Hypoxia prevents etoposide-induced DNA damage in cancer cells through a mechanism involving hypoxia-inducible factor 1

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Abstract
Intratumoral hypoxia is associated with resistance to therapy in many human cancers, and preexposure of tumor cells to hypoxia confers multidrug resistance. Whereas most anticancer drugs kill proliferating tumor cells by causing DNA damage, a role for hypoxia in the prevention and/or repair of drug-induced DNA damage has not been clear. Using the alkaline comet assay, we provide direct evidence that hypoxia-induced resistance to etoposide in human tumor cells (MDA-MB-231 breast carcinoma and DU-145 prostatic adenocarcinoma) is mainly due to prevention of drug-induced DNA damage (i.e., strand breaks) and that the amount of DNA damage present immediately after etoposide exposure is a good independent predictor of clonogenic survival. Our results also revealed that preexposure to hypoxia did not affect the apparent DNA repair capacity of cells. These findings indicate that the extent of DNA damage resulting from etoposide exposure is a more important determinant of survival than subsequent events after DNA damage. Furthermore, immunofluorescence analysis showed that, in a subpopulation of cells, preexposure to hypoxia decreased the levels of topoisomerase IIα, an enzyme that generates DNA strand breaks when poisoned with etoposide. Treatment of cells with small interfering RNA targeting hypoxia-inducible factor 1 prevented the hypoxia-induced decreases in topoisomerase IIα levels, abolished the protective effect of hypoxia against etoposide-induced DNA damage, and inhibited hypoxia-induced etoposide resistance. These findings support a model of hypoxia-induced drug resistance in which etoposide-induced DNA damage is prevented by HIF-1α-dependent adaptations to hypoxia. [Mol Cancer Ther 2009;8(6):1702–13]

Introduction
Most solid tumors contain regions of hypoxia (characterized by oxygen tensions below normal physiologic levels) as a result of an inadequate and irregular blood supply. Consequently, adaptations to hypoxia are necessary for the survival of tumor cells in such microenvironments. Many of the adaptations induced by hypoxia are associated with malignant progression and include an increase in tumor cell invasiveness and metastatic potential, as well as the development of resistance to chemotherapy (1–3). Accordingly, studies have shown that the presence of intratumoral hypoxia is associated with a poor clinical outcome for patients with various types of cancers (4–9).

Hypoxia-induced chemoresistance is an aspect of tumor biology that has received increased attention over the last decade, particularly after the characterization of hypoxia-inducible factor (HIF), a key transcriptional regulator of cellular adaptations to hypoxia (10, 11). Many studies have now shown that HIF activity is necessary for the development of hypoxia-induced drug resistance; however, the precise HIF-dependent mechanisms involved are not well understood (12–16). It has been shown that hypoxia increases the expression of antiapoptotic proteins (13, 17–20) and/or decreases the expression of proapoptotic proteins (12, 13) in various cell lines via both HIF-dependent and HIF-independent mechanisms. It has been proposed that increased cell survival, due to a shift favoring antiapoptotic pathways, is a primary mechanism of hypoxia-induced drug resistance (12, 13). However, alternative mechanisms of cell death can be triggered in the absence of apoptosis (21, 22). There is also experimental evidence that inhibition of apoptosis is not always sufficient to confer resistance to chemotherapeutic agents due to the compensatory activation of cell death mechanisms, such as senescence and mitotic catastrophe (23, 24). Similarly, our laboratory recently reported novel findings that hypoxia-induced chemoresistance in human tumor cells can occur independently of changes in apoptotic potential as a result of escape from drug-induced senescence (16). These findings highlight the importance of nonapoptotic mechanisms in the hypoxia-induced adaptations leading to resistant tumor cell populations.

Successful chemotherapy requires anticancer agents to effectively damage tumor cells beyond a threshold that triggers cell death. Many widely used chemotherapeutic agents cause damage by targeting DNA and/or critical enzymes that regulate DNA topology during replication, transcription, and cell division. Consequently, cell populations that
acquire the ability to attenuate and/or repair drug-induced DNA damage would be expected to tolerate treatment with such anticancer agents. However, despite the importance of DNA damage in chemotherapy, little is known about the effect of hypoxia on the extent of DNA damage caused by anticancer agents or on the ability of tumor cells to repair DNA. Furthermore, the relationship between drug-mediated DNA damage and the development of hypoxia-mediated chemoresistance has not been well characterized. To address these questions, we used the alkaline comet assay to characterize drug-induced DNA damage in human breast and prostate tumor cells preexposed to hypoxia.

Materials and Methods

Cell Culture and Exposure to Hypoxia
Human MDA-MB-231 breast carcinoma cells and DU-145 prostatic carcinoma cells, obtained from the American Type Culture Collection, were maintained in standard culture conditions (5% CO2 in air at 37°C) in RPMI 1640 supplemented with 5% fetal bovine serum (FBS; Invitrogen). To establish hypoxic conditions, cells were placed in airtight plastic chambers flushed with a 5% CO2/95% N2 gas mixture. Oxygen (O2) concentrations within these chambers were maintained at 0.2% using Pro-Ox Model 110 O2 regulators (Biospherix). For specific experiments that required treatment of cells while in hypoxia, cells were incubated in a hypoxic glove box chamber (Coy Laboratory Products, Inc.) maintained at 0.2% O2. Gloved access to the chamber permits manipulation of cells without reoxygenation.

Alkaline Single-Cell Gel Electrophoresis (Comet) Assays
Detailed methods for the alkaline comet assay can be found in the supplementary data. Briefly, cells were incubated for 24 h in standard (20% O2) or hypoxic (0.2% O2) conditions before the culture medium was replaced with either complete medium (for untreated controls) or complete medium containing etoposide (50 μmol/L; BD Pharmingen) for the final hour or final 24 h of culture. DNA damage was measured by the percentage of DNA in tail, and results are presented as frequency distributions of binned data with bin widths of 5% DNA.

Modified comet assays were done to assess the effect of hypoxia on DNA damage in various phases of the cell cycle (see supplementary data for details). Briefly, cells were incubated with bromodeoxyuridine (BrdUrd, 20 μmol/L; BD Pharmingen) for the final hour or final 24 h of a 24-h incubation to allow identification of cells in S or G2 phase, respectively. Cells were washed once with PBS, exposed to etoposide, and processed for comet assays. After the neutralization step, slides were incubated with FITC-conjugated anti-BrdUrd antibody (1:2.5 in PBS/0.5% Tween 20, overnight, 4°C; BD Pharmingen). Slides were then washed in PBS/Tween 20, air-dried overnight, stained with propidium iodide (5 μg/mL in PBS/Tween 20; 10 min), and washed.

Comets were visualized as described earlier, and a comparison of FITC-associated fluorescence with propidium iodide-associated fluorescence was used to identify comets containing BrdUrd-labeled DNA, allowing separate analysis of BrdUrd-positive and BrdUrd-negative populations.

Clonogenic (Colony Formation) Assays
Methods for the clonogenic assay have been described previously (16, 25, 26). Briefly, cells were cultured and treated with etoposide, as described for the comet assay. Cells were then replated in six-well dishes (100–250 per well (nontreated controls) or 1,000–2,500 per well (drug-treated groups)) and cultured in standard conditions for 7 to 14 d. Cells were washed briefly with PBS, fixed with Carnoy’s fixative (1:3 acetic acid/methanol), stained with a dilute crystal violet solution (0.33% w/v), and colonies consisting of 50 or more cells were counted. Studies done using the glove box hypoxic chamber revealed that reoxygenation of hypoxic cells was not required for the induction of drug resistance and that preexposure to hypoxia was the primary determinant of increased resistance (not shown).

Small Interfering RNA Transfection
Knockdown of HIF-1α expression was achieved using Silencer validated small interfering RNA (siRNA) targeted against the human HIF-1α gene (Ambion, Inc.; siRNA ID 42840). Silencer Negative Control siRNA (Ambion) was used to control for nonspecific effects. siRNA (25 nmol/L) was introduced into cells by reverse transfection using siPORT NeoFx reagent (Ambion) for 48 h under standard culture conditions before incubation in hypoxia. Pilot studies performed using Silencer FAM labeled glyceraldehyde-3-phosphate dehydrogenase siRNA (Ambion) under similar conditions demonstrated uptake of siRNA in ~75% to 85% of cells (not shown). Knockdown of HIF-1α expression was confirmed by Western blot analysis, as described previously (16, 27).

Topoisomerase II Immunofluorescence
Analysis of topoisomerase II (topo II) protein levels in situ was determined by immunofluorescence. Cells cultured on glass coverslips were incubated for 24 h in standard or hypoxic conditions, fixed [3.7% formaldehyde onto a Moticam 2000 MCCamera (Motic Instruments, Inc.). Comet analysis was done using CometScore software version 1.5 (Tritek Corporation), scoring ~200 to 350 comets per condition. DNA damage was measured by the percentage of DNA in tail, and results are presented as frequency distributions of binned data with bin widths of 5% DNA.

1 Supplementary material for this article is available at Molecular Cancer Therapeutics Online [http://mct.aacrjournals.org/].
in PBS, 10 min, room temperature (RT), and permeabilized (0.1% Triton X-100, 10 min, RT). Nonspecific binding sites were blocked (3% FBS in PBS, 1 h, RT), and cells were incubated with either topo IIα or topo IIβ antibodies (1:50 in 3% FBS in PBS; BD Biosciences) or negative control mouse IgG2a (DakoCytomation) for 1 h at RT. Coverslips were washed in PBS, incubated with Alexa Fluor 488–conjugated goat anti-mouse antibody (1:500 in 3% FBS in PBS; Invitrogen Molecular Probes, 1 h, RT). Coverslips were washed, incubated with TRITC-conjugated phalloidin (1:200 in 3% FBS in PBS; Sigma-Aldrich Canada Ltd., 1 h, RT), washed again, and mounted on microscope slides using fluorescence mounting medium (DakoCytomation). Cells were viewed by fluorescence microscopy, and quantification of nuclear fluorescence intensity was performed using CometScore software version 1.5 (Tritek Corporation). Selective quantification of the nuclear fluorescence intensity was performed for ~400 to 650 individual cells. After correcting for background fluorescence (based on quantification of cells probed with mouse IgG2a antibodies), results are presented as the frequency distribution of binned fluorescence intensities. To quantify changes in topo II–associated immunofluorescence, individual nuclear fluorescence intensities were arranged in ascending order and the mean intensity of each quartile was calculated for each group.

**Calculations and Statistical Analysis**

For clonogenic assays, plating efficiency was calculated as the number of surviving colonies expressed as a proportion of the total number of cells inoculated. Surviving fractions were determined by dividing the plating efficiency of
drug-treated groups by the plating efficiency of the corresponding untreated control group. Data are reported as the mean surviving fraction from replicates of six and SD (±SD).

To investigate the association between etoposide-induced DNA damage (measured by the comet assay) and survival (measured by clonogenic assays), a threshold for DNA damage associated with cell death was determined using previously reported methods (28, 29). Briefly, each comet assay performed was accompanied by a determination of clonogenic survival for the same population of cells. Individual comets were arranged in ascending order of the percentage of DNA in tail. Based on the rationale that the surviving fraction is most likely represented in the comet assay by the least damaged cells (28, 29), the threshold was defined as the lowest percentage of DNA in tail value necessary to include a fraction of comets corresponding to the surviving fraction. Scatter plots were then prepared for the entire data set, plotting the fraction of cells below the established threshold against the surviving fraction measured for the corresponding population of cells. Linear regression analysis was performed to determine the slope of the best-fit line for each data set, and Spearman and Pearson correlation coefficients were determined.

All statistical analyses were done using GraphPad Prism software version 4.0 (GraphPad Software, Inc.). When two groups were compared, significance was determined by an unpaired, two-tailed t test. When more than two groups were compared, significance was determined by one-way ANOVA followed by a Newman-Keuls multiple comparison test. All tests were two-sided, and differences were considered to be statistically significant at $P < 0.05$.

Results

Preexposure of Human Tumor Cells to Hypoxia Decreases Etoposide-Induced DNA Damage and Increases Clonogenic Survival after Drug Exposure

Chemotherapeutic agent-induced DNA damage (specifically single-strand and double-strand DNA breaks, as well as alkali labile sites) was measured by the alkaline comet assay. This technique is a powerful tool for analyzing DNA strand breaks in individual cells and has been shown, for certain chemotherapeutic agents, to be an effective method of identifying drug-resistant cells in heterogeneous populations (28, 29). Comet assays performed on human MDA-MB-231 breast carcinoma cells revealed that preexposure to hypoxia (and subsequent reoxygenation for 1 h) did not affect basal levels of DNA strand breaks relative to cells maintained in standard conditions, with 90% to 95% of each population displaying <5% DNA in comet tails (Fig. 1A, Untreated). In cells maintained in 20% O$_2$ treatment with etoposide for 1 hour caused numerous strand breaks, with the majority of comets displaying >80% DNA in tail. However, the extent of damage was heterogeneous and small populations of cells displayed low to moderate amounts of damage, with a few cells resembling untreated controls (Fig. 1A, Etoposide, top). In cells preexposed to hypoxia, etoposide treatment also caused considerable damage; however, compared with cells maintained in 20% O$_2$, there was an increase in the fraction of cells with lower levels of damage (Fig. 1A, Etoposide, bottom). Consistent with the measured decrease in DNA damage for the same population of cells, clonogenic assays revealed a 4.4-fold increase in survival for cells preexposed to hypoxia, relative to cells maintained in standard conditions ($P < 0.0001$; $n = 14$; Fig. 1B; surviving fractions shown, 20% O$_2$ = 0.028 and 0.2% O$_2$ = 0.125).

Hypoxia had a similar effect on etoposide-induced DNA damage in other tumor cell lines. In studies performed using human DU-145 prostatic adenocarcinoma cells, the majority of cells maintained in 20% O$_2$ displayed extensive DNA strand breaks after etoposide treatment, whereas preexposure to hypoxia decreased the extent of damage suffered (Fig. 1C) and lead to a 4.7-fold increase in clonogenic survival ($P < 0.0001$; $n = 9$; Fig. 1D; surviving fractions shown, 20% O$_2$ = 0.004 and 0.2% O$_2$ = 0.018). Similarly, human HCT-116 colon carcinoma cells preexposed to hypoxia were characterized by diminished etoposide-induced DNA damage and increased survival, relative to cells maintained in 20% O$_2$ (not shown).

Based on the assumption that cells suffering minimal DNA damage are most likely to survive drug treatment, surviving fractions measured by clonogenic assays were used to identify a “threshold” of DNA damage below which cells could be predicted to survive (28, 29). For example, if 12% of drug-treated cells survived, the threshold was identified as the percentage of DNA in tail value below which the 12% least damaged cells were found in the corresponding comet assay histogram. Using this approach, a threshold of damage at ≤24% DNA in tail was identified for MDA-MB-231 cells. Scatter plots were prepared to compare the “predicted” survival (i.e., the fraction of cells with comets below the threshold) versus the actual surviving fraction for the corresponding population of cells. Overall, there was a good correlation between the clonogenic survival of etoposide-treated MDA-MB-231 cells and the proportion of cells with DNA damage of ≤24% DNA in tail (measured immediately after a 1-hour exposure to etoposide; Spearman correlation coefficient = 0.7357, $P < 0.0001$; Pearson correlation coefficient = 0.7005, $P < 0.0001$; Fig. 2A). Preexposure of MDA-MB-231 cells to hypoxia resulted in a significantly larger fraction of cells with DNA damage below this threshold, relative to cells maintained in 20% O$_2$ ($P = 0.0003$; Fig. 2B). Pooled data from 14 independent experiments revealed that $12.7 \pm 1.4\%$ (mean ± SE) of cells preexposed to hypoxia were below the threshold, compared with $5.8 \pm 0.8\%$ of cells maintained in standard conditions.

For DU-145 cells, the percentage of cells displaying ≤2% DNA in tail after exposure to etoposide correlated well with measured clonogenic survival (Spearman correlation coefficient = 0.8016, $P = 0.0001$; Pearson correlation coefficient = 0.8440, $P < 0.0001$; Fig. 2C). Similar to MDA-MB-231 cells, preexposure of DU-145 cells to hypoxia significantly increased the percentage of cells below the threshold, relative to cells maintained in 20% O$_2$.
After nine independent experiments, 7.4 ± 0.9% of cells preexposed to hypoxia were below the threshold compared with 2.5 ± 0.5% of cells maintained in standard conditions.

To determine whether these findings could be extended to other chemotherapeutics, comet assays and clonogenic assays were performed in MDA-MB-231 and DU-145 cells treated for 1 hour with doxorubicin or mitoxantrone. Overall, in MDA-MB-231 or DU-145 cells cultured in 20% O₂ and subsequently treated with doxorubicin or mitoxantrone, there was a poor correlation between drug-induced DNA strand breaks and measured clonogenic survival (not shown).

Preexposure of Human Tumor Cells to Hypoxia Is Associated with a “Protective” Effect against Etoposide-Induced DNA Damage, Rather than an Increase in DNA Repair

To determine whether preexposure to hypoxia influenced DNA repair capacity, comet assays were performed at multiple intervals using MDA-MB-231 or DU-145 cells cultured in standard conditions after etoposide treatment. The progressive disappearance of DNA strand breaks over time (up to 90 min) was monitored as an indication of DNA repair (30) and quantified by determining the mean percentage of DNA in tail for the most heavily damaged cells (constituting 20% of the total population) at each time point.

**Figure 2.** The extent of DNA damage present immediately after a 1-h exposure to etoposide correlates with the clonogenic survival of drug-treated MDA-MB-231 and DU-145 tumor cells. Alkaline comet assays and clonogenic survival assays were performed on MDA-MB-231 (A, B) and DU-145 (C, D) cells treated with etoposide (50 μmol/L MDA-MB-231) or 125 μmol/L (DU-145) for 1 h in 20% O₂ to assess DNA damage (immediately after drug treatment) and survival (10–14 d later). A and C, the percentages of cells with DNA damage below determined thresholds (≤24% DNA in tail for MDA-MB-231 cells and ≤2% DNA in tail for DU-145 cells) measured in the comet assay were used to predict survival measured by clonogenic assays (data from cells preexposed to 20% O₂ and 0.2% O₂ are shown on the same graph). The best-fit lines after linear regression analysis are indicated and summarized in the attached tables. In both cell lines, the proportion of cells with DNA damage below the corresponding threshold was a good independent predictor of the surviving fraction for the same population of treated cells, independent of the culture conditions before etoposide exposure. B and D, the percentage of cells with etoposide-induced DNA damage below the appropriate threshold were determined for MDA-MB-231 and DU-145 cells preincubated in 20% O₂ or 0.2% O₂ for 24 h and subsequently treated with etoposide (as described for A and C). Compared with cells maintained in standard conditions, preexposure to hypoxia resulted in a significant increase in the fraction of cells below the damage thresholds; *, P < 0.001. Data are presented as the mean percentage of cells below the thresholds (±SE), calculated from 14 (MDA-MB-231) or 9 (DU-145) independent experiments.
Figure 3. The protective effect of hypoxia against etoposide-induced DNA damage is only observed in cells that are not actively synthesizing DNA at the time of drug exposure. MDA-MB-231 (A) and DU-145 (B) cells were cultured in 20% O2 or 0.2% O2 for 24 h and incubated in the presence of BrdUrd during the final hour. Cells were subsequently treated with etoposide [50 μmol/L (MDA-MB-231) or 125 μmol/L (DU-145) for 1 h in 20% O2], and DNA damage and BrdUrd incorporation were assessed by a modified alkaline comet assay. Select analysis of only the cells undergoing active DNA synthesis (BrdUrd-positive cells; top) revealed extensive damage in this population in both cell lines, with nearly every comet containing >60% DNA in tail. Hypoxia-induced differences in the extent of DNA damage were not observed. In contrast, the population of cells not undergoing DNA synthesis (BrdUrd-negative cells; bottom) displayed a full spectrum of damage, with many comets containing moderate to low levels of DNA strand breaks. A protective effect of hypoxia was observed within this population, as evident by an increase in the percentage of cells with ≤45% DNA in tail. Differences in the extent of DNA damage in untreated controls were not observed when BrdUrd-positive and BrdUrd-negative populations were compared. Results are representative of 3 (MDA-MB-231) or 2 (DU-145) independent experiments (see Supplementary Table S11 for a summary of the relative proportions of BrdUrd-negative cells after culture in standard or hypoxic conditions).
point. Differences between cells preexposed to hypoxia and those maintained in standard conditions were not observed in either cell line over the 90-min period examined (Supplementary Fig. S1).

Hypoxia-Induced Changes in Cell Cycle Progression Do not Account for the Protective Effect of Hypoxia Against Etoposide-Induced DNA Damage

The characteristically higher rate of proliferation in tumor cells versus normal nonmalignant populations is often the basis for the “selectivity” of chemotherapeutics targeting cellular components associated with DNA synthesis or cell division (31). To investigate the extent of etoposide-induced DNA damage within various phases of the cell cycle (specifically S-phase versus all other stages), MDA-MB-231 and DU-145 cells were cultured for 24 hours in 20% O2 or 0.2% O2 and labeled with BrdUrd during the final hour. After etoposide treatment, nearly all BrdUrd-positive (i.e., S phase) MDA-MB-231 cells were heavily damaged with comets containing ≥60% DNA in tail (Fig. 3A; n = 3). In contrast, the extent of DNA damage was heterogeneous in the BrdUrd-negative population (i.e., cells in G0, G1, or G2-M), including a small proportion of cells with minimal DNA damage. Pre-exposure to hypoxia increased the proportion of BrdUrd-negative cells with low levels of etoposide-induced DNA breaks but had no effect on the BrdUrd-positive population. Similar results were observed in experiments performed with DU-145 cells (Fig. 3B; n = 2).

Etoposide-resistant cells were found exclusively in the BrdUrd-negative population, raising the possibility that hypoxia-induced resistance was associated with prevention of cell entry into the S-phase. However, in three independent experiments performed with MDA-MB-231 cells there was a trend toward a lower proportion of BrdUrd-negative cells after hypoxia, as 44.5% of cells preexposed to hypoxia were BrdUrd-negative compared with 54.7% of cells maintained in standard conditions (values reported represent the mean of three experiments; see Supplementary Table S1 for individual results). This effect was reproducible, although significant differences were not observed in every experiment. In contrast, the reverse trend was observed in DU-145 cells. In two experiments, 57.5% of the population maintained in standard conditions were BrdUrd-negative, which increased to 64.1% after preexposure to hypoxia (see Supplementary Table S1).

Three cell cycle phases potentially comprise the BrdUrd-negative population under these experimental conditions: G0, G1, and G2-M. Previously, quiescent or noncycling G0 cells have been characterized as being etoposide resistant (32–35), prompting an investigation of the effects of hypoxia on the proportion of noncycling cells. To identify the G0 population, MDA-MB-231 and DU-145 cells were cultured...

Figure 4. The protective effect of hypoxia against etoposide-induced DNA damage is observed in MDA-MB-231 and DU-145 cells that are actively cycling before drug exposure. MDA-MB-231 and DU-145 cells were cultured in 20% O2 or 0.2% O2 for 24 h and incubated in the presence of etoposide during the final 21 h of this incubation. Cells were subsequently treated with etoposide [50 μmol/L (MDA-MB-231) or 125 μmol/L (DU-145), 1 h in 20% O2], and DNA damage and BrdUrd incorporation were assessed by a modified alkaline comet assay. BrdUrd-positive (i.e., cycling) cells displayed a full spectrum of damage, with many comets exhibiting moderate to low levels of DNA strand breaks. In addition, the protective effect of hypoxia was observed within this population, as evident by an increase in the percentage of cells with ≤45% DNA in tail. Results shown are representative of two independent experiments (see Supplementary Table S2 for a summary of the relative proportions of noncycling (BrdUrd negative) cells after culture in standard or hypoxic conditions).
for 24 hours in 20% O₂ or 0.2% O₂ and labeled with BrdUrd during the final 21 hours. Previous studies revealed that a 3-hour incubation in hypoxia is sufficient for cells to become hypoxic before the addition of BrdUrd, whereas 21 hours allows cycling MDA-MB-231 and DU-145 cells to pass through S-phase and incorporate BrdUrd into DNA (not shown). Quantitative analysis revealed that only a small proportion of MDA-MB-231 and DU-145 cells were BrdUrd negative (i.e., noncycling) under standard conditions (1.6% and 3.2% of the total population, respectively; results from two independent experiments are shown in Supplementary Table S2). Exposure of MDA-MB-231 cells to hypoxia increased the noncycling fraction by ∼2-fold (3.1% of the total population). The effect of hypoxia on DU-145 cells was more pronounced, increasing the noncycling population to 10.2% of the total population, representing a 3.2-fold increase (P < 0.005; n = 2). To control for the potential contribution of the G₀ cells to the resistant population, DNA damage was assessed in the cycling (i.e., BrdUrd-positive) population separately. Even after controlling for the increase in noncycling cells, the protective effect of hypoxia against etoposide-induced DNA strand breaks was evident in cycling MDA-MB-231 and DU-145 cells (Fig. 4; n = 2).

**Knockdown of HIF-1α Expression Restores DNA Damage and Etoposide Sensitivity of Cells Preexposed to Hypoxia**

Our previous studies revealed that treatment of MDA-MB-231 cells with siRNA targeting HIF-1α is sufficient to prevent hypoxia-induced resistance to doxorubicin (16). To determine whether the effects of hypoxia on etoposide-induced DNA damage were also dependent on a functional HIF-1 response, MDA-MB-231 cells were transfected with HIF-1α siRNA and evaluated by comet and clonogenic assays. After transfection, successful knockdown of HIF-1α expression was confirmed by Western blot analysis (Supplementary Fig. S2). In the absence of etoposide treatment, basal levels of DNA strand breaks were comparable in untransfected cells and cells transfected with negative control or HIF-1α siRNA and were not affected by preexposure to hypoxia. As observed earlier, preexposure of untransfected MDA-MB-231 cells to hypoxia resulted in a ∼2.4-fold increase in the percentage of cells with ≤24% DNA in tail following pre-exposure to hypoxia (0.2% O₂, 24 h), relative to cells maintained under standard (20% O₂) conditions (see text for details). Similarly, hypoxia increased the percentage of negative control siRNA transfected cells below the defined DNA damage threshold by ∼2.7-fold (P = 0.0315; n = 4). In contrast, the protective effect of hypoxia on etoposide-induced DNA damage was abolished in MDA-MB-231 cells transfected with HIF-1α siRNA. Interestingly, loss of HIF-1α resulted in a modest but reproducible increase (∼1.6-fold) in the basal percentage of cells with minimal drug-induced DNA damage.

Clonogenic assays performed using the same populations of cells analyzed by the comet assay revealed comparable plating efficiencies (i.e., clonogenic survival of untreated cells) for untransfected and siRNA-transfected MDA-MB-231 cells (not shown). Clonogenic survival after exposure to etoposide was in good agreement with the effect of hypoxia on etoposide-induced DNA damage (Table 1). After preexposure to hypoxia, untransfected and negative control siRNA-transfected cells showed ∼3.7-fold and 4-fold higher survival after etoposide treatment, respectively, compared with similarly treated cells maintained in 20% O₂ (P = 0.0264 and P = 0.0041, respectively; n = 4). Hypoxia did not increase the survival of cells treated with HIF-1α siRNA, consistent with the absence of a hypoxia-induced difference in etoposide-induced DNA damage in these cells. Furthermore, after knockdown of HIF-1α expression, the basal increase in cells with minimal drug-induced DNA damage revealed by the comet assay was accompanied by a similar increase in basal etoposide resistance.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of cells with ≤24% DNA in tail*</th>
<th>Fold increase by hypoxia† (P-value‡ shown in brackets)</th>
<th>Surviving fraction§ (Mean ± SE)</th>
<th>Fold increase by hypoxia¶ (P-value∥ shown in brackets)</th>
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<tr>
<td></td>
<td>(20% O₂, Mean ± SE)</td>
<td>(0.2% O₂, Mean ± SE)</td>
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<td>Untransfected</td>
<td>6.90 ± 2.08</td>
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<td>3.74 (0.026)</td>
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<tr>
<td>Negative control</td>
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<td>2.65 (0.032)</td>
<td>0.024 ± 0.005</td>
<td>3.99 (0.004)</td>
</tr>
<tr>
<td>HIF-1α siRNA</td>
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<td>1.04 (0.899)</td>
<td>0.077 ± 0.16</td>
<td>0.81 (0.456)</td>
</tr>
</tbody>
</table>

*Values reported represent the mean percentage of etoposide-treated cells with ≤24% DNA in tail (±SE) of 4 independent comet assays (see text for details regarding siRNA treatment and comet assay conditions).
†The fold increase by hypoxia was determined as the mean fold increase in the percentage of etoposide-treated cells with ≤24% DNA in tail following pre-exposure to hypoxia (0.2% O₂, 24 h), relative to cells maintained under standard (20% O₂) conditions (see text for details).
‡Statistical significance was determined by unpaired t-tests comparing the percentage of cells with ≤24% DNA in tail following pre-exposure to hypoxia versus cells maintained in 20% O₂.
§Values reported represent the mean surviving fraction (±SE) of 4 independent experiments (see text for details regarding siRNA treatment and clonogenic survival assay conditions).
∥The fold increase by hypoxia was determined as the mean fold increase in the clonogenic survival of etoposide-treated cells pre-exposed to hypoxia (0.2% O₂, 24 h), relative to cells maintained under standard (20% O₂) conditions (see text for details).
¶Values reported represent the mean fold increase by hypoxia of etoposide-treated cells pre-exposed to hypoxia (0.2% O₂, 24 h), relative to cells maintained under standard (20% O₂) conditions (see text for details).

Table 1. Treatment of human MDA-MB-231 breast carcinoma cells with HIF-1α siRNA prevents the protective effect of hypoxia against etoposide-induced DNA damage and abolishes hypoxia-induced clonogenic survival

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Hypoxia-Induced Decreases in Nuclear Topo IIα Are Associated with Resistance to Etoposide and Are Prevented by Pretreatment of Cells with HIF-1α siRNA

DNA strand breaks caused by etoposide are thought to occur as a result of poisoning of topo II proteins and stabilization of the cleavable complex formed between DNA and topo II. Accordingly, tumor cells with diminished expression of topo II proteins exhibit resistance to etoposide (36, 37). To investigate possible effects of hypoxia on the regulation of topo II expression, immunofluorescence analysis of topo II protein levels in individual cells was performed after 24 hours of culture in standard or hypoxic conditions. Analysis of MDA-MB-231 cells revealed topo IIα and topo IIβ were localized primarily to the nucleus, with little or no topo II protein

Figure 5. Exposure of MDA-MB-231 tumor cells to hypoxia is associated with an increase in the proportion of cells with low levels of topo IIα protein in the nucleus. A, nuclear topo IIα immunofluorescence analysis of MDA-MB-231 cells after 24 h of culture in standard (20% O2) or hypoxic (0.2% O2) conditions. Data are presented as relative frequency distributions of fluorescence intensity (expressed in arbitrary units and arranged in bins with a width of 5 units), after quantification of 400 to 650 individual cells per condition. The mean fluorescence intensity of cells stained with negative control mouse IgG2a antibodies was used to correct for background fluorescence. Relative to cells cultured in 20% O2, exposure of MDA-MB-231 cells to hypoxia resulted in an increase in the percentage of cells with lower levels of topo IIα, as revealed by a lower mean fluorescence intensity for the first quartile of cells cultured in 0.2% O2 (values reported in the table correspond to the data shown in the graphs and are representative of five independent experiments). B, MDA-MB-231 cells were either transfected with negative control siRNA or HIF-1α siRNA or left untransfected. Forty-eight hours later cells were incubated under standard (20% O2) or hypoxic (0.2% O2) conditions for an additional 24 h before analysis of nuclear topo IIα immunofluorescence. Data are presented as the mean fluorescence intensity (±SE) of the first quartile, pooled from three to five independent experiments (for siRNA-transfected and untransfected cells, respectively). Exposure of untransfected or negative control siRNA-transfected MDA-MB-231 cells to hypoxia resulted in a larger population of cells with low levels of topo IIα, relative to similarly treated cells maintained in 20% O2 (as revealed by a significantly lower mean fluorescence intensity). A similar effect was not observed in cells treated with HIF-1α siRNA. Statistically significant differences in mean fluorescence intensities were determined by one-way ANOVA, followed by a Newman-Keuls multiple comparisons test and are indicated * P < 0.05 relative to similarly treated cells in standard conditions. C, nuclear topo IIβ immunofluorescence analysis in MDA-MB-231 cells after culture, as described in A (frequency distribution bin width, 2.5 units). D, nuclear topo IIβ immunofluorescence analysis of siRNA-transfected MDA-MB-231 cells, as described in B. In contrast to the changes observed in topo IIα, hypoxia did not affect the nuclear levels of topo IIβ, nor did transfection of cells with HIF-1α siRNA. Representative images used for immunofluorescence analysis of MDA-MB-231 cells are shown in Supplementary Fig. S3 (see also Supplementary Figs. S4 and S5 for images and analysis of DU-145 cells from similar experiments).
detected in the cytoplasm (Supplementary Fig. S3). Exposure of cells to hypoxia did not have an obvious effect on the overall subcellular localization of topo II proteins.

Quantitative image analysis revealed that culture of MDA-MB-231 cells in hypoxia for 24 hours led to a decrease in nuclear topo IIα levels in a population of cells. A comparison of the mean fluorescence intensities for the first quartiles of each population distribution revealed a lower mean intensity in cells cultured in 0.2% O2 relative to cells maintained in standard conditions (P < 0.05, n = 5; Fig. 5A and B). Hypoxia had a comparable effect on nuclear topo IIα levels in cells treated with negative control siRNA (P < 0.05, n = 3; Fig. 5B). siRNA-mediated knockdown of HIF-1α expression abolished the hypoxia-induced decrease in nuclear topo IIα levels and resulted in decreased basal topo IIα levels. Under hypoxic conditions, nuclear topo IIα levels in HIF-1α siRNA-treated cells were comparable with the levels observed in untransfected or negative control siRNA-transfected cells maintained in standard conditions (Fig. 5B; n = 3). The effect of hypoxia on topo IIβ levels was less pronounced, and significant differences in mean fluorescence intensity were not observed under any of the conditions investigated (Fig. 5C and D; n = 5).

The effect of hypoxia on topo IIα regulation was also observed in DU-145 cells, with a pronounced increase in the proportion of cells with low nuclear topo IIα levels observed after 24 hours of culture in 0.2% O2 (Supplementary Figs. S4 and S5). Hypoxia-induced changes in topo IIβ levels were not observed.

Discussion
The primary finding in this study is that exposure of tumor cells to hypoxia protects them against etoposide-induced DNA damage and confers drug resistance. This conclusion is based on results showing that preexposure to hypoxia increased the clonogenic survival of human breast carcinoma and prostate adenocarcinoma cells treated with etoposide and that the increase in survival was associated with an increase in the proportion of cells with low levels of DNA damage, specifically strand breaks, as revealed by alkaline comet assays.

The extent of etoposide-induced DNA damage measured immediately after a 1-hour drug treatment was a good indicator of clonogenic survival in the two tumor cell lines investigated, independent of culture conditions before drug exposure. This finding suggests that the extent of etoposide-mediated DNA damage initially inflicted may be a greater determinant of survival, rather than mechanisms activated after DNA damage. This is supported by the findings that hypoxia-induced resistance was not correlated with an increase in the ability of cells to repair damaged DNA. A similar conclusion was drawn from earlier studies using multicellular spheroids treated with other chemotherapeutic agents, including tirapazamine, actinomycin D, 4-nitroquinoline-N-oxide, and RSU1069, wherein the extent of drug-induced DNA damage was found to be a more critical determinant of resistance rather than DNA repair capacity (38).

Although a correlation between the extent of DNA damage and clonogenic survival was observed, this correlation is likely limited by the fact that DNA integrity is only one of many possible factors that determine the ability of a cell to survive, undergo multiple cycles of replication, and form a colony. This is observed even in the absence of drug treatment. When analyzed by the comet assay, the entire population of MDA-MB-231 cells and nearly the whole population of DU-145 cells display DNA damage levels that are below the determined damage thresholds of ≤24% and ≤2% DNA in tail, respectively (Fig. 1). However, when the same populations of untreated cells are reseeded into culture dishes, the observed rate of colony formation (defined as plating efficiency) is typically 70% to 85% for MDA-MB-231 and 35% to 45% for DU-145 cells. These findings support a model in which DNA integrity is required, but not sufficient, for clonogenic growth. Accordingly, the proportion of cells with undamaged DNA would be expected to be greater than the surviving fraction measured for any given population, regardless of drug treatment. The size of the discrepancy between these two measures will be affected by the extent to which additional factors play a role in determining the clonogenic potential of a cell, which in turn may differ under various conditions and from one cell type to another.

The protective effect of hypoxia against DNA damage was dependent in part on cell cycle position as only cells in G0, G1, or G2-M were able to avoid etoposide-induced DNA damage after preexposure to hypoxia. All cells in S-phase suffered extensive damage, regardless of culture conditions before drug exposure. The higher susceptibility of S-phase cells to topo II poisons is likely due to the prompt generation of double-strand breaks after the collision of DNA replication forks with stabilized cleavable topo II-DNA complexes during DNA synthesis (39). In contrast, noncycling cells in G0 have been reported to display resistance to etoposide which has been attributed to the inactive state of DNA in these cells (32-35). Preexposure to hypoxia resulted in an increase in the proportion of noncycling cells (i.e., in G0), which would be expected to contribute to the resistant fraction detected by comet assays. However, cycling cells exposed to hypoxia still suffered less etoposide-induced DNA damage compared with cycling cells maintained in standard conditions, indicating that a mechanism independent of an increase in the G0 population contributes to hypoxia-induced etoposide resistance.

The ability of hypoxia to protect MDA-MB-231 cells against DNA damage and confer chemoresistance required the presence of a functional HIF-1 response which has been associated with a decrease in nuclear topo IIα levels. Several earlier reports have coupled decreased topo IIα expression with acquired etoposide resistance (40-43). Conversely, overexpression of topo IIα in etoposide-resistant brain tumor cells restored drug sensitivity (44), illustrating the relationship between topo IIα levels and etoposide cytotoxicity in cancer. In MDA-MB-231 cells, the loss of HIF-1α also led to a slight increase in basal etoposide resistance, which correlated with decreased topo
Ilα expression under these conditions. This is consistent with the findings of Wang and Minko in which treatment of human ovarian carcinoma cells with HIF-1α antisense oligonucleotides resulted in decreased levels of topo II mRNA under well-oxygenated conditions (45). Together, these data suggest a novel role for HIF-1α in the regulation of basal topo II expression, although further investigation is required to elucidate whether decreased topo II levels are a direct or indirect effect of HIF-1 knockout.

Increased resistance to doxorubicin and mitoxantrone has been observed previously in tumor cells exposed to hypoxia (16), although in the present study a strong correlation between drug-induced DNA strand breaks and clonogenic survival was not observed. In addition to poisoning topo II proteins, these drugs can have other cytotoxic effects, including production of reactive oxygen species, oxidative DNA base damage and lipid peroxidation, formation of drug-DNA adducts and DNA strand crosslinks, DNA alkylating, and direct membrane effects (46–50). Hypoxia-induced resistance to these agents could therefore be associated with resistance to one or several of these alternative cytotoxic effects. Furthermore, DNA strand breaks induced by doxorubicin and mitoxantrone treatment may potentially be masked by DNA strand crosslinks that retard the migration of DNA fragments when a current is applied (47). Thus, the complex pattern of cellular damage caused by these drugs could potentially account for the poor correlation between doxorubicin-induced or mitoxantrone-induced strand breaks and survival.

Overall, the data described here support a model in which the hypoxia-induced chemoresistance phenotype is linked to HIF-1-dependent mechanisms conferring protection against drug-induced DNA damage. Whereas the establishment of a causal relationship between hypoxia-induced decreases in topo II expression and chemoresistance requires further investigation, the present findings reveal the significance of hypoxia-induced adaptations linked to protective mechanisms that lie upstream of DNA damage. Effective disruption of these mechanisms, as demonstrated here through siRNA-mediated knockdown of HIF-1α expression, may represent a feasible strategy for overcoming multidrug resistance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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