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Leptomycin B-induced apoptosis is mediated through caspase activation and down-regulation of Mcl-1 and XIAP expression, but not through the generation of ROS in U937 leukemia cells

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Abstract

Leptomycin B (LMB), which is originally isolated from *Streptomyces*, possesses anti-tumor properties in vivo and in vitro. Though it was previously reported that LMB induces cell cycle arrest and p53-mediated apoptosis in certain cancer cells, however, the mechanism by which LMB induces apoptosis remains poorly understood. Here, we investigated the mechanisms of apoptosis induced by LMB in U937 cells. Treatment with LMB concentration-dependently induced cytotoxicity and apoptosis in U937 cells that correlated temporally with activation of caspases and down-regulation of Mcl-1 and XIAP. LMB did not change the expressions of Bcl-2 or Bax. A broad spectrum caspase inhibitor, z-VAD-fmk, blocked caspase-3 activation and elevated the survival in LMB-treated U937 cells, suggesting that caspase-3 activation is critical for LMB-induced apoptosis. Interestingly, Bcl-2 overexpression that blocked cytochrome c release by LMB effectively attenuated the apoptotic response to LMB, suggesting that LMB-induced apoptosis is mediated through the mitochondrial pathway. Antioxidants or antioxidant enzymes had no effects on LMB-induced apoptosis. Data of flow cytometry analysis using 2′,7′-dichlorofluorescein-diacetate further revealed no reactive oxygen species (ROS) generation by LMB, indicating that apoptosis induced by LMB is ROS-independent. However, the apoptotic response to LMB was not shown in U937 cells pretreated with the sulfhydryl group-containing antioxidant *N*-acetylcysteine (NAC). Further analysis suggested that NAC directly binds LMB and abolishes the apoptotic effects of LMB. Collectively, these findings suggest that LMB potently induces apoptosis in U937 cells, and LMB-induced apoptosis in U937 cells is related with cytochrome c release, activation of caspases, and selective down-regulation of Mcl-1 and XIAP.

Keywords: Leptomycin B; Apoptosis; Caspases; Mcl-1; XIAP; NAC

1. Introduction

Leptomycin B (LMB) was originally discovered as an antifungal antibiotic from *Streptomyces* [1]. Recently, the cellular target of LMB has been identified to be CRM1 protein, which is conventionally recognized to function the maintenance of chromosomal structure [2]. More recently, CRM1 has been found to be a nuclear export receptor which mediates the nuclear-cytoplasmic transport of NES-containing proteins [3,4] or RNAs [5,6]. Because of the high specificity to CRM1, LMB has been widely used by many laboratories in understanding the nuclear export mechanism

Abbreviations: BCR-ABL, breakpoint cluster region-abelson murine leukemia; CRM1, chromosomal region maintenance 1; DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; DEVD-*p*Na, Asp-Glu-Val-Asp-*p*-nitroanilide; DPI, diphenyleneiodonium; IAP, inhibitor of apoptosis protein; LMB, leptomycin B; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NAC, *N*-acetylcysteine; NES, nuclear export sequence; PARP, poly(ADP-ribose)polymerase; ROS, reactive oxygen species; SOD, superoxide dismutase; z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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of proteins or RNAs. However, recent studies have demonstrated that LMB is an effective anti-tumor agent against murine experimental tumors [7] and causes G1 cell cycle arrest in mammalian cells [8]. Furthermore, it has been reported that LMB induces apoptosis in certain types of cancerous cells by nuclear accumulation of NES-containing p53 [9,10] or BCR-ABL [11], whose nuclear sequestration is well correlated with cell death. From the accumulating evidence, it has been considered that cytotoxic and apoptotic effects by LMB are primarily associated with cell cycle arrest and inhibition of CRM1-dependent nuclear export of proteins.

Many therapeutic and chemopreventive agents eliminate cancerous cells by inducing programmed cell death, apoptosis. Apoptosis is an important cellular process to destruct undesired cells during development or homeostasis of multi-cellular organisms and is characterized with distinct morphological changes including plasma membrane bleb, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation [12]. Many proteins are known to involve in the process of apoptosis. Caspases are essential for the execution of cell death by various apoptotic stimuli [13]. Caspase activation is often regulated by various cellular proteins, including members of the IAPs [14] or Bcl-2 family [15]. Apoptosis by anti-cancer agents through lowering expression levels of the members of Bcl-2 or IAPs family has been reported in U937 leukemia cells [16], acute myeloid leukemia cells and chronic lymphocytic leukemia cells [17], or multiple myeloma cells [18]. Furthermore, it has been shown that apoptosis induced by anti-cancer or chemopreventive agents can be mediated through additional apoptotic proteins or pathways, including release of apoptogenic factors such as cytochrome c from the mitochondria to the cytosol [19], or oxidative stress such as reactive oxygen species (ROS) [20,21].

The molecular mechanisms by which LMB induces apoptosis remain poorly understood. In present study, we investigated whether LMB induces apoptosis in U937 leukemia cells, and determined the molecular events that mediate LMB-induced apoptosis in these cells. Our data demonstrate that treatment with LMB induces apoptosis in U937 leukemia cells, and suggest that the apoptosis induced by LMB seems to be related with cytochrome c-mediated activation of caspases and selective down-regulation of XIAP and Mcl-1 expression, but not through the generation of ROS. Moreover, our data suggest that the α,β-unsaturated lactone moiety of LMB, which can interact with the sulfhydryl (SH) group of NAC, appears to be important for the cytotoxic and apoptotic effects induced by LMB.

2. Materials and methods

2.1. Materials

Antibodies of caspase-3, caspase-9, cytochrome c, Mcl-1, XIAP, HIAP-1, Bcl-2, and Bax were bought from PharMingen. Antibody of PARP was purchased from Boehringer Mannheim. MTS reagent was obtained from Promega. DCFH-DA, Vitamin E, ebselen, DPI, allopurinol, NAC, catalase, SOD, and antibody of actin were purchased from Sigma-Aldrich. z-VAD-fmk and Ac-DEVD-*p*Na were obtained from Calbiochem. LMB was kindly provided by Dr. M. Yoshida (RIKEN, Japan).

2.2. Cell culture and transfection

U937 human leukemia cells were maintained at 37 °C in a humidified condition of 95% air and 5% CO_2 in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. U937 cells were transfected with a human *Bcl-2* cDNA (kindly provided by Dr. Rakesh Srivasta, NIH/NIA) and stable clones overexpressing Bcl-2 were selected under antibiotic-containing cell culture media.

2.3. Cell viability and survival assay

For cell viability assay, U937 cells (3×10^5 cells/mL) were seeded in the absence or presence of LMB for the indicated times. After treatment with LMB, cells were washed twice with PBS, followed determination of cell viability with MTS reagent. For cell survival assay, U937 cells (1.5×10^6 cells/mL) were treated for 12 h with LMB alone or LMB–NAC mixture between LMB (20 nM) and NAC in different doses (0.02–20 mM) before addition to cells for 2 h. Cells survived were counted by the trypan blue exclusion methods and expressed as percentage over control.

2.4. DNA fragmentation assay

After treatment with LMB, cells were harvested, washed, and lysed in a buffer [50 mM Tris (pH 8.0), 0.5% sarkosyl, 0.5 mg/mL proteinase K, and 1 mM EDTA] at 55 °C for 3 h, followed addition of RNase A (0.5 μg/mL) and further incubation at 55 °C for 18 h. The lysates were centrifuged at 10,000 × *g* for 20 min. The genomic DNA in the supernatant was extracted with equal volume of neutral phenol–chloroform–isoamyl alcohol mixture (25:24:1), and analyzed by electrophoresis on 1.7% agarose gel. The DNA was visualized and photographed under UV illumination after staining with ethidium bromide (0.1 μg/mL).

2.5. Subcellular fractionation

Cell subcellular fractionation experiment was performed with ApoAlert^R Cell fractionation kit (CLONTECH) in which the efficiency of cell subcellular fractionation (cytosol versus mitochondria) is readily evaluated by the signal of cytochrome c oxidase subunit IV (COX4), a mitochondrial

membrane protein. After LMB treatment, U937 cells were collected, resuspended in PBS, and centrifuged at 600 × *g* for 5 min. The pellets were then lysed in buffer A [0.25 M sucrose, 30 mM Tris–HCl (pH 7.9), and 1 mM EDTA, 1 mM phenylmethysulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 μg/mL aprotinin], and homogenized with a glass dounce homogenizer (40 strokes). After centrifugation at 14,000 rpm for 30 min, the supernatant was collected, and used as the cytosolic fraction. The left over was further extracted in buffer B (buffer A plus 1% Triton X-100 and 1% SDS) with sonication, and used as the mitochondrial fraction. To detect cytochrome c levels in the cytosolic and mitochondrial compartment, an immunoblotting was performed with an anti-cytochrome c antibody.

2.6. Measurement of caspase-3 activity

Cells were collected, washed in PBS, and resuspended in a buffer [25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethysulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin]. Cell lysates were clarified by centrifugation at 12,000 rpm for 10 min, and 50 μg of protein of the supernatant was incubated with 50 μM Ac-DEVD-*p*Na colorimetric substrate at 37 °C for 1 h. The optical density was measured at 405 nm.

2.7. Measurement of ROS generation

The generation of ROS was measured by a flow cytometry analysis using DCFH-DA as a substrate. Briefly, after treatment, cells were harvested, washed twice with PBS, and suspended in PBS (1×10^6 cells/mL). The cell suspension (500 μL) was placed in a tube, loaded with DCFH-DA to a final concentration of 20 μM, and incubated at 37 °C for 15 min. Cells were then exposed with LMB (20 nM) for 0.5–12 h or methylselenocysteine (80 μM, a known ROS inducer [21]) for 0.5 h at 37 °C. The ROS generation was assessed by the DCF fluorescence intensity (FL-1, 530 nm) from 10,000 cells with a FACS Caliber flow cytometer (Becton Dickinson).

2.8. Flow cytometry analysis for measurement of sub-G1 phase

Cells were harvested and washed once with PBS, fixed in ice-cold 70% ethanol and stored at 4 °C. Prior to analysis, cells were washed once again with PBS, suspended in 1 mL of cold propidium iodide solution containing 100 μg/mL RNase A, 50 μg/mL propidium iodide, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40, and further incubated on ice for 30 min in the darkness. Cytometric analyses were carried out with a flow cytometer (FACS Caliber, Becton Dikinson) and CellQuest software. Approximately, 15,000 cells were counted for the analysis.

2.9. Western blot analysis

Whole cell extracts were prepared in a modified RIPA buffer [50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 0.25% sodium-deoxycholate, 1% Triton X-100, 1% NP-40, 1 mM EGTA, protease inhibitor mixture (1×), 1 mM Na_3VO_4, and 5 mM NaF]. The protein concentration was determined with Bradford reagent (Bio-Rad). Approximately, 40 μg of protein was dissolved on 8–12% SDS–PAGE, and transferred to Immobilon P membranes (Millipore). Immunoblotting with appropriate antibodies such as caspase-3 (1:2000), caspase-9 (1:1000), PARP (1:3000), cytochrome c (1:2000), XIAP (1:1000), HIAP-1 (1:1000), Mcl-1 (1:2000), Bax (1:1000), Bcl-2 (1:2000), or Actin (1:10,000) was performed by standard procedures, and proteins were detected using alkaline phosphatase-conjugated secondary antibody (Amersham Pharmacia Biotech). Equal protein loading was determined by an anti-actin Western blot analysis.

3. Results

3.1. Cytotoxic effects of LMB on U937 leukemia cells

To verify LMB-induced cell cytotoxicity, we examined the effects of different concentrations of LMB on U937 cell viability. Our MTS data showed that treatment with LMB for 12 h causes a concentration-dependent reduction of U937 cell viability (Fig. 1A). After 12 h treatment, about 60% loss of cell viability was detected at 20 or 40 nM LMB. Since there was little difference of loss of cell viability between 20 and 40 nM dose of LMB, we used 20 nM concentration of LMB for subsequent studies. In time kinetic studies, we observed that 6 h treatment with LMB at 20 nM induced only 20% loss of viability, but 12 and 24 h treatment with LMB at 20 nM led to about 60% decrease of viability (Fig. 1B). We next tested whether LMB induces DNA fragmentation, one of the hallmarks of apoptosis, in U937 cells. LMB induced a time-dependent progressive accumulation of fragmented DNA, which appeared as a typical ladder pattern of DNA fragmentation due to internucleosomal cleavage associated with apoptosis (Fig. 1C). To quantify the degree of apoptosis, we analyzed the amount of sub-G1 DNA by flow cytometry in U937 cells treated with 20 nM LMB for the indicated times (6, 12, or 24 h). As shown in Fig. 1D, the addition of LMB to U937 cells resulted in increased accumulation of sub-G1 phase in similar time kinetics of LMB-induced loss of cell viability (Fig. 1B) and accumulation of extra-nuclear fragmented DNA (Fig. 1C). Together, these results suggest that the mechanism of cytotoxic effects induced by LMB on U937 cells is related with its apoptosis-inducing activity.

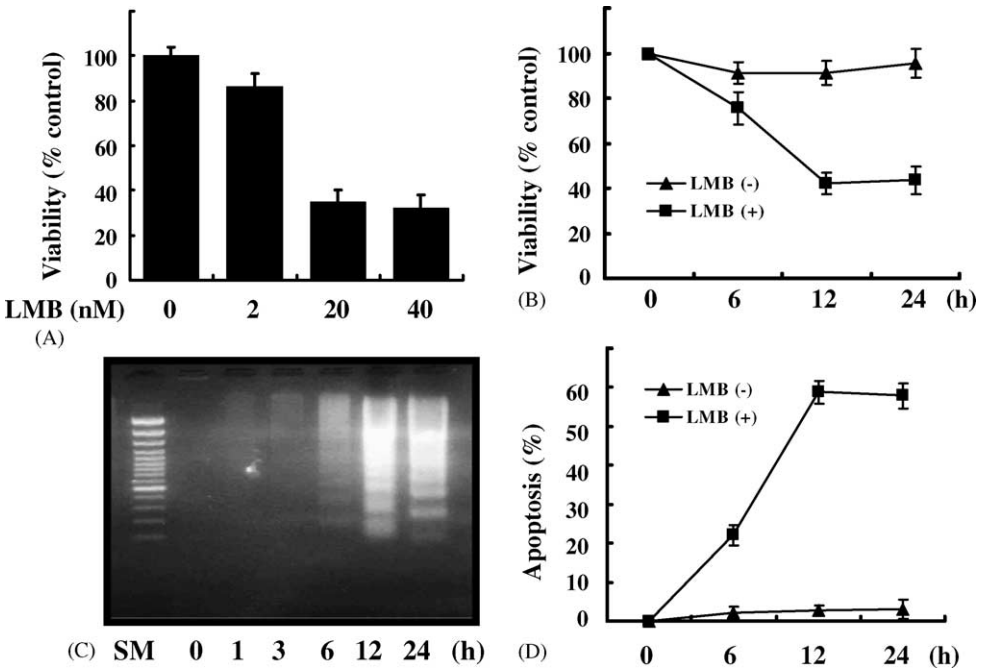

Fig. 1. Effects of LMB on viability, DNA fragmentation, and sub-G1 DNA change in U937 cells. (A) U937 cells were treated with LMB in different concentrations (0, 2, 20, 40 nM) for 12 h, followed measurement of cell viability by a MTS assay. (B) U937 cells were treated without or with LMB (20 nM) for the indicated times (6, 12, or 24 h), followed measurement of cell viability by a MTS assay. Data are mean ± S.E. of three independent experiments. (C) For the analysis of DNA fragmentation, U937 cells were treated with LMB (20 nM) for the indicated times. Extra-nuclear fragmented DNA was extracted, and analyzed on a 1.7% agarose gel. SM indicates a size marker of DNA ladder. (D) To quantify the degree of apoptosis induced by LMB, U937 cells were treated without or with LMB (20 nM) for the indicated times, and evaluated for sub-G1 DNA content by flow cytometer. The graph represents the fraction of apoptotic cells. Data are mean ± S.E. of three independent experiments.

3.2. Caspases play an important role in LMB-induced apoptosis

Caspases are essential for the execution of cell death by various apoptotic stimuli [13]. To determine whether LMB-induced apoptosis is associated with the activation of caspases, we measured the activation of caspases in U937 cells treated with LMB. Activation of caspase-9, -3, or -8 during the apoptotic induction by LMB was evaluated by the decrease in levels of each inactive proform, which gets proteolytically cleaved upon activation. In U937 cells, treatment with LMB resulted in decrease of the inactive proform of both the upstream caspase-9 and the downstream effector caspase-3 in a time-dependent manner (Fig. 2A), whereas there was no change in procaspase-8 by treatment with LMB in U937 cells (data not shown). Activation of caspase often leads to the proteolytic cleavage of several target proteins such as PARP. Subsequent Western blot analysis showed progressive proteolytic cleavage of PARP in U937 cells after treatment with LMB (Fig. 2A). To ascertain the proteolytic activity of caspase-3, we performed an in vitro assay, based on the cleavage of Ac-DEVD-*p*Na by active caspase-3 into chromophore *p*-nitroanilide. Treatment with LMB induced about four-fold increase of caspase-3 activity (Fig. 2B, column 2). To prove that activation of caspases is an essential step in the apoptotic pathway induced by LMB, U937 cells were pretreated with a cell-permeable broad spectrum caspase inhibitor, z-VAD-fmk (100 μM) for 1 h, and then treated with 20 nM LMB for 12 h. Pretreatment with z-VAD-fmk suppressed activation of caspase-3 by LMB (Fig. 2B, column 3). Furthermore, z-VAD-fmk significantly blocked not only PARP cleavage (Fig. 2C) but also DNA fragmentation (Fig. 2D) in LMB-treated U937 cells. These results clearly indicate that LMB-induced apoptosis is associated with the activation of caspases. The major mechanism of processing and activation of caspases is mediated by Apaf-1 [22]. Apaf-1 is activated by binding to cytochrome c from the mitochondria in early stage of apoptosis. Therefore, using subcellular fractionation experiments, we next assessed the extent of cytochrome c translocation from the mitochondria to the cytoplasm in U937 cells in response to LMB. The efficiency of subcellular fractionation (cytosol versus mitochondria) in this study was assessed by the signal of cytochrome c oxidase subunit IV (COX4), a mitochondrial membrane protein. COX4 Western blot clearly demonstrated that this mitochondrial protein was solely detected in the mitochondrial but not in the cytosolic fraction (data not shown), suggesting that the procedure of our cell fractionation experiments is reasonable. A time-dependent increase in cytoplasmic cytochrome c but concomitant decrease in mitochondrial cytochrome c was detected following treatment with

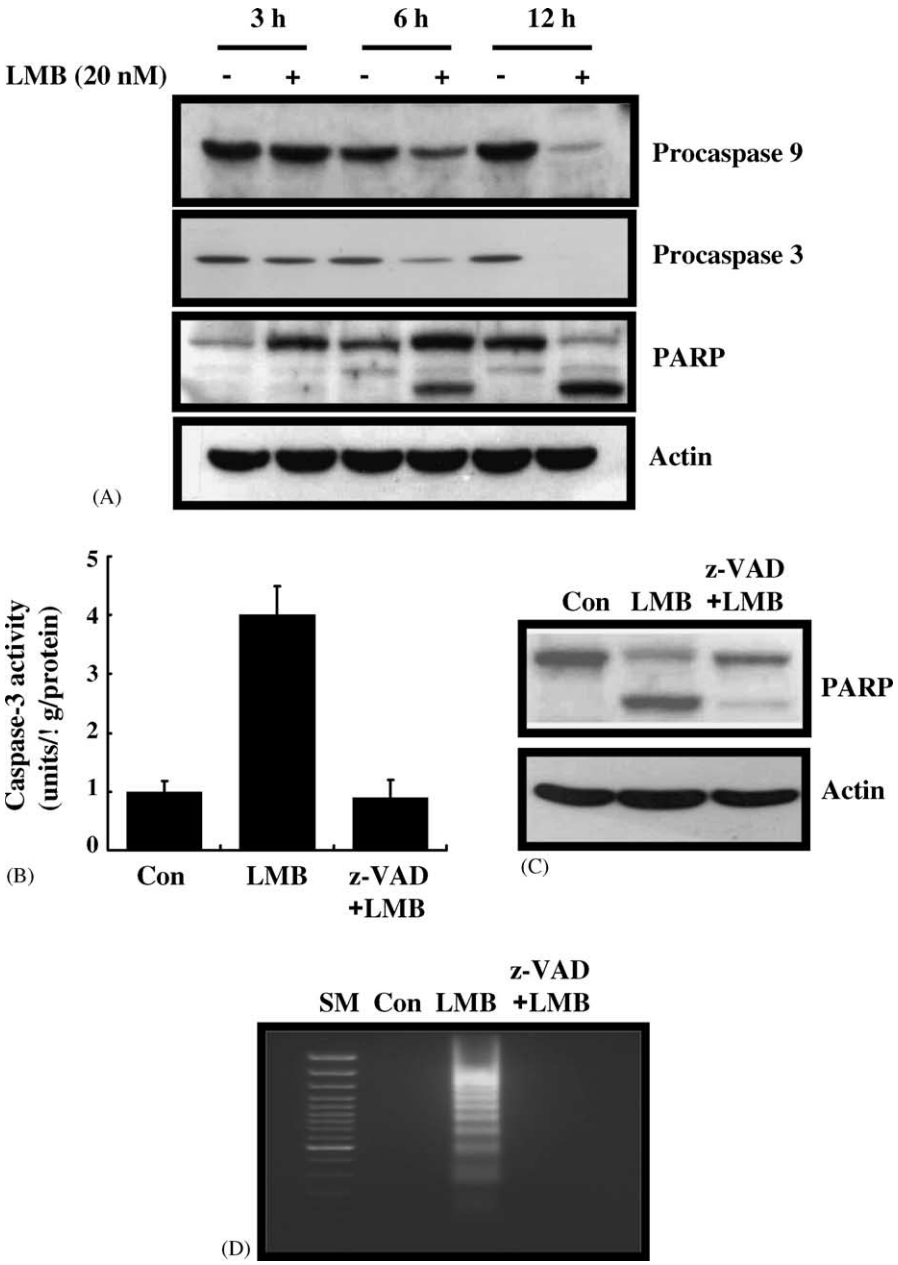

Fig. 2. LMB induces caspase activation that is critical for LMB-induced apoptosis in U937 cells. (A) U937 cells were treated without or with LMB (20 nM) for the indicated times. At each time, whole cell lysates were made, and analyzed for the detection of procaspase-9, procaspase-3, PARP, XIAP, HIAP-1, and actin with respective antibodies. (B) U937 cells were pretreated for 1 h with a pan-caspase inhibitor z-VAD-fmk (100 μM), and then treated with LMB (20 nM) for additional 12 h. Cell lysates were made, and used for the measurement of caspase-3 activity using 50 μM of Ac-DEVD-*p*Na, a caspase-3 substrate. Data are mean ± S.E. of three independent experiments. (C) Whole cell lysates were prepared from U937 cells under same conditions as (A), and used for the detection of PARP cleavage. To confirm equal loading, the same blot was stripped and reprobed with anti-actin antibody. (D) The genomic DNA was extracted from U937 cells grown under same conditions as (A), and run on 1.7% agarose gels for DNA fragmentation.

LMB (Fig. 3A). Interestingly, substantial accumulation of cytoplasmic cytochrome c was detected even at 1 h treatment with LMB, which time kinetic was much earlier than that of the proteolytic activation of caspase-9 and -3 as shown in Fig. 2A. Together, these results evidently demonstrate that the increase of cytochrome c levels in the cytosol in response to LMB is due to the decrease of cytochrome c in the mitochondria, and suggest that LMB-induced apoptosis in U937 cells is mediated through cytochrome c-mediated activation of caspases.

3.3. Changes of the expression of the Bcl-2 and IAP family members in LMB-induced apoptosis

Caspase activation is known to be regulated by various proteins including the IAP or Bcl-2 family [14,15]. To analyze whether LMB induces U937 cell death by alteration of the expression of IAP or Bcl-2 family members, we next examined the expression of IAP or Bcl-2 family members in LMB-treated U937 cells for the different times. As shown in Fig. 3B, the addition of LMB to U937 cells did not change

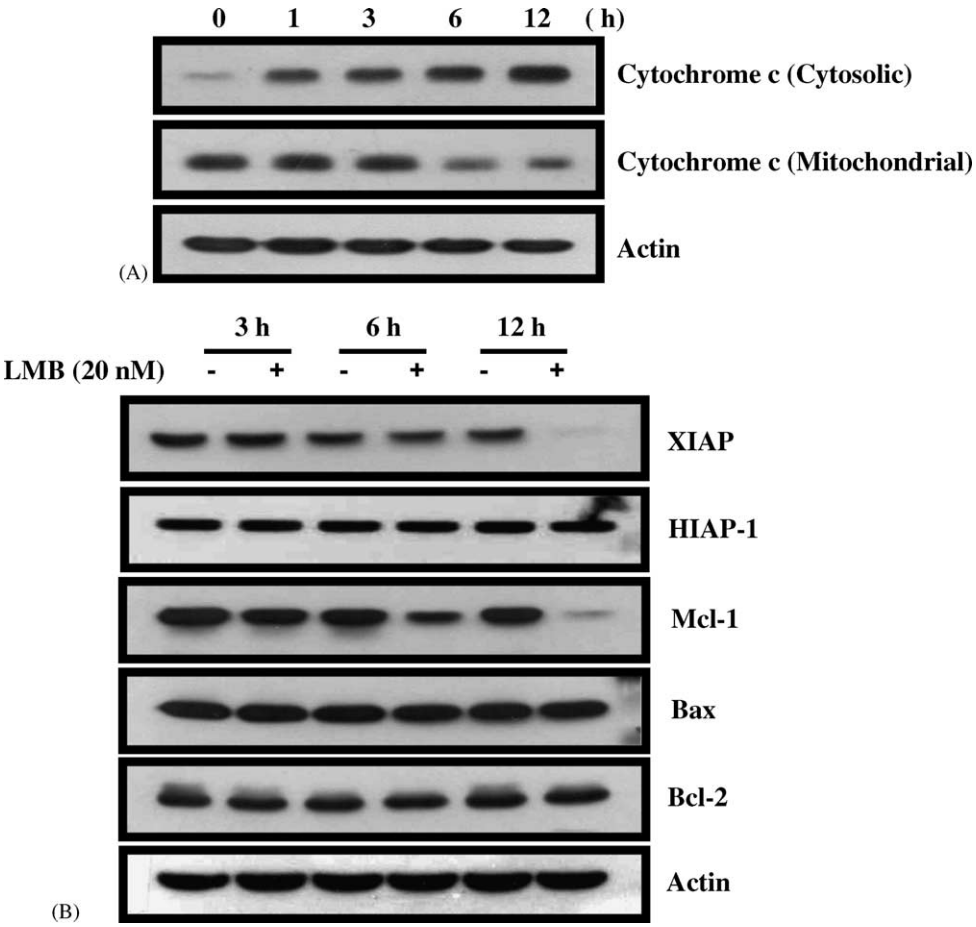

Fig. 3. Cytosolic accumulation of cytochrome c and changes of the expression of apoptotic-related proteins in LMB-treated U937 cells. (A) U937 cells were treated with LMB (20 nM) for the indicated times. At each time, cytosolic and mitochondrial fractions were prepared as described in Section 2, and analyzed for the measurement of cytochrome c levels in each fraction using a specific anti-cytochrome c antibody. Actin was used as a control for equal loading of samples. (B) U937 cells were treated for the indicated times with 20 nM LMB, and whole cell lysates were made. Equal amounts of cell lysates (50 μg) were subjected to electrophoresis and analyzed by Western blotting to measure the expression levels of XIAP, HIAP-1, Mcl-1, Bax, and Bcl-2. To confirm equal loading, actin immunoblotting with an anti-actin antibody was carried out.

the expression level of HIAP-1, but the expression level of XIAP was down-regulated in a time-dependent manner. Treatment with LMB also induced a time-dependent decline in the expression levels of Mcl-1, while the expression levels of Bcl-2 or Bax were not changed by LMB treatment. These results indicate that LMB seems to specifically down-regulate the expression levels of XIAP and Mcl-1 in U937 cells. The down-regulation of XIAP and Mcl-1 occurred in a similar time kinetic with the cleavage of PARP and DNA fragmentation. Therefore, these results suggest that the down-regulation of XIAP and Mcl-1 in response to LMB may have no significant effects on activation of caspases in early apoptotic progress, but their down-regulation may enhance or maintain activation of caspases in late stage of LMB-induced apoptotic pathway.

3.4. Overexpression of Bcl-2 strongly inhibits LMB-induced apoptosis in U937 cells

Although LMB did not change Bcl-2 expression, Bcl-2 is a well-known anti-apoptotic protein and this protein is localized into the mitochondrial membrane. The anti-apoptotic mechanism by Bcl-2 has been associated with increase of the mitochondrial membrane integrity [23,24]. To evaluate the effect of high intracellular levels of Bcl-2 on release of cytochrome c, activation of caspases, and cleavage of PARP during LMB-induced apoptosis, we used U937/vector cells and U937/Bcl-2 cells constitutively expressing high levels of Bcl-2. As shown in Fig. 4A, the expression level of Bcl-2 in U937/Bcl-2 cells was about four-fold higher than that of Bcl-2 in U937/vector cells. As expected, the addition of LMB at 20 nM for 12 h induced release of cytochrome c and activation of caspase-9/-3 in U937/vector cells (Fig. 4A). Furthermore, the exposure of LMB at 20 nM induced apoptosis in U937/vector cells (Fig. 4B), which was determined by measurement of the degree of sub-G1 accumulation. In contrast, Bcl-2 overexpression significantly blocked release of cytochrome c and activation of capase-9/-3 induced by LMB (Fig. 4A), which was consistent with decreased apoptosis in LMB-treated U937/Bcl-2 cells (Fig. 4B). Moreover, while LMB significantly caused

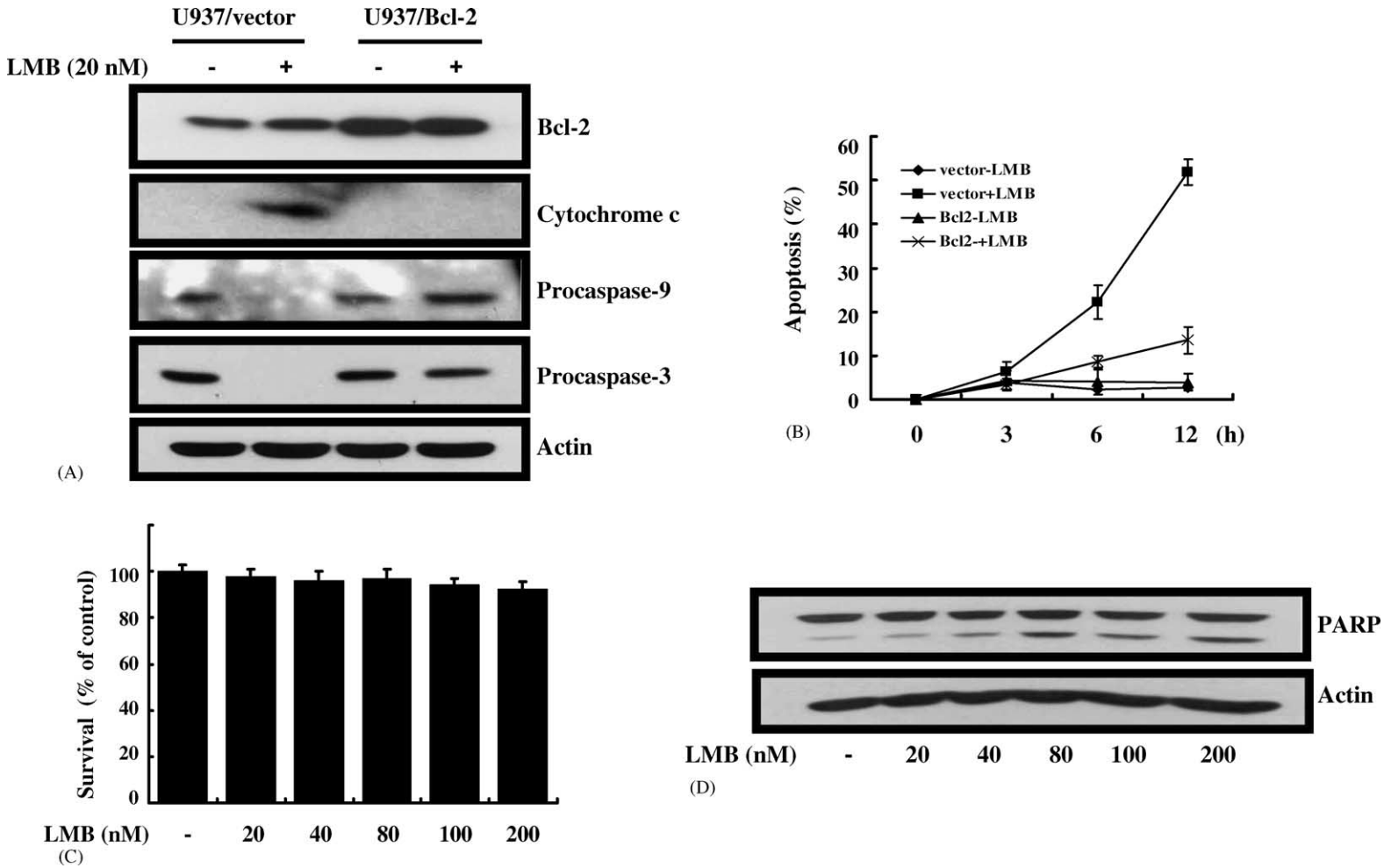

Fig. 4. Effect of Bcl-2 overexpression on LMB-induced apoptosis in U937 cells. (A) U937/vector or U937/Bcl-2 cells were treated with LMB (20 nM) for 12 h. Either cytosolic or whole cell extracts were prepared and analyzed for the detection of either cytochrome c or Bcl-2, procaspase-9, procaspase-3, and actin with respective antibodies. (B) U937/vector cells (U937) or U937/Bcl-2 cells (Bcl-2) were treated without or with LMB (20 nM) for the indicated times (3, 6, or 12 h) and at each time, sub-G1 DNA content was evaluated by flow cytometer. The graph represents the fraction of apoptotic cells. Data are mean ± S.E. of three independent experiments. (C) U937/Bcl-2 cells were treated with LMB (20, 40, 80, 100, or 200 nM) for 12 h, and cells survived were then counted by the trypan blue exclusion method. Data are mean ± S.E. of three independent experiments. (D) Whole cell lysates were prepared from U937/Bcl-2 cells treated with LMB (20, 40, 80, 100, or 200 nM) for 12 h, and used for the detection of PARP cleavage. To confirm equal loading, the same blot was stripped and reprobed with anti-actin antibody.

PARP cleavage in U937/vector cells (Fig. 2A), LMB failed to induce PARP cleavage in U937/Bcl-2 cells (Fig. 4D, column 2). We next determined whether the inhibitory effect of Bcl-2 overexpression on LMB-induced cytotoxicity and apoptosis in U937 cells could be overcome by increasing the concentrations of LMB. The data of cell survival assay and PARP Western blot evidently demonstrated that there was no reduction of cell survival and no significant PARP cleavage in U937/Bcl-2 cells by treatment with increased LMB concentrations (even up to 200 nM) (Fig. 4C and D), suggesting that the effect of Bcl-2 overexpression abolishing LMB-induced cytotoxicity and apoptosis in U937 cells is not overcome by raising the concentrations of LMB. Collectively, these data demonstrate that though LMB does not affect the expression of Bcl-2, Bcl-2 overexpression effectively attenuates LMB-induced apoptosis through blockage of cytochrome c release and activation of caspases, and suggest that LMB may target and damage the mitochondria, thereby leading to release of cytochrome c, followed subsequent activation of caspases.

3.5. Apoptosis induced by LMB is not mediated through the generation of ROS

Many anti-neoplastic agents eliminate tumor cells by inducing apoptosis through oxidative stress such as ROS [20,21]. This led us to examine whether LMB-induced apoptosis is through the generation of ROS. Pretreatments with antioxidants including Vitamin E or ebselen, antioxidant enzymes such as catalase or SOD, and inhibitors of NAPDH or xanthine oxidase (DPI or allopurinol, respectively) did not exert a protective effect on LMB-induced apoptosis in U937 cells (Fig. 5A). Moreover, their treatments did not prevent activation of caspases and PARP cleavage induced by LMB (data not shown). More directly, data of a flow cytometry analysis using DCFH-DA showed that treatment with LMB (20 nM, 0.5 h) did not induce the generation of ROS in U937 cells (Fig. 5B), which was evaluated by the degree of ROS generated in U937 cells treated with methylselenocysteine (80 μM, 0.5 h), a known ROS inducer [21]. Long time treatment with LMB at 20 nM (1–12 h) also did not induce the generation of ROS (data not shown). These results strongly suggest that apoptosis induced by LMB is not mediated through the generation of ROS. However, very interestingly, our data demonstrated that pretreatment with the SH group-containing antioxidant NAC, which is another well-known ROS scavenger, inhibited LMB-induced apoptosis of U937 cells in a concentration-dependent manner (Fig. 5C), and strongly blocked LMB-induced release of cytochrome c, proteolytic activation of caspase-9/-3, and PARP cleavage (Fig. 5D). Therefore, we further investigated the suppressive mechanism of NAC against LMB-induced apoptosis in U937 cells.

3.6. The α,β-unsaturated lactone moiety of LMB seems to mediate the cytotoxic and apoptotic effects by LMB

It has been previously shown that NAC directly binds and inactivates LMB via direct interaction between the SH group of NAC and the α,β-unsaturated lactone of LMB by a Michael-type addition, thereby abolishing the ability of LMB to bind and inactivate CRM1 nuclear export receptor [25]. This led us to investigate whether NAC inhibition of the cytotoxic and apoptotic effects triggered by LMB is through its direct binding to LMB as reported previously. Therefore, an in vitro binding assay was carried out in which LMB (20 nM) was first preincubated for 2 h with NAC in different doses (0.02–20 mM) before addition to cells, and the resultant LMB–NAC mixture was subsequently added to U937 cells for additional 12 h. There was about 60% decrease of the survival in U937 cells treated with LMB that was not preincubated with NAC, but there was great increase of the survival in U937 cells treated with LMB that was preincubated with NAC particularly at higher doses (10 or 20 mM) (Fig. 6A). To assure the binding specificity between LMB and NAC, LMB was also preincubated for 1 h with either Vitamin E (100 μM) or ebselen (20 μM), which lacks the SH group within the own structure, before addition to cells, and the resultant respective mixture was then added to U937 cells. Unlike NAC, preincubation of these compounds did not interfere with the ability of LMB decreasing the survival of U937 cells (Fig. 6A), suggesting the binding specificity between NAC and LMB. Furthermore, there was strong PARP cleavage in U937 cells treated with LMB (20 nM) that was not preincubated with NAC, but there was no PARP cleavage in U937 cells treated with LMB (20 nM) that was preincubated with NAC (20 mM). Strong PARP cleavage was detected in U937 cells treated with LMB (20 nM) that was preincubated with either Vitamin E (100 μM) or ebselen (20 μM) (Fig. 6B). These results clearly indicate that NAC suppressive effect of LMB-induced apoptosis in U937 cells is not through its antioxidant ability but through its direct binding to LMB, which eventually abolishes the cytotoxic and apoptotic effects by LMB. These findings further suggest that the cytotoxic and apoptotic effects by LMB may be mediated through the α,β-unsaturated lactone moiety of LMB, and this moiety seems to be important for the biological activity of LMB (herein LMB cytotoxicity).

4. Discussion

In this report, we have demonstrated that LMB potently induces apoptosis in U937 leukemia cells with the activation of caspases and the down-regulation of Mcl-1 and XIAP. Furthermore, we have shown that Bcl-2 overexpression suppresses LMB-induced apoptosis in U937 cells with inhibition of cytochrome c release and caspase activation,

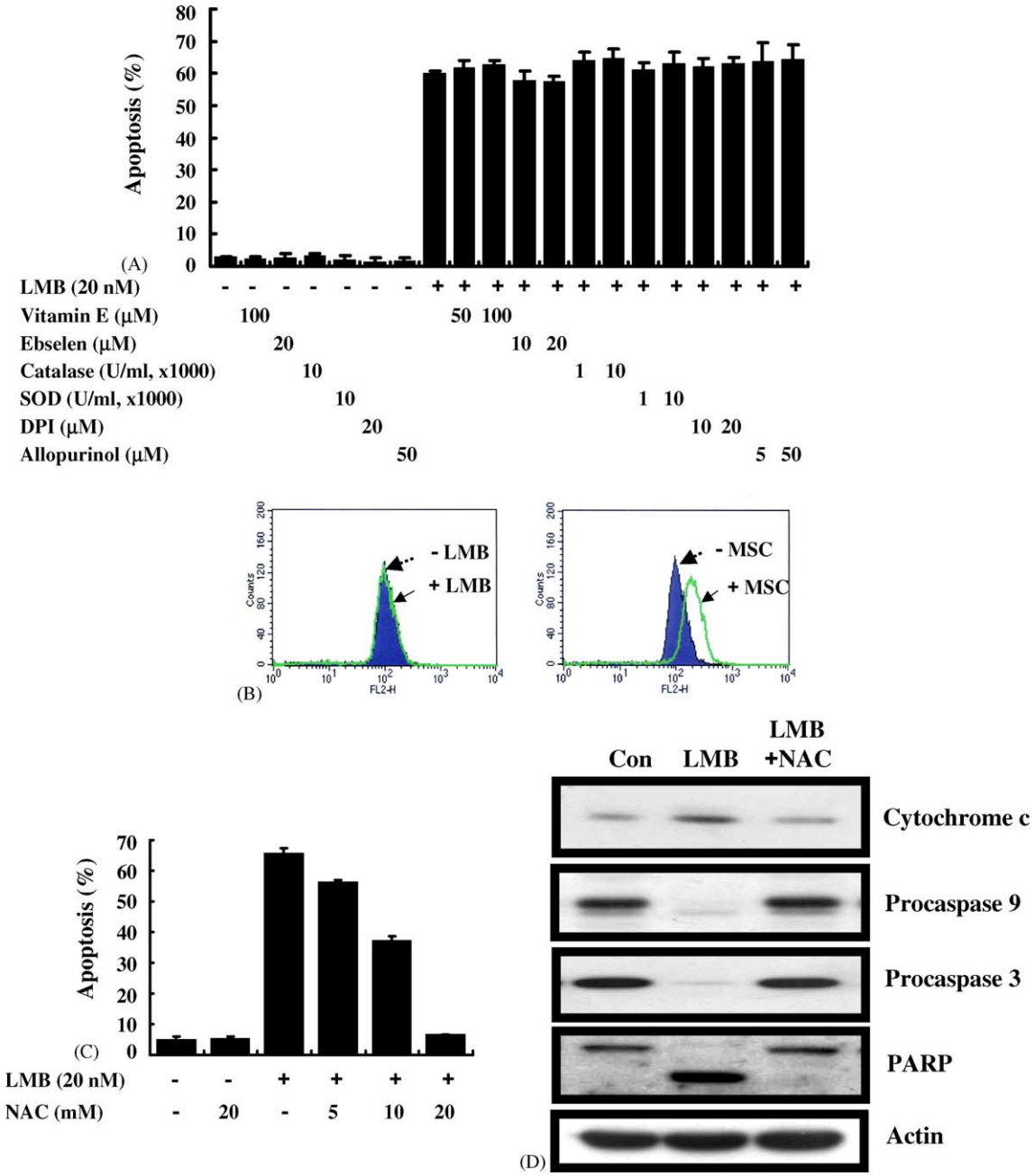

Fig. 5. Effects of Vitamin E, ebselen, catalase, SOD, DPI, allopurinol, or NAC on LMB-induced apoptosis in U937 cells. (A) U937 cells were pretreated for 0.5 h with the indicated dose of Vitamin E, ebselen, catalase, SOD, DPI, or allopurinol, and then treated with LMB (20 nM) for additional 12 h, followed measurement of sub-G1 DNA content by flow cytometer. The graph represents the fraction of apoptotic cells. Data are mean ± S.E. of three independent experiments. (B) U937 cells (1×10^6 cells/mL in PBS) were preloaded with 20 μM of DCFH-DA for 15 min at 37 °C. Five hundred microliter aliquots of cell suspension were treated with LMB (20 nM) or methylselenocysteine (MSC, 80 μM) for additional 0.5 h. DCF fluorescence was then measured using a flow cytometer with FL-1 filter. Data analysis was carried out using CellQuest program. The fluorescence is expressed as a histogram. (C) U937 cells were pretreated for 1 h with the indicated doses of NAC, and treated with LMB (20 nM) for additional 12 h. Change of sub-G1 DNA was then determined by flow cytometer. The graph represents the fraction of apoptotic cells. Data are mean ± S.E. of three independent experiments. (D) Either cytosolic or whole cell lysates were prepared from U937 cells grown under same culture conditions as (C), and analyzed for detection of either cytochrome c or procaspase-9/-3, PARP, and actin using respective antibodies.

suggesting that LMB-induced apoptosis is mediated through the mitochondrial pathway. However, apoptosis induced by LMB was not mediated through the generation of ROS, because various antioxidants do not prevent LMB-induced cytotoxicity. An interesting finding of the present study was that the SH group-containing antioxidant NAC inhibited the ability of LMB to induce apoptosis in U937 cells, and that the α,β-unsaturated lactone moiety of LMB seemed to be important for the cytotoxic and apoptotic effects by LMB. The data presented here may represent hitherto unknown mechanisms by which LMB induces apoptosis in cancer cells.

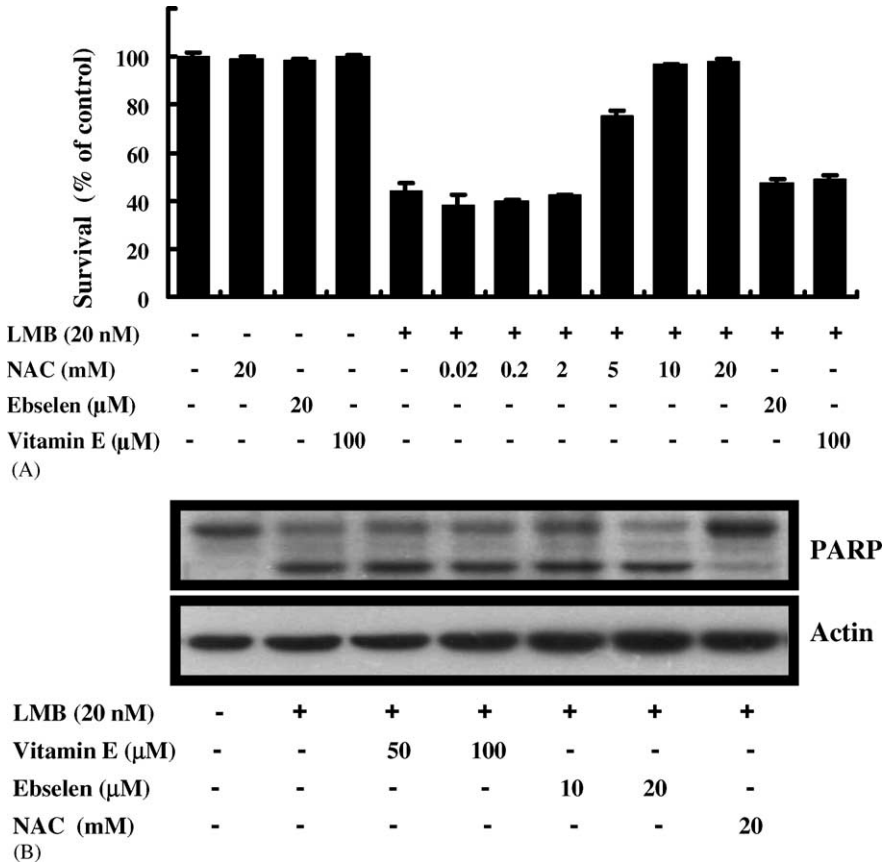

Fig. 6. Suppression of the LMB cytotoxicity by NAC via direct binding. (A) Before addition to U937 cells, LMB was initially pre-incubated with the indicated concentrations of NAC or ebselen at 20 μM or Vitamin E at 100 μM for 2 h. The resultant mixture was then added to U937 cells, and cells survived were counted by the trypan blue exclusion method. Data are mean ± S.E. of three independent experiments. (B) Whole cell lysates were prepared from U937 cells under same conditions as (A), and analyzed for the detection of PARP cleavage. To confirm equal loading, the same blot was stripped and reprobed with anti-actin antibody.

Caspase-3 has been considered as an important cell death-inducing protease that cleaves PARP and other vital proteins [26–28]. In our experiments, LMB induced caspase-3 activation and cleavage of PARP in association with apoptosis in U937 cells, which was inhibited by z-VAD-fmk, a pan-caspase inhibitor, addressing that the caspase activation is critical for the induction of apoptosis in U937 cells in response to LMB. Human IAPs, including XIAP, HIAP-1, HIAP-2, and survivin, have been shown to regulate apoptosis [14]. In particular, XIAP has been reported to directly inhibit members of caspase family including caspase-3 [29] or caspase-9 [30]. Therefore, the present data showing that LMB down-regulated the expression levels of XIAP, but not HIAP-1 in U937 cells suggest that XIAP down-regulation may contribute to the activation of caspases in LMB-induced apoptotic pathway. The members of Bcl-2 family, including Bcl-2, Mcl-1, or Bax, also regulate apoptosis and caspase activation by regulating the mitochondrial membrane integrity [23]. Indeed, down-regulation of the Bcl-2 members has been associated with apoptosis induced by some anti-cancer agents [31,32]. In present study, LMB did not change the expression levels of Bcl-2 and Bax in U937 cells, but did selectively down-regulate the expression level of Mcl-1. A role of Mcl-1 in the survival of both acute myeloid leukemia cells and chronic lymphocytic leukemia cells has been postulated [17,18]. Moreover, it has been shown that Mcl-1 is a critical survival factor for multiple myeloma cells [33] in which the decrease of Mcl-1 expression by anti-sense oligodeoxynucleotides leads to the induction of apoptosis, while overexpression of Mcl-1 delays the activation of caspases. Therefore, the decline of Mcl-1 expression may further contribute to the activation of caspase-3 in LMB-induced apoptotic pathway. It will be interesting to see whether overexpression of Mcl-1 and/or XIAP inhibits or delays the LMB-induced apoptosis in U937 cells, which may directly prove the role of Mcl-1 and/or XIAP in LMB-induced apoptotic pathway. Apoptosis induced by LMB was significantly attenuated in Bcl-2 overexpressing U937 cells. Bcl-2 overexpresion is reported to effectively inhibit cytochrome c release and cytochrome c-mediated caspase activation-dependent apoptosis in cancer cells induced by anti-cancer agents [34]. Supporting this, LMB failed to induce the activation of caspase-9/-3 in Bcl-2 overexpressing U937 cells in which release of cytochrome c by LMB was blocked. Therefore, these transfection data demonstrate that LMB-induced apoptosis and caspase activation is related with the mitochondrial pathway leading to

cytochrome c release, and suggest that overexpression of Bcl-2 may replace the lost Mcl-1 probably at the outer mitochondrial membrane and increase the threshold for LMB-induced cell death. It is interesting to note that the effect of Bcl-2 overexpression cannot be overcome by raising the concentrations of LMB (even up to 200 nM).

Oxidative stress is a common mediator of apoptosis [20,21,35]. However, LMB-induced apoptosis in U937 cells was not mediated through the generation of ROS, based on the facts of no effects by antioxidants systems including Vitamin E, ebselen, catalase, SOD, DPI, or allopurinol on LMB-induced apoptosis in U937 cells and no ROS production in LMB-treated cells. In contrast, the SH group-containing NAC, a well-known ROS scavenger, strongly suppressed LMB-induced apoptosis in U937 cells with inhibition of cytochrome c release, caspase-9/-3 activation, and PARP cleavage. These results led us to further investigate the suppressive mechanism of NAC, which seemed to be independent of its antioxidant ability, against LMB-induced apoptosis. It has been previously shown that NAC directly binds and inactivates LMB via an intermolecular interaction between the SH group of NAC and the α,β-unsaturated lactone of LMB by a Michael-type addition, which eventually abolishes the ability of LMB as an inhibitor of CRM1 [25]. Through an in vitro binding assay using NAC and LMB, in present study, we observed that NAC seemed to directly bind and inhibit the ability of LMB to induce apoptosis in U937 cells, which might be, to our knowledge, the first reporting that NAC directly inhibits the LMB cytotoxic and apoptotic activity against U937 leukemia cells, and the first suggesting that the α,β-unsaturated lactone of LMB might be functionally important for its cytotoxic and apoptotic effect. To assure importance of this moiety mediating LMB-induced apoptosis, it will be interesting to develop analogues of LMB in which this moiety is changed, and to test whether these analogues still hold the similar proapoptotic effects as intact LMB. NAC, Vitamin E, and ebselen are well-known antioxidants that effectively scavenge ROS. However, our data demonstrating that LMB-induced apoptosis in U937 cells is strongly inhibited by NAC, but not by Vitamin E or ebselen which lacks the SH group within the own structure further suggest that the SH moiety of NAC may mediate its suppressive effect on LMB cytotoxicity.

As mentioned earlier, the cellular target of LMB is CRM1 [2]. LMB directly binds the SH group of a certain cysteine residue in CRM1 [25]. Considering that LMB induces apoptosis in some cancer cells by interfering with CRM1-mediated nuclear-cytoplasmic trafficking of proteins including p53 [9,10] or BCR-ABL [11], we cannot rule out the possibility that apoptosis induced by LMB in U937 cells may be through LMB inhibition of CRM1 activity and the resultant CRM1-dependent nuclear export of proteins, whose proper nuclear export may be important for the survival of U937 cells. It will be interesting to test whether CRM1 overexpression inhibits or delays LMB-induced apoptosis. Depletion of cellular GSH levels has been implicated in tumor cell death induced by anticancer agents [36,37]. Considering that NAC, a well-known precursor of cellular glutathione [38], strongly inhibits LMB-induced apoptosis in U937 cells, one can speculate that apoptosis induced by LMB may be through depletion of cellular glutathione. However, it seems not to be the case in LMB-induced apoptosis in U937 cells, based on the facts that the concentration of LMB to induce apoptosis in U937 cells is at nanomolar dose (20 nM), but the GSH content in human tissues is reported to be at the range of 0.1–10 mM [39].

In summary, we have demonstrated that LMB at nanomolar concentration induces apoptosis in U937 leukemia cells. The activation of caspase-3 and the down-regulation of XIAP and Mcl-1 may possibly mediate the LMB-induced apoptosis in U937 cells. The α,β-unsaturated lactone moiety of LMB seems to be important for the cytotoxic and apoptotic effects triggered by LMB. Based on the present data shown here and previous ones by others, LMB may be a potential candidate as an anticancer or chemopreventive agent. Further efforts to explore therapeutic strategies of LMB attacking cancer cells are warranted.

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