LDH-A Knockdown: Changes in The LDH Isoenzyme Profile and Variability in Glioma Response

Masahiro Shindo

Memorial Sloan Kettering Cancer Center

Masatomo Maeda

Memorial Sloan-Kettering Cancer Center

Ko Myat

Memorial Sloan Kettering Cancer Center

Mayuresh Mane

Memorial Sloan Kettering Cancer Center

Ivan J. Cohen

Memorial Sloan Kettering Cancer Center

Kiranmayi Vemuri

Memorial Sloan Kettering Cancer Center

Avi S. Albeg

Memorial Sloan Kettering Cancer Center

Inna Serganova

Memorial Sloan Kettering Cancer Center

Ronald G Blasberg (✉ blasberr@mskcc.org)

Memorial Sloan Kettering Cancer Center

Research

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Abstract

Background: Lactate metabolism in tumors is now recognized as a major energy source and a major gluconeogenic precursor for many tumors, as well as shown to exhibit signaling properties. There is less information on the role of the LDH/lactate axis in brain tumors, although lactate formation in gliomas is associated with poor survival.

Methods: Three murine glioma cell lines (GL261, CT2A, and ALTS1C1) were transduced to knockdown (KD) expression of the murine *LDH-A* gene. The effects of the LDH-A KD were compared to those in control (NC) cells and tumors.

Results: Differences in the expression of LDH-A and LDH-B mRNA, protein, and enzymatic activity were observed in the six cell lines. LDH zymography showed a major difference in LDH subunit distribution between GL261 LDH-A KD and NC tumors, whereas little or no effect of LDH-A KD was observed in CT2A and ALTS1C1 tumors. Tumors LDH-A and LDH-B immunohistochemistry and a Weka segmentation analysis were consistent with isoenzyme patterns and the above analyses. An “inverse” LDH-A/LDH-B staining relationship (high vs low) was observed in many local GL261 tumor regions. In contrast, CT2A tumors showed a more “direct” local LDH-A/LDH-B staining relationship. LDH-A KD prolonged the doubling time of GL261 cells in culture and prevented the formation of subcutaneous flank tumors in immune-competent C57BL/6 mice (GL261 NC tumors had a prolonged growth delay). In nude mice, both LDH-A KD and NC GL261 tumors grew more rapidly than GL261 NC tumors in C57BL/6 mice. No differences between NC and KD cell proliferation (*in vitro*) and tumor growth in C57BL/6 mice (doubling time) were observed for CT2A and ALTS1C1 cells and tumors, consistent with the absence of a difference in their LDH isoenzyme profiles.

Conclusions: These results show the combined impact of a genetic alteration (LDH-A depletion) on the LDH isoenzyme profile, expression of LDH-A vs LDH-B and LDH enzymatic activity, and the immune system (C57BL/6 vs nude mice) on the growth of s.c. located tumors.

Background

Lactate metabolism in tumors has been intensively studied recently: 1) lactate is now considered a major energy source for many tumors; 2) lactate is the major gluconeogenic precursor and 3) lactate exhibits signaling function properties (1). In this study, three murine gliomas (GL261, CT2A, and ALTS1C1) were explored with regards to the impact of LDH-A downregulation on tumor biology, since there has been limited information on the role of the LDH-A/lactate axis in tumors of brain origin (1–4). Lactate formation in gliomas is associated with poor survival and contributes to the suppression of local immunity (5). The relationship between LDH-A expression levels and GBM malignancy, using human glioma cells and its impact on proliferation and apoptosis has been explored (6) (7). It has long been known that many human cancers have higher LDH-A levels compared to normal tissues (8–10). It has also been shown that LDH-A plays an important role in the development, invasion, and metastasis of

malignancies (10–12). The LDH enzyme (EC 1.1.1.27, LDH) is composed of two proteins, LDH-A (predominantly found in skeletal muscle and many solid tumors) and LDH-B (predominantly found in heart muscle and brain). The LDH enzyme is a tetramer and exists in several different electrophoretic forms known as isoenzymes. They catalyze the same biochemical reaction but differ in their kinetic characteristics, physicochemical properties (different net charge), and response to the inhibition by pyruvate (13). The LDH tetrameric enzyme exists in two basic homo-tetrameric forms: i) LDH5 (A₄ or M₄) contains 4 LDH-A subunits, and ii) LDH1 (B₄ or H₄) contains 4 LDH-B subunits. In addition to homo-tetramers, LDH also exists in three hybrid forms, resulting in five structural entities that vary in expression level in different tissues (10). The LDH-A and LDH-B isoforms occupy the mitochondrial compartment, plasma membrane and cytosol (14). Traditionally LDH-A participates in converting pyruvate to lactate, whereas LDH-B has a higher affinity for lactate, converting lactate to pyruvate (15). LDH-B has been considered to facilitate the use of lactate as a carbon energy source. More recently, it has become clear that lactate is both created and consumed in aerobic conditions, and serves as a link between glycolytic and oxidative metabolism (16). Analyses of LDH-A and LDH-B expression levels in tumors have shown that LDH-A is highly expressed in most neoplastic tissues (17–20). However, the role of LDH-B and its regulation is less explored (21, 22).

The role of LDH-A and LDH-B in tumor biology is complex. The relationship between tumor cell growth, the balance between LDH-A and LDH-B, the effects on tumor cell metabolism, and the tumor microenvironment (TME) is variable across different types of gliomas.

Methods

Aim

In this report, we study and compare three murine glioma cell lines and tumors following LDH-A shRNA knockdown (KD). The objective was to explore and compare the effect of LDH-A knockdown (KD) on the expression levels of LDH-A and LDH-B mRNA, protein, LDH enzymatic activity, the effect on the LDH isoenzyme profiles and the impact of these changes on the growth of cancer cells *in vitro* and *in vivo*.

Cells and culture conditions

The GL261 murine glioblastoma cell line was obtained from NCI depository (23, 24). The ALTS1C1 (ALT) murine glioblastoma cell line derived from SV40 large T antigen-transfected astrocytes was kindly provided by Dr. Chiang (Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, Taiwan) (25) and the CT2A high-grade murine astrocytoma cell line was kindly provided by Dr. Seyfried (Biology Department, Boston College, Boston) (26). These cell lines were cultured in DMEM media supplemented with 25 mM glucose, 10% FCS, 4 mM glutamine, and penicillin/streptomycin. LDH-A KD (knock-down) and NC (negative control) cells, derived from each cell line, were grown in the same media and 2.5 mg/L of puromycin.

Generation of LDH-A knockdown and control cell lines

GL261, CT2A and ALTS1C1 cells were transfected with Sure Silencing shRNA plasmids (QIAGEN, Frederick, MD, USA) to specifically knock-down expression of the mouse *LDH-A* gene as described previously (27). Stably transduced clones (KD cell lines) were developed, along with a control (NC) cell line bearing a scrambled shRNA. Based on the previous experience (12, 28), we decided to use the most effective shRNAs (shRNA-2) to develop LDH-A KD cells in murine glioma cells. The transfection of GL261 cancer cells with shRNA-2 resulted in a significant knock-down effect for LDH-A (approximately 10% of that in wild type cells), while bulk CT2A and ALTS1C1 cells transfected with shRNA-2 had a significantly less level of LDH-A knock-down (40-60%). In order to enrich the level of LDH-A knock-down we used a sub-cloning strategy for CT2A and ALTS1C1 cell lines (27).

Western blotting

All immunoblotting experiments were performed as described previously (11, 29). Cell lines underwent protein extraction using RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) with protease & phosphatase inhibitors cocktail (1:100, Thermo Scientific Halt Protease & Phosphatase Inhibitor Single-Use Cocktail). Protein concentrations were determined by Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA). The proteins in equivalent amounts (10-40 µg/well) were separated by electrophoresis in a NuPAGE gradient 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) and were immuno-blotted with anti-LDH-A antibody (#2012S, Cell Signaling Technology, Danvers, MA, USA) at a 1:1,000 dilution and anti-β-actin antibody (Sigma life science, #A2103, USA) at a 1:5,000 dilution antibodies. Bound primary antibodies were visualized with either appropriate horseradish peroxidase-conjugated secondary antibodies (1:2,000) using enhanced chemiluminescence reagent (Western Lightning-ECL) or with Eu-labelled antibody using ScanLater Western Blot Assay kit and SpectraMax ID5 (Molecular Devices, US).

LDH enzyme activity

Total LDH enzyme activity was assessed using the Cytotoxicity Detection Kit PLUS (LDH) (Roche Diagnostics) as described before (27).

Proliferation assay *in vitro*

2×10^5 cells were seeded in 3 mL culture media in 6-well plates, followed by counting cells at 3 different time points, 48, 72, and 96 hours after seeding cells using Countess automated cell counter (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was triplicated, and media were changed every other day.

Animal models

The animal protocol was approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center. Two strains of mice were used in animal experiments. First, 1×10^6 cells in 100 µL PBS were injected into the right flank of immunocompromised Hsd: Athymic Nude Foxn/nu

(Envigo) or athymic *nu/nu* male mice (Charles River Laboratories). Second, 1×10^6 cells in 100 μ L PBS mixed with 100 μ L matrigel were injected subcutaneously into the right flank of immunocompetent C57BL/6 male mice (Charles River Laboratories). The volume (V) of subcutaneous tumors was calculated from caliper measurements, where $V = (\pi/6) \times x \times y \times z$ where x , y , and z are 3 orthogonal diameters. Doubling times were calculated by the equation of trend lines using GraphPad Prism.

LDH zymography

Zymography, a common method to detect isoenzymes, was used to detect tissue-specific differences in LDH isoenzymes. This approach can directly observe 5 isozyme bands in the active state (30). Based on their different electrophoretic motility, all LDH isoenzymes can be identified as LDH1 (B4 or H4), LDH2 (B3A1 or H3M1), LDH3 (B2A2 or H2M2), LDH4 (B1A3 or H1M3), and LDH5 (A4 or M4). The buffer system at pH 8.6 was chosen for the best separation of the five LDH isoenzymes (30-33). Because the B polypeptide has more acidic amino acid residues than the A polypeptide, LDH1/B has the highest migration rate and LDH5/A has the lowest migration rate. The electrophoretic mobilities of the LDH isoenzymes are: LDH 1/B > LDH 2 > LDH 3 > LDH 4 > LDH 5/A.

Immunohistochemical staining and image analyses

Dissected tumors were placed into 4% paraformaldehyde for further immunohistochemistry (IHC). The immunofluorescent (IF) staining was performed at Molecular Cytology Core Facility of MSKCC using Discovery XT processor (Ventana Medical Systems). 5 μ m thick, paraffin-embedded sections were stained for H&E and LDH-A and LDH-B staining. The sections of tumors from nude or immunocompetent mice were stained by anti-LDH-A, anti-LDH-B. The small 5-day tumors from GL261 NC and KD were stained with immune markers: anti-CD68 antibody (Catalog No. TA1518, Boster), anti-CD4 (Catalog No. AF554, R & D Systems) and anti-CD3 antibody (Catalog No. A0452, Dako). Quantification of morphological characteristics was performed using trainable Weka Segmentation (Image J segmentation plugin) to assess the fraction of viable tumor cells, stroma, hemorrhage and necrosis in the H&E sections. The same approach was used to quantify LDH-A and LDH-B staining (28, 34).

Statistical analysis

Results are presented as mean $\pm$ standard error unless otherwise specified. Statistical significance was determined by a two-tailed Student t-test. A p-value of <0.05 was considered significant. All data presented for T cells assessment using IF staining were analyzed using GraphPad Prism (version 7.0; GraphPad Software) and are presented as mean $\pm$ SD. Results were analyzed using the unpaired Student's t-test, and statistical significance was defined as $p < 0.05$.

Results

Effects of LDH-A knockdown on murine glioma cells

We chose three murine brain tumor models to understand the impact of LDH-A downregulation on tumor phenotype and growth potential: GL261 (35), CT2A (26), and ALTS1C1 (25). The wild-type cell lines were transduced with two shRNA retroviral vectors: i) shRNA-2 was used for targeting LDH-A, it was found to provide effective knockdown, based on our previous work (28, 36), and ii) one scrambled control (11). *In vitro* comparisons between the LDH-A knockdown (KD) and the scrambled control (NC) cell lines are shown in **Figures 1 and 2, S-1**. Significant differences were observed between the three control (NC) cell lines with respect to LDH-A and LDH-B mRNA levels (**Fig. 1 A,B; Fig. S-1 A,B**), LDH-A and LDH-B protein expression (**Fig. 1 C-E; Fig S-1 C,D**), and LDH enzyme activity (**Fig. 1F; Fig S-1E**). GL261 NC cells had the lowest expression of both LDH-A and LDH-B protein expression by Western blot assessment (**Fig S-1C, D**) and have lower LDH enzyme activity (**Fig S-1E**) compared with CT2A and ALTS1C1 NC cells. Despite low protein and enzyme levels, GL261 NC cells had the highest LDH-B mRNA level detected by ddPCR (**Fig. 1B, Fig. S-1B**). This suggests up-regulation of LDH-B in GL261 at the mRNA level but not at the protein level (possibly reflecting a lower rate of protein synthesis or more rapid protein degradation). Interestingly, CT2A NC cells had comparatively low LDH-A mRNA expression (**Fig. 1A, Fig. S-1A**) and high LDH-A protein expression on Western blot (**Fig.S-1C**). These results suggest variability in LDH-A and LDH-B synthesis/degradation between the three cell lines.

As expected, following LDH-A shRNA KD, LDH-A mRNA levels, protein expression and enzyme activity were all significantly reduced compared to the control (NC) cell lines. LDH-B was also affected by LDH-A shRNA knock-down but to a variable degree. The most notable difference was a significantly higher LDH-B/LDH-A ratio for mRNA, protein expression and LDH enzyme activity in GL261 KD cells (**Fig. 1 F-H**).

Effects of LDH-A knockdown on *in vitro* and *in vivo* growth profiles

The effect of LDH-A KD on cell proliferation (*in vitro* growth profile plots) was compared to that of control NC cells (**Fig. 2A-C**). The cell proliferation rate (doubling time) was calculated from an exponential fit of the plots (**Fig. 2D**). LDH-A KD prolonged the *in vitro* doubling time only for GL261 cells but had little or no effect on CT2A and ALTS1C1 cells. The doubling times of wild type and NC cells were similar (data are not shown). The differences in the proliferation of cell lines may suggest that there are corresponding differences in their metabolic properties since metabolism and proliferation share common regulatory pathways in cancer cells (37-39).

To study the differences of tumor growth and phenotype in different host organ locations and in both immune competent (C57BL/6) and incompetent (Nude) animals, we initially studied the three gliomas in a subcutaneous (s.c.) location, and subsequently in an intracranial (i.c.) location (to be reported in a following manuscript). The effect of LDH-A KD on corresponding growth profiles of *in vivo* s.c. flank tumors in immune competent C57BL/6 mice are shown (**Fig. 2E-G**), and the tumor doubling times were estimated (**Fig. 2H**). As in the *in vitro* studies, LDH-A KD had a significant effect only on the growth of s.c. GL261 tumors in C57BL/6 mice (**Fig. 2E**). All s.c. GL261 LDH-A KD tumors were suppressed after developing small (<50 mm³) tumors, whereas most GL261 A5NC tumors grew after a long 40-day delay period (**Fig. 2E**). Once GL261 A5NC tumors began to grow in C57BL/6 mice, the subsequent doubling

time was similar to that of wild-type GL261 tumor growth in C57BL/6 mice (data are not shown). No significant difference was observed between the growth profiles of the NC and KD groups of CT2A and ALTS1C1 tumors in C57BL/6 mice (**Fig. 2F, G, H**). Wild-type CT2A and ALTS1C1 tumors grew slightly faster than NC tumors in C57BL/6 mice, but the difference was not statistically significant (data not shown). These results suggest that the LDH-A knockdown did not significantly alter the growth of s.c. CT2A and ALTS1C1 tumors growth in C57BL/6 mice compared with NC control tumors but had a clear effect on GL261 tumor growth in C57BL/6 mice - leading to tumor regression.

In immune compromised nude mice, the growth and doubling times of NC and LDH-A KD GL261, CT2A and ALTS1C1 tumors were similar (**Fig. S2A-D**). Interestingly, GL261 NC tumors grew at a faster rate (doubling time, 3.1 ± 1.5 days) in nude mice than GL261 NC tumors in C57BL/6 mice (7.1 ± 0.3 days) ($p=0.002$). The difference between host animals was reversed with ALTS1C1 NC and CT2A LDH-A KD tumors; tumors grew more slowly in nude compared to C57BL/6 mice. These data demonstrate the variability of the NC and LDH-A KD tumor growth in host animals with different tumor microenvironments and immune responses.

LDH isoenzyme pattern of subcutaneously located murine glioblastoma tumors

LDH zymography is a common method to detect LDH isoenzymes and provides for the direct observation of 5 isozyme bands in the active state (31). The LDH isoenzyme pattern for GL261, CT2A and ALTS1C1 LDH-A KD and NC subcutaneous tumors were compared to each other and to heart and skeletal muscle tissue from the same animals (**Fig. 3A, B**).

All NC tumors have an isoenzyme pattern similar to skeletal muscle (LDH5 and LDH-A dominant), with some formation of LDH 4, 3, 2. CT2A and ALTS1C1 LDH-A KD tumors have a similar LDH isoenzyme pattern as the NC tumors. Only GL261 LDH-A KD tumors were strikingly different, with a LDH isoenzyme pattern similar to the heart, where LDH1, 2, 3, 4 isoenzymes are most highly expressed (40). The LDH isoenzyme ratio of the brain tissue was comparable to ratios found in the heart tissue that presents mostly isoenzymes LDH1-LDH3 and low amounts of the LDH5 isoform (40). These results are consistent with the mRNA and Western blot assays and calculated LDH-B/LDH-A ratios (**Fig. 1**).

LDH-A and LDH-B tumor staining patterns

First, we assessed the structure of subcutaneous GL261 NC and KD tumors growing in nude mice and for CT2A NC and KD tumors growing in C57BL/6 mice by H&E staining. There was a variable pattern of necrosis, stroma and cyst formation for both tumors (**Fig. 4Aa, 4Da**). Second, LDH-A and LDH-B immunohistochemistry also showed a variable pattern of staining for both types of tumors (**Fig. 4Ab,c and 4Db,c**). A Weka analysis (28) showed significantly greater LDH-A staining in both GL261 and CT2A NC tumors compared to the LDH-A KD tumors (**Fig. 4B, 4E, Table S-1**), consistent with the Western blot and LDH enzyme results (**Fig. 1C,E,F**). Considerably greater LDH-B staining was observed in GL261 LDH-A KD compared to NC tumors (**Fig 4C, Table S-1**), but no differences in LDH-B staining were observed

between CT2A LDH-A KD and NC tumors (**Fig 4F, Table S-1**), also consistent with the Western blot results (**Fig.1D,E,H**).

In many, but not all GL261 tumor regions, there was an inverse relationship between LDH-A and LDH-B staining intensity (**Figs. 5A and S-3A**). This inverse relationship was greater for LDH-A KD than NC GL261 tumors. CT2A tumors showed a different relationship; there was a more direct relationship between LDH-A and LDH-B staining intensity (**Figs. 5B and S-3B**).

Early immune cell infiltration of GL261 LDH-A KD and NC s.c. tumors

To compare the extent of early (day 5) immune cell infiltration of GL261 KD and NC s.c. tumors, both GL261 KD and NC cells were implanted s.c. in the same animals (C57BL/6 mice), along with a tumor-free Matrigel plug (**Fig. S-4A**). The size and morphology of the NC and KD tumors were similar (**Fig. S-4B**). Low numbers of CD3⁺ cells were observed in NC tumors (in both high tumor cell density (HD) and low density (LD) tumor regions) at 5 days post-implantation (**Fig. S-4C**). Slightly higher numbers of CD3⁺ cells were observed in HD regions of LDH-A KD tumors compared to NC HD tumor regions. There was also a consistently higher density of CD3⁺ cells in GL261 LDH-A KD HD tumor regions compared to LDH-A KD LD regions in each mouse. Low numbers of CD4⁺ cells were observed in LD tumor cell regions of both NC & KD tumors, 5 days post-implantation (**Fig. S-4D**). Inconsistent CD4⁺ cell staining was observed in GL261 KD HD tumor regions. Low numbers of CD68⁺ cells were observed in both HD and LD tumor cell regions of both NC and KD GL261 s.c. tumors (**Fig. S-4E**). The results of an immunofluorescent staining analysis did not reveal any significant differences (**Fig. S-4F**) but show some trend toward an increase in immune cell infiltration in LDH-A knockdown tumors.

Discussion

Previously, we reported that downregulation of LDH-A expression in 4T1 murine breast cancer cells *in vitro* and in 4T1 tumors located in the mammary fat pad leads to reduced glycolytic flux and increased mitochondrial respiration, leading to slower growth and the delayed onset of (or failure to develop) distant metastases in both immune compromised mice (27) and in immune competent mice (12). We now compare three murine brain tumor models (GL261 (23), CT2A (26) and ALTS1C1 (25) to better understand the impact of LDH-A downregulation (KD) on glioma tumor phenotype and growth potential. We have studied the gliomas in two different body locations (s.c. and i.c.), and in both immune competent and immune compromised animals. In this manuscript, we compare the effects of LDH-A KD on s.c. tumors. In a subsequent manuscript, we focus on the intracranial location of the three tumors and compare the effects of genetic-shRNA LDH-A knockdown and LDH drug-targeted inhibition (41, 42) on tumor cell metabolism, tumor growth and survival time. We also find significantly different effects of LDH-A knockdown on GL261 compared to CT2A and ALTS1C1 cells and tumors.

The six murine glioma cell lines (comparing LDH-A shRNA knockdown (KD) to a scrambled shRNA control (NC)) expressed different levels of LDH-A and LDH-B mRNA, protein and enzymatic activity. In all

cases, the LDH-A KD cells expressed less LDH-A mRNA and protein and had less LDH enzymatic activity than the corresponding NC cell lines. Interestingly, only the GL261 LDH-A KD cell line showed a higher expression of LDH-B mRNA and protein, and the LDH-B/LDH-A mRNA and protein ratios were significantly higher for GL261 LDH-A KD cells compared to the other 5 cell lines (Fig. 1G, H). Also, GL261 had the lowest levels of LDH-A and LDH-B, and LDH enzyme activity of the three cell lines.

LDH is the enzyme catalyzing the final step of glycolysis and contains two subunits A and B, encoded by two genes (43). LDH-A is predominantly found in skeletal muscle and LDH-B is predominantly expressed in the heart and brain. LDH-A and LDH-B can form homo- or hetero-tetramers forming five LDH isoenzymes: LDH-1 (4B), LDH-2 (3B,1A), LDH-3 (2A, 2B), and LDH-5 (4A) (44). These five isoforms catalyze the same overall reaction but differ in their affinity to the substrate, inhibition concentration (Km), isoelectric point and electrophoretic mobility. The five isoforms can be visualized in the active state using LDH zymography (45). A zymogram analysis of the 6 glioma cell lines was performed and showed an LDH-A dominant pattern for 5 of the 6 cell lines (containing mostly LDH5, LDH4 isoenzymes) (Fig. 3). Only GL261 LDH-A KD cells showed a LDH-B dominant pattern (containing mostly LDH1, LDH2 and some LDH3, LDH4 isoenzymes). This major shift in the LDH isoenzyme pattern in GL261 LDH-A KD tumors, (from LDH-A dominant in NC tumors to LDH-B dominant in LDH-A KD tumors), can lead to differences in the kinetics of the LDH enzyme oxidative vs reductive activity and in cell metabolism (45, 46). Similar variations in LDH-A and LDH-B isoforms have been detected in human glioma cells D54MG and U-251MG (6), but this difference was not explored in detail, or related to tumor growth, metabolism, and phenotype.

A careful analysis of the mRNA and protein levels in the six experimental cell lines was performed; it also shows a difference between the levels of LDH-A and LDH-B mRNAs and demonstrates the predominance of LDH-B mRNA in GL261. This difference results in a very high LDH-B/LDH-A ratio for both mRNA and protein levels in GL261 KD cells (Fig. 1G, H). In addition, the concurrent immunoblotting of all samples (with similar amounts of loading) shows the different expression levels of LDH-A and LDH-B in GL261 cells (Fig. 1E). The dominance of LDH-B in GL261 KD cells and LDH-A in CT2A and ALTS1C1 KD and NC cells implies that different murine glioma cells can develop different isoenzyme adaptation profiles. It has been suggested that this difference may relate to the origin of the experimental cells (24).

The LDH-A and LDH-B immunohistochemistry and Weka analysis confirmed the isoenzyme patterns observed with LDH zymography and the above analyses. GL261 and CT2A KD tumors showed significantly less LDH-A staining than their control NC tumors. Also consistent was the significantly greater LDH-B staining only in GL261 LDH-A KD (compared to NC tumors); no significant differences in LDH-B staining was observed between CT2A LDH-A KD and NC tumors. We also noted an “inverse” LDH-A/LDH-B staining relationship (high vs low) in many, but not all GL261 tumor regions. In contrast, CT2A tumors showed a more “direct” LDH-A/LDH-B staining relationship (high vs low) in many tumor regions.

The differences in LDH isoenzyme patterns and LDH-A/LDH-B immunohistochemistry were also reflected in our other experiments. First, LDH-A KD prolonged the doubling time of GL261 cells in culture. Second, GL261 LDH-A KD cells did not establish flank tumors in immune competent C57BL/6 mice, whereas

GL261 NC tumors formed after a 40-day growth delay. Third, both NC and KD GL261 tumors grew more rapidly in nude mice, compared to GL261 NC tumors growing in C57BL/6 mice. These results show the combined impact of both a metabolic alteration (LDH-A KD) and the immune system (C57BL/6 vs nude mice) on the growth of s.c. located tumors. Furthermore, the ability to grow GL261 tumors in nude mice allowed us to compare the isoenzyme profiles of LDH-A KD and NC tumors using zymogram analyses.

The association of LDH-A with cancer metabolism and tumor growth has been studied extensively, and the role of LDH-A is quite different in different tumors (9, 47–50). The association of LDH-B with tumors is much more complex (15). Although LDH-B expression varied in many different tumor types (15) and is overexpressed in some tumors (51–53), LDH-A is more highly expressed in most tumors.

It has been suggested that glioma cells express one of at least two different metabolic phenotypes (6, 54), as reflected in their different metabolic profiles. 251MG and U-87MG glioma cells have been shown to have metabolic characteristics akin to astrocytes (that includes the production of lactate, the storage of glycogen, and the use of lactate to support neurons). These tumors exhibit a glycolytic-dependent phenotype but retain functional oxidative phosphorylation and primarily express LDH-B. In contrast, GL261 and D-54MG glioma and SH-SY5Y neuroblastoma cells display a more oxidative phosphorylation-dependent phenotype, and express both LDH-A and LDH-B isoforms. Therefore, we hypothesized that LDH-A knockdown could cause a shift in the metabolic phenotype of GL261 cells (but not CT2A and ALTS1C1 cells, which remain LDH-A dominant). In the second concurrent paper we show that GL261 tumors transition from an LDH-A dominant to an LDH-B dominant phenotype following LDH-A knockdown and treatment with a specific LDH-A/B inhibitor (GNE-R-140), which changes the metabolic phenotype of cells. These changes in the pattern of LDH isoenzyme expression were not observed in CT2A or ALTS1C1 NC and LDH-A KD tumors. The GL261 LDH-A KD and NC cell and tumor growth profiles were also significantly different (as described above) and reflected the differences in their LDH isoenzyme profiles. In contrast, CT2A and ALTS1C1 LDH-A KD and NC tumors showed little or no difference in their growth profiles, consistent with the absence of a difference in their LDH isoenzyme profiles. It has been suggested that the sole expression of LDH-B might identify an important biological marker of glioma cells that is critical for their progression and that it might afford a new target for anticancer drugs (6).

Conclusions

Genetically altered murine glioma cell lines and tumors (GL261, CT2A and ALTS1C1 - with LDH-A shRNA knockdown (KD) compared to the scrambled shRNA controls - NC) showed significant differences in their levels of LDH-A and LDH-B mRNA, protein, and enzymatic activity. The LDH isoenzyme profiles were significantly different between KD and NC GL261 tumors (LDH-B vs LDH-A dominant), but not in comparable CT2A and ALTS1C1 tumors (all LDH-A dominant). These differences (or lack of differences) correlated with their corresponding LDH-A and LDH-B immunohistochemistry staining pattern, cell proliferation and tumor doubling times. These results show the combined impact of LDH-A depletion

(LDH-A KD vs NC control) and the immune system (C57BL/6 vs nude mice) on the growth of s.c. located tumors.

List Of Abbreviations

1. LDH - Lactate Dehydrogenase
2. shRNA - short hairpin RNA or small hairpin RNA
3. mRNA - messenger RNA
4. s.c. - subcutaneous
5. i.c. - intracranial
6. NC - negative control
7. KD - knockdown
8. TME - tumor microenvironment
9. ddPCR - droplet digital polymerase chain reaction
10. IHC - immunohistochemistry
11. IF - Immunofluorescence
12. ALT - ALTS1C1
13. FCS - Fetal Calf Serum
14. Eu – Europium
15. TAM – Tumor Associated Macrophages

Declarations

Ethics approval and consent to participate - Studies involving animals was approved by the MSKCC IACUC - protocol # 08-07-011

Consent for publication - Not applicable

Availability of data and materials - Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests - There are no financial and non-financial competing interests to be declared

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Author Contributions - Conception and Design, M.S., M.M., I.S., M.M.M., I.C. and R.B.; Development of Methodology, M.S., M.M., I.C., M.M.M., M.K. and I.S.; Acquisition of data, M.S., M.K., M.M., K.V., I.S. and M.M.M.; Writing, review, and/or revision of the manuscript, M.M.M., M.K., M.M., I.S. and R.B.; Administrative, technical, or material support, R.B.; Study supervision, I.S. and R.B.; Pathological

diagnosis, analysis, and interpretation of the immunohistochemical data, M.M.M. and M.K.; and Carrying out experiments and analyzing data, M.M., M.K., M.M.M., K.V., A.A. and I.C.

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Senior author's information - At NIH my focus was radiotracer imaging of metabolism, membrane transport and blood flow, as well as small-molecule, radiotracer probe development for pre-clinical and clinical imaging (quantitative autoradiography, PET, SPECT, and gamma-camera). At MSKCC (1990-present) we were the first group to develop and use radiotracer-PET HSV1-*tk* reporter gene imaging, and one of the first to develop reporter constructs for multi-modality optical and radiotracer imaging, with both constitutive and inducible reporter systems. More recent studies focused on the role of metabolism and the immune system in tumor progression and metastases. We showed an important role for LDH-A and tumor lactate levels in aggressive breast cancer and breast cancer metastases, as well as the impact of LDH-A depletion on the immune system in prostate tumor models. I was founding Editor of Molecular Imaging (2001-2005) and Senior Editor (Imaging) for Clinical Cancer Research (2004-2017), and received several prestigious awards

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Supplementary Information

Figure S-1. Characterization of control (NC) murine glioma cell lines (GL261, CT2A, ALTS1C1) upon LDH-A shRNA knockdown. Comparison of mRNA expression levels of LDH-A and LDH-B by ddPCR, between NC cell lines (A, B); Comparison of western blot analyses for LDH-A and LDH-B protein expression (C, D) $n=3$, $\pm$ SEM; LDH enzyme activity (E); $n=3$, $\pm$ SEM.

Figure S-2. The effect of LDH-A knock-down on s.c. tumor growth in nude mice. Growth profiles of GL261, ALTS1C1 and CT2A tumors (Panels A-C), and estimated tumor doubling times (Panel D). Mean, $\pm$ SD. Comparison between tumor doubling times in nude mice and C57BL/6 mice (Panel E).

Figure S-3A. IHC staining for LDH-A and LDH-B proteins in s.c. GL261 NC and LDH-A KD tumors. A local inverse relationship in LDH-A and LDH-B staining was observed in GL261 NC and LDH-A KD s.c. tumors, growing in immunocompromised nude mice (see Fig. 5).

Figure S-3B. Local IHC staining for LDH-A and LDH-B proteins in s.c. CT2A tumors. A local direct relationship in LDH-A and LDH-B staining was observed in CT2A NC and LDH-A KD s.c. tumors, growing in immunocompromised nude mice (see Fig. 5).

Figure S-4. Effects of LDH-A depletion on the s.c. tumor growth in immune competent mice. The experimental protocol (A). H&E staining (B) and immunofluorescence imaging CD3+, CD4+ T cells and TAM using CD68+ marker (C-E). To set up the experiment NC, LDH-A KD cells as well as Matrigel was implanted in the same mouse to compare the impact on the immune response (A). Three NC and KD tumors were collected on day 5 post-implantation. Quantitative analysis of High and Low Cellular Density regions in tumors was performed using MetaMorph software (F).

Table S-1. Weka analysis for IHC Staining for LDH-A and LDH-B of s.c. GL261 and CT2A tumors. Table summarizes the Weka analysis shown in Figure 5 (Panels B, C, E, F).

Figures

Figure 1

Characterization of murine glioma cell lines (GL261, CT2A, ALTS1C1) following LDH-A shRNA knockdown. LDH-A and LDH-B mRNA levels by ddPCR (A, B); Protein expression on western blot analyses (C-E); and LDH enzyme activity (F) in control NC and LDH-A KD cell lines (GL261, CT2A, ALTS1C1). LDH-B/LDH-A mRNA expression ratios (G) and LDH-B/LDH-A Western blot ratios (H) for control NC and LDH-A KD cell lines (GL261, CT2A, ALTS1C1). n=3, $\pm$ SEM.

Figure 2

The effect of LDH-A knock-down on cells in vitro and s.c. tumor growth, in vivo in immune competent mice. Growth profiles and doubling times of GL261, ALTS1C1 and CT2A cells in vitro (Panels A-D) (Mean $\pm$ SEM) and tumors in C57BL/6 mice (Panels E-H) with and without LDH-A shRNA knockdown (Mean, $\pm$ SD). *Note, the doubling times for GL261 NC tumors were estimated after the initial delay in tumor growth (0~40 days).

Figure 3

Native-polyacrylamide gel electrophoresis LDH zymograms for ex vivo tissue and s.c. GL261, CT2A and ALTS1C1 tumors. Electrophoretic patterns in the heart and skeletal muscle as well as s.c. tumors from NC and LDH-A KD GL261, CT2A and ALTS1C1 tumors (Panel A), corresponding LDH isoform profiles (Panel B); n = 5 independent studies.

Figure 4

Hematoxylin and eosin (H&E) and IHC staining for LDH-A and LDH-B protein expression in s.c. GL261 and CT2A tumors – both LDH-A KD and NC controls. H&E staining for GL261 NC and LDH-A KD tumors (Aa); LDH-A staining (Ab) and LDH-B staining (Ac). GL261 NC and LDH-A KD tumors were grown in immunocompromised nude mice. Quantification of percentage LDH-A tumor expression (B) and

percentage LDH-B tumor expression (C); $\pm$ SEM. A similar presentation is shown for CT2A tumors, grown in immune competent C57BL/6 mice (Panels D-F).

Figure 5

Local intratumoral LDH-A vs LDH-B protein expression relationships in s.c. GL261 and CT2A, NC and LDH-A KD tumors. An inverse local staining (expression) relationship between LDH-A and LDH-B was observed in randomly selected GL261 tumor regions (A), whereas a more direct relationship between LDH-A and LDH-B staining intensity was observed in randomly selected CT2A local tumor regions (B).

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