Hydroxoa-induced apoptosis and Fas/FasL expression in lung epithelial cells

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The treatment of respiratory failure in newborns often involves supplemental oxygen therapy. However, prolonged exposure to supraphysiological oxygen concentrations (hypoxia) causes tissue damage in many organs and tissues and, in the lungs, may lead to the development of acute and chronic lung injury of the premature newborn. The pathological changes in hypoxic lungs are characterized by injury and death of the alveolar endothelial and epithelial cells (8). Hypoxia-induced pulmonary cell death is multimodal and may involve necrosis and apoptosis, two distinct mechanisms of cell death that have specific biochemical, morphological, and functional characteristics (28). In necrosis, acute, nonphysiological injury leads to extensive cell lysis and disruption of the cell membrane. In contrast, apoptosis represents a regulated form of cell death that typically involves the activation of proteases and nucleases within an intact plasma membrane.

Apoptosis has been described as a major death mechanism in hypoxic lung injury in various animal models in vivo (29, 31, 37, 43, 52). In these studies, apoptotic activity was primarily assessed by the TdT-mediated dUTP nick end labeling (TUNEL) assay, which detects DNA fragmentation in tissue sections in situ. Both in newborn and adult animals exposed to hypoxia, TUNEL-positive cells were identified primarily in the distal lung parenchyma, associated with varying degrees of apoptotic activity in the bronchial epithelium (2, 31, 37, 43, 52). The specific lung cell types undergoing apoptosis under hypoxic conditions have not been formally identified by double labeling or ultrastructural analyses in these animal studies. However, in both newborn (37) and adult (2) mice exposed to hypoxia, pulmonary apoptosis was described as occurring primarily along the alveolar surface of the peripheral airways.

In addition, several recent reports have documented elevated alveolar epithelial cell apoptosis in the lungs of ventilated preterm infants with respiratory distress syndrome (RDS) or early bronchopulmonary dysplasia (BPD) (21, 27, 36). Hargitai et al. (21) studied the lungs of ventilated preterm infants at autopsy and documented apoptotic activity, detected by TUNEL assay, in bronchiolar and alveolar epithelial cells in 17 of 24 infants with early or late subacute stages of BPD. Subsequently, Lukkarinen et al. (27) and May et al. (36) reported increased levels of alveolar apoptosis in autopsy lung samples of preterm infants with RDS and formally identified the apoptotic alveolar cells as epithelial by immunohistochemical double-labeling techniques using TUNEL labeling in combination with epithelial markers. It is tempting to speculate that excessive alveolar epithelial cell apoptosis may, at least in part, explain the reduction of alveolar number that is a morphological hallmark of BPD (6, 22, 32).

In contrast to this demonstration of apoptosis in hypoxia-exposed lungs in vivo, the occurrence and mechanisms of lung epithelial cell hypoxia-induced apoptosis in vitro have been less certain. Hypoxia primarily caused necrosis in human alveolar type II cell-like A549 cells and in type I epithelial and murine lung bronchial cells (23, 37, 42). Intriguingly, preconditioning with hypoxia attenuated, rather than induced, oxidant-induced apoptosis in A549 cells (15). Alternatively, hypoxia-induced cell death in the mouse lung and in cultured human A549 cells has been reported to involve a cellular death pathway sharing features of both apoptosis and necrosis (50). Thus the mechanisms of cell death induced by hypoxia...
appear to be complex and may vary depending on the tissue or cell line studied and the culture conditions used.

We previously demonstrated that the Fas/FasL death signaling system is an important developmental regulator of post-canaliculular type II cell apoptosis (9). Fas-mediated apoptosis (39, 47, 49) involves triggering the cell surface “death receptor” Fas (CD95/APO-1), a member of the tumor necrosis factor receptor superfamily. Stimulation of Fas by its natural ligand FasL or Fas-activating antibody results in its trimerization and the recruitment of two key signaling proteins, the adapter protein Fas-associated death domain (FADD, also called MORT-1) and the initiator cysteine protease caspase-8, which form the death-inducing signaling complex. Subsequent activation of the effector caspases through mitochondria-dependent or mitochondria-independent pathways results in activation of caspase-3, the key effector caspase. Activated caspase-3 cleaves a variety of substrates, including DNA repair enzymes, cellular and nuclear structural proteins, endonucleases, and many other cellular constituents.

We showed that alveolar lung remodeling in rabbits and mice is associated with a time-specific increase in type II cell apoptosis (9, 11, 12). This developmental type II cell apoptosis coincided with upregulation of Fas and/or FasL, colocalized to alveolar epithelial type II cells and bronchial epithelial (Clara) cells (9, 11). Receptor stimulation by means of an activating anti-Fas antibody significantly increased perinatal type II cell apoptosis in vitro and in vivo (9). Conversely, administration of a broad-spectrum caspase inhibitor to newborn mice in vivo significantly reduced pulmonary apoptosis, confirming the contribution of the Fas/FasL system in physiological postcanaliculular type II cell apoptosis (9).

The Fas/FasL death signaling system has been implicated in alveolar type II cell apoptosis in various clinical and experimental models of adult lung injury (1, 18, 19, 24–26, 33–35, 40). By analogy, we speculated that Fas/FasL would also be important in hyperoxia-induced perinatal type II cell apoptosis. The aim of the present study was to investigate the time course of death-related events in lung epithelial cells exposed to hyperoxia (24–72 h) with emphasis on determination of the mode of cell death and the temporal expression patterns of key Fas/FasL-related apoptosis-regulating genes.

MATERIALS AND METHODS

Cell Line and Culture Conditions

The murine lung epithelial (MLE) cell line MLE-12 (ATCC no. CRL-2110) is a transformed clonal line representative of alveolar epithelial type II cells (53). We have previously demonstrated that this cell line shows constitutive Fas expression and susceptibility to direct Fas activation (10). MLE-12 cells were grown in 25-cm² tissue culture flasks in DMEM (GIBCO, Franklin Lakes, NJ) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells at 80% confluence were exposed to hyperoxia (95% O₂/5% CO₂) using a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) or room air/5% CO₂ for time intervals ranging from 24 to 72 h with daily media changes.

Alveolar Type II Cell Isolation

Alveolar type II cells were isolated from fetal C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) by a modification of the methods described by Corti et al. (7) and Rice et al. (46), as previously described in detail (9). Briefly, type II cells were isolated by protease digestion and differential adherence to CD45- and CD32-coated dishes. After isolation and purification, type II cells were resuspended in culture medium (HEPES-buffered DMEM, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin). Dishes were coated with Matrigel (BD Biosciences, San Jose, CA). The media were changed after the first day of culture. On day 2 of culture, cells were exposed to hyperoxia or room air, as described above. The study and protocols performed were approved by the Lifespan Animal Welfare Committee (Institutional Animal Care and Use Committee).

Analysis of Cell Death

Lactic acid dehydrogenase (LDH) activity in the cell culture supernatants was measured using a cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN). Medium was removed from normoxic- and hyperoxia-exposed MLE-12 cells at different time points. The samples were incubated with buffer containing NAD⁺, lactate, and tetrazolium. LDH converts lactate to pyruvate, generating NADH. NADH reduces tetrazolium (yellow) to formazan (red), which was detected by absorbance (490 nm).

Analysis of Apoptosis

TUNEL. Quantification of apoptotic cells was achieved with TUNEL, using the In Situ Cell Death Detection Kit (Boehringer Mannheim, Mannheim, Germany), as previously described (4, 9, 11, 12). Negative controls omitted the transferase enzyme. For quantification of positive TUNEL signals, a minimum of 500 cells was counted per well, and the number of apoptotic nuclei per total number of nuclei (apoptotic index) was recorded.

DNA size analysis. The presence of internucleosomal DNA cleavage, characteristic of apoptosis, was investigated by DNA size analysis. For DNA extraction, cell homogenates were incubated in digestion buffer (100 mM NaCl, 10 mM Tris·Cl, pH 8, 25 mM EDTA, pH 8, 0.5% SDS, and 0.1 mg/ml proteinase K). After overnight lysis at 50°C, the samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ammonium acetate/ethanol. Nucleosomal ladders were detected by DNA gel electrophoresis. DNA (5 μg) was loaded on 1.8% agarose ethidium-bromide gels, separated by electrophoresis and visualized with an ultraviolet transilluminator.

Ultrastructural analysis. To characterize further the observed cellular alterations as apoptotic vs. necrotic, hyperoxia-exposed and control MLE-12 cells were studied by electron microscopy. For
Fig. 2. TdT-mediated dUTP nick end labeling (TUNEL). A: TUNEL (left) and 4',6'-diamidino-2-phenylindole (DAPI) staining (right) of MLE-12 cells exposed to hyperoxia for 24 (a, b), 48 (c, d), or 72 h (e, f), or exposed to normoxic conditions (g, h). Omission of transferase enzyme abolished all FITC labeling (not shown). Arrows indicate pyknotic nuclei. B: apoptotic index (percentage of TUNEL-positive nuclei). Values represent means ± SE. *P < 0.001; **P < 0.01 vs. corresponding normoxic control.
ultrastructural studies, MLE-12 cells were grown on Permanox Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY). After 24–72 h of exposure to hyperoxic or normoxic conditions, the cells were fixed with 1.25% glutaraldehyde in 0.15 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide, and dehydrated through graded ethanols. After removal of chambers and gasket, slides were covered with Epoxy 812 resin and placed over a resin-filled slide-duplicating mold in a 60°C oven to polymerize overnight. Ultrathin sections were cut from the resulting cell monolayer, stained with uranyl acetate/lead citrate, and viewed using a Philips 300 electron microscope.

**Western blot analysis of caspase-3 cleavage.** To determine whether the observed apoptosis was caspase mediated, cleavage of pro-caspase-3 was assayed by Western blot analysis of cell lysates. Protein lysates (20 μg) were size fractionated by NU-PAGE Bis-Tris (4–12%) gel electrophoresis (Novex, San Diego, CA) and transferred to polyvinylidene difluoride membranes. Western blots were hybridized consecutively with polyclonal antibodies against the 19-kDa cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) and against the 32-kDa procaspase-3 (H-277). Secondary antibodies were conjugated with horseradish peroxidase, and blots were developed with an enhanced chemiluminescence (ECL) detection assay (Amersham Pharmacia Biotech, Piscataway, NJ).

**Analysis of caspase-3 and caspase-8 activity.** Colorimetric assays (Biosource International, Camarillo, CA) were used to determine caspase-8 and caspase-3 activity in hyperoxic and normoxic cells. Briefly, 2 × 10⁶ cells were incubated with 100 μl of lysis buffer for 10 min at 4°C. Cell lysates were centrifuged at 10,000 g for 1 min, and the supernatants were collected. The supernatant was incubated with 2× reaction buffer (containing 10 mM DTT) and 5 μl of caspase-8 substrate (IEDT-pNA) or caspase-3 substrate (DEVD-pNA) for 2 h at 37°C. Colorimetric reaction was measured at 405 nm. Each experiment was performed in triplicate.

**Analysis of Apoptosis-Related Gene Expression**

**Western blot analysis.** The effects of hyperoxia on Fas/FasL protein levels were evaluated by Western blot analysis of cell lysates, as described in detail elsewhere (11) using polyclonal rabbit anti-Fas (M-20; Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal anti-FasL (BD Transduction Laboratories, Lexington, KY) antibodies. After fractionation by NU-PAGE Bis-Tris (4–12%) gel electrophoresis, protein lysates (20 μg) were transferred to a nitrocellulose membrane. Bands were identified by ECL detection, and their intensities were quantified densitometrically using NIH Image. Band intensity was expressed as the integrated optical density (IOD) of relevant bands normalized to the IOD of actin bands. Specificity controls included preincubation of antibodies with blocking peptides.

![DNA size analysis by agarose gel electrophoresis. Genomic DNA from MLE-12 cells exposed to normoxic or hyperoxic conditions for 24, 48, or 72 h. A distinct nucleosomal ladder pattern is visible in cells exposed to hyperoxia for 48 or 72 h.](image)

**Fig. 3.** DNA size analysis by agarose gel electrophoresis. Genomic DNA from MLE-12 cells exposed to normoxic or hyperoxic conditions for 24, 48, or 72 h. A distinct nucleosomal ladder pattern is visible in cells exposed to hyperoxia for 48 or 72 h.

![Electron microscopic findings. A: MLE-12 cells grown for 48 h under normoxic conditions showing small chromatin aggregates, evenly dispersed over the nucleus. Cytoplasmic organelles are consistent with surfactant-containing lamellar bodies. B: MLE-12 cells exposed to hyperoxia for 48 h showing chromatin condensation in 1 or 2 large-sized dense aggregates (arrows). The plasma and nuclear membranes appear intact, and the cytoplasmic organelles are well preserved. C: MLE-12 cells exposed to hyperoxia for 72 h showing loss of integrity of nuclear and plasma membranes, generalized degenerative changes, and swelling of the cytoplasmic organelles (arrowheads), consistent with necrosis. N, nucleus.](image)

**Fig. 4.** Electron microscopic findings. A: MLE-12 cells grown for 48 h under normoxic conditions showing small chromatin aggregates, evenly dispersed over the nucleus. Cytoplasmic organelles are consistent with surfactant-containing lamellar bodies. B: MLE-12 cells exposed to hyperoxia for 48 h showing chromatin condensation in 1 or 2 large-sized dense aggregates (arrows). The plasma and nuclear membranes appear intact, and the cytoplasmic organelles are well preserved. C: MLE-12 cells exposed to hyperoxia for 72 h showing loss of integrity of nuclear and plasma membranes, generalized degenerative changes, and swelling of the cytoplasmic organelles (arrowheads), consistent with necrosis. N, nucleus.
RNase protection assay. RNase protection assay (RPA) was used to quantify the effects of hyperoxia on Fas/FasL-related mRNA expression, as described elsewhere (9). Total cellular RNA was isolated from cell lysates according to the method of Chomczynski and Sacchi (5). Subsequently, RPA was performed using the RiboQuant RPA kit with the mouse apoptosis-related mAPO-3 multi-probe template set (BD Biosciences, San Diego, CA), which contains DNA templates for caspase-8, FasL, Fas, FADD, Fas-associated phosphatase-1, Fas-associated factor-1, TNF-related apoptosis-inducing ligand, and the “housekeeping” genes L32 and GAPDH. Specific antisense RNA probes were synthesized using [α-32P]UTP by in vitro transcription, and 10-μg samples of total RNA were hybridized for 16 h at 56°C. After RNase and proteinase K treatment, the RNA:RNA duplexes were heat denatured and resolved on a 5% denaturing polyacrylamide gel. Dried gels were exposed to X-AR film (Kodak, Rochester, NY) at −70°C. The resulting bands were scanned and quantified using PhotoShop and NIH Image software (National Institutes of Health, Springfield, VA). Band intensities were normalized to the L32 bands in the same reaction.

Confocal microscopy. The cellular localization of Fas proteins in normoxic and hyperoxic MLE-12 cells at 48 h of culture was studied by confocal microscopy of cells stained by anti-Fas fluorescence immunohistochemistry. Cells were incubated with anti-Fas antibody, followed by Alexa Fluor 488 goat anti-rabbit IgG (H+L; Molecular

Fig. 5. Analysis of caspase-3 and caspase-8 function and processing. A: Western blot analysis of caspase-3 cleavage. Exposure to hyperoxia correlated with the appearance of the 19-kDa subunit of caspase-3, concomitant with decreasing levels of 32-kDa pro-caspase-3. B: analysis of proteolytic activity of caspase-8 (a) and caspase-3 (b) in cell lysates of MLE-12 cells exposed to hyperoxia (●) and compared with controls grown in ambient conditions (○). Values represent means ± SE of at least 3 experiments. *P < 0.01; **P < 0.001 vs. corresponding normoxic control.
Images were acquired with a Nikon PCM 2000 laser scanning confocal microscope (Nikon, Melville, NY) using the argon (488 nm) laser. Serial optical sections were performed with Simple 32 C-imaging computer software (Compix, Cranberry Township, PA). Z-series sections were collected at 0.6-μm with a ×60 Plan Apo lens and a scan zoom of ×2. All images were collected at the same photomultiplier tube settings. Images were processed and reconstructed in NIH Image. Adobe

Fig. 6. Effect of hyperoxia on Fas/FasL expression. A: Western blot analysis of Fas/FasL protein expression in cell lysates of MLE-12 cells exposed to normoxic or hyperoxic conditions for 0–72 h. Appropriately sized bands were detected for FasL (37 kDa) and Fas (45 kDa). Actin served as internal loading control. B: densitometry of Fas (a) and FasL (b) Western blot analysis. Values are means ± SE. Analyses were performed at least in triplicate. *P < 0.05; **P < 0.001 vs. corresponding normoxic control. C: RNase protection assay (RPA) of Fas-related mRNA expression in cell lysates of MLE-12 cells exposed to hyperoxic conditions for 24 or 48 h. Controls were grown in normoxic conditions for 48 h. D: densitometry of RPA analysis of Fas mRNA. Values are means ± SE. Analyses were performed at least in triplicate. *P < 0.01 vs. normoxia (48 h). IOD, integrated optical density; FADD, Fas-associated death domain; FAP, Fas-associated phosphatase-1; FAF, Fas-associated factor-1; TRAIL, TNF-related apoptosis-inducing ligand; TRADD, TNFR-associated death domain; RIP, receptor interacting protein; TNFR, tumor necrosis factor receptor.
Photoshop was used to convert the images to RGB (red, green, blue) and in the assembly of figures.

**Analysis of susceptibility of cells to direct Fas activation.** To determine whether the Fas receptor of hyperoxia-exposed MLE-12 cells was functional in transducing apoptotic signals, cells were incubated with a cross-linking anti-Fas antibody (clone Jo-2, 20 µg/ml, BD Biosciences), as previously described (9). Controls were incubated with isotype-matched hamster IgG (BD Biosciences). The apoptotic response was quantified by caspase assay as described under \textit{Analysis of Apoptosis}.

**Small interfering RNA transfection.** To determine the functional involvement of the Fas/FasL system in hyperoxia-induced apoptosis, we studied the silencing effect of small interfering RNA (siRNA) duplexes targeting the Fas gene. Transfection of siRNA was carried out using Oligofectamine Reagent (Invitrogen/Life Technologies, Carlsbad, CA) in the presence or absence of 100 nM siRNA duplex according to the manufacturer’s protocol. In all transfection experiments, cells were transfected in parallel with target-specific Fas siRNAs and control nonspecific green fluorescent protein (GFP) siRNA duplexes at the same final concentration (100 nM). The siRNAs were selected from the coding region of the target mRNAs and synthesized using 2'-O-ACE-RNA phosphoramidites (Dharmacon Research, Lafayette, CO). The sense and antisense strands of siRNAs were Fas: 5'-P.GUGCAAGUGCAAACCAGACdTdT-3' (sense), 5'-GUCGGUUUGCUUGCAACdDt-3' (antisense) (48); GFP: 5'-P.GCUUCAGUCUCAUGCCACCC-3' (sense), 5'-P.UCCGCUGGCACCCUU3' (antisense) (Dharmacon Research). Cells were assayed 48 h after transfection. Fas gene silencing was confirmed by Western blot analysis of Fas protein expression in at least three independent experiments.

**Statistical Analysis**

Student’s \textit{t}-test was used for comparisons between experimental groups. ANOVA followed by a post hoc Scheffé’s test was used for multiple-group comparisons. Values are expressed as means ± SD or, where appropriate, as means ± SE. The significance level was set at \( P < 0.05 \). Statview software (Abacus, Berkeley, CA) was used for all statistical work.

**RESULTS**

**Assessment of Cell Death by LDH Cytotoxicity Assay**

MLE-12 cells were exposed to 95% O\(_2\) for time intervals ranging between 24 and 72 h. Control cells were exposed to ambient (normoxic) conditions. Cell death was assessed by an LDH cytotoxicity assay (Fig. 1). LDH activity in supernatants of cells grown under normoxia remained relatively stable for the first 48 h of culture. Subsequently, an increase of LDH release was noted, concomitant with, and likely secondary to, visible cellular overgrowth. From as early as 24 h of exposure, the LDH release of hyperoxic cells was significantly higher than that of normoxic cells (\( P < 0.02 \)). At 48 and 72 h, the LDH activity was 50% higher in hyperoxic samples than in normoxic controls (\( P < 0.001 \) (Fig. 1).

**Patterns of Cell Death in Hyperoxia-Exposed MLE-12 Cells Are Time Dependent**

The extent and temporal pattern of apoptosis among cells exposed to hyperoxia was determined using TUNEL assay detection of DNA fragmentation in situ. TUNEL positivity of normoxic cells was low initially and remained at low levels (<5%) throughout the 72-h culture period (Fig. 2). In cells exposed to hyperoxia, in contrast, there was a significant more than five- to eightfold increase of TUNEL positivity at 24 and 48 h compared with normoxic controls (\( P < 0.001 \)). In hyperoxic cells exposed for 72 h, the overall cellularity was much lower than at 24 and 48 h. However, scattered TUNEL-positive cells could readily be identified among the residual adherent cells at this time point (Fig. 2).

Because the TUNEL assay may not distinguish between apoptotic and necrotic cells under certain conditions (16), we also examined the nuclear features of TUNEL-positive cells by
costaining with 4′,6-diamidino-2-phenylindole (Fig. 2). TUNEL-positive nuclei at 24 and 48 h of hyperoxia appeared dense and pyknotic, characteristic of apoptotic cell death (Fig. 2). A large proportion of adherent cells remaining after 72 h of hyperoxia showed nuclear wrinkling and enlargement, indicative of nonapoptotic (necrotic) cell death (Fig. 2).

To confirm further that hyperoxia-exposed MLE-12 cells undergo apoptotic cell death, we performed DNA size analysis by agarose gel electrophoresis of genomic DNA extracted from hyperoxic cells and normoxic controls. As shown in Fig. 3, exposure to hyperoxia was associated with the appearance of a nucleosomal ladder pattern, characteristic of apoptosis. In contrast, no DNA laddering was seen in normoxic cells at any time point.

By electron microscopy, the nuclei of normoxic MLE-12 cells at 24 and 48 h showed small chromatin aggregates evenly dispersed over the nucleus (Fig. 4A). The cytoplasm showed variably sized electron-dense organelles, consistent with surfactant-containing lamellar bodies. Cells exposed to hyperoxia for 24 or 48 h showed highly characteristic condensation of dense chromatin in one or two central or paracentral nuclear foci (Fig. 4B). Occasional nuclear blebbing was seen. Of note, the cytoplasmic organelles of cells exposed to hyperoxia for up to 48 h were intact, and the morphological integrity of the cell membrane was preserved. In contrast, the majority of cells exposed to hyperoxia for 72 h showed ultrastructural evidence of significant cell damage and displayed classic features of necrosis; the cells were larger, the nuclear membrane was folded and irregular, and there was loss of cell membrane integrity. In addition, swelling and injury of cytoplasmic organelles was evident (Fig. 4C).

**Hyperoxia-Induced Apoptosis Is Caspase Mediated**

To determine the involvement of the caspase pathway in the observed hyperoxia-induced apoptotic cell death, we studied the processing of the main executioner caspase, caspase-3, by Western blot analysis of whole cell lysates using antibodies specific for either procaspase or the caspase-3 split products. As seen in Fig. 5A, levels of 19-kDa caspase-3 cleavage products were negligible in normoxic cells at all time points but increased significantly in hyperoxic cells from 24 h on, concomitant with decreasing levels of the 32-kDa procaspase-3.

The effect of hyperoxia on the proteolytic activity of caspase-8 (the key Fas-dependent initiator caspase) and caspase-3 (key executioner caspase) was studied by colorimetric assays using specific caspase substrates. Figure 5B summarizes caspase-8 and caspase-3 activities measured in cell lysates of MLE-12 cells exposed to hyperoxic or normoxic conditions. Hyperoxia induced a significant two- to-threefold increase of apoptotic activity, as determined by caspase-8 assay. In contrast, incubation of hyperoxic MLE-12 cells with Jo-2 did not result in a significant increase of apoptotic activity, as determined by caspase-8 assay. In contrast, incubation of hyperoxic MLE-12 cells with Jo-2 antibody resulted in a significant twofold increase of apoptotic activity (P < 0.02), confirming that hyperoxia-induced Fas is functional.

**Hyperoxia-Induced Apoptosis Is Associated With Increased Fas/FasL Expression**

To determine the signaling pathways mediating hyperoxia-induced apoptosis, we studied the protein and mRNA expression of Fas-related apoptotic signaling molecules during the first 72 h of hyperoxia exposure. Western blot analysis of Fas/FasL expression in MLE-12 cell lysates exposed to ambient or hyperoxic conditions is shown in Fig. 6A; densitometry analysis is shown in Fig. 6B. In agreement with our previous studies (10) and consistent with their derivation from alveolar and bronchial epithelial cells, MLE-12 cells displayed constitutive Fas and FasL protein expression in normoxic conditions. Hyperoxic cells showed a 50% increase of immunoreactive Fas protein levels at 24 h (P < 0.05) and more than threefold higher Fas protein levels at 48 and 72 h (P < 0.001). Protein levels of FasL remained unchanged during the first 24 h of hyperoxia exposure but showed a twofold increase by 48 h (P < 0.05 vs. normoxic control) and a threefold increase by 72 h (P < 0.001) (Fig. 6, A and B).

Steady-state mRNA expression of Fas-related genes was studied using multiprobe template RPA (Fig. 6, C and D). Densitometric analysis showed that the Fas mRNA levels increased synchronously with Fas protein increases, resulting in threefold higher Fas mRNA levels in hyperoxic cells compared with normoxic cells by 48 h of exposure. The Fas mRNA increase was paralleled by similar increases in FADD mRNA, whereas mRNA levels of others Fas-related genes remained unchanged. Although FasL mRNA was not detectable in normoxic cells, faint FasL bands could be visualized in hyperoxic cells (Fig. 6B).

The cellular localization of Fas protein in hyperoxic and normoxic MLE-12 cells was studied by confocal microscopy. Immunofluorescent analysis of anti-Fas stained MLE-12 cells grown under normoxic conditions revealed small amounts of finely dispersed cytoplasmic Fas antigen (Fig. 7). Cells exposed to hyperoxia for 48 h showed markedly increased immunofluorescence intensity of Fas antigen staining in a predominantly cytoplasmic staining pattern (Fig. 7).

To determine whether hyperoxia-induced Fas is functional in transmitting apoptotic signals, the responsiveness of hyperoxic and normoxic MLE-12 cells to direct Fas activation was tested using an agonistic anti-Fas antibody (Jo-2). As shown in Fig. 8, exposure of normoxic MLE-12 cells to Jo-2 did not result in a significant increase of apoptotic activity, as determined by caspase-8 assay. In contrast, incubation of hyperoxic MLE-12 cells with Jo-2 antibody resulted in a significant twofold increase of apoptotic activity (P < 0.02), confirming that hyperoxia-induced Fas is functional.

![Fig. 9. Effect of Fas small interfering RNA (siRNA) transfection on apoptotic activity. A: caspase cleavage. Western blot analysis of Fas protein expression and caspase-3 cleavage in MLE-12 cell lysates transfected with Fas siRNA or nonspecific green fluorescent protein (GFP) siRNA and exposed to normoxic or hyperoxic conditions. Controls were nontransfected MLE-12 cells. Actin served as internal loading control. Bc: densitometry of caspase-3 Western blot analysis. Values are means ± SE. *P < 0.02 vs. nontransfected hyperoxic control. Bf: densitometry of caspase-3 Western blot analysis. Values are means ± SE. *P < 0.05 vs. nontransfected normoxic control; **P < 0.02 vs. nontransfected hyperoxic control. C: TUNEL (left) and DAPI staining (right) of nontransfected MLE-12 cells (a, b) or MLE-12 cells transfected with Fas siRNA (c, d) or mock GFP siRNA (e, f) following 24-h exposure to hyperoxia. Omission of transerase enzyme abolished all FITC labeling (not shown). Arrows indicate pyknotic nuclei. D: apoptotic index (percentage of TUNEL-positive nuclei). Values represent means ± SE. *P < 0.01 vs. nontransfected or GFP-transfected control.](http://ajplung.physiology.org/)}
Fas Gene Silencing Attenuates Hyperoxia-Induced Apoptosis

The functional involvement of Fas/FasL signaling in hyperoxia-induced apoptosis was determined by studying the effects of Fas gene silencing. MLE-12 cells were transfected with siRNA specifically targeting the coding region of the Fas gene. As shown in Fig. 9, Fas siRNA transfection largely prevented hyperoxia-induced Fas upregulation, resulting in 50% lower Fas protein levels in hyperoxic Fas knockdown cells compared with nontransfected hyperoxic controls ($P < 0.02$). Mock transfection with nonspecific GFP siRNA, in contrast, did not affect Fas expression. The effect of Fas silencing on hyperoxia-induced apoptosis was assayed by Western blot analysis of caspase-3 cleavage and by TUNEL assay. Fas silencing resulted in a significant reduction of hyperoxia-induced caspase cleavage and TUNEL reactivity (Fig. 9), confirming the functional role of Fas signaling in hyperoxia-induced cell death. Interestingly, Fas silencing also reduced apoptotic activity in normoxic cells, suggesting that Fas/FasL contributes to the low baseline apoptotic activity in cells cultured under ambient conditions as well.

Hyperoxia-Induced Fas/FasL Gene Expression in Perinatal Murine Type II Cells

To verify the biological relevance of the results obtained in transformed MLE-12 cells, we studied the effects of hyperoxia on apoptotic gene expression in primary murine type II cells isolated at gestational day 18 (saccular stage). In agreement with our observations in MLE-12 cells, hyperoxia induced a significant more than threefold increase in Fas and FasL protein expression in primary type II cells (Fig. 10).

DISCUSSION

We have determined that hyperoxia activates time-dependent cell death in murine type II cell-like MLE-12 cells. Apoptosis was the predominant mode of cell death during the first 48 h of hyperoxia exposure. Characterization of this earliest recognizable form of cell death as apoptotic was based on well-established morphological and biochemical criteria, including DNA size fractionation, ultrastructural evidence of nuclear chromatin condensation with preserved plasma membrane integrity, increased TUNEL positivity, and, finally, increased expression of proapoptotic genes. More prolonged (72 h) exposure to hyperoxia resulted in significant diminution of overall cell number, likely due to detachment of the earlier wave of apoptotic cells. Remaining adherent MLE-12 cells after 72 h of hyperoxia exposure displayed extensive degenerative changes involving cell membranes and cytoplasmic organelles, consistent with necrotic cell death.

We have further demonstrated that the timing of hyperoxia-induced apoptosis coincides precisely with a dramatic upregulation of cellular Fas protein and mRNA expression, associated with increased FasL protein levels. To demonstrate a causative relationship between Fas/FasL upregulation and hyperoxia-induced apoptosis of lung epithelial cells, several approaches were taken. First, we determined by direct receptor activation that hyperoxia-induced Fas was functional in transducing apoptotic signals. Second, we determined that hyperoxia-induced proteolytic activation of caspase-8 and caspase-3, key Fas-dependent initiator and effector caspases, respectively. Finally, conclusive evidence of a direct link between Fas/FasL upregulation and hyperoxia-induced apoptosis was provided by Fas gene silencing using RNA interference (RNAi) strategies (20).

![Fig. 10. Effect of hyperoxia on Fas/FasL expression in murine fetal type II cells. A: Western blot analysis of Fas/FasL protein expression in fetal type II cells isolated at gestational day 18 and exposed to normoxic (48 h) or hyperoxic (24–48 h) conditions. Actin served as internal loading control. B: densitometry of Fas (a) and FasL (b) Western blot analysis. Values are means ± SE. Analyses were performed in triplicate. *$P < 0.001$ vs. normoxic controls.](http://ajplung.physiology.org/)

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RNAi is a sequence-specific, posttranscriptional gene silencing mechanism increasingly utilized as a powerful tool for elucidation of gene function. For the present study, we employed 21 nucleotide siRNAs previously shown to interfere specifically with Fas gene expression. Hyperoxia-induced Fas upregulation was preserved in MLE-12 cells transfected with a control GFP siRNA. In contrast, Fas gene silencing resulted in significant dampening of the apoptogenic effects of hyperoxia. Of note, Fas siRNA transfection did not completely abolish all apoptogenic effects of hyperoxia. Although the persistence of low levels of apoptotic activity in hyperoxia-exposed Fas knockdown cells may be explained by incomplete Fas silencing, it is possible that other apoptotic signaling pathways may participate in the regulation of hyperoxia-induced cell death. Indeed, several recent studies have suggested the involvement of mitochondrial pathways in hyperoxia-induced cell death.

In concordance with our findings, Barazzone et al. (2) reported increased pulmonary Fas mRNA levels in adult mice exposed to 100% oxygen. In addition, others have shown that hyperoxia induces caspase cleavage in vivo in murine lungs (30) and in vitro in A549 cells (50) and that overexpression of the caspase inhibitor FADD-like interleukin-1-β-converting enzyme (FLICE)-like inhibitor protein results in a hyperoxia-resistant phenotype (50). In contrast, Wang et al. (50) have reported that exposure of human lung epithelial cell-like A549 cells to hyperoxia decreased, rather than increased, Fas protein levels. Moreover, Barazzone et al. (2) found no evidence of increased caspase activity in lungs of adult mice exposed to hyperoxia and determined that intravenous administration of a broad-spectrum caspase inhibitor to adult mice did not attenuate hyperoxia-induced alveolar damage (2). Although some of these discrepancies may be explained by technical factors, differences in cell lines, and differences between in vitro and in vivo research, they underscore the complex pathophysiology of hyperoxic lung injury.

Our observation that hyperoxia-induced type II cell apoptosis is, at least in part, mediated by Fas upregulation needs to be reconciled with reports describing the lack of survival advantage in adult Fas-deficient lpr mice exposed to hyperoxia (2). We speculate that alternative death signaling pathways are activated in Fas-null mice exposed to hyperoxia. In support of this notion, in vivo hyperoxia has been shown to induce increased expression of proapoptotic Bax in Fas-deficient lpr mice (50), likely representing compensatory activation of an alternate apoptotic pathway. Similarly, we previously have shown that the Fas-deficient mutant mice may not always be reliable in establishing the functional involvement of the Fas/FasL system in apoptotic processes (9).

Most experiments in this study were conducted in type II cell-like MLE-12 cells. This cell line was produced from lung tumors generated in transgenic mice carrying the simian virus 40 large tumor antigen under transcriptional control of the human surfactant protein C promoter (53). To verify the biological relevance of observations made in transformed cells, we studied the effects of hyperoxia on Fas/FasL expression in freshly isolated murine fetal type II cells. In these cells, as well, hyperoxia induced a marked increase of Fas and FasL protein levels, confirming the validity of results obtained in MLE-12 cells.

We previously have determined that apoptotic activity and Fas/FasL expression are low in fetal murine lungs (9). The observation that hyperoxia can trigger premature upregulation of Fas expression (and apoptosis) in fetal type II cells may have important clinical implications. Several recent reports have shown that the early stages of BPD of the premature newborn are associated with high levels of alveolar epithelial apoptosis (21, 27, 36). It has even been suggested that these supraphysiological levels of apoptosis may contribute to the dysregulated alveolar development that is characteristic of BPD (6, 22, 32), possibly through the induction of fibroblast proliferation and interference with reepithelialization (18).

The molecular regulation of alveolar epithelial cell apoptosis in infants with early BPD remains undetermined. Because BPD is, at least in part, a consequence of hyperoxia-induced lung injury, we speculate that apoptosis in BPD may, at least in part, be mediated by Fas/FasL signaling. By analogy, the Fas/FasL system has been implicated in alveolar epithelial cell loss in various adult acute lung injury models (1, 18, 19, 24–26, 33–35, 40). Paradoxically, apoptosis appears to be essential in the repair process of BPD during the fibrotic response. Apoptosis eliminates excess epithelial cells (3), proliferating mesenchymal cells (45), and inflammatory cells, such as neutrophils, from the alveolar air space and alveolar walls (38). Therefore, apoptosis may have both beneficial and detrimental effects during BPD, depending on its timing, organization, and location in the developing lung.

In summary, we determined that the early effects of hyperoxia (24–48 h) on lung epithelial cells are characterized by caspase-mediated apoptosis, synchronous with Fas/FasL upregulation. The apoptogenic effects of hyperoxia are significantly attenuated by Fas-targeted RNAi strategies, strongly implicating the Fas/FasL death signaling pathway as critical regulator of hyperoxia-induced apoptosis. Similar observations made in freshly isolated fetal type II cells validate the use of MLE-12 cells as a model for studies investigating the molecular regulation of hyperoxia-induced Fas signaling. A better understanding of signaling pathways leading to hyperoxic cell death may provide new approaches to the treatment of hyperoxia-induced lung injury in premature infants, children, and adults.

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