Hypoxia-Mediated Induction of the Polyamine System Provides Opportunities for Tumor Growth Inhibition by Combined Targeting of Vascular Endothelial Growth Factor and Ornithine Decarboxylase

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Abstract

Hypoxia is a hallmark of solid tumors, which may offer opportunities for targeted therapies of cancer; however, the mechanisms that link hypoxia to malignant transformation and tumor progression are not fully understood. Here, we show that up-regulation of the polyamine system promotes cancer cell survival during hypoxic stress. Hypoxia was found to induce polyamine transport and the key enzyme of polyamine biosynthesis, ornithine decarboxylase (ODC), in a variety of cancer cell lines. Increased ODC protein expression was shown in hypoxic, GLUT-1–expressing regions of tumor spheroids and experimental tumors, as well as in clinical tumor specimens. Hypoxic induction of the polyamine system was dependent on antizyme inhibitor (i.e., a key positive regulator of ODC and polyamine transport), as shown by RNA interference experiments. Interestingly, depletion of the polyamines during hypoxia resulted in increased apoptosis, which indicates an essential role of the polyamines in cancer cell adaptation to hypoxic stress. These results were supported by experiments in an in vivo glioma tumor model, showing significantly enhanced antitumor effects of the antiangiogenic, humanized anti–vascular endothelial growth factor (VEGF) antibody bevacizumab when used in combination with the well-established, irreversible inhibitor of ODC, α-difluoromethylornithine. Our results provide important insights into the hypoxic stress response in malignant cells and implicate combined targeting of VEGF and ODC as an alternative strategy to treat cancer disease. [Cancer Res 2008;68(22):9291–301]

Introduction

Low oxygen conditions, or hypoxia, is a hallmark of growing tumors as a result of inadequate oxygen supply from the surrounding vasculature (1, 2). Hypoxia is associated with a more aggressive tumor phenotype, and it may be speculated whether inhibitors of angiogenesis could promote the selection of aggressive and metastasizing cells by augmenting hypoxia in the tumor microenvironment (2–5). The resistance to anti–vascular endothelial growth factor (VEGF) therapy by compensatory up-regulation of other proangiogenic growth factors and by activation of antiapoptotic mechanisms has been referred to as a hypoxic “rescue program” (6). However, the existence of hypoxic regions also provides opportunities for tumor-specific therapies (i.e., by specific targeting of the adaptive response of cancer cells to hypoxia; refs. 1, 5, 7, 8). The adaptation to hypoxia is largely executed by hypoxia-inducible factors (HIF), which are heterodimeric transcription factors composed of a constitutively expressed β-subunit and one of three hypoxia inducible HIF-α subunits (7–9). The hypoxic signaling response primarily serves to protect cells from acute cell death and to restore the supply of nutrients and oxygen to the tumor environment (7, 9). Although targeting of the hypoxia-induced, proangiogenic factor VEGF has been established in the treatment of cancer (10), we are only beginning to understand the molecular mechanisms that link hypoxia to increased tumor aggressiveness.

The polyamines spermidine and spermine and their precursor putrescine are essential for cell transformation and proliferation (11, 12). Recent studies have delineated the existence of an intricate machinery to fine-tune intracellular polyamine levels by endogenous biosynthesis as well as import from the extracellular environment. Antizyme (OAZ) acts as a central regulator of polyamine homeostasis by inactivation of ornithine decarboxylase (ODC; the rate-limiting enzyme of polyamine biosynthesis) as well as inhibition of polyamine import (13, 14). In turn, the ODC homologue OAZ inhibitor (AZIN) sequesters and down-regulates OAZ, resulting in increased levels of ODC and polyamine transport (15).

There is mounting evidence for an important role of the polyamines in cancer disease and other hyperproliferative disorders (11, 12, 16). Overexpression of ODC leads to cellular transformation in vitro and increased susceptibility to tumor development in transgenic mice (17–19). Conversely, loss of one ODC allele or overexpression of OAZ was associated with reduced carcinogenesis (20, 21). Moreover, AZIN has been suggested to have oncogene properties because its overexpression caused increased proliferation and anchorage-independent growth of NIH3T3 fibroblasts (22). ODC is a transcriptional target of the proto-oncogene c-Myc, and back-crossing E2F-Myc mice into the Odc+/− background resulted in the attenuation of Myc-induced lymphogenesis (23). However, although ODC can be transcriptionally regulated by c-myc and potentially other oncogenes, there is currently no unifying explanation to the general induction of polyamines in malignant tissues (24–26).

Importantly, the effects of genetic manipulation of the polyamine system on tumorigenesis can be counteracted or mimicked by α-difluoromethylornithine (DFMO, or eflornithine), which is an irreversible and highly specific inhibitor of ODC activity.

Note: K.J. Svensson and J.E. Welch contributed equally to this work.

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Clinical trials with DFMO have shown no or only limited overall survival benefits in cancer patients, which most probably relates to the cytostatic rather than cytotoxic effect of DFMO in vivo (11). The drug was relatively well tolerated, and more recent studies implicate an interesting role of DFMO as a chemopreventive drug for use in, for example, colorectal cancer, squamous cell carcinomas, and prostate cancer (11, 29). The combined effect of DFMO and antiangiogenic strategies has, to our knowledge, never been tested.

Considering that hypoxia and induction of the polyamines are common features of malignant tissues, we set out to investigate how these phenomena may be interrelated, and whether targeting of the polyamine system is a feasible strategy to interfere with the adaptive response of cancer cells to hypoxic stress.

**Materials and Methods**

**Antibodies and chemicals.** The antibodies anti–HIF-1α (ab1), anti–β-actin, mouse anti-CD31, and anti–GLUT-1 were from Abcam; anti–caspase-7, anti–cleaved caspase-7, anti–caspase-3, anti–cleaved caspase-3, and anti–poly(ADP-ribose) polymerase were from Cell Signaling Technology, Inc.; human anti-CD31 was from DakoCytomation; anti–heparan sulfate antibody (RB-8E12; ref. 30) and rabbit polyclonal anti–ODC (31) were the same as previously described. [3H]-Polyamines and HRP-conjugated secondary antibodies were from Cell Signaling Technology, Inc.; [14C]Polyamines and HRP-conjugated secondary antibodies were from Amersham International, and fluorophore-conjugated secondary antibodies from Invitrogen. DFMO (efloxacin, and TUNEL TMR Kit from Roche Registration Ltd. Lactate dehydrogenase (LDH) assay kit, fine-grade chemicals, cell media, and supplements were from Sigma Chemical Co.

**Cell culture.** HeLa, U-87 MG, C6, LS174T, pc3, and CHO-K1 cell lines were from American Type Culture Collection and cultured in media as recommended by the manufacturer. CHO ODC− where the same as described (32), and MCF-7 was kindly provided by Dr. Åke Borg (Lund University, Lund, Sweden). Routine culture was done in a humidified 5% CO2 incubator at 37 °C, using the respective medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (growth medium). For hypoxia experiments, cells were incubated in a humidified 5% CO2 InVivo; Hypoxia Workstation 400 (Ruskin Technology Ltd.) at 37 °C, and at 0.1% O2 unless stated otherwise. For tumor spheroid formation, the “hanging drop” procedure was used, wherein cells assemble into symmetrical spheroids to a size of ~1 mm over a period of 2 to 3 wk. [3H]Spermine (specific activity, 31 Ci/mmol) uptake experiments were done in extensively preirradiated, subconfluent cells as previously described (33, 34). In all cases, [3H]Spermine uptake was measured over a period of 30 min in serum-free medium without DFMO or any additional polyamine.

**Immunoblot analysis.** Cells were lysed in 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate supplemented with complete mini protease inhibitor cocktail (Roche Diagnostics GmbH). Equal amounts of protein, as determined with BCA Protein Assay Kit (Pierce), were separated by SDS-PAGE in a 4% to 12% gel, electrophoebted onto PVDF membrane, incubated with the indicated primary antibodies, followed by HRP-conjugated secondary antibody, and finally developed using ECL Western blotting substrate (Pierce). Band intensities on X-ray films were quantified by densitometry using TotalLab v1.11 software and normalized to β-actin.

**ODC activity and polyamine levels.** ODC activity was determined by measuring the release of [14C]CO2 from carboxyl-labeled ornithine. Cells were sonicated in ice-cold 0.1 mol/L Tris-HCl (pH 7.5), containing 2.5 mmol/L DTT and 0.1 mmol/L EDTA. The cell homogenates were centrifuged at 30,000 × g for 20 min at 4 °C, and the release of [14C]CO2 from [14C]ornithine in the presence of pyridoxal 5-phosphate (0.5 mmol/L) and ornithine (0.5 mmol/L) was determined by scintillation counting. For polyamine analysis, HeLa cells were collected by scraping, counted, and pelleted at 900 × g for 10 min at 4 °C; polyamine content in cell pellets extracted with 0.2 mol/L perchloric acid was determined by high-performance liquid chromatography (Hewlett Packard 1100) with O-phthaldialdehyde as the reagent (35).

**Immunofluorescence microscopy.** Clinical tumor specimens originating from two moderately differentiated cervical cancers International Federation of Gynecology and Obstetrics stage IB; experimental glioma tumors (see below); and tumor spheres were fixed in formalin, dehydrated with increasing grades of ethanol and xylene, and embedded in paraffin. Antigen retrieval of deparaffinized and rehydrated sections in sodium citrate buffer (pH 6.0) for 15 min at 95 °C to 100 °C was followed by blocking with 2% goat serum in PBS. Tumor sections were incubated with the respective primary antibody at 4 °C overnight, followed by fluorophore-conjugated secondary antibodies for 1 h at room temperature. Negative controls (incubated with serum from the same species as the primary antibody) were stained with secondary antibodies in parallel. Sections were counterstained with Hoescht 33342 nuclear stain, mounted with Permafluor (Beckman Coulter), and analyzed using a Zeiss HBO 100 fluorescence microscope.

**Real-time quantitative PCR.** Cellular RNA was extracted using GenElute Mammalian total RNA kit (Sigma), and cDNA synthesis was done using Superscript III Platinum Two-Step qRT-PCR Kit (Invitrogen). Diluted cDNA was used for quantitative PCR based on SYBR Green 1 chemistry (Sigma) in an ABI PRISM 7900 HT machine (Applied Biosystems). The following primers were used: VEGF, forward 5′-AGCCCTGGCTTGCTGCTTA-3′ and reverse 5′-GTCGTCGGCTTGTTGAGG-5′; ODC, forward 5′-AGTTGAT- GAAATTTGCAAAGC-3′ and reverse 5′-GAAGGCCTGATGGCTAGC-3′; OAZ, forward 5′-AGGAGGACTAAATGCACAG-3′ and reverse 5′- TCTCGTTGGAACAAAGTAGA-3′; and AZIN, forward 5′-TGAATAGGCTTT- TAGTGCAAGG-3′ and reverse 5′-TGTCTTTCAAGGTAGTGCCAAA-3′. Three reference genes (β-actin, forward 5′-AGCACAGAGCTCGCCCTT-3′ and reverse 5′-GGAATCTTCTTGACCCATGC-3′; GAPDH, forward 5′- GAAGGTGAA GTTCGAGGATCA-3′ and reverse 5′-TGGAAGATATGGTAGTGTA-3′ and HPR1, forward 5′-TGCTGACTGCTTATACA-3′ and reverse 5′-CTCGACCAAGAAAGCAAC-3′), which were stable at hypoxic conditions, were used for normalization. The comparative C1 method was used for relative quantification of gene expression on triplicates of each reaction.

**RNA interference.** Cells were transiently transfected with 100 nmol/L predesigned AZIN siRNA (Ambion, ID no. 23268) or nonspecific siRNA (Ambion negative control siRNA #1) using Lipofectamine 2000 reagent (Invitrogen). In some cells, cases were treated with Lipofectamine alone to exclude nonspecific effects of lipopolymyamine on ODC protein level. Following an incubation period of ~24 h in fresh growth medium, cells were cultured at normoxia or hypoxia for another 4 or 8 h and then analyzed for polyamine uptake activity and ODC protein expression, respectively.

**Animal experiments.** Animals were kept under pathogen-free conditions in the isolation facility at the Biomedical Center, Lund University, in accordance with the Swedish guidelines for humane treatment of laboratory animals. The experimental setup was approved by the ethical committee for animal research in Malmö/Lund, Sweden. U-87 MG cells (2.5 × 106 in 200 μL PBS) were injected s.c. into the dorsal region of adult, female SCIDnudeCA mice (n = 30 in experiment 1; n = 25 in experiment 2) with or without pretreatment for 2 d with DFMO (2% w/v) in the drinking water. When the tumors had reached an approximate size of 5 × 5 mm (6 and 11 d after tumor cell injection in experiments 1 and 2, respectively), animals were randomly divided into four groups receiving one of the following treatments: (a) no treatment; (b) DFMO, 2% w/v in the drinking water; (c) antihuman VEGF neutralizing antibody (bevacizumab, Avastin), 200 μg in 200 μL/mouse (~10 mg/kg) administered i.p. twice weekly; or (d) a combination of DFMO and bevacizumab. Animals were sacrificed, and tumor mass was determined after 34 d (experiment 1) or 37 d (experiment 2) of treatment. Dissected tumors were captured using a 1× objective (Leica MZ 125 stereomicroscope) equipped with a digital camera.

**Data analysis.** Data are presented as the mean ± SD. Statistical significance was evaluated with Student’s t test using Microsoft Excel, and P < 0.05 was considered significant.
Results

Effects of hypoxia on the polyamine system in cancer cells.

The effects of hypoxia on polyamine transport were investigated in human cervix cancer (HeLa) cells. We found an inverse correlation between oxygen concentration and spermine uptake; at 0.1% O₂, polyamine import was up-regulated 5-fold as compared with normoxic control (Fig. 1A, left). Spermine uptake followed the Michaelis-Menten kinetics (Fig. 1A, right), and Lineweaver-Burk plot analysis (not shown) suggested that the number of transporters (Vₘₐₓ), rather than their polyamine affinity (Kₘ), was induced by hypoxia. Studies with a variety of human tumor-derived cell lines and C6 rat glioma cells showed that hypoxic induction of spermine uptake is not restricted to HeLa cells, although the effect varied from <2-fold (MCF-7) to >5.5-fold (LS174T; Fig. 1B). Cellular uptake of the other members of the polyamine family, putrescine and spermidine, was also significantly increased by hypoxia (data not shown). HIF-dependent responses to hypoxia (i.e., the induction of VEGF) often display transient kinetics, which also holds true for hypoxia-induced polyamine uptake, showing a peak between 2 and 8 hours and a return to normoxic levels between 8 and 24 hours (Fig. 1C). Moreover, treatment of normoxic cells with either of the HIF-stabilizing agents, cobalt chloride and desferrioxamine (36, 37), significantly increased spermine uptake (data not shown).

The importance of the polyamines in cell homeostasis is reflected by compensatory up-regulation of polyamine transport on inhibition of ODC. We thus hypothesized that induction of polyamine transport by hypoxia is a secondary event to decreased ODC levels. Contrary to this idea, the ODC protein level was substantially increased by hypoxia (3-fold as compared with normoxia), as determined by Western blot analysis. Again, the hypoxia effect was not specific for HeLa cells, as ODC was induced >20-fold in human lung carcinoma (A549) cells in a similar experiment (data not shown). To investigate whether the increased ODC protein level by hypoxia was correlated with an increased formation of active ODC homodimers, we next determined ODC activity in normoxic and hypoxic cells. As shown in Fig. 2, hypoxia caused a transient induction of ODC activity with a peak around 8 h.
Figure 2. Hypoxic induction of ODC activity and polyamines in vitro and increased ODC protein in hypoxic tumor regions in vivo. A, transient induction of ODC activity by hypoxia. HeLa cells were incubated in normoxia or hypoxia for the indicated times and cell lysates were analyzed for ODC activity as described in Materials and Methods. Representative of two independent experiments. Points, mean; bars, SD. B, HeLa cells were incubated in normoxia or hypoxia as described in A, and cell lysates from the 12-h time point were analyzed for the content of the polyamines putrescine, spermidine, and spermine as described in Materials and Methods. *, P < 0.05, versus normoxic control. C, hypoxic regions in cervical cancer samples display increased ODC protein levels. Serial cross sections of formalin-fixed and paraffin-embedded patient tumor specimens were stained with H&E or analyzed by immunofluorescence microscopy using GLUT-1 (hypoxia marker), ODC, or CD31 (endothelial cell/blood vessel marker) antibodies. Top, necrotic tumor area. Bottom, nonhypoxic, vascularized area. D, top, similar staining as in C was done on serial sections of U-87 MG glioma cell tumors established in immunodeficient mice. ODC co-associates with GLUT-1 in proximity to the necrotic tumor area. Representative pictures of nine cross sections from three separate tumors. Bottom, increased ODC expression was shown in the central, hypoxic region of C6 glioma cell spheroids. Representative photomicrographs obtained at ×40 (C) and ×20 (D) magnifications. Red, green, and blue, GLUT-1, ODC, and CD31 stainings, respectively.
of treatment (~10-fold increase as compared with normoxia) in HeLa cells. Consistent with hypoxic induction of polyamine uptake and ODC activity, polyamine content was significantly greater in hypoxic as compared with normoxic cells (Fig. 2B).

These findings were reproduced in vivo; we stained for the HIF-dependent hypoxia marker GLUT-1 (38) in clinical cervical cancer specimens. As expected, GLUT-1 expression was significant in proximity to necrotic areas, whereas the expression was negligible in highly vascularized areas (Fig. 2C, compare top and bottom images). We found a clear association between GLUT-1 and increased staining for ODC and GLUT-1 was similarly found in experimental human glioma cell tumors grown in immunodeficient mice (Fig. 2D, top) and in glioma cell spheroids (Fig. 2D, bottom), indicating that the co-association of ODC and GLUT-1 was related to hypoxia rather than to other factors in the tumor microenvironment.

Hypoxia-mediated induction of the polyamine system depends on antizyme inhibitor. The fact that hypoxia concurrently triggered ODC and polyamine uptake was intriguing, and in the next series of experiments we sought to gain further insight into the mechanism of these results. Mutant cells deficient in ODC activity (ODC−/−) were dependent on substitution with extracellular polyamines to proliferate (32). Spermine uptake was induced ~2-fold above baseline in normoxic ODC−/− cells cultured in relatively polyamine-poor medium, whereas at hypoxia the induction was >10-fold above baseline (Fig. 3A, left). Moreover, in hypoxic cells a substantially higher supply of extracellular polyamines was required to bring the polyamine transport activity down to baseline levels. Consistent with these results, down-regulation of polyamine biosynthesis by DFMO potentiated the hypoxic induction of the polyamine uptake system in HeLa cells. Again, supplementation with extracellular polyamines reversed the hypoxic effect (Fig. 3A, right). The facts that the hypoxic induction of polyamine uptake is further increased in cells with perturbed polyamine biosynthesis and that it is reversed by the addition of extracellular polyamines (Fig. 3A) provide evidence that the hypoxic effects are specifically
related to the supply of polyamine. Conversely, hypoxic up-regulation of ODC was also present in polyamine transport-deficient cells (CHO MGBG; ref. 39; Fig. 3B, left), and polyamine uptake inhibition by anti–heparan sulfate antibody (40) potentiated the hypoxic induction of ODC (Fig. 3B, right). We conclude that the hypoxic induction of neither ODC nor polyamine uptake is secondary to down-regulation of the other. Instead, our data point to a mechanism that acts upstream of both ODC and the polyamine uptake machinery. Moreover, ODC mRNA levels were not significantly affected by hypoxia (Fig. 4A), suggesting posttranscriptional regulation.

The ODC homologue AZIN has been shown to act as a positive, posttranslational regulator of ODC and polyamine uptake through sequestration of OAZ (15). The latter protein is known to promote ODC degradation as well as to down-regulate the polyamine transport system (13, 14). We thus turned our focus to the possible involvement of the OAZ/AZIN pathway. Quantitative real-time PCR experiments showed that OAZ and AZIN mRNA expression levels are not significantly affected by hypoxia (Fig. 4A). Anti-OAZ antibodies are currently not available for immunoblotting experiments. In addition, attempts to obtain reliable AZIN protein expression data were unsuccessful due to very low signal to noise ratios with available antibodies (data not shown). The reasons are probably the low expression and extremely short half-life (0.5–1 hours) of AZIN at physiologic conditions (41). In fact, to our knowledge, immunoblotting for AZIN has only been reported in transfected cells expressing nonphysiologic levels of AZIN (22, 41, 42). We next used RNA interference to down-regulate the endogenous expression of AZIN in normoxic and hypoxic cells. At the conditions used, AZIN mRNA was decreased by ~90% in cells transfected with AZIN siRNA, as compared with untreated cells (control) or cells incubated with either Lipofectamine alone or transfected with scrambled siRNA (Fig. 4B). Interestingly, AZIN down-regulation dramatically decreased hypoxia-mediated...
induction of ODC (∼90% inhibition as compared with scrambled siRNA; Fig. 4C). Moreover, AZIN down-regulation completely reversed the hypoxic induction of polyamine uptake (Fig. 4D).

Together, our data indicate that hypoxia-mediated induction of the polyamine system depends on the regulatory activity of AZIN.

Polyamine deprivation sensitizes cancer cells to hypoxia-induced cell death. The above data show an increased demand for polyamines in hypoxic cells, and we thus hypothesized that the polyamine system has an important role in the adaptive response to hypoxic stress. To test this idea, we initially performed cell proliferation assays at normoxic and hypoxic conditions. As shown in Fig. 5A, hypoxia alone had no significant effect on cell number, whereas, as expected, DFMO reduced cell number by ∼38% as compared with untreated control. Interestingly, DFMO treatment at hypoxic conditions resulted in a substantial reduction of viable cells (∼79% inhibition as compared with control). These results could either reflect an enhanced cytostatic effect of DFMO by hypoxia or that DFMO sensitizes cancer cells to hypoxic cell death. With a few exceptions, ODC inhibition by DFMO is cytostatic rather than cytotoxic, and accordingly, LDH release was only moderately increased by DFMO treatment in normoxic HeLa cells (Fig. 5B). In line with the previous data (Fig. 5A), the effect of hypoxia alone on LDH release in cells with intact polyamine biosynthesis was limited. Interestingly, polyamine deprivation substantially sensitized HeLa cells to hypoxic cell death, and this effect was dependent on the supply of polyamine, as medium supplementation with spermine attenuated LDH release in DFMO-treated, hypoxic cells (Fig. 5B). The latter result excludes the possibility that the hypoxia-specific cytotoxicity of DFMO can be attributed to conversion of DFMO into a nonspecific cytotoxin in oxygen-deprived cells. Similar results were obtained with U-87 MG and MCF-7 cells (data not shown).

Hypoxia-induced cell death involves apoptotic and/or necrotic mechanisms, depending on the cell type studied (43). To gain further insight into the mechanism of hypoxic cell death in...
polyamine-deprived cells, we next determined the activation of caspase-7 and caspase-3 (i.e., major cytoskeletal proteases) that coordinates the downstream events of apoptotic cell death. Consistent with the LDH release experiments and with an apoptotic mechanism, we found significant caspase-7 and caspase-3 activation as well as cleavage of poly(ADP-ribose) polymerase, a substrate of activated caspase-3, in DFMO-treated hypoxic cells, whereas untreated hypoxic cells and DFMO-treated normoxic cells exhibited undetectable or very low levels of active caspase-7 and caspase-3 (Fig. 5C). Similar results were obtained with U-87 MG cells and when cells were evaluated by Annexin V staining (data not shown).

Together, these results indicate that polyamines serve a protective function against hypoxic stress through antiangiogenic mechanisms. To corroborate these findings, cells were treated in the previous experiments and analyzed by the TUNEL apoptosis assay. Consistent with the above data, ODC inhibition by DFMO substantially increased the number of apoptotic cells grown at hypoxic conditions (~4.5-fold as compared with control), whereas the effect of either DFMO or hypoxia alone was almost insignificant (Fig. 5D).

**ODC inhibition enhances the antitumor effect of antiangiogenesis therapy.** The fact that polyamine-deprivation rendered cancer cells more sensitive to hypoxia-induced apoptosis prompted us to investigate the combined effect of antivascular therapy and polyamine deprivation in vivo. Recent studies have shown positive effects of DFMO treatment on the survival of glioma patients when added to conventional cytostatic therapy (44), and other studies have shown that high ODC expression as well as hypoxia is associated with a more aggressive phenotype in glioma tumors (45, 46). Moreover, previous experiments in this study (Fig. 2D) showed upregulation of ODC in hypoxic regions of experimental U-87 MG glioma tumors. We thus decided to test the combined antitumor effect of DFMO and antiangiogenic therapy in a human glioma xenograft model. Initial experiments aimed at establishing the appropriate conditions for antiangiogenic induction of hypoxia; mice bearing U-87 MG glioma tumors were treated with the monoclonal anti-VEGF antibody bevacizumab at a dose (10 mg/kg) previously shown to have antiangiogenic activity and to induce hypoxia in various tumor models (47). We found substantial hypoxic induction in glioma tumors from bevacizumab-treated mice as compared with similarly sized tumors from nontreated control animals (Fig. 6A).

The rationale of the in vivo experiments being that polyamine depletion sensitizes tumors to hypoxic stress, we decided to use a therapeutic, hypoxia-inducing dose of bevacizumab (10 mg/kg) in combination with a dose of DFMO (2% w/v) in the drinking water previously shown to deplete polyamines in mice (27–29). A treatment schedule was thus designed wherein tumor-bearing control animals or DFMO-treated animals were randomized to receive no additional treatment or treatment with bevacizumab. As expected, under these conditions, treatment with DFMO or bevacizumab alone significantly reduced tumor growth (~46% and 47% tumor mass as compared with untreated controls, respectively; P < 0.05). Interestingly, the antitumor effect of bevacizumab was significantly enhanced when given in combination with DFMO (~19% tumor mass as compared with control; P < 0.01; data not shown). Neither of the treatments, alone or in combination, caused any obvious adverse effects (i.e., no changes in body weights or general behavior were registered during the entire course of the experiments). We thus decided to perform a similar experiment wherein tumors were allowed to grow for a longer period of time before the start of bevacizumab treatment. Under these conditions, combined treatment with DFMO and bevacizumab was even more efficient (~3% tumor mass as compared with control; P < 0.01; Fig. 6B), and the tumor size at the conclusion of the study was comparable to the tumor size at the onset of bevacizumab treatment (i.e., there was a complete inhibition of tumor growth). Although bevacizumab treatment alone was also more efficient in this experiment, the antitumor effect was significantly enhanced in combination with DFMO treatment (~23% tumor mass as compared with bevacizumab alone; P < 0.01). The effect of DFMO as single drug was comparable in the two experiments (~46% and 56% tumor mass, respectively, as compared with control). Macroscopically, U-87 MG tumors from mice receiving bevacizumab appeared pale, as indicative of vessel regression and decreased perfusion (Fig. 6B, right). Accordingly, vessel density was significantly decreased in bevacizumab-treated tumors as compared with untreated control and DFMO-treated tumors (Fig. 6C). Notably, whereas DFMO treatment alone had no significant effect on the number of CD31-positive vessels, the addition of DFMO to bevacizumab appeared to reinforce the antiangiogenic activity of the VEGF-blocking antibody. Moreover, consistent with the idea of increased apoptosis in hypoxic cells on polyamine deprivation (Fig. 5), the number of TUNEL-positive cells in tumors from the combination treatment group was significantly increased as compared with tumors from control and single-therapy groups (Fig. 6C).

Discussion

The first successful example of targeting hypoxia-induced pathways in cancer treatment is represented by anti-VEGF...
antibody therapy; however, the potential drawback with these strategies is the selection of malignant cells that adapt to even more hypoxic and acidic conditions. Indeed, hypoxia has been associated with increased tumor aggressiveness through mechanisms promoting cancer cell adaptation to hypoxic stress. An increased understanding of these mechanisms should offer novel approaches to accelerate cancer cell death in hypoxic tumors. Such approaches may include hypoxia-activated prodrugs, HIF-1 small-molecule inhibitors, inhibitors of intracellular pH regulation to reinforce cancer cell acidosis, recombinant anaerobic bacteria, and hypoxia-specific gene therapy (1, 2, 5).

Here, we provide evidence for a novel role of the polyamine system in the hypoxic stress response in cancer cells. The polyamine system is up-regulated by hypoxia in a variety of cancer cell lines, and inhibition of polyamine biosynthesis sensitizes cancer cells to hypoxia-induced apoptosis in vitro. These findings were supported by in vivo experiments, showing that the antitumor effect of bevacizumab was significantly enhanced in mice receiving concomitant treatment with the polyamine biosynthesis inhibitor DFMO. Moreover, immunohistochemistry studies in clinical as well as experimental tumor specimens showed increased ODC levels in hypoxic tumor regions.

In a recent report by Keren-Paz and colleagues (22), it was shown that overexpression of AZIN results in up-regulation of ODC as well as of polyamine uptake. Notably, ODC overexpression alone had the opposite effect on polyamine uptake, as explained by the induction of OAZ, which is known to inhibit both ODC and polyamine uptake (13, 14). OAZ/AZIN–dependent pathways may also operate during hypoxia-mediated induction of the polyamine system, as supported by the following findings in the present study: (a) hypoxia concurrently induced ODC and polyamine uptake; (b) hypoxia had no effect on ODC transcription; and (c) hypoxic induction of the polyamine system was attenuated by RNA interference–mediated down-regulation of AZIN. Moreover, OAZ and AZIN mRNA levels were unaffected by hypoxia. Together, these results suggest that hypoxia triggers an increased AZIN/OAZ ratio by translational and/or posttranslational mechanisms. The transient effect of hypoxia on polyamine uptake and ODC activity and the fact that HIF-stabilizing agents mimicked the hypoxic effects on polyamine uptake point to a potential role of HIF-1α. A direct transcriptional effect of HIF-1α on the expression of ODC, OAZ, or AZIN was clearly not the case (Fig. 4A), which, however, does not exclude the involvement of HIF-1α as a transcription factor because it could act indirectly via activation of genes involved in the regulation of, for example, AZIN. Alternatively, hypoxia may induce HIF-1α–dependent changes that are mediated by interactions that do not require direct transcriptional activity of HIF-1α (e.g., by sequestering the HIF-β subunit ARNT from other transcription factors that heterodimerize with ARNT) thereby leading to inactivation of these factors. The exact mechanisms of hypoxic regulation of the OAZ/AZIN pathway and the potential role of HIF-1α will have to be determined in future studies.

It was previously reported that hypoxic exposure of pregnant rats caused a transient induction of ODC activity in the brain of the fetus (48). In contrast with our data, hypoxia was shown to up-regulate the level of ODC mRNA. However, the studies are not directly comparable, as their study was done on brain tissue exposed to mild, systemic “hypoxia” (~10.5% inspired O2), whereas we have investigated the direct effects of severe hypoxia (0.1–1% O2) generally found in tumor tissue.

Neutralization of VEGF activity by bevacizumab ultimately results in the regression of tumor vasculature, further leading not only to augmented hypoxia and increased cancer cell apoptosis but also to the induction of other proangiogenic factors (6, 47) and prosurvival mechanisms. We have shown an important role of the polyamine system in the hypoxic stress response in cancer cells; DFMO treatment rendered experimental glioma tumors more susceptible to the inhibitory effect of bevacizumab at conditions where tumor hypoxia was augmented by anti-VEGF antibody treatment. Previous reports have suggested that the antitumor effect of polyamine depletion by DFMO may partly be exerted through antiangiogenic actions (27), and that ODC-overexpressing cells form highly vascularized tumors in mice (49). This creates the possibility that ODC induction in response to hypoxia may not only promote tumor cell survival but also counteract some of the antiangiogenic effects of bevacizumab. The fact that DFMO reinforced the antiangiogenic activity of bevacizumab (Fig. 6C) lends some support to this idea. Ongoing work in our group focuses on the role of polyamines in endothelial cell survival and angiogenesis.

Gliomas are the dominating primary tumors of the CNS, and the median survival of high-grade glioma patients is still discouraging with <2% survivors 3 years after diagnosis. Gliomas are highly vascularized, VEGF-driven tumors, and both hypoxia and high ODC expression have been correlated with a more aggressive tumor phenotype (45, 46). A recent phase III trial showed increased survival when DFMO was added to conventional cytostatics as an adjuvant to surgery and radiotherapy in glioma patients (44). Moreover, a phase II trial has shown promising effects of bevacizumab in combination with chemotherapy in patients with recurrent glioma, and a phase III follow-up trial is ongoing (50).

In conclusion, the results of the present study show a role of the polyamine system in the hypoxic stress response and provide a rationale for further in vivo studies combining vascular disrupting agents or antiangiogenic agents with inhibitors of the polyamine system.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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