Preconditioning of the Tumor Vasculature and Tumor Cells by Intermittent Hypoxia: Implications for Anticancer Therapies

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Abstract

Hypoxia is a common feature in tumors associated with an increased resistance of tumor cells to therapies. In addition to O_2 diffusion–limited hypoxia, another form of tumor hypoxia characterized by fluctuating changes in pO_2 within the disorganized tumor vascular network is described. Here, we postulated that this form of intermittent hypoxia promotes endothelial cell survival, thereby extending the concept of hypoxia-driven resistance to the tumor vasculature. We found that endothelial cell exposure to cycles of hypoxia reoxygenation not only rendered them resistant to proapoptotic stresses, including serum deprivation and radiotherapy, but also increased their capacity to migrate and organize in tubes. By contrast, prolonged hypoxia failed to exert protective effects and even seemed deleterious when combined with radiotherapy. The use of hypoxia-inducible factor-1α (HIF-1α)–targeting small interfering RNA led us to document that the accumulation of HIF-1α during intermittent hypoxia accounted for the higher resistance of endothelial cells. We also used an *in vivo* approach to enforce intermittent hypoxia in tumor-bearing mice and found that it was associated with less radiation-induced apoptosis within both the vascular and the tumor cell compartments (versus normoxia or prolonged hypoxia). Radioresistance was further ascertained by an increased rate of tumor regrowth in irradiated mice preexposed to intermittent hypoxia and confirmed *in vitro* using distinctly radiosensitive tumor cell lines. In conclusion, we have documented that intermittent hypoxia may condition endothelial cells and tumor cells in such a way that they are more resistant to apoptosis and more prone to participate in tumor progression. Our observations also underscore the potential of drugs targeting HIF-1α to resensitize the tumor vasculature to anticancer treatments. (Cancer Res 2006; 66(24): 11736-44)

Introduction

Tumor hypoxia may account for resistance to conventional anticancer modalities and is therefore generally associated with bad prognosis (1, 2). Commonly described in tumors, chronic hypoxia refers to the imbalance between O_2 delivery and O_2 consumption (3). Although the high metabolic rate of proliferating tumor cells easily justifies the consumption arm, the sources of chronic deficiencies in tumor O_2 supply are multiple. Diffusion distance from the tumor vasculature, flow resistance, or progressive longitudinal hemoglobin saturation may, for instance, compromise homogenous O_2 delivery to the whole tumor (4). The disorganized tumor neovasculature, however, is generally claimed as the common denominator of the above causes of deficit in O_2 supply. Yet, this view underestimates the temporal dimension according to which these defects may be valid at a given time but not at another one. Indeed, angiogenesis is a dynamic process constantly remodeling the tumor vasculature (5) that per se combat the major cause of its induction (e.g., hypoxia). Also, the more mature tumor vascular compartment, i.e., arterioles, may be subject to vasomotion that also modulates tumor blood flow (6–8). In addition to the alterations in vessel conductance properties, microregional blood instabilities, and in particular alterations in RBC distribution within the vascular trees and partitioning at bifurcations (9–13), may account for or aggravate the transient reduction in pO_2. Altogether, these observations suggest that acute or intermittent hypoxia is a ubiquitous process occurring within most solid tumors (7, 14–16). Periodicities from minutes to days have been identified in mice, rats, and nonrodent species (11–13, 16–20), underlying the many overlapping sources of temporal frequencies of fluctuation in tumor oxygenation (4, 21).

The major phenotypic shift associated with chronic hypoxia is tumor cell resistance to chemotherapy and radiotherapy (14, 22–24) and more invasive and metastatic features (25, 26). Recently, intermittent hypoxia was similarly proposed to favor tumor cell dissemination (27, 28). Still, one obvious difference between chronic and intermittent hypoxia has thus far been largely neglected: The tumor vasculature should be largely influenced by the latter, more precisely by the acute episodes of deep hypoxia. Hence, endothelial cells at the interface between blood and tumor cells are influenced by instabilities in RBC flux, vasomotion, or vascular remodeling, even independently of blood stasis phenomena (29, 30). The possible influence of intermittent hypoxia on the phenotype of endothelial cells lining tumor blood vessels may have significant therapeutic implications. Cyclic hypoxia may, for instance, permit resistance to treatment and thereby affects the survival of hundreds of tumor cells (proportionally dependent on a few vascular endothelial cells).

To explore this concept, we have "preconditioned" endothelial cell to intermittent or prolonged hypoxia and evaluated their resistance to serum deprivation and radiotherapy, as well as their capacity to participate in angiogenesis. We have paradoxically identified the hypoxia-induced factor-1α (HIF-1α; despite the interrupting reoxygenation phases) as a key mediator of the intermittent hypoxia-induced phenotypic shift. Finally, we have

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validated *in vivo*, in tumor-bearing mice, the intermittent hypoxia-mediated protection against radiotherapy-induced apoptosis and extended the paradigm of intermittent hypoxia-induced cell survival to the tumor cell compartment.

Materials and Methods

Cell culture. Transplantable liver tumor (TLT) cells, fibrosarcoma (FsaII), and melanoma (B16-F10) cells were routinely cultured in 175-mm flask in serum-containing DMEM. For terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay, cells were seeded into 16-well Labtek (NUNC, Naperville, IL) and grown to confluence. Human umbilical vein endothelial and bovine aortic endothelial cells were routinely cultured in 60-mm dishes in endothelial growth medium (Clonetics, Walkersville, MD). Two hours before starting the survival experiments, cells were serum starved; for long-term survival studies, culture medium was resupplemented with serum. To reach and control hypoxia conditions, cells were placed in a modular incubator chamber (Billups Rothenberg, Inc., Del Mar, CA) and flushed for 10 minutes with a gas mixture of 5% CO_2-95% N_2; the final medium pO_2 value was consistently measured below the 0.5% to 1% range. The chamber was then sealed and placed at 37°C in conventional cell incubator.

Mouse model. Male NMRI mice (Elevage Janvier, Le Genest-St-Isle, France) were used in experiments with syngeneic TLT liver tumor carcinoma cells (31). Mice received an i.m. injection of 10^5 tumor cells in the posterior right leg. The tumor diameters were tracked with an electronic caliper. When the tumor diameter reached 4.0 ± 0.5 mm, mice were randomly assigned to a treatment group. Unanesthetized mice were placed in a Plexiglas box and exposed to a continuous flow of air or of a defined O_2-N_2-CO_2 gas mixture as previously described by Cairns et al. (27, 28); oxygen levels were followed with an Oxygen Analyzer (Billups Rothenberg). Each procedure was approved by the local authorities according to national animal care regulations.

Radiotherapy and clonogenic assay. Cells or tumors were irradiated in normoxic conditions (21% O_2) using a RT-250 device (Philips); when hypoxia was administered, a period of 1-hour normoxia was consistently observed to avoid any direct influence of the local pO_2 on radiation efficacy. To assess the effects of hypoxia preconditioning on endothelial cell survival following serum deprivation or radiation exposure (2 Gy), clonogenic cell survival assays were done. After a 7-day incubation period, cells were stained with crystal violet and colonies (>50 cells) were counted. To evaluate the effects of intermittent hypoxia preconditioning on tumor growth following the administration of radiotherapy, tumor diameters were tracked daily with an electronic caliper.

Endothelial cell migration and tube formation assays. To evaluate endothelial cell migration, the wound assay model (e.g., scraping of a 0.5-mm-wide line across confluent, serum-starved endothelial cells) was used as described (32). For the quantitative analysis, a migration index was defined as the ratio (expressed as percentage) of the density of migrating cells in the center of the wounded area versus the density of (surviving) cells in a size-matched area of the unwounded region. To assess the formation of capillary-like endothelial tubes, an *in vitro* assay consisting in plating endothelial cells on growth factor–reduced Matrigel (BD PharMingen, Lexington, KY) was used as previously reported (32). The tube formation index (expressed as percentage of the value determined in normoxia) was determined as the length of endothelial tubes per microscopic fields. Cell migration and tube formation were observed using an inverted phase-contrast microscope and were quantified by the analysis of images randomly captured by a video-camera system in three different experiments (20 fields observed per experiment).

Immunoblotting, immunostaining, and TUNEL assay. Endothelial cells were collected and homogenized in a buffer containing protease inhibitors. Total lysates were immunoblotted with HIF-1α antibodies (BD PharMingen) and β-actin antibody (Sigma, Bornem, Belgium). For immunostaining, tumors were cryosliced, probed with a rat monoclonal antibody against CD31 (BD PharMingen), and revealed by a secondary antibody coupled to a FITC fluorophore as previously described (33, 34). Apoptotic cells were probed with an *in situ* cell death detection kit (Roche Diagnostics, Vilvoorde, Belgium) according to the manufacturer's protocol; nuclei were also counterstained by using a 4′,6-diamidino-2-phenylindole (DAPI)–containing mounting medium (Vector Laboratories, Inc., Burlingame, CA). The same technique was applied to cultured tumor cells. Tumor slices or tumor cells were examined with a Zeiss Axioskop microscope equipped for fluorescence. The extent of TUNEL-positive nuclei was evaluated by two blinded investigators. For tumor slices, a scoring procedure was used at high magnification. A value of 0, 1, 2, or 3 was assigned, based on the fraction of apoptotic nuclei. For cultured tumor cells, the number of apoptotic cells was directly counted.

Cell transfections. Endothelial cells were transfected with Lipofectin (Invitrogen) according to the manufacturer's protocol. Two specific small interfering RNA (siRNA) targeting different regions of the HIF-1α transcript were used (AAAGCCTTGGATGGTTTTGTT and AACTGGACACAG-TGTGTTTGA, named sequences *a* and *b*, respectively); the lack of unspecific cytotoxicity was verified using a scramble siRNA in clonogenic assay. We also validated the specificity of the HIF-1α–targeting siRNA used in this study by reversing their effects with a HIF-1α–encoding expression plasmid (ref. 35; transfected by electroporation using the Amaxa device).

Statistical analyses. Data are reported as means ± SE; Student's *t* test and one- or two-way ANOVA were used where appropriate.

Results

Intermittent hypoxia preconditioning promotes endothelial cell survival and angiogenesis. HIF-1α protein expression was used to track the effect of changes in cultured endothelial cell oxygenation. One-hour hypoxia was sufficient to reach a robust expression of HIF-1α in cultured endothelial cells (Fig. 1*A*). The reoxygenation phase caused a rapid abrogation of the induced HIF-1α expression (Fig. 1*B*). Based on these time courses and in accordance with previous measurements by our group of fluctuations in the tumor vasculature occurring at the frequency of 0.5 to 1 cycle per hour (36, 37), we chose to explore the influence of intermittent hypoxia on endothelial cell biology by using a scheme protocol of three cycles of 1-hour hypoxia interrupted by 30-minute periods of reoxygenation.

We first compared the influence of intermittent hypoxia and noninterrupted (prolonged) hypoxia on the ability of endothelial cells to resist proapoptotic stresses, such as serum deprivation and low-dose radiotherapy. Of note, radiotherapy (2 Gy) was always administered in normoxic conditions, i.e., following 1 hour of reoxygenation after the last cycle of intermittent hypoxia or after the end of prolonged hypoxia. Clonogenic assays done on serum-deprived endothelial cell revealed that, whereas the prolonged hypoxic conditions time-dependently reduced cell survival, intermittent hypoxia promoted endothelial cells survival (Fig. 1*C*). When compared with cells maintained in normoxia, even more cells resist serum deprivation when first exposed to intermittent hypoxia (Fig. 1*C*). Figure 1*D* documents that, similarly, intermittent but not prolonged hypoxia preconditioning reduced endothelial cell death in response to 2 Gy ionizing radiations.

To further determine whether the spared endothelial cells following radiotherapy had acquired a specific phenotype, we used two assays aiming to evaluate the capacity of endothelial cells to migrate (see Fig. 2*A*) and to organize in tubes when cultured on Matrigel (see Fig. 2*B*). We found that endothelial cells preexposed to intermittent hypoxia and consecutively to ionizing radiations had a higher angiogenic capacity: both migration (Fig. 2*A* and *C*) and tube formation (Fig. 2*B* and *D*) were increased by ~3-fold (versus nonirradiated, normoxia-exposed endothelial cells). Radiotherapy per se increased the angiogenic index values (see Fig. 2*C* and *D*,

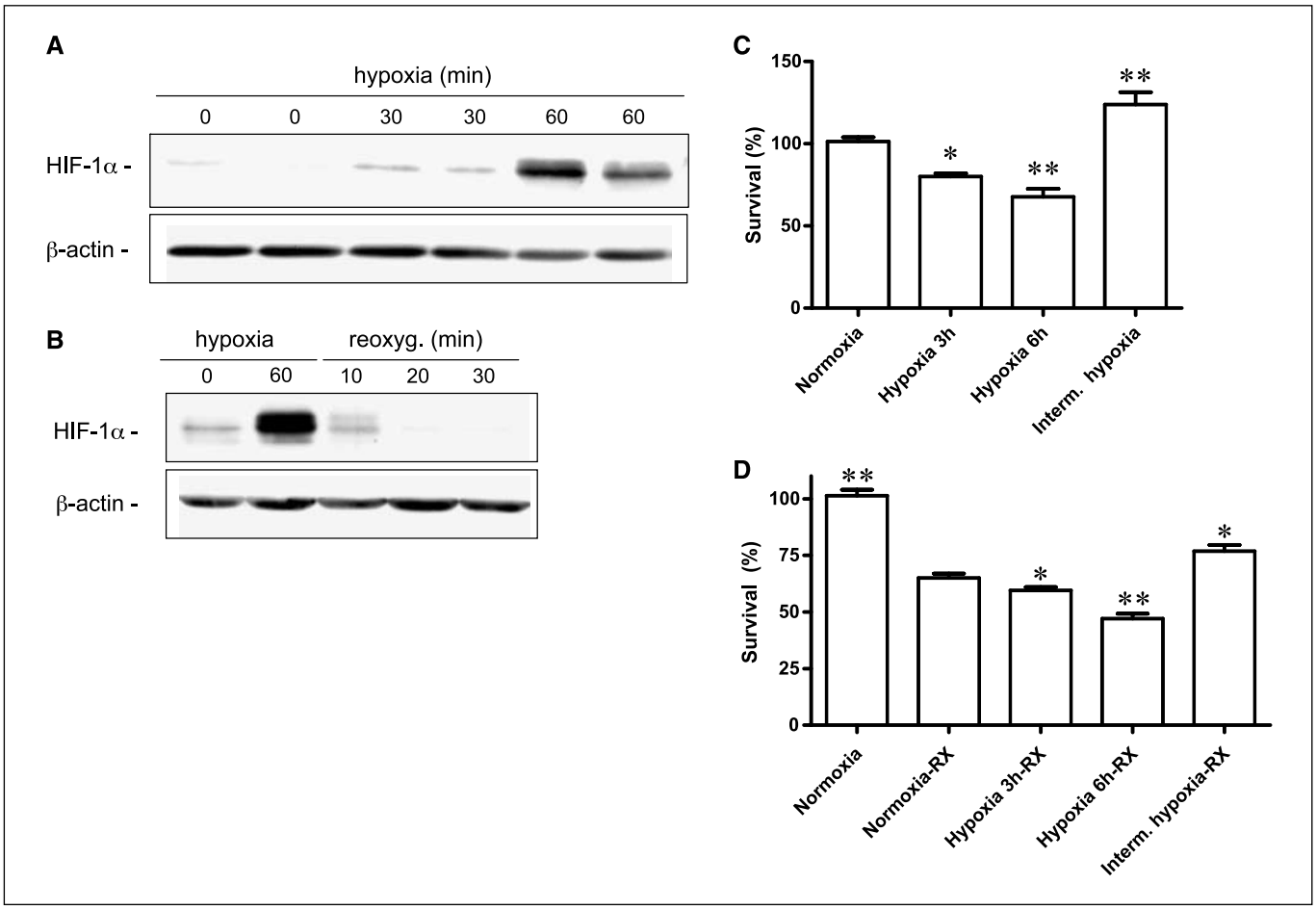

Figure 1. Intermittent hypoxia increases endothelial cell resistance to serum deprivation and radiotherapy. Representative HIF-1α immunoblots from endothelial cell lysates. Endothelial cells were exposed for 0, 30, or 60 minutes to hypoxia (<1% O_2; *A*) and then reoxygenated (*reoxyg.*; 21% O_2) for 10, 20, or 30 minutes (*B*). β-Actin immunoblots are also provided. *C* and *D,* clonogenic survival of endothelial cells after exposure to either continuous 3- and 6-hour hypoxia or intermittent (*Interm.*) hypoxia [i.e., three cycles of 1-hour hypoxia interrupted by a 30-minute reoxygenation]. Effects of serum deprivation (*C*) and exposure to 2 Gy radiation (*RX*) (*D*). *Columns,* % survival of endothelial cells maintained in normoxia; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$ ($n = 4$) versus normoxia (*C*) and versus normoxia + radiotherapy (*D*).

third columns), confirming previous results from our group (32). The combination with the intermittent hypoxia prechallenge was found synergistic; intermittent hypoxia alone had no significant effects. Importantly, 3-hour prolonged hypoxia did not reveal any potentiating proangiogenic effects of radiotherapy (not shown).

HIF-1α mediates the intermittent hypoxia-associated preconditioning effects. Next, we examined whether the transcription factor HIF-1α was involved in mediating the phenotypic shift induced by intermittent hypoxia in endothelial cells. We first evaluated the abundance of HIF-1α protein at different times within the hypoxia-reoxygenation protocol. Figure 3*A* reveals that the expression level of the transcription factor was higher after each new cycle of hypoxia (see also Fig. 3*B* for quantitative data). We also observed that when the HIF-1α induction was blocked by specific silencing siRNA (see Fig. 3*F*), the protection conferred by intermittent hypoxia against serum deprivation (Fig. 3*C*) and radiotherapy (Fig. 3*D*) was lost. Two different siRNAs were used with similar results (see Fig. 3*C*) and transfection with a HIF-1α–encoding plasmid enabled to prevent the siRNA effects (Fig. 3*E*), further validating the HIF-1α dependence of the intermittent hypoxia-protective effects. Figure 3*F* documents the efficacy of the procedure of siRNA cell transduction, leading to a >90% abrogation of HIF-1α expression and the compensatory recombinant expression of HIF-1α.

Intermittent hypoxia influences the survival of both vascular and tumor cells *in vivo*. To verify whether intermittent hypoxia could account for *in vivo* tumor vascular endothelial cell resistance to radiotherapy, we designed experiments where tumor-bearing mice were exposed, before the administration of radiotherapy (10 Gy), to either normal air or 7% O_2 for 3 hours or three cycles of 1 hour 7% O_2 breathing interrupted by 30-minute periods of normal air breathing. The 7% O_2 breathing conditions were previously shown by others (27, 28) and confirmed by us in preliminary studies to lead to a tumor pO_2 strictly <3 mm Hg. Also, mice were allowed to breathe normal air for 1 hour before radiotherapy to avoid confounding effects of remaining local O_2 deprivation; restoration of normal pO_2 levels was verified using the electron paramagnetic resonance technology (not shown). TUNEL assays were done on tumors collected from mice submitted to the above experimental conditions. CD31 costaining was used to determine the extent of TUNEL-positive vascular structures (see Fig. 4*A*). Figure 4*B* shows that intermittent hypoxia reduced by ~2-fold ($P < 0.01$) the extent of TUNEL-positive vascular structures, whereas prolonged hypoxia had the opposite effects,

significantly increasing the extent of apoptosis within the tumor vasculature (+ ~ 75% over normoxic values; $P < 0.01$).

Interestingly, the above observations could be extended to the surrounding tumor tissue. We compared the extent of radiation-induced TUNEL-positive cells into the whole tumor after the different enforced hypoxia prechallenges described above. As depicted in Fig. 5*A*, intermittent hypoxia reduced the extent of radiotherapy-induced apoptosis of tumor cells. Because of the heterogeneous distribution of TUNEL-positive cells (presence of apoptotic hotspots) within the tumor, a scoring procedure was used to quantify these observations. Figure 5*B* confirms a dramatic shift to the left (toward the low-score apoptosis levels) in the intermittent hypoxia conditions ($P < 0.01$). Of note, in the above *in vivo* experiments, prolonged hypoxia (3 hours) did not promote tumor cell survival; a trend toward more apoptosis was even observed when compared with tumor-bearing mice maintained in normoxia (not shown).

We also evaluated the tumor growth delay in irradiated tumor-bearing mice prechallenged or not by the three cycles of 1 hour 7% O_2 breathing. Figure 5*C* showed that the intermittent hypoxia preconditioning led to a higher tumor resistance to radiotherapy (versus normoxic conditions), in good agreement with the lesser apoptosis levels reported in Fig. 5*A*. Finally, to determine the effects of intermittent hypoxia on the intrinsic sensitivity of tumor cells versus the combined effects on both vascular and tumor cells, we repeated these experiments using cultured tumor cells. Figure 5*D* shows that the protective effects of intermittent hypoxia on radiation-induced apoptosis were confirmed *in vitro*.

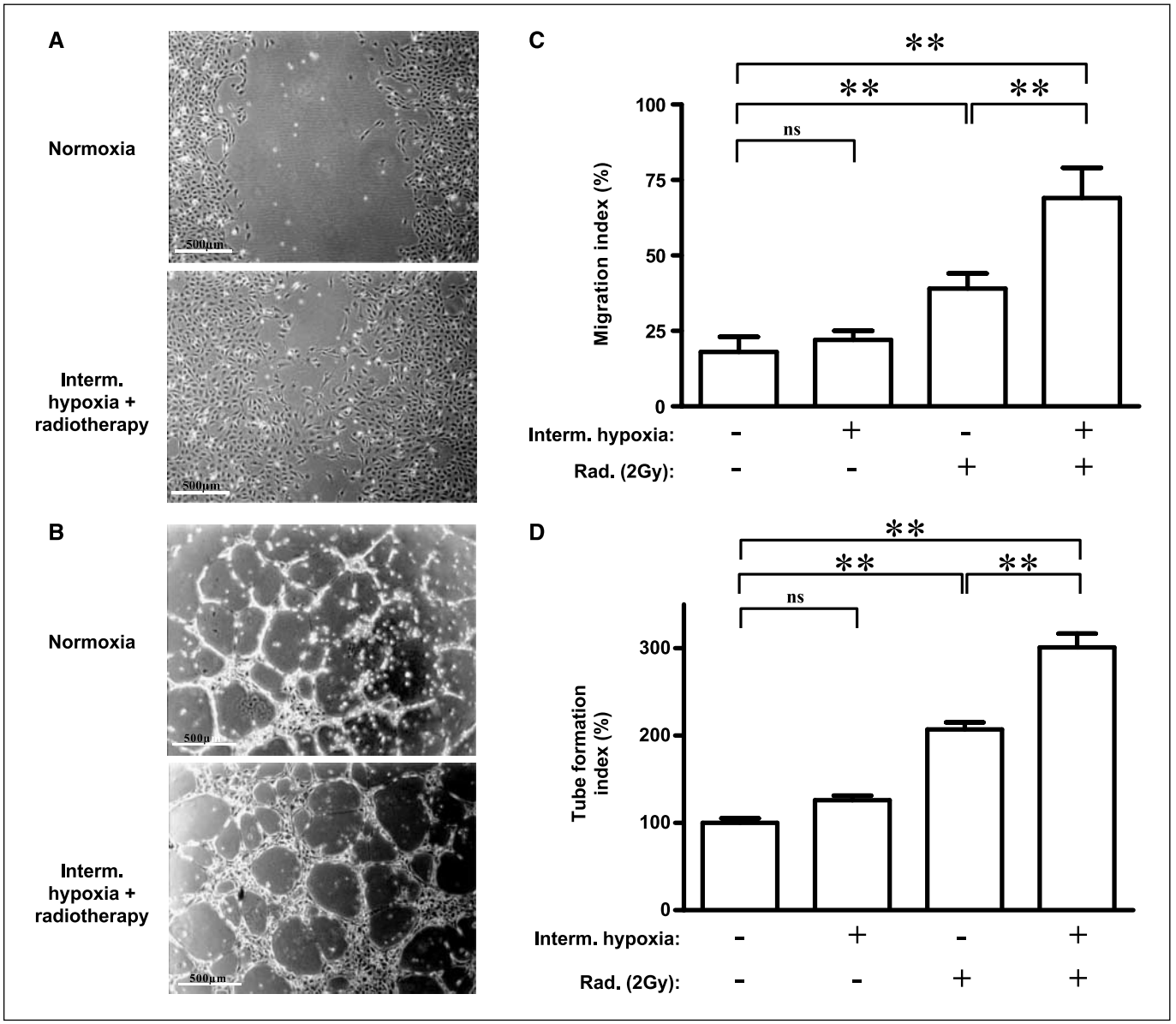

Figure 2. Intermittent hypoxia promotes endothelial cell migration and tube formation. Representative pictures of migrating endothelial cell in the "wound assay" (*A*) and endothelial cell network (tube) formation on Matrigel (*B*); experiments were carried out with cells maintained in normoxia or after challenging the cells with intermittent hypoxia (three cycles of 1-hour hypoxia interrupted by 30-minute reoxygenation) and radiotherapy (*Rad.*; 2 Gy). Quantification is provided as a migration index (*C*) and a tube formation index (*D*), respectively (see Materials and Methods); the extents of migration and tube formation after intermittent hypoxia or radiotherapy alone are also provided. **, $P < 0.01$ ($n = 3$); ns, nonsignificant.

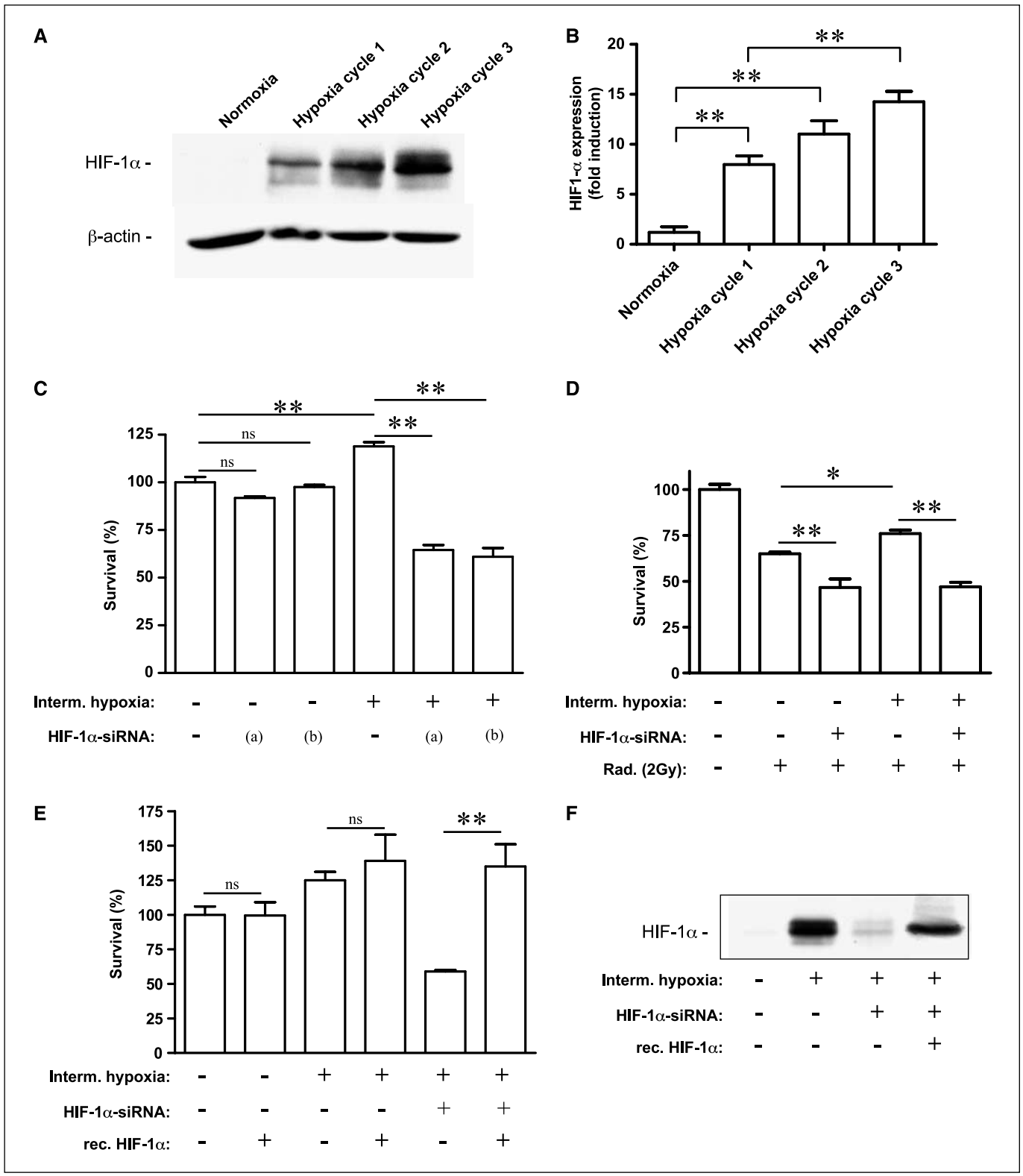

Figure 3. HIF-1α mediates the prosurvival effects of intermittent hypoxia. *A,* representative HIF-1α immunoblots from endothelial cells collected before and at the end of each of the three cycles of 1-hour hypoxia; β-actin immunoblots (used for normalization) are also provided. *B,* the accumulation of HIF-1α during each consecutive hypoxia cycle. **, $P < 0.01$ ($n = 4$). *C, D*, and *E,* the effects of HIF-1α siRNA transduction on the clonogenic survival of endothelial cells maintained in normoxia or exposed to the intermittent hypoxia protocol described above. The effects of serum deprivation (*C*), exposure to 2 Gy radiation (*D*), and concomitant recombinant (*rec.*) HIF-1α expression (*E*). Two different siRNAs, named *a* and *b* (see Materials and Methods for exact sequences), were used in (*C*). *Columns,* % survival of endothelial cells maintained in normoxia; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ns, nonsignificant ($n = 3$–5). *F,* representative HIF-1α immunoblot from endothelial cells exposed to normoxia or intermittent hypoxia, after HIF-1α siRNA and/or HIF-1α–expressing plasmid transduction.

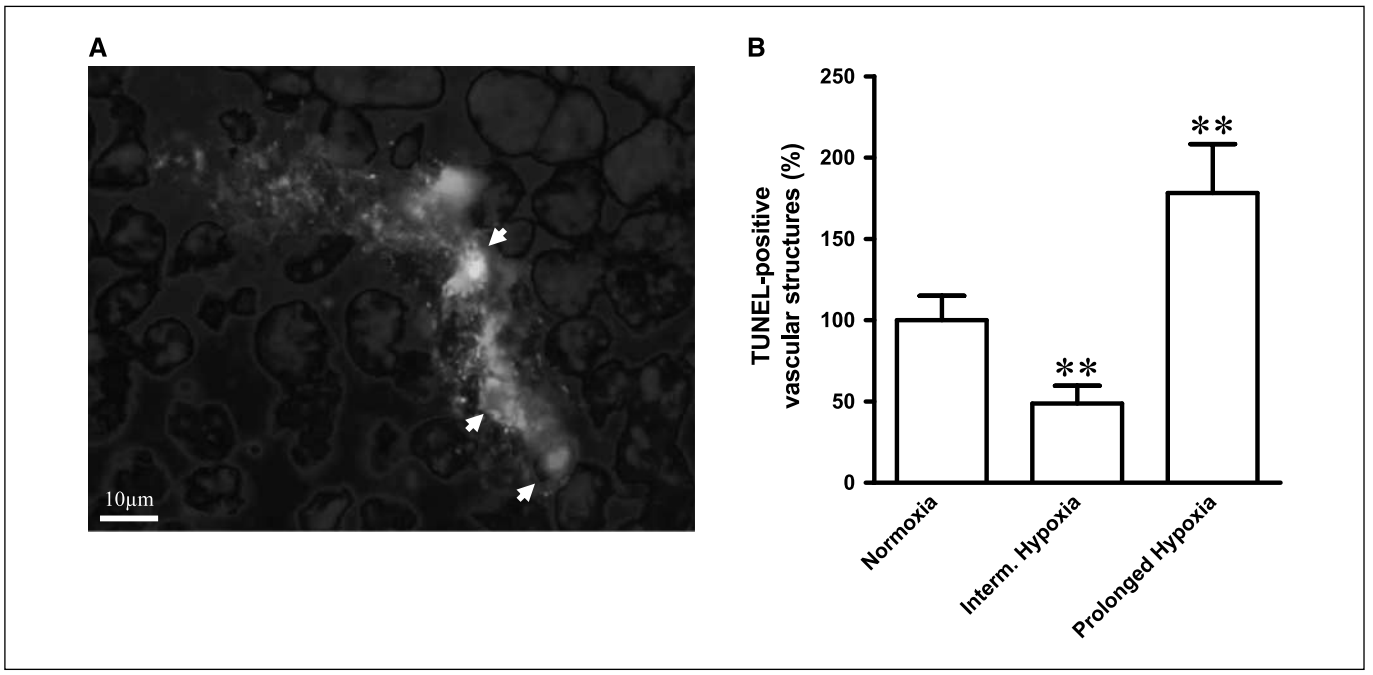

Figure 4. Intermittent but not prolonged hypoxia promotes the tumor vasculature radioresistance. TLT liver tumor–bearing mice were exposed to either continuous 3-hour hypoxia (7% O_2 breathing) or three cycles of 1-hour hypoxia (7% O_2 breathing) interrupted by 30-minute reoxygenation periods (21% O_2). One hour after the end of the hypoxia (or the last hypoxia cycle), mice were locally irradiated (10 Gy). Tumors were collected 24 hours later to evaluate the extent of TUNEL-positive structures on cryosections. *A,* representative picture of TUNEL-positive (*pink*; see also *arrowheads*) nuclei within the CD31-immunostained (*green*) tumor vasculature; the tumor sections were also counterstained with DAPI (*blue*). *B,* the extent of irradiation-induced TUNEL-positive apoptotic cells within the tumor vasculature after either intermittent or prolonged hypoxia. *Columns,* % TUNEL-positive vascular cells in tumor-bearing mice that were maintained in normoxia; *bars,* SE. **, $P < 0.01$ ($n = 4$; 10 high-magnification fields were considered per experiment).

Interestingly, melanoma B16 and fibrosarcoma FsaII cells (more and less radiosensitive than transplantable liver tumor hepatocarcinoma cells, respectively) revealed a similar increased resistance to radiotherapy when first preexposed to intermittent hypoxia.

Discussion

A major finding of this study is that intermittent hypoxia preconditions endothelial cells in such a way that they become more resistant to stress than cells exposed to prolonged hypoxia or even maintained in normoxia. Intermittent hypoxia is nowadays recognized as a hallmark of many tumors as acknowledged by a variety of approaches, including mismatch staining (14, 38), oxygen-sensing microelectrodes (16), intravital microscopy (39), or more recently magnetic resonance imaging (19, 37). It has now become evident that tumor hypoxia is not simply a phenomenon alternating with normoxia depending on the angiogenic status of the tumors (8, 40). Intermittent hypoxia, also known as acute or cyclic hypoxia, may result from the transient occlusion of blood vessels and, more generally, from temporal instabilities in RBC flux (4, 7). These microregional blood instabilities may directly arise from disproportionate cell partitioning at capillary bifurcations but also from more dynamic processes like arteriolar vasomotion and vascular remodeling (see Introduction). Investigations on the relevance of intermittent hypoxia already led to the identification of a more invasive phenotype of tumor cells (27, 28). Here, we provide evidence that intermittent hypoxia also participates in the tumor resistance to radiotherapy and, importantly, that a part of these effects proceeds through endothelial cells lining tumor blood vessels. These cells are usually described as genetically stable and thereby do not lead, as reported for tumor cells, to the clonal selection of cells more adapted to apoptosis. However, the principle of intermittent hypoxia has introduced the concept that vascular cells, although directly located at the interface with the bloodstream, may also be exposed to a hypoxic environment (thus far reserved to tumor cells at a certain distance from tumor blood vessels).

The resistance to proapoptotic serum deprivation and radiotherapy was substantiated by using clonogenic assays that integrate the cell death occurring after several cell divisions. Furthermore, promigratory and proangiogenic properties were also conferred to endothelial cells by intermittent hypoxia. The effects of intermittent hypoxia reinforce the angiogenic potential of endothelial cells surviving ionizing radiations (32). By exposing animals bearing tumors to cycles of hypoxia reoxygenation, we further found a net lower rate of apoptotic nuclei in vascular structures (versus mice maintained in a normoxic atmosphere or prolonged hypoxia). Altogether, these data emphasize the link between intermittent hypoxia and resistance to antitumor treatments through the preservation (or lesser alteration) of the tumor vascular compartment. Importantly, we have also found that intermittent hypoxia protected tumor cells against irradiation-induced cell death (see Fig. 5). This was verified *in vivo* by documenting a higher postradiation rate of tumor growth in mice preexposed to intermittent hypoxia but also *in vitro* using three tumor cell lines with distinct intrinsic radiosensitivity. These data extend the paradigm of intermittent hypoxia as a trigger of prosurvival pathways to both endothelial cells and tumor cells,

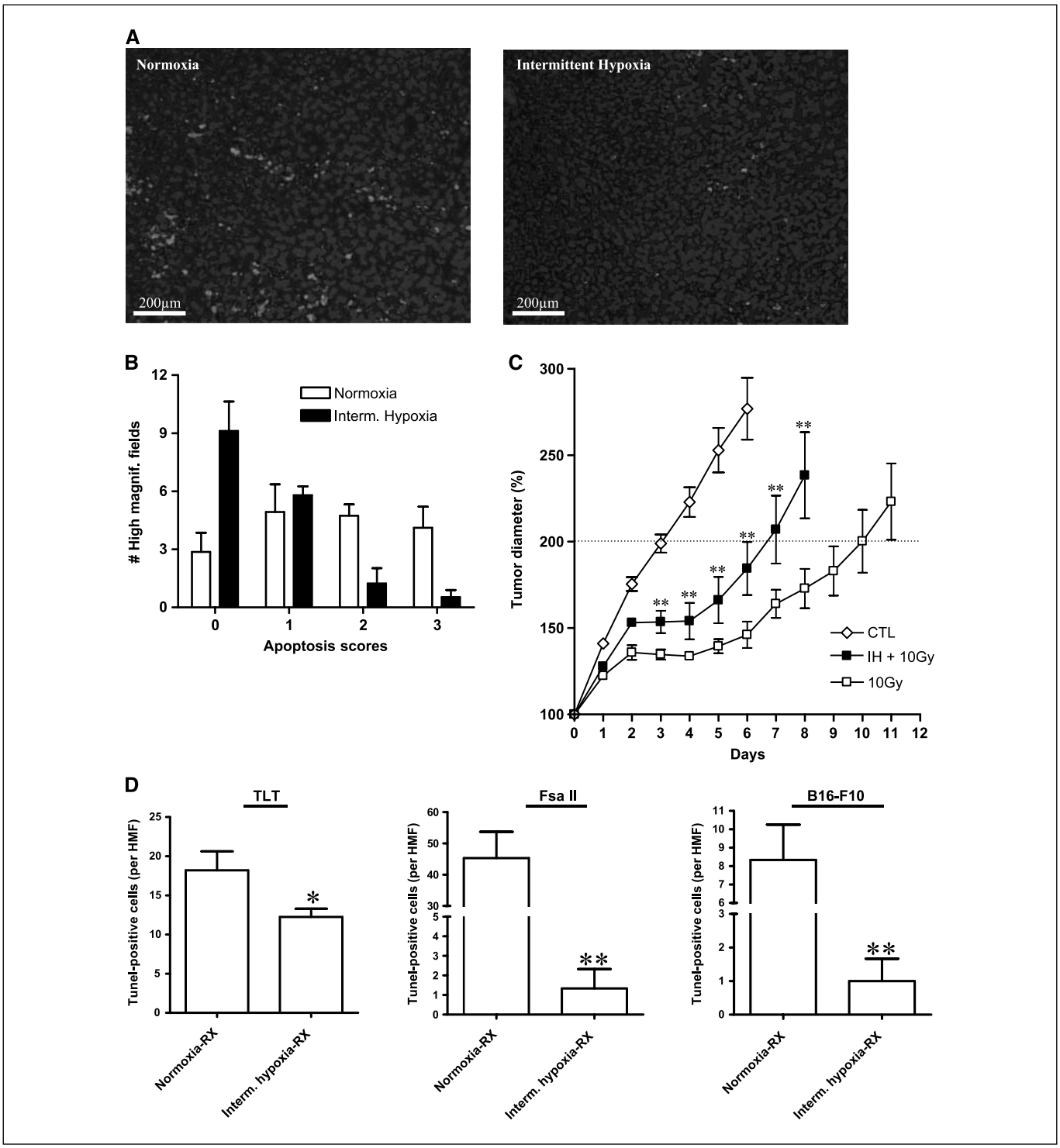

Figure 5. Intermittent hypoxia promotes tumor cell radioresistance both *in vitro* and *in vivo*. TLT liver tumor–bearing mice were either maintained in normoxia or exposed to the intermittent hypoxia protocol described in Fig. 4 legend. One hour after the last hypoxia cycle, mice were locally irradiated (10 Gy). Tumors were either collected 24 hours later to evaluate apoptosis on cryosections or measured daily to evaluate tumor growth delay. *A,* representative pictures of TUNEL-positive (*pink*) tumor cell nuclei (from mice maintained in normoxia or preexposed to intermittent hypoxia); the tumor sections were also counterstained with DAPI (*blue*). *B,* the extent of irradiation-induced TUNEL-positive apoptotic tumor cells (CD31-costained cells were excluded from the counting procedure), evaluated by a scoring procedure to integrate the variability of the pattern observed in a maximum of tumor cryoslices (80–100 high-magnification fields evaluated by two blinded investigators). *Columns,* % of high-magnification fields [where the indicated score was attributed from 0 (no or very few apoptotic nuclei) to 3 (>50% apoptotic nuclei)]; *bars,* SE. The extent of apoptosis is significantly higher in cells not preconditioned by intermittent hypoxia. *C,* tumor growth delay when radiotherapy (10 Gy) is administered to mice prechallenged by intermittent hypoxia (*IH*; ■) or maintained in normoxia (□); tumor growth in untreated mice (◇; $n = 8$). *D,* the radiosensitivity of cultured tumor cells [transplantable liver tumor (*TLT*) hepatocarcinoma, FsaII fibrosarcoma, and B16 melanoma] was also examined *in vitro* after exposure to the intermittent hypoxia protocol described in Fig. 3. One hour after the last hypoxia cycle, tumor cells were irradiated (10 Gy) and analyzed 24 hours later by TUNEL assay. *Columns,* number of irradiation-induced TUNEL-positive nuclei per high-magnification fields; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$ ($n = 4$; 10 high-magnification fields were considered per experiment).

with the caveat that increased endothelial cell death also indirectly alters the tumor cell survival.

A second key finding of the current study is the involvement of HIF-1α in mediating the prosurvival effects of the intermittent hypoxia preconditioning. We first documented that HIF-1α progressively accumulates with the number of cycles of hypoxia (see Fig. 3*A*). Whether this is due to a lack of induction of prolyl hydroxylase domain proteins, as observed with prolonged hypoxia (41), or is due to another stabilizing mechanism is currently under investigation. Nevertheless, the role of HIF-1α was ascertained by the ability of specific siRNA to prevent the protective effects of intermittent hypoxia versus serum deprivation and low-dose radiotherapy (and by the capability of recombinant HIF-1α to reverse the siRNA effects; see Fig. 3*E*). Transcriptomic studies are ongoing in our laboratory to identify downstream targets within the many genes with hypoxia-responsive elements containing promoters (42). Of note, two major angiogenic axes, namely those dependent on vascular endothelial growth factor and basic fibroblast growth factor, are, however, likely not be involved in the observed HIF-1α–driven survival because we failed to detect significant changes in the transcript expression of these growth factors and their receptors (not shown).

Although drugs targeting HIF-1α, including strategies based on the siRNA technology (43), are currently developed to block the various adaptive processes induced by hypoxia or oncogenes in tumor cells (44), our data extend their therapeutic potential to the tumor vasculature compartment. Our study indicates that HIF-1α siRNA, despite having no effect on the survival of endothelial cells maintained in normoxia, reduced the intermittent hypoxia-driven cell survival to a lower level than that of cells maintained in normoxia (see Fig. 3*C*). When combined to radiotherapy, the HIF-1α siRNA also reduced cell survival to a larger extent than radiotherapy alone (see Fig. 3*D*). These observations indicate that if the HIF-1α induction is prevented, deleterious effects of intermittent hypoxia are unmasked. This is yet more relevant in the context of radiotherapy because HIF-1α was recently shown to be induced in response to the radiation exposure (45).

In conclusion, this study unravels the influence of intermittent hypoxia on the phenotype of the tumor vasculature. Cyclic modifications of tumor pO_2 may be considered together with the hypoxia-driven clonal selection of apoptosis-resistant cells as a source of resistance to conventional anticancer therapies. Although the nature of the transcriptomic changes within the tumor vasculature in response to repeated changes in tumor oxygenation warrants further investigation, this process was found to be largely HIF-1α dependent. Together with the observation that intermittent hypoxia promotes the survival not only of endothelial cells but also of tumor cells, our findings provide a new rationale to use HIF-1α–targeting drugs as an adjuvant modality to conventional radiotherapy and chemotherapy.

Acknowledgments

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References

1. Harris AL. Hypoxia—a key regulatory factor in tumour growth. Nat Rev Cancer 2002;2:38–47.
2. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 2001;93:266–76.
3. Gulledge CJ, Dewhirst MW. Tumor oxygenation: a matter of supply and demand. Anticancer Res 1996;16:741–9.
4. Dewhirst MW. Mechanisms underlying hypoxia development in tumors. Adv Exp Med Biol 2003;510:51–6.
5. Jain RK. Molecular regulation of vessel maturation. Nat Med 2003;9:685–93.
6. Intaglietta M, Myers RR, Gross JF, Reinhold HS. Dynamics of microvascular flow in implanted mouse mammary tumours. Bibl Anat 1977;15:273–6.
7. Dewhirst MW, Kimura H, Rehmus SW, et al. Microvascular studies on the origins of perfusion-limited hypoxia. Br J Cancer Suppl 1996;27:S247–51.
8. Feron O. Targeting the tumor vascular compartment to improve conventional cancer therapy. Trends Pharmacol Sci 2004;25:536–42.
9. Chaplin DJ, Hill SA. Temporal heterogeneity in microregional erythrocyte flux in experimental solid tumours. Br J Cancer 1995;71:1210–3.
10. Lanzen J, Braun RD, Klitzman B, Brizel D, Secomb TW, Dewhirst MW. Direct demonstration of instabilities in oxygen concentrations within the extravascular compartment of an experimental tumor. Cancer Res 2006;66:2219–23.
11. Braun RD, Lanzen JL, Dewhirst MW. Fourier analysis of fluctuations of oxygen tension and blood flow in R3230Ac tumors and muscle in rats. Am J Physiol 1999;277:H551–68.
12. Kimura H, Braun RD, Ong ET, et al. Fluctuations in red cell flux in tumor microvessels can lead to transient hypoxia and reoxygenation in tumor parenchyma. Cancer Res 1996;56:5522–8.
13. Kiani MF, Pries AR, Hsu LL, Sarelius IH, Cokelet GR. Fluctuations in microvascular blood flow parameters caused by hemodynamic mechanisms. Am J Physiol 1994;266:H1822–8.
14. Chaplin DJ, Olive PL, Durand RE. Intermittent blood flow in a murine tumor: radiobiological effects. Cancer Res 1987;47:597–601.
15. Coleman CN. Hypoxia in tumors: a paradigm for the approach to biochemical and physiologic heterogeneity. J Natl Cancer Inst 1988;80:310–7.
16. Brurberg KG, Graff BA, Rofstad EK. Temporal heterogeneity in oxygen tension in human melanoma xenografts. Br J Cancer 2003;89:350–6.
17. Gilead A, Neeman M. Dynamic remodeling of the vascular bed precedes tumor growth: MLS ovarian carcinoma spheroids implanted in nude mice. Neoplasia 1999;1:226–30.
18. Patan S, Tanda S, Roberge S, Jones RC, Jain RK, Munn LL. Vascular morphogenesis and remodeling in a human tumor xenograft: blood vessel formation and growth after ovariectomy and tumor implantation. Circ Res 2001;89:732–9.
19. Baudelet C, Ansiaux R, Jordan BF, Havaux X, Macq B, Gallez B. Physiological noise in murine solid tumours using T2*-weighted gradient-echo imaging: a marker of tumour acute hypoxia? Phys Med Biol 2004;49:3389–411.
20. Brurberg KG, Skogmo HK, Graff BA, Olsen DR, Rofstad EK. Fluctuations in pO_2 in poorly and well-oxygenated spontaneous canine tumors before and during fractionated radiation therapy. Radiother Oncol 2005;77:220–6.
21. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004;4:891–9.
22. Kirkpatrick JP, Cardenas-Navia LI, Dewhirst MW. Predicting the effect of temporal variations in PO2 on tumor radiosensitivity. Int J Radiat Oncol Biol Phys 2004;59:822–33.
23. Rofstad EK, Maseide K. Radiobiological and immunohistochemical assessment of hypoxia in human melanoma xenografts: acute and chronic hypoxia in individual tumours. Int J Radiat Biol 1999;75:1377–93.
24. Durand RE. Intermittent blood flow in solid tumours—an under-appreciated source of "drug resistance." Cancer Metastasis Rev 2001;20:57–61.
25. Subarsky P, Hill RP. The hypoxic tumour microenvironment and metastatic progression. Clin Exp Metastasis 2003;20:237–50.
26. Rofstad EK. Microenvironment-induced cancer metastasis. Int J Radiat Biol 2000;76:589–605.
27. Cairns RA, Hill RP. Acute hypoxia enhances spontaneous lymph node metastasis in an orthotopic murine model of human cervical carcinoma. Cancer Res 2004;64:2054–61.
28. Cairns RA, Kalliomaki T, Hill RP. Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. Cancer Res 2001;61:8903–8.
29. Dewhirst MW, Ong ET, Klitzman B, et al. Perivascular oxygen tensions in a transplantable mammary tumor growing in a dorsal flap window chamber. Radiat Res 1992;130:171–82.
30. Sorg BS, Moeller BJ, Donovan O, Cao Y, Dewhirst MW. Hyperspectral imaging of hemoglobin saturation in tumor microvasculature and tumor hypoxia development. J Biomed Opt 2005;10:44004.
31. Taper HS, Woolley GW, Teller MN, Lardis MP. A new transplantable mouse liver tumor of spontaneous origin. Cancer Res 1966;26:143–8.
32. Sonveaux P, Brouet A, Havaux X, et al. Irradiation-induced angiogenesis through the up-regulation of the nitric oxide pathway: implications for tumor radiotherapy. Cancer Res 2003;63:1012–9.

33. Brouet A, Dewever J, Martinive P, et al. Antitumor effects of *in vivo* caveolin gene delivery are associated with the inhibition of the proangiogenic and vasodilatory effects of nitric oxide. FASEB J 2005;19:602–4.

34. Sonveaux P, Martinive P, Dewever J, et al. Caveolin-1 expression is critical for vascular endothelial growth factor-induced ischemic hindlimb collateralization and nitric oxide-mediated angiogenesis. Circ Res 2004;95:154–61.

35. Michel G, Minet E, Mottet D, Remacle J, Michiels C. Site-directed mutagenesis studies of the hypoxia-inducible factor-1α DNA-binding domain. Biochim Biophys Acta 2002;1578:73–83.

36. Baudelet C, Gallez B. How does blood oxygen level-dependent (BOLD) contrast correlate with oxygen partial pressure (pO_2) inside tumors? Magn Reson Med 2002;48:980–6.

37. Baudelet C, Cron GO, Ansiaux R, et al. The role of vessel maturation and vessel functionality in spontaneous fluctuations of T2*-weighted GRE signal within tumors. NMR Biomed 2006;19:69–76.

38. Bennewith KL, Durand RE. Quantifying transient hypoxia in human tumor xenografts by flow cytometry. Cancer Res 2004;64:6183–9.

39. Erickson K, Braun RD, Yu D, et al. Effect of longitudinal oxygen gradients on effectiveness of manipulation of tumor oxygenation. Cancer Res 2003;63:4705–12.

40. Moeller BJ, Cao Y, Vujaskovic Z, Li CY, Haroon ZA, Dewhirst MW. The relationship between hypoxia and angiogenesis. Semin Radiat Oncol 2004;14:215–21.

41. D'Angelo G, Duplan E, Boyer N, Vigne P, Frelin C. Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation. J Biol Chem 2003;278:38183–7.

42. Dachs GU, Tozer GM. Hypoxia modulated gene expression: angiogenesis, metastasis and therapeutic exploitation. Eur J Cancer 2000;36:1649–60.

43. Zhang X, Kon T, Wang H, et al. Enhancement of hypoxia-induced tumor cell death *in vitro* and radiation therapy *in vivo* by use of small interfering RNA targeted to hypoxia-inducible factor-1α. Cancer Res 2004;64:8139–42.

44. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–32.

45. Moeller BJ, Cao Y, Li CY, Dewhirst MW. Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. Cancer Cell 2004;5:429–41.