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Bcl-2 overexpression prevents daunorubicin-induced apoptosis through inhibition of XIAP and Akt degradation

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Abstract

Daunorubicin (DNR) induces apoptosis in the human myeloid leukemia cells by activation of neutral sphingomyelinease and ceramide production. In the present study, we determined the effect of the antiapoptosis protein Bcl-2 on caspase-3 activation, phospholipase C-γ1 (PLC-γ1) degradation and cytochrome *c* release during the DNR-induced apoptosis. Treatment with 3 μM DNR for 12 hr produced morphological features of apoptosis and DNA fragmentation in U937 cells, which was associated with caspase-3 activation and PLC-γ1 degradation. Induction of apoptosis was also accompanied by release of cytochrome *c*, down-regulation of X-linked inhibitor of apoptosis protein (XIAP), and inactivation of Akt, which was blocked by the pan-caspase inhibitor z-VAD-fmk. DNR-induced caspase-3 activation, PLC-γ1 degradation and apoptosis were significantly attenuated in Bcl-2 overexpressing U937/Bcl-2 cells. Ectopic expression of Bcl-2 appeared to inhibit DNR-induced apoptosis by interfering with inhibition of XIAP and Akt degradation.

Keywords: Daunorubicin; Apoptosis; Caspase; XIAP; Akt; Bcl-2

1. Introduction

Many antitumor agents, which interact with different targets, can kill chemosensitive leukemic cells *via* an apoptotic process [1,2]. Apoptosis is a genetically encoded cell death program characterized by distinct set of morphological and biochemical changes [3–6]. The apoptotic pathway, in general, can be affected by different factors such as the tumor cell type, differentiation status, growth factors, or oncogenes [4]. Several genes have been identified as either inducers or repressors of apoptosis. Bcl-2, initially identified at the breakpoint of the t(14;18) chromosomal translocation that occurs in the majority of non-Hodgkin's B-cell lymphomas [7,8], can protect cells against apoptosis induced by such diverse stimuli as viral infection, hypoxia, ionizing radiation or chemotherapeutic agents [9–13]. The precise biochemical mechanisms by which Bcl-2 family proteins affect cell survival and death, however, remain unclear. *In vivo* and *in vitro* studies have shown that Bcl-2 regulates intracellular Ca^{2+} levels and prevents the loss of mitochondrial membrane potential induced by pro-apoptotic stimuli [14]. It has been suggested that Bcl-2 may act as an ion channel and regulates the release of cytochrome *c* from mitochondria [15–17]. The release of cytochrome *c* triggers the formation of a complex containing Apaf-1 and procaspase-9 in the presence of dATP, resulting in caspase-9 activation [18,19]. Once activated, initiator caspases in turn activate the effector caspases, such as caspase-3 and -7 [18,19]. The active effector caspases promote apoptosis by cleaving cellular substrates leading to the morphological and biochemical features of apoptosis.

DNR, an anthracycline antibiotic, is a broad-spectrum antitumor chemotherapeutic drug that is used to treat a variety of malignant tumors including leukemia. It has been reported that DNR kills the leukemic cells by

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Abbreviations: DNR, daunorubicin; RT-PCR, reverse transcriptase-polymerase chain reaction; DEVD-*p*NA, Asp-Glu-Val-Asp-chromophore *p*-nitroanilide; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; PLC-γ1, phospholipase C-γ1; IAP, inhibitor of apoptosis; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ROS, reactive oxygen species.

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inhibiting topoisomerase II, DNA damage and generation of reactive oxygen species (ROS) [20–23].

In the present study, we have found the inhibitory effect of ectopic expression of Bcl-2 on DNR-induced apoptosis and down-regulation of XIAP and Akt during DNR-induced apoptosis.

2. Materials and methods

2.1. Cell line and culture

The human leukemia cell line U937 was purchased from the American Type Culture Collection, and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 μg/mL penicillin at 37° and 5% CO_2. Bcl-2 overexpressing U937 cells were generated using a pMAX vector containing the human Bcl-2 gene (provided by Dr. Rakesh Srivastava, NIH/NIA). U937 cells (2×10^6 cells/mL) in RPMI 1640 were transfected by preincubation with 15 μg Bcl-2 plasmid for 10 min at room temperature followed by electroporation at 500 V, 700 μF. The sample was immediately placed on ice for 10 min, and after 10 mL complete medium was added, the cells incubated at 37° for 24 hr. The cells were selected in a medium containing 0.7 μg/mL geneticin (G418 sulfate) for 4 weeks. Single cell clones were obtained by limiting dilution and subsequently analyzed for an increase in Bcl-2 protein expression relative to the identically cloned empty vector control.

2.2. Drugs and materials

DNR (Calbiochem) was directly added to cell cultures at the indicated concentrations. Anti-cIAP1, anti-cIAP2, anti-Bcl-2, anti-Bcl-xL, anti-Hsp70, and anti-PLC-γ1 antibodies were purchased from Santa Cruz Biotechnology. Antibodies against the following protein were purchased from the indicated suppliers; caspase-3 and cytochrome *c* from PharMingen, actin from Sigma and XIAP from R&D systems. C2-ceramide was purchased from Calbiochem.

2.3. Western blotting

Cellular lysates were prepared by suspending 1×10^6 cells in 100 μL lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenymethylsulfonyl fluoride and 20 μM leupeptin, adjusted to pH 7.2). Cells were disrupted by sonication and extracted at 4° for 30 min. Proteins were electrotransferred to Immobilon-*P* membranes (Millipore). Detection of specific proteins was carried out with the ECL Western blotting kit according to the manufacturer's instructions.

2.4. Cell count and flow cytometry analysis

Cell counts were performed using a hemocytometer. Approximately, 1×10^6 U937 cells were suspended in 100 μL PBS and 200 μL 95% ethanol was added while vortex-mixing. Cells were incubated at 4° for 1 hr and washed once with PBS. Cells were resuspended in 250 μL 1.12% (w/v) sodium citrate buffer (pH 8.4) together with 12.5 μg RNase. Incubation was continued at 37° for 30 min. Cellular DNA was then stained by applying 250 μL of propidium iodide (50 μg/mL) for 30 min at room temperature. The stained cells were analyzed by the fluorescent activated cell sorting (FACS) on a FACScan flow cytometer for a relative DNA content based on red fluorescence.

2.5. DNA fragmentation assay

After treatment with DNR, U937 cells were lysed in a buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10,000 *g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) and analyzed electrophoretically on 2% agarose gels containing 0.1 μg/mL ethidium bromide.

2.6. RNA isolation and RT-PCR

Total RNA was isolated according to the previously published method [24]. Single-strand cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Gibco-BRL). The mRNAs for Akt and XIAP and actin were amplified by PCR with specific primers. The sequence of the sense and antisense primers for Akt were 5′-CAACTTCTCTGTGGCGCAGTG-3′ and 5′-GACAGGTGGAAGAA CAGCTCG-3′, respectively. The sequence of the sense and antisense primers for XIAP were 5′-CTTGAGGAGTGTCTGGTAA-3′ and 5′-GTGACTAGAT GTCCACAAGG-3′, respectively. Conditions for PCR reaction were 1× (94°, 3 min); 35× (94°, 45 s; 58°, 45 s; and 72°, 1 min) and 1× (72°, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

2.7. Caspase-3 activity assay

To evaluate caspase-3 activity, cell lysates were prepared after their respective treatment with daunorubicin. Assays were performed in 96-well microtiter plates by incubating 20 μg cell lysates in 100 μL reaction buffer (1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 10% glycerol) containing the 5 μM caspase-3 substrate (DEVD-*p*NA). Lysates were incubated at 37° for 2 hr. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

2.8. Analysis of cytochrome c release

Cells (2×10^6) were harvested, washed once with ice-cold PBS and gently lysed for 2 min in 80 μL ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 20 mM Tris–HCl pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/mL pepstatin A, 10 μg/mL leupeptin, 2 μg/mL aprotinin). Lysates were centrifuged at 12,000 *g* at 4° for 10 min to obtain the supernatants (cytosolic extracts free of mitochondria) and the pellets (fraction that contains mitochondria). The resulting cytosolic fractions were used for Western blot analysis with an anticytochrome *c* antibody.

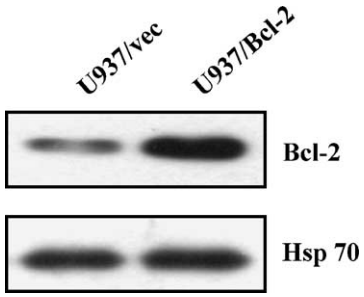

Fig. 1. Overexpression of Bcl-2 in U937 cells. Immunoblot analysis of cell lysate (50 μg) from control (U937/vector) or Bcl-2 transfected (U937/Bcl-2) cells with anti-Bcl-2 antibody. The blot was stripped of the bound antibody and reprobed with anti-Hsp70 antibody to confirm equal loading.

3. Results

3.1. Expression levels of Bcl-2 overexpressing cells

In order to evaluate the functional role played by Bcl-2 in preventing apoptosis induced by the antitumor agent DNR, we first established Bcl-2 overexpressing cells. U937 cells were transfected with an expression vector containing *bcl*-2 cDNA. After 4 weeks, geneticin (G418 sulfate) resistant cells were isolated and the relative expression level of Bcl-2 was determined by Western blot. Immunoblot analysis revealed that U937/Bcl-2 cells exhibited approximately 4-fold increase in Bcl-2 expression compared with cells containing empty-vector only (Fig. 1). In contrast, levels of Hsp70 expression remained same in U937/Bcl-2 and U937/vector cells.

3.2. DNR-induced apoptosis in U937/vector and U937/Bcl-2 cells

To determine whether DNR-induced apoptosis was associated with the activation of caspase-3, we measured the expression level and activity of caspase-3 in U937/vector and U937/Bcl-2 cells that had been exposed to various concentrations of DNR. Caspase-3 is activated

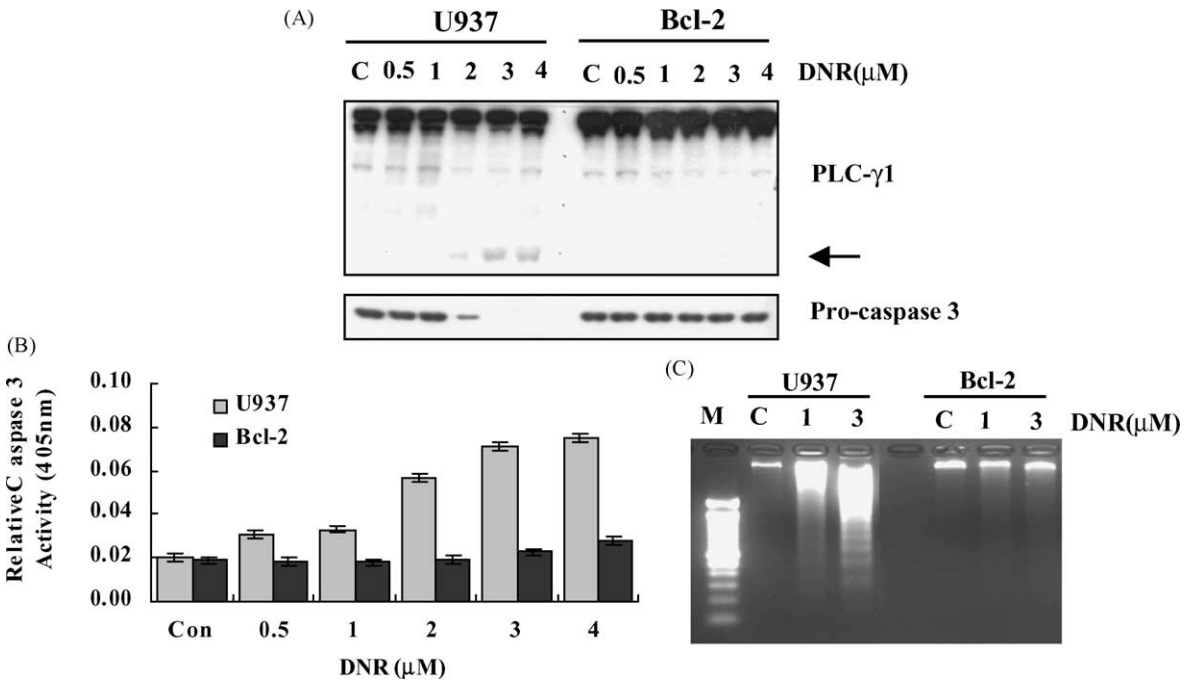

Fig. 2. DNR-induced apoptosis in U937/vector and U937/Bcl-2 cells. (A) Cells were treated with the indicated concentrations of DNR. Equal amounts of cell lysates (50 μg) were subjected to electrophoresis and analyzed by Western blot for caspase-3 and PLC-γ1. The proteolytic cleavage of PLC-γ1 is indicated by an arrow. (B) U937/vector and U937/Bcl-2 cells were treated with the indicated concentrations of DNR for 12 hr and harvested in lysis buffer. Enzymatic activities of caspase-3 were determined by incubation of 30 μg total protein with 200 μM chromogenic substrate (DEVD-*p*NA) in a 100 μL assay buffer for 2 hr at 37°. The release of chromophore *p*-nitroanilide (*p*NA) was monitored spectrophotometrically (405 nm). Data are mean values obtained from three independent experiments and bars represent standard deviations. (C) Inhibition of DNR-induced genomic DNA fragmentation by Bcl-2 overexpression cells. U937/vector and U937/Bcl-2 cells were treated as above. DNA was extracted and analyzed by 2% agarose gel electrophoresis.

by proteolytic processing of the 32 kDa form into two smaller subunits. Activity of caspase-3 during DNR-induced apoptosis was measured by a decrease in proenzyme level using Western blot analysis and a proteolytic activity with a chromogenic substrate. As shown in Fig. 2A, treatment with DNR resulted in a decrease in the level of caspase-3 in U937/vector cells exposed to 2–4 μM DNR for 12 hr. In contrast, the expression levels of caspase-3 were not significantly altered in DNR-treated U937/Bcl-2 cells. Subsequent Western blotting demonstrated proteolytic cleavage of PLC-γ1, a downstream target of activated caspase-3 *in vivo* [25], in U937/vector cells after 12 hr of 0.5–4 μM DNR. This cleavage of PLC-γ1 is dose-dependent in U937/vector

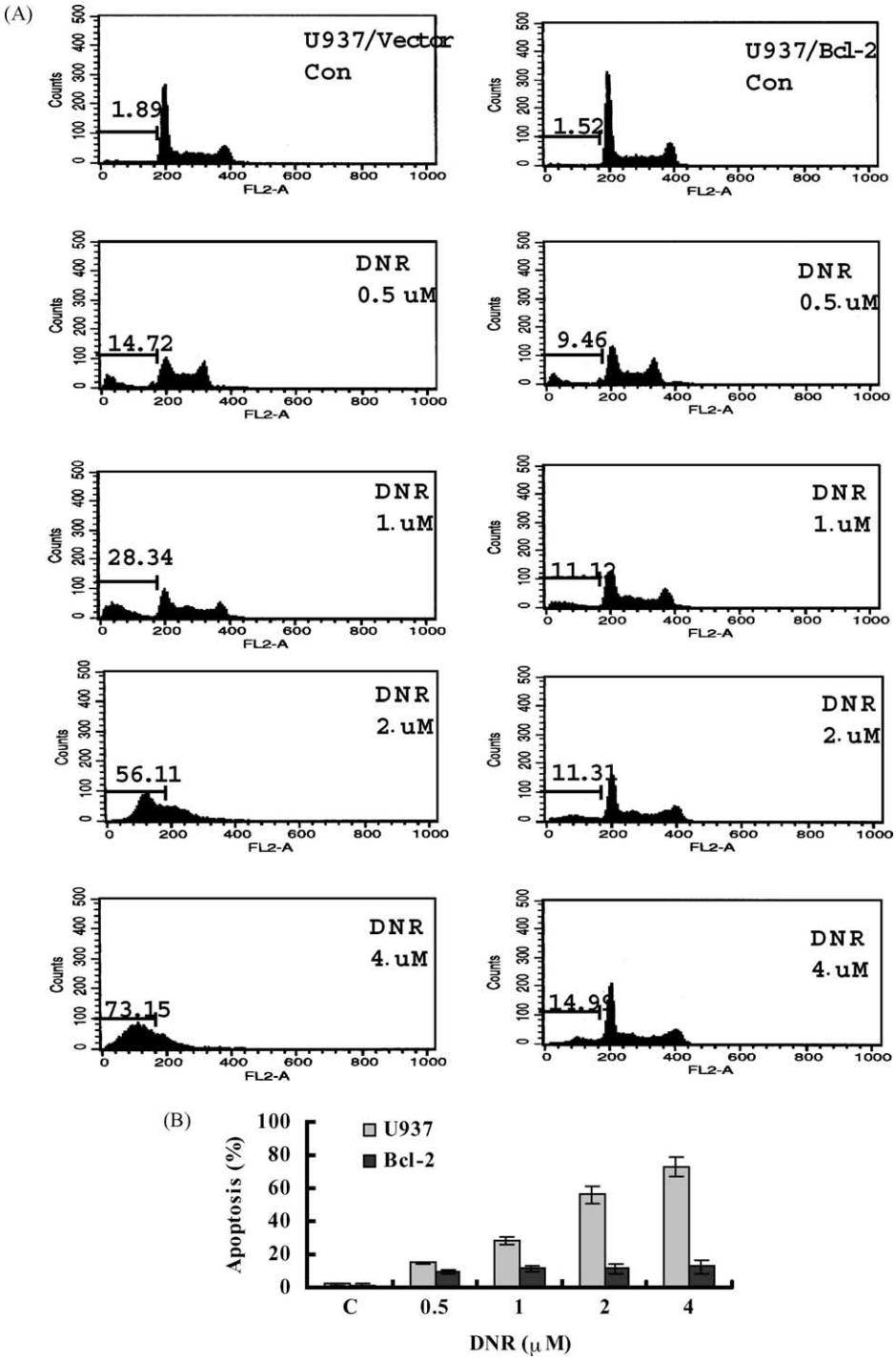

Fig. 3. Flow cytometric analysis of apoptotic cells. (A) Cells were treated for 12 hr with the indicated concentrations of DNR and their DNA content was measured after propidium iodide staining. (B) The proportion of apoptotic cells is indicated. Data are mean values obtained from three independent experiments and bars represent standard deviations.

cells, but not in U937/Bcl-2 cells (Fig. 2A). To verify and quantitate the proteolytic activity of caspase-3, we performed an *in vitro* assay based on the proteolytic cleavage of DEVD-*p*NA by caspase-3 into the chromophore *p*-nitoranilide (*p*NA). U937/vector cells showed an increase in DEVD-*p*NA cleavage after 12 hr exposure to various concentrations of DNR. In contrast, the levels of caspase-3 were not significantly altered in DNR-treated U937/Bcl-2 cells (Fig. 2B). Another hallmark of apoptosis is the degradation of chromosomal DNA at internucleosomal linkage. We analyzed DNA fragmentation induced by DNR in both U937/Bcl-2 and U937/vector cells. Agarose gel electrophoresis analysis showed the lack of internucleosomal DNA fragmentation in U937/Bcl-2 cells treated with 1 and 3 μM DNR for 12 hr, while a typical ladder pattern of DNA fragmentation was observed in U937/vector cells (Fig. 2C). In order to quantify the degree of apoptosis, we analyzed the amount of sub-G1 DNA by flow cytometry. U937/Bcl-2 and U937/vector cells were exposed to various concentrations of DNR for 12 hr. As shown in Fig. 3, DNR treatment to U937/vector cells resulted in a markedly increased accumulation of cells in the sub-G1 phase, which occurred in a dose-dependent manner. In contrast, overexpression of Bcl-2 reduced DNR-induced accumulation of sub-G1 phase. Taken together, these results indicate that apoptosis induced by DNR in U937 cells can be blocked by Bcl-2.

3.3. Modulation of Bcl-2 and IAP protein families in DNR-induced apoptosis in U937/vector and U937/Bcl-2 cells

We also examined whether DNR induces cell death by modulating the expression of Bcl-2 family members, which ultimately determine the cellular response to apoptotic stimuli. Treatment of U937/Bcl-2 and U937/vector cells with DNR at concentrations that are sufficient to induce apoptosis failed to significantly alter the expression of the Bcl-2, Bcl-xL and Bax protein after 12 hr (Fig. 4A). To determine whether activity of caspase-3 was associated with the expression levels of caspase inhibitors in DNR-induced apoptosis, we measured the expression levels of IAP family proteins in U937/Bcl-2 cells and U937/vector cells that had been exposed DNR. As shown in Fig. 4B, DNR treatment resulted in a decrease in levels of cIAP1 and XIAP, but not cIAP2 in U937/vector cells exposed to 2–4 μM DNR for 12 hr. In contrast, the decrease of cIAP1 and XIAP in U937/Bcl-2 cells was significantly inhibited (Fig. 4B). There was no significant difference in expression levels of cIAP2 between U937/Bcl-2 cells and U937/vector cells after DNR treatment. These results indicate that the elevated caspase-3 activity in DNR-treated U937 cells is associated with down-regulation of cIAP1 and XIAP, but not cIAP2. To further study the relationship between total XIAP protein and XIAP mRNA in U937 cells, we measured XIAP mRNA levels by reverse transcriptase-polymerase

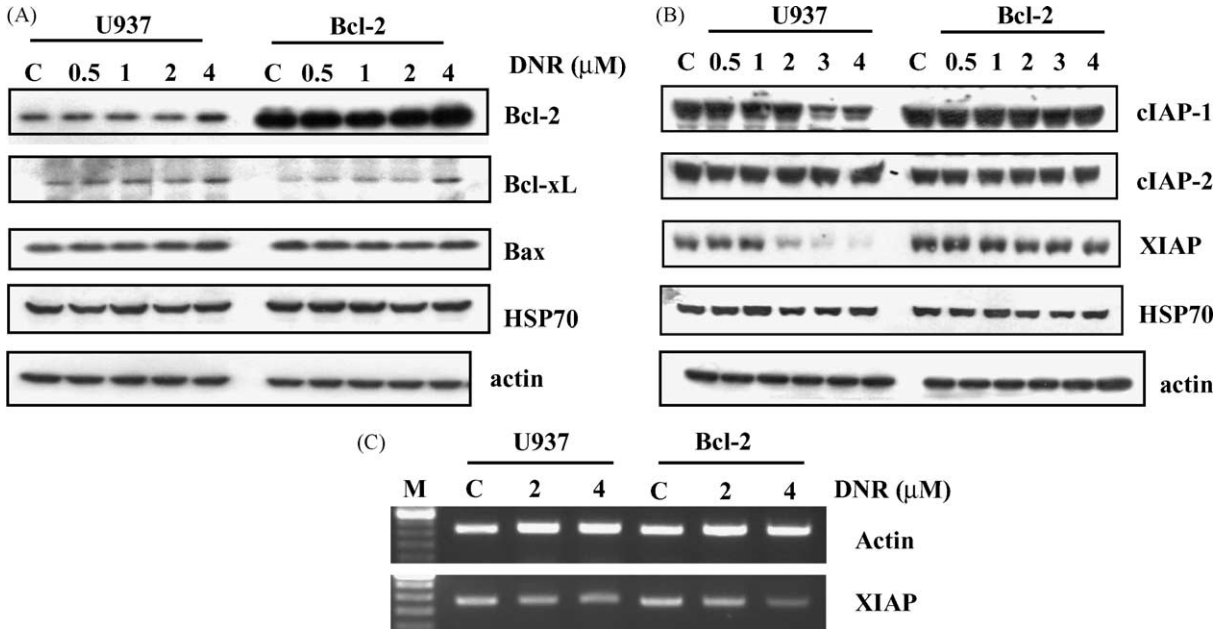

Fig. 4. The expression levels of the apoptosis-related proteins by treatment with DNR in U937/vector and U937/Bcl-2 cells. (A) U937/vector and U937/Bcl-2 cells were treated with indicated concentrations of DNR. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies (anti-Bcl-2, anti-Bcl-xL, or anti-Bax). Membrane was probed with anti-Hsp70 and anti-actin antibody to serve as control for the loading of protein level. A representative result is shown; two additional experiments yielded similar results. (B) U937/vector and U937/Bcl-2 cells were treated with indicated concentrations of DNR. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies (anti-cIAP1, anti-cIAP2, or anti-XIAP). A representative result is shown; two additional experiments yielded similar results. (C) XIAP mRNA is not down-regulated by DNR treatment. U937/vector and U937/Bcl-2 cells were incubated for 16 hr with various concentrations of DNR and RT-PCR analysis was performed.

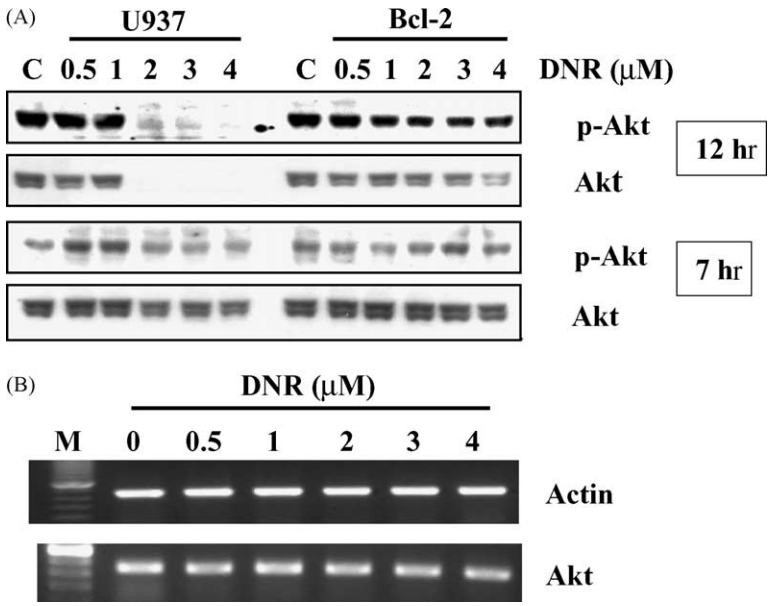

Fig. 5. The phosphorylation and expression levels of Akt in U937/vector and U937/Bcl-2 cells by treatment with DNR. (A) U937/vector and U937/Bcl-2 cells were treated with indicated concentrations of DNR for 7 and 12 hr, respectively. Equal amounts of cell lysates (40 μg) were treated as above and probed with specific antibodies (antiphospho-Akt and anti-Akt). A representative result is shown; two additional experiments yielded similar results. (B) Akt mRNA is not down-regulated by DNR treatment. U937 cells were incubated for 16 hr with various concentrations of DNR and RT-PCR analysis was performed. A representative study is shown; two additional experiments yielded similar results.

chain reaction (RT-PCR) and found that XIAP mRNA levels remain constant through the DNR treatment at different doses (Fig. 4C). These data suggest that DNR-mediated degradation of total XIAP protein is regulated at the post-transcriptional levels.

3.4. DNR induces degradation of Akt in U937 cells

Akt has been described as a downstream effector of PI3K that mediates survival signaling. We examined whether Akt could be activated U937 cells in response to DNR. The activation of Akt is dependent on phosphorylation at two sites Ser-473 and Thr-308. We determined the expression and phosphorylation levels of Akt in U937 cells and Bcl-2 overexpressing cells after treated with various concentrations of DNR for 12 hr. As shown in Fig. 5A, the expression and phosphorylation levels of Akt were significantly decreased in response to 2 μM DNR in U937 cells, but not in U937/Bcl-2 cells after 12 hr. However, treatment of U937 cells and U937/Bcl-2 cells with concentrations of DNR did not significantly alter the expression and phosphorylation levels of Akt after 7 hr (Fig. 5A). Moreover, Akt mRNA levels remained same in a DNR dose-dependent manner (Fig. 5B). These data strongly suggest that DNR-mediated degradation of total Akt protein is also subjected to post-transcriptional regulation.

3.5. DNR-induced inhibition of Akt and XIAP in U937 cells requires caspase-3

To address the possible role of caspase cleavage as a mechanism for degradation of Akt and XIAP protein in

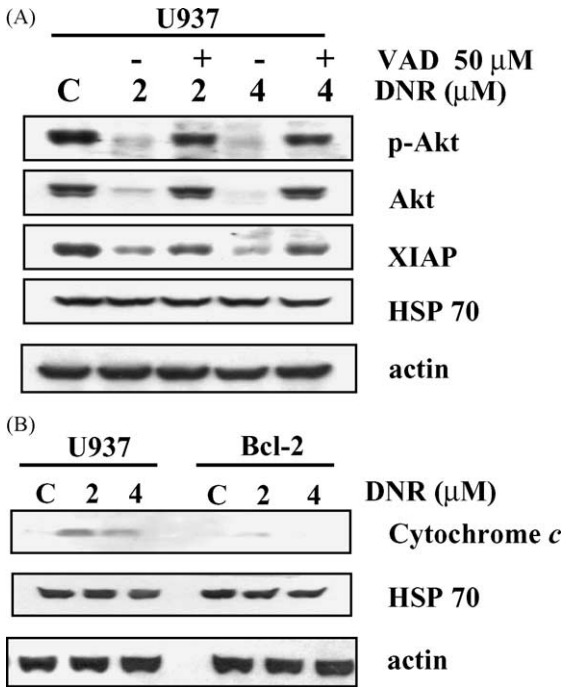

Fig. 6. Release of cytochrome *c* in U937/vector and U937/Bcl-2 cells by treatment with DNR. (A) U937 cells were incubated with 50 μM z-VAD-fmk or solvent for 30 min before treatment with 2 or 4 μM DNR. Equal amount of cell lysate were subjected to electrophoresis and analyzed by Western blot for phospho-Akt, Akt, XIAP and Hsp70. A representative result is shown; two additional experiments yielded similar results. (B) U937/vector and U937/Bcl-2 cells were treated with indicated concentrations of DNR. Cytosolic extracts were prepared as described in Section 2. Thirty micrograms of cytosolic protein was resolved on 12% SDS–PAGE and then transferred to nitrocellulose, and probed with specific anticytochrome *c* antibody, or with anti-Hsp70 and anti-actin antibody to serve as control for the loading of protein level.

DNR-induced apoptosis, we used a pan-caspase inhibitor, z-VAD-fmk for the further study. As shown in Fig. 6A, DNR treatment in U937 cells led to degradation of Akt protein and XIAP protein, and z-VAD-fmk pretreated cells significantly attenuated the down-regulation of Akt and XIAP protein. These data clearly indicate that DNR-induced apoptosis is associated with degradation of Akt and XIAP. There has been accumulating evidence that mitochondria play an essential role in mediating apoptosis by releasing apoptogenic effectors, such as cytochrome *c* and apoptosis-inducing factor (AIF). Cytochrome *c* directly activates caspases by binding to Apaf-1 in the presence of ATP. Western blot analysis with cytosolic fractions revealed the release of cytochrome *c* in DNR-treated U937/vector and U937/Bcl-2 cells. Interestingly, the overexpression of antiapoptotic Bcl-2 in U937 cells significantly blocked DNR-induced release of cytochrome *c* from the mitochondria into the cytoplasm (Fig. 6B).

4. Discussion

Our study shows that DNR induces apoptosis in U937 cells. Proapoptotic concentrations of DNR induce early oxidative stress, which results in cytochrome *c* release, activation of caspase-3, PLC-γ1 cleavage and degradation of Akt. Furthermore, ectopic expression of Bcl-2 significantly attenuates DNR-induced apoptosis in U937 cells by inhibition of caspase-3 and sustained expression of the XIAP caspase inhibitors.

Since the discovery of Bcl-2, several theories have been proposed to unravel the antiapoptotic properties of this protein [14–16]. The antiapoptotic function of Bcl-2 may be explained by its ability to control several key steps of death signaling. Bcl-2 can form ion channels in biological membranes, and its ion channel activity may control apoptosis by influencing the permeability in the intracellular membranes and cytochrome *c* release into cytoplasm [26,27]. The release of cytochrome *c* triggers the activation of caspase-3 and fragmentation of DNA. However, overexpression of Bcl-2 protein may rescue cells from apoptosis by blocking the release cytochrome *c* from mitochondria and maintaining membrane integrity, or by a yet unrecognized function of the Bcl-2 protein [15,16].

One possible explanation for caspase-3 inactivation by DNR in Bcl-2 overexpressing cells is associated with sustained expression levels of XIAP. The caspase-inhibitory functions of XIAP are subject to multiple regulatory mechanisms. XIAP is inactivated by at least two mechanisms; proteolytic cleavage and interaction with XIAP-binding proteins such as Smac [28].

The mechanism by which PI3K or its products interfere with DNR-induced apoptosis is still unresolved. However, from other studies, one can speculate that the PI3K protective function includes Akt-mediated phosphorylation of Bcl-2 family proteins [29], inhibition of caspase activity [30,31], or stimulation of protein kinase C isoform activities [32,33]. Zhou *et al.* have reported that cell-permeable ceramide decreases the Akt activity, suggesting a possible cross-talk between shingomyelin-ceramide and PI3K-Akt pathways in DNR-treated cells [34]. Akt enhances cell proliferation and inhibits apoptosis in cancer cells, although the precise mechanism how it exerts its antiapoptotic effects is not clear. Although precisely how DNR decreases XIAP and Akt content in U937 cells is not known, it has been shown to activate down-stream caspases. Because XIAP and Akt are a substrate of caspase-3 and -7 [35,36], it is possible that the observed decrease in XIAP and Akt content could be a consequence of caspase-mediated processing after DNR treatment. The down-regulation of XIAP and Akt was blocked by a caspase inhibitor, indicating that XIAP and Akt were cleaved by caspases during apoptosis.

In summary, our studies demonstrate that DNR treatment induced cytochrome *c* release in U937 cells, which was associated with down-regulation of XIAP and inactivation of Akt. Moreover, overexpression of Bcl-2 attenuates DNR-induced apoptosis and prevents the release of cytochrome *c* from the mitochondria, sustained expression levels of XIAP. Therefore, it is likely that Bcl-2 overexpression inhibits DNR-induced apoptosis by a mechanism that interferes with down-regulation of XIAP and Akt involved in execution of apoptosis. Thus, Bcl-2 may be an important determinant of clinical response and prognosis in chemotherapy of leukemia.

Acknowledgments

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