

Hypoxia-induced resistance to anticancer drugs is associated with decreased senescence and requires hypoxia-inducible factor-1 activity

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Abstract

Hypoxia in solid tumors is associated with the development of chemoresistance. Although many studies have focused on the effect of hypoxia on drug-induced apoptosis, the effect of nonapoptotic pathways on hypoxia-induced drug resistance has not been previously investigated. Here, we determined the effects of hypoxia on multiple forms of drug-induced death in human MDA-MB-231 breast carcinoma cells. Clonogenic assays showed that preexposure to hypoxia leads to resistance to various classes of chemotherapeutic agents, including anthracyclines (daunorubicin and doxorubicin), epipodophyllotoxins (etoposide), and anthracenediones (mitoxantrone). Results revealed a high degree of heterogeneity in nuclear and cytoplasmic alterations in response to acute drug exposure; however, the majority of exposed cells displayed morphologic and biochemical changes consistent with drug-induced senescence. Hypoxia decreased only the proportion of cells in the senescent population, whereas the small proportion of cells exhibiting features of apoptosis or mitotic catastrophe were unaffected. Similar results were obtained with human HCT116 colon carcinoma cells, indicating that the protective effect of hypoxia on drug-induced senescence is not unique to MDA-MB-

231 cells. Treatment of MDA-MB-231 cells with small interfering RNA targeting the α -subunit of hypoxia-inducible factor-1 (HIF-1), a key regulator of cellular adaptations to hypoxia, prevented hypoxia-induced resistance. HIF-1 α small interfering RNA also selectively abolished the hypoxia-induced changes in the senescent population, indicating that the increased survival was due to protection against drug-induced senescence. These results support a requirement for HIF-1 in the adaptations leading to drug resistance and reveal that decreased drug-induced senescence is also an important contributor to the development of hypoxia-induced resistance. [Mol Cancer Ther 2008;7(7):1961–73]

Introduction

Regions of hypoxia are present in many solid tumors due to an inadequate and poorly formed vasculature. Consequently, tumor cells often acquire the ability to adapt to hypoxia so that their internal oxygen homeostatic balance is maintained. Increasing evidence from experimental and clinical studies has revealed that tumor cell adaptations to hypoxia are closely linked to malignant progression and contribute to the development of resistance to ionizing radiation and chemotherapy (1–6).

Cellular adaptations to hypoxia involve the coordinated expression of a large and diverse group of genes, many of which are transcriptionally regulated by hypoxia-inducible factor-1 (HIF-1; ref. 7). HIF-1 is a transcription factor composed of HIF-1 α and HIF-1 β subunits. HIF-1 β is constitutively expressed and control of HIF-1 function occurs primarily through the oxygen-dependent degradation of the α -subunit. On activation, HIF-1 binds to *cis*-acting hypoxia response elements to induce the expression of target genes, several of which have physiologic relevance to malignant progression.

Recent investigations into the role of HIF-1 in the development of drug resistance have focused on determining whether there is a link between hypoxia and escape from drug-induced apoptosis (8–13). Unruh et al. (8) reported that loss of HIF-1 α expression predisposed cells to apoptosis induced by chemotherapeutic agents and ionizing radiation under standard and hypoxic culture conditions. Human colon cancer cells exposed to hypoxia *in vitro* or grown as tumor xenografts have been shown to express decreased levels of the proapoptotic factors Bid, Bad, and Bax compared with well-oxygenated cells (9). Decreased levels of proapoptotic proteins were correlated with lower levels of etoposide-induced apoptosis and attributed to both HIF-1-dependent and HIF-1-independent mechanisms. Genetic approaches and small-molecule inhibitors targeting HIF-1 have proven effective at decreasing

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hypoxia-induced resistance to chemotherapeutics in colon (10), neuroblastoma (11), and non-small cell lung cancer cells (12), thereby highlighting the importance of HIF-1 in the acquisition of drug resistance.

A common paradigm is that chemotherapeutic agents stimulate cancer cells to undergo apoptosis, implying that impairment of apoptotic pathways may be sufficient to account for the development of chemoresistance. However, following DNA damage, cells can be eliminated through other forms of programmed death, including autophagy, mitotic catastrophe, and necrosis. In addition, several chemotherapeutic agents are known to induce senescence in a variety of cancer lines *in vitro*, resulting in irreversible arrest of cell proliferation (14–19). Analysis of human breast tumor specimens indicates that drug-induced senescence also occurs *in vivo* and is a physiologically relevant response to DNA damage resulting from cancer therapy (18). In fact, there is evidence that in response to some chemotherapeutic agents mitotic catastrophe and senescence are more prominent than apoptosis (15, 18).

Highlighting the importance of nonapoptotic mechanisms of death in the response of tumor cells to chemotherapeutic agents are results of experiments showing a lack of correlation between specific inhibition of apoptosis and overall response to anticancer therapy. Although stable overexpression of the antiapoptotic Bcl-2 protein in HeLa cells nearly abolished etoposide-induced apoptosis, there was no effect on survival when compared with controls (20). Bcl-2 overexpression prevented the appearance of apoptotic cells but instead led to the formation of enlarged, multinucleated cells characteristic of mitotic catastrophe. Similarly, overexpression of P-glycoprotein markedly diminished the sensitivity of HeLa cells to the induction of apoptosis induced by ionizing radiation without affecting overall survival (21). Analysis revealed that the inhibition of apoptosis was accompanied by compensatory increases in cell death due to mitotic catastrophe and senescence. Conversely, knock-down of the expression of the antiapoptotic molecule Bcl-X_L in colorectal cancer cells resulted in a switch from drug-induced senescence to apoptosis following treatment with the topoisomerase I inhibitor SN38 (22). These studies suggest that, in cells incurring sufficient levels of DNA damage, suppression of a single death pathway may be inadequate to enable survival as alternative death or nonproliferative pathways can be activated.

The effect of nonapoptotic pathways on hypoxia-induced drug resistance has not been previously investigated. Furthermore, it is unclear whether decreased levels of drug-induced apoptosis are sufficient to account for the increased survival following exposure to hypoxia or whether this increase in survival may be due to a hypoxia-mediated down-regulation of alternative mechanisms of cell death. To address these questions, we determined the response of human breast and colon cancer cells to multiple chemotherapeutic agents following pre-exposure to hypoxia and assessed the role of HIF-1 in the various pathways of drug-induced cell death.

Materials and Methods

Cell Culture and Exposure to Hypoxia

Human MDA-MB-231 breast carcinoma cells were obtained from the American Type Culture Collection and human HCT116 colon carcinoma cells were kindly provided by Dr. Xiaolong Yang (Department of Pathology and Molecular Medicine, Queen's University, Kingston, Ontario, Canada). Cells were maintained in monolayer cultures in a standard CO₂ incubator (5% CO₂ in air at 37°C) in either RPMI 1640 supplemented with 5% fetal bovine serum (MDA-MB-231) or McCoy's 5A medium supplemented with 10% fetal bovine serum (HCT116; all tissue culture media were purchased from Invitrogen). The MDA-MB-231 cell line is derived from an adenocarcinoma pleural effusion and carries an activating K-ras, codon 12 mutation; it is also p53 mutant and p16 null, carries wild-type Rb, and is estrogen receptor negative. The HCT116 cell line is derived from a colon adenocarcinoma, carries an activating K-ras mutation, and expresses wild-type p53. To establish hypoxic conditions, cells were placed in airtight plastic chambers that were flushed with a 5% CO₂/95% N₂ gas mixture. Oxygen concentrations within these chambers were maintained at 0.2% using Pro-Ox Model 110 O₂ regulators (BioSpherix).

Clonogenic (Colony Formation) Assays

All drugs used in the clonogenic assays were purchased from Sigma-Aldrich Canada Ltd. and the methods for this assay have been described previously (1–3). Briefly, after 24 h of standard or hypoxic incubation, culture medium was replaced with either complete medium (for nontreated controls) or complete medium containing one of the following chemotherapeutic agents: daunorubicin, doxorubicin, etoposide, or mitoxantrone, or staurosporine at the concentrations indicated in the figure legends. Drug exposure was performed under standard culture conditions for 1 h (or 24 h for staurosporine treatment); cells were then washed once in PBS, harvested by trypsinization, counted using a hemocytometer, and replated (six replicates per condition) in six-well tissue culture plates. After an additional 7 to 10 d of culture, cells were fixed with an acetic acid/methanol (1:3) solution and stained with a dilute crystal violet (0.33%, w/v) solution, and surviving colonies consisting of ~50 or more cells were counted. Pilot studies performed using a hypoxic chamber (Coy Laboratory Products, Inc.) that enables manipulation of cells while under hypoxic conditions 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 to doxorubicin (data not shown).

Microscopic Analyses and Terminal Deoxynucleotidyl Transferase – Mediated dUTP Nick End Labeling Assays

To characterize morphologic changes following exposure to anticancer drugs, cells were treated as described above for the clonogenic assays. However, following drug exposure, they were instead replated onto glass coverslips in six-well dishes and cultured for up to 8 d in a standard incubator (37°C; 5% CO₂). To retain the less adherent dead

or dying cells, we developed an agarose overlay method. Briefly, following replating of cells and an additional 5 h of culture to allow for the cells to adhere to the coverslips, the culture medium was replaced with a thin layer of warm (37°C) complete medium containing 0.5% SeaPlaque low melting temperature agarose (Lonza) and allowed to polymerize briefly at room temperature before returning to a standard incubator. Pilot studies confirmed that the agarose overlay did not cause restriction of cell proliferation or alterations in cell morphology. At select time points, cells were fixed for 15 min at 4°C using 3.7% formaldehyde in PBS, allowing the formaldehyde to penetrate through the agarose layer without disrupting the cells below. Fixative was removed and wells were gently washed in PBS at 4°C twice for 15 min each. Coverslips were carefully excised from the agarose overlay, washed once in PBS, and either assayed for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (as a marker for drug-induced apoptosis) or stained for senescence-associated β -galactosidase (SA- β gal) activity and counterstained with hematoxylin to assess other morphologic features.

TUNEL assays were performed using the DeadEnd Fluorometric TUNEL System (Promega/Fisher Scientific) according to the manufacturer's instructions. Following the enzymatic reactions, cells were counterstained with 4',6-diamidino-2-phenylindole (Vectashield, Vector Laboratories, Inc.) and the percentage of TUNEL-positive cells was determined using fluorescence microscopy scoring a minimum of 200 cells randomly sampled for each experimental condition.

SA- β gal staining was performed using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Fermentas Canada, Inc.) at pH 6.0 as previously described (23). Characterization of cell morphology and SA- β gal activity was determined by bright-field microscopy. A minimum of 200 cells was scored at random for each slide according to a variety of morphologic criteria, including (a) overall size of the nuclear compartment (normal, enlarged, or shrunken), (b) the appearance of the nucleus (single, binucleated, multinucleated or micronucleated, or fragmented), (c) general chromatin appearance (regular and evenly stained or condensed), (d) overall size of the cytoplasmic compartment (normal, enlarged, or shrunken), (e) SA- β gal staining (positive or negative), and (f) general appearance of the cell membrane (defined as "blebbed" or ruptured). The intention of morphologic scoring was to follow the fate of plated cells over time to determine whether cells had undergone cell death, become permanently arrested, or remained viable (i.e., survived) following drug treatment. In contrast to the arrested or dead cells, surviving cells underwent proliferation leading to the formation of colonies with progressively increasing cell numbers throughout the time course. Despite being composed of multiple cells at the time of fixation, each individual colony represented an individual surviving cell plated at the beginning of the time course experiment and was therefore counted as such.

Flow Cytometry

For analysis of cellular complexity and cell division by flow cytometry, cells were treated as described above for the clonogenic assays, trypsinized, and labeled with the fluorescent membrane-binding molecule PKH67 (Sigma-Aldrich Canada) according to the manufacturer's instructions. Cells were replated and incubated under standard culture conditions in the dark. Adherent and nonadherent cells were collected at various time points, fixed in 2% paraformaldehyde, and stored at 4°C protected from light. PKH67 fluorescence and 90° light scatter were monitored with a Beckman Coulter EPICS Altra HSS flow cytometer. Excitation was performed by an argon laser at a wavelength of 488 nm, the emitted fluorescence was collected at 525 \pm 10 nm, and at least 10,000 events per sample were analyzed.

Small Interfering RNA Transfection

Validated Silencer HIF-1 α Small Interfering RNA (siRNA; ID 42840) and Silencer Negative Control siRNA 1 were purchased from Ambion, Inc. siRNA was introduced into cells by reverse transfection using siPORT NeoFX reagent (Ambion) according to the manufacturer's instructions. The siRNA treatments were carried out for 48 h under standard culture conditions before incubation in hypoxia.

Western Blot Analysis of HIF-1 α Levels

Following incubation under various conditions, cells were frozen immediately after removal from the Pro-Ox chamber or incubator by rapidly discarding the medium and placing the culture plates in a liquid nitrogen bath. The levels of HIF-1 α in cells were determined by Western blot analysis as previously described (24). To control for even sample loading, membranes were blotted with anti- β -actin antibody (1:5,000; Sigma-Aldrich Canada).

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 \pm SE.

Statistical analyses were conducted using GraphPad Prism software version 4.0 (GraphPad Software, Inc.). Statistical significance was determined by an unpaired, two-tailed *t* test and differences were considered to be significant at *P* < 0.05.

Results

Preexposure to Hypoxia Increases the Clonogenic Survival of Human MDA-MB-231 Breast Carcinoma Cells Treated with Chemotherapeutic Agents

Exposure of MDA-MB-231 cells to hypoxia (0.2% O₂) for 24 h before drug treatment leads to significantly (*P* < 0.01) increased resistance to multiple anticancer drugs (daunorubicin, doxorubicin, etoposide, or mitoxantrone) relative to cells maintained under standard culture conditions (20% O₂; Fig. 1). The effect of hypoxia on survival was highly

reproducible and the mean increase in survival was 4.0-fold for daunorubicin ($n = 6$), 3.5-fold for doxorubicin ($n = 17$), 4.8-fold for etoposide ($n = 7$), and 3.6-fold for mitoxantrone ($n = 4$).

Hypoxia Does Not Alter MDA-MB-231 Apoptotic Cell Death Induced by Treatment with Chemotherapeutic Agents or Staurosporine

The chemotherapeutics used in this study are thought to exert their anticancer effects in part through inhibition of topoisomerase II, leading to the formation of stable DNA-topoisomerase II complexes and DNA strand breaks and initiation of the apoptotic cascade. To assess the effects of hypoxia on the apoptotic response of drug-treated MDA-MB-231 cells, we used the TUNEL assay, which preferentially detects DNA strand breaks in apoptotic cells. Very few TUNEL-positive cells were detected at early time points (1 and 2 days following drug treatment), but 4 days following drug treatment, a small population of TUNEL-positive cells became evident and progressively increased throughout the time course (Fig. 2A). At 8 days following drug treatment, <20% of the total population showed TUNEL-positive staining for all of the four chemotherapeutics tested (Fig. 2B). More importantly, when compared with cells maintained under well-oxygenated conditions, the proportion of TUNEL-positive cells following preexposure to hypoxia were not statistically different for any of the drugs tested.

To further characterize the effects of hypoxia on the apoptotic response of MDA-MB-231 cells, clonogenic assays were conducted using staurosporine, a known inducer of apoptosis. The majority of MDA-MB-231 cells treated with staurosporine showed morphologic features characteristic of cells undergoing apoptosis, including a high degree of cell shrinkage, chromatin condensation, disintegration of the cell membrane to form structures resembling apoptotic bodies, and TUNEL-positive staining (Fig. 2C). In contrast to the chemotherapeutic agents used in this study, preexposure to hypoxia did not increase the clonogenic survival of staurosporine-treated MDA-MB-231 cells relative to cells maintained under standard culture conditions (Fig. 2D).

Treatment of MDA-MB-231 Cells with Chemotherapeutic Agents Predominantly Leads to the Appearance of a Senescence-like Phenotype

To investigate the role of nonapoptotic forms of programmed cell death and arrest, we examined changes in MDA-MB-231 cell morphology following treatment with chemotherapeutic agents. Overall, treated cells showed a high degree of morphologic heterogeneity, including enlarged and shrunken cells, multinucleated or micronucleated cells, cells with blebbed or ruptured plasma membranes, and various combinations of these features (Fig. 3A). A small proportion of enlarged cells contained several completely or partially separated micronuclei with

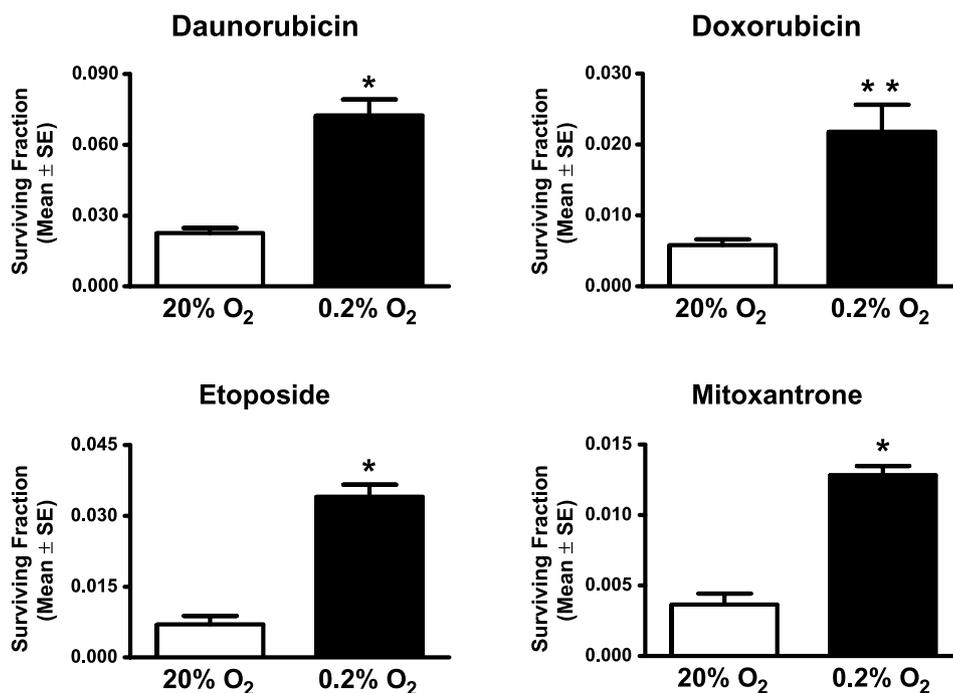


Figure 1. Effect of hypoxia on the survival of human MDA-MB-231 breast cancer cells treated with chemotherapeutic agents. Clonogenic assays were conducted on MDA-MB-231 cells preincubated for 24 h in standard (20% O₂) or hypoxic (0.2% O₂) conditions and subsequently treated with various anticancer agents for 1 h under standard culture conditions as described in Materials and Methods. Compared with the survival of cells incubated in 20% O₂, the survival of MDA-MB-231 cells preincubated in 0.2% O₂ was significantly higher following treatment with daunorubicin (5 μmol/L), doxorubicin (5 μmol/L), etoposide (50 μmol/L), or mitoxantrone (1 μmol/L). Columns, mean surviving fractions; bars, SE. Results are representative of a minimum of five independent experiments. *, $P < 0.0001$; **, $P = 0.0021$.

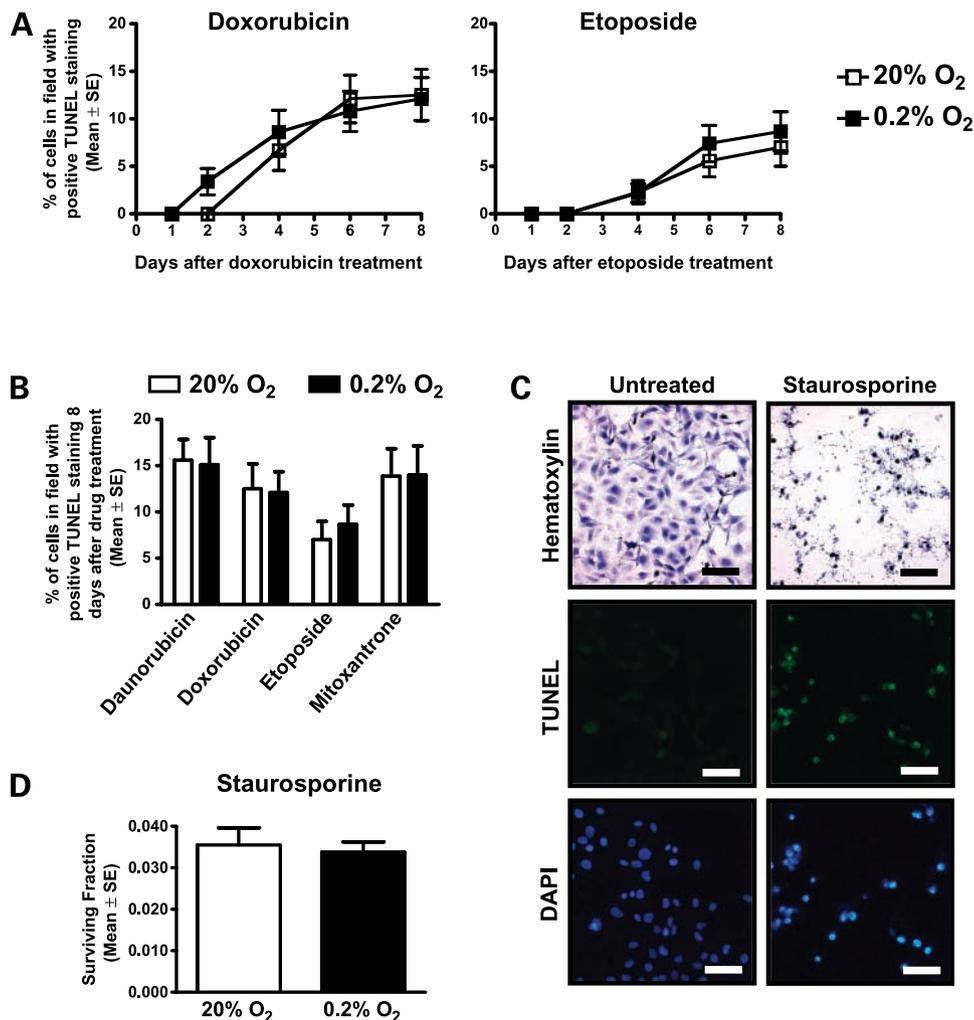


Figure 2. Effect of hypoxia on drug-induced apoptosis in MDA-MB-231 cells. **A**, time course of appearance of TUNEL-positive cells following treatment with doxorubicin (5 $\mu\text{mol/L}$) or etoposide (50 $\mu\text{mol/L}$). No statistically significant differences were found between the TUNEL-positive fractions of cells preexposed to hypoxia and those maintained in standard culture conditions. *Points*, mean percentage of cells scored in the microscopic field; *bars*, SE. **B**, mean percentage of TUNEL-positive MDA-MB-231 cells after 8 d of culture following a 1-h treatment with daunorubicin (5 $\mu\text{mol/L}$), doxorubicin (5 $\mu\text{mol/L}$), etoposide (50 $\mu\text{mol/L}$), or mitoxantrone (1 $\mu\text{mol/L}$); no statistically significant differences between cells exposed to 20% O₂ and 0.2% O₂ were observed. **C**, MDA-MB-231 cells were cultured on glass coverslips and left untreated (*left*) or treated for 24 h with 0.5 $\mu\text{mol/L}$ staurosporine (*right*) before fixing and counterstaining with hematoxylin (*top*), analyzed by TUNEL assay (*middle*), or counterstained with 4',6-diamidino-2-phenylindole (DAPI; *bottom*). Cells were visualized using bright-field (hematoxylin) or fluorescence microscopy (TUNEL and 4',6-diamidino-2-phenylindole). *Scale bar*, 100 μm . Microscopic analysis indicated that this treatment was sufficient to induce apoptosis in the majority of cells, as determined by the degree of cell shrinkage, chromatin condensation, the disintegration of the cell membrane to form apoptotic bodies, and TUNEL-positive staining. **D**, clonogenic assays were conducted on MDA-MB-231 cells preincubated for 24 h in standard (20% O₂) or hypoxic (0.2% O₂) conditions followed by treatment with staurosporine (0.5 $\mu\text{mol/L}$, 24 h in 20% O₂). In contrast to the effect of hypoxia on cell survival following treatment with the conventional chemotherapeutic agents used in this study, preexposure to hypoxia (versus 20% O₂) did not increase the survival of MDA-MB-231 cells following treatment with staurosporine. Results are representative of three independent experiments (differences not statistically significant).

evenly stained chromatin, features often reported for cells undergoing mitotic catastrophe. A smaller fraction of cells seemed to have a shrunken cytoplasm with condensed chromatin and blebbing of the plasma membrane disintegrating into structures resembling apoptotic bodies. However, the predominant morphologic change was the emergence of enlarged and flattened cells, a phenotype associated with the onset of drug-induced senescence (15). Consistent with the observed morphologic changes, many

enlarged and flattened cells stained positive for SA- β gal activity (Fig. 3A), a histochemical biomarker of senescent cells (15, 23).

Based on the appearance of cells with a senescence-like morphology following drug treatment, we used flow cytometry to confirm additional features associated with senescence, such as a loss of proliferative capacity and an increase in cellular complexity (15, 25, 26). MDA-MB-231 cells were treated as outlined for the clonogenic assay and

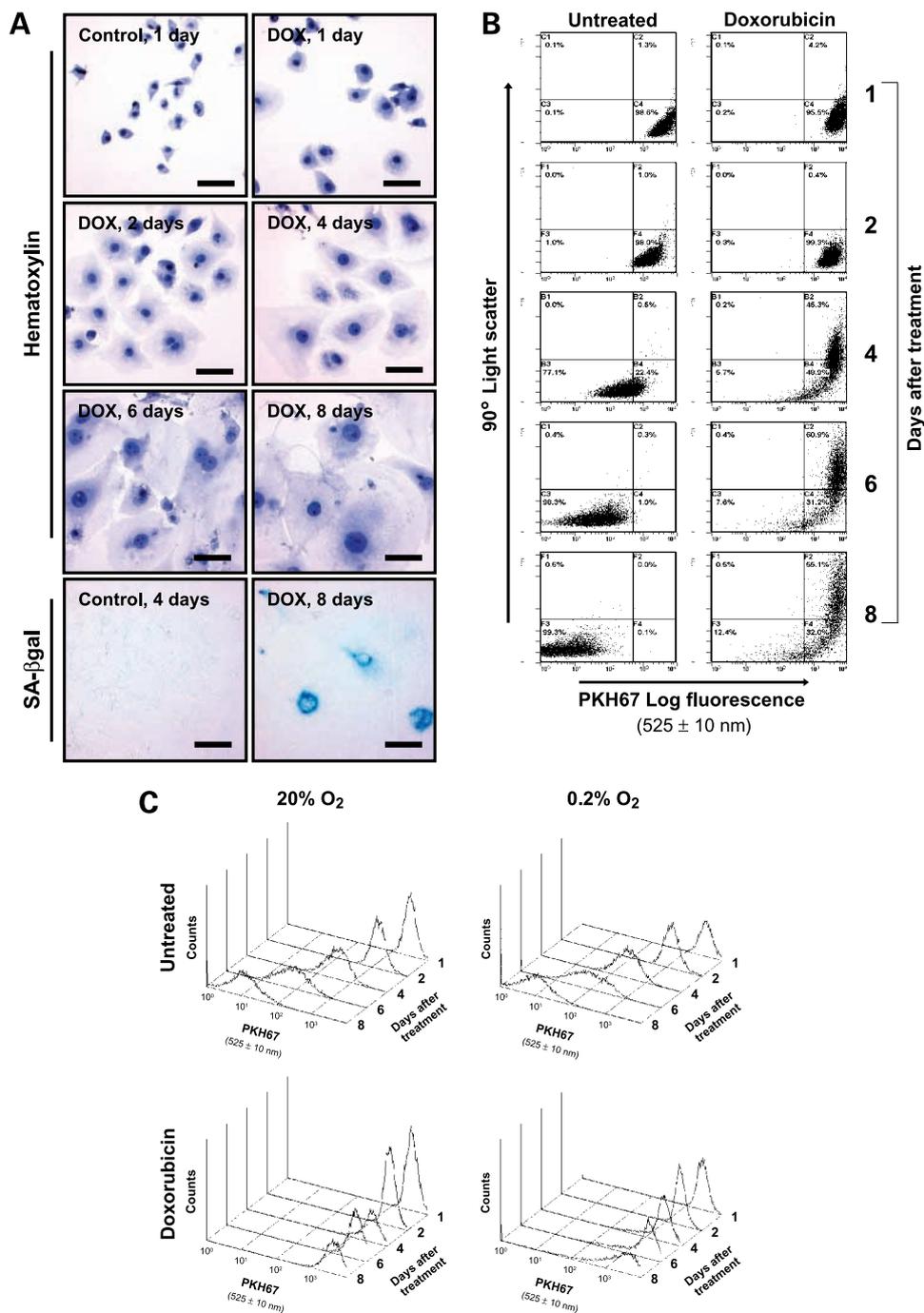


Figure 3. Changes in MDA-MB-231 cell morphology induced by treatment with chemotherapeutic agents. **A**, MDA-MB-231 cells were cultured on glass coverslips under standard conditions (20% O₂) for up to 8 d following exposure to doxorubicin (DOX; 5 μmol/L, 1 h in 20% O₂), fixed, and counterstained with hematoxylin or stained for SA-βgal activity. Cells were visualized using bright-field microscopy. Scale bar, 100 μm. Similar changes in MDA-MB-231 cell morphology and SA-βgal-positive staining were observed following exposure to daunorubicin, etoposide, and mitoxantrone (data not shown). Results are representative of five independent experiments. **B**, flow cytometric analysis of PKH67-labeled MDA-MB-231 cells following treatment with doxorubicin. MDA-MB-231 cells were preincubated for 24 h under standard (20% O₂) or hypoxic (0.2% O₂) conditions and subsequently treated with doxorubicin (5 μmol/L, 1 h in 20% O₂) before labeling of the plasma membrane with PKH67. Cells were then cultured in drug-free medium under standard conditions for up to 8 d. Dot density plots of PKH67 fluorescence (X axis) versus 90° light scatter (Y axis) for untreated and doxorubicin-treated cells reveal a progressive increase in cell size for doxorubicin-treated cells at consecutive time points following treatment. **C**, PKH67 fluorescence profiles for untreated control cells (top) and for doxorubicin-treated cells (bottom) at consecutive time points following treatment. Untreated cells exhibited progressive loss of fluorescence intensity due to cell proliferation, whereas doxorubicin treatment almost completely inhibited proliferative capacity. Note the small population of drug-treated cells that retained proliferative capacity following preexposure to hypoxia (bottom right, days 4–8). For flow cytometry experiments, a minimum of 10,000 cells was analyzed for all conditions and results are representative of two independent experiments.

then labeled with the lipophilic fluorescent compound PKH67, which is incorporated into the membrane of labeled cells and subsequently distributed evenly between daughter cells during mitosis. Measurement of PKH67

fluorescence is used to identify populations of cells that have undergone division, indicated by decreased PKH67-associated fluorescence intensity, whereas increases in 90° light scatter are a reflection of increases in cell size (25) and

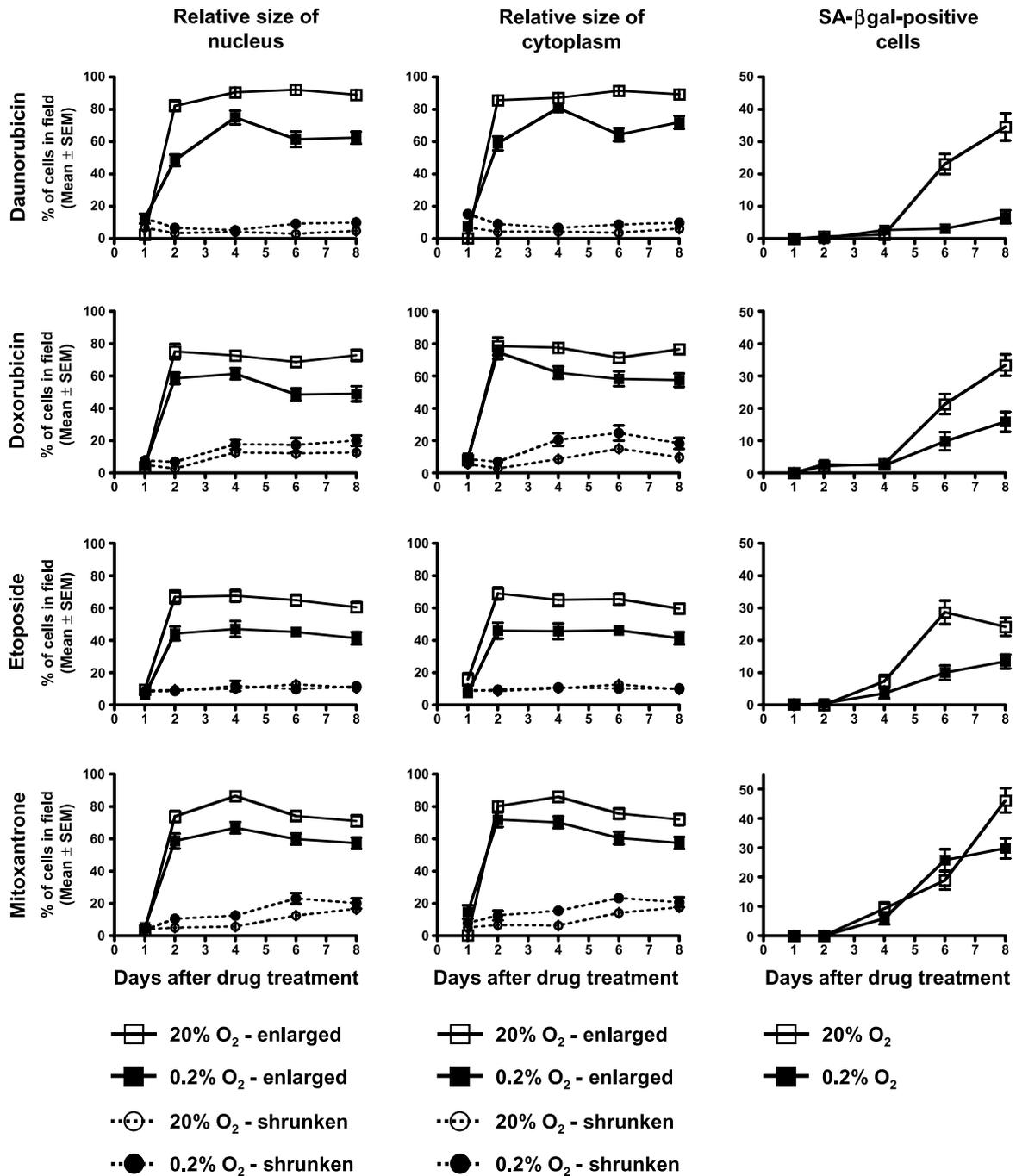


Figure 4. Characterization of chemotherapy-induced changes in MDA-MB-231 cell morphology following preexposure to hypoxia. Morphologic analyses were conducted on MDA-MB-231 cells treated with anticancer agents following 24 h of culture under standard (20% O₂) or hypoxic (0.2% O₂) conditions. Time courses of drug-induced changes in the fraction of cells with enlarged or shrunken nuclei, enlarged or shrunken cytoplasm, and detectable SA-βgal activity. The complete analysis from morphologic scoring is shown in the Supplementary Data. Compared with cells incubated in 20% O₂, preexposure to hypoxia led to a significant reduction in the proportion of drug-treated cells with enlarged nuclei ($P < 0.01$), enlarged cytoplasm ($P < 0.01$), and SA-βgal activity ($P < 0.005$) for all of the chemotherapeutic agents investigated (reported P values correspond to differences at day 8 following drug exposure). Points, mean percentage of cells scored in the microscopic field; bars, SE. Results are representative of two independent experiments.

granularity/complexity (26). In untreated control cells, PKH67 fluorescence progressively declined over time, and after 8 days of culture, 99.7% to 99.8% of cells had reduced fluorescence intensity compared with cells on day 1 (Fig. 3B, compare *bottom left quadrants* of each dot plot; $n = 2$). In contrast, doxorubicin treatment largely prevented the gradual decrease in PKH67 intensity, with only 4.4% to 12.9% of cells showing decreased PKH67 intensity 8 days after drug treatment. Furthermore, a large proportion of doxorubicin-treated cells exhibiting high PKH67 fluorescence showed elevated 90° light scatter (Fig. 3B, compare *top right quadrants* of each dot plot). The increase in cell size and complexity was detectable 4 days after treatment and progressively increased at later time points (55.1–57.4% by day 8). Changes in cell size or complexity were not observed for untreated controls.

Flow cytometry was also used to assess the proliferative capacity of MDA-MB-231 cells following preexposure to hypoxia. Compared with untreated control cells maintained under standard conditions (Fig. 3C, *top left*), preexposure to hypoxia had no apparent effect on the proliferative capacity of MDA-MB-231 cells on reoxygenation (Fig. 3C, *top right*). Similar to cells cultured at 20% O₂, doxorubicin treatment of cells preexposed to hypoxia resulted in substantial retention of PKH67 fluorescence intensity; however, a larger proportion of cells with decreased fluorescence were evident compared with cells cultured at 20% O₂ (Fig. 3C, *bottom right*), consistent with the hypoxia-induced increase in clonogenic survival observed under similar conditions (Fig. 1). Four days after doxorubicin treatment, this population of lower-intensity cells became distinct from the peak of growth-arrested cells and progressively increased over subsequent time points (identified as a lower intensity shoulder on the left side of the histograms for days 4–8).

Preexposure to Hypoxia Protects MDA-MB-231 Cells from Drug-Induced Senescence

Based on the predominant induction of senescence in MDA-MB-231 cells exposed to chemotherapeutic agents, we hypothesized that the increase in clonogenic survival following preexposure to hypoxia was associated with a decrease in the proportion of cells undergoing drug-induced senescence. To test this hypothesis, we performed quantitative microscopic analyses to determine which morphologic features were altered in the population of cells preexposed to hypoxia. Cell morphology was scored at multiple time points following drug treatment according to criteria that encompassed morphologic and biochemical alterations in the nucleus, cytoplasm, and plasma membrane (see Materials and Methods for details).

Overall, the progression of morphologic changes was similar for all four chemotherapeutic agents tested (Fig. 4; Supplementary Fig. S1).³ For cells maintained under well-oxygenated conditions within 2 days of drug exposure, the

majority of the population (~60–80% of total cells scored) were characterized by an enlarged nucleus and enlarged cytoplasmic compartment. SA- β gal activity was detectable 4 days after drug treatment with the proportion of SA- β gal-positive cells increasing to approximately 30% to 40% of the total population by day 8. For all chemotherapeutics, preexposure to hypoxia led to significant decreases in the proportion of cells with enlarged nuclei and enlarged cytoplasmic compartments, as well as significant decreases in the proportion of SA- β gal-positive cells (scoring results and P values are summarized in Supplementary Table S1).³ Hypoxia did not significantly affect the proportion of cells with shrunken nuclei or cytoplasm, which represented approximately 5% to 20% of the total population by day 8, consistent with the observed fraction of TUNEL-positive cells (Fig. 2B). Similarly, preexposure to hypoxia did not significantly affect the proportions of cells with binucleated, multinucleated, or fragmented nuclei, condensed chromatin, or blebbed or ruptured cell membranes (Supplementary Fig. S1 and Supplementary Table S1).³

Preexposure of Human HCT116 Colon Carcinoma Cells to Hypoxia Increases Clonogenic Survival and Protects Cells from Drug-Induced Senescence

To determine whether a similar decrease in drug-induced senescence occurs in other human tumor cell lines exposed to hypoxia, clonogenic assays and morphologic analyses were performed using HCT116 cells, a cell line previously reported to undergo doxorubicin-induced senescence (15). Similar to MDA-MB-231 cells, exposure of HCT116 cells to hypoxia (0.2% O₂) for 24 h before drug treatment led to significantly ($P < 0.0001$) increased resistance to daunorubicin, etoposide (Fig. 5A), and doxorubicin (data not shown). The mean increase in survival following preexposure to hypoxia was 3.9-fold for daunorubicin ($n = 2$), 8.0-fold for etoposide ($n = 3$), and 2.3-fold for doxorubicin ($n = 7$). After 8 days of culture following drug treatment, the majority of HCT116 cells were characterized by an enlarged and flattened morphology, accompanied by expression of SA- β gal (Fig. 5B). Quantitative microscopic analyses confirmed that, by day 8, approximately 60% to 75% of the total population of cells maintained in 20% O₂ were characterized by an enlarged nucleus and cytoplasm and approximately 35% to 60% of the total population were SA- β gal positive (Fig. 5C; Supplementary Table S2).³ Preexposure to hypoxia significantly reduced the proportion of cells with enlarged nuclei (~25–30% decrease), enlarged cytoplasm (~24–31% decrease), and detectable SA- β gal activity (~32–50% decrease), relative to cells maintained at 20% O₂. Hypoxia-induced differences in the proportion of drug-treated cells with apoptotic-like morphologies were not observed.

HIF-1 α Is Required for Hypoxia-Induced Doxorubicin Resistance and Escape from Drug-Induced Senescence

To investigate the role of HIF-1-mediated gene transcription in the development of the hypoxia-induced drug resistance phenotype, we introduced into MDA-MB-231 cells siRNA targeting HIF-1 α and assessed clonogenic

³Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

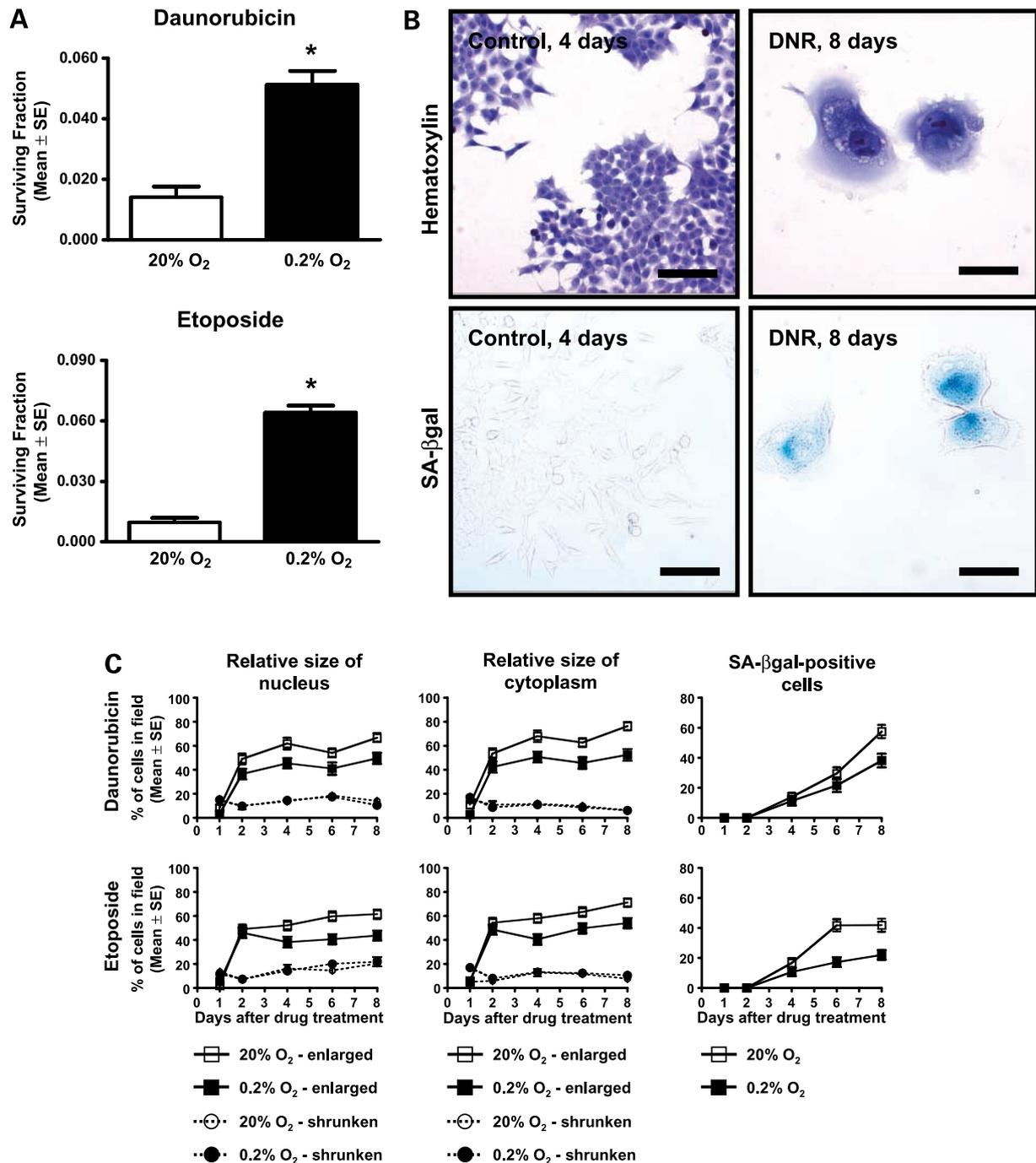


Figure 5. Effect of hypoxia on the clonogenic survival and cell morphology of human HCT116 colon cancer cells treated with chemotherapeutic agents. **A**, clonogenic assays were conducted on HCT116 cells preincubated for 24 h in standard (20% O₂) or hypoxic (0.2% O₂) conditions and subsequently treated with various anticancer agents as described in Materials and Methods. Compared with the survival of cells incubated in 20% O₂, the survival of HCT116 cells preincubated in 0.2% O₂ was significantly higher following treatment with daunorubicin (7.5 μmol/L) or etoposide (60 μmol/L). *, $P < 0.0001$. Columns, mean surviving fraction; bars, SE. Results are representative of two to three independent experiments. **B**, HCT116 cells were cultured on glass coverslips under standard conditions (20% O₂) for 4 to 8 d following exposure to daunorubicin (DNR; 7.5 μmol/L, 1 h in 20% O₂), fixed, and counterstained with hematoxylin or stained for SA-βgal activity. Cells were visualized using bright-field microscopy. Scale bar, 100 μm. Similar changes in HCT116 morphology were observed following treatment with either etoposide (60 μmol/L) or doxorubicin (7.5 μmol/L; data not shown). **C**, morphologic analyses were conducted on HCT116 cells treated with anticancer agents following 24 h of culture under standard (20% O₂) or hypoxic (0.2% O₂) conditions as described for MDA-MB-231 cells in Fig. 4. Compared with cells incubated in 20% O₂, preexposure to hypoxia led to a significant decrease in the proportion of drug-treated cells with enlarged nuclei, enlarged cytoplasm, and SA-βgal activity for both chemotherapeutic agents investigated ($P < 0.005$ for differences observed after 8 d following drug exposure). Points, mean percentage of cells scored in the microscopic field; bars, SE. Results are representative of two independent experiments.

survival and cell morphology following drug treatment. Exposure of untransfected cells to hypoxia resulted in a substantial increase in the levels of HIF-1 α protein that was unaffected by transfection of negative control siRNA (Fig. 6A). In contrast, HIF-1 α protein was barely detected in cells treated with HIF-1 α siRNA. Hypoxia increased the survival of untransfected and negative control siRNA-transfected cells treated with doxorubicin (Fig. 6B; untransfected cells: mean 3.5-fold increase, $P < 0.0001$, $n = 17$; negative control siRNA-transfected cells: mean 5.7-fold increase, $P < 0.0001$, $n = 5$). Pretreatment of MDA-MB-231 cells with HIF-1 α siRNA abolished the hypoxia-mediated increase in doxorubicin resistance with no significant difference in relative survival compared with similarly treated cells maintained at 20% O₂ ($n = 5$). Transfection with either negative control siRNA or HIF-1 α siRNA had no significant effect on overall plating efficiency of control cells (no doxorubicin treatment; data not shown) or survival of doxorubicin-treated cells preincubated at 20% O₂ (Fig. 6B) when compared with untransfected MDA-MB-231 cells. Furthermore, treatment of MDA-MB-231 cells with HIF-1 α siRNA was also sufficient to prevent hypoxia-induced increases in etoposide resistance (data not shown), indicating that the effects of HIF-1 α knockdown on resistance are not specific to doxorubicin.

To identify the population of cells affected by HIF-1 α siRNA treatment, microscopic analyses were performed following standard clonogenic assays. Cells were scored in the three morphologic categories showing hypoxia-induced differences. As observed previously, preexposure of untransfected cells to hypoxia significantly reduced the proportion of the total population with enlarged nuclei (39.0–44.6% decrease, $n = 2$) and enlarged cytoplasm (38.2–44.6% decrease), as well as the fraction of cells with positive SA- β gal staining (49.6–68.6% decrease; Fig. 6C; Supplementary Table S3).³ Similarly, in cells transfected with negative control siRNA, preexposure to hypoxia led to a ~23% decrease in cells with enlarged nuclei, a ~25% decrease in cells with enlarged cytoplasm, and a 25% to 43% decrease in SA- β gal-positive cells ($n = 2$). In contrast, the effects of hypoxia on drug-induced morphologic changes were abolished by loss of HIF-1 α expression, as the proportion of enlarged or SA- β gal-positive cells were not statistically different from HIF-1 α siRNA-treated cells maintained under standard (20% O₂) culture conditions (Fig. 6C). The proportion of cells with shrunken nuclei and cytoplasm were not statistically different under any of the conditions examined.

Discussion

This study shows that hypoxia is able to increase tumor cell resistance to chemotherapeutic agents by preventing drug-induced senescence independently of changes in the apoptotic fraction. This conclusion is based on results showing that preexposure to hypoxia increased the survival of human breast and colon carcinoma cells following exposure to various anticancer drugs and that

the increase in survival was associated with a selective decrease in the proportion of cells undergoing senescence, the predominant response triggered by the chemotherapeutic agents used. Although a small population of MDA-MB-231 and HCT116 cells underwent apoptosis following drug treatment (as determined by cell shrinkage, chromatin condensation, membrane blebbing, or TUNEL staining), the absence of hypoxia-induced changes in the proportion of apoptotic cells suggests that hypoxia did not disrupt the balance of proapoptotic and antiapoptotic factors and that impairment of apoptosis was unlikely to account for the observed increase in survival. This is consistent with the inability of hypoxia to induce resistance of MDA-MB-231 cells to apoptosis induced by staurosporine.

Senescence is characterized by an irreversible arrest of the cell cycle and can be induced by various forms of stress, including telomere dysfunction, oxidative damage, DNA damage, and aberrant expression of oncogenic proteins such as Ras (27). In addition to a loss of proliferative capacity, senescent cells show a characteristic phenotype defined by the development of an enlarged and flattened morphology, accompanied by increased granularity and the detection of SA- β gal activity at pH 6.0 (15). Senescence is generally categorized as either replicative senescence, a physiologic process triggered to limit the life span of nonmalignant cells, or accelerated senescence, associated with a rapid onset of terminal proliferation arrest in response to damage to the cell. Initiation of replicative senescence is thought to occur when chromosomal telomeres reach a critical length following progressive erosion over multiple cycles of replication (27). Forced expression of the catalytic subunit of telomerase in normal human cells is sufficient to maintain telomere length, prevent the induction of senescence, and extend the replicative life span (28, 29). Recent evidence indicates that hypoxia can increase telomerase activity through HIF-1-mediated transcriptional activation of telomerase gene promoters and alternative splicing that favors the production of a more active telomerase variant (30–33). In this way, the hypoxic microenvironment of solid tumors may contribute to malignant progression through selection of cells with elevated telomerase activity that enables them to escape replicative senescence. In contrast, drug-induced senescence is not prevented by overexpression of telomerase and seems to occur in a telomere length-independent manner (19). Accordingly, elevated telomerase activity is unlikely to account for hypoxia-induced resistance. Nevertheless, there is evidence that exposure to some chemotherapeutic agents, such as doxorubicin, can result in preferential DNA damage (single- and double-strand breaks) in telomere-associated regions, leading to a loss of protection of the telomere cap, which may signal the initiation of accelerated senescence (19). It is not clear whether the hypoxia-induced increase in telomerase activity is accompanied by additional changes in telomere structure that may contribute to the protection of chromosomes in these regions and confer resistance to such DNA-damaging agents.

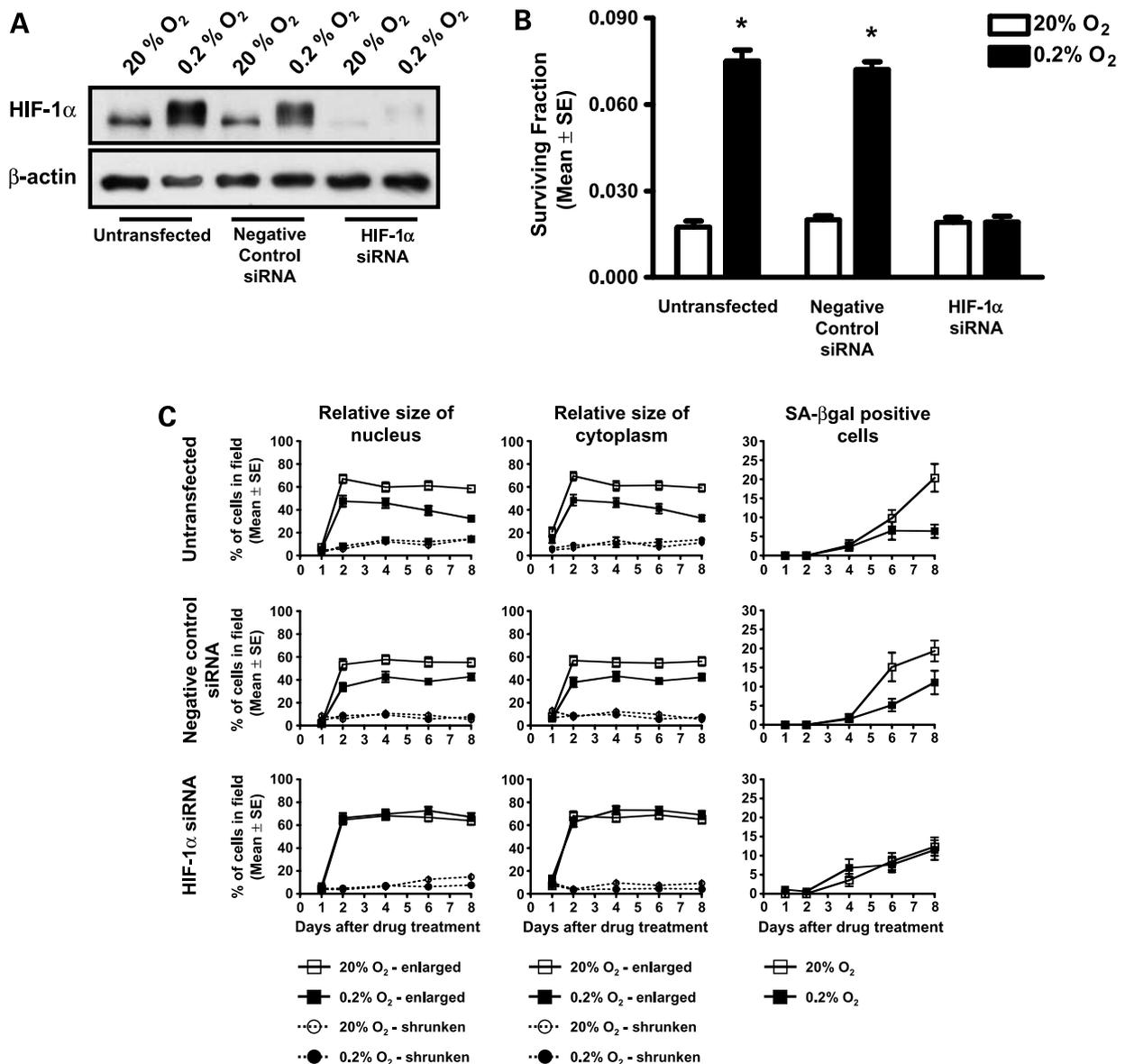


Figure 6. Effect of siRNA on HIF-1 α accumulation, hypoxia-induced resistance to doxorubicin, and doxorubicin-induced morphologic changes in MDA-MB-231 cells. Cells were either transfected with negative control siRNA or HIF-1 α siRNA or left untransfected. Forty-eight hours later, they were incubated under standard (20% O₂) or hypoxic (0.2% O₂) conditions for an additional 24 h before analysis by Western blot, clonogenic assay, or microscopic scoring of cell morphology. **A**, cell lysates were analyzed by immunoblot assay using anti-HIF-1 α or anti- β -actin monoclonal antibodies. **B**, following the treatment outlined above, cells were exposed to 5 μ mol/L doxorubicin for 1 h and clonogenic survival was assessed. Compared with the survival of cells incubated in 20% O₂, the survival of untransfected or negative control siRNA-transfected cells preexposed to 0.2% O₂ was significantly higher. *, $P < 0.0001$. In contrast, the survival of HIF-1 α siRNA-transfected cells following preexposure to hypoxic conditions was not significantly different compared with the survival of cells maintained under standard conditions. **Columns**, mean surviving fractions; **bars**, SE. Results are representative of five independent experiments. **C**, cells were treated as described in **B** and cultured on glass coverslips under standard conditions (20% O₂) for up to 8 d. Graphs indicate the time course of drug-induced changes in the fraction of cells with enlarged or shrunken nuclei, enlarged or shrunken cytoplasm, and detectable SA- β gal activity. The complete analysis from morphologic scoring is shown in the Supplementary Data. Preexposure of untransfected and negative control siRNA-transfected cells to hypoxia led to significant reductions in the percentages of cells with enlarged nuclei ($P < 0.05$), enlarged cytoplasm ($P < 0.01$), and SA- β gal activity ($P < 0.05$) following doxorubicin treatment (reported P values correspond to differences at day 8 following drug exposure). Transfection of cells with HIF-1 α siRNA completely prevented the hypoxia-induced decreases in the proportions of cells with enlarged nuclei, enlarged cytoplasm, or SA- β gal activity. **Points**, mean percentage of cells scored in the microscopic field; **bars**, SE. Results are representative of two independent experiments.

In contrast to the well-characterized family of proteins regulating the apoptotic response, the signaling pathways and molecular regulators involved in drug-induced senescence have yet to be clearly defined. The p53-p21-p16

cascade is thought to play an important role in initiating and maintaining terminal growth arrest following the onset of DNA and other cellular damage (14, 18, 27). Although there are conflicting data about the requirement for

functional p53 in drug-induced senescence, characteristic morphologic changes and detection of SA- β gal activity have been reported in p53-deficient as well as p21- and p16-deficient colon cancer cells, suggesting that these proteins may function as positive regulators of the response rather than absolute requirements (14, 16). Our findings support a model in which the induction of drug-induced senescence, as well as escape from it, can occur independently of p53 activity as the data reveal that hypoxia protects both p53 wild-type HCT116 and p53-mutant MDA-MB-231 cells from drug-induced senescence.

Recent studies have shown that cells cultured under hypoxic conditions can acquire the ability to delay the onset of replicative senescence through a mechanism mediated, at least in part, by HIF-1-dependent up-regulation of hypoxia response element-containing genes such as macrophage migration inhibitory factor (34, 35). Although hypoxia may potentially act in a similar fashion to prevent the initiation or maintenance of drug-induced senescence, an alternative mechanism for hypoxia-induced resistance is proposed. As outlined in Introduction, there is evidence that suppression of a single pathway of cell death may be insufficient for the acquisition of drug resistance in damaged cells due to the activation of alternative death or nonproliferative pathways (20–22). A corollary to this hypothesis is that the hypoxia-induced increase in survival observed in tumor cells is a consequence of increased protection against, or repair of, drug-induced damage rather than the result of an acquired inability to undergo cell death or senescence due to hypoxic alterations in the signaling pathways regulating these nonproliferative fates. This hypothesis is supported by preliminary data revealing that preexposure of MDA-MB-231 or HCT116 cells to hypoxia results in decreased levels of etoposide-induced DNA damage (specifically DNA strand breaks measured by comet assays) relative to cells maintained in standard culture conditions.⁴ The effect of hypoxia on DNA damage is observed immediately following a 1-h drug treatment under identical conditions to those used in clonogenic assays, indicating that cells exposed to hypoxia have already acquired a survival advantage over well-oxygenated cells several hours (or days) before the initiation of downstream signaling pathways regulating cell arrest or programmed cell death. Our findings are supported by evidence that cells expressing HIF-1 α have increased double-strand break repair efficiency and elevated resistance to carboplatin, etoposide, and ionizing radiation compared with HIF-1 α -deficient cells (8). Further studies investigating the effects of hypoxia on the mechanisms regulating DNA damage and repair are under way in our laboratory.

In conclusion, our findings support a requirement for HIF-1 activity in the adaptations leading to the drug resistance phenotype and reveal that hypoxia can protect

tumor cells from drug-induced senescence leading to the development of hypoxia-induced resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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