Autophagy Inhibition Sensitizes Colon Cancer Cells to Antiangiogenic and Cytotoxic Therapy

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Abstract

**Purpose:** Autophagy is a critical survival pathway for cancer cells under conditions of nutrient or oxygen limitation, or cell stress. As a consequence of antiangiogenic therapy, solid tumors encounter hypoxia induction and imbalances in nutrient supply. We wished to determine the role of autophagy in protection of tumor cells from the effects of antiangiogenic therapy and chemotheraphy. We examined the effect of inhibiting autophagy on hypoxic colon cancer cells *in vitro* and on bevacizumab- and oxaliplatin-treated mouse xenografts *in vivo*.

**Experimental Design:** The autophagic response to hypoxia and DNA-damaging agents was assessed by fluorescent microscopic imaging, autophagy-related gene expression, and by electron microscopic ultrastructural analysis. Pharmacologic and molecular approaches to autophagy inhibition were taken in a panel of colon cancer cell lines. Mouse xenograft models were treated with combinations of oxaliplatin, bevacizumab, and chloroquine to assess effects on tumor growth reduction and on pharmacodynamic markers of autophagy inhibition.

**Results:** Autophagy was induced in colon cancer models by exposure to both hypoxia and oxaliplatin. Inhibition of autophagy, either with chloroquine or by downregulation of beclin1 or of ATG5, enhanced sensitivity to oxaliplatin under normal and hypoxic conditions in a synergistic manner. Both bevacizumab and oxaliplatin treatments activate autophagy in HT29 murine xenografts. The addition of chloroquine to bevacizumab-based treatment provided greater tumor control in concert with evidence of autophagy inhibition.

**Conclusions:** These findings implicate autophagy as a mechanism of resistance to antiangiogenic therapies and support investigation of inhibitory approaches in the management of this disease.

Introduction

Colorectal cancer is the third most common malignancy and the second most common cause of cancer-related death in the United States. The application of both chemotherapy and targeted therapies including antiangiogenic therapy has increased the survival of patients with colorectal cancer (1–4). However, despite the success of antiangiogenic therapy regimens in advanced disease, the use of bevacizumab with chemotherapy did not confer a survival benefit in disease confined to lymph nodes (stage III), and the optimal use of this modality remains to be defined. It is critical to unravel the mechanisms that underlie the resistance of colon cancer cells to antiangiogenic therapy.

In solid tumors, intratumoral hypoxic regions are implicated in poor prognosis and chemotherapy resistance (5, 6). Hypoxic regions in the tumors are also associated with metabolic alterations as an adaptive response to nutrient deprivation (7, 8). By inhibiting the tumor vasculature, bevacizumab, and other antiangiogenic agents cause substantial alterations in the tumor microenvironment (9). We have previously shown that bevacizumab treatment induces hypoxia in colon cancer xenografts, and that tumor shrinkage as a consequence of treatment is variable (10, 11). The degree to which hypoxia is induced in different models is similar: the production of tumor shrinkage by bevacizumab, however, depends on susceptibility to hypoxia-induced cell death (11). An emerging concern with antiangiogenic therapy is that under this selection pressure, more aggressive tumor behavior may ensue (12, 13). Furthermore, cells in hypoxic environments have been shown to accumulate mutations and more aggressive growth characteristics (14, 15). Therefore, targeting this hypoxic population may have implications beyond immediate tumor shrinkage.
Translational Relevance

The combination of cytotoxic chemotherapy and bevacizumab is the backbone of therapy for advanced colorectal cancer. Understanding the basis of resistance is the key to development of newer and more effective therapies. Our previous work suggests that the induction of hypoxia by bevacizumab contributes to its interaction with chemotherapy, and may both increase cell kill and at the same time promote the acquisition of resistance—much like cytotoxics. We show here that one of the ways in which colon cancer cells become resistant to bevacizumab and oxaliplatin is through induction of an autophagy program. In an animal model, inhibition of autophagy is associated with greater sensitivity to both oxaliplatin and bevacizumab individually, and especially in combination. These data may point the way to more effective colon cancer therapy, and clinical studies are in progress to that end.

Both hypoxia and nutrient deprivation are known to induce the process of autophagy, which has recently emerged as a critical cellular process in cancer cell survival (16, 17). Autophagy is a highly conserved catabolic process whereby long-lived proteins and organelles are engulfed in double-membrane structures called autophagosomes and targeted to the lysoosomes for degradation for energy production (17, 18). Some 20 autophagy-related proteins (ATG) have been identified, initially from yeast genetic studies, followed by characterization of their mammalian homologs (17, 19, 20). Autophagy seems to play a role at multiple levels of tumor development and may have a protective role in carcinogenesis (21). Autophagy is also a consequence of cytotoxic drug treatment, and more recently, has been appreciated as a means by which cells might survive the stress of cellular insults, and so become resistant to treatment (22). Evidence supports a role for inhibiting autophagy to enhance the effectiveness of cancer treatment and genetic manipulation of cells to impede autophagy sensitizes resistant cells to treatment (23).

Autophagy is often localized to hypoxic regions of the tumor, where its induction is thought to facilitate cell survival (24–26). Hypoxia-induced autophagy mechanisms have been shown to depend on the Bcl-2 family member BNIP3 in the induction of cell death in apoptosis-competent cells (27–29). The displacement of beclin1 from beclin1/Bcl-2 complexes permits the rapid activation of autophagy (28). Interestingly, silencing of BNIP3 by histone deacetylation and methylation has been shown in colon cancer cell lines, and so other mechanisms must be sought in this disease (30). Hypoxia-induced autophagy has also been shown to contribute to chemoresistance in hepatocellular carcinoma cells (31). Accordingly, a strategy directed to autophagy inhibition has the potential to overcome a key means of survival of cancer cells (22, 32).

In this study, we examined the effect of hypoxia and oxaliplatin on autophagy regulation in colon cancer cells and explored the impact of autophagy inhibition by chloroquine on oxaliplatin- and hypoxia-induced cytotoxicity. We found a synergistic interaction of oxaliplatin and chloroquine on several colon cancer cell lines under oxic and hypoxic environments, and confirmed the role of autophagy inhibition in this interaction through silencing of beclin1 and ATG5 expression by RNA interference. We validated the interaction by showing a marked enhancement of tumor control by the incorporation of chloroquine with bevacizumab and oxaliplatin therapy in a colon cancer mouse xenograft model. On the basis of these findings a clinical trial of autophagy reversal is in progress in colorectal cancer.

Materials and Methods

Cell lines and treatments

Colon cancer cell lines HT29, HCT116, HCT15, SW620, KM12, BE, WiDr, and LoVo were obtained from American Type Culture Collection. HCT116 p53+/− cells were provided by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD). The WiDrOXL27 cell line was generated by an incremental increase in exposure of oxaliplatin to WiDr cells and maintained in culture with a high concentration of oxaliplatin. For hypoxia treatments, the colon cancer cell lines were exposed to hypoxia (0.1% O2 for 24 hours), then returned to a standard culture environment (5% CO2, 95% N2 and 5% CO2, 95% air) for a further 48 hours. For chemotherapy treatments, cells in exponential growth were treated in hypoxia or normoxia followed by 48 hours of normal culture conditions, as previously described (10, 11). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Oxaliplatin and chloroquine were obtained from Sigma.

In vitro cytotoxicity assay and combination index analysis

Cytotoxicity was determined using the MTT assay (10). Briefly, after plating and overnight incubation at a density of 3,000 cells per well in 96-well plates, cells were subjected to hypoxia for 24 hours in an anaerobic chamber (Forma Scientific Inc.), which was gassed using oxygen-poor (less than 1 part per 10 billion) 95% N2 and 5% CO2, and then cultivated for 2 more days under normal conditions, followed by MTT assay. The absorbance at 570 nm was determined using a microplate reader (Elx800, BioTek Instruments, Inc.). The data shown are the mean ± SD of 2 independent experiments carried out in triplicate. The combination index (CI) analysis was conducted using the method of median effect method by Chou and Talalay (33, 34). Initially, the IC50 values were calculated for chloroquine and oxaliplatin for colon cancer cell lines using standard algorithms. The ratio for combination of drugs was determined on the basis of IC50 values. The CI values for synergy, additivity and antagonism were determined using commercially available Compusyn software (34). The CI is a quantitative measure of the degree of interaction between...
1 or more drugs. When it equals to 1, it denotes additivity, >1 antagonism, <1 synergism, and <0.3 strong synergism. The CI was calculated under the assumption of a mutually nonexclusive drug interaction.

RNA interference

The cells were transfected with 50 nmol/L of control (ON-TARGETplus Nontargeting Pool; Dharmacon) or beclin1 siRNAs (ON-Target plus SMARTpool; Dharmacon) with Lipol ectamine RNAiMAX (Invitrogen) reagent using reverse transfection methods as described by the manufacturer. The sequences of the sense strands of the RNAs used in this study were as follows: beclin1 siRNA-1 GAIU ACC CAC GUC UIU A, beclin1 siRNA-2 GGA ACU CAC AGC UCC AUU A, beclin1 siRNA-3 CUA AGG AGC UGC CGU UAI A, beclin1 siRNA-4 GAG AGG AGC UGC CGU UIU A, and control siRNAs of ON-TARGETplus Nontargeting Pool from Dharmacon. The sequences of the sense strands of the RNAs used in this study for ATG-5 knockdown were as follows: ATG5 siRNA-1 GCC AGU AUC CAA UIU GGU U, ATG5 siRNA-2 GCA GAA CCA UAC UIU UUG C, ATG5 siRNA-3 UGA CAG AUU UGA CCA GGU UIU, ATG5 siRNA-4 ACA AAG AUU UGC UUG GAG A, ATG5 siRNA-3 UGA CAG AUU UGA CCA GGU UIU, ATG5 siRNA-4 ACA AAG AUU UGC UUG GAG A, ATG5 siRNA-3 UGA CAG AUU UGA CCA GGU UIU, ATG5 siRNA-4 ACA AAG AUU UGC UUG GAG A, HT29, HCT116, KM12, BE, SW620, and HCT15 colon cancer cell lines were initially cultured in 12-well plates and transfected with either 50 nmol/L of control siRNA pool, or with beclin1 or ATG5 siRNAs SMARTpool, for 48 hours. We confirmed downregulation of beclin1 and ATG5 proteins by Western blot analysis. For oxaliplatin sensitivity, cells were seeded in 96-well plates and reverse transfected with 50 nmol/L of either control siRNA pool or beclin1 or ATG5 siRNA SMARTpool. After 24 hours of transfection, cells were treated with oxaliplatin under hypoxic (24 hours) or normal conditions. Oxaplatin cytotoxicity was determined by MTT assay as described earlier.

GFP-LC3 assay

HT29 cells were stably transfected with GFP-LC3 plasmids and selected with G418. GFP-positive cells were further sorted by flow cytometry. HT29-LC3 cells were seeded in 6-well plates with coverslip and treated with hypoxia. The cells were fixed in 4% paraformaldehyde and LC3 puncta were measured by light fluorescence and confocal microscopic counting of cells with GFP-LC3 puncta. A minimum of 50 to 100 cells per sample will be counted in triplelicate samples per condition per experiment. To visualize autolysosomes in live cells, HT-29 cells expressing GFP-LC3 protein delivered by retroviral construct (Addgene) were seeded on glass slides 24 hours before hypoxic treatment (24 hours) with or without oxaliplatin (1 μmol/L). At the end of treatment, LysoTracker Red DND-99 (Invitrogen) was added for 1 hour at 1 μmol/L. Cells were removed from the hypoxia chamber, immediately washed twice with PBS and observed live, using EVOSFluorescent microscope (AMG) equipped with GFP and red fluorescent protein (RFP) filters. Images taken consequently with each filter were digitally overlapped to assess formation of autolysosomes.

Western blotting

Proteins from a total cell extract (20 μg/lane) were separated by electrophoresis in a 12% SDS-polyacrylamide gel and transferred to a Hybond-P membrane (Amersham). Western blotting was carried out using LC3-III (Novus Biologicals), beclin1, Atg7, (Santa Cruz Biotechnology Inc.), and ATG5 (Cell Signaling Technology) were used as primary antibodies (Calbiochem) and horseradish peroxidase–conjugated second antibody (Santa Cruz Biotechnology Inc.) was used to detect signals. The actin antibody (Santa Cruz Biotechnology Inc.) was used as protein-loading control. The ECL-plus detection system (Amersham) was used to develop the signal.

Transmission electron microscopy

For transmission electron microscopy (TEM) quantitation of autophagosomes, tumors were fixed with 2.5% glutaraldehyde/2% formaldehyde with 0.1 mol/L sodium cacodylate and stored at 4°C until embedding. Tumors were embedded in LX-112 medium (Ladd), and sections were cut ultra thin (90 nm), and placed on uncoated copper grids. The sections were stained with 0.2% lead citrate and 1% uranyl acetate. Images were examined with a JEOL-1010 electron microscope (JOEL) at 80 kV. For quantitation of cells using electron microscopy of tumor tissues, high-powered micrographs (×12, 000-20,000) of 25 single cells from multiple distinct low-powered fields in each tumor were obtained. For quantification of autophagic vesicle, cells with more than 3 to 4 double-membrane vesicles were scored as positive for autophagosomes.

Tumor growth and antiangiogenic therapy

To assess the efficacy of autophagy inhibition by chloroquine in antiangiogenic and oxaliplatin combination therapy in mouse xenograft model, adult (8–10 weeks of age) female severe combined immunodeficient mice (C.B.17 SCID) were used. To generate tumors, HT29 cells (2 × 10⁶ cells) were injected subcutaneously into the left flank of the mice. When the tumors reached approximately 400 mm³, mice were divided into 8 groups (6 mice per group) for treatment with bevacizumab (Genentech, Inc.), oxaliplatin, the combination of bevacizumab and oxaliplatin, chloroquine, the combination of bevacizumab and chloroquine, the combination of oxaliplatin and chloroquine, the combination of bevacizumab, oxaliplatin, and chloroquine, and a vehicle control group. Mice in the bevacizumab treatment group received 5 mg/kg of bevacizumab by intraperitoneal injection every 3 days for 27 days. The oxaliplatin treatment group was injected intraperitoneally with 5 mg/kg oxaliplatin per week for 2 weeks. The chloroquine treatment group was injected intraperitoneally 3.5 mg/kg every day for 27 days. The combination treatment groups received bevacizumab (every 3 days, 5 mg/kg for 27 days), oxaliplatin (weekly for 2 weeks, 5 mg/kg), and chloroquine (every day, 3.5 mg/kg). The control group received saline intraperitoneally every day. Tumor volume and body weight were measured every 3 days. Tumor volume was calculated using the formula $V = \frac{AB^2}{2}$, where $A$ and $B$ are the longest and shortest diameters, respectively.
A is the largest diameter and B is the smallest diameter. Tumor growth delay was calculated as the difference in the time for control and treated tumors to grow from 400 to 800 mm³. Mice were sacrificed after treatments on day 27 for tumor processing for quantitation of autophagy by TEM. All animal experiments were carried out according to an approved University of Pennsylvania Institutional Animal Care and Use Committee.

Tissue immunohistochemistry

Immunohistochemistry was conducted using the antigen retrieval protocol followed by primary antibody incubation as previously described (11). Rabbit anti-human LC3 antibody (Cell Signaling Technology) was used to detect the expression of LC3 in HT29 xenografts. For blood vessels and Ki-67 markers detection, primary antibodies of CD31 (Abcam) and Ki-67 (Dako) were used. Immunohistochemistry images were visualized using a Leica DMRBE upright microscope with QImaging MicroPublisher 5.0 RTV color camera with objective lens PL FluotAR 20x/0.5 (Leica). Images were captured using iVision acquisition software, which were processed with Adobe Photoshop software (Adobe Systems). Density of tumor blood vessels was determined by counting per ×40 high-power field, as described earlier (11). For quantitative analysis, ImageJ software, which measures the intensity of staining through threshold analysis, coupled with the Color Deconvolution plug-in (http://www.dentistry.bham.ac.uk/landinig/software/cdeconv/cdeconv.html), was used to quantify LC3 immunoreactivity in xenograft samples.

Statistical analysis

Tumor volume was represented as mean ± SD. Statistical analysis was conducted using ANOVA. A P value less than 0.05 was considered significant.

Results

Hypoxia and oxaliplatin treatment induce autophagy in colon cancer cells

To determine the importance of autophagy in colon cancer therapy, we began by examining the occurrence of autophagy in hypoxia- and oxaliplatin-treated colon cancer cells. We generated stable GFP-LC3–expressing HT29 cells by transfection and G418 selection. The LC3 proteins were measured by examining GFP-LC3 as punctate aggregations under fluorescence microscopy, and the conversion of LC3-I protein to modified form of LC3-II by Western blot analysis. The reason for the selection of HT29 cells for our study is that we have previously extensively characterized these cells for oxaliplatin and hypoxic cell signaling pathways and their ability to undergo antiangiogenic therapy in a mouse model (10, 11). HT29 cells were monitored for GFP-LC3 dots after 24 hours of hypoxia (0.1%) and oxaliplatin (IC50 dose) treatments. Clearly more cells with the punctate structures of autophagosomes are observed in hypoxia- and oxaliplatin-treated cells (Fig. 1A). Staining of cells for lysosomes and overlapping of images further showed induction of autophagy by both treatments, especially by hypoxia and the combination (Fig. 1A). Quantitation of cells positive for punctate structures revealed the induction of autophagy by oxaliplatin and hypoxia (Fig. 1B). We confirmed autophagy induction in HT29 cells through electron microscopy ultrastructural analyses. As illustrated in Fig. 1C and D and Supplementary Fig. S1, hypoxia and oxaliplatin significantly induce autophagosomal vesicles in HT29 cells (P < 0.05). To further confirm autophagy induction, protein extracts were made after hypoxia and oxaliplatin treatments for 24 and 48 hours. Western blot analyses show increased LC3-II and LC3-I proteins following treatment. The induction of LC3 proteins is robust in hypoxic cells, and in cells treated with combined hypoxia and oxaliplatin, as seen in Fig. 1E. The autophagy-related genes beclin1 (BECN1) and ATG-7 were also induced with hypoxic and in combination treatments.

Autophagy-inhibition sensitizes colon cancer cells to hypoxia and oxaliplatin cytotoxicity

To understand the role of autophagy induction in hypoxia- and oxaliplatin treatments, we have used an autophagy inhibition strategy with pharmacologic inhibitors 3-MA, bafilomycin A1, and chloroquine, and RNA interference technology for beclin1 and ATG5 knockdown. The autophagy inhibitor bafilomycin A1, which inhibits autophagosomal and lysosomal fusion, showed increased LC3-II accumulation in oxaliplatin- and hypoxia-treated cells, indicating enhanced autophagy flux under these conditions (Fig. 2A–C). As expected, bafA1 has increased LC3 protein accumulation in Western blot analysis and inhibited autophagy as evidenced by reduction of p62 protein degradation in hypoxic treatments (Fig. 2D). The use of bafA1 in MTT assays at IC10 concentration, sensitized colon cancer cells to oxaliplatin in hypoxia (Fig. 2E). Another autophagy inhibitor, 3-MA, which inhibits autophagy at initiation of double membrane encapsulation, also showed autophagy inhibition as evidenced by LC3 downregulation and p62 accumulation in hypoxia treatment (Fig. 3A). 3-MA also sensitized hypoxia-induced oxaliplatin resistance at IC10 concentration and showed synergy with oxaliplatin cytotoxicity in colon cancer cells (Fig. 2B and C). To further understand the mechanistic role of hypoxia-induced autophagy, we have used the inhibitor YC-1, which was used in other studies for pharmacologic inhibition of hypoxia-inducible factor-1α (HIF-1α; ref. 29). YC-1 treatment showed inhibition of HIF-1α protein but not the autophagy inhibition or sensitization of oxaliplatin resistance as evidenced by LC3 and p62 protein levels and also in MTT-based cell survival assays (Supplementary Fig. S2).

Becin1 knockdown sensitizes colon cell lines to oxaliplatin

We also sought to downregulate autophagy by targeting the essential autophagy gene, beclin1. We used siRNA to decrease the levels of beclin1 in cells and found that protein levels were considerably depleted by the intervention (Fig. 3D). As expected, knockdown of beclin1 in these cell lines caused marked inhibition of the accumulation of the
autophagy markers LC3-II and ATG5, both upregulated by hypoxia in the parental line (Fig. 3D). We went on to transfect a series of colon cancer cell lines, and to determine the effect of beclin1 downregulation on sensitivity to oxaliplatin under oxic and hypoxic conditions (Table 1). As expected, 5 of 6 lines show resistance to oxaliplatin under hypoxic conditions. In 4 of 6 lines, beclin1 downregulation resulted in enhancement of sensitivity to oxaliplatin by 2-fold or more. In 3 of the lines, the sensitization was greater under hypoxic than under oxic conditions, whereas in the rest the degree of sensitization was equivalent. In no case was there a protective effect of downregulating beclin1. To confirm that this inhibitory activity was a consequence of autophagy downregulation, we also targeted the autophagy-related protein ATG-5 through RNA interference. We found comparable enhancement of sensitivity to oxaliplatin under both oxic and hypoxic conditions to that with beclin1 knockdown (Supplementary Fig. S3 and Table S1). The results support targeting autophagy as a means to increase the effectiveness of colorectal cancer treatment.

**Effect of autophagy inhibition by chloroquine in hypoxia and oxaliplatin-induced cytotoxicity**

To determine the role of autophagy in colon cancer cell survival during hypoxia and oxaliplatin treatment, we used the known autophagy inhibitor chloroquine in MTT assays. The addition of chloroquine at 1, 3, and 5 μmol/L increased the sensitivity to oxaliplatin under oxic conditions in all of the cell lines (Fig. 4). It will be seen that in 3 of the lines, chloroquine treatment has a further sensitizing effect under
hypoxic conditions. We conducted isobologram analysis (Supplementary Fig. S4) to characterize the interaction between these interventions. Chloroquine showed a synergistic interaction with oxaliplatin under both oxic (median CI = 0.75) and hypoxic conditions (median CI = 0.58) in 6 of 8 colon cancer cell lines tested (Table 2). These data indicate a strong interaction between the treatments. The lower CI under hypoxic conditions suggests that sensitization of these tumors may occur both to DNA-damaging agents and to hypoxia.

**Autophagy inhibition by chloroquine sensitizes mouse colon cancer xenografts to bevacizumab**

To determine the effect of chloroquine on bevacizumab, oxaliplatin, and in the combination therapy on colon cancer xenografts in vivo, mice bearing HT29 colon adenocarcinoma xenografts were randomly divided into 8 groups for treatments as follows: PBS-treated control, chloroquine alone, bevacizumab alone, bevacizumab and chloroquine in combination, oxaliplatin alone, oxaliplatin and chloroquine in combination, bevacizumab and oxaliplatin in combination, and bevacizumab, oxaliplatin, and chloroquine in combination. Chloroquine treatment alone decreased the tumor growth somewhat compared with PBS alone control tumor growth (Fig. 5A). The tumor growth delay was for 4 days in chloroquine alone treatment (P < 0.01). A much greater impact was seen with the combinations of chloroquine with both oxaliplatin and bevacizumab in combination. Tumor growth delay of 7.2 days was observed in the bevacizumab alone group (P < 0.01), compared with 12 days with bevacizumab and chloroquine (P < 0.01). Tumor growth delay was 6 days for oxaliplatin alone treatment (P < 0.05) and 9 days for oxaliplatin and chloroquine (P < 0.05). When all the treatments were combined, effects were even more dramatic: the growth delay was 15 days for bevacizumab and oxaliplatin (P < 0.01) and 23 days for bevacizumab, oxaliplatin, and chloroquine treatment (P < 0.01). Therefore, in all treatments, the strategy of incorporating chloroquine increased the tumor growth delay significantly, suggesting that autophagy inhibition may have an impact on both cytotoxic and antiangiogenesis effectiveness (Table 3).
Bevacizumab and oxaliplatin treatments induce autophagy in mouse HT29 xenografts

Mouse tumors treated with bevacizumab and oxaliplatin were also processed for electron microscopy analyses. As depicted in Fig. 5B, bevacizumab- and oxaliplatin-treated tumors examined by electron microscopy had more cells with vacuolar structures typical of autophagosomes than untreated or chloroquine-treated tumors. The quantitation of autophagosomal cells revealed significantly increased autophagy in bevacizumab- and oxaliplatin-treated tumors when compared with PBS alone controls (Fig. 5C). In tumors treated in addition with
chloroquine, we observed a reduced number of autophagosomal cells (Fig. 5C). Incorporating chloroquine with bevacizumab and oxaliplatin treatments inhibited autophagosomal induction when compared with treatments without chloroquine (Fig. 5B and Supplementary Fig. S5). In the case of the triple (bevacizumab/oxaliplatin/chloroquine) combination, we did not observe a decrease compared with bevacizumab/oxaliplatin alone, and a Table 1. Sensitization of colon cancer cells to oxaliplatin by beclin1 knockdown

<table>
<thead>
<tr>
<th></th>
<th>Oxaliplatin (IC50 in μmol/L)</th>
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<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>si-Ctrl</td>
<td>si-BECN1</td>
<td>si-Ctrl</td>
</tr>
<tr>
<td>1. HT29</td>
<td>0.71</td>
<td>0.36 (1.9)</td>
<td>1.6</td>
</tr>
<tr>
<td>2. HCT116</td>
<td>0.98</td>
<td>0.12 (8.1)</td>
<td>1.51</td>
</tr>
<tr>
<td>3. KM12</td>
<td>7.5</td>
<td>0.16 (47)</td>
<td>7.9</td>
</tr>
<tr>
<td>4. BE</td>
<td>1.56</td>
<td>0.62 (2.5)</td>
<td>4.52</td>
</tr>
<tr>
<td>5. SW620</td>
<td>0.38</td>
<td>0.32 (1.2)</td>
<td>0.82</td>
</tr>
<tr>
<td>6. HCT15</td>
<td>2.9</td>
<td>2.7 (1.1)</td>
<td>10.9</td>
</tr>
</tbody>
</table>

NOTE: Beclin1 knockdown enhances oxaliplatin sensitivity in colon cancer cells under oxic and hypoxic conditions. Parentheses indicate the fold enhancement of oxaliplatin sensitivity.

Figure 4. Autophagy inhibition by chloroquine (CQ) sensitizes colon cancer cells to hypoxia and oxaliplatin. Inhibition of autophagy by chloroquine sensitizes colon cancer cells to oxaliplatin under oxic and hypoxic conditions. HT29 cells were tested for the effect of autophagy inhibition by chloroquine. The concentration of chloroquine at 1, 3, and 5 μmol/L enhanced the cytotoxicity of oxaliplatin under normal and hypoxic conditions.
Discussion

Autophagy is increasingly recognized as a contributor to the malignant phenotype, and as a possible mechanism for treatment failure in cancer (21, 22, 24–26). We hypothesized, on the basis of our previous findings (10, 11) that the hypoxic effects induced by bevacizumab in tumors in vivo were critical to its therapeutic action and that autophagy might attenuate that response. We thought further that as one response to DNA-damaging drugs is the induction of autophagy, its inhibition could sensitize colon tumors to oxaliplatin. We confirm that induction of autophagy is a consequence of exposure both to oxaliplatin as well as to hypoxia in colon cancer cell lines. We note that the exposure to both hypoxia and oxaliplatin produces additive effects in autophagy protein expression (Fig. 1E), whereas autophagosomes production seems to be maximal with either stress alone (Fig. 1D). Similar findings are obtained with bafilomycin A (Fig. 2). The quantitative pharmacodynamic implications of this finding remain to be resolved, but it likely reflects a maximum effect level under these conditions. It is also worth noting that a basal level of autophagy is present in all of the cell lines as evidenced by activation of LC3-II and beclin 1—in this we find colon cancer cells to be similar to pancreatic cancer models as described recently by Yang and colleagues (35). In that study, progressive activation of autophagy accompanied the progression of lesions through stages of increasing malignancy. In colon cancer also, activation of the autophagy pathway is a feature of the neoplastic phenotype, both in polyps and cancers (36).

Accumulating evidence also implicates autophagy in angiogenesis (37). Hypoxic endothelial cells derived from mice with heterozygous beclin1 disruption showed greater proliferative and functional responses than those from wild-type animals, a finding associated with a switch in hypoxia-response gene expression in these cells from HIF-1α to HIF-2α, the implications of which remain to be defined (38). In contrast, studies in bovine aortic endothelial cells with disrupted ATG5 showed reduced angiogenesis under conditions of nutrient deprivation (39). Plausibly, autophagic responses from nutrient deprivation may differ from those under hypoxia, and the microenvironment of tumors, in which autophagy is reproducibly engaged in all of the cell lines as evidenced by activation of LC3-II and beclin 1—in this we find colon cancer cells to be similar to pancreatic cancer models as described recently by Yang and colleagues (35). In that study, progressive activation of autophagy accompanied the progression of lesions through stages of increasing malignancy. In colon cancer also, activation of the autophagy pathway is a feature of the neoplastic phenotype, both in polyps and cancers (36).

Table 2. Synergistic interaction of chloroquine and oxaliplatin in colon cancer cells

<table>
<thead>
<tr>
<th></th>
<th>Chloroquine (IC50 concentration, µmol/L)</th>
<th>Oxaliplatin (IC50 concentration, µmol/L)</th>
<th>CI50</th>
<th>Chloroquine (IC50 concentration, µmol/L)</th>
<th>Oxaliplatin (IC50 concentration, µmol/L)</th>
<th>CI50</th>
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<tbody>
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<td>1.</td>
<td>WiDr</td>
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<td>1.04</td>
<td>0.71</td>
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<td>1.26</td>
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<td>2.</td>
<td>HT29</td>
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<td>0.82</td>
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<td>1.6</td>
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<tr>
<td>3.</td>
<td>BE</td>
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<td>1.02</td>
<td>0.81</td>
<td>5.16</td>
<td>9.7</td>
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<td>SW620</td>
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<td>0.75</td>
<td>15.43</td>
<td>0.8</td>
</tr>
<tr>
<td>5.</td>
<td>HCT116</td>
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<td>1.04</td>
<td>0.89</td>
<td>20.84</td>
<td>1.36</td>
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<tr>
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<td>22.1</td>
<td>5.9</td>
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<tr>
<td>7.</td>
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<td>0.45</td>
<td>34.5</td>
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<td>8.</td>
<td>HCT15</td>
<td>27.56</td>
<td>2.65</td>
<td>0.59</td>
<td>27.2</td>
<td>18.6</td>
</tr>
</tbody>
</table>

NOTE: Synergistic interaction between chloroquine and oxaliplatin in colon cancer cells. IC50 values for chloroquine and oxaliplatin were calculated using MTT assays. CI was calculated as described in Materials and Methods.
our contention that induction of hypoxia and the tumors response to it plays a key role in determining outcome of bevacizumab therapy.

We report here that the autophagy inhibitor chloroquine sensitizes colon cancer models to oxaliplatin under hypoxic conditions in vivo and reduces the tumor growth of colon cancer xenografts in bevacizumab- and oxaliplatin-treated mice. In vitro, where such interactions can be explored in some detail, chloroquine enhanced the activity of both treatments individually, whereas the greatest synergy was observed with the 3 drug combination. These experiments cannot distinguish between the effects of inhibiting autophagy and some possible unrelated cytotoxic action of chloroquine. On the other hand, chloroquine has been widely used for many years in malaria prophylaxis, without significant toxicity, a fact that may argue against a significant cytotoxic effect of the drug. To rule out such an effect, we undertook a molecular approach to autophagy inhibition through downregulation of beclin1, a key mediator of the autophagy response. We found that the cells in which beclin1 was knocked down showed susceptibility to oxaliplatin similar to the degree of interaction with chloroquine, and that the potentiation of drug effect was greater under hypoxic conditions. We confirmed the autophagy-specificity of these findings by experiments in which ATG5 was knocked down showed susceptibility to oxaliplatin similar to the degree of interaction with chloroquine, and that the potentiation of drug effect was greater under hypoxic conditions. We confirmed the autophagy-specificity of these findings by experiments in which ATG5 was knocked down showed susceptibility to oxaliplatin similar to the degree of interaction with chloroquine, and that the potentiation of drug effect was greater under hypoxic conditions. 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Table 3. Effect of chloroquine on bevacizumab, oxaliplatin, and the combination therapy of bevacizumab and oxaliplatin on the growth delay of HT29-derived tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor growth delaya</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Chloroquine</td>
<td>HT29</td>
<td></td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>HT29</td>
<td></td>
</tr>
<tr>
<td>Bevacizumab + chloroquine</td>
<td>HT29</td>
<td></td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>HT29</td>
<td></td>
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<tr>
<td>Oxaliplatin + chloroquine</td>
<td>HT29</td>
<td></td>
</tr>
<tr>
<td>Bevacizumab + oxaliplatin</td>
<td>HT29</td>
<td></td>
</tr>
<tr>
<td>Bevacizumab + oxaliplatin + chloroquine</td>
<td>HT29</td>
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</tr>
</tbody>
</table>

*aTumor growth delay was calculated as time (d) needed for treated tumors to grow from 400 to 800 mm³ minus the time needed for control tumor to grow the same size control. P values are treated versus untreated tumors.
autophagy is engaged by each cell stress may be different, and so possibly be amenable to more targeted intervention.

The mechanisms critical to cell death in this setting remain to be elucidated. Frezza and colleagues (44) showed induction of a catabolic metabolism profile in hypoxic (1% O₂) HCT116 colon cancer cells, and that this was sufficient to sustain ATP levels, albeit at reduced levels. These changes were accompanied by induction of autophagy, inhibition of which depleted ATP formation and led to cell death. Although oxidative phosphorylation was impaired, preservation of mitochondria suggested that mitophagy was not associated with the induction of hypoxic cell death. More profound hypoxia may be required for engagement of this process (45).

Previous studies of hypoxia-induced signaling events leading to autophagy identified BNIP3 as a key component of the cell’s detection and response to a hypoxic environment (28, 46). However, BNIP3 protein levels in the colon cancer cell lines studied here (HT29, HCT116) were unde-
tectable (data not shown), consistent with previous reports of its silencing by aberrant methylation and histone deacetylation in colon and gastric cancer (30, 47). In the glioblastoma work of Hu and colleagues, BNIP3 was a key mediator of the autophagy response (29). When present, BNIP3 activates beclin1 by displacing its binding partners Bcl-2 and Bcl-XL, but in these colon cancer models, the basis for this signaling remains to be elucidated. Clearly, BNIP3 is not required for signaling to autophagy in this model: the additional participation of bcl-2 family proteins is being studied in human tumor samples. These findings may permit selection of patients for autophagy inhibition to sensitize tumors to therapy.

An issue of clinical importance is to define the molecular characteristics of colon cancers that might respond. The

Figure 6. A, immunohistochemical detection of LC3 in bevacizumab-treated HT29 tumors. Representative pictures of LC3-staining (scale bars, 20 μm) from tumor samples harvested at 21 days after initiating treatment with bevacizumab alone, or oxaliplatin, or both, in combination with chloroquine (CQ). B, quantification LC3 staining by ImageJ analysis. C, immunohistochemical staining for blood vessels by CD31 antibodies. D, quantification of CD31 per high-power field (hpf) analysis. E, immunohistochemical staining for cell proliferation marker Ki-67. F, quantification of Ki-67–positive cells.
known link between disruption of growth and survival genes and metabolic responses renders this a potentially fruitful area of investigation in this context. We observe that in HCT116 and its derivative with biallelic deletion of p53 (Table 2), chloroquine synergizes with oxaliplatin in HCT116 under hypoxic conditions but is only additive in its p53−/− derivative. A number of investigators have questioned the strength of such findings to define p53-specificity of responses. We note that many of the models in which autophagy inhibition affords a therapeutic benefit (SW620, KM12, and HCT15) are p53-deficient, and one might be wary of over-interpreting the HCT116 data.Ultimately, this will be an important question to address in clinical trials.

The contribution of hypoxia to the effectiveness of anti-angiogenic therapy has not yet been definitively established. The work of Goel and colleagues showed that the tumor vasculature is often disorganized, defective in maturation, and functionally impaired (48). They showed that an early effect of antiangiogenic therapy is normalization of such vasculature, with restoration of normal permeability and that this may improve oxygenation in the short-term. These findings were recently corroborated in an elegant electron paramagnetic resonance study by Matsumoto and colleagues (49) in a sarcoma model treated with sunitinib, and there is evidence for therapeutic benefit of vascular normalization (50). However, in the longer term, with continued treatment both groups found vascular attenuation and nutritional impairment of the treated tumors. Hence, these findings in colon cancer models that induction of hypoxia may ultimately complement the effects of chemotherapy and increase tumor cell kill, are not inconsistent with those that find early normalization of vasculature. Furthermore, the therapeutic effects of chloroquine in vivo, with or without oxaliplatin, provide further support for a model in which the induction of hypoxia in colon tumors may confer therapeutic benefit, overriding any potential hypoxic cell resistance (10, 11). On the basis of this notion, we have in progress a clinical trial of hydroxychloroquine with chemotherapy and bevacizumab in patients with advanced colorectal cancer.

Disclosure of Potential Conflicts of Interest
P.J. O’Dwyer has a commercial research grant from Genentech, Inc. and is a consultant/advisory board member of the same. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Selvakumaran, I.A. Vasilevskaia, P.J. O’Dwyer
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Selvakumaran, R.K. Amaravadi, P.J. O’Dwyer
Writing, review, and/or revision of the manuscript: M. Selvakumaran, R.K. Amaravadi, I.A. Vasilevskaia, P.J. O’Dwyer
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.J. O’Dwyer
Study supervision: P.J. O’Dwyer

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References


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