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Hyperoxia-Induced DNA Damage Causes Decreased DNA Methylation in Human Lung Epithelial-Like A549 Cells

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ABSTRACT

The effect of hyperoxia on levels of DNA damage and global DNA methylation was examined in lung epithelial-like A549 cells. DNA damage was assessed by the single-cell gel electrophoresis (comet assay) and DNA methylation status by the cytosine extension assays. Cells exposed to ionizing radiation (0, 1, 2, 4, or 8 Gy) showed increasing rates of percentage of DNA in the tail and tail length with increasing radiation dose. When cells were exposed to room air (normoxia) for 1 day and 95% O_2 (hyperoxia) for 1, 2, 3, 4, and 5 days, data indicated that hyperoxia caused time-dependent increases in levels of (a) single strand breaks, (b) double strand breaks, and (c) 8-oxoguanine. Decreased DNA methylation also was observed at day 5 of hyperoxic exposure, suggesting that hyperoxia-induced DNA damage can influence patterns of DNA methylation in a lung-derived cell line. *Antioxid. Redox Signal.* 6, 129–136.

INTRODUCTION

SUPPLEMENTAL OXYGEN is used clinically in the treatment of premature neonates due to their inefficient respiration. However, prolonged treatment can cause acute lung injury, also known as respiratory distress syndrome. If such injury persists, and continuous oxygen support is necessary, the acute lung injury can evolve into chronic lung injury, also known as bronchopulmonary dysplasia.

Direct and indirect evidence supports the hypothesis that hyperoxia induces the generation of reactive oxygen species, which can further mediate pulmonary oxygen toxicity (2). These oxidants can attack DNA directly, causing oxidation of bases and sugars, depurination, depyrimidation, and phosphodiester single and double strand breaks, or indirectly, by oxidizing lipids that, in turn, damage DNA. These processes contribute to lung cytotoxicity (for review, see 34). Recently, investigations of hyperoxia-dependent lung injury in newborn rodents concluded that oxidative DNA damage observed in the model was neutrophil-dependent (4). The role of neutrophils in hyperoxic lung injury has been the subject of a large number of previous investigations. Many studies have concluded that both production of reactive oxygen species and lung injury can proceed independently from inflammation. In this study, we used a simplified cell culture model to examine the potential for hyperoxia to cause DNA damage in a lung-derived cell.

DNA methylation is the covalent binding of a methyl group to a cytosine molecule (thus making a methylcytosine molecule) within CpG dinucleotide sequences (25). Its effects on the mammalian genome can include modifying the chromatin into a transcriptionally inactive state. Absence of methylation at critical sites can promote transformation of a normal cell into a malignant one (5, 15). In tumor cells, global hypomethylation has been observed within protooncogenes (19) accompanied by region-specific hypermethylation (CpG islands) of tumor suppressor genes (24, 42). Methylation-associated gene silencing events have been shown to involve promoter (8), as well as actively transcribed (29) regions of genes. This can provide tumor cells with a growth advantage.

DNA damage has been shown to interfere with the ability of the nucleic acid template to serve as a substrate for DNA methyltransferases. For example, x-ray-induced abasic sites and

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single strand breaks can diminish the methyl group-accepting capacity of DNA (46). In addition, both UV (6) and γ-irradiation (26) can elicit global DNA hypomethylation.

The presence of hyperoxic DNA damage due to generation of oxidative stress could facilitate changes in DNA methylation status, with potentially important effects on cell survival. Although the impact of certain DNA-damaging agents has been assessed, the effect of hyperoxia-induced oxidative stress on DNA methylation status has not been investigated in cells derived from the lung. This is important because DNA damage can alter methylation patterns such that gene expression can be influenced. Therefore, we also examined the effects of hyperoxic exposure (95% O_2) on DNA methylation status in addition to DNA damage in lung epithelial-like (A549) cells.

MATERIALS AND METHODS

Cell culture

A549 lung epithelial-like carcinoma cells were grown in 100-mm Falcon tissue culture dishes in 10 ml of F-12K media (GibcoBRL, Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C, under a humidified atmosphere of air containing 5% CO_2. Cell cultures were passaged by trypsinization and subcultured at a plating density of 500,000 cells/plate.

Cell exposures to hyperoxia or radiation

For all exposures, cells were trypsinized and plated in 100-mm dishes at a density of 5×10^5 cells/plate. After 24 h, the cultures were given 10 ml of fresh media and placed in a Plexiglas box. The box was sealed and gassed with 95% O_2/5% CO_2 for 20 min. The oxygen concentrations that result from such exposures, at the cellular level, have recently been reported (3). Importantly, exposure to 95% O_2, at Denver atmospheric pressure, is equivalent to only 79% oxygen at sea level pressure. For radiation exposures, cells were placed at a density of 2×10^5 cells/plate in 100-mm dishes and allowed to grow overnight. To test for DNA damage, cells were exposed to 0, 1, 2, 4, or 8 Grey (Gy) of cesium-137 at a dose rate of 2.2 Gy/min. Following exposure, cells were harvested by trypsinization.

DNA extraction and precipitation

DNA was extracted by using the E.Z.N.A Tissue DNA Kit (Omega Bio-Tek Inc., Doraville, GA, U.S.A.) according to the manufacturer's protocol. Precipitation was performed by adding 44.4 μl of 3 *M* sodium acetate buffer (pH 5.2) to the dissolved DNA solution. Then 800 μl of ice-cold methanol was added, and the solution was mixed for 5 min, after which it was placed on ice for 1 h. DNA was recovered by centrifuging at 12,000 *g* for 10 min at 4°C. The supernatant was removed, and the pellet was washed twice with 500 μl of 70% ethanol and centrifuged at 12,000 *g* for 2 min at 4°C. The DNA pellet was left at room temperature to allow any methanol residue to evaporate. DNA was redissolved in 20 μl of TE buffer (10 m*M* Tris-Cl, 1 m*M* EDTA at pH 8.0).

Cytosine-extension assay

Assessment of random DNA methylation status was accomplished by using the cytosine-extension assay as described previously (39). In brief, 1 μg of genomic DNA was digested overnight at 37°C, with 10 U of *Hpa II* endonuclease (New England Biolabs, Beverly, MA, U.S.A.) in 1× nuclear extract (NE) buffer 1 (New England Biolabs; 10 m*M* Bis-Tris propane-HCl, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol at pH 7.0). Total reaction volume was 20 μl. A second aliquot, which served as background control, contained all components except the restriction enzyme. The cytosine-extension assay was performed in a 25-μl reaction volume containing the following: 0.5 μg of genomic DNA, 1× polymerase chain reaction buffer II (without $MgCl_2$), 25 m*M* $MgCl_2$, 0.5 U of AmpliTaq DNA polymerase (PerkinElmer), and 0.3 μl of [^{3}H]dCTP (14.2 Ci/mmol; NEN Life Science Products). The mixture, without the DNA, was incubated at 95°C for 7 min, and then DNA was added. The reaction was covered with mineral oil and incubated at 55°C for 1 h. Samples were placed on ice, and 25 μl of the reaction mixture was applied onto Whatman DE 81 ion-exchange filter paper (Fisher Scientific). Filters were washed three times (10 min each time) with 0.5 *M* sodium phosphate buffer (pH 7.5) at room temperature. For the purpose of a positive control, 10 μg of genomic DNA from control cells was methylated *in vitro* in the presence of *S*-adenosylmethionine (160 μ*M*) (New England Biolabs), 20 U of CpG methylase (New England Biolabs), 1× NE buffer 2 (New England Biolabs; 50 m*M* NaCl, 10 m*M* Tris-HCl, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol at pH 7.9), and dH_2O up to 50-μl reaction volume. The mixture was incubated for 2 h at 37°C. Disintegrations per minute (dpm) were obtained with the use of a scintillation counter (Beckman, model no. LS5000TD).

Single-cell gel electrophoresis (SCGE) assay

DNA damage was examined by using the Reagent Kit for Single Cell Gel Electrophoresis Assay (Trevigen Inc., Gaithersburg, MD, U.S.A.) according to the manufacturer's protocol. Cells were resuspended in phosphate-buffered saline (PBS) at 1×10^5/ml and combined with low-melting-point agar at 42°C at a ratio of 1:10 (vol/vol). Immediately, 75 μl of this suspension was pipetted onto a CometSlide (microscope slides specially treated to promote adherence of low-melting-point agar). Slides were placed at 4°C in the dark for 30 min, followed by immersion in prechilled lysis solution (2.5 *M* NaCl, 100 m*M* EDTA, pH 10, 10 m*M* Tris base, 1% sodium lauryl sarcosinate, and 1% Triton X-100) at 4°C for 60 min. Slides were then immersed in freshly prepared alkali solution (0.3 *M* NaOH and 200 m*M* EDTA, pH >13) for 60 min at room temperature in the dark. For the detection of double strand breaks, slides were removed from the alkali solution and immediately washed in 1× TBE buffer (90 m*M* Tris base, 90 m*M* boric acid, and 3 m*M* EDTA, pH 8.0) for 5 min, two times. Slides were then transferred to a horizontal electrophoresis apparatus where 1× TBE buffer was poured until slides were covered. Power supply was set to 1 V/cm (measured electrode to electrode), and the voltage was applied for 10 min at 4°C. After electrophoresis, slides were dipped in 70% ethanol for 5 min and stored overnight at room temperature. For the detection of single strand breaks, slides were removed from the

alkali solution and transferred to a horizontal electrophoresis apparatus. Alkali solution was poured until the fluid level covered the slides. Voltage was set to 1 V/cm, 300 mA for 30 min at 4°C. Then slides were dipped in 70% ethanol for 5 min and stored overnight at room temperature. For both single and double strand breakage, detection slides were stained with 50 µl of diluted SYBR Green dye [1 µl of the dye in 10 µl of TE buffer (10 m*M* Tris-Cl, pH 7.5, 1 m*M* EDTA)].

Visual scoring

As described in detail previously (37, 38), visual scoring of DNA damage on each slide was determined for 100 randomly selected nucleoids (state of cells after being lysed) at a magnification of 200×. Nucleoids were categorized into five classes (0, 1, 2, 3, and 4) representing an increasing extent of DNA damage. Each nucleoid was assigned a numeric value according to its class. The overall score for each slide ranged from 0 up to 400 arbitrary units (AU).

Computer-based scoring

Computer scoring was performed on 50 cells for each treatment group, for the determination of both tail length and percentage of DNA in tail. Percentage of DNA in tail refers to the total cell DNA that migrated into the comet tail. The equation used was: (tail DNA/total DNA) × 100. Tail length measures the length of the tail from the edge of the head eclipse to the tip of the tail. In brief, each nucleoid was captured as a digital image using an Olympus Vanox-T fluorescence microscope attached to a digital camera (Cooke, Auburn Hills, MI, U.S.A.). The UV filter used was of 360-nm excitation/457-nm emission, and the magnification was set at 200×. Images were recorded using the Slide Book 2.6.5.5 software (Intelligent Imaging Innovations, Denver, CO, U.S.A.) on a Macintosh G3 computer. The software used to assess DNA damage was a modified version of the Comet 1.4 macro (23) and can be found at http://rsbweb.nih.gov/pub/nih-image/user-macros/ under the filename: elc_comet11.txt. Assessment of overall DNA damage was made in ~20 randomly selected cells.

Assessment of 8-oxoguanine levels

Cell levels of 8-oxoguanine were determined by using the Fluorogenic OxyDNA Assay Kit (Calbiochem Inc., San Diego, CA, U.S.A.) according to the manufacturer's protocol. After hyperoxic exposures, cells were harvested by trypsinization (1–2 × 10^6 cells/sample), centrifuged at 230 *g*, and resuspended in 2 ml of PBS. Cells were fixed in 4% paraformaldehyde, and then incubated on ice for 15 min, followed by centrifugation and resuspension in 70% ice-cold ethanol. Cells were stored at −20°C overnight. The next day cells were centrifuged and resuspended first in PBS and then in wash solution (25× Tris-buffered saline containing 1% Tween 20 and thimerosal; diluted 1:25 in dH_2O). Nonspecific binding sites were blocked with 50 µl of blocking solution (diluted 1:10 in wash solution) for 1 h at 37°C. At the end of the incubation period, 3 ml of wash solution was added and cells were mixed, centrifuged, and resuspended. Then cells were incubated with 100 µl of fluorescein isothiocyanate conjugate (diluted 1:10 with wash solution) for 1 h in the dark at room temperature. Cells were centrifuged and resuspended in wash solution. Finally, cells were resuspended in 1 ml of PBS and analyzed on a FACS CALIBER flow cytometer (BD Instruments, San Diego, CA, U.S.A.) operated by Cell Quest software and incorporating an argon laser (488 nm, 15 mW) for excitation. For the purpose of a positive control, cells were treated with a bolus addition of 10 m*M* hydrogen peroxide (H_2O_2) for 1 h at 37°C in the absence of serum.

Statistical analysis

All calculations were performed using the JMP software (SAS Institute, Cary, NC, U.S.A.). Means were compared by one-way analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons. A value of $p < 0.05$ was considered significant.

RESULTS

DNA strand break measurements in irradiated A549 cells

DNA architecture persists as supercoiled loops attached to the nuclear matrix. If cells are permeabilized with a detergent, and nuclear proteins are extracted with a high salt concentration, the DNA remains within a nucleus-like structure called the nucleoid. Thus, undamaged cells appear as intact nucleoids (comet heads) without tails. However, if the DNA contains breaks, their presence relaxes supercoiling, and the loops are now free to extend outside the bounds of the matrix instead of being attached to nuclear matrix (31). Figure 1A shows increased migration of DNA from the nucleus of representative cells with increasing doses of ionizing radiation.

To evaluate the SCGE assay and the computer-based scoring software in detecting increasing levels of DNA strand breaks, A549 cells were exposed to 0, 1, 2, 4, and 8 Gy of ionizing radiation and the SCGE assay was performed. Ionizing radiation increased the mean DNA percentage in comet tails in a dose-dependent manner [1 Gy (8.7% ± 0.9), 2 Gy (10.0 ± 0.9), 4 Gy (13.3 ± 1.4), 8 Gy (22.7 ± 1.9)] when compared with unexposed cells [0 Gy (5.4 ± 0.9)] (Fig. 1B). The alternative measurement of tail length by software analysis was comparable to tail DNA percentage measurements [0 Gy (1.3 ± 0.24), 1 Gy (3.0 ± 0.3), 2 Gy (4.1 ± 0.3), 4 Gy (6.3 ± 0.6), 8 Gy (10.4 ± 1.2)] (Fig. 1C). Assessment of radiation-induced DNA damage by visual scoring revealed a comparable dose-dependent increase in strand breaks (data not shown).

Hyperoxia-induced DNA damage in A549 cells

DNA strand breaks were assessed by alkaline (single strand breaks) and neutral (double strand breaks) SCGE assays. The mean level of single strand breaks in room air-exposed cells was 30.2 ± 4.5 AU, whereas 24 h of hyperoxic exposure significantly increased those levels (95.4 ± 30.7 AU). Exposure to hyperoxia for longer durations further increased the levels of single strand breaks [2 days (161.2 ± 10.7 AU), 3 days (188.2 ± 13.1 AU), 4 days (208.3 ± 15.4 AU), 5 days (232.7 ± 12.2 AU)] (Fig. 2). Levels of double strand breaks also were determined over a similar time course of hyperoxic exposures. Hyperoxia-exposed cells had increased levels of dou-

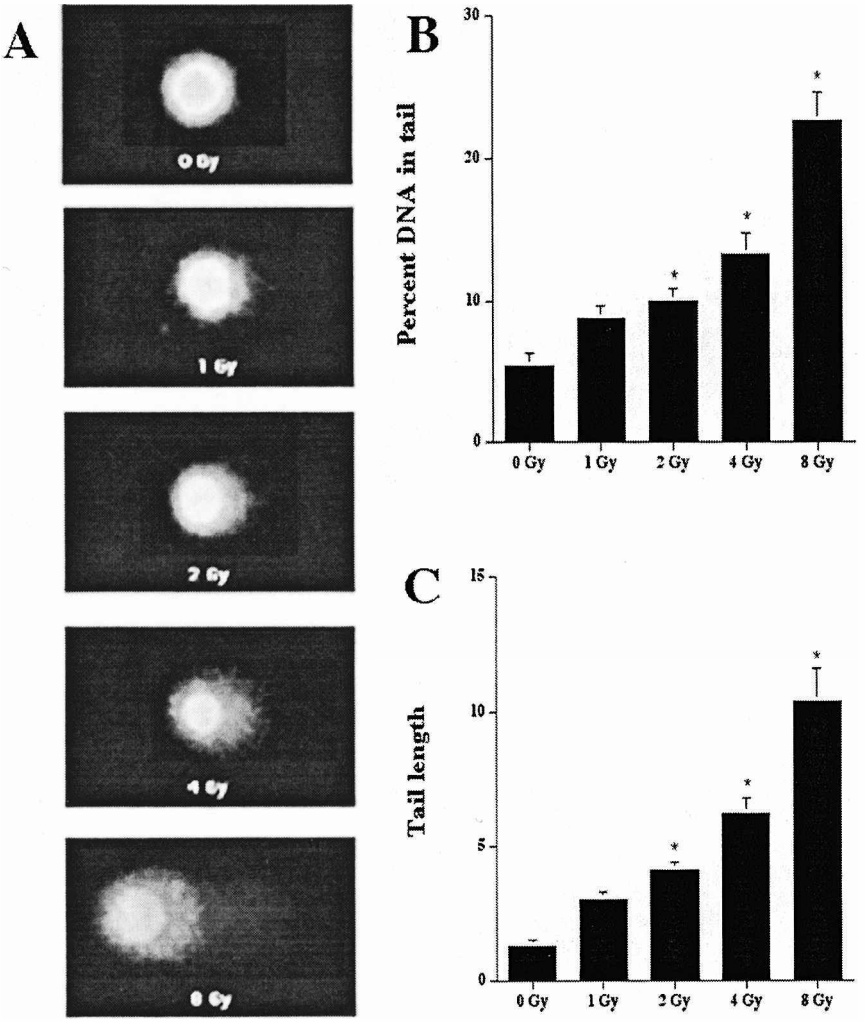

FIG. 1. **(A)** SCGE (comet) assay. A549 cells were exposed to 0, 1, 2, 4, or 8 Gy before being embedded in low-melting-point agarose. Cells were electrophoresed, stained with a SYBR Green dye, and visualized under a fluorescence microscope. Undamaged nuclear DNA (0 Gy) appears as an intact nucleoid (comet head) without a tail. In the presence of breakage, the supercoiling nature of DNA relaxes and extends to form a tail structure. Note the increased extension of the DNA from the comet head to the comet tail with increasing radiation dose. **(B)** Determination of percent DNA in tail in A549 cells using a computer scoring approach. Cells were exposed to 0, 1, 2, 4, and 8 Gy of ionizing radiation. $*p < 0.05$ versus 0 Gy by one-way ANOVA with Tukey's test for multiple comparison; $n = 18$ for 0 Gy, $n = 23$ for 1 Gy, $n = 24$ for 2 Gy, $n = 22$ for 4 Gy, $n = 23$ for 8 Gy. **(C)** Determination of tail length in A549 cells using a computer scoring approach. Cells were exposed to 0, 1, 2, 4, or 8 Gy of ionizing radiation. $*p < 0.05$ versus 0 Gy by one-way ANOVA with Tukey's test for multiple comparison; $n = 18$ for 0 Gy, $n = 23$ for 1 Gy, $n = 24$ for 2 Gy, $n = 22$ for 4 Gy, $n = 23$ for 8 Gy.

ble strand breaks [1 day (21.0 ± 2.0 AU), 2 days (48.7 ± 2.6 AU), 3 days (42.8 ± 4.4 AU), 4 days (37.7 ± 9.3 AU), 5 days (63.2 ±5.6 AU)] when compared with room air-exposed cells (14.7 ± 2.2 AU) (Fig. 3). Thus, hyperoxia elicited a time-dependent increase in both single and double strand breaks. The absolute level of single strand breaks was greater than that of double strand breaks at each time point, with single strand breaks being three to four times greater at the final time point.

8-Oxoguanine levels in A549 cells exposed to hyperoxia

Levels of 8-oxoguanine, in room air- and hyperoxia-exposed cells, were detected by flow cytometry after direct binding of an antibody to 8-oxoguanine moieties in the DNA of fixed cells. Room air-exposed cultures had a basal level of 18.0 ± 5.1% of 8-oxoguanine-positive cells (Fig. 4). In response to hyperoxia, a significant increase in 8-oxoguanine-positive cells was not evident until after 4 days (29.7 ± 1.5%) and 5 days (34.9 ± 1.5%) of exposure. In contrast, significant increases in levels of 8-oxoguanine-positive cells were evident within 1 h after treatment with 10 m*M* H_2O_2 (Fig. 4).

Effect of hyperoxia on DNA methylation

For the determination of global DNA methylation, the methylation-sensitive restriction enzyme *Hpa II* was used. This enzyme cleaves at unmethylated CpG dinucleotide sequences. This leaves a guanine molecule "overhanging." As a result, [^{3}H]dCTP is incorporated at those unmethylated (cleaved)

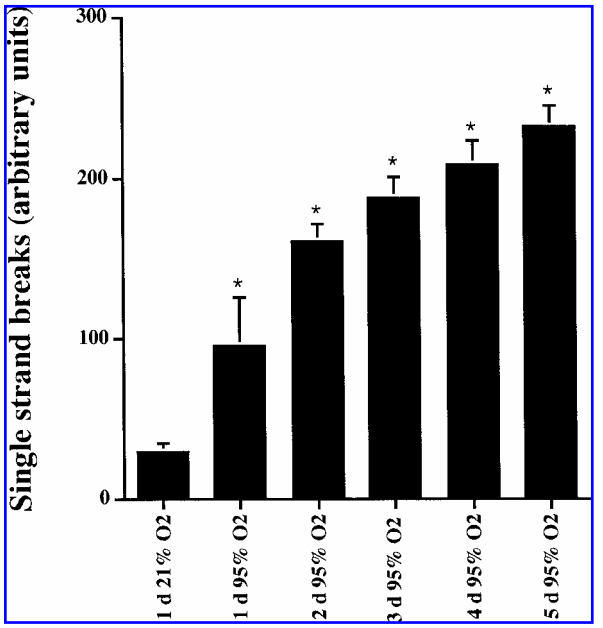

FIG. 2. Determination of DNA single strand breaks in hyperoxia-exposed A549 cells. Cells were exposed to either 1 day of 21% O_2 or 1, 2, 3, 4, and 5 days of 95% O_2. DNA damage was scored visually on a scale of 0–400 AU. *$p < 0.05$ versus 21% O_2 by one-way ANOVA with Tukey's test for multiple comparison; $n = 5$ per group.

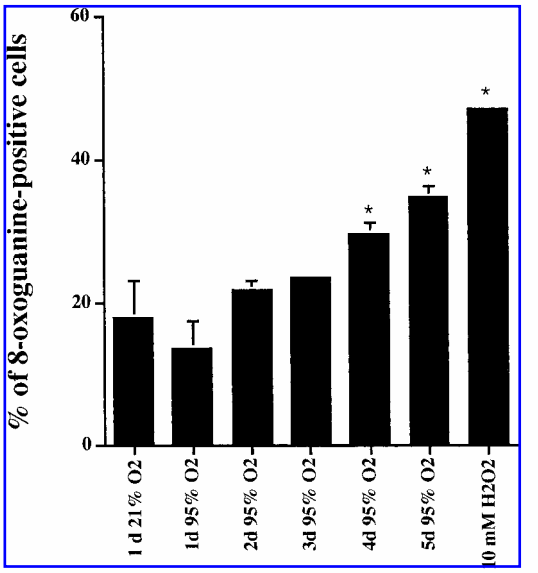

FIG. 4. Effect of hyperoxia on the percentage of 8-oxoguanine-positive A549 cells. Cell cultures were exposed to either 1 day of 21% O_2 or 1, 2, 3, 4, and 5 days of 95% O_2. The rightmost column is the positive control and indicates cells being treated with a bolus addition of 10 m*M* H_2O_2 (see Materials and Methods). *$p < 0.05$ versus 21% O_2 by one-way ANOVA with Tukey's test for multiple comparison; $n = 5$ per group.

sites. Thus, the extent of [^{3}H]dCTP incorporation reflects the number of unmethylated sites. Conversely, low [^{3}H]dCTP incorporation indicates a high degree of DNA methylation. During hyperoxia studies, a positive control was included consisting of extensively methylated genomic DNA. The level of incorporation in room air-exposed cells was lower (1,437.2 ± 211.3 dpm) than after 5 days of exposure to hyperoxia (3,349.5 ± 118.5 dpm). This indicates that levels of methylation declined

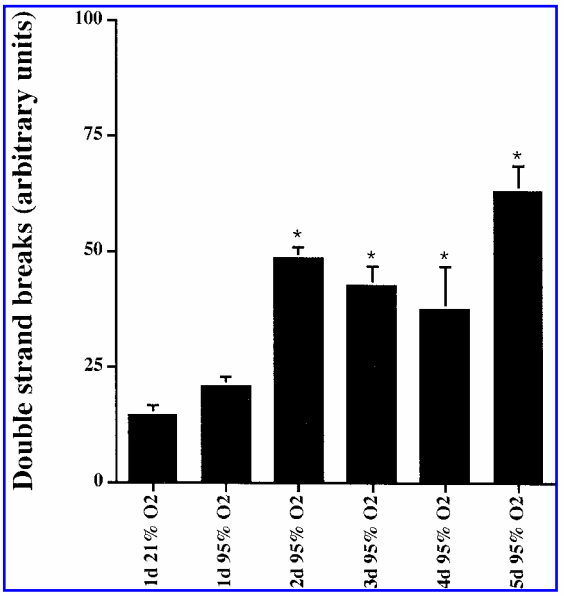

FIG. 3. Determination of DNA double strand breaks in hyperoxia-exposed A549 cells. Cells were exposed to either 1 day of 21% O_2 or 1, 2, 3, 4, and 5 days of 95% O_2. DNA damage was scored visually on a scale of 0–400 AU. *$p < 0.05$ versus 21% O_2 by one-way ANOVA with Tukey's test for multiple comparison; $n = 5$ per group.

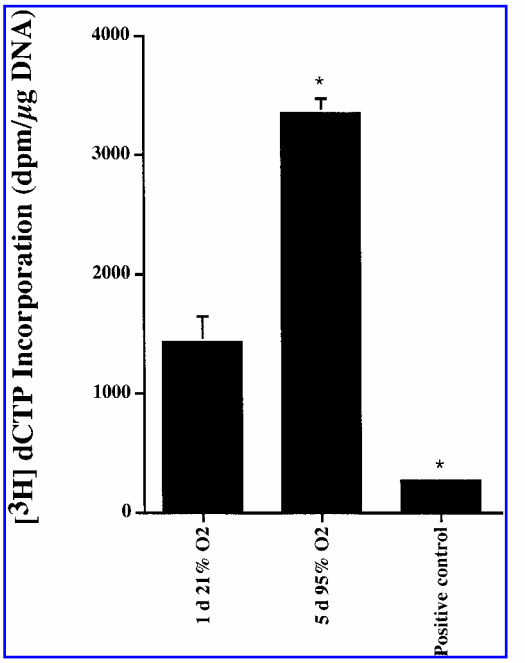

FIG. 5. Differences in DNA methylation between 21% O_2- and 95% O_2-exposed cells. Cells were exposed to 1 day of 21% O_2 and 5 days of 95% O_2. Positive control indicates 10 μg of genomic DNA from control cells methylated *in vitro* (see Materials and Methods). *$p < 0.05$ versus 21% O_2 by one-way ANOVA with Tukey's test for multiple comparison; $n = 5$ per group.

during exposure. Such levels were significantly reduced in control cells under high methylation conditions (266.0 ± 36.5 dpm) (Fig. 5; positive control). Statistically significant changes in DNA methylation status were not observed at earlier time points than day 5 of exposure (data not shown). Thus, statistically significant changes in 8-oxoguanine levels preceded those in global DNA methylation.

DISCUSSION

The purpose of this investigation was to investigate the nature and time course of DNA damage due to hyperoxia using assays for both oxidative and global DNA damage, and to assess for potentially associated changes in DNA methylation status, in a pulmonary epithelial cell line (A549). The SCGE (comet) assay was validated in a study in our lab using a wide range of ionizing radiation because the dose dependency of this agent in causing DNA damage is well established (16). Our results (Fig. 1B and C) confirm a dose-dependent increase of DNA single strand breaks with increasing doses. Such increase was quantified by using two computer-based scoring parameters: (a) percentage of DNA in tail and (b) tail length. Although both are among those parameters most commonly used (10), percentage of tail DNA is most useful because it is linearly related to breakage frequency over a wide range of damage (11). The alternative to computer-based scoring is visual scoring wherein nucleoids are classified by visual inspection into categories of increasing damage (Fig. 1A). Although subjective and semiquantitative in nature, it is a widely used method that in experienced hands has a strong linear relationship with the computer scoring (10).

For many years, hyperoxia has been considered to be a cause of DNA damage and mutagenesis (12). Recent studies have been able to link hyperoxic lung injury with DNA damage as indicated by increases in p53 activity (35). It remains unclear whether DNA damage contributes to the acute lung injury associated with exposure to hyperoxia. In addition, it is uncertain what type(s) of DNA lesion(s) are involved. A recent study has shown that when newborn rats were treated with 95% O_2 for 8 days, a significant neutrophil-mediated DNA damage was observed, as reflected by elevated levels of lung 8-oxoguanine. The authors concluded that such damage may contribute to the abnormal lung development seen in hyperoxia-induced oxidative stress (4). Under our "neutrophil-free" experimental conditions, we were able to detect DNA single strand breaks as early as 1 day after onset of hyperoxic exposure (Fig. 2), whereas increased double strand breaks were significantly elevated after 2 days of exposure (Fig. 3). The apparent lag in the levels of 8-oxoguanine in hyperoxia-exposed cells relative to single strand breaks (Fig. 4) may be due to an early elevation being obscured by a relatively high background innate to this assay. These *in vitro* observations demonstrate that hyperoxia has a direct genotoxic effect in addition to any indirect neutrophil-derived flux of reactive oxygen species. Lung epithelial-like A549 cells are known to be growth-arrested almost immediately after onset of hyperoxic exposure (24 h), initially at the S phase and later at the G_1 (40). Whether and how the presence of such DNA damage is interfering with the cell growth machinery is an evolving story. However, current evidence indicates that DNA damage due to hyperoxia can contribute to cellular injury (47), growth arrest (41), and cell death (36).

The presence of 8-oxoguanine as a biomarker of oxidative DNA damage (Fig. 4) could be of great importance due to its potential to interfere with DNA methylation. A recent study has demonstrated that when either of the guanines of the CCGG recognition site for initiation of enzymatic methylation is substituted with 8-hydroxyguanine, the binding of the DNA methyltransferase to the oligomer is altered, thereby preventing methylation at this site (45). Thus, DNA damage during hyperoxia may result in alterations to the DNA template leading to a decline in global methylation within the genome. This may lead to aberrant expression of genes that are ordinarily methylated and silenced. Alternatively, the observed hyperoxia-induced hypomethylation may be the result of the direct oxidation of 5-methylcytosine molecules on DNA by oxygen radicals. Such radicals can react with 5-methylcytosine, forming a 5-methylcytosine glycol, an intermediate product that deaminates to form thymine glycol. The thymine glycol moiety can pair with adenine resulting in a C- to T-transition. Such transitions are thought to contribute 40–60% of the mutations caused by reactive oxygen species (28). Substantial evidence indicates that mitochondrial reactive oxygen species production and damage occur very early in hyperoxia (1, 17, 20, 21, 43). Surprisingly, hyperoxia, even at sea level pressure, does not appear to cause damage to mitochondrial DNA (30). Because the mitochondrion is the predominant source of reactive species in hyperoxia (18), as well as the site of greatest damage (44), this finding suggests that DNA damage and cell injury may not have a cause–effect relationship. Further, the possibility is suggested that DNA damage might be a result of, or at least be potentiated by, growth arrest and/or altered bioenergetics.

Ongoing experiments in our laboratory are focused on the mechanisms by which hyperoxia can induce DNA damage and hypomethylation. This is potentially important because although *de novo* methylation of CpG dinucleotides requires cell division (44), such division does not take place in hyperoxia. Conversely, studies have shown that decreased DNA methylation after treatment with 5-aza-2-deoxycytidine slows the growth of human tumor cell lines (7). In addition, inhibition of DNA methyltransferase 1 induces transcription of the gene p21 (32), activates the p53 DNA damage response pathway (27), and triggers an S-phase arrest (33). Therefore, it may be that hyperoxia-induced growth arrest is the result of the direct inhibition of DNA methylation by inhibition of DNA methyltransferase 1. If this is true, the growth arrest observed may protect the genome against more extensive DNA demethylation. Furthermore, it will be of interest to determine DNA methylation status in other lung cell types during exposures to hyperoxia. Very current evidence indicates that the methylation status of DNA may be affected not only by methylation, but also by a dynamic process of demethylation (14). The latter may be affected by *S*-adenosylmethionine and *S*-adenosylhomocysteine, and our studies are directed at the metabolism of these compounds in oxidative stress.

To summarize, we have shown evidence of elevated levels of DNA single and double strand breaks in a lung-derived epithelial-like cell, suggesting direct genotoxicity by hyper-

oxia. This confirms the type of DNA lesions caused by hyperoxia that have been observed in other cell types (8, 9, 13, 22). In addition, increased levels of 8-oxoguanine appear independently from the presence of inflammation and could interfere with the ability of the cell to maintain existing methylation status. This could contribute, in turn, to altered gene expression patterns in hyperoxia. This is, to our knowledge, the first report to indicate that hyperoxia can alter DNA methylation.

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ABBREVIATIONS

ANOVA, analysis of variance; AU, arbitrary units; dCTP, deoxycytidine 5′-triphosphate; dpm, disintegrations per minute; Gy, Grey; H_2O_2, hydrogen peroxide; NE, nuclear extract; PBS, phosphate-buffered saline; SCGE, single-cell gel electrophoresis; TBE, Tris-borate-EDTA; TE, Tris-EDTA.

REFERENCES

1. Ahmad S, White CW, Chang LY, Schneider BK, and Allen CB. Glutamine protects mitochondrial structure and function in oxygen toxicity. *Am J Physiol Lung Cell Mol Physiol* 280: L779–L791, 2001.
2. Albertine K and Plopper C. DNA oxidation or apoptosis? Will the real culprit of DNA damage in hyperoxic lung injury please stand up? *Am J Respir Cell Mol Biol* 26: 381–383, 2002.
3. Allen CB, Schneider BK, and White CW. Limitations to oxygen diffusion and equilibration in in vitro cell exposure systems in hyperoxia and hypoxia. *Am J Physiol Cell Mol Physiol* 281: L1021–1027, 2001.
4. Auten RL, Whorton MH, and Mason SN. Blocking neutrophil influx reduces DNA damage in hyperoxia-exposed newborn rat lung. *Am J Respir Cell Mol Biol* 26: 391–397, 2002.
5. Ballestar E and Esteller M. The impact of chromatin in human cancer: linking DNA methylation to gene silencing. *Carcinogenesis* 23: 1103–1109, 2002.
6. Becker FF, Holton P, Ruchirawat M, and Lapeyre J-N. Perturbation of maintenance and de novo DNA methylation in vitro by UVB (280–340 nm)-induced pyrimidine photodimers. *Proc Natl Acad Sci U S A* 82: 6055–6059, 1985.
7. Bender CM, Pao MM, and Jones PA. Inhibition of DNA methylation by 5-aza-2-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res* 58: 95–101, 1998.
8. Cacciuttolo MA, Trinh L, Lumpkin JA, and Rao G. Hyperoxia induces DNA damage in mammalian cells. *Free Radic Biol Med* 14: 267–276, 1993.
9. Christie NA, Slutsky AS, Freeman BA, and Tanswell AK. A critical role for thiol, but not ATP, depletion in 95% O_2-mediated injury of preterm pneumocytes in vitro. *Arch Biochem Biophys* 313: 131–138, 1994.
10. Collins A, Dusinska M, Franklin M, Somorovska M, Petrovska H, Duthie S, Fillion L, Panayiotidis M, Raslova K, and Vaughan N. Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environ Mol Mutagen* 30: 139–146, 1997.
11. Collins AR, Dusinska M, Gedik GM, and Stetina R. Oxidative damage to DNA: do we have a reliable biomarker? *Environ Health Perspect* 104: 465–469, 1996.
12. Conger A and Fairchild L. Breakage of chromosomes by oxygen. *Proc Natl Acad Sci U S A* 38: 289–299, 1952.
13. Crapo JD, Barry BE, Foscue HA, and Shelburne J. Structural and biochemical changes in rat lungs occurring during exposures to lethal and adaptive doses of oxygen. *Am Rev Respir Dis* 122: 123–143, 1980.
14. Detich N, Hamm S, Just G, Knox DJ, and Szyf M. The methyl donor *S*-adenosylmethionine inhibits active demethylation of DNA: a candidate novel mechanism for the pharmacological effects of *S*-adenosylmethionine. *J Biol Chem* 278: 20812–20820, 2003.
15. Ehrlich M. DNA methylation in cancer: too much, but also too little. *Oncogene* 21: 5400–5413, 2002.
16. Epe B. DNA damage induced by photosensitizers and other photoreactive compounds. In: *DNA and Free Radicals: Techniques, Mechanisms and Applications,* edited by Aruoma OI and Halliwell B. Saint Lucia, London: OICA International, 1998, pp. 63–96.
17. Freeman BA and Crapo JD. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J Biol Chem* 256: 10986–10992, 1981.
18. Freeman BA and Crapo JD. Biology of disease: free radicals and tissue injury. *Lab Invest* 47: 412–426, 1982.
19. Garcea R, Daino L, Pascale R, Simile M, Puddu M, Ruggiu M, Seddaiu M, Satta G, Sequenza M, and Feo F. Protooncogene methylation and expression in regenerating liver and preneoplastic liver nodules induced in the rat by diethylnitrosamine: effect of variations of *S*-adenosylmethionine:*S*-adenosylhomocysteine ratio. *Carcinogenesis* 10: 1183–1192, 1989.
20. Gardner PR, Nguyen DD, and White CW. Aconitase is a sensitive and critical target of oxygen poisoning in cultured mammalian cells and in rat lungs. *Proc Natl Acad Sci U S A* 91: 12248–12252, 1994.
21. Gardner PR, Raineri I, Epstein LB, and White CW. Superoxide radical and iron regulate aconitase activity in mammalian cells. *J Biol Chem* 270: 13399–13405, 1995.
22. Gille JJ, Mullaart E, Vijg J, Leyva AL, Artwert F, and Joenje H. Chromosomal instability in an oxygen-tolerant variant of Chinese hamster ovary cells. *Mutat Res* 219: 17–28, 1989.
23. Helma C and Uhl M. A public domain image-analysis program for the single cell gel electrophoresis (comet) assay. *Mutat Res* 466: 9–16, 2000.
24. Herman JG. Hypermethylation of tumor suppressor genes in cancer. *Semin Cancer Biol* 9: 359–367, 1999.

25. Jeltsch A. Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. *Chembiochem* 3: 274–293, 2002.
26. Kalinich JF, Catravas GN, and Snyder SL. The effect of γ radiation on DNA methylation. *Radiat Res* 117: 185–197, 1989.
27. Karpf AR, Moore BC, Ririe TO, and Jones DA. Activation of the p53 DNA damage response pathway after inhibition of DNA methyltransferase by 5-aza-2-deoxycytidine. *Mol Pharmacol* 59: 751–757, 2001.
28. Lee D-H, O'Connor TR, and Pfeifer GP. Oxidative DNA damage induced by copper and hydrogen peroxide promotes CG to TT tandem mutations at methylated CpG dinucleotides in nucleotide excision repair-deficient cells. *Nucleic Acids Res* 30: 3566–3573, 2002.
29. Liang G, Salem C, Yu M, Nguyen H, Gonzales F, Nguyen T, Nichols P, and Jones P. DNA methylation differences associated with tumor tissues identified by genome scanning analysis. *Genomics* 53: 260–268, 1998.
30. Lightfoot RT, Khov S, and Ischiropoulos H. Transient injury to rat lung mitochondrial DNA after exposure to hyperoxia and inhaled nitric oxide. *Am J Physiol Lung Cell Mol Physiol* 286: L23–L29, 2003.
31. McKelvey-Martin VJ, Green MHL, Schmezer P, Pool-Zobel BL, De Meo MP, and Collins A. The single cell gel electrophoresis assay (comet assay): a European review. *Mutat Res* 288: 47–63, 1993.
32. Milutinovic S, Knox JD, and Szyf M. DNA methyltransferase inhibition induces the transcription of the tumor suppressor p21 (WAF1/CIP1/sdi1). *J Biol Chem* 275: 6353–6359, 2000.
33. Milutinovic S, Zhuang Q, Niveleau A, and Szyf M. Epigenomic stress response. Knockdown of DNA methyltransferase 1 triggers an intra-S-phase arrest of DNA replication and induction of stress response genes. *J Biol Chem* 278: 14985–14995, 2003.
34. O'Reilly M. DNA damage and cell cycle checkpoints in hyperoxic lung injury: braking to facilitate repair. *Am J Physiol Lung Cell Mol Physiol* 281: L291–L305, 2001.
35. O'Reilly MA, Staversky RJ, Watkins RJ, and Finkelstein JN. Exposure to oxygen induces p53 expression in mouse lung epithelium. *Am J Respir Cell Mol Biol* 18: 43–50, 1998.
36. O'Reilly MA, Staversky RJ, Finkelstein JN, and Keng PC. Activation of the G2 cell cycle checkpoint enhances survival of epithelial cells exposed to hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 284: L368–L375, 2003.
37. Panayiotidis M and Collins A. Ex vivo assessment of lymphocyte antioxidant status using the comet assay. *Free Radic Res* 27: 533–537, 1997.
38. Panayiotidis M, Tsolas O, and Galaris D. Glucose oxidase-produced H_2O_2 induces Ca^{2+}-dependent DNA damage in human peripheral blood lymphocytes. *Free Radic Biol Med* 26: 548–556, 1999.
39. Progribny I, Yi P, and James SJ. A sensitive new method for rapid detection of abnormal methylation patterns in global DNA and within CpG islands. *Biochem Biophys Res Commun* 262: 624–628, 1999.
40. Rancourt RC, Keng PC, Helt CE, and O'Reilly MA. The role of $p21^{CIP1/WAF1}$ in growth of epithelial cells exposed to hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 280: L617–L626, 2001.
41. Rancourt RC, Hayes DD, Chess PR, Keng PC, and O'Reilly MA. Growth arrest in G1 protects against oxygen-induced DNA damage and cell death. *J Cell Physiol* 193: 26–36, 2002.
42. Robertson KD and Jones PA. DNA methylation: past, present and future directions. *Carcinogenesis* 21: 461–467, 2000.
43. Turrens JF, Freeman BA, and Crapo JD. Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes. *Arch Biochem Biophys* 217: 411–421, 1982.
44. Velicescu M, Weisenberger DJ, Gonzales FA, Tsai YC, Nguyen CT, and Jones PA. Cell division is required for de novo methylation of CpG islands in bladder cancer cells. *Cancer Res* 62: 2378–2384, 2002.
45. Weitzman SA, Turk PW, Milkowski DH, and Kozlowski K. Free radical adducts induce alterations in DNA cytosine methylation. *Proc Natl Acad Sci U S A* 91: 1261–1264, 1994.
46. Wilson VL and Jones PA. Inhibition of DNA methylation by chemical carcinogens in vitro. *Cell* 32: 239–246, 1983.
47. Wu M, He YH, Kobune M, Xu Y, Kelley MR, and Martin WJ 2nd. Protection of human lung cells against hyperoxia using the DNA base excision repair genes hOgg1 and Fpg. *Am J Respir Crit Care Med* 166: 192–199, 2002.

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2. George WM Millington. 2008. Epigenetics and dermatological disease. *Pharmacogenomics* **9**:12, 1835-1850. [CrossRef]
3. Igor Koturbash, Kristy Kutanzi, Karl Hendrickson, Rocio Rodriguez-Juarez, Dmitry Kogosov, Olga Kovalchuk. 2008. Radiation-induced bystander effects in vivo are sex specific. *Mutation Research/ Fundamental and Molecular Mechanisms of Mutagenesis* **642**:1-2, 28-36. [CrossRef]
4. Malgorzata Romanowska, Anna Maciag, Andrew L. Smith, Janet R. Fields, Laura W. Fornwald, Keith D. Kikawa, Kazimierz S. Kasprzak, Lucy M. Anderson. 2007. DNA damage, superoxide, and mutant K-ras in human lung adenocarcinoma cells. *Free Radical Biology and Medicine* **43**:8, 1145-1155. [CrossRef]
5. I Koturbash, R E Rugo, C A Hendricks, J Loree, B Thibault, K Kutanzi, I Pogribny, J C Yanch, B P Engelward, O Kovalchuk. 2006. Irradiation induces DNA damage and modulates epigenetic effectors in distant bystander tissue in vivo. *Oncogene* **25**:31, 4267-4275. [CrossRef]
6. Mihalis I. Panayiotidis, Sally P. Stabler, Aftab Ahmad, Aglaia Pappa, Leighton H. Legros, Daniel Hernandez-Saavedra, B. Kelly Schneider, Robert H. Allen, Vasilis Vasiliou, Joe M. McCord, Malak Kotb, Carl W. White. 2006. Activation of a novel isoform of methionine adenosyl transferase 2A and increased S-adenosylmethionine turnover in lung epithelial cells exposed to hyperoxia. *Free Radical Biology and Medicine* **40**:2, 348-358. [CrossRef]
7. Igor Koturbash, Igor Pogribny, Olga Kovalchuk. 2005. Stable loss of global DNA methylation in the radiation-target tissue—A possible mechanism contributing to radiation carcinogenesis?. *Biochemical and Biophysical Research Communications* **337**:2, 526-533. [CrossRef]
8. Kumuda C. Das . 2004. Redox Control of Premature Birth and Newborn Biology. *Antioxidants & Redox Signaling* **6**:1, 105-107. [Citation] [Full Text PDF] [Full Text PDF with Links]