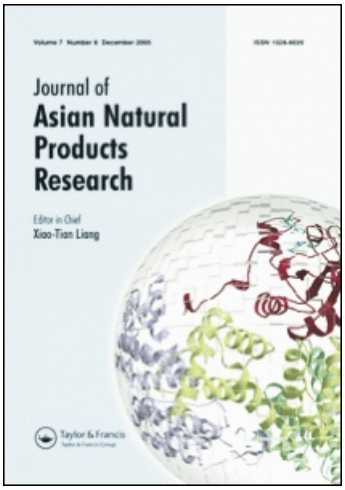

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Resveratrol induces apoptosis and differentiation in acute promyelocytic leukemia (NB4) cells

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Resveratrol induces apoptosis and differentiation in acute promyelocytic leukemia (NB4) cells

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Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a naturally occurring phytoalexin found in grapes and wine, and has been reported to exert a variety of important pharmacological effects. We have investigated the activity of resveratrol on proliferation and differentiation of the acute promyelocytic leukemia cell line NB4. The growth inhibitory properties of resveratrol appear to be due to its induction of apoptotic cell death, as determined by morphological changes, DNA fragmentation, increased proportion of the subdiploid cell population and decreased mitochondrial transmembrane potential ($\Delta\psi_m$). Colorimetric assay for activity of caspase-3 showed an obvious increase in caspase-3 activity in cells after treatment with resveratrol. However, the expression levels of protein Bcl-2 and Bax show no significant change in response to resveratrol treatment. These results suggest that apoptosis of NB4 cells induced by resveratrol requires caspase-3 activation and is related to the mitochondrial transmembrane potential. The combination of resveratrol and all-*tran*-retinoic acid (ATRA) induced 100% of the NB4 cells to become NBT-positive, whereas only a small part of cells became positive for NBT after a similar exposure to either resveratrol or ATRA alone. Thus, resveratrol may be useful in treating acute promyelocytic leukemia.

Keywords: Resveratrol; Apoptosis; Differentiation; Mitochondrial transmembrane potential; Caspase-3; Bcl-2; Bax

1. Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a phytoalexin present in grapes and other natural products, has been suggested as a potential cancer chemopreventive agent based on its striking inhibitory effects on diverse cellular events associated with tumor initiation, promotion, and progression [1]. An accumulation of evidence has shown that the anti-cancer activities of resveratrol are related to its ability to cause cell-cycle arrest in the G1 phase or in the S-G2 phase transition [2,3], or to trigger apoptosis in various cancer cell lines [4–8]. However, the precise mechanisms of resveratrol are not well understood.

Apoptosis is a major form of cell death, which is characterized by a series of tightly regulated stereotypic molecular events [9–12]. It plays an essential role as a protective mechanism against neoplastic development in organism by eliminating genetically damaged cells or excess cells that have been improperly induced to divide by a mitotic stimulus

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[13,14]. Several pathways regulate apoptosis during development, tumorigenesis, and chemical treatments. The expression of many genes is critical in the regulation of apoptosis, such as caspase cascades and Bcl-2 family proteins. The caspase family of aspartate-specific cysteine proteases is emerging as the executioner of apoptosis. Caspase-3 is activated in various cell types during apoptosis. Bcl-2 and Bax, two commonly studied apoptosis regulatory proteins, have been clearly associated with regulation of survival in several neoplastic cells. A decrease in Bcl-2 or an increase in Bax is associated with the initiation of cell death [15,16].

Acute promyelocytic leukemia (APL) is a special type of acute myelocytogenic leukemia. All-*trans*-retinoic acid (ATRA) induces complete remission in APL patients *via* induction of differentiation. However, the administration of ATRA might be associated with various side effects. Besides RA syndrome [17], the resistance to ATRA is another major problem [18]. Novel effective drugs alone or combination with ATRA are needed for ATRA-resistant or refractory APL patients.

In this study, we investigated the possible cellular and molecular mechanisms of resveratrol by using NB4 cells, an APL cell line with chromosome translocation *t* (15;17) from a relapsed APL patient [19], as an *in vitro* model. The results show that resveratrol can induce apoptosis in NB4 cells, which is possibly associated with the loss of mitochondrial transmembrane potential ($\Delta\psi_m$) and activation of caspase-3. Moreover, our study showed that the combination of resveratrol and ATRA was effective for the induction of differentiation of NB4 cells.

2. Results and discussion

2.1 Growth inhibition of NB4 cells by resveratrol

Resveratrol-induced NB4 cell growth inhibition was evaluated by using the trypan blue assay. The growth of NB4 cells was markedly inhibited with resveratrol treatment at 6.25–50 μmol L^{-1} (figure 1).

2.2 Induction of apoptosis by resveratrol

Typical apoptotic features, including apoptotic body, chromatin condensation and nuclear fragmentation were observed in NB4 cells after 24 h treatment with 50 μmol L^{-1} of

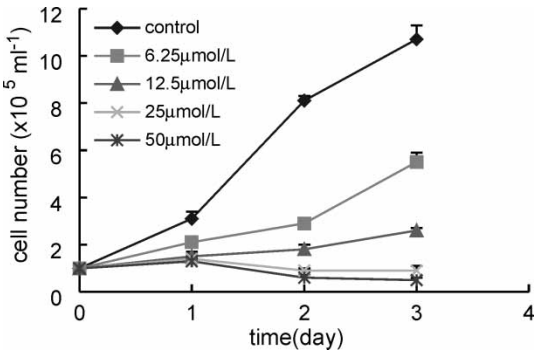

Figure 1. Growth inhibition of NB4 cells treated with different concentrations of resveratrol for 3 days. Data are presented as mean ± SD ($n = 3$).

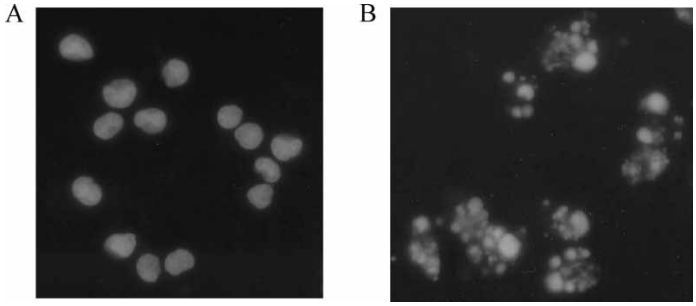

Figure 2. Resveratrol-induced morphological changes characteristic of apoptosis in NB_4 cells. Fluorescence microscopic examination of untreated cells (A) or those treated with 50 μmol L^{-1} resveratrol (B) for 24 h and stained with Hoechst 33258.

resveratrol (figure 2). The apoptotic changes were confirmed by DNA fragmentation analysis by means of agarose-gel electrophoresis. A typical DNA "ladder" was detected in NB4 cells after treatment with 25 and 50 μmol L^{-1} resveratrol (figure 3). The percentage of apoptotic cells evaluated by flow cytometry showed that induction of apoptosis in NB4 cells was dose-dependent (figure 4).

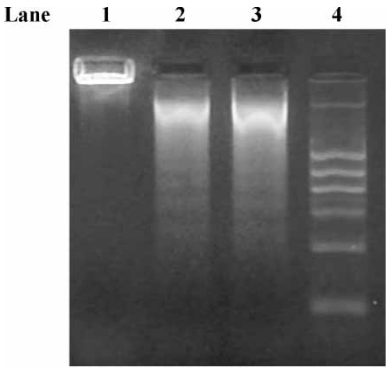

Figure 3. Agarose-gel electrophoresis of DNA from NB4 cells treated for 24 h with 25 and 50 μmol L^{-1} resveratrol. Lane: (1) control, (2) 25 μmol L^{-1}, (3) 50 μmol L^{-1}, (4) DNA marker.

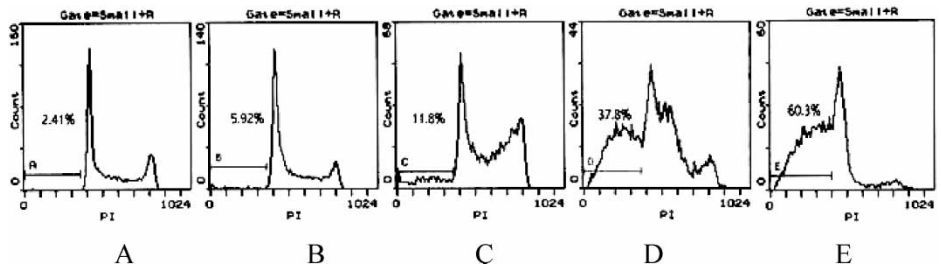

Figure 4. Flow cytometric analysis of DNA cleavage in resveratrol-treated cells. Cells were exposed to different concentrations of resveratrol for 24 h and stained with PI for DNA content analysis. (A) Control, and (B) 6.25, (C) 12.5, (D) 25, (E) 50 μmol L^{-1} resveratrol.

2.3 Induction of differentiation in NB4 cells

Differentiation was monitored by NBT reduction activity, resveratrol (12.5 μmol L^{-1}) treatment 5 days did not induce the reduction of NBT in NB4 cells; however, the combination of resveratrol (12.5 μmol L^{-1}, 5 days) and ATRA (10 nmol L^{-1}, 5 days) induced 100% of the NB4 cells to reduce NBT and the cells to show monocytic morphologic maturation with increased cytoplasm and polygonal nuclei, whereas only 10% of the cells became NBT-positive after exposure to ATRA alone (figures 5 and 6).

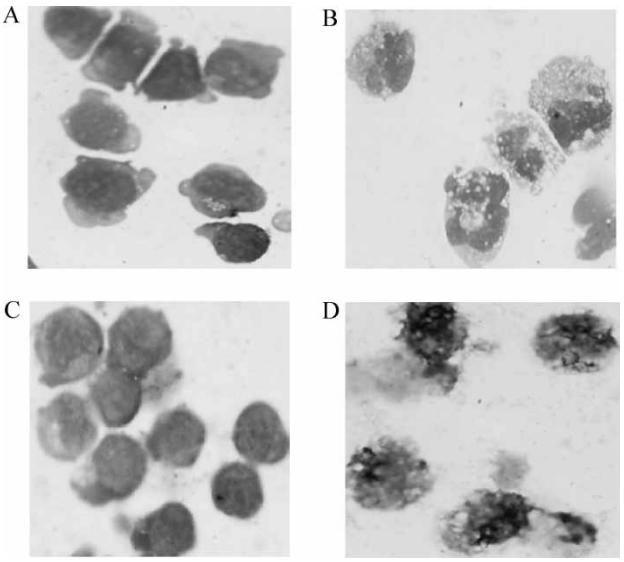

Figure 5. Morphological changes of NB4 cells after exposure to resveratrol and ATRA. (A) Wright-Giemsa staining of untreated NB4 cells; (B) Wright-Giemsa staining of NB4 cells after treatment with resveratrol (12.5 μmol L^{-1}) and ATRA (10 nmol L^{-1}) for 5 days. Nitroblue tetrazolium reduction assay either before (C) or after (D) resveratrol and ATRA treatment.

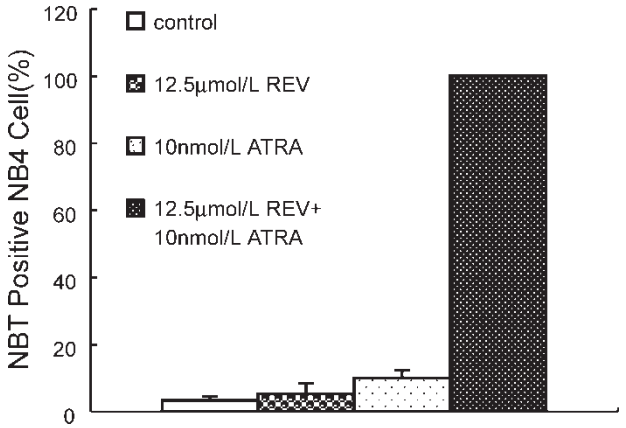

Figure 6. Nitroblue tetrazolium (NBT) positivity (%) of NB4 cells. Data are presented as mean ± SD ($n = 3$).

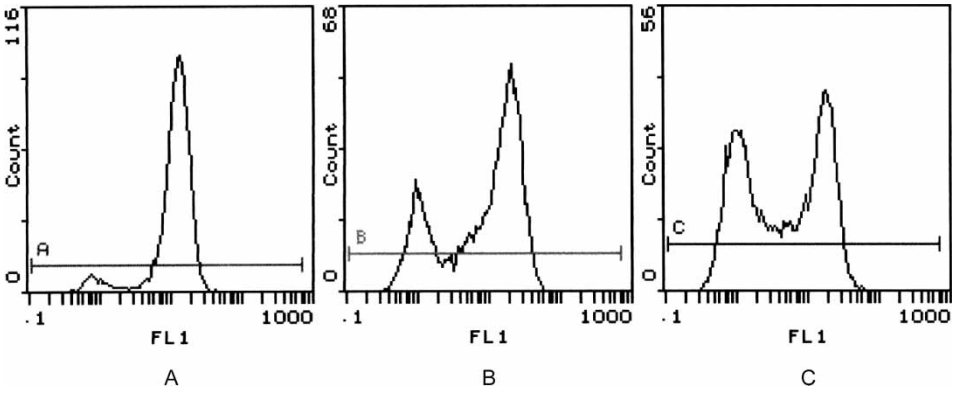

Figure 7. Flow-cytometric analysis of $\Delta\psi_m$ estimated by the Rh123 intensity. NB4 cells were treated with 25 and 50 μmol L^{-1} resveratrol for 24 h. Rh123 fluorescence of NB4 cells was analyzed by flow cytometry. (A) Control, and (B) 25, (C) 50 μmol L^{-1} resveratrol.

2.4 Effect of resveratrol on mitochondrial transmembrane potential $\Delta\psi_m$

We examined $\Delta\psi_m$ collapse by flow cytometry using Rh123, a lipophilic fluorochrome taken up by mitochondria in proportion to the $\Delta\psi_m$. After treatment with 25 and 50 μmol L^{-1} resveratrol for 24 h, low Rh123 staining NB4 cells indicated the loss of $\Delta\psi_m$ in a concentration-dependent manner (figure 7).

2.5 Resveratrol exposure results in activation of caspase-3

To investigate the pathways involved in resveratrol-induced apoptosis, the levels of caspase-3 activity were evaluated using the colorimetric substrate Ac-DEVD-pNA, a substrate specific for caspase-3. Incubation of NB4 cells with different concentration of resveratrol for different times resulted in an increased capacity of the cell lysated to cleave the chromogenic substrate compared with untreated cells. However, cells pretreated with Z-VAD-fmk, the general inhibitor of caspases, before the addition of resveratrol, completely inhibited

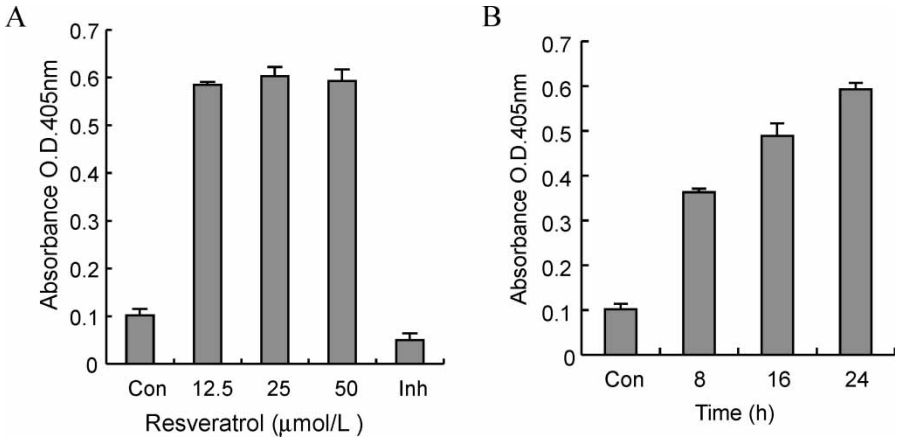

Figure 8. Resveratrol stimulates caspase-3 activity in NB4 cells. (A) Cells treated with different concentrations of resveratrol for 24 h (Inh: Z-VAD-fmk + 50 μmol L^{-1} resveratrol); (B) cells treated with 50 μmol L^{-1} resveratrol for different times. Data are presented as mean ± SD ($n = 3$).

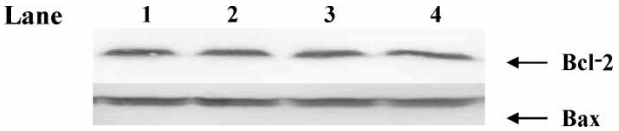

Figure 9. Western blot analysis of Bcl-2 and Bax in NB4 cells treated with different concentration of resveratrol for 24 h. Lane: (1) Control, and (2) 12.5, (3) 25, (4) 50 μmol L^{-1} resveratrol.

caspase-3 activity by resveratrol (figure 8). These data demonstrate that activation of caspase-3 is involved in resveratrol-induced apoptosis.

2.6 Western blot analysis of Bcl-2 and Bax

To investigate the possible involvement of Bcl-2 and Bax in the resveratrol-induced apoptosis, proteins from resveratrol-treated NB4 cells were analyzed using Western blot analysis. The cells were treated with different concentrations of resveratrol for 24 h. As compared with untreated cells, no significant changes in Bcl-2 or Bax were noted (figure 9). These data suggest that resveratrol-induced apoptosis in NB4 cells does not occur as a consequence of Bcl-2 protein modulation.

3. Discussion

Apoptosis is the most common and distinct form of cell death, involving a series of steps and acts as physiological suicide mechanism to preserve tissue homeostasis through proper cell turnover. There is ample evidence that naturally occurring compounds and many chemotherapeutic agents with antitumor effects can trigger the apoptosis of cancer cells [21]. The effects of resveratrol on programmed cell death are well documented by studies on various cellular or animal models [22,23]. Our present results are the first to show apoptosis induction by resveratrol in NB4 cells. The apoptotic effects of resveratrol in NB4 cells were demonstrated by flow cytometry analysis, DNA ladder formation and morphological evaluation as well.

The mechanism of apoptosis is remarkably conserved and arbitrated with the expected greater complexity. Accumulating evidence has proved that caspase and Bcl-2 families play key roles in opposite sides of cell death or survival in response to the numerous stimuli from inside or outside the cells. Caspases are at the heart of the apoptotic machinery. Several caspases are key executioners of apoptosis mediated by various inducers, including antitumor agents. Of these, caspase-3 is an important key executioner of apoptosis, being responsible either partially or wholly for proteolytic cleavage of numerous substrates. Our experiments have shown that caspase-3 activity in NB4 cells increased following treatment with resveratrol and was reverted efficiently by a caspase inhibitor (Z-VAD-fmk).

Mitochondria are essential in the propagation of apoptosis. An early stage apoptotic stimuli alters the mitochondrial transmembrane potential ($\Delta\psi_m$); the disruption of $\Delta\psi_m$ generally defines an early but already irreversible stage of apoptosis. The treatment of proliferating NB4 cells for 24 h with 25, 50 μmol L^{-1} resveratrol induces significant variations in the $\Delta\psi_m$ measured by flow cytometry using fluorochrome Rh123. We have shown that resveratrol induces a collapse of the $\Delta\psi_m$ in a dose-dependent manner. The collapse of $\Delta\psi_m$ results in an uncoupling of the respiratory chain and the efflux of small molecules (*e.g.* cytochrome c and calcium) and certain proteins, including caspase-2 and -9,

as well as the apoptosis-inducing factor that can, in turn, stimulate the proteolytic activation of caspase-3 [24]. Taken together, these results suggest that resveratrol-induced apoptosis is associated with the loss of $\Delta\psi_m$ and the activation of caspase-3.

The Bcl-2 family and its relationship to apoptosis in various cell types have been extensively investigated. The ratio of Bcl-2 to Bax determines survival or death following an apoptotic stimulus [25]. Bcl-2 inhibits induction of apoptosis and promotes cell survival under normal and neoplastic conditions. Our results show that there was no significant change in Bcl-2 after incubating NB4 cells with resveratrol. This indicates that Bcl-2 might not be involved in the apoptotic effects associated with resveratrol. Similar to Bcl-2, the Western blot analysis in our study did not demonstrate significant change in the major proapoptotic mediator, Bax. These findings exclude a possible role for Bax in the initiation of resveratrol-induced apoptosis in NB4 cells.

In summary, resveratrol can inhibit the cellular proliferation of NB4 cells *via* apoptosis, which is associated with the loss of mitochondrial transmembrane potential and the activation of caspase-3. Neither Bcl-2 nor Bax appears to be involved in this apoptosis. We also found that resveratrol enhanced the ATRA-induced differentiation of NB4 cells compared with ATRA alone. Our findings suggest that resveratrol merits further investigation as a therapeutic agent for the treatment of APL.

4. Experimental

4.1 Cell culture and growth inhibition assay

Human acute promyelocytic leukemia NB4 cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Cell growth was assessed in triplicate by trypan blue dye exclusion assay at the indicated times.

4.2 Drugs and reagents

Resveratrol was kindly provided by Professor Lin Mao, Institute of Materia Medica, Chinese Academy of Medical Science. All-*trans*-retinoic acid (ATRA), nitroblue tetrazolium (NBT), Rhodamine 123 (Rh123) and propidium iodide (PI) were from Sigma. Monoclonal antibodies to Bcl-2 and Bax were from Santa Cruz Biotechnology.

4.3 Morphological examination

A total of 10^6 cells were treated with 50 μmol L^{-1} resveratrol at 37°C for 24 h; the cells were then fixed with 4% formaldehyde in PBS, stained by Hoechst 33258 for 30 min. The slides were observed under fluorescent microscopy.

4.4 DNA fragmentation

NB4 cells (1×10^6 cells mL^{-1}) were treated with 25 and 50 μmol L^{-1} resveratrol for 24 h. Cellular DNA was extracted by the method described previously [20]. Electrophoresis was

performed in 1.8% agarose gel. The DNA was visualized and photographed under UV light after staining with ethidium bromide.

4.5 DNA content analysis and determination of mitochondrial transmembrane potentials ($\Delta\psi_m$) by flow cytometry

Cells treated with resveratrol were harvested by centrifugation and washed twice with cold PBS and resuspended at 1×10^6 cells mL^{-1} in 70% ethanol for 12 h, and then treated with 50 $\mu g\, mL^{-1}$ RNase A for 30 min at 37°C. Propidium iodide was added to the solution at a final concentration of 50 $\mu g\, mL^{-1}$. After 1 h incubation in the dark at 4°C, the distribution of cells with different DNA contents was determined by flow cytometry. To determine $\Delta\psi_m$, cells were washed twice with PBS and incubated with Rh123 (1 $\mu g\, mL^{-1}$) at 37°C for 30 min. The Rh123 intensity was determined by flow cytometry.

4.6 Cell differentiation studies

Cells were seeded at a cell density of 10^5 cells mL^{-1}. After treatment with resveratrol, ATRA for 5 days, cell differentiation was assessed by their ability to produce superoxide, as measured by the reduction of NBT, and a morphological examination was made on slides stained with Wright-Giemsa.

4.7 Caspase-3 activity assay

Activation of caspase-3 was determined by colorimetric assay using the caspase-3 activation kit from R&D Systems following the manufacturer's protocol. The cells were incubated for 24 h with and without resveratrol. All protein lysates were precleared by centrifugation at 12,000 rpm for 15 min at 4°C. Lysates were incubated with and without caspase-3 substrate for 4 h at 37°C before measurement on a microplate reader set at a wavelength of 405 nm. The relative increase in caspase-3 activity in resveratrol-treated cells compared with untreated cells was calculated after subtracting the background measurement obtained from lysate containing no substrate.

4.8 Western blot analysis

After treatment with different concentrations of resveratrol, about 2×10^7 cells were washed twice with PBS and lysed in 200 μL lysis buffer (0.1 M NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu g\, mL^{-1}$ aprotinin) at 4°C for 30 min. Lysates were centrifuged at 12,000 rpm for 10 min, and aliquots of the supernatant were boiled in SDS sample loading buffer for 5 min before electrophoresis on 12% SDS-polyacrylamide gel. After overnight transfer of SDS-polyacrylamide gel to nitrocellulose membrane, the blots were blocked with 5% non-fat dry milk in TBS buffer (50 mmol L^{-1} Tris–HCl, pH 7.6, 0.15 mmol L^{-1} NaCl) for 2 h at room temperature and then washed in TBS buffer. Nitrocellulose membranes were incubated for 1 h at room temperature with a mouse bcl-2, bax monoclonal antibody. Blots were rinsed with TBS and the immunocomplex was visualized by colorization with horseradish peroxidase-conjugated second antibody and diaminobenzine substrates.

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