Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 1297–1306
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Inhibition of bleomycin-induced cell death in rat alveolar macrophages and human lung epithelial cells by ambroxol

Jun Sik Hong, Hyun Hee Ko, Eun Sook Han, Chung Soo Lee*

Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, South Korea

Received 10 March 2003; accepted 18 June 2003

Abstract

The mitochondrial permeability transition is recognized to be involved in toxic and oxidative forms of cell injury. In the present study, we investigated the effect of ambroxol against the cytotoxicity of bleomycin (BLM) by looking at the effect on the mitochondrial membrane permeability in alveolar macrophages and lung epithelial cells. Alveolar macrophages or lung epithelial cells exposed to BLM revealed the loss of cell viability and increase in caspase-3 activity. Ambroxol (10–100 μM) reduced the 75 mU/mL BLM-induced cell death and activation of caspase-3 in macrophages or epithelial cells. It reduced the condensation and fragmentation of nuclei caused by BLM in macrophages. Ambroxol alone did not significantly cause cell death. Treatment of alveolar macrophages with BLM resulted in the decrease in transmembrane potential in mitochondria, cytosolic accumulation of cytochrome *c*, increase in formation of reactive oxygen species (ROS) and depletion of GSH. Ambroxol (10–100 μM) inhibited the increase in mitochondrial membrane permeability, ROS formation and decrease in GSH contents due to BLM in macrophages. Ambroxol exerted a scavenging effect on hydroxyl radicals and nitric oxide and reduced the iron-mediated formation of malondialdehyde and carbonyls in liver mitochondria. It prevented cell death due to SIN-1 in lung epithelial cells. The results demonstrate that ambroxol attenuates the BLM-induced viability loss in alveolar macrophages or lung epithelial cells. This effect may be due to inhibition of mitochondrial damage and due to the scavenging action on free radicals.

Keywords: Bleomycin; Ambroxol; Alveolar macrophages; Lung epithelial cells; Mitochondrial membrane permeability; Cell death

1. Introduction

BLM, an antineoplastic drug, is used in the treatment of lymphomas, squamous cell carcinomas and testicular tumors. However, the clinical use of BLM is often limited by a pneumonitis that can progress to interstitial pulmonary fibrosis [1]. The infiltration of inflammatory cells into the lung is demonstrated in the BLM-treated animals, and alveolar macrophages are suggested to play an important role in the development of lung fibrosis by releasing a variety of substances, including cytokines, ROS and reactive nitrogen species [2,3]. The lung tissues of BLM-treated rats reveal the decreased levels of cellular antioxidants that may lead to pulmonary fibrosis [4,5].

The exposure of cells to BLM results in the fragmentation of DNA, leading to cell death [6,7]. BLM causes the apoptotic cell death in alveolar macrophages, PC3 cells and human umbilical vein endothelial cells [7–9]. The expressions of caspase-1 and -3 are upregulated in the lung epithelial cells and alveolar macrophages in the BLM-treated mice [10]. Many anticancer drugs have been demonstrated to kill cancer cells by inducing apoptosis, and the dysregulation of apoptosis may initiate a cancer formation [11]. The silica-induced apoptosis in alveolar macrophages is postulated to induce the inflammatory responses in lung fibrosis and the cancer formation [12,13]. These findings support that an injury of alveolar macrophages due to BLM may be involved in the pathogenesis of lung fibrosis [8].

In the presence of oxygen and a reducing agent, the ferrous ion–BLM complex becomes activated and functions mechanically as a ferrous oxidase, transferring electrons from ferrous ion to molecular oxygen to produce

* Corresponding author. Tel.: +82-2-820-5659; fax: +82-2-815-3856.
E-mail address: leecs@cau.ac.kr (C.S. Lee).

Abbreviations: AMB, ambroxol; BLM, bleomycin sulfate; SOD, superoxide dismutase; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; $DCFH_2$-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescin; PMSF, phenylmethylsulfonylfluoride; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); MTT, 1,3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide; MDA, malondialdehyde; SIN-1, 3-morphosydnonimine; DMEM, Dulbecco's modified Eagle's medium; PBS, Dulbecco's phosphate buffered saline; FBS, fetal bovine serum; ROS, reactive oxygen species.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00448-9

ROS that cause scission of DNA [14]. The compounds that exert antioxidant abilities may have a therapeutic usefulness to reduce or prevent the toxicity of anticancer drugs [15]. Ambroxol (*trans*-4 [(2-amino-3,5-dibromobenzyl)amino] cyclohexanol HCl), which is used as an expectorant, has been found to improve the clinical course of respiratory distress syndrome, including bronchopulmonary dysplasia [16] and to reduce postoperative pulmonary complication [17]. It reveals antioxidant action and anti-inflammatory effects. It reduces oxidative damage of tissue components caused by ROS and attenuates the production of cytokines and free radicals in activated neutrophils and macrophages [18,19]. It has been shown that ambroxol reduces the silica-induced cell death in alveolar macrophages [20].

There is evidence that the defects in mitochondrial function are involved in the induction of cell death [21,22]. The membrane permeability transition of mitochondria is recognized as a central event in the course of toxic and oxidative forms of cell injury [23]. Ambroxol reduces the infiltration of inflammatory cells into the lung and attenuates changes in phospholipid composition of lung surfactant in rats treated with BLM [24,25]. However, the effect of ambroxol on the mitochondrial damage due to BLM, especially change in the mitochondrial membrane permeability, has not been clarified. Thus, the effect of ambroxol on alveolar macrophages and pulmonary epithelial cells against the cytotoxicity of BLM was assessed by looking at the effect on the mitochondrial transmembrane potential, cytochrome *c* release, ROS formation, GSH contents and caspase-3 activity in cells and by observing the scavenging action on free radicals.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (weighing between 230 and 270 g each, about 8 weeks old) were housed in a controlled environment and provided with standard rodent chow and water. Animals were cared for in accordance with the NIH guidelines of U.S.A. and the guide of Korean Academy of Medical Sciences.

2.2. Materials

Ambroxol, BLM, SOD (from bovine erythrocytes; 2500–7000 U/mg of protein), catalase (from bovine liver; 10,000–25,000 U/mg of protein), Hoechst 33258, $DiOC_6(3)$, $DCFH_2$-DA, PMSF, DTNB, glutathione reductase (from spinach), MTT, 2-deoxy-D-ribose, 2,4-dinitrophenylhydrazine, dimethyl sulfoxide, dithiothreitol, nitrate reductase (from *Escherichia coli*), NADPH, flavin adenine dinucleotide, SIN-1, GSH (reduced form), rutin trihydrate, Ficoll-Hypaque solution and DMEM were purchased from Sigma–Aldrich Inc. Quantikine® M rat/mouse cytochrome *c* assay kit was purchased from R&D systems, ApoAlert™ CPP32/Caspase-3 assay kit from CLONTECH Laboratories Inc., and FBS from Life Technologies. The polystyrene 24- and 96-well plates (Corning®) were purchased from Corning Incorporated, and 15 mL polypropylene tubes (Falcon®) used in the isolation of cells from Becton Dickinson.

2.3. Isolation of rat alveolar macrophages

Male Sprague–Dawley rats were anesthetized by intraperitoneal injection of 40 mg/kg of pentobarbital sodium and then killed by cervical dislocation. After this, a tracheal cannula was immediately inserted through an incision in the neck, and 6 mL of cold Ca^{2+}, Mg^{2+}-free PBS, pH 7.4 was instilled the lung via a syringe attached to the cannula [20]. Instillation of PBS was repeated three times to obtain macrophages. Cell suspensions were treated with hypotonic solution (0.2% saline) for 20 s for the lysis of erythrocytes, and then added equal amount of 1.6% saline to make an isotonic state. The cell pellets suspended in Ca^{2+}, Mg^{2+}-free PBS were placed on a Ficoll-Hypaque gradient and were centrifuged at 400 *g* for 45 min at 4°. Macrophages were collected from the interphase of the gradient. The cells were washed with DMEM and were suspended in the same solution.

2.4. Isolation of rat liver mitochondria

Mitochondria were isolated from the liver of Sprague–Dawley rats by differential centrifugation [26]. The livers were finely minced in ice-cold homogenization medium (210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 5 mM HEPES, pH 7.4). The tissue suspended in nine volumes of homogenization medium was homogenized with tissue homogenizer (Polytron Model PT-20, Brinkmann Instruments). Tissue debris and nuclei were separated from the homogenates by centrifugation at 1000 *g* for 10 min at 4°. The supernatants were carefully collected and centrifuged at 10,000 *g* for 10 min at 4°. The mitochondrial pellet was washed with the EDTA-free homogenization medium and was suspended in a KCl–Tris medium (120 mM KCl and 50 mM Tris–HCl, pH 7.4). Protein concentration was determined by the method of Bradford described in Bio-Rad protein assay kit (Bio-Rad Laboratories).

2.5. Cell culture of pulmonary epithelial cells

The WI-26 VA4 normal human embryo pulmonary epithelial cell was obtained from the Korean cell line bank (Seoul, South Korea). Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in 5% CO_2 atmosphere at 37°. The culture medium was changed every 3 days, and the cells were subcultured about once a week. Cells ($1–2 \times 10^7$) were plated on polystyrene 60 mm × 15 mm cell culture dishes (Corning Incorporated) 48–72 hr before experiments. Cells were washed with DMEM containing 1%

FBS and replated onto 96-well plates at a density of 4×10^4 cells per well in a volume of 200 μL (or various numbers of cells/mL in 24-well plates). Cells were treated with BLM in DMEM containing 1% FBS for 24 hr at 37°.

2.6. Cell viability assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [27]. Cells (4×10^4 of macrophages or lung epithelial cells) were treated with BLM in the presence of ambroxol for 24 hr at 37°. The medium (200 μL) was treated with 10 μL of 10 mg/mL MTT solution for 2 hr at 37°. Culture medium was removed, and 100 μL of dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Spectra MAX 340). Cell viability was expressed as a percent of the control culture value.

2.7. Morphological observation of nuclear change

Macrophages (1×10^6 cells/mL) were treated with BLM in the presence of ambroxol for 24 hr at 37°, and the nuclear morphological change was assessed using Hoechst dye 33258 [28,29]. Macrophages were incubated with 1 μg/mL Hoechst 33258 for 3 min at room temperature, and nuclei were visualized using an Olympus Microscope with a WU excitation filter.

2.8. Measurement of apoptosis by using a caspase-3 activity assay

Apoptosis in macrophages or pulmonary epithelial cells was assessed by measuring the activity of caspase-3, which is considered to be involved in programmed cell death [22]. Cells (2×10^6 cells/mL) were treated with BLM in the presence of ambroxol for 24 hr at 37°. The effect of ambroxol on apoptosis in the BLM-treated cells was determined as described in user's manual of ApoAlert™ CPP32/Caspase-3 assay kit. The supernatant obtained by a centrifugation of cells dissolved was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) and was incubated for 1 hr at 37°. Absorbance of the chromophore *p*-nitroanilide produced was measured at 405 nm. The standard curves were obtained from absorbances in the *p*-nitroanilide standard reagent diluted with cell lysis buffer (up to 20 nM). One unit of the enzyme was defined as 1 nmol of chromophore *p*-nitroanilide produced.

2.9. Flow cytometric measurement of mitochondrial transmembrane potential

Change in the mitochondrial transmembrane potential during the BLM-induced apoptosis in macrophages was quantified by flow cytometry with the cationic lipophilic dye $DiOC_6(3)$ [30,31]. Macrophages (1×10^6/mL) were treated with BLM in the presence of ambroxol for 4 hr at 37°. $DiOC_6(3)$ (40 nM) was added to the medium, and incubation was performed for 15 min at 37°. After centrifugation at 1500 *g* for 5 min, the supernatants were removed, and the pellets were resuspended in PBS containing 0.5 mM EDTA. For analysis, a FACScan cytofluorometer (Becton Dickinson) with argon laser excitation at 501 nm was used to assess 10,000 cells from each sample.

2.10. Measurement of cytochrome c release

Cytochrome *c* released into the cytosol of alveolar macrophages was assessed by using a solid phase ELISA kit for the detection of rodent cytochrome *c*. Cells (5×10^5/mL) harvested by centrifugation at 800 *g* for 10 min were washed twice with PBS and resuspended in 250 mM sucrose, 20 mM HEPES–KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol and 0.1 mM PMSF. Cells were further homogenized by successive passages through a 26-gauge needle. The homogenates were centrifuged at 100,000 *g* for 30 min. The supernatant obtained was used for analysis of cytochrome *c*. The supernatants were added into the 96-well microplates coated with monoclonal antibody specific for rat/mouse cytochrome *c* that contain cytochrome *c* conjugate. The procedure was performed as described in the assay kit. Absorbance of samples was measured at 450 nm in a microplate reader. Like samples, the diluted solutions of cytochrome *c* standard were added to the microplates coated with monoclonal antibody, and the standard curve was constructed. The amount was expressed as ng/mL by using a standard curve.

2.11. Measurement of intracellular ROS formation

The dye $DCFH_2$-DA, which is oxidized to fluorescent DCF by hydroperoxides, was used to measure relative levels of cellular peroxides [32]. After exposure to BLM, 4×10^4 macrophages were incubated with 50 μM dye for 30 min at 37° and were then washed with PBS. The cell suspensions were centrifuged at 412 *g* for 10 min, and medium was removed. Cells were dissolved with 1% Triton X-100, and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (SPECTRAFLUOR, TECAN).

2.12. Measurement of total glutathione

The total glutathione (GSH + GSSG) was determined using glutathione reductase [33]. Macrophages (3×10^5/mL) were treated with BLM for 24 hr at 37°. The cell suspensions were centrifuged at 412 *g* for 10 min in a microplate centrifuge, and medium was removed.

Cells were dissolved with 2% 5-sulfosalicylic acid (100 μL) and then incubated in 100 μL of the solution containing 22 mM sodium EDTA, 600 μM NADPH, 12 mM DTNB and 105 mM NaH_2PO_4, pH 7.5 at 37°. Twenty microliters of glutathione reductase (100 U/mL) was added to the mixture, which was further incubated for 10 min. Absorbance was measured at 412 nm using a microplate reader.

2.13. Measurement of malondialdehyde, carbonyl groups and degraded 2-deoxy-D-ribose

Lipid peroxidation of liver mitochondria was determined by measuring the MDA chromogen formation using thiobarbituric acid [34]. Liver mitochondria are frequently used in the measurement of the function and oxidative tissue injury [26,35]. Thus, the antioxidant ability of ambroxol was assessed by looking at the effect on oxidative damage of liver mitochondria. The mitochondria (0.4 mg of protein/mL) suspended in the reaction mixture containing 120 mm KCl and 50 mM Tris–HCl, pH 7.4 were treated with 10 μM $FeSO_4$ and 100 μM ascorbate for 30 min at 37°. Absorbance was measured at 532 nm, and the MDA concentration was determined using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

The protein oxidation products in mitochondria were quantified by carbonyl assay using 2,4-dinitrophenylhydrazine [36]. One milliliter of reaction mixture containing 1 mg of protein was treated with 10 μM $FeSO_4$ and 100 μM ascorbate for 30 min at 37°. The reaction mixtures incubated with 2 mM 2,4-dinitrophenylhydrazine were sequentially treated with 20 and 10% trichloroacetic acid. The pellets were washed with the solution (mixture of ethanol and ethyl acetate (1:1, v/v)) and were dissolved in 6 M guanidine HCl solution. Absorbance was measured at 370 nm, and carbonyls were determined using a molar extinction coefficient of $2.2 \times 10^4\ M^{-1}\ cm^{-1}$.

The scavenging effect of ambroxol on hydroxyl radicals was assessed by measuring the formation of MDA chromogen due to degradation of 2-deoxy-D-ribose [37]. The reaction mixtures contained 2 mM 2-deoxy-D-ribose, 50 μM $FeCl_3$, 50 μM EDTA, 500 μM H_2O_2, 100 μM ascorbate, 120 mM KCl and 50 mM NaH_2PO_4, pH 7.4. After a 30-min incubation at 37°, the reaction was stopped by adding 1% thiobarbituric acid in 50 mM NaOH and 2.8% trichloroacetic acid. Absorbance was measured at 532 nm.

2.14. Statistical analysis

The values are expressed as means ± SEM. Statistical analysis was performed by one-way analysis of variance. The analysis of variance justifies post hoc comparisons between the different groups was conducted by using the Duncan's test for multiple comparisons. A probability of $P < 0.05$ was considered to be statistically significant.

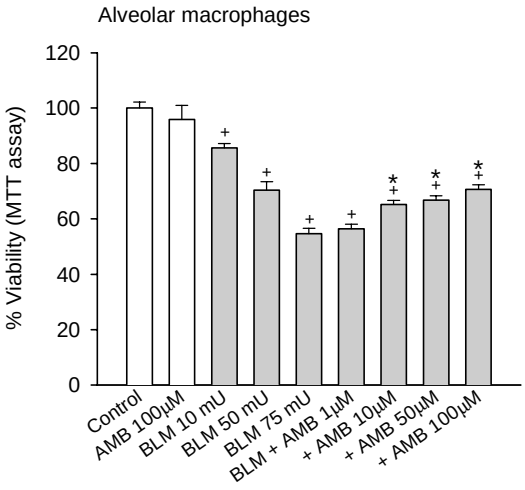

Fig. 1. Effect of ambroxol on BLM-induced loss of cell viability in alveolar macrophages. Cells (4×10^4) were treated either with various amounts of BLM or with 75 mU/mL BLM in the presence of 1–100 μM ambroxol (AMB) for 24 hr at 37°, and then mixtures were treated with MTT for 2 hr. The values are expressed as percentage of control and represent means ± SEM (N = 8). $^{+}P < 0.05$, significantly different from the control; $^{*}P < 0.05$, significantly different from 75 mU/mL BLM alone.

3. Results

3.1. Effect of ambroxol on BLM-induced cell death and caspase-3 activation

The treatment of BLM caused cell death in alveolar macrophages in a concentration-dependent manner (Fig. 1). Macrophages treated with 75 mU/mL BLM for 24 hr revealed a 45% of cell death in the MTT assay. The depressant effect of ambroxol on the BLM-induced loss of cell viability was investigated in alveolar macrophages. Ambroxol (10–100 μM) reduced the BLM-induced cell death in alveolar macrophages but did not completely suppress cytotoxicity of BLM (Fig. 1). To assess the cytotoxic effect of ambroxol itself, macrophages were treated with ambroxol in the absence of BLM. In this study, ambroxol (100 μM) alone did not significantly cause cell death.

The cytotoxic effect of BLM was assessed in lung epithelial cells. When lung epithelial cells were treated with 75 mU/mL BLM for 24 hr, a 51% of cell death occurred. Ten micrograms/mL SOD, a scavenger of superoxide radical, and 10 μg/mL catalase, a scavenger of hydrogen peroxide, inhibited the cytotoxic effect of BLM. As in alveolar macrophages, ambroxol reduced the BLM-induced cell death in lung epithelial cells in a concentration-dependent manner but did not completely prevent cytotoxicity of BLM (Fig. 2). Ambroxol alone did not induce cell death in lung epithelial cells.

To clarify the inhibitory effect of ambroxol against cytotoxicity of BLM, we investigated the effect on the nuclear morphological changes observed in the BLM-treated cells. Nuclear staining with Hoechst 33258 demonstrated that

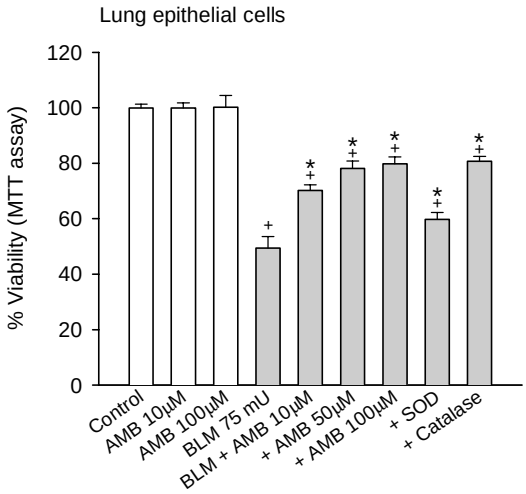

Fig. 2. Effect of ambroxol on loss of cell viability in lung epithelial cells treated with BLM. Lung epithelial cells (4×10^4 cells) were treated with 75 mU/mL BLM, 10–100 μM ambroxol (AMB) and 10 μg/mL antioxidant enzymes (SOD and catalase) for 24 hr at 37°, and then mixtures were treated with MTT for 2 hr. The values are expressed as percentage of control and represent means ± SEM (N = 6). $^{+}P < 0.05$, significantly different from the control; $^{*}P < 0.05$, significantly different from 75 mU/mL BLM alone.

control macrophages had regular and round-shaped nuclei. In contrast, the condensation and fragmentation of nuclei characteristic of apoptotic cells were evident in macrophages treated with 75 mU/mL BLM for 24 hr at 37° (Fig. 3). Ambroxol (100 μM) reduced the BLM-induced nuclear damage while the nuclear morphology in cells

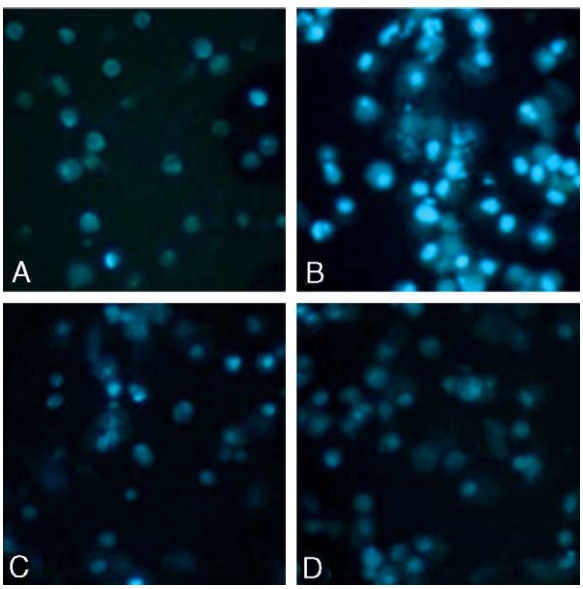

Fig. 3. Effect of ambroxol on the apoptotic cell death induced by BLM. Alveolar macrophages (1×10^6 cells) were treated with 75 mU/mL of BLM and 100 μM ambroxol for 24 hr at 37°. Cells were observed by fluorescence microscopy after nuclei staining with Hoechst 33258. Microscopic morphology of the control cells (A), cells treated with BLM alone (B), cells treated with BLM and 100 μM ambroxol (C), and cells treated with 100 μM ambroxol alone (D). All the subparts are representative of four different experiments.

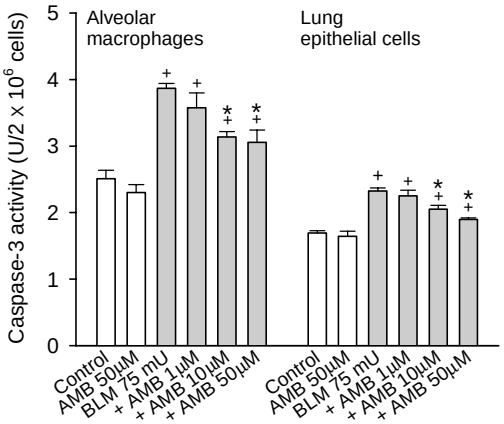

Fig. 4. Effect of ambroxol on BLM-induced increase in caspase-3 activity. Alveolar macrophages or lung epithelial cells (2×10^6 cells) were treated with 75 mU/mL BLM and 1–50 μM ambroxol (AMB) for 24 hr at 37°. Caspase-3 activity was measured using the assay kit. The values are means ± SEM (N = 5). $^{+}P < 0.05$, significantly different from the control; $^{*}P < 0.05$, significantly different from 75 mU/mL BLM alone.

exposed to ambroxol alone was similar to that in the control cells.

Apoptosis in alveolar macrophages or lung epithelial cells was assessed by measuring the activity of caspase-3 [38]. Control alveolar macrophages and lung epithelial cells had caspase-3 activities of 2.51 and 1.70 U in 2×10^6 cells, respectively. Treatment of macrophages or epithelial cells with 75 mU/mL BLM for 24 hr revealed an increase in caspase-3 activity (3.87 U in macrophages and 2.32 U in lung epithelial cells). Ambroxol (10 and 50 μM) depressed the increase in caspase-3 activation in both cells exposed to BLM while ambroxol alone did not affect the caspase-3 activity (Fig. 4). As in cell viability, ambroxol did not completely abolish caspase-3 activation due to BLM.

3.2. *Effect of ambroxol on change in mitochondrial membrane potential, cytochrome c release, ROS production and GSH depletion due to BLM*

Disruption of the mitochondrial transmembrane potential has been recognized to be a general feature of apoptosis [21]. Looking at the effect on the mitochondrial membrane potential we assessed the cytotoxic effect of BLM. Change in the mitochondrial transmembrane potential in macrophages treated with BLM was quantified by flow cytometry with the cationic lipophilic dye $DiOC_6(3)$. When macrophages were treated with 75 mU/mL BLM for 4 hr at 37°, the increase in the percentage of depolarized cells (characterized by low values of the transmembrane potential) was observed. Ambroxol (10 and 100 μM) reduced the BLM-induced increase in depolarized cells while the transmembrane potential in the presence of ambroxol alone was similar to that in the controls (Fig. 5). Compared to 10 μM concentration, 100 μM ambroxol did not completely nullify loss of the mitochondrial transmembrane potential due to BLM.

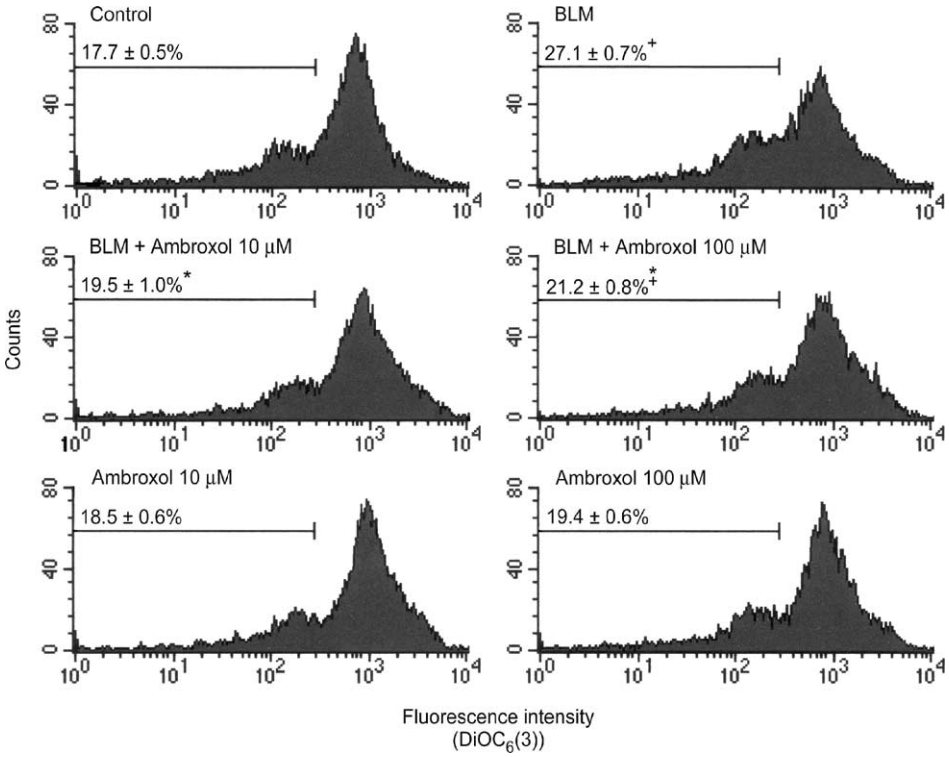

Fig. 5. Effect of ambroxol on loss of the mitochondrial transmembrane potential due to BLM. Alveolar macrophages (1×10^6 cells) were treated with 75 mU/mL of BLM in the presence of ambroxol (10 and 100 μM) for 4 hr at 37°, and then mixtures were treated with 40 nM $DiOC_6(3)$. The values are means ± SEM of the percentage-depolarized cells in three independent experiments. $^{+}P < 0.05$, significantly different from the control; $^{*}P < 0.05$, significantly different from 75 mU/mL BLM alone.

Loss of the mitochondrial membrane potential causes a release of cytochrome *c* from mitochondria into the cytosol, which may be involved in apoptotic cell death [22]. The BLM-induced cell death was assessed by measuring the cytochrome *c* released into the cytosol. BLM is found to induce the highest accumulation of cytochrome *c* at 4 hr of incubation in PC3 cells [9]. Thus, the effect of ambroxol on the release of cytochrome *c* was investigated in alveolar macrophages incubated with BLM for 4 hr. Treatment of alveolar macrophages with 75 mU/mL BLM for 4 hr revealed a 6.2-fold increase in the cytosolic cytochrome *c* (Fig. 6). Ambroxol (10–100 μM) reduced the BLM-induced release of cytochrome *c* while ambroxol alone did not significantly cause cytochrome *c* release. The BLM-induced release of cytochrome *c* was not completely inhibited by the addition of ambroxol.

In order to assess the involvement of ROS in the BLM-induced cell death, we investigated the production of ROS within cells by monitoring conversion of $DCFH_2$-DA to DCF. The macrophages exposed to 75 mU/mL BLM for 24 hr caused a significant increase in DCF fluorescence by 52%. The BLM-induced increase in DCF fluorescence was reduced by 10 μg/mL SOD or 10 μg/mL catalase (Fig. 7A). Ambroxol depressed the increase in DCF fluorescence induced by BLM in a concentration-dependent manner, and at 100 μM a 69% of inhibition was observed. Ambroxol (10–100 μM) also reduced change in DCF fluorescence in the control cells without treatment of BLM, and incubation of 100 μM ambroxol for 24 hr revealed a 13% of inhibition.

Reduction of intracellular GSH increases the sensitivity of cells against oxidants [35,39]. GSH content in cells correlates with cell viability [40]. Thus, the cytotoxic effect of BLM was assessed by its effect on the GSH contents. The thiol content in macrophages was 11.06 ± 0.29 nmol

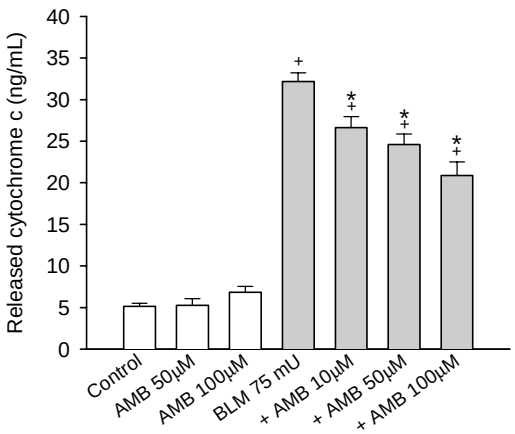

Fig. 6. Effect of ambroxol on BLM-induced release of cytochrome *c*. Alveolar macrophages (5×10^5 cells) were treated with 75 mU/mL of BLM and 10–100 μM ambroxol (AMB) for 4 hr at 37°. The supernatants of disrupted cells were used to assay for cytochrome *c* as described in Section 2. The values are expressed as ng/mL in 5×10^5 cells and represent means ± SEM (N = 5). $^{+}P < 0.05$, significantly different from the control; $^{*}P < 0.05$, significantly different from 75 mU/mL BLM.

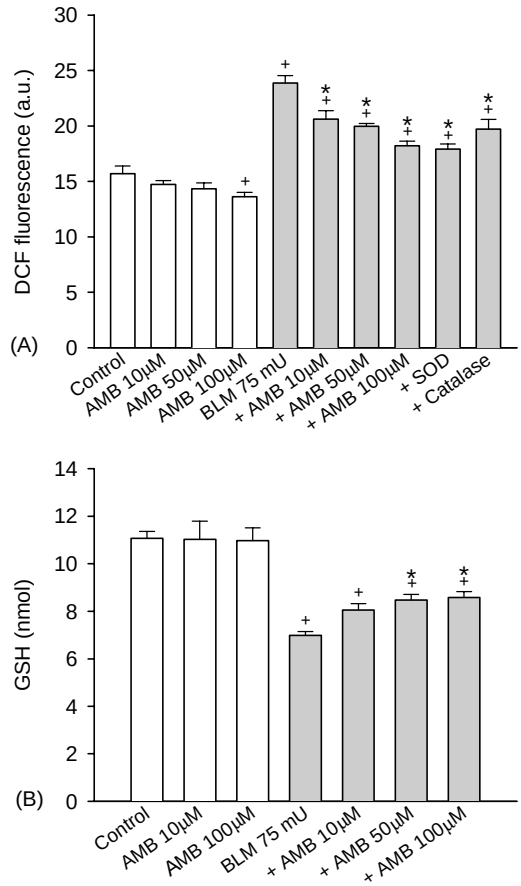

Fig. 7. Effect of ambroxol on ROS formation and GSH depletion due to BLM. Alveolar macrophages (4×10^4 cells in the assay of ROS formation, and 3×10^5 cells in the assay of GSH contents) were treated either with 75 mU/mL of BLM, 10–100 μM ambroxol (AMB) and 10 μg/mL antioxidant enzymes or with ambroxol alone for 24 hr at 37°. The values are expressed as arbitrary units of fluorescence in ROS formation (A) and nmol in GSH contents (B). Data represent means ± SEM (N = 6). $^{+}P < 0.05$, significantly different from the control; $^{*}P < 0.05$, significantly different from 75 mU/mL BLM.

per 3×10^5 cells. Incubation of 75 mU/mL BLM for 24 hr depleted a 4.09 nmol of GSH. Ambroxol significantly prevented the BLM-induced depletion of GSH in macrophages but did not completely interfere with it (Fig. 7B). Ambroxol alone (10 and 100 μM) did not reduce the GSH contents in macrophages.

3.3. Effect of ambroxol on damaging action of ROS and reactive nitrogen species

Looking at the effect on the iron-mediated oxidative damage of mitochondria we assessed the antioxidant effect of ambroxol. Incubation of liver mitochondria with 10 μM $FeSO_4$ and 100 μM ascorbate caused formations of MDA, as product of lipid peroxidation, and carbonyls, as product of protein oxidation. Ambroxol (10 and 100 μM) and various antioxidants (10 μg/mL SOD, 10 μg/mL catalase, 10 mM dimethyl sulfoxide and 10 mM sodium formate) reduced the peroxidative effect of iron and ascorbate (Table 1). The inhibitory effect of ambroxol in the presence of antioxidant against iron-mediated peroxidation was less than the sum of each effect of compounds.

The inhibitory effect of ambroxol against toxicity of BLM was elucidated with the scavenging action on ROS. The scavenging effect of ambroxol on hydroxyl radicals was assessed by measuring a 2-deoxy-D-ribose degradation that is caused by Fe^{3+}, EDTA, H_2O_2 and ascorbate [37]. The 50 μM Fe^{3+}, 50 μM EDTA, 500 μM H_2O_2 and 100 μM ascorbate-induced deoxyribose degradation was significantly inhibited by 10 mM of hydroxyl radical scavengers (dimethyl sulfoxide, mannitol or sodium formate). Ambroxol (10 and 100 μM) significantly reduced the iron and EDTA-mediated degradation of 2-deoxy-D-ribose (Table 1). The effect of co-added ambroxol and antioxidant against the deoxyribose degradation was less than the sum of each effect of compounds.

The effect of ambroxol on the damaging action of reactive nitrogen species was determined by measuring the effect on the SIN-1-induced cell death. The SIN-1 releases the superoxide radicals and nitric oxide, which results in the formation of peroxynitrite, a strong oxidant [41]. Ambroxol (10 and 100 μM), 10 μg/mL SOD and 50 μM rutin (a scavenger of nitric oxide) significantly reduced cell death due to 500 μM SIN-1 in lung epithelial cells (Table 1). The inhibitory effect of co-added ambroxol and radical scavenger against cell death was less than the sum of each effect of compounds.

4. Discussion

The BLM-induced DNA damage followed by cell death has been recognized to be mediated by free radicals that are liberated from phagocytic cells or from the drug itself [8,42]. The silica-induced apoptotic cell death in alveolar macrophages appears to induce the fibrosis and cancer formation in the lung [13]. In a number of cells, there is evidence that mitochondrial dysfunction and increased formation of ROS are involved in cell death [22]. The ROS produced by doxorubicin disrupts the mitochondrial membrane potential, which leads to injury of myocytes [43]. It has been demonstrated that BLM causes a release of cytochrome *c* into the cytosol during the induction of apoptosis in PC3 cells [9]. Despite these reports, it is uncertain whether the cytotoxic effect of BLM is mediated by the mitochondrial permeability transition.

A significant cytotoxic effect of BLM on cell viability in alveolar macrophages and lung epithelial cells was demonstrated by using MTT assay and by observing nuclear morphological changes with Hoechst 33258 stain. Loss of the mitochondrial membrane potential causes the release of cytochrome *c* from mitochondria to the cytosol, followed by the activation of caspase-3 that is involved in apoptotic cell death [22,44]. The condensation and fragmentation of nuclei (Fig. 3) and a significant increase in

Table 1
Effect of ambroxol on damaging action of ROS and reactive nitrogen species

Antioxidants	MDA (nmol)	Carbonyls (nmol)	Deoxyribose degradation (A_{532})	Cell viability (%)
Control	6.53 ± 0.19	3.40 ± 0.10	0.926 ± 0.022	56.1 ± 1.7
Ambroxol 1 μM	6.12 ± 0.19	3.09 ± 0.26	0.900 ± 0.038	60.5 ± 1.8
Ambroxol 10 μM	4.63 ± 0.15^{+}	2.19 ± 0.08^{+}	0.788 ± 0.019^{+}	66.8 ± 1.8^{+}
Ambroxol 100 μM	1.45 ± 0.07^{+}	1.15 ± 0.04^{+}	0.634 ± 0.011^{+}	93.1 ± 1.7^{+}
SOD 10 μg/mL	4.72 ± 0.21^{+}	2.28 ± 0.09^{+}	–	61.6 ± 1.2^{+}
Catalase 10 μg/mL	4.87 ± 0.13^{+}	1.53 ± 0.06^{+}	–	–
DMSO 10 mM	5.41 ± 0.15^{+}	1.93 ± 0.07^{+}	0.136 ± 0.002^{+}	–
Sodium formate 10 mM	5.82 ± 0.17^{+}	2.02 ± 0.10^{+}	0.258 ± 0.005^{+}	–
Mannitol 10 mM	–	–	0.201 ± 0.006^{+}	–
Rutin 100 μM	–	–	–	75.6 ± 1.3^{+}
Ambroxol 10 μM + SOD 10 μg/mL	3.47 ± 0.11$^{+,*}$	2.30 ± 0.09^{+}	–	62.9 ± 2.0^{+}
Ambroxol 10 μM + catalase 10 μg/mL	4.06 ± 0.10$^{+,*}$	1.45 ± 0.11$^{+,*}$	–	–
Ambroxol 10 μM + DMSO 10 mM	4.24 ± 0.10*	1.95 ± 0.15*	0.134 ± 0.002$^{+,*}$	–
Ambroxol 10 μM + sodium formate 10 mM	4.65 ± 0.11^{+}	1.96 ± 0.11^{+}	0.220 ± 0.002$^{+,*}$	–
Ambroxol 10 μM + rutin 100 μM	–	–	–	77.1 ± 2.0$^{+,*}$

Control represents the oxidation of mitochondrial lipids and proteins caused by 10 μM iron plus 100 μM ascorbate, the deoxyribose degradation caused by iron plus EDTA, and the percent of live cells in lung epithelial cells treated with 500 μM SIN-1 for 24 hr at 37° as described in Section 2. The values are means ± SEM (N = 5–8).

$^{+}$ $P < 0.05$, significantly different from the control.

* $P < 0.05$, significantly different from 10 μM ambroxol alone.

caspase-3 activity (Fig. 4) indicated the apoptotic death in alveolar macrophages and lung epithelial cells treated with BLM. It has been suggested that anticancer drugs cause cell injury by altering the mitochondrial membrane permeability [43,44]. However, because some anticancer drugs cause apoptosis without the cytosolic accumulation of cytochrome *c*, the role of cytochrome *c* in cell death due to anticancer drugs has not been clearly elucidated [9]. Thus, we performed this study to define involvement of the mitochondrial membrane permeability change in the cytotoxic effect of BLM. The present results suggest that the loss of the mitochondrial membrane potential and the release of cytochrome *c* into the cytosol caused by BLM may elicit apoptotic cell death in alveolar macrophages. Inhibition of mitochondrial respiration leads to the diminished ATP production, increased ROS formation and eventually apoptotic or necrotic cell death [23,45]. To explore role of ROS in the cytotoxic effect of BLM, we investigated the formation of intracellular ROS by monitoring increase in DCF fluorescence. The increased formation of ROS observed in alveolar macrophages treated with BLM seems to be mediated by inhibition of mitochondrial respiratory chain. In this study, the inhibitory effect of antioxidant enzymes (SOD and catalase) suggests that BLM itself liberate ROS.

GSH effectively defends cells against ROS and regulates the redox state of many cellular substances [35]. In addition, a reduction in GSH may impair hydrogen peroxide clearance and promote formation of hydroxyl radicals, particularly in the presence of iron. Depletion of mitochondrial GSH has been demonstrated to increase ROS formation [40]. Drops in GSH levels and concomitant increase in ROS are found during the apoptotic process [46]. Decrease in mitochondrial GSH also enhances the sensitivity of hepatocytes against the cytotoxic action of respiratory chain inhibitor [47]. In the present study, the GSH contents in alveolar macrophages treated with BLM were significantly reduced as in a cell viability assay. Thus, the results indicate that the BLM-induced cell death in alveolar macrophages (or lung epithelial cells) may be due the change in the mitochondrial membrane permeability, enhanced formation of ROS and depletion of GSH.

Ambroxol, a bromhexine metabolite, is demonstrated to attenuate the stimulated responses in phagocytic cells [18] and reduce the silica-induced cell death in alveolar macrophages [20]. However, effect of ambroxol on the mitochondrial membrane permeability change in alveolar macrophages or lung epithelial cells has not been clarified. As previously mentioned the mitochondrial dysfunction and enhanced production of free radicals are considered to cause cell death. Thus, we explored the effect of ambroxol on the membrane permeability change in mitochondria that may lead to cell death. The present findings suggest that ambroxol may reduce the BLM-induced cell death by attenuating the loss of the mitochondrial transmembrane potential, cytochrome *c* release, increased formation of ROS and decrease in GSH contents. The murine peritoneal macrophages preincubated with 100 μM ambroxol for 15 min, followed by PBS wash, reduced the superoxide production due to incubation of 10 ng/mL interleukin-1 for 4 hr at 37° by 11% (not shown). Ambroxol appears to depress the stimulated responses in rat alveolar macrophages exposed to silica by inhibition of the activities of protein kinases and the calcium transport [20]. Thus, it is estimated that ambroxol may cross the cell membrane and act at intracellular sites, which reveals an inhibitory effect on the cytotoxic action of anticancer drugs, including BLM. The elevated cytosolic Ca^{2+} level and increase in

formation of ROS due to opening of the mitochondrial permeability transition pore cause a cell death [21,40]. Ambroxol is demonstrated to reduce the elevation of cytosolic Ca^{2+} level in rat alveolar macrophages exposed to silica [20]. These reports suggest that the depressant effect of ambroxol on the BLM-induced mitochondrial damage and ROS formation appears to be partially mediated by the inhibitory effect on altered Ca^{2+} transport in cells.

The BLM-induced production of nitric oxide in mice was suppressed by administration of taurine [48]. Curcumin, which shows anti-inflammatory and antioxidant effect, significantly reduced the production of TNF-α and free radicals in rat alveolar macrophages treated with BLM [49]. The present results also suggest that the BLM-induced cell death in alveolar macrophages and lung epithelial cells are caused by oxidative stress. Thus, observing the antioxidant effect we assessed the protective effect of ambroxol on cells against the toxicity of BLM. Ambroxol depresses the respiratory burst in activated phagocytic cells [18,50]. However, the scavenging effect of ambroxol on the superoxide radicals and hydrogen peroxide has been uncertain. As previously mentioned BLM is well known to cause scission of DNA by interacting with iron and oxygen. Thus, we investigated the effect of ambroxol on oxidative damage of liver mitochondria due to the iron in the presence of ascorbate that produce a significant pro-oxidant effect. Inhibition of the iron-mediated formation of MDA and carbonyls in mitochondria clearly reveals the antioxidant ability of ambroxol. The degradation of 2-deoxy-D-ribose induced by Fe^{3+}, EDTA, H_2O_2 and ascorbate is used as a sensitive detection method to assay hydroxyl radicals and is reduced by the hydroxyl radical scavengers, including mannitol [37]. The scavenging action of ambroxol on hydroxyl radicals was suggested by inhibition of the deoxyribose degradation. The increased production of nitric oxide in bronchoalveolar lavage fluid and the expression of inducible nitric oxide synthase mRNA in the lung tissues are found in BLM-treated mouse, which is probably proceeded to lung fibrosis [3,48]. The present study revealed that ambroxol may prevent cell death due to reactive nitrogen species. The action of ambroxol as a free radical scavenger could also be supported by the result that ambroxol in the presence of antioxidant did not reveal an enhanced inhibitory effect against the damaging action of iron or SIN-1. These results suggest that ambroxol may prevent the formation of ROS by reducing the BLM-induced mitochondrial damage and scavenge free radicals liberated from inhibition of the mitochondrial respiration or from BLM itself.

Overall, therefore, we conclude that ambroxol may reduce the BLM-induced viability loss in alveolar macrophages or lung epithelial cells through the inhibition of the mitochondrial damage and scavenging action on free radicals. Ambroxol seems to exert a protective effect on lung cells against the toxic action of BLM.

Acknowledgments

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ1-PG3-20802-0013) to C.S. Lee.

References

[1] Jules-Elyse K, White DA. Bleomycin-induced pulmonary toxicity. Clin Chest Med 1990;11:1–20.
[2] Laskin DL, Pendino KJ. Macrophages and inflammatory mediators in tissue injury. Annu Rev Pharmacol Toxicol 1995;35:655–77.
[3] Yamazaki C, Hoshino J, Sekiguchi T, Hori Y, Miyauchi S, Mizuno S, Horie K. Production of superoxide and nitric oxide by alveolar macrophages in the bleomycin-induced interstitial pneumonia mice model. Jpn J Pharmacol 1998;78:69–73.
[4] Chandler DB. Possible mechanisms of bleomycin-induced fibrosis. Clin Chest Med 1990;11:21–30.
[5] Desai VG, Casciano D, Feuers RJ, Aidoo A. Activity profile of glutathione-dependent enzymes and respiratory chain complexes in rats supplemented with antioxidants and treated with carcinogens. Arch Biochem Biophys 2001;394:255–64.
[6] Beinert T, Binder D, Stuschke M, Jorres RA, Oehm C, Fleischhacker M, Sezer O, Mergenthaler HG, Werner T, Possinger K. Oxidant-induced lung injury in anticancer therapy. Eur J Med Res 1999;25:43–53.
[7] Mailloux A, Grenet K, Bruneel A, Beneteau-Burnat B, Vaubourdolle M, Baudin B. Anticancer drugs induce necrosis of human endothelial cells involving both oncosis and apoptosis. Eur J Cell Biol 2001;80:442–9.
[8] Hamilton RF, Li L, Felder TB, Holian A. Bleomycin induces apoptosis in human alveolar macrophages. Am J Physiol 1995;269:L318–25.
[9] Tang DG, Li L, Zhu Z, Joshi B. Apoptosis in the absence of cytochrome *c* accumulation in the cytosol. Biochem Biophys Res Commun 1998;242:380–4.
[10] Kuwano K, Kunitake R, Maeyama T, Hagimoto N, Kawasaki M, Matsuba T, Yoshimi M, Inoshima I, Yoshida K, Hara N. Attenuation of bleomycin-induced pneumopathy in mice by a caspase inhibitor. Am J Physiol (Lung Cell Mol Physiol) 2001;280:L316–25.
[11] Williams GT. Programmed cell death: apoptosis and oncogenesis. Cell 1991;65:1097–8.
[12] Iyer R, Holian A. Involvement of the ICE family of proteases in silica-induced apoptosis in human alveolar macrophages. Am J Physiol 1997;273:L760–7.
[13] Shi X, Castranova V, Halliwell B, Vallyathan V. Reactive oxygen species and silica-induced carcinogenesis. J Toxicol Environ Health 1998;1:181–97.
[14] Burger RM, Projan SJ, Horwitz SB, Peisach J. The DNA cleavage mechanism of iron-bleomycin. Kinetic resolution of strand scission from base propenal release. J Biol Chem 1986;261:15955–9.
[15] Conklin KA. Dietary antioxidants during cancer chemotherapy: impact on chemotherapeutic effectiveness and development of side effects. Nutr Cancer 2000;37:1–18.
[16] Wauer RR, Schmalisch G, Bohme B, Arand J, Lehmann D. Randomized double blind trial of ambroxol for the treatment of respiratory distress syndrome. Eur J Pediatr 1992;151:357–63.
[17] Fegiz G. Prevention by ambroxol of bronchopulmonary complications after upper abdominal surgery: double-blind Italian multicenter clinical study versus placebo. Lung 1991;169:69–76.
[18] Cho Y, Jang YY, Han ES, Lee CS. The inhibitory effect of ambroxol on hypochlorous acid-induced tissue damage and respiratory burst of phagocytic cells. Eur J Pharmacol 1999;383:83–91.
[19] Gillissen A, Nowak D. Characterization of *N*-acetylcysteine and ambroxol in anti-oxidant therapy. Resp Med 1998;92:609–23.

[20] Kim YK, Jang YY, Han ES, Lee CS. Depressant effect of ambroxol on stimulated functional responses and cell death in rat alveolar macrophages exposed to silica *in vitro*. J Pharmacol Exp Ther 2002;300: 629–37.
[21] Bernardi P. The permeability transition pore. Control points of a cyclosporine A-sensitive mitochondrial channel involved in cell death. Biochim Biophys Acta 1996;1275:5–9.
[22] Chandra J, Samali A, Orrenius S. Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 2000;29:323–33.
[23] Chakraborati T, Das S, Mondal M, Roychoudhury S, Chakraborati S. Oxidant, mitochondria and calcium: an overview. Cell Signal 1999; 11:77–85.
[24] Luisetti M, Poena V, Salmona M, Pagnoni AM, Villani F, Knerich R, Genghini M, Abba L, Pozzi E. Ambroxol and pulmonary toxicity induced by antineoplastic drugs. Int J Clin Pharmacol Res 1986; 6:129–36.
[25] Stettner S, Ledwoztw A. The effect of ambroxol on bleomycin-induced changes in phospholipid composition of rat lung surfactant. Acta Physiol Lung 1995;83:181–7.
[26] Moreno AJM, Madeira VMC. Mitochondrial biogenetics as affected by DTT. Biochim Biophys Acta 1991;1060:166–74.
[27] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[28] Oberhammer FA, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, Schulte-Hermann R. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β1. Proc Natl Acad Sci USA 1992;89:5408–12.
[29] Lotharius J, Dugan LL, O'Malley KL. Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. J Neurosci 1999;19:1284–93.
[30] Isenberg JS, Klaunig JE. Role of the mitochondrial membrane permeability transition (MPT) in rotenone-induced apoptosis in liver cells. Toxicol Sci 2000;53:340–51.
[31] Lizard G, Miguet C, Bessede G, Monier S, Gueldry S, Neel D, Gambert P. Impairment with various antioxidants of the loss of mitochondrial transmembrane potential and of the cytosolic release of cytochrome *c* occuring during 7-ketocholesterol-induced apoptosis. Free Radic Biol Med 2000;28:743–53.
[32] Fu W, Luo H, Parthasarathy S, Mattson MP. Catecholamines potentiate amyloid β-peptide neurotoxicity: involvement of oxidative stress, mitochondrial dysfunction, and perturbed calcium homeostasis. Neurobiol Dis 1998;5:229–43.
[33] van Klaveren RJ, Hoet PHM, Pype JL, Demedts M, Nemery B. Increase in γ-glutamyltransferase by glutathione depletion in rat type II pneumocytes. Free Radic Biol Med 1997;22:525–34.
[34] Gutteridge JMC, Rowley DA, Halliwell B. Superoxide dependent formation of hydroxyl radicals and lipid peroxidation in the presence of iron salts. Detection of 'catalytic' iron and anti-oxidant activity in extracellular fluids. Biochem J 1982;201:605–9.
[35] Halliwell B, Gutteridge JMC. Antioxidant defences. In: Halliwell B, Gutteridge JMC, editors. Free radicals in biology and medicine, 3rd ed. New York: Oxford University Press; 1999. p. 105–245.
[36] Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz A-G, Ahn B-W, Shaltiel S, Stadman ER. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1993;186:464–78.
[37] Aruoma OI. Deoxyribose assay for detecting hydroxyl radicals. Methods Enzymol 1994;233:57–66.
[38] Stennicke HR, Salvesen GS. Caspases: preparation and characterization. Companion Methods Enzymol 1999;17:313–9.
[39] Rahman Q, Abidi P, Afaq F, Schiffmann D, Mossman BT, Kamp DW, Athar M. Glutathione redox system in oxidative lung injury. Crit Rev Toxicol 1999;29:543–68.
[40] Jurma OP, Hom DG, Andersen JK. Decreased glutathione results in calcium-mediated cell death in PC12. Free Radic Biol Med 1997;23: 1055–66.
[41] Pryor WA, Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am J Physiol 1995;268:L699–722.
[42] Hagimoto N, Kuwano K, Nomoto Y, Kunitake R, Hara N. Apoptosis and expression of Fas/Fas ligand mRNA in bleomycin-induced pulmonary fibrosis in mice. Am J Respir Cell Mol Biol 1997;16:91–101.
[43] Xu M, Ashraf M. Melatonin protection against lethal myocyte injury induced by doxorubicin as reflected by effects on mitochondrial membrane potential. J Mol Cell Cardiol 2002;34:75–9.
[44] Amarante-Mendes GP, Kim CN, Liu L, Huang Y, Perkins CL, Green DR, Bhalla K. Bcr-Abl exerts its antiapoptotic effect against diverse apoptotic stimuli through blockade of mitochondrial release of cytochrome *c* and activation of caspase-3. Blood 1998;91:1700–5.
[45] Zamzami N, Hirsch T, Dallaporta B, Petit PX, Kroemer G. Mitochondrial implication in accidental and programmed cell death: apoptosis and necrosis. J Bioenerg Biomembr 1997;29:185–93.
[46] Tan S, Sagara Y, Liu Y, Maher P, Schubert D. The regulation of reactive oxygen species production during programmed cell death. J Cell Biol 1998;141:1423–32.
[47] Garcia-Ruiz C, Colell A, Morales A, Laplowitz N, Fernandez-Checa JC. Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and activation of transcription factor-kappa B: studies with isolated mitochondria and rat hepatocytes. Mol Pharmacol 1995;48:825–34.
[48] Gurujeyalakshmi G, Wang Y, Giri SN. Suppression of bleomycin-induced nitric oxide production in mice by taurine and niacin. Nitric Oxide 2000;4:399–411.
[49] Punithavathi D, Venkatesan N, Babu M. Curcumin inhibition of bleomycin-induced pulmonary fibrosis in rats. Br J Pharmacol 2000;131:169–72.
[50] Gillissen A, Bartling A, Schoen S, Schultze-Werninghaus G. Antioxidant function of ambroxol in mononuclear and polymorphonuclear cells *in vitro*. Lung 1997;175:235–42.