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Metabolic challenge of proneural glioblastoma cancer stem cell lines does not induce a mesenchymal transformation in vitro

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Abstract: The histologically defined tumor entity glioblastoma (GBM) comprises different molecular subgroups. On a transcriptional level, at least two subgroups were uniformly identified: proneural and mesenchymal GBM. Treatment of GBM with radiotherapy and anti-angiogenic drugs may trigger molecular subtype transformation with proneural tumors shifting towards a mesenchymal phenotype. Because both treatment modalities also result in dramatic changes of the metabolic microenvironment of GBM, we hypothesized if the major determinants in local microenvironment—hypoxia and (lactate) acidosis—may induce consistent changes in the phenotype of primary glioblastoma stem cell lines in vitro. A panel of 5 different proneural GBM stem cell lines (GSC) lines was systematically challenged for 7 days with acidosis (pH 5.5-7.4), sodium lactate (0-60 mM), lactic acid (0-15 mM), or with 72 h hypoxia (2% oxygen). Acidosis significantly improved survival but inhibited proliferation of GSC lines. Sodium lactate inhibited proliferation without affecting survival. Neither acidosis nor sodium lactate did consistently change growth pattern or stem cell marker expression. Hypoxia and lactate induced a more proneural-like phenotype with increased Olig2 and CD133 expression in 1 GSC line, but did not consistently alter marker expression in the other 4 GSC lines. None of the 5 GSC lines investigated showed changes indicative for a proneural-to-mesenchymal transformation towards a more malignant phenotype. Taken together, our data does not exclude the possibility that metabolic factors like lactate acidosis may induce a more mesenchymal phenotype. Nevertheless, data suggest that therapy-induced metabolic changes in the microenvironment are likely not of crucial importance to our understanding of mesenchymal transformation after radio-and anti-angiogenic therapy for most GBM.

Keywords: Metabolic microenvironment, glioma stem cells, CD133, CD44, hypoxia, lactate

Introduction

Glioblastoma (GBM) is the most common primary malignant brain tumor with an average survival of affected patients of about 15 months [1]. All GBM share same histological features but differ substantially on a molecular level. Various attempts have been made to divide GBM into different groups based on their profile on mRNA, protein, genome, and stem cell level [2-7]. Although all studies varied with respect to their results and the number of subgroups identified, all studies agreed on the existence of two major groups: a (pro-) neural and a mesenchymal phenotype, which account for the majority of all GBM. These two groups were initially named because of the expression of marker genes resembling different stages of neurogenesis [3]. The subtypes differ substantially with respect to tumor cell properties (e.g. differences in WNT-, TGF-beta, hippo-signaling) but possibly also with respect to local microenvironment, amongst others extend of immune infiltration [8, 9]. It remains controversial if both subgroups have prognostic significance for patients treated with standard therapy [3, 7,
Differences of both groups in response to immunotherapy have, though, been observed [11].

GBM are characterized by massive angiogenesis, and new antiangiogenic therapies raised hopes to improve the patients’ outcome. Glioma-induced angiogenesis can be visualized using MRI as tumor core with pathological blood vessels and contrast enhancement [12-15]. The most thoroughly investigated substance-bevacizumab (BEV)-induces often a dramatic response of the contrast-enhancing tumor in MRI and substantially improves the progression free survival (PFS) of patients irrespective of the molecular subtype [13, 16, 17]. However, BEV failed in recent clinical studies to improve overall survival [16]. The combination of an impressive improvement of PFS without improvement in overall survival suggests a malignant transformation of the tumor under therapy. This malignant transformation seems to depend on the molecular subtype: a retrospective analysis of the AVAGlio [16] data could recently show, that tumors with a mesenchymal subtype did not benefit from BEV treatment, while patients with proneural GBM did [17].

If and how a transformation of the molecular phenotype influences these differences remains controversial. Piao et al. showed data suggesting that treatment failure after BEV may be caused by a proneural-to-mesenchymal transformation [18]. A molecular analysis of the molecular phenotypes by DeLay et al. before and after therapy showed transition of the phenotype after BEV treatment in 8 of 13 tumors investigated [19]. However, they found a BEV induced a transformation in both directions (i.e. proneural-to-mesenchymal and vice versa). A transformation of the molecular phenotypes was also described for recurrent gliomas after radiotherapy by Phillips et al.: 8 out of 26 paired samples investigated changed the molecular phenotype mainly from a proneural to a mesenchymal profile [3]. In conclusion, these and other reports [20] support the hypothesis that a transformation of the molecular subtype may contribute to treatment failure.

Treatment with BEV and radiotherapy results in relevant changes of the important determinants of the metabolic microenvironment [21]. Impairing and destroying tumor angiogenesis results in tissue hypoxia, increased glycolysis, and lactate acidosis. Conversely, metabolic changes have been postulated determinants of treatment failure/success under BEV treatment [22]. It is therefore possible that these factors may be the crucial trigger for this transformation of the molecular phenotype.

GSC lines became an accepted and widely used in vitro model to investigate GBM biology. GSC lines are more favorable compared to serum-cultured cell lines because they are genetically stable and closely resemble the initial tumor also after many passages in vitro [23]. Like GBM, GSC lines derived from tumors are heterogeneous, and, as in the parental tumors, GSC lines with a proneural and a mesenchymal transcription profile have been described [6, 8, 24]. Both types of GSC differ with respect to marker expression, although, to date, no unambiguous marker for the two major subtypes has been identified. Recently, several studies independently suggested CD133 and CD44 as markers for proneural and mesenchymal GSC [6, 25, 26]. In vivo, Olig2 and DLL3 have been proposed to be indicative for proneural and YKL-40 for mesenchymal GBM [3, 8]. In vitro, these GSC lines may differ with proneural GSC showing a neurosphere-like growth pattern and mesenchymal GSC having a more adherent growth pattern [24, 27]. However, the analysis of the growth pattern is not without ambiguity because culture conditions (e.g. continuous selection of spheres, laminin coating, or frequent use of trypsin) change the growth pattern without affecting the molecular subtype.

The molecular mechanisms of transformation from a proneural to a more mesenchymal phenotype were recently investigated using GSC lines. These studies showed that alterations of TGF-beta [8, 28-30], TNF-alpha/NFkB, and MET-signaling [31, 32] were of importance especially for proneural-to-mesenchymal transformation. The putative mechanisms of the mesenchymal-to-proneural transformation after BEV treatment are to date unknown.

In contrast to in vivo studies, the study of GSC allows to investigate changes in the tumor cells only. This is relevant because invading neural stem cells or mesenchymal cells may substantially modulate the molecular phenotype of tumor samples without a relevant change of bona fide tumor cells. We therefore used a panel of GSC lines to address the question if metabolic challenge induces a proneural-to-
mesenchymal transformation in proneural GSC lines to substantiate or to falsify the hypothesis that metabolic changes may induce a mesenchymal transformation of GBM cells.

**Materials and methods**

**Cell culture**

All GSC lines were derived from primary tumor tissue dissociated direct after operation as described previously [24, 33]. All primary tumors were IDH wildtype GBM diagnosed according to the WHO classification [34]. The generation of GSC was approved by the local Ethic Committees of Southern Denmark and at the University Hospital Regensburg. GSC lines were cultured in DMEM-F12 supplemented with 20 ng/ml EGF, 20 ng/ml bFGF, B27 (1:50), and antibiotics as described previously [6, 24].

**Microarray analysis**

Microarray analyses were previously described in [6, 24, 35]. In brief, total RNA was isolated using the Qiagen RNeasy kit. The generation of labeled cRNA and its hybridization to U133 Plus 2 GeneChip arrays (Affymetrix) were done and scanned at the Kompetenzzentrum für Fluoreszenz Bioanalytik (KFB; Regensburg, Germany) and the Department of Clinical Genetics (Odense University Hospital, Odense, Denmark) according to standard procedures [24]. Analyses were performed using BRB-Array Tools developed by Dr. Richard Simon and BRB-Array Tools Development Team. Additional microarrays of established GSC lines and published by Gunter et al. [27] and Lottaz et al. [6] were used for analysis. Arrays were normalized using RMA. Supervised clustering was performed using the 24 genes signature published by Lottaz et al. [6].

**Metabolic challenge**

Acidosis: To induce acidosis, DMEM-F12 medium was acidified using 37% HCl until the pH given was reached. For the experiments, the growth factors 20 ng/ml EGF, 20 ng/ml bFGF, B27 (1:50), and antibiotics were added.

Lactate acidosis: For lactate acidosis, sodium lactate was added to standard culture medium in the concentrations given in the experiments. Due to the high concentrations used (up to 60 mM) we chose to use glucose to as control for osmolality.

Hypoxia: GSC lines were cultured as small spheres in T25 flasks. They were cultured under moderate hypoxia (2% O₂, 5% CO₂, 94% N₂) in a Sanyo O₂/CO₂ incubator (Sanyō Electric Co. Ltd., Japan) for 24 h and 72 h. After the treatment the cells were re-seeded as single cells in 6-well plates in pairs (20.000 in each well in the case of R28 24 h and 50.000 for the rest). After 3 and 7 days pictures were taken and the media was changed.

**Flow cytometry**

Flow cytometry was performed according standard protocols. At the time points indicated, cells were trypsinized and harvested as single cells for cytometric analysis. Each sample/treatment was split into 3 FACS samples for Isotype control (mlgG2b-PE, 1:100), CD133/CD-133/2-PE (1:100, Miltenyi Biotech), and CD44-FITC (1:100, BD), and PI staining (Sigma Aldrich). 10,000 single cells were counted for each sample in a FACS Canto analyzer and the data were analyzed using FACS Diva software.

**Western blot**

Western blot was performed according to standard protocols. At the time points indicated, cells were harvested and total protein lysates were made using Cell Lysis Reagent (Sigma) containing both Protease Inhibitor Cocktail Set I and Phosphatase Inhibitor Cocktail Set II (Calbiochem). The extracts were separated by SDS-PAGE (4-12% Bis-Tris-gel, Novex) and transferred to a 0.45 µm PVDF membrane (Invitrogen). The Novex Sharp Pre-stained protein standard was used to assess the protein size. The primary antibodies used were anti-YKL-40 (Quidel, 1:100), anti-Olig2 (Bio Labs 1:100), and β-actin (N-term, AC-15 Sigma 1:10,000). The secondary antibodies were rabbit IgG HRP-conjugated (Olig2, YKL-40) and mouse IgG HRP-conjugated (β-actin).

**Results**

**Characterization of the GSC lines**

All GSC lines used were previously characterized in detail [6, 24, 35]. We recently could show that GSC can be classified as “proneural-
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Like" and "mesenchymal-like" using a 24-gene signature [6]. Supervised clustering of mRNA expression profile of the 5 GSC lines used and a panel of 20 previously characterized GSC lines showed that all GSC lines were classified as "proneural-like" based on these signature genes (Figure 1). With respect to marker expression, 4 out of the 5 GSC lines expressed the surface epitope CD133, and all GCS lines expressioned the transcription factor Olig2, which are both marker proteins associated with

Figure 1. Classification of previously published GSC lines by Gunther et al. [27], Lottaz et al. [6], and the GSC lines used (marked in yellow) according to the "Lottaz signature".

Table 1. Marker profile

<table>
<thead>
<tr>
<th></th>
<th>CD133</th>
<th>Olig2</th>
<th>CD44</th>
<th>YKL40</th>
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<tr>
<td>R28</td>
<td>+++</td>
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<td>T78</td>
<td>++</td>
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<td>T86</td>
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<tr>
<td>T111</td>
<td>(+)</td>
<td>+</td>
<td>++</td>
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</tr>
</tbody>
</table>

+++>50% positive cells/very strong signal WB; ++10-50% positive cells/strong signal WB; +1-10% positive cells/weak signal WB; (+) 0.1-1% positive cells/very weak signal WB.
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“proneural-like” GSC [6, 25]. However, 4 out of 5 GCS lines also expressed CD44, and 2 out of 5 were positive for YKL-40, which are both putative markers for “mesenchymal-like GSC”. Table 1 gives an overview over the marker profile of the GSC lines investigated.

Effects of low pH on 5 GSC lines

Low pH (due to lactate acidosis) and low oxygen pressure/tissue hypoxia are the major components of the cellular metabolic microenvironment and are closely related. We therefore

Figure 2. Effects of acidosis on proliferation, survival, CD133/CD44 expression, growth pattern. (A) Cell number; (B) Proportion of viable cells; (C) Proportion of CD133 expressing cells; and (D) proportion of CD44 expressing cells after treatment with pH 6.5 and pH 5.5 for 7 days are given; (E) Representative pictures of the growth pattern of GSC lines R28 are shown.
investigated the effects of chronic expose with major metabolic determinants-acidosis, lactate concentration, lactate acidosis, and hypoxia-on survival, proliferation, and in vitro phenotype of a panel of 5 different GSC lines. To differentiate the effects of lactate acidosis and low pH, we...
Figure 4. Effects of lactate acidosis on proliferation, survival, CD133/CD44 expression, growth pattern, Olig2/YKL-40 expression. (A) Cell number; (B) Proportion of viable cells; (C) Proportion of CD133 expressing cells; and (D) proportion of CD44 expressing cells after treatment with 5 mM and 15 mM lactate for 7 days are given; (E) Representative pictures of the growth pattern of GSC lines R28 are shown; (F) Western blot for Olig2 and YKL-40 expression of the panel of GSC lines treated with 15 mM lactate for 7 days (C = control, L = treatment with 15 mM lactate).
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challenged the 5 GSC lines using standard serum free medium acidified using HCl to pH 6.5 and 5.5 for 7 days. Acidification significantly and dose-dependently reduced the prolifera-

Figure 5. Effects of acute hypoxia on proliferation, survival, CD133/CD44 expression, growth pattern. (A) Cell number; (B) Proportion of viable cells; (C) Proportion of CD133 expressing cells; and (D) proportion of CD44 expressing cells after treatment with 2% O\textsubscript{2} for 72 h days are given; (E) Representative pictures of the growth pattern of GSC lines R28 are shown; (F) Western blot for Olig2 and YKL-40 expression of the panel of GSC lines treated with hypoxia (2% oxygen) for 72 h (c = control, H = hypoxia).
tion of GSC lines (Figure 2A). Remarkably, acidification also increased survival as determined by a significantly increased proportion of viable cells as determined by PI staining (Figure 2B). Despite these marked effect, acidification did neither significantly changed expression of the surface marker of CD133 and CD44 (Figure 2C, 2D), nor the growth pattern of the GSC lines (Figure 2E).

Effects of sodium lactate on 5 GSC lines

Lactate acidosis is a major contributor to the metabolic microenvironment especially in tumors. To dissect the effects of lactate alone and lactate acidosis, we treated the panel of 5 GSC with increasing doses of sodium lactate. As expected, sodium lactate significantly inhibited proliferation of GSC lines (Figure 3A) without consistently affecting survival of the cells (Figure 3B). With respect to stem cell marker expression, sodium lactate did not induce significant and consistent changes in the expression of CD133 and CD44 (Figure 3C, 3D). Further, the growth pattern of the GSC was unaffected (Figure 3E).

Effects of lactate acidosis on GSC lines

Due to the “Warburg effect”, lactate acidosis represents a hallmark of cancer and is also present in GBM [36-38]. In vitro, increasing doses of lactate acidosis did not elicit homogeneous effects in the 5 GSC lines screened. Proliferation was impaired in two GSC lines (T86, T78), which were also particular sensitive to lactate and acidosis induced growth inhibition (Figure 4A). The concentrations used for 1 week (5 mM and 15 mM lactate) did not increase proliferation of a single GSC line and did not consistently affect survival (Figure 4A, 4B). With respect to stem cell marker expression, lactate acidosis induced CD133 expression in the GSC line T78. The other GSC lines did not show a relevant change in proneural (CD133, Figure 4C) and mesenchymal (CD44, Figure 4D) marker expression. Growth pattern was slightly altered in one GSC line (T111) towards a more adherent growth pattern (Figure 4E). Because of the slight effects seen in these experiments, we performed confirmatory experiments investigating the expression of YKL-40 and Olig2 by Western blot. Both are proposed marker proteins for mesenchymal and proneural-like GSC lines. Lactate acidosis induced an increased Olig2 expression in the GSC line T78, which also showed an increase of CD133 expression, and in the GSC line R28, which did not show a relevant change of CD133 expression. The expression level of YKL-40 was not substantially altered by lactate acidosis.

Short-term and long-term effects of hypoxia on GSC lines

Hypoxia is the other determinant of the metabolic microenvironment and the effects of hypoxia on GSC lines have been extensively studied [35, 39, 40]. For this experiment, GSC lines were incubated with 2% oxygen for 72 h and analyzed direct after exposure and 7 days after re-plating of treated cells. There were no consistent changes in all 5 GSC lines. Hypoxia foster proliferation in GSC lines R28, T78, and T86 as compared to control conditions, the proliferation of GSC T87 was impaired under hypoxia but cells recovered rapidly (Figures 5A, 5B and 6A, 6B). Notably, 2% hypoxia did not induce cell death but seemed to have protective properties (Figure 5B). Only the phenotype of GSC line T111 was substantially altered: 72 h hypoxia slightly reduced the number of cells but the cells kept proliferating substantially slower than control cells (Figures 5A, 6A). The reduced proliferation of GSC lines T111 was paralleled by a decrease of CD133 and an increased expression of CD44 but without a change of the growth properties, loss of Olig2 expression or increase of YKL-40 expression (Figures 5C-F, 6C-E). In the other 4 GSC lines, hypoxia slightly increased the expression of CD133 in two GSC lines. Because of the effects seen in the GSC lines T111, we chose to study the expression of Olig2 and YKL-40 in all GSC lines upon hypoxia. Hypoxia substantially induced Olig2 expression in the GSC line T78 and slightly increased Olig2 expression in R28 [41]. With respect to YKL-40, an increase of the expression level was seen in R28, T78 showed minimal reduction (Figure 5F).

Discussion

A substantial part of GBM patients showed shrinkage of the contrast-enhancing part of the tumor upon anti-angiogenic therapy. Unfortunately, this did not translate into a longer survival and may change the tumors’ growth pattern [42]. These clinical phenomena, also seen with other anti-angiogenic treatments,
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A. Cell count 7 days after hypoxia

B. Cell death 7 days after hypoxia

C. CD44 positive cells 7 days after hypoxia

D. CD133 positive cells 7 days after hypoxia

E. D3 after treatment | D7 after treatment

control | treated
may suggest a fundamental change of tumor properties. Piao et al. postulated that a proneural-to-mesenchymal transformation causes the acquisition of a more mesenchymal phenotype and thereby contributes to the resistance against anti-angiogenic therapy [18, 43]. Given that the mesenchymal phenotype may also be associated with a worse prognosis [3], this mesenchymal transformation may explain the more aggressive behavior of recurrent GBM especially after anti-angiogenic treatment. Data by DeLay et al. could not support the concept proposed by Piao et al. but the mesenchymal signature of a tumor does not necessarily only depend on tumor cells but also on cells within the tumor stroma, like endothelial cells.

In vitro, we could anecdotally observe a transformation of the expression profile of long-term GSC lines (unpublished observation). We therefore aimed at establishing an in vitro model allowing investigating how metabolic factors modulate the properties of proneural GSC lines. We screened at total of 8 GSC lines, 5 GSC lines were investigated extensively. We could not observe any evidence for a transformation into a more mesenchymal subtype. Thus, we would like to contribute to the ongoing discussion on the relevance and pathomechanism of the postulated mesenchymal transformation, by providing evidence that chronic changes in the metabolic microenvironment were not able to induce a relevant mesenchymal transformation in our panel of proneural GSC lines. Quite contrary to this hypothesis, we found a GSC line (T78), which showed a more proneural-like phenotype with induction of Olig2 and CD133 expression. We did not go into detail if this increase of marker expression actually corresponded to induction of “stemness” or a more pronounced proneural phenotype. Either way there was no indication for a more mesenchymal phenotype. Furthermore, we did not make microarray analysis after metabolic challenge of the GSC lines. The analysis of the cell cultures did neither indicate a change of the growth pattern, nor a remarkable increase of migrating cells. Thus, microarray analysis would not have contributed to our understanding of the lack of a mesenchymal transformation.

Of course, our experiment does not exclude the possibility that a proneural-to-mesenchymal transformation does exist. We could have used more GSC lines and we did not test all possible conditions: We neither used extreme conditions, nor did we investigate the effects of very long treatment (>1 week). Even if the investigation of 20 GSC lines would have shown substantial effects in 1-2 GSC lines under extreme conditions, our major conclusion would have remained unchanged: A substantial proneural-to-mesenchymal transformation triggered by chronic physiological metabolic changes is not common and therefore likely not relevant for the vast majority of patients.

In summary, our experiments did not provide any evidence in support of the concept postulating that the proneural-to-mesenchymal transformation induced by changes of the local metabolic microenvironment is common and relevant.

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Disclosure of conflict of interest

None.

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Figure 6. Long-term effects of hypoxia on proliferation, survival, CD133/CD44 expression, growth pattern. (A) Cell number, (B) Proportion of viable cells, (C) Proportion of CD133 expressing cells, and (D) proportion of CD44 expressing cells 7 days after treatment 2% hypoxia for 72 h are given. (E) Representative pictures of the growth pattern of GSC lines R28 7 days after hypoxia are shown.
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