Hyperoxia inhibits proliferation of Mv1Lu epithelial cells independent of TGF-β signaling

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Hyperoxia inhibits proliferation of Mv1Lu epithelial cells independent of TGF-β signaling. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L1172–L1178, 1999.—High concentrations of O2 inhibit epithelial cell proliferation that resumes on recovery in room air. To determine whether growth arrest is mediated by transforming growth factor-β (TGF-β), changes in cell proliferation during exposure to hyperoxia were assessed in the mink lung epithelial cell line Mv1Lu and the clonal variant R1B, which is deficient for the type I TGF-β receptor. Mv1Lu cells treated with TGF-β accumulated in the G1 phase of the cell cycle as determined by propidium iodide staining, whereas proliferation of R1B cells was unaffected by TGF-β. In contrast, hyperoxia inhibited proliferation of both cell lines within 24 h of exposure through an accumulation in the S phase. Mv1Lu cells treated with TGF-β and exposed to hyperoxia accumulated in the G1 phase, suggesting that TGF-β can inhibit the S phase accumulation observed with hyperoxia alone. Cyclin A was detected in cultures exposed to room air or growth arrested by hyperoxia while decreasing in cells growth arrested in the G1 phase by TGF-β. Finally, hyperoxia failed to activate a TGF-β-dependent transcriptional reporter in both Mv1Lu and R1B cells. These findings reveal that simple growth arrest by hyperoxia involves a defect in S phase progression that is independent of TGF-β signaling.

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been extensively studied. SV40T-T2 cells rapidly growth arrest when exposed to hyperoxia and resume proliferation on recovery in room air (5). Hyperoxia increased mRNA levels of TGF-β1, TGFβ-1, and TGFβ-1(4, 6). Moreover, hyperoxia increased the expression of p21 that bound and inhibited the activity of cyclin E-Cdk2 complexes. It was concluded that hyperoxia inhibited proliferation through TGF-β signaling because increased cyclin E-Cdk2 activity was observed in cells exposed to both hyperoxia and neutralizing antibodies to TGF-β. Although these studies suggested that hyperoxia inhibits proliferation through TGF-β signaling, they never demonstrated that administration of neutralizing antibodies resulted in normal cell proliferation in the presence of hyperoxia. In addition, it remains to be determined where in the cell cycle hyperoxia inhibits proliferation.

The present study investigates the effects of hyperoxia on proliferation of the mink lung epithelial cell line Mv1Lu, which is markedly growth arrested by TGF-β (31). This cell line was chosen because chemically induced mutant lines that are unresponsive to TGF-β have also been identified (31). The RIB cell line is a member of the R class of mutant Mv1Lu cells that have lost expression of TβR-I. In the present study, we tested the hypothesis that hyperoxia inhibits proliferation through TGF-β signaling by analyzing proliferation of Mv1Lu and RIB cells exposed to hyperoxia. Our findings reveal that hyperoxia caused both cell lines to cease proliferation in the S phase of the cell cycle independent of TGF-β signaling.

MATERIALS AND METHODS

Cell culture. Mv1Lu (mink lung adenocarcinoma) cells were obtained from Dr. Anita Roberts (National Cancer Institute, National Institutes of Health, Bethesda, MD), and RIB (chemically induced mutant Mv1Lu) cells were obtained from Dr. Joan Massague (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY). The cells were incubated at 37°C in 5% CO2 in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 50 U/ml of penicillin, and 50 µg/ml of streptomycin (GIBCO BRL). The cells were maintained in tissue culture flasks and routinely passage every 3 days.

For exposures to hyperoxia or TGF-β, the cells were trypsinized, counted with a hemacytometer, and plated in 100-mm dishes at a density of 5 x 10^5 overnight. The medium was replenished the next morning, at which time the cells were treated with 5 % porcine TGF-β1 obtained from R&D Systems (Minneapolis, MN) and/or exposed to hyperoxia in a Plexiglas box (Belco Glass, Vineland, NJ). The box was sealed and flooded with 95% O2-5% CO2 at 15 min at a flow rate of 5 l/min. O2 concentrations were monitored with a miniOxi analyzer from Catalyst Research (Owings Mills, MD). The cells were harvested at various times with 0.25% trypsin, counted with a hemacytometer, and stained for viability with 0.5% trypan blue or 10 µg/ml of propidium iodide.

Flow cytometry. Cells were trypsinized, resuspended in their original medium, and centrifuged at 300 g. The medium was removed, and the cells were fixed in 75% ethanol for 24 h. The cells were resuspended in 1 ml of RNase (1 mg/ml) for 30 min, centrifuged, and resuspended in 0.5 ml of propidium iodide (10 µg/ml). The samples were analyzed on an Epics Profile (Coulter Electronics, Hialeah, FL) set to collect 10,000 events. DNA histograms were analyzed, and the percentages of G1, S, and G2/M phase cells were determined according to the mathematical model of Fried et al. (31). Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining was performed with the Apo-BrdU kit obtained from Phoenix Flow Systems (San Diego, CA), and fluorescent-positive cells were measured by flow cytometry. As a positive control for TUNEL staining, the cells were exposed to 5 Gy of 137Cs at a dose rate of 3.7 Gy/min and recovered in room air for 24 h.

Western blot analysis. The cells were harvested at 4°C by scraping in 50 mM Tris, pH 7.4, 150 mM sodium chloride, 2 mM EDTA, 25 mM sodium fluoride, 25 mM β-glycerophosphate, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 0.3% Nonidet P-40, 10 µg/ml of leupeptin, 10 µg/ml of pepstatin, and 10 µg/ml of aproatin. The cell lysates were cleared by centrifugation, and protein concentrations were determined with a modified Lowry assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The lysates were diluted in 1× Laemmli buffer (1× is 62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromphenol blue, and 5% β-mercaptoethanol). Proteins (10 µg/ml) were separated by size on polyacrylamide-SDS gels and transferred to nitrocellulose. The membranes were blocked in PBS containing 5% nonfat dry milk overnight at 4°C before incubation with an anti-cyclin A antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1,000 dilution for 1 h at room temperature. Nonspecific interactions were removed by washing in PBS containing 0.05% Tween 20 before the blots were incubated in goat anti-rabbit peroxidase-conjugated secondary antibody at 1:5,000 (Jackson Immunoresearch Laboratories, West Grove, PA). The blots were extensively washed again, and the conjugates were visualized with chemiluminescence (Amersham, Arlington Heights, IL) by exposure to Kodak Bio-Max film. The blots were rebotted with anti-β-actin antibody (Sigma, St. Louis MO) at 1:5,000 dilution as a loading control. TGF-β-inducible luciferase reporter assays. The cells were transfected with the TGF-β-inducible reporter plasmids using calcium phosphate as previously described (21). The p3TP-Lux reporter contains three 12-O-tetradecanoylphorbol 13-acetate (TPA) response elements (TRE) from the human collagenase promoter and the TGF-β responsive element from the plasminogen activator inhibitor-1 promoter linked upstream to the adenovirus E4 minimal promoter (31). Transfection efficiencies were normalized with the pRL-TK vector (Promega, Madison, WI) that expresses the Renilla luciferase gene. Luciferase assays were performed with the dual-luciferase reporter assay system (Promega) and measured with a luminoimeter from Tropix (Bedford, MA).

Statistical analyses. Values are expressed as means ± SD. Group means were compared by ANOVA with Fisher’s post hoc analysis with StatView software for Macintosh, with P < 0.05 being considered significant.

RESULTS

Effects of TGF-β on cell cycle distribution of Mv1Lu and RIB cells. Mv1Lu and RIB cells were irradiated with 30 Gy (n = 5) with the adenovirus E4 minimal promoter. Flow cytometric analysis revealed that asynchronous cultures of Mv1Lu cells cultured in room air contained cells in all phases of the cell cycle. Approximately 55% of the cells were in the G1 (diploid) phase, 30% in the G2/M phase, and 15% in the S phase.

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phase, and 15% in the G2 (4n) phase (Table 1). Mv1Lu cells treated with TGF-β accumulated in the G1 phase of the cell cycle, with a significant decrease in the percentage of cells in the S and G2 phases. In fact, nearly 90% of TGF-β-treated cells accumulated in the G1 phase. Although asynchronous cultures of R1B cells had a similar cell cycle distribution as Mv1Lu cells when cultured in room air, they were unaffected by exposure to TGF-β (Table 1). These findings confirm a previous study (31) that demonstrated that the Mv1Lu clonal variant R1B cell line is unresponsive to TGF-β.

Hyperoxia inhibits Mv1Lu and R1B proliferation. Subconfluent cultures of Mv1Lu and R1B cells were exposed to room air or hyperoxia to determine whether hyperoxia inhibited their proliferation. The cells were harvested every 24 h and counted with a hemacytometer. Mv1Lu and R1B cultures had an increasing number of cells over time when incubated under normoxic conditions (Fig. 1). In contrast, the cell number did not increase in cultures exposed to hyperoxia. The effects of hyperoxia on total cell number were distinguishable within the first 24 h of exposure.

Changes in total cell number in the presence of hyperoxia could be due to decreased cell proliferation or increased cell death. Although Mv1Lu and R1B cells remained attached to their plates for the first 72 h of exposure, trypan blue dye exclusion was used to quantitatively measure cell viability. More than 90% of the cells exposed to hyperoxia for up to 72 h continued to possess good membrane integrity based on their ability to exclude dye (Fig. 2A). Similarly, R1B cells maintained membrane integrity over this time period (data not shown). Although membrane integrity remains a reasonable method to determine the viability of necrotic cells, it does not adequately detect apoptotic cells (25). TUNEL staining was used to assess apoptosis in Mv1Lu cells exposed to hyperoxia. Minimal TUNEL-positive cells were observed in cultures exposed to room air or hyperoxia (Fig. 2B). Hyperoxia also does not induce DNA laddering in Mv1Lu cells (data not shown). As a positive control for apoptotic cell death, Mv1Lu cells were exposed to 5 Gy of ionizing radiation. In contrast to the effects of hyperoxia, nearly 90% of Mv1Lu cells exposed to radiation became TUNEL positive. TUNEL staining was not measured in R1B cells because this finding and a previous study (16) have shown that hyperoxia kills cells in vitro through necrosis. Collectively, our findings suggest that the effects of hyperoxia on cell number were not due to increased cell death.

Mv1Lu and R1B cells were exposed to room air or hyperoxia for 24 h and analyzed by flow cytometry to determine whether hyperoxia altered their cell cycle distribution. Asynchronous cultures of Mv1Lu and R1B cells cultured in room air had cells in all phases of the cell cycle (Fig. 3). In contrast, cultures of both cell lines exposed to hyperoxia for 24 h had a marked increase in the percentage of cells in the S phase associated with a decrease in the percentage of cells in the G1 phase. In fact, the percentage of cells in the S phase nearly doubled after 24 h of exposure (Table 1). Continued exposure to hyperoxia for 48 and 72 h resulted in a...
Differential effects on the cell cycle by TGF-β and hyperoxia. Asynchronous cultures of Mv1Lu cells were then exposed to hyperoxia in the absence and presence of 5 ng/ml of TGF-β1 to determine whether TGF-β could maintain a G1 phase growth arrest in the presence of hyperoxia. Changes in the percentage of Mv1Lu cells in the S phase were time dependent as noticed by the continual increase in the number of cells with a DNA content of 2n and 4n (Fig. 4, left). Approximately 80% of the cells were in the S phase based on propidium iodide staining after 72 h of hyperoxia. In contrast, cells exposed to hyperoxia and treated with TGF-β accumulated in the G1 phase and remained predominantly in the G1 phase even after 72 h of hyperoxia (Fig. 4, right). Although the percentage of cells retained in the G1 phase with TGF-β treatment decreased from 90% after 24 h to ~70% after 72 h, it was still significantly greater than the percentage of cells exposed to hyperoxia alone. TGF-β inhibited S phase entry of cells exposed to hyperoxia for 3 days when the medium was replenished every 24 h as well as when the cells were treated once at the beginning of the exposure (data not shown).

The expression of cyclin A was also determined as further evidence that TGF-β and hyperoxia inhibit proliferation through distinct mechanisms. Cyclin A is expressed by cells in the S and early G2 phases and is decreased in Mv1Lu cells treated with TGF-β (27, 29). Mv1Lu cells were exposed to room air, TGF-β, hyperoxia, or TGF-β and hyperoxia for 24 h. Cyclin A was readily detected in asynchronous cultures growing under normoxic conditions and in cultures growth arrested by hyperoxia (Fig. 5). In contrast, cyclin A abundance was decreased to nearly undetectable levels in cultures exposed to TGF-β or TGF-β and hyperoxia. Thus cyclin A was detected in asynchronous cultures...
Effect of hyperoxia on a TGF-β-dependent transcriptional reporter. These observations demonstrate that TGF-β inhibits proliferation in the G1 phase, whereas hyperoxia causes cells to accumulate in the S phase. Furthermore, they suggest that TGF-β signaling can overcome the effects of hyperoxia because cells treated with TGF-β and hyperoxia accumulate in the G1, not in the S, phase. To confirm that TGF-β and hyperoxia signaling through distinct pathways, the TGF-β-responsive luciferase reporter gene p3TP-Lux was used to measure TGF-β-dependent transcriptional responses (31). Mv1Lu cells were transfected with this plasmid and exposed to room air, TGF-β, hyperoxia, or TGF-β and hyperoxia for 24 h. Room air-exposed cells had minimal luciferase activity that was markedly induced by treatment with TGF-β (Fig. 6). Cells exposed to hyperoxia alone also had minimal luciferase activity that was not significantly different from that in room air-exposed cells. However, cells exposed to both TGF-β and hyperoxia had induced luciferase activity, consistent with the ability of TGF-β to signal even in O₂-exposed cultures. As a control for reporter specificity, R1B cells were transfected and exposed to room air, TGF-β, hyperoxia, or TGF-β and hyperoxia. Minimal luciferase activity was detected in room air-exposed cultures and was not induced by TGF-β or hyperoxia (Fig. 6).

**DISCUSSION**

The present study extends previous observations on the growth-arresting activities of hyperoxia in other cell lines by showing that hyperoxia caused Mv1Lu and R1B cells to accumulate in the S phase of the cell cycle. These changes were independent of TGF-β signaling because hyperoxia inhibited proliferation of the TGF-β-unresponsive cell line R1B. Mv1Lu cells exposed to room air or hyperoxia expressed cyclin A, whereas cells treated with TGF-β accumulated in the G1 phase and had reduced levels of cyclin A. Moreover, Mv1Lu cells exposed to both TGF-β and hyperoxia accumulated in the G1 phase, with decreased expression of cyclin A, suggesting that TGF-β signaling can overcome the growth-arresting activities of simple hyperoxia. This was confirmed by demonstrating functional activity of a TGF-β-dependent transcriptional reporter in Mv1Lu cells cultured in room air or hyperoxia. However, hyperoxia by itself was unable to activate TGF-β-dependent transcription. Collectively, these observations suggest that TGF-β does not participate in the growth-arresting activities of hyperoxia. However, TGF-β may modify the cellular response to hyperoxia by preventing S phase entry.

Previous studies with rat SV40T-T2 cells demonstrated that their proliferation was inhibited by hyperoxia. Although growth arrest was associated with increased mRNA expression of histone and thymidine kinase mRNAs, they were not efficiently translated, which could account for the failure of these cells to proliferate (5). In addition, hyperoxia increased mRNA levels of TGF-β1, TβR-I, and TβR-II (4, 6). Hyperoxia also increased p21, which inhibited the kinase activity of cyclin E-Cdk2 complexes (6). The authors concluded that TGF-β participates in mediating the growth-arresting activities of hyperoxia because SV40T-T2 cells cultured with a neutralizing antibody to TGF-β had a modest increase in cyclin E-Cdk2 activity. Unfortunately, these studies never measured cell cycle progression or determined whether addition of the neutralizing TGF-β antibody resulted in an increase in total cell number. Based on the present findings, it is possible that blocking TGF-β activity resulted in more cells exiting the G1 phase and entering the S and/or G2/M phases where they arrested. Alternatively, hyperoxia may inhibit cell proliferation by different mechanisms that are cell-type dependent. For example, the effects of hyperoxia on SV40T-T2 cells may be unique to these cells because SV40 and other DNA tumor proteins are
known to rearrange cyclin and Cdk partners (32). In contrast, there is no evidence that the Mv1Lu cells, which are derived from the fetal mink lung, express viral genes (14).

The present study reveals that Mv1Lu cells exposed to hyperoxia accumulate predominantly in the S phase of the cell cycle. Although G1 and G2 cell cycle checkpoints in response to DNA damage have been studied extensively, less is known about the existence of an S phase checkpoint. Cells exposed to ionizing radiation growth arrest in the G1 phase through the DNA damage-dependent accumulation of the tumor suppressor p53, which transcriptionally increases p21 (10, 17, 20). In addition to blocking S phase entry, p21 also binds proliferating cell nuclear antigen, which participates in both DNA replication and repair (18). Additional studies in yeast have identified DNA polymerase-ε as a DNA damage sensor that may integrate DNA replication and repair (19). Ionizing radiation also induces a G2 phase checkpoint that is controlled by RAD53 and other related kinases (29). In contrast, ultraviolet B radiation, which creates DNA adducts, prolonged G1 and S phase progression in neonatal rat keratinocytes (24). Similarly, alkylating agents slow S phase progression in Saccharomyces cerevisiae (23). Because DNA replication was dependent on the MEC1 and RAD53 genes, the authors concluded that alkylating agents inhibited proliferation through activation of a checkpoint control rather than through failure to bypass DNA lesions.

Checkpoints were defined as places in the cell cycle where progression was dependent on completion of the previous phases (12). Failure to complete the previous phase resulted in a transient growth arrest during which the cells were able to conclude incomplete biochemical processes. Although hyperoxia alone is not cytotoxic, it is converted to genotoxic ROS that would be predicted to elicit the classic G1 phase checkpoint involving p53 and p21 or the G2 phase checkpoint involving RAD53 (20, 29). The present finding that cells exposed to hyperoxia fail to progress through the S phase is consistent with an inability to appropriately replicate DNA. Further studies are needed to determine whether this is due to a global shutdown in cellular function or a novel DNA damage checkpoint.

The present study found that TGF-β could maintain the G1 phase checkpoint in cells exposed to hyperoxia, thereby preventing the entry and subsequent arrest in the S phase caused by hyperoxia alone. It is widely believed that the cells arrested in the G1 or G2 phase will have a greater capacity to survive genotoxic stress compared with cells in the S phase when DNA replication is occurring (29). However, yeast cells arrested in the S phase with the alkylating agent methylmethane sulfonate have enhanced survival when challenged with ionizing radiation (23). The authors suggest that methylmethane sulfonate may protect against radiation-induced DNA damage because DNA repair events are coupled with replication. This hypothesis is consistent with the observation that DNA polymerase-ε and p21 coordinate DNA repair and replication (18, 19). The concept that resistance to genotoxins may be coupled to the cell cycle is also consistent with a recent study (15) where confluent (growth-arrested) cultures of small-airway epithelial cells were more resistant to hyperoxic injury than subconfluent (proliferating) cultures. Although these studies use different cell types and DNA-damaging agents, they suggest that cells in different phases of the cell cycle are not equally injured by genotoxins. Thus cells arrested in the G1 phase by TGF-β may have an altered ability to survive exposure to hyperoxia than cells arrested in the S and G2 phases by hyperoxia alone. Because TGF-β decreases antioxidant enzyme expression and enhances the cytotoxicity of hydrogen peroxide in A549 cells, future experiments must take into account the multitude of biological activities associated with this cytokine in addition to its ability to simply inhibit proliferation (3).

In summary, the findings in this study reveal that hyperoxia induced Mv1Lu and R1B cells to accumulate in the S phase independent of TGF-β signaling. TGF-β induced a G1 phase growth arrest that blocked entry into and subsequent arrest in the S phase caused by hyperoxia. These changes in cell cycle progression were not associated with decreased cell viability. It is worth noting that TGF-β expression increased within 1–3 h of exposure to hyperoxia in pulmonary epithelial cells of adult mice (22). Although the role that TGF-β plays in hyperoxic lung injury remains to be determined, this finding is interesting because it suggests that TGF-β may prevent S phase entry of pulmonary cells exposed to hyperoxia in vivo.

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