Hypoxia Enhances Metastatic Efficiency in HT1080 Fibrosarcoma Cells by Increasing Cell Survival in Lungs, Not Cell Adhesion and Invasion

Li Zhang\textsuperscript{1,2} and Richard P. Hill\textsuperscript{1,2,3}

\textsuperscript{1}Research Division, Ontario Cancer Institute/Princess Margaret Hospital and Departments of \textsuperscript{2}Medical Biophysics and \textsuperscript{3}Radiation Oncology, University of Toronto, Toronto, Ontario, Canada

Abstract

This study examined possible mechanisms for hypoxia-induced metastasis of tumor cells in vivo and in vitro. Tumor hypoxia has been strongly associated with tumor resistance to therapy and malignant progression such as increased metastatic potential (1–3). For tumor cells to form metastases, they have to intravasate into the circulatory system, survive the mechanical shearing force in the circulation, arrest and survive at the remote sites, and then initiate growth and continue to proliferate (4, 5). Although the mechanisms involved in hypoxia-induced metastatic efficiency are not completely understood, studies have suggested that exposure to hypoxia may have an effect on all these steps of the metastatic process. In addition, hypoxia may also promote angiogenesis at primary sites to produce new lymphatic and blood vessels that provide a route for tumor cells to enter the circulation system.

Studies have shown that metastasis is an inefficient event and that the mechanisms for the inefficiency can be cell type specific. For example, in vivo video microscopy and cell fate analysis using mouse melanoma B16F10 cells suggested that metastatic inefficiency is primarily due to regulation of the balance between tumor cell growth and death at secondary sites; the failure of growth initiation of arrested tumor cells plays an important role (4). Studies using transformed rat embryo fibroblasts have shown that apoptosis of lung-arrested tumor cells after i.v. injection is an early event involved in metastatic inefficiency (6). Both experimental and clinical studies have indicated that metastatic efficiency of tumor cells can be associated with their resistance to apoptosis (7–9). Because hypoxia can regulate cell growth through various pathways (10), it is possible that hypoxia may affect metastatic efficiency by modifying the balance between tumor cell death and growth. Although direct evidence to support this hypothesis is still needed, studies have shown that gene products, such as vascular endothelial growth factor (VEGF), a protein up-regulated by hypoxia, can act not only as an angiogenic factor but also as a survival factor for some human tumor cells (11), and blocking VEGF with neutralizing antibody has been reported to significantly decrease metastatic efficiency of some human melanoma cell lines (12, 13). Recently, lysyl oxidase (LOX) has been reported to be essential in hypoxia-induced metastasis, and one of the postulated mechanisms is to allow the growth and proliferation of tumor cells at metastatic sites (14, 15).

Hypoxia-induced LOX can also modify tumor cell motility and invasion; thus, it may increase tumor cell metastatic efficiency by affecting both intravasation and extravasation (14). Other studies have also reported that hypoxia may have an effect on intravasation and extravasation by modulating tumor cell motility and invasion potential through different mechanisms [e.g., through the regulation of protease activity on extracellular matrix (ECM) degradation; refs. 1, 2]. Hypoxia can also enhance the expression of autocrine motility factor, and by doing so increase motility of human pancreatic cancer cells (16). In addition, exposure to 3% oxygen was found to activate transcription of the Met proto-oncogene and consequently to promote the invasive growth of the tumor cells (17). Furthermore, hypoxia-inducible factor-1 (HIF-1), a protein that can be induced by hypoxia through the stabilization of its subunit HIF-1\(\alpha\), can regulate cell invasion in colon carcinoma (18). Recently, it has been reported that hypoxia can stimulate carcinoma invasion by stabilizing microtubules and by promoting the Rab11 trafficking of integrin \(\alpha_{\text{d}}\beta_{4}\) (19).

Hypoxia may also affect tumor cell arrest at metastatic sites by modifying their adhesion potential. Although it has been reported that the arrest of tumor cells is a passive process due to the size restriction of capillaries smaller than tumor cells, there is evidence suggesting that cancer cells may undergo adhesive arrest in the...
liver in precapillary vessels, when the endothelium has been activated by the cytokine interleukin-1α (20). In addition, it has been reported that certain types of tumor cells can be arrested in lungs by attaching to relatively larger pulmonary blood vessels, where the cells are unlikely to be trapped by size limitation (21). This attachment is mediated through the interaction between integrin α5β1 on tumor cells and laminin-5 in blood vessel basement membrane (22). Integrins are proteins that have been shown to play an essential role in cell adhesion to ECM and other cells and to be involved in tumor progression (23). The expression of some integrins can be regulated by hypoxia through the induction of HIF-1 or the activation of extracellular signal-regulated kinase (ERK) 1/2 and p38 mitogen-activated protein kinase, thereby potentially modulating cellular adhesion (24–26). These studies suggest that hypoxia might promote tumor cell arrest by increasing tumor cell adhesion potential through the induction of integrins to increase metastases.

In this study, we investigated mechanisms that may contribute to hypoxia-enhanced metastasis using a green fluorescent protein (GFP)–labeled human fibrosarcoma HT1080 cell line. We observed that the main effect of hypoxic exposure on this cell line was increased survival of cells in the lungs, suggesting that by increasing tumor cell survival alone, hypoxia is able to enhance tumor cell metastatic efficiency. This was further supported by a finding that treatment with farnesylthiosalicylic acid (FTS) both reduced metastatic efficiency in hypoxia preexposed cells and inhibited the effect of hypoxia on tumor cell survival in lungs.

Materials and Methods

Cell culture and hypoxic treatment. HT1080-GFP cells were kindly provided by Dr. Ruth Muschel (Radiobiology Research Institute, Churchill Hospital, Headington, Oxford, United Kingdom). The cells were cultured in α-MEM medium (Invitrogen) supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS). For all experiments, 2 × 10^5 to 5 × 10^5 cells were plated in 10-cm dishes and incubated in 5% CO2 and air at 37°C. To render cells hypoxic, dishes were placed in a plastic chamber (Billups-Rothenberg) flushed with a gas mixture of 5% CO2 and a designated percentage of O2 balance N2. It took 3 to 6 h to achieve severe hypoxia (≤5 mmHg) in medium when 0% O2 was used. For reoxygenation, cells were incubated in a tissue culture incubator. The average rate of oxygen increase was at ~3 mmHg/min during the first 30 min of reoxygenation. FTS was purchased from Toronto Research Chemicals, Inc.

Assays for experimental lung metastasis. Severe combined immuno-deficient mice were housed in the specific pathogen-free colony of the Ontario Cancer Institute under conditions approved by the Canadian Council on Animal Care. For the lung colony assay, each mouse received 5 × 10^5 cells through the tail vein and was killed 28 days later. The lungs were fixed in Bouin’s solution. The pulmonary tumors on the surface were counted under a dissecting microscope. The number of injected viable cells was determined using clonogenic assays. The plating efficiencies for different treatment groups, which varied from 60% to 90%, were then used to calculate metastatic efficiency.

Clonogenic assays for viability of lung-arrested tumor cells. For the viability assay of the arrested tumor cells, 10^5 or 10^3 cells per mouse were injected i.v. To prepare lung single-cell suspensions, the lungs were removed, minced, and incubated in 4.5 mL PBS. 0.5 mL 0.05% trypsin-EDTA (Invitrogen), and 150 Kunitz units/ml DNase I (Sigma-Aldrich) for 30 min at 37°C with agitation, and then the tissue pieces were incubated in α-MEM medium containing 0.25% collagenase type 4 (Sigma-Aldrich) and 300 Kunitz units/ml DNase I for 1 h at 37°C with agitation. The cells were dispersed by shaking and then passed through a 40-μm cell strainer to collect a single-cell suspension. A total of 5 × 10^4 lung-derived cells were plated into 10-cm tissue culture dish. Colonies were counted 10 days later.

Plating efficiency assay. Tumor cells from the same population as i.v. injected cells or cells for other assays were plated at 100 tumor cells/10-cm tissue culture dish. The colonies formed on dishes were fixed, stained, and counted at 10 days later to determine plating efficiency.

Western blot analysis. Cells were washed with cold PBS and lysed in 0.5 to 1.0 mL lysis buffer [50 mmol/L HEPES (pH 8.0), 10% glycerol, 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 1.5 mmol/L MgCl2, 100 mmol/L NaF, 10 mmol/L Na3PO4, freshly added protease inhibitors]. The lysates were scraped into tubes, incubated on ice for 1 h, and then centrifuged for 10 min at 10,000 rpm and 4°C to collect the supernatant. Whole-cell lysates were resolved on 10% SDS-polyacrylamide gels and transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences). The antibodies used were polyclonal anti-integrin α5, anti–integrin β3, anti–β3(α5)–integrin (Santa Cruz Biotechnology, Inc.), anti–phospho-ERK1/2, anti–phospho-Akt (Cell Signal Technology), and anti-tubulin (Abcam) and monoclonal anti-MDM2 (Oncogene/Calbiochem), anti-p35 (Santa Cruz Biotechnology), and anti–HIF-1α (Abcam). The hybridization and detection were done according to the instructions from the antibody manufacturers.

Invasion assay. Invasion assays were done using a Cell Invasion Assay kit (Chemicon). Cells were harvested and resuspended in medium (2% FBS) at 5 × 10^3 cells/mL. An aliquot of the cell suspension (200 μL) was added to inserts coated with a matrix of reconstituted basement membrane proteins (ECMatrix). Complete medium was added as chemoattractant into a 24-well plate (500 μL/well), in which the coated inserts were placed. At 24 and 48 h of incubation, the cells that had invaded through the inserts were detached and lysed, and a CyQuant GR Dye was added. The fluorescence intensity was measured with a SpectraFluor Plus plate reader (Tecan) using 485/535 nm filter set.

Adhesion assay. Cell adhesion potential was tested using an Integrin-Mediated Cell Adhesion kit, a CytoMatrix Screen kit (Chemicon), or 96-well plates coated with laminin-5 (Biosignet) at 2 μg/mL overnight. Cells were harvested and resuspended in complete medium at 5 × 10^5 cells/mL. An aliquot of the cell suspension was added to a coated well (100 μL/well of a 96-well plate). The cell number and incubation time were determined in preexperiments. After 2 h of incubation at 37°C in a cell culture incubator, the plate was rinsed three times with PBS containing 1 mmol/L CaCl2 (200 μL/well), stained with 0.2% crystal violet in 10% ethanol, and rinsed again, and the dye was extracted into a mixture of 50 μL of 0.1 mol/L NaOH/NaPO4 (pH 4.5) and 30 μL of 50% ethanol. The absorbance at 560 nm was measured as aforementioned.

Proliferation (WST-1) assay. Cells (1 × 10^4–5 × 10^5) were seeded into a 96-well plate and cultured under normal or hypoxic conditions. On the day of the assay, 10 μL/well of the WST-1 labeling solution (EMD Biosciences) were added. After 2 h of incubation at 37°C in a cell culture incubator, the absorbance was measured as aforementioned at 450 nm with the reference wavelength 620 nm.

Immunohistochemistry. A total of 10^3 cells per mouse were injected i.v. The lungs were removed and fixed in formalin and later embedded in paraffin. The paraffin-embedded lungs were cut into 4-μm-thick sections, dewaxed, dehydrated, and microwave heat treated for epitope retrieval. Sections were treated with a protein blocker (Signet Labs) before incubating with antibody to GFP (Abcam), Ki67 (DAKO), or cleaved caspase-3 (Cell Signaling). The sections were then incubated for 30 min each with antibody to GFP, Ki67 (DAKO), or cleaved caspase-3 (Cell Signaling). The sections were then incubated for 30 min each with biotinylated secondary antibody (Vector Laboratories) and hors eradish peroxidase–labeling reagent (ID Labs). The incubations were all at room temperature. Reaction products were revealed by 3,3′-diaminobenzidine, counterstained, and mounted in Permount (Fisher). For double staining, the images were immediately captured using a ScanScope scanner (Aperio Technologies), and then the coverslips and mounting medium were removed to do second round staining. Alkaline phosphatase–streptavidin (Vector Laboratories) and a freshly prepared Vector red solution (Vector Laboratories) were used to reveal reaction products.

Real-time quantitative PCR. Total RNA was isolated using an RNeasy Miniprep kit (Qiagen). For quantitative PCR, 2 μg of total RNA were reverse transcribed using an Omniscript kit (Qiagen), and 1 μL of the reverse transcription products was mixed with PCR primers, double-distilled water,
and SYBR Green PCR Master Mix (Applied Biosystems) to a total volume of 12 μL. The reaction condition was as follows: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 60 s. The reactions were run and analyzed with an ABI Prism 9700 Sequence Detector (Applied Biosystems).

**Statistics.** The t test (two tailed) was done for comparisons between two groups. Kruskal-Wallis statistic or ANOVA was used to detect significant changes among multiple groups followed by Dunn's test for multiple comparisons against one single control group.

**Results**

**Hypoxia pretreatment increased experimental metastatic efficiency in HT1080-GFP cells.** Severe chronic hypoxia (5% CO₂ and 95% N₂, 24 h) pretreatment significantly increased (P < 0.0025) experimental lung metastatic efficiency in HT1080-GFP cells by 3- to 4-fold (Fig. 1A). Reoxygenation for 6 to 18 h showed a trend for further increase, but it was not statistically significant compared with that of groups without reoxygenation. After 24 h of reoxygenation, the metastatic efficiency started to return toward control level. At 48 h of reoxygenation, the metastatic efficiency was similar to that of normoxic control groups.

Because tumor cells exposed to chronic anoxia or severe hypoxia in a solid tumor may lie far from functional vasculature, and thus may be unable to contribute significantly to the development of metastasis, we examined the effect of hypoxia on tumor cell metastatic efficiency at higher oxygen concentrations. As shown in Fig. 1B, the lung experimental metastatic efficiency was increased in response to a wide range of low oxygen preexposure at concentrations from 0% to 2%, with the largest increase occurring at 0.2% oxygen (P < 0.01 for 0.2% versus 0% and P < 0.05 for 0.2% versus 2%), although the difference between 0.2% and 1% oxygen treatments was not statistically significant. The concentration of 0.2% O₂ was used in all subsequent experiments. Figure 1C shows that HT1080-GFP cell viability was not significantly changed after hypoxia treatment as tested by clonogenic assay.

**Hypoxic exposure did not affect adhesion or invasion potential in HT1080-GFP cells.** Previously, we showed that hypoxia increased metastasis in a mouse fibrosarcoma cell line (KHT-C) by up-regulating Mdm2 and suppressing p53 activity (27). However, this mechanism does not explain hypoxia-enhanced metastatic efficiency in the human fibrosarcoma cell line (HT1080). As shown in Fig. 2A, p53 protein level was increased by hypoxia. The accumulation of MDM2 protein, a target gene of p53 transactivity, was observed in HT1080-GFP cells after 5 Gy of ionizing radiation even with hypoxia pretreatment, suggesting that hypoxia may induce, rather than suppress, p53 activity in this cell line.

Because previous studies using this HT1080-GFP cell line have shown that lung metastasis can originate from the proliferation of endothelium-attached tumor cells and that the vascular attachment of these tumor cells rather than size limitation was observed as the mechanism of lung arrest (21), we investigated whether hypoxia was able to increase the adhesion potential of these cells. First, we examined the expression level of αvβ3 integrin after hypoxia treatment, based on the observation by Wang et al. (22) that lung arrest of the HT1080-GFP cells was mediated through an interaction between αvβ3 integrin on tumor cells and laminin-5 in exposed pulmonary base membranes. We did not detect any change of integrin αv or β3 expression in HT1080-GFP cells after hypoxia treatment with or without reoxygenation (Fig. 2B).

In addition to integrin αvβ3, other integrins can also play important roles in cell adhesion and may be regulated by hypoxia (25, 28). Furthermore, hypoxia can increase cell adhesion by activating integrins through a mechanism that does not involve the regulation of integrin expression at either mRNA or protein level (24). Thus, we did adhesion assays to test cell adhesion potential using antibodies to various integrins and using different ECM proteins, including fibronectin, vitronectin, laminin, collagen-1, and collagen-4. The tested integrins (integrin αvβ3, integrin αvβ5, and all β3-containing integrins) have been shown to play important roles in mediating cell adhesion to ECM (22–25). Figure 2C (top) shows that hypoxia pretreatment, with or without reoxygenation, did not change HT1080-GFP cell adhesion potential mediated by any one of the integrins. There was also no difference observed between normoxic and hypoxia-pretreated HT1080-GFP cells with or without reoxygenation in their adhesion to ECM proteins, including fibronectin, vitronectin, laminin, collagen-1, and collagen-4 (Fig. 2C, bottom). Finally, the adhesion of HT1080-GFP cells to laminin-5 was not affected by hypoxia (please see below). Taken together, these data suggested that hypoxia did not change adhesion potential of HT1080-GFP cells.

Extravasation is another step in the metastatic cascade that can be potentially modulated by hypoxia (5). Because at the time of extravasation, tumor cells are usually exposed to normal physiologic oxygen concentration, an invasion assay on tumor cells pretreated with hypoxia was done under normoxic conditions to mimic the effect of hypoxia on tumor cell extravasation. Normoxic or hypoxia-pretreated cells that migrated through the ECM-coated inserts were quantified at 24 and 48 h after seeding. The numbers of migrated cells were normalized against the plating efficiencies ofoxic or hypoxia-pretreated cells seeded directly in tissue culture dishes and is presented as relative fluorescence units in Fig. 2D. In this assay, the number of the cells migrated through the coated inserts is dependent on both ECM degradation and cell motility, the combination of which determines the efficiency of tumor cell extravasation. Again, no difference was detected between control and hypoxia-pretreated HT1080-GFP cells at both time points, suggesting that hypoxia did not affect tumor cell invasion in this model. Although the oxic or hypoxia-pretreated cells had been grown for 24 and 48 h on ECM-coated inserts and might have proliferated, the proliferation rates of these cells are unlikely to cause any change in the number of migrated cells because no significant difference in proliferation rates was observed between oxic and hypoxia-pretreated HT1080-GFP cells (Fig. 3C).

**Hypoxia pretreatment increased the survival of lung-arrested HT1080-GFP cells.** Experimental and clinical studies indicate that metastatic potential can be associated with tumor cell resistance to apoptosis in certain types of tumors (6–8, 29–31). Furthermore, our previous studies showed that hypoxia can increase metastatic efficiency by inhibiting an apoptotic response in KHT-C and SCC VII cells (27). Thus, we investigated the effect of hypoxia on survival of HT1080-GFP cells arrested in mouse lungs. Similar to previous results in KHT-C cells, apoptotic cell death was observed in lung-arrested HT1080-GFP cells (Fig. 3A), suggesting a role of apoptosis in the death of HT1080-GFP cells after lung arrest. Clonogenic assays on cells derived from lungs of mice injected i.v. with tumor cells showed that more clonogenic tumor cells were recovered from the lungs of mice injected with hypoxia-pretreated tumor cells than from those injected with normoxic control cells (10⁵ or 10⁶ cells/mouse injected; Fig. 3B). In addition, this increase is likely due to a better survival rather than enhanced proliferation of hypoxia-pretreated cells because a large difference in proliferation rate would be required to explain the big difference observed.
Furthermore, hypoxia did not promote cell proliferation in this cell line (Fig. 3C), and at 24 h after injection, the tumor cells are unlikely to have undergone significant proliferation. This was further confirmed by Ki67 immunohistochemistry staining on sections of lungs removed from mice at 24 h after tumor cell injection. As shown in Fig. 3D, Ki67-positive staining was not correlated with GFP-positive staining (for HT1080-GFP cells) in mouse lungs at 24 h after injection of oxic or hypoxia-preexposed HT1080-GFP cells, suggesting that the injected tumor cells did not initiate proliferation in mouse lungs during the first 24 h after injection.

**FTS inhibited the effect of hypoxia on tumor cell survival and lung experimental metastasis.** Blum et al. (32) reported that a Ras inhibitor FTS inhibited the phosphorylation but not the total amount of Akt or ERK protein and down-regulated HIF-1α at the protein level by inhibiting the Akt/mammalian target of rapamycin (mTOR) pathway, causing cell death in glioblastoma cells, suggesting that this could potentially be used as a cancer therapeutic agent targeting HIF-1α activation. We tested whether FTS was able to block the effects of hypoxia on the HT1080-GFP cells. We observed that FTS treatment was able to inhibit the effect of hypoxia on increased experimental metastatic efficiency (Fig. 4A). Surprisingly, FTS treatment of the cells during hypoxic exposure inhibited hypoxia-induced VEGF but not hypoxia-induced carbonic anhydrase 9 (CA9) or LOX (Fig. 4B). Furthermore, Fig. 4C shows that the accumulation of HIF-1α was not correlated with Akt activation. This suggests that the mechanism of the action of FTS is unlikely to be through inhibition of HIF-1α accumulation by targeting the Akt/mTOR pathway. Further investigation showed that the phosphorylation of Akt or ERK was not changed by hypoxic exposure alone or by FTS. Reoxygenation was required for Akt activation, and this activation was inhibited by FTS (Fig. 4C and D); however, these changes were not observed in ERK phosphorylation. Because HIF-1α has a very short half-life under oxic condition, the accumulation of HIF-1α was observed only in cells treated with hypoxia without reoxygenation (Fig. 4C) and it was not inhibited by FTS treatment (Fig. 4D), suggesting that other mechanisms and/or transcription factors may be involved in the inhibitory effect of the drug on hypoxia-increased metastases in HT1080-GFP cells.

To determine the role of tumor cell survival after lung arrest in hypoxia-increased metastasis, we further tested whether FTS could affect the survival and adhesion potential of HT1080-GFP cells. FTS treatment did not affect the in vitro viability of either oxic or hypoxic HT1080-GFP cells (Fig. 5A) nor did it inhibit the adhesion of oxic or hypoxic cells to laminin-5 (Fig. 5B, top) or to some other ECM proteins (Fig. 5B, bottom). However, if the cells were treated with FTS during hypoxic exposure and then injected i.v. into mice, the hypoxia-enhanced survival of lung-arrested cells was inhibited, whereas no effect was observed in FTS-treated normoxic cells (Fig. 5C). These data support the linkage between the effect of hypoxia on the survival of lung-arrested tumor cells and hypoxia-increased experimental metastatic efficiency.

![Figure 1](https://example.com/f1.jpg)

**Figure 1.** Hypoxia pretreatment increased the metastatic efficiency of HT1080-GFP cells. HT1080-GFP cells were grown under control conditions or pretreated with hypoxia and various periods of reoxygenation and then injected i.v. into animals to generate lung colonies. An in vitro clonogenic assay was done on the same cell populations to determine the number of actual viable cells injected, and the plating efficiency determined (C) was used to calculate metastatic efficiency. The metastatic efficiency shown is the mean from four independent experiments, and five mice were used per group for each experiment. The error bars represent SEs. A, the cells were grown under oxic control conditions (CON) or anoxic conditions for 24 h without reoxygenation (0) or reoxygenated for 6, 12, 18, 24, and 48 h (6, 12, 18, 24, and 48) before i.v. injection. B, the cells were grown under oxic control conditions (CON) or hypoxic conditions for 24 h (H24) at designated oxygen concentrations (0%, 0.2%, 1%, or 2%), and then the cells were injected i.v. without reoxygenation. C, cells from the same populations as in (A and B) were plated at 100 cells per dish to determine the plating efficiencies of HT1080 cells after different hypoxia and reoxygenation treatments. *, P < 0.025, Kruskal-Wallis statistic; P < 0.05 for H24(0%) versus CON, H24(0%)/O6 versus CON, and H24(0%)/O18 versus CON; P < 0.01 for H24(0%)/O18 versus CON, H24(0.2%) versus CON, and H24(1%) versus CON, Dunn’s test; P < 0.01 for 0.2% versus 0% and P < 0.05 for 0.2% versus 2%, ANOVA.
Discussion

It has been well shown that most solid tumors grow in a unique microenvironment characterized by an abnormal vascular structure, which leads to an insufficient supply of oxygen and nutrients to tumor cells. Tumor hypoxia is a consequence of this abnormal vasculature and promotes tumor malignant progression such as increased metastasis (1, 2). A better understanding of the mechanisms involved in the hypoxia-induced increase in

Figure 2. The effect of hypoxia on HT1080-GFP cell adhesion potential. HT1080-GFP cells were cultured under normal oxic conditions or exposed to hypoxia (0.2% O2) with or without reoxygenation before the cells were harvested or the assays were done. A, HT1080-GFP cells were irradiated with 5 Gy, and protein was harvested from nonirradiated cells or irradiated cells at 3 and 5 h after irradiation. The protein levels of p53 and MDM2 were examined using immunoblotting analysis. β-Actin was probed as a loading control. The numbers under each band are the relative intensity versus the nonirradiated control band corrected to this loading control. B, the cell lysates were harvested from cultured HT1080-GFP cells grown under oxic conditions (CON), exposed to hypoxia for 24 h (H24), or 24 h of hypoxic exposure followed by 24 h of reoxygenation (H/O). The protein levels were examined using Western blot analysis. CA9 was used as a positive control for hypoxia treatment and β-actin as a loading control. Results shown are representative of three separate experiments. C, for adhesion assays, the cells were collected after growth under oxic conditions (CON), 24 h of hypoxic exposure (H24), or 24 h of hypoxic exposure followed by 24 h of reoxygenation (H24/O24). Top, cells were counted and then seeded into 96-well plate format stripes coated with antibodies that recognize integrin αvβ3 (α5b3), integrin αvβ5 (α5b5), and all β1-containing integrins (β1). Noncoated stripes were used as negative controls. Bottom, cells were seeded into plates coated with different ECM proteins, including fibronectin, vitronectin, laminin, collagen-1, and collagen-4. Bovine serum albumin (BSA)–coated stripes were used as negative controls. A total of 5 × 10⁴ cells per well were seeded and incubated at 37°C for 2 h as being optimized by preexperiments to ensure that the number of the attached cells is within the linear range. The number of seeded viable cells was determined by clonogenic assay and used for normalization. The number of the cells attached to the wells was determined by staining the attached cells and then extracting the dye into a solution to measure the absorbance. Data presented were corrected according to plating efficiency and were the average of four repeats. Columns, absorbance; bars, SE. D, for invasion assays, HT1080-GFP cells were cultured under oxic control condition (CON) or pretreated with hypoxia (0.2% O2) for 24 h (H24) before the cells were collected for the invasion assay. The cells were counted and seeded into the inserts containing ECM-coated membrane and incubated under normal tissue culture conditions. A clonogenic assay was done on control or hypoxia-pretreated groups at the time of the assay. The number of the cells that invaded through the ECM-coated membrane at 24 and 48 h was determined by staining the cells with a fluorescent dye and then measuring the fluorescence intensity. The data were normalized according to plating efficiency and are the average of four repeats. Columns, relative fluorescence units (RFU); bars, SE.
metastatic disease is needed to improve the outcome of patients with hypoxic tumors. This study investigated the effect of hypoxia on various stages of metastatic process using an GFP-labeled human fibrosarcoma cell line HT1080. We found that increased survival of tumor cells under secondary stress conditions after hypoxic exposure may be enough to cause increased metastases. More importantly, by inhibiting the enhanced tumor cell survival, the hypoxia-increased metastatic efficiency was prevented.

Another important biological event involved in metastatic growth is angiogenesis. Angiogenesis can be stimulated by hypoxia through the induction of growth factors such as VEGF. In a primary tumor, angiogenesis may provide escape routes for dissociated tumor cells, thereby promoting intravasation. At later stages of metastatic progression, angiogenesis is required to sustain the growth of micrometastases to develop into clinically detectable macrometastases (4, 33). However, the effect of hypoxia on angiogenesis and intravasation is unlikely to play a role in the increased lung colonies observed in this study because tumor cells were injected i.v. into mice, and also the effect of hypoxia on lung metastasis in this cell line is transient and lost after 48 h of reoxygenation (Fig. 1A).

Angiogenesis is unlikely to be required at this early stage. For the same reason, other mechanisms that affect intravasation, for example hypoxia-regulated expression of urokinase-type plasminogen activator or matrix metalloproteinases (1), are unlikely to be involved.

The results in this study are consistent with our previous findings in a murine fibrosarcoma cell line KHT-C (27), in which we showed that lung-arrested KHT-C cells underwent apoptosis and that the altered response of KHT-C cells to apoptosis, which involves hypoxia-induced up-regulation of Mdm2 and the consequent inhibition of p53 activity, can contribute to increased

Figure 3. Hypoxia increased in vivo survival of HT1080-GFP cells after lung arrest. A, formalin-fixed lung sections from mice injected i.v. with HT1080-GFP cells were stained with cleaved caspase-3 antibody (brown) to detect apoptotic cells or stained with GFP antibody (pink) to identify HT1080-GFP tumor cells in lungs. The cells costained by both antibodies represent the apoptotic tumor cells in mouse lungs (arrows). B, HT1080-GFP cells were cultured under oxic control conditions (CON) or pretreated with hypoxia (0.2% O2) for 24 h (H24), and then 10^5 (top) or 10^6 (bottom) cells were injected i.v. into each mouse. A clonogenic assay was done on cells from the same populations to ensure that similar numbers of viable control and hypoxic cells were injected. The number of viable HT1080-GFP cells recovered from lungs was determined by clonogenic assay. The proportions of viable HT1080-GFP cells recovered at different times were normalized against the number of viable cells recovered at 30 min after i.v. injection. Points, average from four experiments (two mice were used per experiment for each group); bars, SE. C, HT1080-GFP cells were cultured under oxic control conditions (CON) or pretreated with hypoxia (0.2% O2) for 24 h (H24). A proliferation assay using WST-1 was done right after hypoxia treatment (time point 0 on X-axis) or after the treated cells were cultured for 24 and 48 h (time point 24 and 48 on X-axis) under normal cell culture conditions. A clonogenic assay was done from hypoxic or control cells at time point 0, and the plating efficiency was used for normalization of the proliferation rate. Points, average of four independent treatments; bars, SE. D, formalin-fixed lung sections from mice at 24 h after i.v. injection with HT1080-GFP cells were stained with GFP antibody to identify tumor cells or stained with Ki67 antibody to detect cell proliferation in lungs. The images shown are representative from 10 mice injected with oxic control (5 mice) or hypoxia-pretreated (5 mice) HT1080-GFP cells, with two sections from each lung. *, P < 0.001 for comparisons at all time points in (B, top). **, P < 0.005 for comparison at 48 h and P < 0.001 for comparisons at the other time points in (B, bottom).
metastatic efficiency. Apoptosis was also observed to occur in the lung-arrested HT1080-GFP cells, and hypoxia pretreatment increased the survival of these cells (Fig. 3), indicating apoptotic potential as an important mechanism involved in the effect of hypoxia on tumor metastatic progression in this model. However, the molecular mechanisms involved are likely to be different in these two models because activation rather than suppression of p53 by hypoxia was observed in HT1080-GFP cells (Fig. 2A). Further studies are needed to understand the molecular mechanisms involved in the HT1080-GFP model. Equally, the importance of the hypoxia-induced increase in lung survival also needs to be investigated in different types of tumor cells to determine whether it is a cell type specific effect of hypoxia.

An interesting and clinically relevant aspect of these results is that the increased metastatic efficiency can be due to enhanced survival potential achieved by altered response to apoptosis, which may confer resistance to radiotherapy and some chemotherapies (34). More importantly, this can occur after up to 24 h of reoxygenation (Fig. 1A). The existence of hypoxic tumor cells thus may contribute to both the failure of local control and the increased incidence of metastatic disease. Moreover, these effects are transient, suggesting that long-term hypoxic exposure to select for mutants is not required. Thus, it could be easier for the tumor cells to acquire this transient more aggressive phenotype. This model therefore may provide a useful tool to study and test for the efficacy of new therapeutic regimens to target the malignant progression promoted by hypoxia.

In this context, the potential use of FTS to inhibit the effect of hypoxia on tumor cells was tested. FTS has been reported to be a Ras-dislodging antagonist through the inhibition of Ras methylation, which is one of the Ras post-translational modifications required for its membrane localization (35). FTS has been shown to inhibit cell growth and induce apoptosis in several different tumor types by blocking Ras downstream pathways (32, 36–39). As a promising agent for cancer therapy in tumors with mutant activated Ras, FTS may also have the additive benefit of controlling hypoxia-induced tumor progression due to the important role of Ras activation in hypoxia-regulated gene expression. For example, hypoxia-dependent up-regulation of osteopontin, which is linked to Ras activation in hypoxia-regulated gene expression. For example, hypoxia-dependent up-regulation of osteopontin, which is linked to Ras activation in hypoxia-regulated gene expression. For example, hypoxia-dependent up-regulation of osteopontin, which is linked to Ras activation in hypoxia-regulated gene expression. For example, hypoxia-dependent up-regulation of osteopontin, which is linked to Ras activation in hypoxia-regulated gene expression.
Therefore, to achieve a better control of hypoxia-induced tumor progression, Ras activation can potentially be an effective target. However, whether or how Ras activation maybe involved in hypoxia-increased metastatic efficiency in HT1080-GFP cells still needs to be determined.

The data in Figs. 4 and 5 show that FTS successfully inhibited the hypoxia-induced increase in tumor cell survival and metastatic efficiency in HT1080-GFP cells, but no effect was observed on oxic cells, suggesting that this inhibitory effect of FTS on metastatic efficiency is by targeting hypoxia-induced proteome changes in this cell line, at least at the tested concentrations. Despite this finding, our initial studies show no evidence for an effect of FTS on down-regulation of HIF-1α in hypoxic HT1080 cells (Fig. 4D). Thus, a much more detailed investigation of the mechanisms of action of FTS in HT1080-GFP cells is needed. Currently, we are investigating the involvement of other Ras downstream pathways, such as p38 and c-Jun NH2-terminal kinase pathways, which have also been shown to regulate cell apoptosis (49, 50). The FTS inhibition of hypoxia-upregulated VEGF also merits further study. The potential clinical use of FTS as an antimetastatic agent needs to be further studied to determine if it can inhibit metastases by oxic tumor cells at different concentrations.

Overall, although hypoxia has been reported to modify many aspects of tumor cell behavior that may be important in the metastatic process, we have shown that they are not all required for hypoxia to increase metastatic efficiency using human fibrosarcoma HT1080-GFP cell line. By promoting the survival of arrested cells alone, hypoxia is able to promote metastatic progression. These results provide important guidelines for developing new therapeutic strategies. For example, metastasis can originate intravascularly from the proliferation of these tumor cells attached to the endothelium without the need for extravasation (21); therefore, targeting tumor cell invasion alone may not be effective to control metastatic progression in patients with similar tumors. This study confirms that a transient alteration in the apoptotic response of cells can play an important role in metastasis formation. This aspect needs to be considered in the development of cancer therapies targeting metastatic development.

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Figure 5. The effect of FTS on HT1080-GFP cell survival and adhesion potential. HT1080-GFP cells were cultured under normoxic control conditions (CON) or treated with hypoxia (0.2% O2) for 24 h (H24). FTS was added while the cells were exposed to hypoxia for the duration of 24 h and then removed by changing medium right after the cells were returned to oxic conditions for the following assays. A, the viability of FTS-treated or nontreated cells was determined by clonogenic assay. Columns, average of plating efficiencies from four independent experiments; bars, SE. C, the adhesion assays were done as described previously on normoxic control (C) or hypoxic (H) cells with or without FTS. The tested ECM proteins were laminin-5 (top) and fibronectin (F), vitronectin (V), laminin (L), collagen-1 (C1), and collagen-4 (C4) (bottom). FBS was used as a blank control. Data were corrected according to the plating efficiency of the cells from the same cell populations. Columns, average of three independent experiments; bars, SE. D, 105 control or hypoxic cells treated with 25 μmol/L FTS (+FTS) or nontreated cells were i.v. injected into mice. The lungs were removed at 30 min or 24 h after the injection to make lung single-cell suspensions. The number of viable tumor cells recovered from the lungs was determined by clonogenic assay and normalized by the plating efficiency of the cells before injection. Columns, average of eight mice from two independent experiments; bars, SE. **, P < 0.01, treated versus control.
References

1. Sabursky P, Hill RP. The hypoxic tumour microenvi-
2. Rofstad EK. Microenvironment-induced cancer me-
4. Chambers AF, Groom AC, MacDonald IC. Dissemina-
9. Chambers AF, Groom AC, MacDonald IC. Dissemina-
17. Zhang I, Hill RP. Hypoxia enhances metastatic effi-
28. Morgan MA, Dolg O, Reuter CW. Cell-cycle-depen-
31. Santen RJ, Lynch AB, Neal LR, McPherson RA, Yue W. Farnesylisoalcoholic acid: inhibition of prolifera-
35. Zhu Y, DenvarHT, Cao H, et al. Hypoxia upregulates osteopontin expression in NIH-3T3 cells via a Ras-
37. Koong AC, Chen EY, Giaccia AJ. Hypoxia causes the activation of nuclear factor-κB through the phosphory-
41. Iyer NV, Kotch JJ, Agani F, et al. Cellular and developmental control of O2 homeostasis by hypoxia-
45. Danterm A, Beda E, Risau W. Activator-protein-1 binding potentiates the hypoxia-induciblefactor-1-me-
48. Olson JM, Hallahan DR, p38 MAP kinase: a conver-
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