Hypoxia-Induced Autophagy Promotes Tumor Cell Survival and Adaptation to Antiangiogenic Treatment in Glioblastoma


Abstract

Antiangiogenic therapy leads to devascularization that limits tumor growth. However, the benefits of angiogenesis inhibitors are typically transient and resistance often develops. In this study, we explored the hypothesis that hypoxia caused by antiangiogenic therapy induces tumor cell autophagy as a cytoprotective adaptive response, thereby promoting treatment resistance. Hypoxia-induced autophagy was dependent on signaling through the hypoxia-inducible factor-1α (HIF-1α)/AMPK pathway, and treatment of hypoxic cells with autophagy inhibitors caused a shift from autophagic to apoptotic cell death in vitro. In glioblastomas, clinically resistant to the VEGF-neutralizing antibody bevacizumab, increased regions of hypoxia and higher levels of autophagy-mediating BNIP3 were found when compared with pretreatment specimens from the same patients. When treated with bevacizumab alone, human glioblastoma xenografts showed increased BNIP3 expression and hypoxia-associated growth, which could be prevented by addition of the autophagy inhibitor chloroquine. In vivo targeting of the essential autophagy gene ATG7 also disrupted tumor growth when combined with bevacizumab treatment. Together, our findings elucidate a novel mechanism of resistance to antiangiogenic therapy in which hypoxia-mediated autophagy promotes tumor cell survival. One strong implication of our findings is that autophagy inhibitors may help prevent resistance to antiangiogenic therapy used in the clinic. Cancer Res; 72(7); 1–11. ©2012 AACR.

Introduction

The hypothesis that tumor progression can be curbed by antiangiogenic agents targeting abnormal tumor vessels has been confirmed by preclinical evidence and clinical trials (1). However, these initial successes were tempered by the failure of angiogenesis inhibitors to produce enduring clinical responses. For example, in clinical trials of VEGF-neutralizing antibody bevacizumab in glioblastoma, 40% to 60% of tumors progressed after initially successful treatment (2), consistent with the development of resistance to antiangiogenic therapy, a state exhibiting a poor prognosis and poor response to available treatments (3). The molecular basis of acquired resistance to antiangiogenic treatments causing this lack of sustained responses remains undefined. We hypothesized that the devascularization caused by antiangiogenic therapy increases tumor hypoxia and that this hypoxia mediates resistance to antiangiogenic therapy.

Recent reports suggest that hypoxia activates autophagy, a lysosomal degradation pathway which may promote tumor cell survival (4). The mechanisms by which hypoxia induces autophagy need clarification, but the finding that BNIP3, a hypoxia-inducible factor-1α (HIF-1α) downstream target gene, is essential to hypoxia-induced autophagy suggests one possible mechanism (5).

During autophagy, a crescent-shaped structure, the isolation membrane, forms in the cytoplasm and closes around components targeted for destruction, leading to formation of the autophagosome, which fuses with the lysosome to become an autolysosome, leading to enzymatic degradation of autophagosome contents (6). While autophagosomes were initially identified in dying cells, a phenomenon that led to the term “autophagic cell death” to describe a cell death mode distinct from apoptosis, subsequent studies have shown that autophagy can allow cells to cope with stressors by destroying damaged proteins and organelles as a survival-promoting mechanism (7–9). During autophagosome formation, the ATG7 protein is essential for autophagosome membrane expansion (10).

While autophagosomes sequester cytosolic material non-specifically in a process called nonselective autophagy, additional evidence shows that a process of selective autophagy also occurs in which autophagic degradation of specific protein aggregates or organelles targeted for destruction occurs. Selective autophagy degrading–specific proteins is associated with degradation of p62 (11), a protein complex that binds ubiquitinated protein aggregates to target them for
degradation, or BNIP3, a marker of autophagic destruction of mitochondria. Nonselective autophagy can involve microtubule-associated protein light chain 3 (LC3), a protein that, after conversion from its cytosolic form LC3-I to its autophagosomal membrane–associated form LC3-II, is ultimately degraded by lysosomal enzymes in autolysosomes during nonselective autophagy, causing the total amount of LC3 (LC3-I plus LC3-II) to drop (6).

We report here that increasing tumor hypoxia occurs during antiangiogenic therapy and increases tumor cell autophagy as a cell survival mechanism, a novel resistance mechanism to antiangiogenic therapy. We then investigated the role of hypoxia-upregulated pathways in promoting autophagy. We showed the translational impact of this novel mechanism of resistance to antiangiogenic therapy by showing in animal models that pharmacologic or genetic autophagy disruption prevented hypoxia-associated resistance to antiangiogenic therapy. On the basis of the targetable novel mechanism of resistance to antiangiogenic therapy described here, combining antiangiogenic therapy with autophagy inhibition is a therapeutic strategy warranting further investigation in malignant cancers like glioblastomas.

Materials and Methods

Cells and reagents

Cell lines and protocols for primary tumor cell isolation are described in Supplementary Methods. A total of 300,000 cells per well were plated in 12-well plates overnight in Dulbecco's Modified Eagle's Medium with 10% FBS, then cultured under normoxia (5% CO2, 20% O2, 74% N2) or hypoxia (5% CO2, 1% O2, 94% N2) in a humidified O2 control incubator (Sanyo) never opened during incubation. Cells were incubated for 3, 6, 16, and 24 hours in dimethyl sulfoxide (DMSO; control), bafilomycin A1 (BafA1, 1 nmol/L), 3-methyladenine (3-MA, 1 mmol/L), chloroquine (10 or 25 μmol/L), or YC-1 (10 μmol/L). Relative cell numbers were measured by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). For apoptosis assays, one million cells per well were plated in 6-well plates overnight, then treated with DMSO, 1 mmol/L 3-MA, or 1 mmol/L BafA1 for 24 hours. Apoptotic and necrotic cells were measured by FITC-Annexin V Apoptosis Detection Kits (BD Pharmingen), with FACS carried out with a BD FACS Calibur II machine with FlowJo software (Tree Star, Inc.).

Western blotting

Western blotting was carried out as described in the Supplementary Methods.

Cell proliferation and apoptosis assays

A total of 5,000 cells per well in 96-well plates in triplicate were plated overnight, then treated with DMSO, 3-MA (1 mmol/L), or BafA1 (5 nmol/L) under normoxia or hypoxia for 48 hours. For extended treatment (72 hours), cells were treated with DMSO, BafA1 (1, 2.5 nmol/L), or chloroquine (10 or 25 μmol/L). Relative cell numbers were measured by ATG7 expression by Western blotting.

Real-time reverse transcriptase PCR

Real-time reverse transcriptase PCR is described in Supplementary Methods.

siRNA transfection

A total of 300,000 cells per well in 12-well plates were transfected overnight using METAFECTENE transfection reagent (Biontex Laboratories) with 20 nmol/L of negative control, HIF-1α, or AMPKα siGENOME SMARTpool siRNAs (Dharmacon, Inc.), which combine 4 siRNAs into a single pool. After culture with fresh medium for 24 hours, knockdown was confirmed by Western blotting.

Short hairpin RNA–mediated ATG7 knockdown

U87MG and SF8557 cells were infected with SMARTvector2.0 shRNA lentiviral particles (Thermo Scientific) expressing non-targeting negative control (5'-005000-01) or 3 human ATG7 short hairpin RNAs (shRNA; SH-020112-01, SH020112-02, and SH020112-03) in the presence of 4 μg/mL polybrene. After 48 hours, subcultured cells were selected in 1 μg/mL puromycin for 1 week. Lysates from stably selected cells were assessed for ATG7 expression by Western blotting.

Human tissue and xenograft immunohistochemistry

Paraffin-embedded sections and xenografts were processed as described in Supplementary Methods. Photographs were taken with a Zeiss Axioskop 2 and a Zeiss Axiocam color CCD. Staining quantification is described in Supplementary Methods.

Statistics

Two group comparisons of nonnormal values were carried out using the Wilcoxon signed rank (paired samples, e.g., pre- and posttreatment) or Wilcoxon rank-sum (unpaired) tests. For subcutaneous tumor volumes, initial comparison of 3 or more groups used the Kruskal–Wallis test (nonparametric alternative to ANOVA), followed by subsequent post hoc testing using the Wilcoxon rank-sum test for 2 group analyses. Kaplan–Meier analysis was used to compare survival between groups. P < 0.05 was considered significant, except for the post hoc Wilcoxon rank-sum testing, for which a Bonferroni correction for multiple testing required using αk < 0.01 (i.e., 0.01/k, where k = 5) to define significance. Error bars are SDs,
except for tumor volumes, whose error bars are standard errors. Experiments were repeated in triplicate with similar results each time, with figures containing representative experiments.

Results

Patient tumors resistant to antiangiogenic therapy exhibit increased hypoxia compared with before treatment

Review of an institutional database of 234 bevacizumab-treated glioblastomas from 2006 to 2010 revealed 6 cases meeting the following criteria: (i) after initial radiographic response to bevacizumab as monotherapy (n = 2) or combined with topoisomerase inhibitor irinotecan (n = 4), these cases exhibited the nonenhancing FLAIR bright growth on MRI seen in bevacizumab-treated glioblastomas (12, 13); (ii) surgical resection of the bevacizumab-resistant tumor occurred within 28 days of last bevacizumab dose; and (iii) archived paired pre- and posttreatment tissue was available. Tumor vessel density, assessed by CD31 immunostaining, decreased nearly 60% after bevacizumab resistance compared with pretreatment specimens from these patients (P < 0.001), meanwhile hypoxia-inducible genes HIF-1α and carbonic anhydrase 9 (CA9; ref. 14) immunostaining increased nearly 70% (Fig. 1, P < 0.05) and 80% (Fig. 1, P < 0.05), respectively. These trends persisted when separating cases into those growing during bevacizumab monotherapy (70% decreased vessel density, 72% increased HIF-1α immunostaining, and 40% increased CA9 immunostaining) versus those growing during bevacizumab plus irinotecan, (50% decreased vessel density, 67% increased HIF-1α immunostaining, and double the CA9 immunostaining), suggesting that, while irinotecan inhibition of HIF-1α expression (15) was more than offset by bevacizumab-induced devascularization and hypoxia-inducible gene expression.

Hypoxia induces autophagy in human glioblastoma cell lines

Briefly culturing U87MG and T98G glioma cell lines in hypoxia caused at least 2 autophagy-associated changes that progressively accumulated at 3, 6, 16, and 24 hours: degradation of total LC3 (LC3-I plus LC3-II) and p62 were seen in U87MG and T98G cells (Fig. 2A–C). Of note, LC3-II expression increased over time in normoxia, consistent with basal autophagy due to metabolite accumulation (16), but this autophagy was clearly increased by hypoxia. Minimal contribution of transcriptional changes to hypoxia-induced autophagy was suggested by the finding that hypoxia-associated alterations in levels of LC3A, LC3B, and p62 transcripts were insignificant (P = 0.1–0.9, Supplementary Fig S1) and did not correlate with hypoxia duration, consistent with prior reports (17, 18). Similar results were observed in U251, U138MG, A172, and G55 glioblastoma cell lines (Figs. 2D, Supplementary Fig. S2A). LC3-I to LC3-II conversion, a third autophagy-associated change (19), was seen in hypoxic T98G (Fig. 2B), U251 (Fig. 2D), and G55 (Supplementary Fig. S2A) cells. We further confirmed hypoxia-

Figure 1. Glioblastoma (GBM) progressing during antiangiogenic therapy exhibit decreased vessel density, increased hypoxia, and increased BNIP3 staining compared with pretreatment glioblastomas from the same patients. A, representative immunostaining for endothelial marker CD31 (top row), hypoxia markers HIF-1α (second row; blue, Hoescht nuclear staining; green, HIF-1α), and CA9 (third row) and autophagy-mediating BNIP3 (bottom row; blue, Hoescht nuclear staining; green, BNIP3) in this glioblastoma resistant to bevacizumab (right) compared with paired specimens from the same patient before treatment (left). CA9 and BNIP3 stainings are from adjacent sections. B, vessel density decreased (P < 0.001), hypoxia markers HIF-1α (P < 0.05), hypoxia marker CA9 staining increased (P < 0.05), and BNIP3 immunostaining increased (P < 0.001) in 6 glioblastomas after bevacizumab resistance compared with paired pretreatment specimens. Magnification, ×40; scale bar, 200 μm. hpf, high-power field.
induced autophagy when we identified that hypoxia upregulated BNIP3 expression in all human glioblastoma cell lines examined (Fig. 2E).

**Hypoxia upregulates autophagy in primary glioblastoma cells**

Because primary cells might respond differently to hypoxia than cell lines, we studied the ability of hypoxia to induce autophagy-associated protein changes in primary glioblastoma cells from freshly resected human glioblastomas. Hypoxia induced the same autophagy-associated changes found in cell lines, degradation of p62 and total LC3, in the primary human glioblastoma cells SF8244, SF8167, SF8106, and SF7796, with 2 of these primary human glioblastoma cells also exhibiting hypoxia-induced LC3-I to LC3-II conversion (Figs. 2F, Supplementary Fig. S2A). Because the ATG4 protease modifies LC3 (20), we investigated ATG4 expression in cells exhibiting (T98G, SF8167, and SF7796) or not exhibiting (SF8106) hypoxia-induced LC3-I to LC3-II conversion (Figs. 2F, Supplementary Fig. S2A). ATG4A and ATG4B homologues were not detectable in these cells (data not shown), whereas ATG4C expression decreased slightly with hypoxia in T98G and the 3 primary glioma cells (Supplementary Fig. S3), suggesting that ATG4C did not contribute to the hypoxia-induced LC3-I to LC3-II conversion occurring in many glioma cells.

**Increased expression of autophagy mediators in human glioblastomas after bevacizumab resistance**

We used immunohistochemistry to identify hypoxic areas and areas that stained positive for autophagy mediator BNIP3 in 6 bevacizumab-resistant glioblastomas and the paired pretreatment glioblastomas from these same patients. The increased hypoxia of these specimens after bevacizumab resistance compared with before was quantified earlier. While the core of hypoxic areas in these bevacizumab-resistant glioblastomas was often necrotic, the hypoxic periphery of these necrotic areas stained positive for autophagy-mediating BNIP3 (Fig. 1A). Tumors exhibited increased BNIP3 immunoreactivity after bevacizumab failure in a manner reflecting the greater hypoxia seen after resistance to antiangiogenic therapy (Fig. 1A), with image analysis revealing 55% more BNIP3 immunostaining in the 6 glioblastomas after bevacizumab resistance than in tumors from the same patients before bevacizumab treatment (Fig. 1B, P < 0.001). These trends persisted when separating cases into those treated with bevacizumab.
monotherapy (53% increased BNIP3 immunostaining) versus bevacizumab plus irinotecan (57% increased BNIP3 immunostaining).

Autophagy inhibitors disrupt hypoxia-induced glioblastoma autophagy

Early autophagy inhibitor 3-MA and late autophagy inhibitor BafA1 both blocked hypoxia-induced p62 degradation in cultured U87MG cells. 3-MA inhibited conversion of LC3-I to LC3-II, whereas BafA1 increased LC3-I to LC3-II conversion (Fig. 3A), reflecting the fact that these inhibitors disrupt autophagy either before (3-MA) or after (BafA1) LC3-I to LC3-II conversion. Similar effects were seen in U373 cells transduced to express a GFP-LC3 fusion protein, with hypoxia increasing autophagy, as assessed by the number of cells with punctate green staining, a marker reduced by early autophagy inhibitors, and Western blotting for free GFP released by autophagic degradation, a marker reduced by early and late autophagy inhibitors (16). Early autophagy inhibitor 3-MA lowered the number of cells with punctate green staining in hypoxia, and late autophagy inhibitor BafA1 maintained the high number of cells with punctate green staining seen in hypoxia (Figs. 3B and C, P < 0.01). Similarly, hypoxia increased free GFP identified by Western blotting over 3-fold, with free GFP lowered by early (3-MA) or late (BafA1) autophagy inhibitors, particularly the latter (Supplementary Fig. S4).

Inhibiting hypoxia-induced autophagy increases cell death

Next we measured cell survival under hypoxic culturing conditions in the presence of either 3-MA or BafA1. BafA1 significantly decreased the number of viable U87MG and T98G cells in hypoxia (Fig. 4A, P < 0.05), with 3-MA having slightly less inhibitory effects on cell viability (Fig. 4A, P = 0.06). Having shown a survival-promoting effect of hypoxia-induced autophagy, we characterized the type of cell death occurring in hypoxia when autophagy was inhibited. We conducted flow cytometry after using Annexin V (AnnV) and propidium iodide (PI) to label hypoxic U87MG cells that had been treated with and without autophagy inhibitors. In the presence of 3-MA or BafA1, hypoxia significantly increased the number of cells that were AnnV<sup>+</sup>PI<sup>+</sup> (late-stage apoptosis; BafA1, P < 0.01; 3-MA, P < 0.05), whereas the number of AnnV<sup>+</sup>PI<sup>−</sup> cells in early apoptosis did not change (Fig. 4B, P > 0.05). The percentage of total PARP that was active or cleaved, an apoptosis marker, increased in hypoxic cells treated with autophagy inhibitors, with lesser effects seen in normoxia (Fig. 4C), suggesting that the cell death promoted when autophagy was inhibited was apoptotic.
Dependence of various aspects of hypoxia-induced autophagy on HIF-1α or AMPK

Pathways activated by hypoxia in tumor cells that can contribute to autophagy include those mediated by HIF-1α, activated during physiologic hypoxia (O₂, 0.1%–3%), or by HIF-1α-independent 5′ AMP-activated protein kinase (AMPK), activated during anoxia (<0.01% O₂; ref. 4). At 1% O₂, both HIF-1α and AMPK were activated in U87MG and T98G cells, with HIF-1α activation occurring earlier than AMPK activation (Fig. 5A). Furthermore, siRNA-mediated knockdown of AMPK or HIF-1α blocked hypoxia-mediated LC3-I to LC3-II conversion and total LC3 degradation but neither siRNA affected hypoxia-mediated p62 degradation (Fig. 5B). Similarly, HIF-1α inhibitor YC-1 (21) blocked hypoxia-mediated total LC3 degradation and LC3-I to LC3-II conversion but did not affect hypoxia-mediated p62 degradation (Fig. 5C). We then investigated the role of HIF-1α or AMPK in the hypoxia-mediated BNIP3 expression identified earlier. Hypoxia-induced BNIP3 expression was reduced with HIF-1α inhibition achieved through HIF-1α siRNA (Fig. 5B) or YC-1 (Fig. 5D). In contrast, AMPK inhibition achieved through AMPK siRNA failed to alter hypoxic induction of BNIP3 (Fig. 5B). Therefore, hypoxia-mediated LC3-I to LC3-II conversion depended on HIF-1α, but AMPK, hypoxia-mediate BNIP3 expression depended on HIF-1α not AMPK, and hypoxia-mediated p62 degradation occurred independent of these pathways.

Combined inhibition of autophagy and angiogenesis exerts a potent antitumor effect in vivo

3-MA and BafA1 are typically used as autophagy inhibitors in vitro (22), but chloroquine or hydroxyl-chloroquine, late autophagy inhibitors like BafA1, are used to inhibit autophagy in vivo (23), partly because they are the only U.S. Food and Drug Administration (FDA)-approved autophagy inhibitors. Like BafA1, chloroquine blocked hypoxia-induced p62 degradation, but by blocking autophagy after LC3-I to LC3-II conversion, caused more LC3-I to LC3-II conversion to occur in cultured U87MG, GBM39, and G55 glioma cells (Supplementary Figs. S2B and S5A), and decreased the viability of U87MG (P<0.05, Supplementary Fig. S2B) and G55 (P<0.05, Supplementary Fig. S2C) in hypoxia compared with normoxia. We also examined the effect of chloroquine on BNIP3 expression in 5 cell lines and xenograft-derived cells and found that, whereas hypoxia increased BNIP3 expression in all cells, chloroquine minimally affected BNIP3 expression under normoxia or hypoxia (Supplementary Fig. S5C), consistent with prior in vitro reports (24), and suggesting that late autophagy inhibitor chloroquine exerted its effects downstream of BNIP3 upregulation.

We then investigated whether chloroquine counteracted the survival-promoting effects of hypoxia-induced autophagy caused by antiangiogenic treatment by treating subcutaneous tumors derived from GBM39 primary glioma cells with autophagy inhibitor chloroquine and/or antiangiogenic agent bevacizumab. After 4 weeks, tumor volumes differed between the 4 treatment groups (P<0.05) and, compared with PBS, neither chloroquine nor bevacizumab inhibited tumor growth (P=0.3–0.8). Combined therapy (bevacizumab + chloroquine) inhibited tumor growth in a prolonged and significant manner versus either agent alone (P<0.01, bevacizumab vs. bevacizumab + chloroquine; P<0.005, chloroquine vs. bevacizumab + chloroquine; Fig. 6A). Bevacizumab-treated tumors, with or without combined chloroquine, exhibited 4- to 6-fold reduced vessel density (P<0.01) and over double increased hypoxic area (P<0.05), compared with PBS-treated tumors or tumors treated with chloroquine monotherapy (Fig. 6B), confirming that antiangiogenic therapy induced devascularization and
hypoxia. While bevacizumab monotherapy increased BNIP3 expression nearly 2-fold over than PBS or chloroquine treatment ($P < 0.05$), adding chloroquine to bevacizumab reduced BNIP3 expression to levels comparable with PBS- or chloroquine-treated tumors (Fig. 6B, $P < 0.05$). Cell death in these xenografts was characterized using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining to detect cells in late apoptosis, and staining increased over 2-fold in chloroquine-treated xenografts compared with PBS-treated xenografts ($P < 0.01$) and nearly 4-fold in bevacizumab plus chloroquine-treated xenografts compared with bevacizumab-treated xenografts (Fig. 6B, $P < 0.05$).

Similar sustained tumor growth inhibition in combined treated tumors versus eventual accelerated growth in bevacizumab-treated tumors was noted in subcutaneous U87MG tumors ($P < 0.005$ for 4 group comparison; $P < 0.01$, bevacizumab vs. bevacizumab + chloroquine after 8 and 11 treatment days; Supplementary Fig. S7A) human glioma cell lines. For U87MG-derived xenografts, prolonged treatment of the bevacizumab monotherapy and bevacizumab plus chloroquine groups for 2 additional weeks increased the separation between the 2 groups, with bevacizumab-treated tumors exhibiting an increased growth rate, and the combined treatment tumors exhibiting sustained growth suppression ($P < 0.01$, Supplementary Fig. S6B). Immunohistochemistry of treated U87MG xenografts revealed similar findings as seen with GBM39–decreased vessel density and increased hypoxia in bevacizumab-treated xenografts, increased BNIP3 expression in bevacizumab-treated xenografts, and increased TUNEL staining in chloroquine-treated xenografts (Supplementary Fig. S6C). Western blotting of protein from subcutaneous U87MG tumors revealed increased LC3-I to LC3-II conversion and G55 ($P < 0.001$ for 4 group comparison; $P < 0.01$, bevacizumab vs. bevacizumab + chloroquine after 8 and 11 treatment days; Supplementary Fig. S7A) human glioma cell lines.

Figure 5. Hypoxia upregulates HIF-1α expression and AMPK phosphorylation, which contribute to some aspects of hypoxia-induced autophagy. A, U87MG and T98G cells cultured in hypoxia exhibited time-dependent activation of HIF-1α and AMPK (with AMPK activation assessed by detecting phosphorylated AMPK in the first row), with HIF-1α activation occurring before AMPK activation. B, siRNA-mediated knockdown of AMPK and HIF-1α in hypoxic U87MG cells exhibited reduced LC3-I to LC3-II conversion and reduced total LC3 degradation but neither siRNA affected hypoxia-mediated p62 degradation, whereas only HIF-1α siRNA reduced hypoxia-induced BNIP3 expression. C, similarly, YC-1 (a HIF-1α inhibitor) blocked hypoxia-mediated LC3-I to LC3-II conversion and total LC3 degradation without affecting hypoxia-mediated p62 degradation in T98G cells. D, in U87MG cells cultured for 24 hours in hypoxia, YC-1 blocked hypoxia-mediated BNIP3 upregulation.
after bevacizumab treatment, consistent with autophagy, and after chloroquine treatment, consistent with our \textit{in vitro} data reflecting the fact that chloroquine is a late autophagy inhibitor (Supplementary Fig. S6D).

Another patient specimen–derived subcutaneous xenograft, SF8244, exhibited similar sustained lack of growth in combined treated tumors versus eventual accelerated growth in bevacizumab-treated tumors ($P < 0.01$ for 4 group comparison, Supplementary Fig. S7B). Delayed chloroquine addition to bevacizumab-treated SF8244 tumors that had reached volumes averaging 400 mm$^3$ reduced tumor volume whereas bevacizumab-bevacizumab-treated tumors continued exponential growth ($P < 0.001$, Supplementary Fig. S7B), suggesting that inhibiting autophagy upon initiation of resistant growth could still suppress antiangiogenic therapy resistance. Chloroquine alone did not affect tumor growth compared with PBS in any xenografts ($P = 0.4–0.7$).

**Knockdown of essential autophagy gene \textit{ATG7} promotes bevacizumab responsiveness \textit{in vivo}**

Because chloroquine could exert nonspecific effects, to more precisely define the contribution of autophagy to antiangiogenic therapy resistance, we engineered U87MG and SF8557 glioma cells to stably express 3 different shRNAs targeting autophagy-mediating gene \textit{ATG7} (Supplementary Fig. S8A). Cells expressing the shRNA causing greatest ATG7 knockdown exhibited inhibition of 2 hypoxia-mediated autophagy-associated protein changes, p62 degradation, and LC3-I to LC3-II conversion (Supplementary Fig. S8B). We treated subcutaneous tumors derived from U87MG/shControl and U87MG/shATG7 cells and intracranial tumors derived from SF8557/shControl and SF8557/shATG7 cells with PBS or bevacizumab. Whereas subcutaneous U87MG/shControl (Fig. 6C) and intracranial SF8557/shControl (Fig. 6D) tumors exhibited no response to bevacizumab ($P = 0.3–0.8$), all subcutaneous U87MG/shATG7 tumors regressed to cure (Fig. 6C, $P < 0.001$) and intracranial SF8557/shATG7 tumors exhibited 90% long-term survival (Fig. 6D) with bevacizumab treatment ($P = 0.003$). Immunostaining subcutaneous and intracranial shRNA–transduced tumors except for bevacizumab-treated subcutaneous U87MG/shATG7 tumors, which were cured, revealed that bevacizumab decreased vascularity and increased hypoxia in shControl- and shATG7–transduced ectopic and orthotopic tumors ($P < 0.05$, Supplementary Fig. S9), consistent with our \textit{in vivo} results with other bevacizumab-treated tumors, BNIP3 expression increased with bevacizumab treatment of shControl- and shATG7-transduced tumors ($P < 0.05$, Supplementary Fig. S9), with the former consistent with our \textit{in vivo} results and the latter consistent with a prior report (25).

**Discussion**

Cells exposed to various stressors undergo a process of self-digestion known as autophagy, during which cytoplasmic cargo sequestered inside double-membrane vesicles are delivered to the lysosome for degradation. Several \textit{in vitro} studies suggest that, while autophagy initially prevents cancer cell survival, once a tumor develops, autophagic self-catabolization of damaged organelles promotes cell survival by allowing tumor cells to survive the hypoxia and the nutrient and growth factor deprivation (7–9) found in the tumor microenvironment. Suggestion that autophagy promotes tumor cell survival \textit{in vivo} comes from the correlation of immunostaining for autophagy-promoting BNIP3 with poor cancer survival (26, 27). Several cancer therapies induce autophagy (28–30), and the autophagic response to some treatments is cytoprotective (31).

Because of the failures of conventional DNA-damaging chemotherapy, antiangiogenic therapy has been investigated, with efficacy showed in several cancer clinical trials. However, this efficacy is often transient with acquired resistance to the contribution of autophagy to antiangiogenic therapy common (32). While antiangiogenic therapy can transiently normalize structural and functional abnormalities in tumor vessels (33), the long-term effect of antiangiogenic therapy is tumor devascularization, which ultimately worsens tumor hypoxia.

We hypothesized that hypoxia, as occurs after antiangiogenic therapy (34), would promote autophagy as a cytoprotective adaptive mechanism. Others have shown that hypoxia upregulates autophagy-associated factors, like BNIP3 (35), a finding supported by the identification of BNIP3 expression in perinecrotic regions of patient tumor specimens (36), but whether the response is cytoprotective and which pathways are involved remain undetermined.

The cytoprotective nature of autophagy during hypoxia induced by antiangiogenic therapy was verified by our \textit{in vitro} data showing decreased survival of cells treated

---

*Figure 6. Autophagy inhibitor chloroquine (Chloro) combined with bevacizumab (Bev) inhibits GBM39 tumor growth \textit{in vivo}. A, subcutaneous tumors in athymic mice were treated with PBS, chloroquine, bevacizumab, and chloroquine plus bevacizumab. After 4 weeks, there were significantly different tumor volumes amongst groups ($P < 0.05$). Compared with PBS (black), neither chloroquine (pink) nor bevacizumab (blue) inhibited tumor growth ($P = 0.3–0.8$). Combined therapy with bevacizumab and chloroquine (red) inhibited tumor growth in a prolonged and statistically significant manner relative to either agent alone ($P < 0.01$, bevacizumab vs. chloroquine; $P < 0.005$, chloroquine vs. bevacizumab + chloroquine), B, vessel density (CD31 staining, red) decreased in bevacizumab-treated tumors ($P < 0.01$). Hypoxia (CA9 staining, green) increased in bevacizumab-treated tumors ($P < 0.05$); BNIP3 expression increased with bevacizumab treatment ($P < 0.05$), an increase eliminated by adding chloroquine to bevacizumab ($P < 0.05$). TUNEL staining (red) increased in chloroquine-treated tumors ($P < 0.05$). 4′, 6-Diamidino-2-phenylindole (DAPI) nuclear counterstaining is blue. Bevacizumab plus chloroquine–treated tumors were small enough that the entire tumor fit one field of view. Magnification, x20; scale bar, 200 μm. C, when subcutaneous U87MG/shControl and U87MG/shATG7 xenografts were treated with PBS or bevacizumab, U87MG/shATG7 tumors completely regressed with bevacizumab treatment ($P < 0.001$), whereas U87MG/shControl xenografts were minimally responsive ($P = 0.8$). D, intracranial SF8557/shATG7 xenografts exhibited 90% long-term survival with bevacizumab treatment, whereas PBS treatment of intracranial SF8557/shATG7 xenografts led to 18-day median survival ($P = 0.003$). Intracranial SF8557/shControl xenografts exhibited 15-day median survival with PBS, similar to their 18-day median survival with bevacizumab ($P = 0.3$).*
with autophagy inhibitors in hypoxic conditions, particularly with late autophagy inhibitors, and more so at 72 hours (Supplementary Fig. S5B) than 48 hours (Fig. 4A) and our in vitro data showing increased TUNEL staining in chloroquine plus bevacizumab–treated xenografts compared with bevacizumab–treated xenografts (Fig. 6B, Supplementary Fig. S6C), suggesting an increased number of apoptotic cells during combined treatment. Of note, while chloroquine consistently exerted antitumor effects in hypoxic conditions in vitro and when combined with antiangiogenic therapy in vivo, it promoted tumor growth, albeit in a manner not quite statistically significant, in normoxic U87MG cells (Supplementary Fig. S5B) and as monotherapy compared with PBS in G55 xenografts (Supplementary Fig. S7A). Similarly, in addition to potentiating the response to antiangiogenic therapy, ATG7 knockdown caused faster in vivo growth of PBS-treated tumors than wild-type tumors. These findings illustrate the dual functions of autophagy—a tumoricidal effect under normoxic unstressed conditions, such that autophagy inhibition under those conditions can actually promote tumor growth, versus a tumor-protective effect upon exposure to stressors like the hypoxia caused by antiangiogenic therapy. These dual functions of autophagy suggest that inhibiting autophagy may be of limited clinical value alone but, when used with antiangiogenic therapy, could provide a therapeutic benefit. These findings also suggest that the effect we observed in vivo was not the additive effect of combining 2 antitumor agents but instead reflected the ability of one therapeutic modality, antiangiogenic treatment, to turn another modality, autophagy inhibition, with mild tumor-promoting effects into a true antitumor strategy.

The tumor response to hypoxia activates several factors, including HIF-1α, felt to activate at moderate hypoxia (0.1%), and HIF-1α–independent AMPK, felt to activate at anoxia (0.01%; ref. 4). We found that at 1% oxygen, a concentration more typical of glioblastomas than 0.1% or 0.01% (37), both HIF-1α and AMPK were activated, with HIF-1α activated earlier than AMPK, suggesting that, different durations of hypoxia, not just different hypoxia levels, may differentially activate these pathways. Both HIF-1α and AMPK could contribute to autophagy, with mTOR inhibition a possible mechanism (38–41). We found that hypoxia-mediated LC3-I to LC3-II conversion and overall LC3 degradation depended on both HIF-1α and AMPK, hypoxia-mediated BNIP3 expression depended on HIF-1α not AMPK, and hypoxia-mediated p62 degradation was independent of HIF-1α and AMPK. While LC3 contributes to nonselective autophagy (degradation of bulk cytoplasmic contents including organelles), p62 degradation and BNIP3 expression are more involved in selective autophagy destroying ubiquitinated proteins and mitochondria, respectively. Future studies will need to clarify mediators of hypoxia-induced p62 degradation.

Interestingly, chloroquine minimally affected BNIP3 expression in our cultured cells, consistent with prior reports using cultured colon carcinoma cells treated with BafA1, another late autophagy inhibitor (42), and suggesting that chloroquine inhibited autophagy downstream of BNIP3 expression. In contrast, chloroquine lowered BNIP3 expression in bevacizumab–treated xenografts. The differences between these in vitro and in vivo results could reflect as yet uncharacterized factors in the microenvironment absent in cultured cells, or could reflect the longer treatment duration tumors were exposed to in vivo than in culture, potentially increasing cell death and reducing in vivo BNIP3 expression. Tumors derived from cells transduced to express shRNA targeting essential autophagy gene ATG7 exhibited slightly increased BNIP3 expression, consistent with a prior report in which genetic disruption of ATG7 eliminated autophagy but led to a slight increase in BNIP3 expression that could not trigger autophagy in the setting of ATG7 loss (25).

Our findings are significant because we show that targeting autophagy through pharmacologic or genetic means disrupts antiangiogenic therapy resistance in vivo. While some of these observations were made in ectopic subcutaneous tumors, because of our findings of the importance of hypoxia in resistance to antiangiogenic therapy and reports that orthotopic murine intracranial tumors exhibit less hypoxia than ectopic subcutaneous tumors and that the hypoxia of the latter more closely resembles human glioblastoma (43, 44), our findings in subcutaneous tumors should be pertinent to glioblastoma.

Chloroquine, a clinically approved antimalaria drug, has been studied in a randomized glioblastoma trial combining chloroquine with conventional treatment with a benefit not quite significant (45). Currently more than 20 phase 1/II cancer clinical trials involving chloroquine or hydroxychloroquine are open nationwide (24). Furthermore, while chloroquine plus antiangiogenic therapy in our xenografts was not curative, chloroquine exerts numerous nonspecific effects, incompletely disrupts autophagy, and achieves maximal plasma concentration (46) 10-fold lower than the concentrations inhibiting hypoxia-induced autophagy in vitro. Thus, our finding that genetic disruption of essential autophagy gene ATG7 dramatically increased response to antiangiogenic therapy from no response to curative, suggests that long-term evaluation of autophagy inhibitors in treating antiangiogenic therapy resistance will require more specific and potent autophagy inhibitors currently being developed (24).

Disclosure of Potential Conflicts of Interest
A. Jahangiri is a Howard Hughes Medical Institute Fellow. No potential conflicts of interest were disclosed by the other authors.

Grant Support
The work was supported by funding to MKA from the American Brain Tumor Association, the James S. McDonnell Foundation, the NIH (5K02NS64167-2), and the UCSF Brain Tumor SPORE. A. Jahangiri is a Howard Hughes Medical Institute Research Fellow.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 22, 2011; revised January 25, 2012; accepted February 14, 2012; published OnlineFirst March 23, 2012.
References


www.aacrjournals.org
Cancer Res; 72(7) April 1, 2012
OF11

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2012 American Association for Cancer Research.
Hypoxia-Induced Autophagy Promotes Tumor Cell Survival and Adaptation to Antiangiogenic Treatment in Glioblastoma


Cancer Res  Published OnlineFirst March 23, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-3831

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/02/21/0008-5472.CAN-11-3831.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.