have demonstrated elevated levels of XIAP in patients with acute myelogenous leukemia (AML), indicative of a poor prognosis *(40)*. Moreover, RA is clinically utilized in the treatment of AML *(22,23)*. The observation that retinoids induce apoptosis in T-cells by activation of caspases-2 and -3 further supports a role for RA and its derivatives in cancer therapy *(41)* and, together with the present results, suggests that XIAP may be a downstream target in the apoptotic pathway activated by RA.

In previous studies, Kobayashi et al. *(42)* showed that a murine homolog of the IAP, survivin (TIAP), was upregulated by concanavalin A or α-CD3 antibody in synchronized mouse fibroblast cell lines during S to G2/M phase cell-cycle progression. They proposed that during proliferation, the cells might be protected from apoptosis by induction of IAPs such as TIAP. Since PRL stimulated *XIAP* expression within 12 h in the Nb2 cells, a time coincident with the entry of Nb2 cells into S phase, we conclude that the antiapoptotic effect of PRL is accompanied by its regulation of this gene together with other previously described suppressor genes. Therefore, we suggest that PRL-stimulated *XIAP* expression protects cells from apoptotic signals encountered as they traverse the later phases of the cell cycle.

Materials and Methods

Hormones and Other Supplies

Ovine PRL (NIDDK oPRL-20, AFP10677C) was obtained from the National Hormone and Pituitary Program (Bethesda, MD). The human XIAP cDNA utilized has been previously described *(18)*. APC was obtained from Biomol Research Labs. Unless indicated otherwise, all other reagents were molecular biologic grade and obtained from Sigma (St. Louis, MO).

Culturing of Nb2 Lymphoma Cells

The PRL-dependent rat T-lymphoma cell line, Nb2-11, and the PRL-independent subline, Nb2-SFJCD1, were obtained from Dr. P. W. Gout (Vancouver, British Colombia, Canada). Cells were maintained at 37ºC in Fischer's medium containing 10% fetal bovine serum (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) *(43)*. Exponentially proliferating Nb2-11 cells (Log) were arrested in the early G1 phase of the cell cycle by culturing for 18–24 h in lactogen-free medium (i.e., Fischer's medium supplemented with 2-ME, antibiotics, and 10% nonmitogenic gelding serum [ICN, Irvine, CA; assay medium]) prior to each experiment. Proliferation of Nb2-SFJCD1 cells was arrested by pretreatment with differentiating agents including sodium butyrate (2 m*M*, 72 h), all *trans*-retinoic acid (10 μ*M*, 72 h), or 1,25-dihydroxycholecalciferol (vitamin D, 100 n*M*, 24 h). Prior to each experiment, differentiating agents were removed by washing; the cells were subsequently resuspended in assay medium, similar to the Nb2-11 cell experiments, prior to the addition of PRL.

To evaluate PRL responsiveness of RA-treated Nb2-SFJCD1 cells, the effect of hormone stimulation was determined on cell population density. Exponentially proliferating cells were cultured in the presence of RA (10 μ*M*, 72 h). The vitamin was removed by washing, and cells (1.2×10^5 cells/well) were treated with increasing concentrations of PRL. After 48 h, population density was determined by electronic cell counting.

To evaluate PRL-regulated expression of *XIAP* mRNA, stationary Nb2-11 (0 h) and Nb2-SFJCD1 cells pretreated with differentiating agent (0 h) were stimulated with 20 ng/ml of PRL. Cultures were harvested at multiple time points through 24 h after the addition of hormone; expression of the *XIAP* transcript was evaluated by Northern blot analysis. To assess the relationship between XIAP mRNA expression and cell-cycle progression, stationary Nb2-11 cells were synchronized at G1/S interphase by incubating for 12 h with APC (0.5 μg/mL) and PRL (20 ng/mL); then cultures were harvested at various time points.

XIAP Transfection

The full-length XIAP cDNA was ligated into the *Eco*RI/*Xho*I multiple cloning site of pcDNA 3.1 expression vector (Invitrogen, San Diego, CA), which contains a cytomegalovirus promoter for constitutive expression. Following amplification, the construct was transfected into Nb2-11 cells (1×10^7) by electroporation using an ECM 600 instrument (300 V/950 μF, 20 s; Genetronics, San Diego, CA). Transfectants were selected by culturing in the presence of G418 added to the medium. Control Nb2-11 cells were transfected with an empty pcDNA 3.1 vector. Overexpression of XIAP in the stable transfectants was verified by Northern blot analysis.

Northern Blotting Procedures

Total RNA was isolated from 2×10^7 cells per treatment condition using RNAzol (Tel-Test, Friendswood, TX), quantitated spectrophotometrically, denatured in formaldehyde, fractionated on 1% agarose gels, and transferred to Immobilin-Ny$^+$ (Millipore). Equal loading per lane was verified by densitometric analysis of ethidium bromide–stained 18S and 28S RNA, which was visualized and photographed under ultraviolet illumination. Membranes were hybridized utilizing an *XIAP* cDNA probe labeled with [^{32}P] deoxy-CTP (Amersham, Arlington Heights, IL).

Determination of Thymidine Incorporation

To determine the optimal concentration of APC that synchronized Nb2 cells at G1/S, pulse-labeling experiments

using [^{3}H]-thymidine (SA, 86.0 Ci/mmol; Amersham) were performed by adding 0.5 µCi/well at 4-h intervals to sextuplicate cultures of Nb2 cells treated with varying concentrations of APC and PRL (20 ng/mL). At each time point, cells were harvested onto glass-fiber filters using an automated cell harvester (Cambridge Technology, Watertown, MA), precipitated with ice-cold 10% trichloroacetic acid (TCA), and washed with ethanol. Radioactivity in TCA-insoluble material was determined by liquid scintillation spectroscopy.

Flow Cytometric Analysis

Cell-cycle evaluation of treated cells was conducted by flow cytometric analysis of propidium iodide–stained cell nuclei. Aliquots of 1–2 × 10^6 cells were centrifuged at 200*g* and fixed by resuspension in cold 70% ethanol. The cells were washed and resuspended in cold phosphate-buffered saline (PBS), then exposed to 1.0 mg/mL of RNase. After 1 h of incubation at 37ºC, the cells were washed twice with cold PBS and resuspended in a hypotonic propidium iodide solution (50 ng/mL). The stained cells were analyzed using an Elite flow cytometer (Coulter Electronics) at 488 nm.

Nb2-11 cells (1 × 10^6/sample), lactogen deprived for 24 h or treated with DEX (100 n*M*) for 12 h, were harvested by centrifugation (2000*g*, 10 min, 4ºC). Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated annexin V in PBS-bovine serum albumin (BSA) (0.5% BSA) for 30 min at 4ºC. Negative controls were treated as just described but without the addition of annexin V. After 30 min, the cells were washed twice with PBS by centrifugation and resuspended in PBS. Fluorescence was analyzed using an Epics XL (Beckman coulter) flow cytometer with a 15-mW laser at 488 nm and a filter for FITC at 525 nm.

DNA Fragmentation Analysis

Apoptosis was induced in XIAP overexpressing and vector-only transfected Nb2-11 cells by PRL deprivation. Cells (3 × 10^6) were harvested after 24 and 48 h by resuspension in PBS and fixed in 70% ethanol. The fixed cells were centrifuged and then lysed using 0.2 *M* sodium dihydrogen phosphate and 0.1 *M* citric acid. The lysates were centrifuged and the supernatant was dried; treated with NP-40, RNase A (1 mg/mL), and proteinase K (1 mg/mL); and resolved on 1.8% agarose gels. The ethidium bromide–stained gel was photographed under ultraviolet illumination.

Statistical Analyses

For Northern blotting procedures, equal loading per lane was verified by densitometric analysis of ethidum bromide staining of 18S and 28S rRNA. Where applicable, data are represented as the means ± SE. Differences among treatment groups in experiments performed on Nb2-SFJCD1 cultures treated with vitamin D and NaBT were evaluated by analysis of variance (ANOVA), followed by linear trend analysis. Statistical analysis of the Nb2-11 and Nb2-SFJCD1 cultures treated with RA was conducted by ANOVA followed by Dunnett's posttest for multiple comparisons.

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References

1. Gout, P. W., Beer, C. T., and Noble, R. L. (1980). *Cancer Res.* **40,** 2433–2436.
2. Fleming, W. H., Pettigrew, N. M., Matusik, R. J., and Freisen, H. G. (1982). *Cancer Res.* **42,** 3138–3141.
3. Cox, J. H., Lamb, J. R., Bal, V., Butcher, G. W., Howard, J. C., Owen, M. J., and Ivanyi, J. (1989). *J. Immunol.* **66,** 83–89.
4. Richards, J. F., Beer, C. T., Bourgeault, C., Chen, K., and Gout, P. W. (1982). *Mol. Cell Endocrinol.* **26,** 41–49.
5. Fletcher-Chiappini, S. E., Compton, M. M., LaVoie, H. A., Day, E. B., and Witorsch R. J. (1993). *Proc. Soc. Exp. Biol. Med*. **202,** 345–352.
6. Witorsch, R. J., Day, E. B., LaVoie, H. A., Hashemi, N., and Taylor, J. K. (1993). *Proc. Soc. Exp. Biol. Med.* **203,** 454–460.
7. LaVoie, H. A. and Witorsch, R. J. (1995). *Proc. Soc. Exp. Biol. Med.* **209,** 257–269.
8. Krumenacker, J. S., Buckley, D. J., Leff, M. A., McCormack, J. T., de Jong, G., Gout, P. W., Reed, J. C., Miyashita, T., Magnuson, N. S., and Buckley, A. R. (1998). *Endocrine* **9(2),** 163–170.
9. Gout, P. W. (1987). *Cancer Res.* **47,** 1751–1755.
10. Zhang, M., Blake, M. J., Gout, P. W., Buckley, D. J., and Buckley, A. R. (1999). *Cell Growth Differ.* **10,** 759–767.
11. Leff, M. A., Buckley, D. J., Krumenacker, J. S., Reed, J. C., Miyashita, T., and Buckley, A. R. (1996). *Endocrinology* **137,** 5456–5462.
12. Hockenbery, D. M. (1992). *Semin. Immunol.* **4(6),** 413–420.
13. Moroy, T., Grzeschiczek, A., Petzold, S., and Hartmann, K. U. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 10,734–10,738.
14. Buckley, A. R., Buckley, D. J., Leff, M. A., Hoover, D. S., and Magnuson, N. S. (1995). *Endocrinology* **136,** 5252–5259.
15. Duckett, C. S., Nava, V. E., Gedrich, R. W., Clem, R. J., Van Dogen, J. L., Gilfillan, M. C., Shiels, H., Hardwick, J. M., and Thompson, C. B. (1996). *EMBO J.* **15,** 2685– 2694.
16. Clem, R. J., Fechheimer, M., and Miller, L. K. (1991). *Science* **254,** 1388–1390.
17. Clem, R. J. and Miller, L. K. (1994). *Mol. Cell. Biol.* **14,** 3212–3222.
18. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997). *Nature* **388,** 300–303.
19. Deveraux, Q. L., Leo, E., Stennicke, H. R., Welsh, K., Salvesen, G. S., and Reed, J. C. (1999). *EMBO J.* **18(19),** 5242–5251.
20. Wagenknecht, B., Glaser, T., Naumann, U., Kugler, S., Isenmann, S., Bahr, M., Korneluk, R., Liston, P., and Weller, M. (1999). *Cell Death Differ.* **6(4),** 370–376.
21. Buckley, A. R., Leff, M. A., Buckley, D. J., Magnuson, N. S., de Jong, G., and Gout, P. W. (1996). *Cell Growth Differ.* **7,** 1713–1721.
22. Imaizumi, M. and Breitman, T. R. (1987). *Eur. J. Haematol.* **38(4),** 289–302.
23. Leung, M. F. and Wong, K. F. (1999). *J. Hematother.* **8(3),** 275–279.
24. Miyaura, C., Abe, E., Kuribayashi, T., Tanaka, H., Konno, K., Nishi, Y., and Suda, T. (1981). *Biochem. Biophys. Res. Commun.* **102(3),** 937–943.

25. Pedrali-Noy, G. and Spadari, S. (1979). *Biochem. Biophys. Res. Commun.* **88,** 1194–2002.
26. Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H., and Mano, Y. (1978). *Nature* **275(5679),** 458–460.
27. Hubscher, U., Kuenzle, C. C., Limacher, W., Sherrer, P., and Spadari, S. (1979). *Cold Spring Harbor Symp. Quant. Biol.* **439(1),** 625–629.
28. Datta, R., Oki, E., Endo, K., Biedermann, V., Ren, J., and Kufe, D. (2000). *J. Biol. Chem.* **275(41),** 31,733–31,738.
29. Borden, K. L. (1998). *Biochem. Cell. Biol.* **76,** 351–358.
30. Sun, C., Cai, M., Gunasekara, A. H., Meadows, R. P., Wang, H., Chen, J., Zhang, H., Wu, W., Xu, N., Ng, S. C., and Fesik, S. W. (1999). *Nature* **401(6755),** 818–822.
31. Stehlik, C., de Martin, R., Kumabashiri, I., Schmid, J. A., Binder, B. R., and Lipp, J. (1998). *J. Exp. Med.* **188(1),** 211–216.
32. Hofer-Warbinek, R., Schmid, J. A., Stehlik, C., Binder, B. R., Lipp, J., and de Martin, R. (2000). *J. Biol. Chem.* **275,** 22,064–22,068.
33. Kawakami, A., Nakashiman, T., Sakai, H., Urayama, S., Yamasaki, S., Hida, A., Tsuboi, M., Nakamura, H., Ida, H., Migita, K., Kawabe, Y., and Eguchi, K. (1999). *Blood* **94(11),** 3847–3854.
34. Zhang, H. G., Huang, N., Liu, D., Bilbao, L., Zhang, X., Yang, P., Zhou, T., Curiel, D. T., and Mountz, J. D. (2000). *Arthritis Rheum.* **43(5),** 1094–1105.
35. Sonoda, Y., Matsumoto, Y., Funakoshi, M., Yamamoto, D., Hanks, S. K., and Kasahara, T. (2000). *J. Biol. Chem.* **275(21),** 16,309–16,315.
36. Asselin, E., Mills, G. B., and Tsang, B. K. (2001). *Cancer Res.* **61,** 1862–1868.
37. Krumenacker, J. S., Narang, V. S., Buckley, D. J., and Buckley, A. R. (2001). *J. Neuroimmunol.* **113,** 249–259.
38. Tran, J., Rak, J., Sheehan, C., Saibil, S. D., LaCasse, E., Korneluk, R. G., and Kerbel, R. S. (1999). *Biochem. Biophys. Res. Commun.* **264(3),** 781–788.
39. Gum, J. R., Kam, W. K., Byrd, J. C., Hicks, J. W., Sleisenger, M. H., and Kim, Y. S. (1987). *J. Biol. Chem.* **262,** 1092–1097.
40. Tamm, I., Kornblau, S. M., Segall, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D. A., Tudor, G., Qui, Y. H., Monks, A., Andreeff, M., and Reed, J. C. (2000). *Clin. Cancer Res.* **6(5),** 1796–1803.
41. Piedrafita, F. J. and Pfahl, M. (1997). *Mol. Cell. Biol.* **17,** 6348–6358.
42. Kobayashi, K., Hatano, M., Otaki, M., Ogasawara, T., and Tokuhisa, T. (1999). *Dev. Biol.* **96(4),** 1457–1462.
43. Gout, P. W., Beer, C. T., and Noble, R. L. (1980). *Cancer Res.* **40,** 2433–2436.
44. Clevenger, C.V., Thickman, K., Ngo, W., Chang, W., Takayama, S., and Reed, J. C. (1997). *Mol. Endocrinol.* **11(5),** 608–618.