Cellular Oxygen Toxicity

OXIDANT INJURY WITHOUT APOPTOSIS*

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All forms of aerobic life are faced with the threat of oxidation from molecular oxygen (O₂) and have evolved antioxidant defenses to cope with this potential problem. However, cellular antioxidants can become overwhelmed by oxidative insults, including supraphysiologic concentrations of O₂ (hyperoxia). Oxidative cell injury involves the modification of cellular macromolecules by reactive oxygen intermediates (ROI), often leading to cell death. O₂ therapy, which is a widely used component of life-saving intensive care, can cause lung injury. It is generally thought that hyperoxia injures cells by virtue of the accumulation of toxic levels of ROI, including H₂O₂ and the superoxide anion (O₂⁻), which are not adequately scavenged by endogenous antioxidant defenses. These oxidants are cytotoxic and have been shown to kill cells via apoptosis, or programmed cell death. If hyperoxia-induced cell death is a result of increased ROI, then O₂ toxicity should kill cells via apoptosis. We studied cultured epithelial cells in 95% O₂ and assayed apoptosis using a DNA-binding fluorescent dye, in situ end-labeling of DNA, and electron microscopy. Using all approaches we found that hyperoxia kills cells via necrosis, not apoptosis. In contrast, lethal concentrations of either H₂O₂ or O₂⁻ cause apoptosis. Paradoxically, apoptosis is a prominent event in the lungs of animals injured by breathing 100% O₂. These data indicate that O₂ toxicity is somewhat distinct from other forms of oxidative injury and suggest that apoptosis in vivo is not a direct effect of O₂⁻.

All aerobic life forms face the threat of oxidation from molecular oxygen (O₂) (1, 2). Perhaps to cope with this threat, families of enzymatic and nonenzymatic antioxidants have evolved (3). Antioxidants prevent the accumulation of toxic levels of oxygen-derived free radicals, also called reactive oxygen intermediates (ROI), which can wreak havoc upon cells by modifying proteins (4), lipids (5), and DNA (6). Yet because O₂ is vital to human life, O₂ therapy is used in the treatment of patients can be damaged, probably because lungs receive the highest O₂ exposure (7, 8).

Cell culture experiments indicate that cells are usually killed when exposed to >40% O₂ (9). Exposure to hyperoxia is associated with increased levels of ROI (2), which is reflected by the accumulation of oxidatively damaged cellular macromolecules (1, 10, 11) and chromosomal breakage (12). In cultured, transformed alveolar epithelial cells, lethal exposure to hyperoxia (95% O₂) is also associated with cell cycle arrest at G1 (13) and inactivation of aconitase (14). Paradoxically, apoptosis is a prominent event leading up to the death of epithelial cells by hyperoxia. Rather, direct O₂ toxicity appears to result in necrosis. Paradoxically, apoptosis is a notable feature of hyperoxic lung injury in vivo.

MATERIALS AND METHODS

Cell Culture—Human lung adenocarcinoma A549 cells (ATCC CCL 185) were grown in F12K medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in 95% room air, 5% CO₂ in a humidified chamber. Subconfluent cultures were used in all experiments, and cells adhered overnight prior to experimental treatment. Cells were cultured in sealed chambers flushed with 95% O₂, 5% CO₂. Control cells were cultured in 95% room air, 5% CO₂. Some cell cultures were treated with H₂O₂ or paraquat (Sigma). At each time point, cell viability was determined by the exclusion of Trypan blue dye and counted using a hemacytometer. Media and oxidants were refreshed each day when cells were cultured for several days.

Assays of Apoptosis—Cells were seeded on coverslips and treated in an identical manner as in the cytotoxicity assay. The protocol utilized for the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end-labeling) staining of tissue sections was as described previously (22) except that proteinase K treatment was omitted. TUNEL reagents including rhodamine-conjugated anti-digoxigenin Fab fragment were obtained from Boehringer Mannheim. Cells were double-labeled with 2 μg/ml Hoechst 33258 (Polysciences, Warrington, PA) for 2 min at room temperature. Tissue sections (4–5 μm) were mounted onto slides pretreated with 3-aminopropyltriethoxysilane (Digene Diagnostics, Inc., Beltsville, MD). Slides were baked for 30 min at 60 °C and then washed twice in fresh

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†The abbreviation used is: ROI, reactive oxygen intermediates.
xylenes for 5 min each to remove paraffin. The slides were rehydrated through a series of graded alcohols and then washed in distilled water for 3 min in each. For TUNEL staining of the tissue sections, the sections were double-labeled with 2 μg/ml 4',6-diamidine-2-phenylindole-dihydrochloride (Boehringer Mannheim).

For electron microscopy assays, cell cultures were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at 4 °C. The cells were then postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in LX112 (Ladd Corp., Burlington, VT). Thin sections (60 nm) were cut, stained with uranyl acetate and lead citrate, and examined on a Zeiss EM 10 transmission electron microscope.

Animals and Tissue Preparation—Male C57 Bl/6j mice (8 to 10 weeks of age) were exposed to 100% O2 in plexiglass chambers as described previously (22). Controls were kept in room air. Animals were killed by cervical dislocation, and lungs were fixed by perfusion of 10% buffered formalin at 20 cm H2O pressure and embedded in paraffin as described previously (22).

Image Analysis—Conditions were established enabling apoptotic nuclei to be unambiguously and objectively scored by computer-assisted image analysis using the Image 1 system (Universal Imaging, West Chester, PA). At least 1000 nuclei were counted from a minimum of two independent experiments for each time point. Between 10 and 50 random viewing fields were counted on each coverslip. To quantify the extent of apoptosis, cells were double-labeled with rhodamine-conjugated anti-digoxigenin for TUNEL and Hoechst 33258. Positive nuclei were evident with rhodamine fluorescence using the G-2A filter (Nikon Inc.).

RESULTS

Because the lung is a sensitive target of O2 toxicity, we focused on a lung epithelial cell model. A549 cells, derived from alveolar type II cells, have been extensively studied with respect to their responses to hyperoxia and other oxidant injuries (14, 19). Cells were cultured either in 95% room air or 95% O2.

Table I indicates that cells cultured in hyperoxia showed overt signs of death (Trypan blue exclusion) by day 4 and were 69% dead by day 7. The remainder of the cells died over the next 3 days (data not shown). The kinetics of A549 cell death in hyperoxia are similar to those reported for other cell types (23–25). Because cell death from other oxidants occurs via apoptosis (16–18), we tested whether hyperoxia also induces apoptosis.

One means of determining the number of cells undergoing apoptosis in cell culture utilizes the DNA-binding dye Hoechst 33258, which fluoresces brightly under UV excitation when bound. Nuclei that are condensed during apoptosis are much smaller and brighter than those in nonapoptotic cells. Fig. 1 shows that the nuclei of cells exposed to hyperoxia were larger than controls with no apparent increase in fluorescent staining. In contrast, cells exposed to the oxidants H2O2 or paraquat (which generates intracellular O2-) underwent apoptosis, as shown by their characteristically shrunken and intensely-fluorescent nuclei (Fig. 1).

We also utilized the in situ TUNEL assay to study apoptosis in these cultures. This assay labels 3'-OH ends of DNA in chromatin, which result from endonucleolytic cleavage occurring during apoptosis (26, 27). Fig. 1 shows that cells cultured in hyperoxia were TUNEL negative. In contrast, a large population of cells exposed to the other oxidants was clearly TUNEL positive.

Another means of assessing whether cells are apoptotic is to study their morphology by electron microscopy. Fig. 2 shows that when cells were exposed to 95% O2 for 6 days they became swollen, with enlarged nuclei and mitochondria. By contrast, cells that were exposed to paraquat or H2O2 had condensed chromatin, which is a hallmark of apoptosis. These data confirm the results obtained using light microscopy and further indicate that hyperoxia did not result in apoptosis.

If the kinetics of cell death is correlated with the extent of apoptosis at each time point, it may be concluded that the cells...
have died via apoptosis. Conversely, if there is no correlation, cells would have died via necrosis, not apoptosis. To quantify the extent of apoptosis, we used computer-aided image analysis. Fig. 1 shows that when apoptosis was induced by exposure to H$_2$O$_2$ or paraquat (panels C and D), a large population of cells with shrunken and brightly fluorescent nuclei were clearly distinguished from untreated cells (panel A). Using this approach, we established an objective threshold for the area of apoptotic nuclei. Cells were scored as apoptotic only when their nuclear area was beneath that threshold. Similar quantitative analyses have been achieved by fluorescence-activated cell sorting of nonadherent cells (28), although we have found that computer-aided image analysis is well suited to adherent cells. Table I shows that there was no correlation between cell death and the extent of apoptosis at any time during hyperoxic exposure, as determined either by image analysis after Hoechst staining or by counting TUNEL-positive nuclei. In contrast, cell death and apoptosis were tightly correlated in cultures exposed either to 5 mM H$_2$O$_2$ or 20 mM paraquat.

Hyperoxic cell death is also different than death from other oxidants in terms of the kinetics of cell killing. At the oxidant doses typically studied (6, 17, 18, 29, 30) and used here, cells are killed in a matter of hours, while it took days for hyperoxia. To determine if oxidant-induced apoptosis occurs when the rate of cell death is substantially reduced, experiments were performed at much lower concentrations of H$_2$O$_2$ and paraquat. Similar to cell death by hyperoxia, we observed virtually no apoptosis at much lower oxidant concentrations (see Table I). It should be noted that unlike hyperoxia, these low oxidant doses were not 100% lethal, and a subpopulation of cells began to adapt and divide (data not shown).

After determining that apoptosis did not occur in cells exposed to hyperoxia in vitro, we became interested in hyperoxic lung injury in vivo. Mice were exposed to 100% O$_2$ for 48 h, a time at which they begin showing overt signs of lung injury (22). TUNEL assays of tissue sections were used to assess apoptosis in the lungs. Fig. 3 shows a typical result with an obvious increase in the number of TUNEL-positive nuclei in the hyperoxic lung. Therefore, in contrast to direct exposure of cells in culture to hyperoxia, apoptosis was clearly induced in the lungs of hyperoxic mice in vivo.
Apoptosis appears to be the major mode of cell death when cells experience lethal oxidative insult from exposure to oxidants, including \( \text{H}_2\text{O}_2 \) and superoxide (16–20). Interestingly, even cells that undergo apoptosis following nonoxidative insults, such as steroid treatment or viral infection, have been shown to accumulate lipid peroxides, which is evidence of oxidative damage (31–33). Moreover, apoptosis can be prevented in such cells by the overexpression of cellular antioxidant enzymes or the oncogene Bcl-2, which is thought to be an antioxidant (34). Taken together, there is a tight correlation between oxidative damage to cells and apoptosis, and lipid peroxidation may be a key step leading to apoptosis in some cases and resulting from it in others. For these reasons, we anticipated that another form of oxidant injury, \( \text{O}_2 \) toxicity, would also result in apoptosis. Hyperoxia is lethal to cells and has been shown to cause the accumulation of apoptosis-inducing reactive oxygen intermediates, such as \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \). To test if hyperoxia causes apoptosis, we exposed alveolar epithelial cells to 95% \( \text{O}_2 \) and found that these cells did not die via apoptosis. Using three assays, we found no evidence for apoptosis caused by hyperoxia. We have made virtually identical observations in HeLa cells (data not shown), suggesting that these results are consistent in other epithelial cells and possibly in other cell types as well. In sharp contrast, cells exposed to lethal concentrations of two other oxidants, \( \text{H}_2\text{O}_2 \) or paraquat (which generates intracellular \( \text{O}_2^- \)), were found to undergo typical apoptosis, consistent with previous observations by others. Under the light microscope, hyperoxic cells and their nuclei both appeared swollen. In EM, the mitochondria also appeared swollen, and the chromatin was not condensed, although it was highly condensed when the cells became apoptotic by other oxidants. Overall, hyperoxic cells had a morphology typical of necrosis. The simplest conclusion is that the mode of epithelial cell death by hyperoxia is necrosis.

That apoptosis does not occur by molecular \( \text{O}_2 \) but can occur by peroxide and superoxide radicals suggests that hyperoxia is distinct from other forms of lethal oxidative insult, causing a different mode of cell death. It is also likely that at least some of the organellar and macromolecular sites of \( \text{O}_2 \) damage are different from sites affected by other oxidants, since molecular \( \text{O}_2 \) is not as reactive as oxygen-derived free radicals, diffuses throughout the cell, and can target virtually all organelles and cytosolic molecules. One possible explanation for the lack of apoptosis in hyperoxia is that one or more steps in the oxidative damage-induced pathway to apoptosis might be sensitive to direct oxidation by high levels of molecular \( \text{O}_2 \). This would predict that hyperoxia can inhibit apoptosis in some cases, which could be tested in mutant, hyperoxia-resistant cell lines. Consistent with this possibility is the observation that poly(A)DP-riboseylation is defective in hyperoxia-injured cells (35). Poly(A)DP-ribose polymerase may be activated by cleavage for apoptosis to occur in some systems (36). However, hyperoxia clearly does not inhibit apoptosis in all cases, since apoptosis occurs in lungs of animals injured by in vivo \( \text{O}_2 \) toxicity.

The occurrence of apoptosis in hyperoxic lung is somewhat paradoxical, in light of the absence of apoptosis in cultured hyperoxic cells. Several possible explanations may account for this difference. First, the cell types that undergo apoptosis in lung have not yet been identified, and it is possible that nonepithelial cells become apoptotic. We believe that this is unlikely, however, since we have observed widespread apoptosis in many cell types of severely injured hyperoxic lungs, including airway and alveolar epithelium. Likewise, capillary endothelial cells, which appear to be the cell type most sensitive to hyperoxia in vivo, are described as becoming swollen during hyperoxic injury (37), consistent with cell death via necrosis. A second possibility is that apoptosis in hyperoxic lungs might require paracrine interactions or cell junctions among several cell types in the intact organ. A third and likely possibility is that apoptosis in hyperoxia-injured lung may be a downstream phenomenon, occurring as a result of the release of mediators that are known to induce apoptosis in some systems, or oxidants released by inflammatory cells. Supporting this notion is the observation that apoptosis occurs in a model of acute lung injury and inflammation (38).

When the dose of either \( \text{H}_2\text{O}_2 \) or paraquat was reduced to the point that the rate of cultured cell death was slowed considerably (from hours to days), the mode of cell death was changed, and many fewer cells underwent apoptosis. Moreover, the morphology of many of these cells was consistent with death via necrosis (data not shown). It is noteworthy that cell culture in low level oxidants is also associated with adaptation of clones or populations of cells, which maintain a selective advantage and continue to divide (39, 40). In contrast, this has not been shown to occur when unadapted cells are placed in 95% \( \text{O}_2 \), which is uniformly lethal.

In summary, lethal exposure of cultured lung epithelial cells to hyperoxia causes cell necrosis. In contrast, exposure to high levels of the other oxidants, \( \text{H}_2\text{O}_2 \) and superoxide, cause death via apoptosis, suggesting that lethal doses of \( \text{O}_2 \) cause cell death via mechanisms that are distinct from death caused by lethal doses of other oxidants.

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