Abstract

Hypoxia-inducible factor 1 (HIF-1) is a master transcription factor that controls cellular homeostasis. Although its activation benefits normal tissue, HIF-1 activation in tumors is a major risk factor for angiogenesis, therapeutic resistance, and poor prognosis. HIF-1 activity is usually suppressed under normoxic conditions because of rapid oxygen-dependent degradation of HIF-1α. Here, we show that, under normoxic conditions, HIF-1α is upregulated in tumor cells in response to doxorubicin, a chemotherapeutic agent used to treat many cancers. In addition, doxorubicin enhanced VEGF secretion by normoxic tumor cells and stimulated tumor angiogenesis. Doxorubicin-induced accumulation of HIF-1α in normoxic cells was caused by increased expression and activation of STAT1, the activation of which stimulated expression of iNOS and its synthesis of nitric oxide (NO) in tumor cells. Mechanistic investigations established that blocking NO synthesis or STAT1 activation was sufficient to attenuate the HIF-1α accumulation induced by doxorubicin in normoxic cancer cells. To our knowledge, this is the first report that a chemotherapeutic drug can induce HIF-1α accumulation in normoxic cells, an efficacy-limiting activity. Our results argue that HIF-1α-targeting strategies may enhance doxorubicin efficacy. More generally, they suggest a broader perspective on the design of combination chemotherapy approaches with immediate clinical impact. Cancer Res; 73(20); 1–13. ©2013 AACR.
only, factor stabilizing HIF-1α. Hypoxia-mimetic drug cobalt chloride, iron chelators, nitric oxide (NO), free radicals, and genetic alterations can enhance HIF-1 expression under normoxic conditions (1, 3, 9, 12–14).

Doxorubicin (adriamycin) is a first-line chemotherapeutic drug for treating a wide spectrum of cancers. Lee and colleagues elegantly showed that doxorubicin inhibited HIF-1α transcriptional activity by blocking the binding of hypoxia-induced HIF-1α to DNA (15). HIF-1α knockdown or inhibition increases the sensitivity of hypoxic tumor cells to doxorubicin (16, 17). These studies suggest a synergistic anticancer effect by combining chemotherapy with HIF-1α inhibition to target hypoxic tumor cells. In contrast to considerable investigations into the interplay between chemotherapeutic drug and HIF-1α under hypoxic conditions, so far it is unknown whether chemotherapeutic drugs could regulate HIF-1α expression under normoxic conditions. One reason for this dearth is the technical difficulty of sensitively detecting HIF-1α in cells exposed to both normoxia and chemotherapy – two adverse conditions for HIF-1α expression because of oxygen-dependent HIF-1α degradation and impaired protein synthesis machinery due to cytotoxicity. To elucidate the effects of chemotherapy on HIF-1α expression in normoxic tumor cells, we used a 4T1 mouse breast tumor cell line (4T1ODD-luc) that was stably transduced with a fused HIF-1α reporter gene consisting of a mouse HIF-1α ODD domain and a firefly luciferase (9). This reporter cell line allows noninvasive monitoring HIF-1α expression with high sensitivity (9). Previous studies have identified that doxorubicin concentrations in 4T1 and MCF-7 tumors ranged between 0 and 12 μg/mL (18, 19) and 0 and 8 μg/mL (20), respectively. By treating 4T1ODD-luc and MCF-7 cells with doxorubicin at concentrations within these ranges, we found increased HIF-1α expression under normoxic conditions. We then investigated the effects of this normoxic HIF-1α accumulation on VEGF secretion and tumor angiogenesis after doxorubicin chemotherapy. More importantly, we identified the underlying mechanism that was the activation of the STAT1–iNOS–NO–HIF-1α signaling pathway. In addition, we explored therapeutic strategies to suppress doxorubicin-induced normoxic HIF-1α accumulation. This work has important implications for trials targeting HIF-1α, which is upregulated not only by hypoxia but also by chemotherapy.

Materials and Methods

Cell culture

4T1 mouse breast tumor cells and MCF-7 human breast cancer cells were obtained from the Duke University cell culture facility in 2006. 4T1ODD-luc HIF-1α reporter cells were obtained from Chuan-Yuan Li’s laboratory in 2006. 4T1ODD-luc and MCF-7 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS and 1% antibiotic–antimycotic under normoxic conditions (95% air, 5% CO2). Then, 5 × 104 4T1ODD-luc or 1 × 106 MCF-7 cells per 10-cm dish were plated for the Western blots. Similarly, 1 × 106 4T1ODD-luc cells per 10-cm dish or 2.5 × 105 MCF-7 cells per well of six-well plates were plated overnight for fluorescence-activated cell sorting (FACS). The cells were cultured in fresh medium with drugs the next day and continued for 24, 48, or 72 hours. Doxorubicin (Bedford Laboratories) concentrations are provided in figures, or is 1 μg/mL (1.72 μmol/L) for 4T1 cells and 0.5 μg/mL (0.86 μmol/L) for MCF-7 cells. The concentrations of 1400W and EGCG (Cayman Chemical) were 10 μg/mL and 15 μmol/L, respectively. An equal volume of solvent was used as the negative control. Hypoxic culture condition was 0.5% O2, 5% CO2, and N2 balanced for 48 hours.

Bioluminescent imaging

HIF-1α ODD-luciferase reporter activity was quantified by the Xenogen IVIS bioluminescence imaging system (9). For this, 1 × 107 4T1ODD-luc cells per well of 12-well plates were cultured with 2 mL culture medium overnight. The cells were treated the next day with fresh medium containing doxorubicin (0.1, 1, or 10 μg/mL; 0.172–17.2 μmol/L) or an equal volume of control vehicle for 24, 48, or 72 hours under normoxic conditions. Alternatively, 1 × 107 4T1ODD-luc cells were treated with 0.1, 1, or 10 mmol/L l-NAME (Sigma-Aldrich) or control vehicle ± 1 μg/mL doxorubicin for 24, 48, or 72 hours. Cell reporter activity and tumor volume were measured on day 0 before treatment and at multiple time-points posttreatment. The mice were imaged 10 minutes after intraperitoneal (i.p.) injection of luciferin (150 mg/kg). Tumor bioluminescence intensity was normalized to tumor volume at each time point.

Animal studies

In a mouse model, 5 × 106 4T1ODD-luc cells with 200 μL PBS were injected orthotopically in the right thoracic mammary fat pad of each female Ncr/nu nude mouse (3). Tumor volume was calculated as: volume = length × width2 × π/6. When tumor size reached seven millimeter in diameter, animals were randomized and injected with 100 μL saline (control) or a maximum tolerated dose (MTD) of doxorubicin (10 mg/kg) via tail vein.

ELISA

A total of 1 × 107 4T1ODD-luc cells per well of 12-well plate were cultured with one milliliter medium overnight. The cells were treated with 0, 0.1, 1, or 10 μg/mL of doxorubicin for 24, 48, and 72 hours. VEGF in culture medium was quantified by a mouse VEGF ELISA kit (R&D Systems).

Real-time PCR

Total RNA was prepared using the miRVana extraction kit (Applied Biosystems). One microgram of total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad). Real-time PCR was conducted on a ABI7900HT Fast Real-Time PCR System using Power SYBRGreen PCR Mix (Applied Biosystems). PCR products were verified by melting curves. The threshold cycle (Ct) values for each gene were normalized to expression levels of β-actin. The β-actin primers were: forward 5′-GTTACTGCTGCTTGCTTCTAC-3′; reverse 5′-GACTCATCGACTCTCTGTGG-3′. The mouse iNOS primers were: forward 5′-CTGTAAGACCTTGTGATGGCAGAGG-3′; reverse 5′-CTGGATAGCCTATATGGCTTG-3′.
Western blot analysis
Protein samples from 4T1ODD-luc cells and MCF-7 cells were respectively collected on days 2 and 3 posttreatment. Nuclear proteins were extracted using a NucBuster kit (Novagen). Equal amounts of protein samples were loaded and detected with corresponding antibodies: monoclonal mouse anti-HIF-1α antibody (NB100-105, 1:500 dilution; Novus Biologicals), rabbit polyclonal anti-HIF-1α antibody (NB100-654, 1:500 dilution; Novus Biologicals), mouse anti-α-tubulin antibody (1:10,000 dilution; Sigma-Aldrich), monoclonal mouse anti-β-actin antibody (A2228, 1:5,000 dilution; Sigma-Aldrich), polyclonal rabbit anti-iNOS antibody (1:500 dilution; Assay Designs), polyclonal sheep anti-histone H1 antibody (NB100-748, 1:500 dilution; Novus Biologicals), rabbit anti-STAT1/ phosphor-STAT1 (Tyr701)/phosphor-STAT1 (Ser727) antibodies (1:1,000 dilution; Cell Signaling Technology), and monoclonal rabbit anti-jAK2/phospho-jAK2 (Tyr1007/1008) antibodies (1:1,000 dilution; Cell Signaling Technology).

Immunohistochemical staining
Five to six orthotopic 4T1ODD-luc tumors per group were collected on days 0, 1, 4, 7, and 16. Hypoxia marker pimonidazole (NPI) and perfusion marker Hoechst33342 (Sigma-Aldrich) were administered as described previously (14). Thereafter, 10-μm frozen sections were made. HIF-1α fluorescent staining was conducted with anti-HIF-1α antibody (Novus Biologicals). Direct pimonidazole labeling was done using Hypoxyprobe (NPI) with Zenon Alexa Fluor555 Mouse IgG Labeling Kit (Invitrogen). Scanned images of each entire tumor section were composited by Metamorph to compare the tumor area in each field. Tumor-activated macrophages (TAM) were stained with rat anti-mouse CD68 antibody (Serotec) and Alexa488-conjugated anti-rat IgG antibody (Invitrogen).

FACS analysis
Single-cell suspensions were made after mild trypsinization at each time point and incubated with the NO-specific probe DAF-FM diacetate according to the manufacturer’s instructions (Invitrogen). FACS was conducted on a FACSCalibur flow cytometer (Becton Dickinson). The cells without DAF-FM probe and the cells exposed to 0.2 mmol/L S-nitrosoglutathione (GSNO) for four hours were the negative and positive controls, respectively.

Cell viability assay
Controls, respectively. Cell viability assay of cultured with 100 μL medium overnight. We replaced the medium with a fresh one containing control vehicle (PBS), 10 μg/mL 1400 W, or 15 μmol/L EGCG, ± 1 μg/mL doxorubicin the next day. The treatments continued for 24, 48, and 72 hours. Daily cell viability was quantified and normalized to the value on day 0. WST-1 cell viability assay (Roche Applied Science): cells were plated in a 96-well plate overnight and were treated with 1 μg/mL doxorubicin, t-NAMe (0.1, 1.0, or 10 mmol/L), or a combination of these compounds the next day. At 24, 48, and 72 hours posttreatment, the WST-1 reagent was added to the wells and cell viability was assessed by a spectrometer according to the manufacturer's instructions.

STAT1 silencing
A total of 1 × 10⁶ 4T1 cells were plated in 10-cm dishes and cultured in 5 mL medium overnight. The cells were then transfected with either the STAT1 siRNA (ON-TARGETplus SMARTpool, mouse STAT1) or the scrambled siRNA control (ON-TARGETplus Non-targeting siRNA; Thermo Fisher Scientific) with the Lipofectamine 2000 transfection reagent (Invitrogen). The cells were treated with 0.5 μg/mL doxorubicin or a vehicle control (PBS). Approximately 24 hours after transfection and drug treatment, the media was replaced with fresh media and drug. Cells were harvested 48 hours posttreatment for assessment of STAT1 and HIF-1 protein expression. These experiments were repeated four times.

Results
Doxorubicin increases HIF-1α level in tumor cells both in vitro and in vivo
To determine whether doxorubicin affects HIF-1α level in normoxic tumor cells, we treated 4T1ODD-luc reporter cells with increased doxorubicin concentrations (0, 0.1, 1, or 10 μg/mL) for 24, 48, and 72 hours. All three concentrations of doxorubicin induced significant increases in HIF-1α reporter activity 48 hours posttreatment (Fig. 1A and B). However, 1 μg/mL of doxorubicin induced the most potent upregulation of HIF-1α reporter activity on both 48 hours and 72 hours posttreatment. To confirm doxorubicin-induced increase in normoxic HIF-1α accumulation, we detected HIF-1α protein expression by Western blots, which showed enhanced HIF-1α expression in 4T1ODD-luc cells and MCF-7 cells posttreatment (Fig. 1C). This doxorubicin-induced normoxic HIF-1α accumulation was also verified by a hypoxia-induced HIF-1α control (Supplementary Fig. S1). These results reveal that doxorubicin upregulates HIF-1α expression in tumor cells under normoxic conditions in vitro.

To further determine whether doxorubicin may affect HIF-1α level in tumors, we intravenously injected MTD of doxorubicin into female nude mice with orthotopic 4T1ODD-luc tumors. Mean bioluminescence intensities in doxorubicin-treated tumors were significantly higher than controls. The major time window of the increase in HIF-1α reporter activity in vivo was from day 3 through day 5 after doxorubicin
treatment (Fig. 1D and E). These findings suggest that doxorubicin chemotherapy upregulates HIF-1α level in tumor cells in vivo.

Because hypoxia and poor perfusion are common causes for HIF-1α upregulation in tumors, we next sought to identify whether the earlier doxorubicin-induced in vivo HIF-1α upregulation was due to potential changes in tumor hypoxia or perfusion after doxorubicin therapy. We compared the positive-area fractions of HIF-1α, hypoxic marker pimonidazole, and perfusion dye Hoechst 33342 in whole frozen sections of 4T1ODD-luc tumors at multiple time points posttreatment. Doxorubicin significantly increased tumor HIF-1α fraction on posttreatment days 1, 4, and 7 when compared with control treatment (Fig. 2A and B). The increased HIF-1α fractions in doxorubicin-treated tumors confirmed the enhanced HIF-1α reporter activities as described earlier (Fig. 1D and E). In contrast, there was no difference in either the pimonidazole or perfused tumor fractions (Hoechst 33342 labeling) between the doxorubicin- and the saline-treated tumors (Fig. 2C and Supplementary Fig. S2). These results suggest that doxorubicin-induced HIF-1α upregulation was not caused by aggravated tumor hypoxia or decreased perfusion.

Doxorubicin-induced HIF-1α upregulation stimulates VEGF secretion by tumor cells in vitro and tumor angiogenesis in vivo

One important consequence of HIF-1α upregulation is to form HIF-1 to promote the expression of VEGF – one of the most potent angiogenic factors (1, 2). Because doxorubicin upregulates tumor cell HIF-1α both in vitro and in vivo, we asked whether this doxorubicin-induced HIF-1α may stimulate VEGF secretion by tumor cells and promote tumor angiogenesis. Cell counting showed that the 0.1, 1, and 10 μg/mL doxorubicin treatments significantly reduced surviving cell numbers, which were less than 40% of the control cell numbers at all three time points posttreatment (Fig. 3A). However, despite a much smaller surviving cell number, the
Doxorubicin Induces Normoxic HIF-1α Accumulation in Tumor Cells

**Figure 2.** The upregulated HIF-1α expression in doxorubicin-treated 4T1ODD-luc tumors was not due to hypoxia. A, representative immunofluorescent stainings showing the distribution of HIF-1α (green) and pimonidazole (red) in two adjacent entire tumor sections five days posttreatment. Scale bars, 1 mm. B, HIF-1α fraction in 4T1ODD-luc tumors on days 0, 1, 4, 7, and 16 posttreatment (n = 5, mean ± SE). *P < 0.05; **P < 0.01, Student t test. C, pimonidazole fraction in 4T1ODD-luc tumors on days 0, 1, 4, 7, and 16 posttreatment (n = 5, mean ± SE). *P > 0.05, Student t test.

Doxorubicin-treated cells secreted at least 87% of the amount of VEGF secreted by the control cells on day 1 (Fig. 3B). This pattern persisted on days 2 and 3 when, despite less than 30% of relative surviving cell number post doxorubicin treatment compared with control treatment, the VEGF secreted by this small fraction of surviving cells was more than 60% of the amount of VEGF secreted by the control cells. In other words, the surviving doxorubicin-treated cells secreted an increased amount of VEGF relative to control cells. The other piece of evidence was that 1 μg/mL doxorubicin did lead to higher VEGF secretion than 0.1 μg/mL doxorubicin on both days 1 and 2 (Fig. 3B). Therefore, these data support that doxorubicin stimulates VEGF secretion in surviving tumor cells. Because tumor vascular index rather than tumor size is the most reliable prognostic indicator for tumor relapse after chemotherapy, we then compared the relative tumor vascular fraction in tumors treated with doxorubicin versus saline to see whether the doxorubicin-induced increase in VEGF secretion by tumor cells may affect tumor angiogenesis. The relative tumor vasculature fraction in doxorubicin-treated tumors was significantly higher compared with the control tumors four days after a single MTD treatment (Fig. 3C and D). These findings suggest that doxorubicin not only upregulates HIF-1α expression and promotes VEGF secretion in surviving tumor cells, but also stimulates tumor angiogenesis shortly after treatment.

**NO and nitric oxide synthase play important roles in doxorubicin-induced normoxic HIF-1α accumulation**

Because the earlier results suggest that hypoxia is not the cause for doxorubicin-induced HIF-1α upregulation, we sought to identify other nonhypoxia factors that may upregulate HIF-1α. Kimura and colleagues reported that NO donors stimulated HIF-1α expression and VEGF reporter activity in normoxic tumor cells (22). In addition, Metzen and colleagues found that NO impaired HIF-1α degradation under normoxic conditions (23). Based on this converging evidence, we investigated whether doxorubicin could increase intracellular NO level in normoxic 4T1ODD-luc cells. The NO-specific fluorescent probe DAF-FM diacetate showed that doxorubicin significantly increased the intracellular NO level compared with control treatment (Fig. 4A). Higher concentrations of doxorubicin led to higher intracellular NO level. The earlier HIF-1α bioluminescent reporter assay (Fig. 1A) and Western blots (Supplementary Fig. S3) also showed that higher concentrations of doxorubicin led to more HIF-1α expression. The parallel changes in intracellular NO level and HIF-1α expression suggest that NO may play an important role in doxorubicin-induced normoxic HIF-1α accumulation. Nitric oxide synthases (NOS) are enzymes that catalyze the synthesis of NO from L-arginine. The NOS family includes inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). To identify whether NOS participated in the doxorubicin-induced normoxic HIF-1α accumulation, we treated 4T1ODD-luc cells with increasing concentrations of general NOS inhibitor L-NAME with or without doxorubicin (Fig. 4B). In the control group without doxorubicin, increasing concentrations of L-NAME did not cause any difference in HIF-1α reporter activity at each time point (Fig. 4B and C). Doxorubicin alone significantly increased HIF-1α reporter activity in the normoxic 4T1ODD-luc cells (Fig. 4B and D, empty column). This finding is consistent with the results in Fig. 1A and B. When L-NAME was combined with doxorubicin, the doxorubicin-induced enhancement of HIF-1α reporter activity was significantly suppressed (Fig. 4D). In addition, we conducted a quantitative WST-1 cell viability assay to compare the surviving fraction of tumor cells at 24, 48, and 72 hours after these treatments (Supplementary Fig. S4). Statistical...
analysis revealed that the combination of doxorubicin + L-NAME (0.1, 1, 10 mmol/L) treatments did not increase cell death compared with the treatment with doxorubicin alone (Supplementary Fig. S4). This result rules out increased cell death as a possible cause of decreased HIF-1α reporter activity in the cells treated with doxorubicin + L-NAME (Fig. 4D). Therefore, the earlier experiments suggest that NOS and NO participate in doxorubicin-induced normoxic HIF-1α accumulation.

STAT1 and iNOS signaling pathway participates in doxorubicin-induced normoxic HIF-1α accumulation

iNOS is the isoenzyme most commonly associated with carcinogenesis and tumor progression. Because iNOS overexpression led to HIF-1α accumulation in kidney cells (24), we sought to determine the role of iNOS in the doxorubicin-induced normoxic HIF-1α accumulation in tumor cells. Quantitative real-time PCR showed that doxorubicin significantly enhanced iNOS transcription compared with control treatment (Fig. 5A). Western blots further confirmed that doxorubicin stimulated iNOS protein expression under normoxic conditions (Fig. 5B). Because STAT1 is a transcription factor required for iNOS transcription and activation (25), we then sought to determine whether STAT1 is an upstream regulator enhancing iNOS expression in response to doxorubicin treatment. Western blots showed that doxorubicin not only stimulated the expression of STAT1 but also promoted STAT1 activation through the phosphorylation of Tyr701 and Ser727 residues (Fig. 5C).

The JAK protein family member JAK2 regulates neoplasm, blood cell development, and immune function in response to growth factors and pro-inflammatory cytokines. As an upstream kinase for STAT phosphorylation, JAK2 needs to be activated first by phosphorylation. We conducted a Western blot to determine whether JAK2 might be required for the earlier STAT1 phosphorylation. Because doxorubicin decreases total JAK2 expression and there is no JAK2 phosphorylation after doxorubicin or control treatment (Supplementary Fig. S5), JAK2 does not participate in the doxorubicin-stimulated STAT1 phosphorylation. The roles

Figure 3. Doxorubicin stimulates VEGF secretion by 4T1ODD-luc cells and causes a resurgent tumor angiogenesis. A, relative viable cell numbers of all doxorubicin-treated groups were less than 40% compared with the control ($n = 3$, mean $\pm$ SE). $P < 0.05$ compared with the control group, one-way ANOVA. B, mouse VEGF ELISA showing VEGF concentrations in the cell culture medium of 4T1ODD-luc cells treated with doxorubicin (0.1, 1, and 10 μg/ml) versus control treatment ($n = 3$, mean $\pm$ SE), $^* P < 0.005$, one-way ANOVA. C, representative fluorescent staining of vasculature in 4T1 ODD-luc tumors four days posttreatment. Scale bar, 100 μm. D, relative tumor vascular fractions in 4T1ODD-luc tumors ($n = 5$ in doxorubicin groups on days 0, 4, 7, and 16, $n = 6$ in all other groups, mean $\pm$ SE), $^* P < 0.05$, Student t test.
of other JAK family members or other non-JAK family kinases in this process merit further investigation. To confirm the importance of the STAT1-iNOS signaling pathway in doxorubicin-induced normoxic HIF-1α accumulation, we investigated whether disruption of iNOS or STAT1 would suppress doxorubicin-induced HIF-1α upregulation. To this end, 4T1ODD-luc cells were treated with either the iNOS-specific inhibitor 1400W or the STAT1-interfering chemical EGCG with or without combined doxorubicin treatment (26). Without doxorubicin, 1400W or EGCG only suppressed the basal-level iNOS expression compared with control treatment. When combined with doxorubicin, both 1400W and EGCG effectively suppressed doxorubicin-induced iNOS upregulation in 4T1ODD-luc cells (Fig. 5D, Supplementary Fig. 5A and 5B). Western blots further confirmed that doxorubicin-induced upregulations of STAT1 expression and activation were suppressed by EGCG (Fig. 5E). These results suggest that the STAT1–iNOS signaling pathway participates in doxorubicin-induced normoxic HIF-1α accumulation.

To determine whether inhibition of iNOS could suppress the doxorubicin-induced increase in intracellular NO synthesis, we treated 4T1ODD-luc and MCF-7 cells with doxorubicin in the presence or absence of 1400W. FACS analysis showed that 1400W significantly suppressed the doxorubicin-induced upregulation of NO synthesis in both cell lines (Fig. 6A and B). To determine whether inhibition of STAT1 could suppress the doxorubicin-induced increase in intracellular NO level, we treated both cell lines with doxorubicin in the presence or absence of EGCG. FACS analysis showed that EGCG also significantly suppressed the doxorubicin-induced upregulation of NO synthesis in both cell lines (Fig. 6C and D). Because inhibition of either iNOS or STAT1 can suppress doxorubicin-induced NO upregulation, we then sought to determine whether 1400W or EGCG could suppress doxorubicin-induced normoxic
HIF-1α accumulation. Western blots showed that 1400W and EGCG suppressed doxorubicin-induced HIF-1α upregulation in both cell lines under normoxic conditions (Fig. 6E and F). To verify the role of STAT1 in doxorubicin-induced HIF-1α upregulation, we knocked down STAT1 by siRNA. STAT1 siRNA decreased 51% of STAT1 expression and 42% of HIF-1α expression compared with the scrambled siRNA control after doxorubicin treatment (Fig. 6G and H). This finding is consistent with the earlier result after inhibition of STAT1 by EGCG and confirms that STAT1 is important for doxorubicin-induced HIF-1α upregulation. These results support the conclusion that doxorubicin induces normoxic HIF-1α accumulation by activating the STAT1–iNOS–NO–HIF-1α signaling pathway. Therefore, STAT1 and iNOS are rational targets to suppress the upregulated HIF-1α expression in doxorubicin-treated normoxic tumor cells. Because of the pleiotropic effects of STAT1 and iNOS in apoptosis (27, 28), we took additional caution to estimate the cytotoxicity of doxorubicin combined with EGCG or 1400W against tumor cells. Compared to the initial cell viability right before treatment, the relative cell viabilities of doxorubicin, doxorubicin + 1400W, or doxorubicin + EGCG treatment were individually 37.13 ± 1.60%, 36.54 ± 1.13%, and 87.62 ± 3.77% 48 hours posttreatment, respectively; and were 7.83 ± 0.47%, 7.83 ± 0.20%, and 29.77 ± 1.79% 72 hours posttreatment, respectively. Doxorubicin combined with EGCG or 1400W still maintained significant cytotoxicity against tumor cells compared to control treatment (Fig. 7A). Although there was slightly higher cell viability after Dox + EGCG treatment, there was no attenuation in cytotoxicity after Dox + 1400W treatment compared with Dox treatment.

Discussion

There has been intense interest in developing novel therapeutic strategies to target HIF-1α in cancer therapy for three main reasons: (i) HIF-1α expression has been found in the majority of tumors because of hypoxia, which is usually absent in normal tissues (29). This differential expression of HIF-1α between malignant and normal tissues allows inhibition of HIF-1α to target cancer cells while sparing normal tissues. (ii) Because HIF-1 is a master regulator for many aspects in cancer biology, inhibition of HIF-1α leads to the disruption of multiple important mechanisms for tumor cell survival, angiogenesis, and progression. (iii) Inhibition of HIF-1α may exploit tumor hypoxia by converting it from a treatment obstacle into a targeting advantage (30). Recent studies have shown that many anticancer therapies such as radiotherapy, photodynamic therapy, and hyperthermia can upregulate HIF-1α expression in tumor cells (3, 9, 31, 32). Although these findings improved the understanding of HIF-1α stabilization induced by other factors rather than hypoxia, very little is known about the effects of chemotherapeutic agents on HIF-1α expression in normoxic cancer cells. Nevertheless, it is important to address this question, not only because HIF-1α is a major determinant for cancer cell homeostasis under cytotoxic stress (1, 13), but also because normoxic HIF-1α accumulation is sufficient to initiate tumor angiogenesis and to promote cancer cell chemoresistance (14). The majority of
chemotherapeutic drugs exert most of their cytotoxic effects on normoxic cells rather than hypoxic cells. The reduced cytotoxicity against hypoxic tumor cells is because of multiple mechanisms including limited penetration distance of drugs in hypoxic tumor regions (33), a low proliferative fraction in hypoxic tumor cell populations, and stimulated detoxification/chemoresistance machinery (30). Therefore, it is generally believed that normoxic tumor cells are more sensitive to chemotherapy than hypoxic tumor cells. However, this paradigm is true only when normoxic tumor cells are exposed to enough high drug concentration to kill them before they activate homeostatic, survival, and antiapoptotic signaling pathways. Without a sufficiently high drug concentration locally, even a normoxic tumor microenvironment does not guarantee that all tumor cells are sensitive to chemotherapy, especially when treated tumor cells activate survival molecules such as HIF-1. To determine the effects of doxorubicin on normoxic HIF-1α accumulation, we selected several doxorubicin concentrations in the ranges of in vivo drug concentrations in 4T1 and MCF-7 tumors (18–20). This study reveals that surviving normoxic tumor cells are able to accumulate HIF-1α, which promotes VEGF secretion after doxorubicin treatment. Cell viability assay showed that 37.13% and 7.83% of doxorubicin-treated cells survived for 48 and 72 hours.
respectively after doxorubicin treatment (Fig. 7A). Many studies have proven that even less than five percent of the entire cancer cell population, when it survives, is enough to result in cancer relapse. Because HIF-1 is a key transcriptional factor regulating hundreds of downstream genes, identification of key molecular mechanisms for normoxic HIF-1α accumulation in tumor cells during chemotherapy may provide important insights into how tumor cells respond to chemotherapy, gain survival advantage, and develop therapeutic resistance causing tumor relapse. This study takes a major step toward addressing this very important issue. The significance of doxorubicin-induced normoxic HIF-1α accumulation in tumor cells and the underlying mechanism are explicated further.

First, we found that the STAT1–INOS–NO–HIF-1α signaling pathway is important for doxorubicin-induced normoxic HIF-1α accumulation (Fig. 7B). STAT1 is a transcription activator for INOS expression and NO synthesis (25). Previous studies have shown that NO enhances the expression and activity of HIF-1α under normoxic conditions through three mechanisms: (i) NO inhibits prolyl hydroxylase activity to stabilize HIF-1α (23, 34); (ii) NO S-nitrosylates the Cys533 in HIF-1α ODD domain to prevents HIF-1α degradation (9); and (iii) NO inhibits FIH enzyme activity to abolish the FIH-mediated asparagine hydroxylation of HIF-1α and activates the transcription of HIF-1 downstream genes (35). The time-course of in vivo HIF-1α upregulation in 4T1ODD-luc tumors is three to five days after doxorubicin treatment, which overlaps the time of HIF-1α upregulation induced by radiotherapy (4–7 days postradiotherapy; ref. 9). Li and colleagues found that ionizing radiation stimulated TAMs to synthesize NO. The exogenous NO produced by TAMs then diffused into 4T1ODD-luc reporter cells and S-nitrosylated Cys533 in HIF-1α ODD domain to stabilize HIF-1α under normoxic conditions (9). The use of the same 4T1ODD-luc reporter cell line allowed us to compare the NO-producing cells between doxorubicin chemotherapy and radiotherapy. The absence of TAMs in our in vitro study (Fig. 1 and Figs. 4–6) and the nonsignificant difference in activated TAM fractions (TAM-marker CD68 staining) between the doxorubicin-treated tumors and the control tumors in our in vivo study (Supplementary Fig. S7) strongly suggest that the NO synthesized in doxorubicin-treated tumor cells is sufficient to elicit normoxic HIF-1α accumulation independent of other exogenous NO-producing cells such as TAMs. This work also shows that doxorubicin dosages have different effects on tumor HIF-1 activity. Lee and colleagues elegantly reported that low-dose metronomic doxorubicin therapy (1 mg/kg/day for five days) inhibited HIF-1 transcriptional activity in tumor xenografts (15). The dose of doxorubicin in their metronomic therapy was only 1/10 of the single bolus MTD of doxorubicin (10 mg/kg) in this study. Low-dose metronomic doxorubicin therapy inhibits HIF-1 activity, whereas treatment with doxorubicin at MTD induces HIF-1. Therefore, doxorubicin may play multiple roles to regulate HIF-1 expression and activity depending on dosing, timing, and oxygen tensions. In addition to this mechanism, previous studies suggest that other important mechanisms may also contribute to doxorubicin-induced normoxic HIF-1α accumulation: (i) the degradation of HIF-1α depends on HIF-PH, which requires both Fe2⁺ and molecular oxygen (7, 8). When Fe2⁺ is removed by chelating agents such as desferrioxamine (DFX) or substituted by Co2⁺ or Ni2⁺, HIF-1α degradation is inhibited. Therefore, DFX and CoF3 are hypoxia mimetics. Because doxorubicin is a strong iron chelator (36), iron deficiency inhibits HIF-PH activity causing normoxic HIF-1α accumulation. (ii) Sinha and colleagues elegantly showed that iron-mediated electron transfer from doxorubicin to molecular oxygen generated free radicals/reactive oxygen species (ROS; ref. 37). Many studies have proven that free radical species and ROS upregulate HIF-1α level (3, 32). We previously provided the first direct in vivo evidence showing that free radicals, ROS, and/or reactive nitrogen species upregulate HIF-1α in tumors (3). Therefore, doxorubicin may induce normoxic HIF-1α accumulation through the generation of free radicals and ROS. The regulatory mechanisms of doxorubicin on signaling pathways, cell toxicity, and metabolism are complex because of its multiple effects on diversified molecular targets including iron
chelation, generation of free radicals/ROS, DNA binding, alkyla-
tion, and inhibition of topoisomerase II (38). Our data add
important new content to this body of knowledge – not only is
the biology relevant to tumors and to therapeutics, but also
exploitation of these findings may hold promise for other
clinical benefits such as reduction of cytotoxicity to normal
tissues.

Second, this work unveils hypoxia-independent VEGF secre-
tion and tumor angiogenesis induced by doxorubicin. Hypoxia
is one important factor for HIF-1 activation, VEGF expression,
and angiogenesis (1, 30, 39). Other nonhypoxia factors such as
radiation therapy and hyperthermia can also upregulate
HIF-1α and VEGF (3, 9, 32). VEGF stimulates tumor growth
by autocrine and paracrine mechanisms (40, 41) because
VEGF is not only a potent proangiogenic factor for tumor
endothelial cells but also a key survival factor for tumor cells
including 4T1 and MCF-7 cells (40, 42). Here, we show that
doxorubicin enhances VEGF secretion in surviving normoxic
tumor cells and stimulates posttreatment tumor angiogenesis
by activating the STAT1–iNOS–NO–HIF-1α–VEGF signaling
pathway (Fig. 7B), which is different from those mechanisms
underlying HIF-1α upregulation induced by radiotherapy
or hyperthermia (9, 32, 43). These new findings also suggest
the importance of combining conventional chemotherapy
such as doxorubicin with anti-VEGF/antiangiogenic therapy
to inhibit tumor VEGF secretion and angiogenesis not
only caused by hypoxia, but also induced by doxorubicin
chemotherapy.

Third, our data indicate that the p53 tumor-suppressor
gene may not be required in doxorubicin-induced activation
of the STAT1–iNOS–NO–HIF-1α signaling pathway. p53 muta-
tion is the most common genetic lesion in human cancers. In
a previous clinical study, Yamaguchi and colleagues showed
that iNOS expression was significantly correlated with tumor
progression by stimulating angiogenesis and there was no
correlation between iNOS and P53 expression (44). The
4T1O0DD-luc cell line is p53 null, whereas MCF-7 expresses
wild-type p53 (45, 46). The HIF-1α upregulation in both
p53-null and p53 wild-type tumor cell lines indicates that
P53 tumor suppressor might not be necessary for doxorubi-
cin-induced normoxic HIF-1α accumulation (Fig. 1C). In addi-
tion, the successful suppression of HIF-1α upregulation by
EGCG or 1400W in both cell lines suggests that the STAT1–
iNOS-targeting strategy does not depend on wild-type P53
expression (Fig. 6E and F). Therefore, doxorubicin chem-
otherapy combined with STAT1 or iNOS inhibitors might be
used to treat both p53 wild-type and p53-mutant tumors.

Finally, our findings raise thought-provoking questions
with regard to chemoresistance. STAT1 is associated with
acquired resistance to doxorubicin (47). However, the
molecular mechanism of STAT1-related therapeutic resis-
tance has not been well understood. Unveiling the activation
of the STAT1–iNOS–NO signaling pathway in doxorubicin-
induced normoxic HIF-1α accumulation may provide
new insight into the roles of STAT1 in chemoresistance.
Muerkoster and colleagues found that pancreatic cancer
cells acquired chemoresistance when treated with the
low-dose chemotherapeutic drug etoposide. Etoposide sti-
mulates intracellular synthesis of NO, which inactivates
caspases causing chemoresistance (48). Inhibition of iNOS
by 1400W rescues caspase activity and enhances tumor
cell chemosensitivity (48). Therefore, iNOS is a key deter-
nant for acquired chemoresistance. Here, we discovered
that doxorubicin also upregulates iNOS expression and
intracellular NO levels (Figs. 5 and 6). Together with
Muerkoster’s findings, it appears that different types of
tumor cells may share this iNOS–NO pathway in response
to chemotherapeutic drugs such as doxorubicin and etopo-
side. Because both doxorubicin and etoposide inhibit topo-
isomerase II, it is worthwhile to further investigate whether
this pathway is a common tumor response to topoisomer-
ase-targeted chemotherapeutic drugs. In addition, we found
that 1400W suppressed the expression and activation of
STAT1 (Fig. 5E). One potential possibility might be that
1400W inhibited iNOS and lowered intracellular NO levels,
thereby restoring caspase activity in a similar manner as that
found in Muerkoster’s study (48). The restored caspase
activity and apoptosis after 1400W treatment might sup-
press the expression and activation of STAT1. The other
potential possibility is based on the finding that NO stimu-
lates STAT1 activation (49). Therefore, the iNOS-specific
inhibitor 1400W might attenuate STAT1 activation by sup-
pressing intracellular NO synthesis. It would be intriguing
to distinguish and verify the above two hypotheses in future.

The mechanistic elucidation of doxorubicin-induced
normoxic HIF-1α accumulation significantly advances under-
standing of tumor response to cytotoxic drugs, which may
help to optimize conventional chemotherapy by the use
of HIF-1 inhibitors. This therapeutic strategy is supported by
a recent study of Zhang and colleagues showing that combined
therapy with HIF-1 inhibitor and doxorubicin achieves signif-
icantly improved tumor control (50). With regard to clinical
significance, this study suggests that combination chemother-
apy with HIF-1α inhibition and doxorubicin would target
not only hypoxic tumor cells but also the heretofore over-
looked normoxic tumor cells.

Disclosure of Potential Conflicts of Interest
JM. Provenzale is a professor of radiology at Emory University School
of Medicine and has a consulting/advisory board relationship with Bayer Phar-
macuticals. No potential conflicts of interest were disclosed by the other authors.

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