Hydrogen Peroxide-Induced Apoptosis and Necrosis in Human Lung Fibroblasts: Protective Roles of Glutathione

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ABSTRACT—Although reactive oxygen species (ROS)-related cell damage has been implicated in pathogenesis of fibrogenetic pulmonary disorders, features of ROS-mediated cell death in human lung fibroblasts are not completely understood. We therefore examined the effects of hydrogen peroxide (H_2O_2) on cell growth kinetics in human lung fibroblasts (HFL-1 cells) and tested the roles of antioxidants on the H_2O_2-induced cell death (i.e., necrosis and apoptosis) in HFL-1 cells. We found that the relatively low concentrations of H_2O_2 ranging from 10 μM to 100 μM induced predominantly apoptosis, whereas higher concentration of H_2O_2 ranging 1 mM–10 mM induced predominantly necrosis in HFL-1 cells. Extracellular supplementation of glutathione (GSH) in culture media significantly abolished the H_2O_2-induced cell death, whereas GSH-depleted cells by pretreatment with buthionine sulfoxime (BSO) were likely to undergo cell death caused by a lower concentration of H_2O_2 than normal HFL-1 cells without BSO treatment. These results indicate that H_2O_2 induces both necrosis and apoptosis of human lung fibroblasts at least in part through the action of ROS and that modulation of the ROS production inside and outside of cells may influence the cell survival during oxidative insults.

Keywords: Hydrogen peroxide, Apoptosis, Necrosis, Antioxidant, Lung fibroblast

Because human lung fibroblasts (HFL) play a pivotal role in many airway diseases including pulmonary hypertension, pulmonary fibrosis, and diffuse alveolar damage by thickening and/or remodeling of airways and parenchyma (1–5), kinetics of HFL following lung injuries may be very important for the understanding of the mechanism of airway remodeling. Evidence of oxidative reactions is often associated with fibrogenesis occurring in the lung (6). Moreover, many experimental and clinical data support that oxidative stress is involved in the pathogenesis of a number of airway diseases (7–12). Thus, reactive oxygen species (ROS)-mediated cell death (e.g., apoptosis) of airway cells including lung fibroblasts may have a contributory role in the development of oxidant-stress associated pulmonary diseases. Although ROS is known to play a central role in many apoptotic pathways (13–15), there is some evidence suggesting that ROS-mediated cell damage exhibits not only apoptotic cell death but also necrotic cell death (15–17). Because the ROS-induced cell death mediated by pathways of necrosis can be contrasted with those of apoptosis and because the intensity of the oxidative stress may determine which pathway triggered (15), the antioxidant status

may be responsible for the features of ROS-induced cell damage, both apoptosis and necrosis, in HFL. However, the protective roles of antioxidants against oxidative stress have not been fully elucidated in human lung fibroblasts.

The purpose of this study was to identify the features of hydrogen peroxide (H_2O_2)-induced cell damage, both apoptosis and necrosis, in human lung fibroblasts. In the initial study, we compared the effects of concentrations of H_2O_2 on cell growth kinetics of HFL-1 in cultures. Next, we examined the relationship between the concentrations of H_2O_2 and the occurrence of types of cell death in fibroblasts. Finally, we examined the roles of antioxidants in the H_2O_2-induced cell death in HFL-1 cells.

MATERIALS AND METHODS

Cell cultures

In the present study, normal diploid human fetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Rockville, MD, USA). All experiments using HFL-1 were performed on a single clone, between passage 13 to 14 (18). The cells used in the cur-

rent study did not show any evidence of differentiation into myofibroblasts, i.e., positive immunostaining of α-smooth muscle actin (α-SM), whereas the cells treated with platelet-derived growth factor (PDGF)/activin A showed a significant number of α-SM-positive cells (19). The cells were fed on alternate days with Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies, Inc., Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (Interon Inc., Purchase, NY, USA).

Materials

Hydrogen peroxide (H_2O_2), γ-L-glutamyl-L-cysteinyl-glycine (glutathione, GSH), L-buthionine-[S,R]-sulfoximine (BSO) and all other reagents, unless indicated otherwise, were obtained from Sigma Co. (St. Louis, MO, USA).

Kinetics of cell proliferation and viability after H_2O_2 exposure in HFL-1

The cell proliferation rate in HFL-1 was determined by counting cells every other day for 5 days after exposure of the cells to H_2O_2. The cell numbers of five dishes for each group were calculated using a standard hemacytometer following the detachment by 0.1% trypsin plus 1 mM ethylenedinitrilo-tetraacetic acid (EDTA) in phosphate-buffered saline (PBS) (trypsin-EDTA solution). Cell viability was assessed by trypan blue exclusion. Fibroblasts were plated at 5×10^4 cells per dish in 12-well supports (Flow Laboratories, Inc., Mclean, VA, USA), allowed to attach for 12 hr, and then non-adherent cells removed by gentle washing with PBS. One day after plating, the total cell number/dish equaled approximately $6-7 \times 10^4$ (n=5). Dishes were then randomly assigned to one of six experimental groups: H_2O_2 concentration was 10 μM, 100 μM, 1 mM or 10 mM or the vehicle control (DMEM + 0.4% FBS media). Cells were exposed to a concentration of H_2O_2 for 4 hr at 37°C in air plus 5% CO_2, following which the cells were washed with PBS and fed with fresh DMEM containing 0.4% FBS. The control group was exposed to 0.5 ml of vehicle (DMEM + 0.4% FBS) alone for comparable time periods and washed as above.

Detection of apoptosis

DNA staining with Hoechst 33258 dye: One day after seeding HFL-1 (1×10^4/well) in each well of a 4-well chamber slide, cells in one well were exposed to a concentration of H_2O_2 for 4 hr and the other dish was exposed to vehicle for 4 hr. At 24 hr after H_2O_2 or vehicle exposure, cells on the culture slide were washed two times with PBS and then immediately fixed with 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.2) at 4°C for 1 hr. The fixed cells on the slide chamber were stained with Hoechst 33258 dye (10 μg/ml). Apoptotic cell death, necrotic cell death and live cells were determined by the features of nuclear DNA stained with Hoechst 33258 dye.

Morphologic characteristics of cells after H_2O_2 exposure in HFL-1: Further morphologic characterization of cells was performed by transmission electron microscopy (TEM). One day after seeding HFL-1 (1×10^5/dish) on six 35-mm dishes, cells in one dish were exposed to a concentration of H_2O_2 for 1 hr, and the other dish was exposed to vehicle for 4 hr. At 24 hr after H_2O_2 exposure, non-adherent cells in the culture dish were harvested by centrifugation at 800 r.p.m. ($200 \times g$). The cell culture medium of the vehicle-exposed dish was harvested similarly and in addition, the adherent cells of the vehicle-exposed dish were collected using trypsin-EDTA solution. The harvested cells were immediately fixed with 2% glutaraldehyde and 2% PFA in 0.1 M phosphate buffer (pH 7.2) and then postfixed with 1% buffered osmium tetroxide (OsO_4) in 0.1 M phosphate buffer at 4°C for 1 hr. After alcohol dehydration, the cells were embedded in Epon Resin according to the standard method and sectioned using a LKB-Huxley ultramicrotome to final thickness of 60–90 nm. Sections were stained with uranyl acetate and lead acetate and then examined in a Hitachi H-7100 transmission electron microscope (Hitachi, Tokyo).

Quantification of apoptosis using TUNEL staining in a culture dish: To determine the ratio of apoptotic cells to the total cells in a culture dish, modified terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) was performed using Apoptag® (Oncor Co., Gaithersburg, MD, USA) (20). HFL-1 cells were plated at 3×10^5 cells per dish in 6-well culture dishes (Costar). One day after seeding cells, the cells in each of the 15 wells were exposed to H_2O_2 for 4 hr or vehicle. At 24 hr after H_2O_2 exposure, the cells were harvested using trypsin-EDTA solution and then stained by the modified TUNEL method. In brief, the harvested cells were fixed by 2% PFA on ice for 15 min. Then the cells were washed with PBS and pelleted ($200 \times g$ for 5 min). After centrifugation, the supernatant was removed and the cells were resuspended in 32 μl of equilibration buffer. After centrifugation at $200 \times g$ for 5 min, the supernatant was removed, and the cells were incubated with 8 μl of TdT enzyme with 19 μl of reaction buffer at 37°C for 1 hr. The reaction was terminated by addition of 20 μl of stop buffer with 680 μl of distilled water. After centrifugation, the supernatant was removed, and the cells were resuspended in 20 μl of anti-digoxigenin-fluorescein with 38 μl of blocking solution and then incubated at room temperature for 30 min. After the incubation, 10 μl of 0.1% triton X-100 in PBS was added to the cell suspen-

sion. The cells were then pelleted by centrifugation and the supernatant removed. The cells were resuspended in 200 μl of PBS containing 1 μg of propidium iodide (PI) and 10 μg of RNase (Boehringer Mannheim Co., Tokyo), allowed to stand of PBS for 15 min, and then washed with PBS. The anti-digoxigenin-fluorescein–positive cells (apoptotic cells) were counted using a hemacytometer under a fluorescent microscope (Nikon Microphot, EPI-FL3; Nikon, Tokyo). Total cell numbers were determined by counting cells with PI staining under the microscope. The ratio of apoptotic cells to total cells in each sample (minimum of 500 cells counted in each sample) was calculated.

Effect of extracellular GSH supplementation on H₂O₂-induced cell death in HFL-1

The HFL-1 cells were incubated with 0.1–10 mM GSH in 0.4% FBS + DMEM for 1 hr and then the cells were exposed to a concentration of H₂O₂ for 4 hr. At 24 hr after H₂O₂ exposure, the cells were harvested using trypsin-EDTA solution and then stained with trypan blue. The viability was measured by the trypan blue exclusion method.

Effect of intracellular GSH depletion on H₂O₂-induced cell death in HFL-1

Intracellular GSH in HFL-1 cells was decreased by pretreatment with BSO in 0.4% FBS + DMEM for 24 hr and then the cells were exposed to a concentration of H₂O₂ for 4 hr. At 24 hr after H₂O₂ exposure, the cells were harvested using trypsin-EDTA solution and then stained with trypan blue.

Statistical analyses

Data were presented as the mean±S.D. Statistical analyses were performed using analysis of variance (ANOVA) with Fisher's protected least significant difference method or Student's *t*-test by Stat View 4.0 (Abacus Concepts, Inc., Barkeley, CA, USA). A P<0.05 was considered to be statistically significant.

RESULTS

Growth kinetics of HFL-1 after exposure of cells to H₂O₂

The kinetics of cell proliferation after exposure of HFL-1 cells to H₂O₂ is depicted in Fig. 1. The growth curves of H₂O₂-exposed cells were shifted to the right in a H₂O₂-concentration-dependent manner compared with that of vehicle-exposed cells. While the log phase growth started at 3 days after exposure of HFL-1 cells to lower concentrations of H₂O₂, no growth was investigated in the cells exposed to H₂O₂ at the higher concentration. The

viability of cells as indexed by trypan blue exclusion was considerably lower in H₂O₂-exposed cells than that in vehicle-exposed cells (Fig. 2). The effect of exposure time of H₂O₂ on viability of HFL1-cells is shown in Fig. 3. Cell viability decreased as a function of incubation time with H₂O₂ up to about 4 hr, with very little or no further decrease after that for each H₂O₂ concentration.
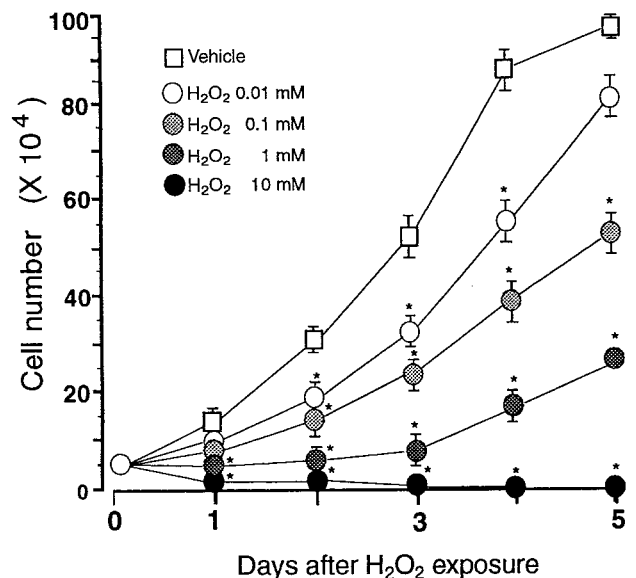

Fig. 1. Kinetics of cell proliferation after exposure to H₂O₂ or vehicle (DMEM + 0.4% FBS) in human lung fibroblasts (HFL-1). The value of cell numbers on each day was expressed as the mean±S.D. of the cell populations of three dishes in each group. *P<0.05, statistically different from the cell number following vehicle exposure at the same time.
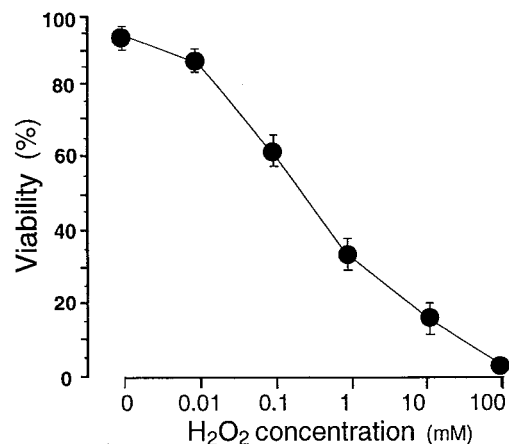

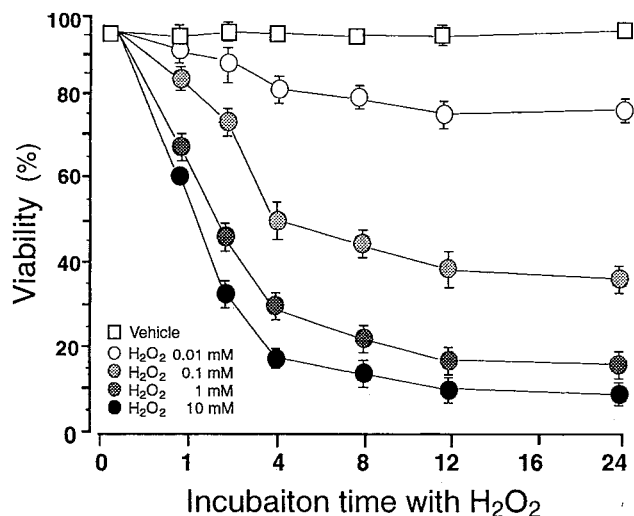**Fig. 2.** Viability of HFL-1 cells after exposure to a concentration of H₂O₂. Each value is a mean±S.D. (n=5).

Fig. 3.   Viability of HFL-1 cells after a variable time of exposure to H_2O_2. Each value is a mean±S.D. (n=5).
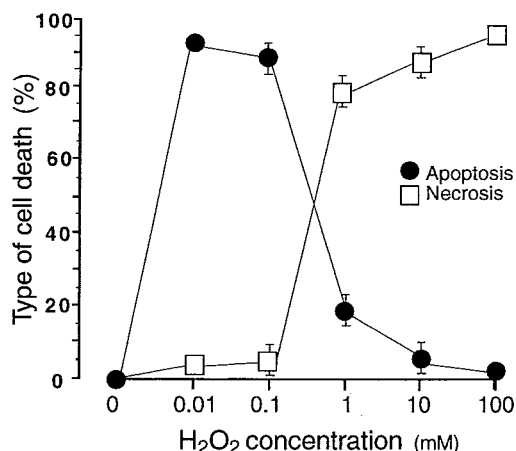

Fig. 5.   The percentage of apoptotic cells or necrotic cells to the total number of dead cells in HFL-1 cells after exposure to a concentration of H_2O_2. Each value is a mean±S.D. (n=5).

Detection of apoptosis in HFL-1 after exposure of cells to H_2O_2

The examination of DNA staining with Hoechst 33258 dye in HFL-1 cells revealed the different nuclear features of cells exposed to H_2O_2 (Fig. 4). The nucleus of HFL-1 exposed to 10 μM and 100 μM H_2O_2 had features consistent with those of apoptosis (i.e., nuclear condensation and fragmentation) (Fig. 4). However, HFL-1 exposed to

higher concentrations of H_2O_2 had features characteristics of necrosis such as expansion without nuclear condensation. The ratio of the number of cells exhibiting necrotic cell death or apoptotic cell death to the total number of dead cells was quantitated by the counting cells on the slide dish following staining with Hoechst 33258 dye (Fig. 5).

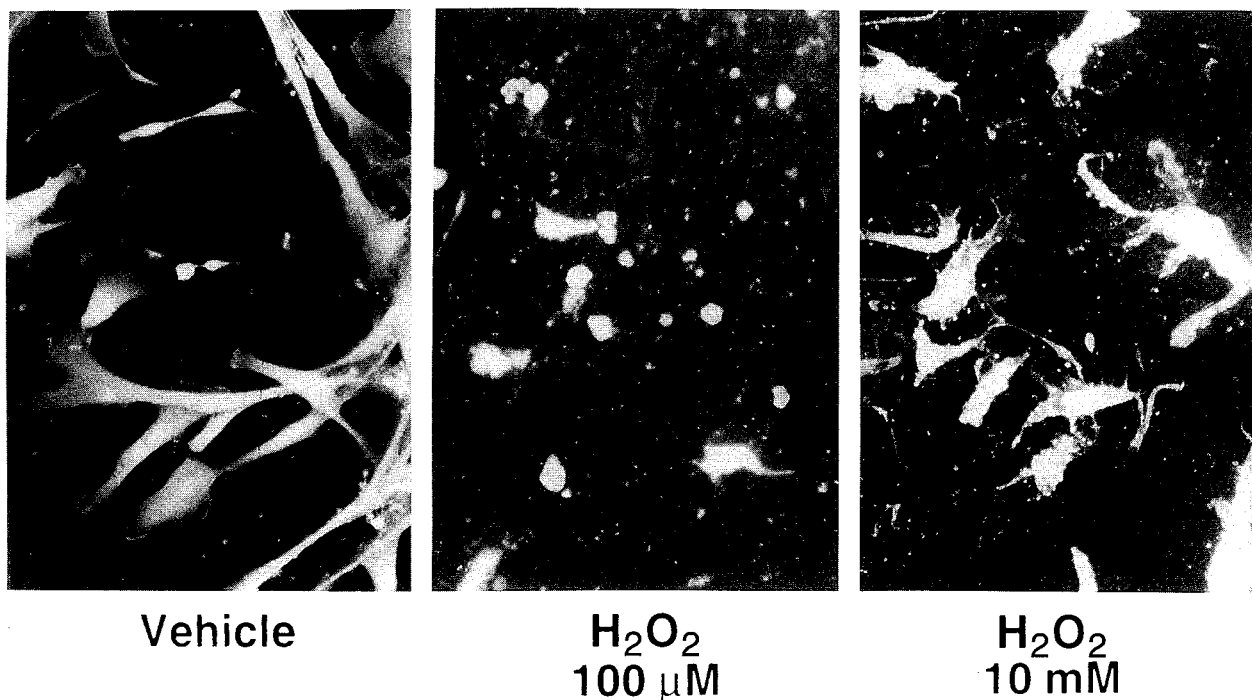Transmission electron micrographs of vehicle-exposed HFL-1 cells and H_2O_2-exposed HFL-1 cells are shown in

Fig. 4.   DNA-stained HFL-1 cells after exposure to H_2O_2 or vehicle.

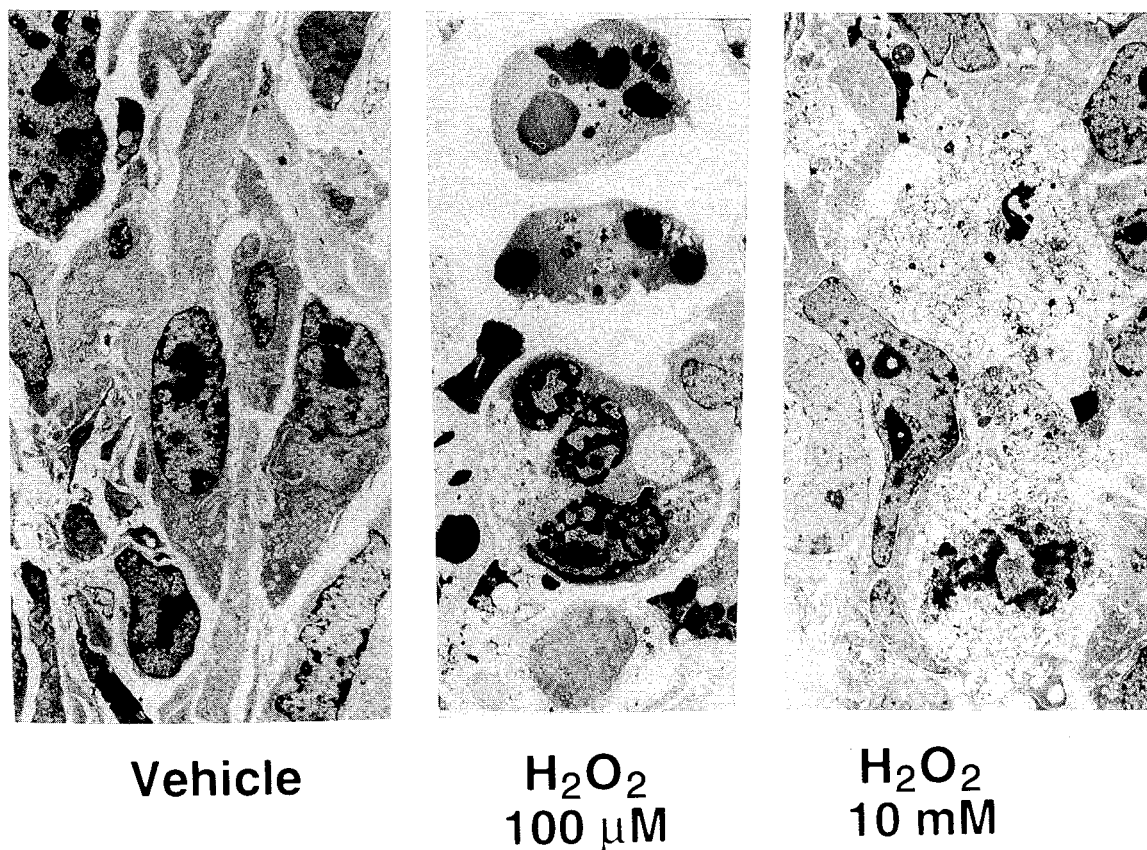**Vehicle**   **H₂O₂ 100 μM**   **H₂O₂ 10 mM**

Fig. 6. Transmission electron micrographs of HFL-1 cells after exposure to H_2O_2 or vehicle.

Fig. 6. Compared with the vehicle-exposed cells, HFL-1 exposed to a concentration of 100 μM H_2O_2 had the features of apoptosis such as cell shrinkage, nuclear conden-

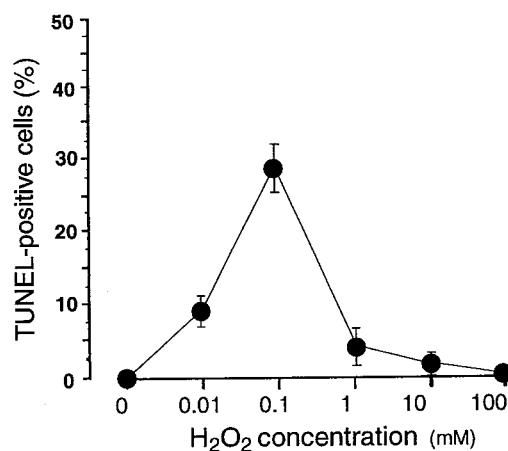sation, nuclear fragmentation and apoptotic body (Fig. 6). However, HFL-1 exposed to a higher concentration of

Fig. 7. Effect of H_2O_2 exposure on induction of apoptosis in HFL-1 cells. The ratio of TUNEL-positive cells (apoptotic cells) to total cells was measured in HFL-1 cells with exposure to H_2O_2. Each value is a mean$\pm$S.D. (n=5).
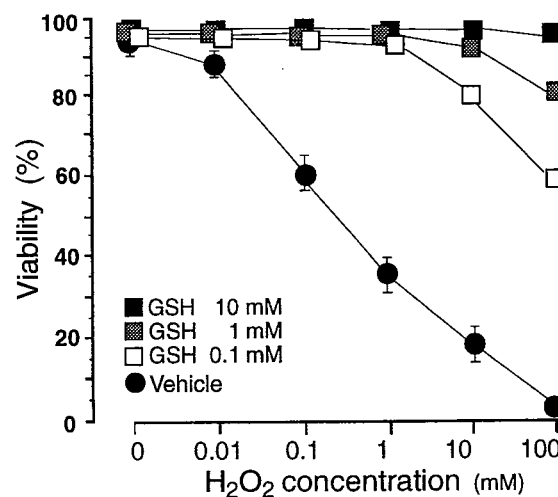

Fig. 8. Effect of extracellular supplementation of glutathione (GSH) on H_2O_2-induced cell death in HFL-1 cells. Viability of HFL-1 cells after exposure to a concentration of H_2O_2 with GSH was determined. Each value is a mean$\pm$S.D. (n=5).
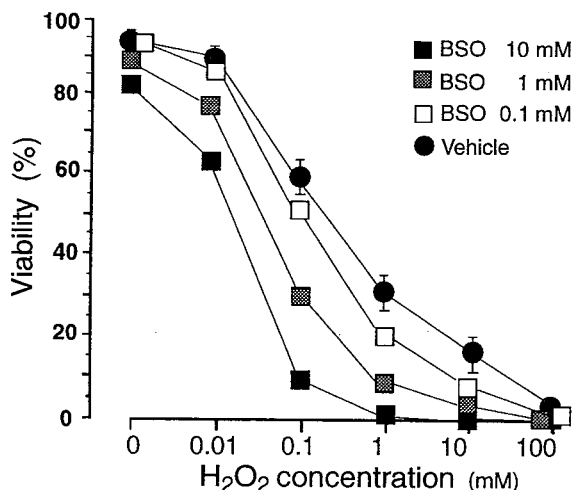
Fig. 9. Effect of pretreatment with L-buthionine-[*S,R*]- sulfoximine (BSO) on H$_2$O$_2$-induced cell death in HFL-1 cells. Viability of HFL-1 cells pre-treated with BSO after exposure to a concentration of H$_2$O$_2$ was measured. Each value is a mean±S.D. (n=5).

H$_2$O$_2$ showed features of necrosis such as cell expansion and nuclear enlargement without fragmentation (Fig. 6).

The percentage of apoptotic cells was further quantitated by DNA strand breaks detected by digoxygenin-conjugated dUTP labeling (TUNEL) (Fig. 7). The ratio of apoptotic cells (TUNEL-positive cells) to total cells including dead cells and live cells was increased in the population of cells exposed to H$_2$O$_2$ at a concentration of 10 μM or 100 μM. However, the apoptotic cells were decreased after exposure of HFL-1 cells to H$_2$O$_2$ at a concentration of 1 mM or 10 mM, whereas necrotic cells, which were not stained with TUNEL, but determined with Hoechst stained cell expansion, were markedly increased in culture (Fig. 7).

Effect of extracellular GSH supplementation on H$_2$O$_2$-induced cell death in HFL-1

The viability of HFL-1 cells exposed to H$_2$O$_2$ was markedly improved with extracellular supplementation of GSH in culture media (Fig. 8). Ten millimolar GSH in culture media totally abolished the H$_2$O$_2$-induced cell death in HFL-1, suggesting extracellular supplementation of GSH prevented the cells from H$_2$O$_2$-induced apoptosis and/or necrosis.

Effect of intracellular GSH depletion on H$_2$O$_2$-induced cell death in HFL-1

There was an approximately 15% decrease in the ability of the cells to exclude trypan blue after the pretreatment with the high concentration of BSO (10 mM) (Fig. 9). Although the GSH depletion alone has a marginal effect on cell viability in culture, GSH depletion plus

H$_2$O$_2$ exposure considerably affected the cell survival of HFL-1 (Fig. 9). The H$_2$O$_2$-induced cell death in HFL-1 cells following the treatment with BSO was more frequently investigated at the lower concentration of H$_2$O$_2$, compared with the cells untreated with BSO.

DISCUSSION

All aerobic mammals face the threat of oxidation from molecular oxygen. Although oxygen is vital to the life of humans, an excess amount of oxygen (e.g., hyperoxia) is toxic to normal cells and organs in humans (21, 22). Because lungs receive the highest oxygen among the organs, the lungs of critically ill patients with exposure to high concentration of oxygen is likely to be damaged. Thus, the lung is amongst the targets of oxidant stress and many airway cells are considerably injured by excess production of highly toxic oxidants (6, 7, 23).

The present study demonstrates that the lower concentrations (0.01–0.1 mM) of exogenous H$_2$O$_2$ induces apoptosis in human lung fibroblasts, resulting in the slower cell growth of fibroblasts. Furthermore, the higher concentrations of H$_2$O$_2$ (1–10 mM) caused a greater magnitude of cell death, which exhibited the features of necrosis, in fibroblasts. The results indicate that the degree of oxidative insults determines which pathway (i.e., apoptotic or necrotic pathway) is triggered. It has been reported that H$_2$O$_2$ can induce apoptotic and necrotic cell death in association with the degree of the oxidative insult in tumor cells and lymphoblastoid cells (14, 15). However, the interaction between the H$_2$O$_2$-related oxidative injury and cell death in normal cells is not fully elucidated. Our results indicated that two distinct patterns of cell death were detected; the higher concentrations of H$_2$O$_2$ (>10 mM) rapidly killed targets with no evidence of apoptosis; lower concentrations of H$_2$O$_2$ induced cell death with morphologic evidences of apoptosis such as cell shrinkage, nuclear condensation and fragmented nucleus. This evaluation is further consistent with the increased rate of DNA strand breaks in the lower concentrations of H$_2$O$_2$-exposed cells as determined by the TUNEL assay. Thus, it is likely that apoptosis plays an important role in the cytotoxicity to whole fibroblasts induced by 0.01 and 0.1 mM of H$_2$O$_2$, but not by the higher concentrations of H$_2$O$_2$. Several variables may be relevant to these observations. First, it is possible that the differences in types of cell death reflects differences in interactions between H$_2$O$_2$ and cellular levels of anti-oxidants. Michiels and coworkers have reported that antioxidant enzymes accelerate or inhibit cell proliferation in human fibroblasts (23). Furthermore, Hockenbery and coworkers have suggested that antiapoptotic protein BCL-2, which inhibits reactive oxygen intermediates

(ROI)-induced apoptosis, can act as an antioxidant (13). Second, the relationship between the oxidative insults and the susceptibility of cell death was not the same among different cells. Kazzatz and coworkers have reported that H$_2$O$_2$ at the high concentration of 5 mM induces apoptosis in human airway epithelial cells (24), whereas the current study suggests that H$_2$O$_2$ at 1–10 mM induces necrosis rather than apoptosis. The different results in oxidant-induced cell death between epithelial cells and fibroblasts can not be clearly explained by the present study; however, the cell integrity may explain the different responses to the exogenous oxidative insults. Because the airway epithelial cells are terminally differentiated cells, but fibroblasts can be differentiated into myofibroblasts, it may be that well differentiated cells like epithelial cells are resistant to oxidative insults compared with poorly differentiated cells. Third, the signal transduction may be different between the cells exposed to a low concentration of H$_2$O$_2$ and cells exposed to a high concentration of H$_2$O$_2$. Hampton and Orrenius have reported that H$_2$O$_2$ has two distinct effects on the activity of caspases, which become activated during apoptosis, in Jurkat T-lymphocytes (25). While lower concentrations of H$_2$O$_2$ can activate the caspases and cause apoptosis in the T-lymphocytes, higher concentrations of H$_2$O$_2$ did not trigger caspase activity and the cells died by necrosis (25). Although it is not clear that this strategy works on other cells including lung fibroblasts, the different triggering of cell signals by various concentrations of H$_2$O$_2$ may explain the induction of different types of cell death of HFL exposed to higher and lower concentrations of H$_2$O$_2$.

Our results also indicate that an antioxidant agent may protect against all forms of oxidant-mediated cell death (i.e., apoptosis and necrosis). One of the mechanisms thought to trigger programmed cell death is the accumulation of ROS. The increase in ROS levels has been correlated with cell death caused by growth factor deprivation in neural cells (26). Accordingly, the addition of antioxidants (13, 27) or the overexpression of superoxide dismutase (15) have proved to be protective against cell death in different systems. In the current study, cells treated with BSO, a GSH-depleting agent, were susceptible to H$_2$O$_2$ in contrast to BSO-untreated ones. Therefore, GSH depletion may play some role in H$_2$O$_2$-mediated cell death in HFL by modulation of increase in ROS.

It has been well understood that oxidative stress is involved in the pathogenesis of many airway diseases (1–12, 25). In pulmonary fibrosis and the other inflammatory lung disorders, lung fibrogenetic changes are associated with oxidative reactions (6). Thus, ROS-mediated cell damage and/or death of lung fibroblasts may be important for remodeling of the structure of airways.

Recently, Uhal and coworkers have demonstrated that fibroblasts exposed to hyperoxia and paraquat release soluble factors capable of inducing cell death (i.e., apoptosis) of alveolar epithelial cells (28). These data suggest that ROS may directly induce apoptosis in airway epithelial cells and that fibroblasts injured by ROS during oxidative stress may also induce apoptosis of airway epithelial cells, resulting in further airway damage. Inversely, the modality of reduction in ROS inside and outside of cells by using antioxidants may decrease cell death in both HFL and airway epithelial cells. GSH is the most abundant intracellular antioxidant in mammals and plays an important role in various cellular functions including regulation of protein function and maintenance of the immune system (29–31). Because GSH and N-acetylcysteine can be administered to patients with pulmonary diseases, these agents may be possible means to prevent the oxidant-induced cell death.

In conclusion, H$_2$O$_2$ induces both necrosis and apoptosis of HFL and the supplementation of antioxidants may in part inhibit H$_2$O$_2$-mediated cell death of the fibroblasts. Thus, the administration of antioxidants may be a candidate therapy for pulmonary fibrotic disorders in terms of ameliorating airway remodeling.

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