VEGF-Targeted Therapy Stably Modulates the Glycolytic Phenotype of Tumor Cells

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Abstract

Anti-VEGF therapy perturbs tumor metabolism, severely impairing oxygen, glucose, and ATP levels. In this study, we investigated the effects of anti-VEGF therapy in multiple experimental tumor models that differ in their glycolytic phenotypes to gain insights into optimal modulation of the metabolic features of this therapy. Prolonged treatments induced vascular regression and necrosis in tumor xenograft models, with highly glycolytic tumors becoming treatment resistant more rapidly than poorly glycolytic tumors. By PET imaging, prolonged treatments yielded an increase in both hypoxic and proliferative regions of tumors. A selection for highly glycolytic cells was noted and this metabolic shift was stable and associated with increased tumor aggressiveness and resistance to VEGF blockade in serially transplanted mice. Our results support the hypothesis that the highly glycolytic phenotype of tumor cells studied in xenograft models, either primary or secondary, is a cell-autonomous trait conferring resistance to VEGF blockade. The finding that metabolic traits of tumors can be selected by antiangiogenic therapy suggests insights into the evolutionary dynamics of tumor metabolism. Cancer Res; 75(1). 120–133. © 2014 AACR.

Introduction

Among various angiogenic factors, VEGF plays a crucial role in tumor angiogenesis (1). Neutralization of the VEGF pathway impairs tumor growth and this notion has provided ground for development of antiangiogenic drugs (2). However, both preclinical models and clinical trials have shown that benefits from first-generation angiogenesis inhibitors are generally short-term, due to the development of intrinsic as well as acquired resistance (3, 4). Apart from vascular resistance, due to bypassing of VEGF blockade by proangiogenic factors produced by either tumor cells, stromal cells or various types of bone marrow-derived cells, resistance has also been associated with selection of clones resistant to hypoxia, acquisition of an invasive phenotype, and overexpression of c-Met (3, 5, 6).

Metabolism of tumors differs remarkably from that of the tissues of their origin. Tumor cells exhibit altered pathways of metabolism in the tumor microenvironment associated with acquisition of an invasive phenotype (7). Metabolic alterations in cancer cells may derive from genetic changes associated with cell transformation such as dysregulated activity of HIF1 (9), loss of p53 or altered expression of metabolic enzymes (10), or be triggered by inhibition of mitochondrial function (11). Moreover, changes in tumor metabolism can also follow reduction in energy sources (O2, glucose, glutamine) in the tumor microenvironment associated with antiangiogenic therapy (12–14). Although in patients anti-VEGF therapy is generally combined with chemotherapy, in preclinical studies its administration as monotherapy is a strategy to better discern downstream effects of antiangiogenic drugs from cytotoxic drugs. We recently reported that short-term anti-VEGF therapy causes severe impairment of glucose and ATP levels in tumors (14). In this study, we also found that the response to short-term
VEGF blockade is strongly dependent on the glycolytic phenotype of the tumor. Specifically, highly glycolytic tumor xenografts developed large necrotic areas following short-course VEGF neutralization, whereas xenografts characterized by relatively low glycolytic activity remained substantially viable (14). To investigate whether metabolic features could affect the therapeutic outcome, here we analyzed long-term effects of anti-VEGF treatment on tumor xenografts characterized by different levels of glucose addiction. The results show that highly glycolytic tumors rapidly acquire resistance to VEGF neutralization. Moreover, based on results of metabolic assays in four different tumor models, we report for the first time that anti-VEGF therapy causes stable selection of highly glycolytic tumor cells, indicating that the metabolic phenotype of tumors can change following administration of antiangiogenic drugs.

Materials and Methods

Cell culture and treatments

Several tumor cell lines representative of highly glycolytic (OC316 and MCF7) or poorly glycolytic (IGROV1 and SKOV3) cells were used in this study (Supplementary Fig. S1; ref. 14). IGROV1 and MCF7 cells were generated from ATCC, OC316 cells were provided by S. Ferrini (IST, Genoa, Italy), SKOV3 cells was kindly provided by S. Canevani (INT, Milan, Italy). IGROV1, OC316 and SKOV3 were grown in RPMI1640 (Euroclone) supplemented with 10% fetal calf serum (FCS; Life Technologies), 1% HEPES (10 mmol/L, Cambrex Bioscience), 1% L-glutamine (2mmol/L), 1% sodium pyruvate (1 mmol/L), and 1% antibiotic-antimycotic mix (Gibco-BRL). MCF7 cells were grown in DMEM (Euroclone) supplemented with 10% FCS and 1% antibiotic-antimycotic mix. Cultures were maintained at 37˚C in a humidified 5% CO2/95% air atmosphere. Where specified, tumor cells were treated with 2-deoxyglucose (2-DG; Sigma-Aldrich) at 6 g/L for 72 hours before apoptosis evaluation. Hypoxic treatment (0.5% O2) was achieved by incubating cells in an InVivo2 300 hypoxic chamber (Ruskinn Technology).

In vivo experiments

Procedures involving animals and their care conformed with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 12 December, 1987). For tumor establishment, 8-week-old SCID and BALB-neuT mice, respectively, and mice were sacrificed at the end of the incubation period.

To generate the PDOVCA37 xenograft, following informed consent, ascitic fluid of one patient with ovarian cancer was obtained from the Medical Oncology Unit of our Institute. Tumor cells (2 × 106) were subcutaneously injected into both flanks with 0.3–0.5 × 10⁹ tumor cells mixed at 4˚C with liquid Matrigel (Becton Dickinson). Tumor volume (mm³) was calculated as previously reported (15).

In a set of experiments, 20-week-old female BALB-neuT mice bearing measurable mammary tumors (3 mm diameter) were used (18). Founder BALB-neuT male mice were kindly provided by Biogem following agreement with Dr. Guido Forni (University of Torino, Torino, Italy). BALB-neuT mice were bred in house with BALB/c female mice. Female offsprings were then screened for the presence of Her2/ neu oncogene as previously described (19).

Anti-human VEGF monoclonal antibody (mAb; bevacizumab) or anti-human/mouse VEGF mAb (B20-4.1.1; ref. 20) were administered intraperitoneally at 100 µg/dose bi- or triweekly to SCID and BALB-neuT mice, respectively, and mice were sacrificed 48 hours after the last treatment. Control mice received intraperitoneal injections of PBS.

Reverse transcription PCR and real-time PCR assay

Total RNA was extracted using the RNEasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized from 0.5 to 1 µg of total RNA using the Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed with SYBR Green dye and Gene Amp 5700 Sequence Detection System (PE Biosystems). Cycling conditions were 10 minutes at 95˚C, 40 cycles of 15 seconds at 95˚C, and 1 minute at 60˚C. Each sample was run in duplicate. For all genes evaluated, mRNA was normalized to β2-microglobulin (B2M) mRNA by subtracting the cycle threshold (Ct) value of B2M mRNA from the Ct value of the gene of interest (Ct). Fold difference (2⁰−ΔΔCt) was calculated by subtracting the ΔCt (treated sample) to ΔCt (reference sample), to generate a ΔΔCt. PCR efficiency was in the range 95% to 105%. Primers used for real-time PCR are listed under Supplementary Table S1.

Glucose and lactate measurements

Glucose and lactate concentrations in supernatants were determined by colorimetric methods on an automated analyzer (Dimension Rxl, Dade Behring). Values were normalized to cells number at the end of the incubation period.

Oxygen consumption rate and extracellular acidification rate analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed in real-time with a XF24 Extracellular Flux Analyzer (Seahorse Biosciences) as described (11). Briefly, cells (2.5 × 10⁶/well) were plated in RPMI medium supplemented with 10% FBS. The next day, cells were placed in a running DMEM medium (supplemented with 25 mmol/L d-glucose, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, and without serum and bicarbonate) and preincubated for 30 minutes at 37˚C in atmospheric CO2 before starting metabolic measurements. At the end of the experiment, OCR and ECAR values were normalized for the protein content of each sample. Accurate titration with the uncoupler FCCP was performed for each cell type, to utilize the FCCP concentration (400 nmol/L) that maximally increases OCR without being toxic.

Positron emission tomography studies

Cell proliferation and regional tissue hypoxia were investigated using [18F]-fluorothymidine (FLT) and [18F]-
fluoroazomycin arabinoside (FAZA) as radioligands, respectively, and YAP-(S)-PET II (ISE, Pisa, Italy) small animal scanner (21, 22). Further details of PET studies are reported under Supplementary Data.

**Induced metabolic bioluminescence imaging**

Freeze-clamped tumors were excised and cut into serial cryosections for structural hematoxylin and eosin staining and metabolic measurements. For quantitative measurement of ATP, lactate and glucose, the method of metabolic imaging with induced bioluminescence (imBI) was applied, as described before (23–25). Further details of imBI are reported under Supplementary Data.

**Statistical analysis**

Results were expressed as mean value ± SD. Statistical comparison between two sets of data was performed using either the unpaired Student t test (two-tailed) or, in the case of analysis of glucose and lactate levels in supernatants, the Mann–Whitney test (two-tailed). To analyze PET data, ANOVA followed by the Bonferroni post hoc test for multiple comparisons was used. Differences were considered statistically significant at \( P < 0.05 \).

Further experimental details are available under Supplementary Data.

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**Results**

The glycolytic phenotype of tumors modulates resistance to anti-VEGF therapy

Following establishment of tumor xenografts, mice were treated with two or three weekly administrations of the anti-VEGF antibody bevacizumab for 4 weeks. Results show marked growth arrest of poorly glycolytic IGROV-1 tumors, whereas highly glycolytic OC316 xenografts after an initial shrinkage became substantially resistant to antiangiogenic therapy according to measurements of tumor size, although at sacrifice of the mice tumors were still smaller compared with controls (Fig. 1). Ad interim histologic analysis performed in samples analyzed after 7 days of treatment showed higher levels of necrosis in highly glycolytic OC316 compared with poorly glycolytic IGROV-1 tumors (data not shown), in line with our previous observations (14). In contrast, analysis of tumor samples collected at day 28 disclosed abundant necrotic areas in all anti-VEGF-treated tumors (Supplementary Fig. S2A). Microvessel density was significantly lower in tumors treated with anti-VEGF at day 28 after treatment (Supplementary Fig. S2B), indicating that tumor angiogenesis was still partially dependent on VEGF at the end of treatment. These results were further validated by anti-VEGF treatment of additional tumor xenografts. MCF7 and SKOV3 represent other examples of relatively highly and poorly glycolytic tumor cell lines, respectively, as shown by glucose consumption and lactate consumption.
production levels in vitro (Supplementary Fig. S1A) as well as sensitivity to glucose starvation/hypoxia and treatment with 2-DG (Supplementary Fig. S1B). Following anti-VEGF therapy, SKOV3 tumors were almost completely starved, whereas MCF7 xenografts became rapidly resistant (Fig. 1). Since these results could also in part depend on the different genetic background of the various tumor cell lines, we sought to corroborate them by using pairs of isogenic cells with different metabolic profiles. To this end, we exploited tumor cells bearing reduced expression of both AMPKα1 and α2 subunits. Ground for this experiment was provided by our previous study, which indicated that AMPK silencing in IGROV-1 cells leads to acquisition of highly glycolytic features (14) as well as by a recent study that reported that AMPK is a negative regulator of aerobic glycolysis in tumor cells (26). As expected, double silencing of AMPKα1 and α2 by shRNA delivery was followed by reduced AMPKa RNA and protein levels in IGROV-1 cells (Supplementary Fig. S3A) and was associated with significantly increased levels of glucose consumption and lactate production in vitro (Supplementary Fig. S3B) and much higher cell death under glucose starvation or hypoxia compared to control cells (Supplementary Fig. S3C). Proliferation and viability of IGROV-1 shAMPKα1/α2 cells under standard culture conditions were similar to that of control cells (not shown). However, tumors formed by IGROV-1 shAMPKα1/α2 cells had somewhat accelerated growth compared with control tumors (Supplementary Fig. S3D). Following treatment with bevacizumab, tumors formed by IGROV-1 cells transduced by an irrelevant shRNA regressed similarly to parental IGROV-1 tumors; in contrast, tumors formed by IGROV-1 cells bearing attenuated AMPKα1/α2 levels initially regressed but subsequently became resistant to anti-VEGF therapy (Supplementary Fig. S3D). Altogether, these findings suggest that high glycolytic phenotype of tumor cells with therapeutic responses to VEGF blockade in SCID mice.

Selection of highly glycolytic tumor cells following VEGF blockade

Our previous study indicated that the amount of tumor necrosis following VEGF blockade in part depends on the glycolytic phenotype of tumor cells, being much lower in poorly glycolytic than in highly glycolytic xenografts (14). The observation of large necrotic areas in IGROV-1 tumors following long-term anti-VEGF therapy (Supplementary Fig. S2A) suggested that the low glycolytic phenotype of these tumor cells could be modulated by anti-VEGF therapy. To investigate this possibility, we stained tumor sections with anti-monocarboxylate transporter 4 (MCT4), a lactic acid transporter that clearly distinguishes OC316 from IGROV-1 cells in vitro (27). As shown in Fig. 2A, this glycolytic marker was expressed at much higher levels in OC316 than in IGROV-1 tumors. The latter had negligible MCT4 expression following short-term (7 days) anti-VEGF therapy whereas after prolonged anti-VEGF treatment (28 days) MCT4 expression increased compared to control tumors (Fig. 2A). Similar results were found in SKOV-3 tumors following antiangiogenic therapy, although in this case prominent up-regulation of MCT1 - another member of the MCT family - was observed (Fig. 2A). Importantly, marked MCT1 expression was also found in clinical liver metastasis samples of colorectal cancer treated with chemotherapy plus bevacizumab (Fig. 2A), suggesting that this phenomenon occurs also in patients treated with anti-VEGF drugs. In experimental tumors, HIF1α—a transcription factor that is strongly stabilized under hypoxic conditions and regulates expression of several glycolysis-associated transcripts, including MCT4 (28)—was found mainly expressed in the nuclei of tumor cells after anti-VEGF therapy compared with prevalently cytoplasmic expression in control samples (Fig. 2B). These data indicate a metabolic shift in tumors treated with anti-VEGF therapy and suggest that HIF1α-mediated up-regulation of glycolysis could in part account for increased expression of lactate transporters in tumors.

Anti-VEGF-treated tumors contain a highly proliferative and hypoxic core

Since metabolic reprogramming, hypoxia and cell proliferation are strictly intertwined (9), we next investigated the proliferative activity of tumors treated with anti-VEGF therapy. To this end, [18F]FLT PET was exploited to functionally measure the dynamics of proliferation in IGROV-1 and OC316 xenografts. To evaluate the extension and distribution of cell phenotypes in tumors, we measured the volume of distribution of [18F]FAZA, as a marker of hypoxic regions within tumors. In both xenografts, 7 days after anti-VEGF therapy, the volume of highly proliferative regions was similar to pretreatment values. At the end of therapy (day 28), in IGROV1 tumors, nevertheless a cytostatic effect in tumor volume measured at caliper, we found increased [18F]FLT distribution compared with previous measurements (Fig. 3A), indicating that tumors treated with bevacizumab were composed by highly proliferative cells. This was also accompanied by a progressive increase of tumor hypoxic areas compared with pretreatment values, as shown by results of [18F]FAZA PET (Fig. 3A). PET analysis disclosed similar changes in proliferation and hypoxic areas also in OC316 tumors, that acquired resistance to VEGF neutralization (Fig. 3B). We observed a significant difference in tumor volume measured at caliper between treated and control group at 28 days, although tumor size increased compared with baseline. In addition, a concomitant enlargement of highly proliferative and hypoxic areas was noted.

Proliferation was further studied in tumor sections by staining with antiphosphohistone3 (p-H3) antibody, an in situ marker of cell proliferation. Results showed significant—reduced numbers of p-H3+ cells in anti-VEGF-treated IGROV-1 at 7 days after treatment, whereas numbers comparable with control IGROV-1 were found at day 28 (Supplementary Fig. S4). Altogether, these results suggest that continuous VEGF blockade eventually selects for a population of cells with high proliferative activity embedded in an hypoxic microenvironment, which may account for the increased expression of glycolysis-associated markers observed.

VEGF blockade is associated with stable modulation of aerobic glycolysis in tumor cells

To better characterize metabolic changes observed after prolonged (28 days) anti-VEGF therapy in tumors, we set up ex vivo cultures of tumor cells. Measurement of glucose consumption and lactate production indicated that cultures from anti-VEGF-treated IGROV-1 tumors had slightly but
significantly higher glucose consumption and lactate production rates compared with \textit{ex vivo} cultures from control tumors (Fig. 4A), along with increased cell death under glucose starvation, suggesting selection of glucose-addicted cells (Fig. 4B). We also found increased expression of several glycolysis-associated transcripts including \textit{GLUT3}, \textit{PKF}, \textit{LDHA}, and \textit{MCT4} transcript (Fig. 4C) and protein (Supplementary Fig. S5). Stimulated by these findings, we performed bioenergetic analysis of \textit{ex vivo} cultures of IGROV-1 tumors. Parental IGROV-1 cells, which were previously characterized by the same technique (27), were used as controls. Notably, growth in mice as tumor xenografts did not change \textit{per se} the bioenergetic features of IGROV-1 cells, whereas after anti-VEGF treatment, cells became much more glycolytic (Fig. 4D). In
In fact, OCR and ECAR did not substantially change in IGROV-1 cells after formation of tumor xenografts (compare wt and control IGROV-1 traces in the left and right panels of Fig. 4D), whereas treatment with bevacizumab (i) strongly inhibited OCR, and the effect of the ATP synthase inhibitor oligomycin indicated a specific downmodulation of coupled respiration, i.e., the fraction of oxygen consumption coupled to ATP synthesis; (ii) abolished any respiratory reserve, as the uncoupler FCCP, which at the reported concentration stimulates the maximal OCR, could not further increase oxygen consumption with respect to the basal value; this observation implies that cells acquire a Warburg phenotype, as they must utilize glycolytic ATP for any energy demand that exceeds their basal respiratory activity; (iii) increased ECAR, also after inhibition of respiratory complexes by rotenone/antimycin, which indicates that glycolysis is enhanced and scarcely funneled towards the Krebs cycle, leading to an enhancement of pyruvate conversion into lactate. This latter observation is in accordance with increased lactate production measured in supernatants of cultures obtained from anti-VEGF-treated mice (Fig. 4A) and with increased LDHA and MCT4 expression levels (Fig. 4C). Overall, these results show that anti-VEGF therapy selected a highly glycolytic subpopulation of tumor cells, a conclusion further supported by bioenergetic analysis of ex vivo cultures of OC316 tumors, which became resistant to anti-VEGF therapy (Supplementary Fig. S7).

Mechanistically, we measured decreased expression of the mitochondrial complex NDUFS1 in cell cultures established from bevacizumab-treated tumors as well as reduced mitochondrial mass (Fig. 4E), which is consistent with decreased mitochondrial respiration. On the other hand, we did not detect increased HIF1α activity in normoxic cultures of IGROV-1 cells compared with the parental cell line (Supplementary Fig. S6A), indicating that these metabolic changes were not caused by persistent HIF1α signaling. Moreover, c-MYC, pAKT (Ser473 and Thr308) and pAMPK (Thr172) protein levels were comparable in ex vivo cultures from anti-VEGF-treated and control tumors (Supplementary Fig. S6B and S6C), suggesting that these well-established regulators of the Warburg effect (10) did not account for increased aerobic glycolysis.
Figure 4.
Stable upmodulation of the glycolytic phenotype following anti-VEGF therapy. A, measurement of glucose consumption and lactic acid production by ex vivo cultures of IGROV-1 cells established from IGROV-1 tumors untreated (control) or treated for 28 days with anti-VEGF therapy (bevacizumab). Average volumes of control and bevacizumab tumors at sacrifice were 442.29 ± 197.19 mm$^3$ and 90.83 ± 80.00 mm$^3$, respectively. Ex vivo cultures were maintained in flasks at least 10 to 15 days before analysis. Cells were plated in P6 wells at 1.5 ± 10$^5$ cells/well, incubated for 72 hours in vitro under normoxic conditions, and metabolic parameters were quantified by an automated analyzer. Mean ± SD values of five different samples for each group is shown. *, P < 0.05, t test. B, measurements of apoptosis by Annexin V staining of ex vivo cultures of IGROV-1 cells under glucose starvation. Columns report the mean values ± SD of five different samples for each group. **, P < 0.01, t test. (Continued on the following page.)
controls. Columns report the mean values/C6 of duplicate determinations of four samples for each group. 

Anti-VEGF therapy causes a metabolic shift in clinically relevant tumor models

To strengthen these findings, we treated with bevacizumab a patient-derived ovarian cancer xenograft, which was tagged with the luciferase gene to enable noninvasive monitoring of tumor growth by optical imaging. Following an initial response, mainly consisting of reduced tumor burden, tumors became substantially resistant to VEGF blockade, as shown by optical imaging and measurements of tumor weight and volume of ascitic fluid at sacrifice (Fig. 5A and B). As found in the subcutaneous tumor models, metabolic flux analysis of ex vivo cultures established from these bevacizumab-treated tumors showed increased ECAR and reduced OCR compared with cultures from control tumors (Fig. 5C).

To rule out the possibility that these metabolic changes could only occur in the context of xenograft models, we validated our hypothesis in the BALB-neuT transgenic model of breast cancer (18). To this end, we treated 20-week-old BALB-neuT mice bearing measurable breast tumors (Supplementary Fig. S8A) with B20-4.1.1, an antibody neutralizing both human and mouse.

Figure 5.
Resistance to anti-VEGF therapy is associated with stable upmodulation of the glycolytic phenotype in patient-derived ovarian cancer xenografts. A, in order to track resistance to bevacizumab by optical imaging, PDOVCA37 cells were labeled with the luciferase gene and intraperitoneally injected into SCID mice (1 × 10⁷ cells/mouse; n = 6 mice/group). When tumors became detectable by IVIS imaging (pre), mice were treated with bevacizumab (100 μg/dose administered every 2–3 days) for 2 weeks, until resistance appeared. Left, representative images acquired at 7 and 14 days after anti-VEGF treatment of six representative control and six bevacizumab-treated mice. Right, quantitative analysis of luciferase activity at various time points of measurement (n = 6 mice/group). Statistically significant differences in average radianc in the two groups of samples are indicated (, P < 0.05, t test). B, columns indicate tumor weight and ascitic fluid volume of control and bevacizumab-treated tumors at sacrifice. C, bioenergetic analysis of ex vivo cultures of PDOVCA37 tumors. Representative OCR and ECAR traces obtained from monolayers of PDOVCA37 cells obtained from mouse xenografts untreated (control) or treated with anti-VEGF mAb (bevacizumab). Subsequent additions of the ATP synthase inhibitor oligomycin, of the uncoupler FCCP, of the ETC complex I inhibitor rotenone, and of the respiratory complex III inhibitor antimycin A were carried out. Data are mean ± SD values of ten replicates normalized to protein content. The experiment was repeated in five independent ex vivo cultures per group with similar results.

(Continued.) C, quantitative RT-PCR analysis of glycolysis-associated transcripts in ex vivo cultures from IGROV-1 tumors. Columns show mean ± SD values of duplicate determinations of four samples for each group. , P < 0.05, t test. Expression levels of the various transcripts in ex vivo IGROV-1 control samples were set at 1 to calculate relative variations. D, bioenergetic analysis of ex vivo cultures of IGROV-1 tumors. Representative OCR and ECAR traces obtained from monolayers of IGROV-1 cells either kept in culture, or obtained from mouse xenografts untreated (control) or treated with anti-VEGF mAb (bevacizumab). Subsequent additions of the ATP synthase inhibitor oligomycin, of the uncoupler FCCP, of the ETC complex I inhibitor rotenone, and of the respiratory complex III inhibitor antimycin A were carried out. Data are mean ± SD values of ten replicates normalized to protein content. The experiment was repeated in five independent ex vivo cultures per group with similar results. E, Western blot analysis of NDUFS1 levels in ex vivo cultures established from these bevacizumab-treated tumors showed increased ECAR and reduced OCR compared with cultures from control tumors (Fig. 5C).

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Figure 6.
The metabolic shift induced by anti-VEGF therapy is stable in vivo. A, quantification of lactate and glucose levels in IGROV-1 tumor xenografts by imBI. Left, hematoxylin and eosin (H&E) staining and color-coded distributions of lactate and glucose in sequential cryosections from representative tumors. As some tumors contain large central necrosis, glucose was at background levels within these large central areas. However, high glucose concentrations are clearly seen in the tumor periphery. This nicely illustrates the importance of structure-associated data acquisition with imBI. The concentration values were color-coded, with each color corresponding to a defined concentration range in μmol/g. (Continued on the following page.)
VEGF (20). Measurements of tumor volume and number of tumors per mouse showed that in this HER-2-driven spontaneous tumor model antiangiogenic therapy delayed tumor growth for about 24 days, followed by secondary resistance (Supplementary Fig. S8B). When ex vivo cultures of anti–VEGF-treated tumors were analyzed, increased ECAR, and reduced OCR were measured compared with ex vivo cultures from control tumors (Supplementary Fig. S8C).

Altogether, metabolic analysis in four different tumor models strongly support the conclusion that anti-VEGF therapy causes stable modulation of aerobic glycolysis in tumor cells.

Anti–VEGF-treated tumor cells maintain increased glycolysis in vivo, are highly tumorigenic, and show resistance to anti-VEGF therapy in vivo

To investigate whether upregulation of the glycolytic phenotype was stable and it was maintained in vivo, we subcutaneously injected tumor cells from ex vivo cultures of IGROV-1 tumors into naïve SCID mice. Measurement of glucose and lactate levels by imBi analysis disclosed significantly lower glucose levels and a trend towards higher lactate concentrations in tumors formed by IGROV-1 cells from anti–VEGF-treated mice compared with control tumors (Fig. 6A).

To strengthen these findings, we also investigated whether interruption of anti-VEGF therapy would affect the glycolytic phenotype of tumor cells. To this end, 7 days after anti-VEGF treatment mice bearing established OC316 xenografts were randomized to either continue for additional 21 days (on therapy group) or interrupt (off therapy group) anti-VEGF treatment (Fig. 6B). Seahorse analysis of ex vivo cultures from these tumors disclosed that even short-term anti-VEGF therapy is associated with increased glycolytic activity in the OC316 model. Moreover, similar metabolic profiles were found in "on therapy" versus "off therapy" samples, indicating that interruption of anti-VEGF therapy did not reverse the highly glycolytic phenotype associated with antiangiogenic therapy (Fig. 6B).

Finally, we investigated whether this metabolic switch was associated with distinctive patterns of tumor growth and response to anti-VEGF therapy. Accelerated kinetics of tumor growth was observed following injection of IGROV-1 cells from anti–VEGF-treated tumors (Fig. 7A), a finding that was confirmed also in the case of OC316 tumors (Fig. 7A). Notably, tumors formed by comparatively highly glycolytic IGROV-1 or OC316 cells from bevacizumab-treated mice were substantially less responsive to VEGF blockade compared with control tumors (Fig. 7B), thus indicating that these cell-autonomous metabolic traits confer resistance to VEGF blockade.

Cell tracing studies support in vivo selection of highly glycolytic tumor cells by anti-VEGF therapy

Finally, to determine whether tumor cells bearing highly glycolytic features are positively selected by anti-VEGF therapy, we performed cell tracing studies. In these experiments, we subcutaneously injected SCID mice with mixtures of tumor cells labeled by a fluorescent reporter (EGFP). MIX1 and MIX2 were obtained by in vitro mixing of unlabeled IGROV-1 cells in a 1:1 proportion with EGFP+/bevacizumab-treated (highly glycolytic) or control (poorly glycolytic) IGROV-1 cells, respectively. Thirty-five to 43 days later, when average tumor volume was approximately 100 mm³ (Fig. 7C), mice were either sacrificed or treated with bevacizumab according to the usual schedule for 28 days. The percentage of EGFP+ cells in tumors was measured by flow cytometry either before or after anti-VEGF therapy. As shown in Fig. 7D, in the case of MIX2 we found 28.43% ± 8.36% EGFP+ cells in pre-bevacizumab tumor samples and these numbers changed minimally after anti-VEGF therapy (29.13% ± 5.22%). The percentage of EGFP+ cells was reduced compared with the preinjection value (50%), probably due to partial downregulation of transgene expression. In contrast, in the case of MIX1, the percentage of EGFP+ cells in tumors significantly increased after anti-VEGF therapy (37.85% ± 10.33%) with respect to pretreatment values (18.95% ± 3.22%; Fig. 7D). This result supports the hypothesis that anti-VEGF therapy fosters selection of tumor cells endowed with certain metabolic features.

Discussion

Several hypotheses have been proposed to explain resistance to antiangiogenic therapies (3, 4). As these drugs generally lack direct cytotoxic effects, their therapeutic activity is assumed to be due to their indirect effects, such as depletion of oxygen and key substrates needed to sustain tumor cell proliferation and growth (29). As tumors exhibit heterogeneous metabolic profiles (30), we sought to investigate whether the metabolic phenotype of tumor cells might influence therapeutic responses to antiangiogenic drugs.

On the basis of our previous finding that glucose levels are dramatically impaired in experimental tumors treated with anti-VEGF therapy (14), we initially hypothesized that glucose-addicted tumors could better respond to antiangiogenic drugs. However, we observed that while various glucose-dependent tumor xenografts are initially starved due to induction of large necrotic areas, they subsequently become rapidly resistant to VEGF blockade. This finding was confirmed by analysis of response to anti-VEGF therapy of xenografts formed by variants of IGROV-1 cells, which selectively differ in the levels of expression of MIX1, the percentage of EGFP+ cells in tumors significantly increased after anti-VEGF therapy (37.85% ± 10.33%) with respect to pretreatment values (18.95% ± 3.22%; Fig. 7D). This result supports the hypothesis that anti-VEGF therapy fosters selection of tumor cells endowed with certain metabolic features.

Resistance to VEGF blockade was associated with selection of tumor cells with increased proliferation, as shown by [18F]FLT PET analysis (Fig. 3) and also by measurement of cell proliferation in ex vivo cultures from bevacizumab-treated versus control tumors (Supplementary Fig. S4). In this respect, highly proliferative...
Tumor rims were previously reported following treatment of experimental tumors with vascular damaging drugs (32). Altogether, these results suggest that necrosis induced by anti-VEGF therapy triggers proliferation in the surviving tumor cells. Metabolic features of tumors, and in particular their glycolytic phenotype, have a substantial impact on the development of tumor necrosis following antiangiogenic treatment. This increased cellular turnover could accelerate Darwinian selection of a population of cells resistant to hypoxia and acidosis (33), thereby influencing the kinetics of secondary resistance.

On the other hand, other factors could also contribute to relapse, including recruitment by the necrotic tissue of circulating endothelial progenitor cells (34) and other specialized myeloid cells releasing proangiogenic factors that could bypass VEGF neutralization (35). Indeed, some variations in tumor-associated myeloid cells, mainly reflecting increased levels of CD11b+/Gr1+ cells, were measured in tumors treated with anti-VEGF therapy (data not shown). Moreover, lactate, which accumulates in highly glycolytic tumors, is a pleomorphic tumor-promoting factor that stimulates angiogenesis by NFκB activation in endothelial cells (36). However, it should be noted that in our models microvessel density of anti-VEGF-resistant tumors was significantly reduced compared with controls, indicating that the tumor vasculature was still dependent on VEGF. Therefore, therapeutic resistance did not appear to be primarily related to renewed vascularization due to recruited myeloid cells or a switch to alternative angiogenic factors, as reported in other models of resistance to VEGF blockade (37–39).

Figure 7.
Tumor cells from anti-VEGF-treated mice have increased tumorigenic capacity, are resistant to bevacizumab, and are positively selected by anti-VEGF therapy. A, kinetics of tumor development following subcutaneous injection of IGROV-1 (left) or OC316 (right) tumor cells (4 × 10⁷ cells/flank) derived from bevacizumab-treated tumors compared with control tumors (n = 5 mice/group). *, P < 0.05, t test. B, effects of multiple injections of the anti-VEGF mAb bevacizumab (arrows, 100 μg/dose administered every 2–3 days) on tumor size. Tumor xenografts derived from ex vivo cultures of anti-VEGF-treated IGROV-1 or OC316 tumors are resistant to anti-VEGF therapy (right) compared with xenografts derived from control cells (left; n = 5 mice for group). *, P < 0.05, t test. C, kinetics of tumor development following subcutaneous injection of a 1:1 mix of EGFP-tagged IGROV-1 cells derived from bevacizumab-treated tumors and unlabeled control tumor cells (MIX1) or a 1:1 mix of EGFP-tagged IGROV-1 cells from control tumors and unlabeled control tumor cells (MIX2) and effects of the anti-VEGF mAb bevacizumab (100 μg/dose administered every 2–3 days) on tumor size. MIX1 and MIX2 tumors (n = 4 samples/group) were collected either before (time points 1 and 2 for MIX1 and MIX2, respectively) or after (time points 3 and 4 for MIX1 and MIX2, respectively) bevacizumab administration and the percentage of EGFP+ cells in tumors was quantified by flow cytometry. D, tumor cells with peculiar metabolic features are positively selected by anti-VEGF therapy. Left, quantification of EGFP+ IGROV-1 cells in MIX1 and MIX2 tumors before or after bevacizumab administration. Columns report the mean values ± SD of four different samples for each group. *, P < 0.05, t test. Right, representative histogram plots of EGFP+ cells for each of the experimental groups (1, 2, 3, and 4) described in C.
We concede that the clinical relevance of these observations remains to be established. First of all, treatment-induced necrosis may eventually trigger an immune-mediated response in patients, which cannot be seen in immunodeficient mice, and this could restrain tumor growth. Moreover, bevacizumab is invariably given to patients in combination with chemotherapy and cytotoxic drugs that could potentially kill highly proliferating tumor cells resistant to anti-VEGF therapy, thus preventing tumor relapse (31).

The most important conclusion of this study, grounded on experimental work in both tumor xenografts and a spontaneous tumor model, is that antiangiogenic therapy favors emergence of a glucose-dependent metabolic phenotype. This is likely promoted by chronic hypoxia and induction of the HIF1α-driven transcriptional program, which involves activation of glycolysis (28). Indeed, we observed a dramatic increment in tumor hypoxic regions with [18F]FAZA PET and also nuclear accumulation of HIF1α, which could drive the metabolic switch in the tumor microenvironment and favor evasive mechanisms (40). However, the stable metabolic changes measured in vitro were not due to stabilization of HIF1α levels in tumor cells, increased c-MYC and pAKT levels, or loss of AMPK activity (Supplementary Fig. S6). The discrepancy between HIF1α expression levels in vivo versus in vitro conditions indicates that accumulation of this transcription factor in tumor cells remains hypoxia-dependent.

Although increased expression of glycolysis-associated markers following bevacizumab has been observed in other studies (41), here we show for the first time that anti-VEGF therapy caused a stable modification of the metabolic phenotype of the tumor. This was supported by (i) the finding that ex vivo cultures from anti–VEGF-treated tumors had different metabolic features with respect to those established from control tumors (Fig. 4), (ii) results of im81 analysis, which showed that xenografts formed by tumor cells from anti–VEGF-treated mice maintained a highly glycolytic phenotype (Fig. 6A), and (iii) results of on/off therapy experiments (Fig. 6B).

These stable metabolic modifications could be due to either selection of a pre-existing subpopulation of highly glycolytic tumor cells or, alternatively, be accounted for by epigenetic reprogramming of cell metabolism, as shown in the model presented in Supplementary Fig. S9. With regard to the first possibility, angiogenesis inhibitors alter the environment through increased hypoxia, glucose deprivation, and acidosis, which produce strong Darwinian forces that rapidly promote adaptive phenotypes (33). In this respect, it has been reported that glucose deprivation provides a strong selection for activated oncogenes, such as KRAS (42). Moreover, Xu and colleagues recently described mitochondrial defects in ex vivo cultures of LoVo cells from bevacizumab-treated tumors (43), and we also found reduced mitochondrial mass and decreased expression of NDUF51, a component of respiratory complex I, in ex vivo cultures of bevacizumab-treated IGROV-1 cells (Supplementary Fig. S5). In support of this possibility, cell tracing experiments demonstrated that EGFP-labeled highly glycolytic tumor cells are positively selected by anti-VEGF therapy (Fig. 7).

Alternatively, new findings have uncovered regulation of glucose metabolism by sirtuins 1, 3, 4, and 6 (44), and epigenetic variations in sirtuins levels could possibly contribute to the phenomenon observed. In preliminary experiments, however, we found that SIRT3, SIRT4, and SIRT6 were barely expressed in IGROV-1 and OC316 cells, whereas SIRT1 was expressed at comparable levels in IGROV-1 and OC316 cells from bevacizumab-treated tumors compared with control cultures (not shown). Therefore, although other epigenetic mechanisms could be involved, dysregulated expression of sirtuins is not likely to account for the metabolic shift observed in these tumor models.

Metabolic evolution of tumors following antiangiogenic therapy is a novel concept that could have relevant translational implications. A metabolic shift involving downregulation of genes involved in the tricarboxylic acid cycle, decreased AMPK and PTEN protein levels, upregulation of the pentose phosphate pathway was recently described in aggressive renal cell carcinoma (45) and fits with increased kinetics of tumor growth by anti-VEGF-treated tumor cells observed in this study. Moreover, as glucose-addicted tumors have dismal prognosis (25, 46) and could be relatively resistant to cytotoxic therapies (47, 48), this phenomenon might contribute to explain the relatively short-term benefits of antiangiogenic therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
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