Colorectal Cancer Cells Refractory to Anti-VEGF Treatment Are Vulnerable to Glycolytic Blockade due to Persistent Impairment of Mitochondria

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Abstract
Antiangiogenesis therapy has shed new light on cancer treatment, but its effectiveness, especially for overall patient survival, is still controversial. Here, we show that antiangiogenesis treatment causes a persistent suppression of mitochondria biogenesis in colorectal cancer cells, which renders them more sensitive to glycolytic blockade therapy. We first analyzed bevacizumab-resistant colon cancer xenografts by two-dimensional Blue Native/SDS-PAGE and found a serious and persistent loss of mitochondrial protein complex I. Further metabolic assays revealed significantly impaired mitochondrial function and hyperactive glycolysis, which were concomitant with the upregulation of HIF-1 and Hsp70. The treatment of bevacizumab-resistant cells with the glycolysis inhibitor 3-BrPA caused cell senescence in vitro. Intraperitoneal injection of 3-BrPA to xenograft mice bearing bevacizumab-resistant cells also resulted in smaller tumor volume and longer survival. These data provide direct evidence for the mitochondrial destruction of bevacizumab-resistant tumor cells and suggest that glycolysis blockade may potentiate the therapeutic effect of antiangiogenesis treatment.

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Introduction
Colorectal cancer is currently the third most diagnosed cancer in men and the second in women worldwide, and its targeted therapy is under extensively study (1). Given the pivotal role of VEGF-regulated angiogenesis in colorectal cancer progression, anti-VEGF therapeutic strategies hold promise for the treatment of advanced colorectal cancer (2). Bevacizumab, an antibody against VEGF, is currently used in first-line metastatic colorectal cancer treatment in combination with other chemotherapeutic reagents (3). However, the effectiveness of bevacizumab, especially on the overall survival of patients with metastatic colorectal cancer, has been a topic of much debate (4–6). To further improve the effect of anti-VEGF treatment, it is worthy to study the characteristics of colorectal cancer cells that eventually acquire resistance to antiangiogenesis reagents and develop effective complementary therapeutic strategies.

Accumulating evidence is pointing to a potential role of hypoxic metabolism in the acquired resistance to bevacizumab treatment. The increased dependence of cells on glycolytic pathway for ATP generation is known as one of the most fundamental metabolic alterations during malignant transformation. Hypoxia has been shown to promote tumor progression by accumulating the hypoxia-inducible factor-1α (HIF-1α), which regulates proliferation and angiogenesis (7, 8). Recent studies suggested that a successive hypoxic microenvironment induced by antiangiogenesis treatment can increase the population of tumor stem cells in breast cancer (9) and glioblastoma (10). The anaerobic metabolism of tumors refractory to antiangiogenesis therapy provides a biochemical basis for the design of complementary therapeutic strategies by pharmacological inhibition of glycolysis.

In the present study, we evaluate the extent and reversibility of mitochondria damage in bevacizumab-resistant colorectal cancer cells, aiming to provide mechanistic basis for glycolysis inhibition as a complementary therapeutic strategy. We detected the destruction of mitochondria protein complex I in bevacizumab-resistant (Bev-R) colorectal cancer xenograft and found this characteristic defect still persisted even after Bev-R cells were brought to optimal oxygen-supplied environment. The glycolysis inhibitor 3-bromopyruvic acid (3-BrPA) showed significant inhibitory effect on the growth of bevacizumab-resistant cells both in vitro and in vivo, suggesting a potential therapeutic value for glycolysis inhibitors to aid anti-VEGF in the treatment of colorectal cancers.
Materials and Methods

Reagents and antibodies
Bevacizumab and 3-bromopyruvate were, respectively, purchased from Roche and Sigma-Aldrich. The antibodies specific for mitochondria (MTC02, a mitochondrial marker antibody raised by semipurified mitochondrial preparation), HIF1α (MGC3 mouse monoclonal), Hsp70 (A3A mouse monoclonal), and Vimentin (V9 mouse monoclonal) were purchased from Abcam, and the antibodies for actin (C-2 mouse monoclonal) and MT-ND2 (C-16 rabbit polyclonal) are commercially available from Santa Cruz. The fluorescently labeled secondary antibodies were purchased from Invitrogen.

Cell culture and proliferation assay
The human colon carcinoma LoVo cells were purchased from the American Type Culture Collection and were passaged in our laboratory for less than 6 months. Cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with glutamine, pyruvate, antibiotics, and 10% fetal calf serum in a humidified atmosphere containing 5% CO2 at 37°C. The proliferation of cells was determined based on cell counting. At each time point, cells were trypsinized, diluted, and counted by an automatic cell counter (Scepter, Millipore).

Mitochondrial function assay
Mitochondrial function assay was conducted according to the product manual of ToxGlo assay (Promega). Production of ATP via glycolysis using galactose-containing media yields no net ATP, and cells primarily use mitochondrial oxidative phosphorylation to produce ATP. Therefore, the level of ATP can well reflect the function of mitochondria in cells cultured with galactose-containing media (11, 12). To measure mitochondrial function, equal number of LoVoBev-R and control cells was plated in 96-well plates, and 10 mmol/L galactose was used to generate ATP using a luminescent assay.

Glycolytic activity assay
Glycolysis of cells was determined by measuring the amount of lactate, the end product of glycolysis, using a commercially available kit (#600450, Cayman Chemical Company). The assay was conducted according to the product manual. Briefly, cells were seeded in a 96-well plate at a density of 50,000 cells/well in DMEM containing 25 mmol/L glucose, 10% FBS. After 24 hours, culture supernatant (10 μL) was removed from each well and added to reaction solution. The mixture was incubated with gentle shaking on an orbital shaker for 30 minutes at room temperature, and the absorbance at 490 nm was detected with a plate reader.

Western blot analysis
Cell lysate proteins were separated by SDS-PAGE (10% gels) and transferred to nitrocellulose membranes. Protein amounts were quantified using the Bradford method, and equal protein amounts were loaded to the gel. Membranes were blocked in Tris-buffered saline with 0.05% Tween 20 (TBST) containing 5% nonfat dry milk powder for 1 hour. Western blots were probed with primary antibodies for 1 hour, washed 3 times with TBST, and then incubated with the appropriate secondary antibodies for 30 minutes. Membranes were then washed with TBST 3 times, before developing with SuperSignal West Dura chemiluminescent substrate.

Immunofluorescent staining
Cells were removed from incubator and washed with PBS, followed by fixation using 4% formalin for 15 minutes. After permeabilization and blocking with 0.2% Triton X-100 and 1% BSA in PBS for 1 hour, the first antibody was diluted at 1:100 and incubated with the slide for 40 minutes. The secondary antibodies were diluted at 1:1,000 and incubated with the slides for 15 minutes. The fluorescent images were taken using a confocal microscope (Nikon).

Quantitative reverse transcription PCR
Quantitative reverse transcription PCR (qRT-PCR) was carried out using the following oligonucleotides and PCR conditions: 2 minutes at 95°C (20 seconds at 95°C, 20 seconds at the annealing temp, and 30 seconds at 72°C) × 35, and 10 minutes at 72°C. Primers were as follows: actin, forward: 5'-CATCAGTTATGGCAGAGACAGC-3', reverse: 5'-ACGCGGCTCAAGCCAGTCC-3'; Hsp70, forward: 5'-GCCGAGAAGGACGAGTTTGA-3', reverse: 5'-GCCGAGAAGGACGAGTTTGA-3'; HIF-1α, forward: 5'-GCCGAGAAGGACGAGTTTGA-3', reverse: 5'-GCCGAGAAGGACGAGTTTGA-3'; and MT-ND, forward: 5'-GCCGAGAAGGACGAGTTTGA-3', reverse: 5'-GCCGAGAAGGACGAGTTTGA-3'.

Creation of xenograft model
All animal experiments were carried out in the experimental animal platform of Tongji University (Shanghai, China). Protocols were conducted in accordance with Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by The Ministry of Science and Technology of the People’s Republic of China. Nude mice were grafted subcutaneously in the left flank with 5 × 106 LoVo cells in 0.3 mL of sterile normal saline solution. Mice were randomized into control and experimental groups (each containing 5 mice) when the tumor volume reached approximately 100 mm3. Tumors were measured with a caliper and calculated using the formula: 0.5 × a × b², where a was the length and b was the width of the tumor.

Drug treatment
In the experimental and control groups, bevacizumab (5 mg/kg) and PBS were respectively injected intraperitoneally twice a week. Tumors were measured by calipers.
twice weekly. At the end of drug treatment, the mice were
humanely euthanized and tumors were, respectively,
harvested. Tumor tissues were dissociated mechanically
and enzymatically to obtain a single-cell suspension.
Tumors were minced by scalpel and incubated in Med-ium
199 (Invitrogen) mixed with collagenase/hyaluronidase
(Sigma Aldrich) at 37°C for 60 minutes. The tissues
were further dissociated by pipette trituration and then
passed through a 40-μm nylon mesh to produce a
single-cell suspension, which was used for subsequent
culture and treatments. Cells derived from experimental
and control groups of xenografts were, respectively,
mixed and incubated (named as LoVo Bev-R and
LoVo control cells).

For in vitro treatment with 3-BrPA, xenograft-derived
cells (LoVo Bev-R and LoVo control) were, respectively,
cultured in media containing 150 μmol/L of 3-BrPA. For
in vitro 3-BrPA treatment, mice implanted with LoVo Bev-R
and LoVo control cells were injected intraperitoneally
5 mg/kg 3-BrPA once per day when the xenograft
volume reached 300 mm³. The tumor volume was mea-
sured every 2 days after the first 3-BrPA injection.

Two-dimensional BN/SDS-PAGE
The two-dimensional (2D) BN/SDS-PAGE was con-
ducted according to the product manual of the Native-
PAGE Novex Bis-Tris Gel System (Invitrogen). Briefly,
the 1D Blue Native-PAGE was conducted with a buffer
system containing Coomassie G250 but without SDS.
Cells were lysed using 18 mmol/L 3-[3-cholamidoprop-
yl]dimethylammonio]-1-propanesulfonic acid (CHAPS)
in TBS with DNase and protease inhibitors (Roche) for 30
minutes on ice, and a sample buffer containing 10%
glycerol and 1% Coomassie was added to the lysate before
digested with an enzyme mixture to obtain single-cell
suspensions (LoVo Bev-R). The cells derived from 5 bev-
acizumab-resistant xenografts were mixed and cultured
under an optimized oxygen-supplied condition for 3
weeks, followed by 2D Blue Native/SDS-PAGE (2D
BN/SDS-PAGE) to examine the status of mitochondrial
protein complexes. Cells were also derived from xeno-
grafts in the control group and analyzed using the same
procedure. The 1D Blue Native-PAGE fractionated pro-
tein complexes without disrupting native protein bind-
ing, whereas the 2D SDS-PAGE broke down the com-
plexes to display individual subunits. As shown in Fig.
1A and B, the LoVo Bev-R cells showed dramatic decrease
of a protein complex with MW of over 1,000 kDa as
compared with LoVo cells from control xenografts. For
2D PAGE revealed multiple subunits with various
molecular sizes that constituted this large complex (Fig.
1C and D). We blotted these proteins to PVDF mem-
brane and conducted N-terminal protein sequencing on
2 selected spots, which turned out to be MT-ND2 and
NDUFV2, 2 components of the mitochondria protein
complex I (NADH dehydrogenase; Fig. 1E and F).

To probe the status of mitochondria in LoVo Bev-R cells,
we conducted immunofluorescence using a specific mark-
er for mitochondria. A substantial decrease of mitochon-
dria content was detected in LoVo Bev-R cells as compared
with control LoVo cells, as indicated by the lower staining
intensity and fewer mitochondrial structures in LoVo Bev-R
cells (Fig. 2A). Moreover, the structure of the mitochon-
dria in LoVo Bev-R cells was punctuate-like, and this was in
contrast to the thinned, filamentous morphology of con-
trol LoVo cells (Fig. 2A). Combining the result of 2D
PAGE, these data suggested a substantial disorder of the
mitochondria structure in bevacizumab-resistant LoVo
cells.

Suppression of angiogenesis potentiated glycolytic
activity of cancer cells
Given the vital role of NADH dehydrogenase (com-
plex I) in mitochondrial electron transport chain, we
examined the extent of mitochondria impairment in
LoVo Bev-R cells. The mitochondria functional assay sug-
gested a dramatic decrease of mitochondria activity in
LoVo Bev-R cells, which only accounted for 10% of the
level in control cells (Fig. 2B). We further determined
the glycolytic activity of LoVo Bev-R cells and found it
increased by 5-folds as compared with control LoVo
cells (Fig. 2C). Western blot and qRT-PCR assays showed
decreased level of MT-ND2 in LoVo Bev-R cells
(Fig. 3), which was consistent with the result from 2D
PAGE. Moreover, the mRNA and protein levels of HIF-1
and Hsp70 were significantly increased in LoVo Bev-R
cells (Fig. 3), suggesting that HIF-1 may be relevant to
the activation of glycolytic pathway. Note these results
were obtained from LoVo Bev-R cells cultured for 3 weeks
under optimal oxygen-supplied condition. The persis-
tence of increased glycolysis caused by anti-VEGF treat-
ment supports the model that cells progressively evolve


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toward malignancy during adaptation to the hypoxic microenvironment (13).

Glycolytic blockade induced cell senescence and growth suppression in vitro

As the vital energy source that supported the fast proliferation of LoVoBev-R cells was glycolysis-generated ATP, it is thus plausible to use glycolysis inhibitors to suppress the growth of these hypoxia-adapted cells. The small compound 3-BrPA, a synthetic brominated derivate of pyruvic acid, is a potent inhibitor of glycolysis capable of inducing severe ATP reduction and cell death in cancer cells with mitochondrial defects or under hypoxic conditions (14, 15). A recent study suggested that 3-BrPA could induce the covalent modification of HKII protein and directly trigger its dissociation from mitochondria, thus providing a potential mechanism for the anti-glycolytic effect of 3-BrPA (16). We treated the LoVoBev-R cells in vitro using 150 μmol/L 3-BrPA and analyzed the effect of glycolysis inhibition on cell...
proliferation. The presence of 3-BrPA induced progressive senescent morphology of LoVoBev-R cells with the formation of large and flat cell bodies (Fig. 4A). After 8 days of incubation, all cells were out of the cell cycle and showed giant nuclei within enormous cytoplasmic masses (Fig. 4A). Consistently, cell number counting also suggested that LoVoBev-R cells stopped proliferation after 6 to 8 days of incubation with 3-BrPA. On the contrary, the control LoVo cells without preselection by bevacizumab showed few senescent cells till the end of incubation, and the proliferation rate of control LoVo cells was also significantly faster than that of LoVoBev-R cells (Fig. 4B).

3-BrPA reduced tumor size and improved survival of xenograft-transplanted mice

We further tested the effect of 3-BrPA on LoVoBev-R cells in a xenograft mouse model. The LoVoBev-R and control cells were respectively implanted into the left flank of 15 nude mice, and 3-BrPA was injected intraperitoneally after the xenograft volume reached 300 mm³. We measured tumor sizes 6 days after injection of 3-BrPA and found that the LoVoBev-R xenografts showed very limited growth (~350 mm³). On the contrary, the tumor sizes in the control group increased by over one fold (Fig. 4C and D). Moreover, the survival of mice bearing LoVoBev-R xenografts was significantly longer than those control mice, suggesting a consistent effect of 3-BrPA as observed in vitro.

Discussion

Bevacizumab is the first-in-class VEGF inhibitor that was initially approved by the U.S. Food and Drug Administration in 2004 for the treatment of metastatic colon cancer and other solid tumors. However, recent clinical data concluded that incorporating bevacizumab into adjuvant regimens does not prolong disease-free survival (DFS) or overall survival (OS) of patients with colorectal cancers (17). In this study, we provide mechanistic evidence for the involvement of persistent mitochondrial impairment in the acquired resistance to anti-VEGF treatment. The mitochondria of LoVoBev-R cells showed severe loss of the mitochondrial complex I (NADH dehydrogenase) as revealed by 2D BN/SDS-PAGE. NADH dehydrogenase is the first enzyme (complex I) of the mitochondrial electron transport chain, and its proper functionality is vital for ATP generation through the aerobic respiration pathway. We also showed by immunofluorescence that the amount of mitochondria significantly decreased in LoVoBev-R cells, concomitant with the loss of filamentous morphology and the appearance of fragmented, punctate-like structure of mitochondria. It was thus not surprising that the mitochondrial function in LoVoBev-R cells was dramatically decreased as compared with control cells. However, the fast proliferation of LoVoBev-R cells in vitro suggested they might consume comparable amount of ATP as the control cells. This was explained by the hyperactivation of glycolysis in the LoVoBev-R cells, thus suggesting a substantial shift of metabolistic pathway in bevacizumab-resistant colorectal cancer cells.

The dependence of LoVoBev-R cells on glycolytic metabolism was persistent and was not affected by the supplement of oxygen in the culture condition. This could be explained by the sustained upregulation of HIF-1α, which is known to stimulate expression of glycolytic enzymes and decreases reliance on mitochondrial oxidative phosphorylation in tumor cells (18). Moreover, both Western blotting and qPCR suggested the upregulation of Hsp70 in the bevacizumab-resistant LoVo cells. The molecular chaperone Hsp70 is a transcriptional target of HIF-1, which can help cancer cells to overcome hypoxic stress and survive (19). These findings provided mechanistic insight into how colorectal cancer cells evade from anti-VEGF treatment and evolve toward malignant phenotypes.

The severe and persistent dependence of bevacizumab-resistant cells on glycolytic metabolism provides a venue for complementary therapeutic strategy. Our

Figure 3. Expression levels of MT-ND2, HIF-1 and Hsp70 in control and LoVoBev-R cells. A, Western blot analysis of control and LoVoBev-R cells. The cells were lysed and analyzed by SDS-PAGE, and the blotted membrane was probed with antibody for MT-ND2, HIF-1α, Hsp70, and actin to show the protein expression level. B–D, mRNA levels of MT-ND2, HIF-1α, and Hsp70 as determined by qRT-PCR. Total mRNA was isolated from control and LoVoBev-R cells, and cDNAs were synthesized using random primers. The levels of MT-ND2, HIF-1α, and Hsp70 mRNA were determined by qPCR. All values were normalized with the expression level of actin.
data suggested that the glycolytic inhibitor, 3-BrPA, could significantly suppress the proliferation of LoVo^Bev-R^ cells and induce their senescence in vitro. The therapeutic effect of 3-BrPA has been reported previously in many cancers (20), and here we show that 3-BrPA is especially effective against the colorectal cancer cells that have acquired resistance to anti-VEGF therapy. The hypoxic microenvironment induced by bevacizumab may select cells that have evolved with hyperactive glycolytic metabolism, and blockade of glycolysis could thus deprive the major source of ATP and drive cells to senescence. Our in vivo data showed that injection of 3-BrPA to mice carrying bevacizumab-resistant colorectal cancer tumor resulted in significantly decreased tumor volume and longer survival, thus indicating glycolysis inhibition as a potential complementary strategy for antiangiogenesis therapy.

Taken together, our study provided direct evidence for the involvement of persistent mitochondria impairment and hyperactive glycolysis in the acquired resistance of colorectal cancer cells against anti-VEGF treatment. We also showed that glycolysis blockade strongly suppressed the proliferation of colorectal cancer cells that were refractory to bevacizumab treatment. In future studies, it is interesting to examine the effects of other glycolysis inhibitors on bevacizumab-resistant colorectal cancer cells. It would also be meaningful to study whether a synergistic therapeutic effect could be achieved by combination of bevacizumab and 3-BrPA in clinical colorectal cancer cases.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Xu
Development of methodology: J. Xu, J. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Xu, J.-Y. Fang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Xu, X. Zhou
Writing, review, and/or revision of the manuscript: J. Xu, J. Wang, B. Xu, H. Ge, J.-Y. Fang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Zhou
Study supervision: J.-Y. Fang

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