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Residual Tumor Cells Are Unique Cellular Targets in Glioblastoma

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Abstract

Residual tumor cells remain beyond the margins of every glioblastoma (GBM) resection. Their resistance to postsurgical therapy is considered a major driving force of mortality, but their biology remains largely uncharacterized. In this study, residual tumor cells were derived via experimental biopsy of the resection margin after standard neurosurgery for direct comparison with samples from the routinely resected tumor tissue. In vitro analysis of proliferation, invasion, stem cell qualities, GBM-typical antigens, genotypes, and in vitro drug and irradiation challenge studies revealed these cells as unique entities. Our findings suggest a need for characterization of residual tumor cells to optimize diagnosis and treatment of GBM.

Despite combining surgical, radio-, and chemotherapeutic efforts, progression of disease always occurs in patients suffering from glioblastoma (GBM), leading to a poor median overall survival time of 14.6 months.^{1–3} A major element of GBM aggressiveness is the infiltrative nature of tumor cells,⁴ which migrate away from a hypothetical point of origin,

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Auxiliary data, figures, tables, and an extended Subjects and Methods section can be found in the online version of this article.

Additional Supporting Information may be found in the online version of this article.

Potential Conflicts of Interest

Nothing to report.

populating surrounding native brain parenchyma. Even if a complete, standard tumor resection is accomplished, as revealed by postoperative magnetic resonance imaging,³ at least a few malignant cells always stay behind (Supplementary Fig 1). These residual cells subsequently become exposed to standard and experimental therapy, although surprisingly little is known about their biology. To date, GBM diagnosis, experimental analysis, and the definition of treatment strategies are mainly based on data acquired from tissue removed via standard resection.^{5–8} This practice implies that malignant cells of the resected tissue and residual cells share the same biological properties. Data from earlier histological and microarray studies on the GBM periphery/infiltration zone may already question this assumption.^{9–11} However, a standardized comparison of residual cells and cells from the resected GBM tissue has not yet been performed. Here, we isolated, enriched, and characterized for the first time vital residual GBM cells harvested from brain tissue surrounding the resection cavity. Comparative analysis revealed that residual cells could be considered as distinct malignant subentities, and that their characteristics cannot be predicted by analysis of routinely resected GBM tissue.

Subjects and Methods

More extensive details on subjects and methods are given in the online Supplemental Material.

Subjects

Paired tissue samples were received from 33 GBM patients (Supplementary Table 1; Supplementary Fig 1). One sample was obtained during the early stages of resection from the solid tumor core (GBM center). A second, experimental biopsy was obtained from tissue surrounding the resection cavity after completion of standard neurosurgery (GBM periphery). This study was approved by the local ethics committee; all patients provided written informed consent.

Tissue Handling and Cell Culture

Tissue was minced and allocated into 3 representative fractions for histological, molecular, and cell culture studies. Defined serum-free media conditions were used according to Lee et al⁷ for in vitro experimentation. Methods for a standardized, methylcellulose-based neurosphere assay were previously described.¹² For flow cytometry, CD133/1 and CD133/2 antibodies were used according to the manufacturer's suggestions (Miltenyi Biotec, Bergisch Gladbach, Germany). Histology, fluorescence in situ hybridization, and immunocytochemistry of fixed samples were performed using standard protocols.

Molecular Analysis

Genotyping of 620,901 single nucleotide polymorphisms (SNPs) was conducted using the Illumina (San Diego, CA) Human610-Quad BeadChip according to the manufacturer's Infinium II protocol. Chromosomal aberrations were identified by examination of logR ratios and B-allele frequencies. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis was performed on an iCycler iQ multicolour real-time PCR detection system (Biorad, Oberkochen, Germany), applying standard Taqman or Sybr-green detection

protocols. Custom-made or predesigned primers (Invitrogen, Carlsbad, CA) were used for transcript analysis (Supplementary Table 2).

Statistics

For center versus periphery comparison of mean values, the Student *t* test (cell culture experiments) or the Wilcoxon signed-rank test (qRT-PCR data) were applied (SPSS v.17.0, SPSS Inc., Chicago, Ill; level of significance set to $p < 0.05$).

Results

Histological analysis of paired tissue samples from 33 GBM patients (27–79 years old at the time of surgery, Supplementary Table 1) revealed an abundant presence of tumor cells in tissue from routine resection (GBM center) compared to sparsely distributed residual cells in tissue from the resection margin (GBM periphery; Fig 1A, B). This indicated a need for isolation and enrichment of center and residual GBM cells for standardized comparisons on a functional level. For experimentation, we used recently suggested methods for in vitro derivation of primary GBM cultures that retain original tumor characteristics.⁷ To verify this approach, and to investigate for a previously undemonstrated degree of tumor cell enrichment under these conditions, we examined passage 7 ± 2 cells from a total of 3 paired tissue samples (#'s 021, 023, 035; shown is #023). Genotype analysis confirmed that cells expanded from both tumor regions retained GBM-typical alterations (see Fig 1C, D). Fluorescence in situ hybridization studies furthermore directly revealed a striking enrichment of tumor cells and patient-specificity in cultures from both tumor regions (see Fig 1E, F). Thus, subsequent comparative analysis of center and residual GBM populations was conducted between cell culture passages 5 and 10.

Our in vitro evaluation exposed residual cells as a rapidly proliferating and highly invasive population compared to GBM center cells (Fig 2A, B). In contrast, GBM center cultures revealed an increased frequency of self-renewing and multipotent cells (see Fig 2C, D). Eleven pairs of passage 5 cultures were studied under proliferative low-density conditions in a standardized neurosphere assay. Primary neurosphere formation was commonly observed in 21/22 (95%) cases. However, a total of 73% (8/11) of the center versus only 18% (2/11) of the residual cultures contained multipotent cells that continuously self-renewed (the attributed features of stem cells). This specific topography of proliferation and self-renewal was similarly observed when comparing several probes per location from the same patient ($n = 5$; multiple center vs periphery biopsies; Supplementary Fig 2). In line with these observations, quantitative RT-PCR analysis of neural stem/progenitor-typical⁶ transcripts revealed significantly increased levels of Sox2 and Nestin as well as a trend for increased levels of Musashi-1 in GBM center cultures (see Fig 2E; Supplementary Fig 2).

We next tested if the observed dissimilarity of center versus residual cells could similarly be revealed by in vitro drug and irradiation challenge studies. Five paired cell samples (#'s 013, 021, 023, 035, 046) were investigated as previously described.^{6,13} We observed case-specific patterns of cellular responses to irradiation,² temozolomide,² and lomustine¹⁴; however, in 16/25 (64%) of all comparative measurements, residual cells responded dissimilarly from their corresponding center GBM cells (Supplementary Fig 3).

Intraindividual diversity of GBM center and residual cells was additionally revealed by expression analysis of prominent molecular targets (Fig 3, Supplementary Fig 2).^{15,16} In most cases (43/60 comparative measurements), mRNA levels varied >50% between the paired cultures (see Fig 3A, B; highlighted in red). However, 4 of the investigated markers revealed particularly distinguishing features of residual and center GBM cells. A complementary expression pattern was noted for vascular endothelial growth factor receptor-2 and CD133 (significantly increased in center cultures), as well as for platelet-derived growth factor receptor-B and urokinase plasminogen activator receptor (significantly increased in residual cells).

Discussion

We have demonstrated for the first time that residual cells are distinct from the cells found in routinely resected GBM tissue. Specifically, we have shown that they vary in their content of stem/progenitor cells, their proliferative and invasive capacity, their marker and molecular target profiles, and in their sensitivity to in vitro drug and irradiation challenges. Thus, one may speculate that residual cells represent distinct, malignant GBM subentities. For our study, residual GBM cells were derived from the surgery site at the time of diagnosis and primary treatment. Because it is these cells that would have become exposed to adjuvant radio- and chemotherapy, and because recurrence of disease always occurs (often at the resection margin), their characterization may open new avenues for future GBM diagnosis and treatment.

Furthermore, their characterization may lead to a more comprehensive understanding of all aspects of the disease (ie, GBM initiation, propagation, maintenance, and recurrence). One purpose of our study was to evaluate the presence of stem/progenitor cells that have been assigned an important role in the pathogenesis of GBM.^{5–7,17} We could confirm this currently prevailing view, demonstrating clonogenic, self-renewing, and multipotent (stem) cells in almost every patient. However, these qualities as well as increased levels of typical neural stem cell-related transcripts were most frequently confined to the center biopsy specimens. In contrast, residual cells presented as highly proliferative and invasive entities showing a very limited potential for continuous self-renewal. Others have suggested that the stem cell population is inherently resistant to therapy and hence an important disease reservoir.^{5,6} The findings reported here challenge the field to concisely define the role of brain tumor stem cells in the processes of GBM initiation, maintenance, and recurrence. Based on the stem cell model of tumorigenesis,¹⁷ residual cells fulfill the functional criteria of transit amplifiers rather than representing stem cells. This view would allow the speculation that stem-like cells nurture the expanding tumor mass, generating rapidly dividing, invasive progeny that populate the surrounding periphery.

A second purpose of our study was to investigate whether the distinct functional characteristics of residual GBM cells could influence current and potential future therapeutic considerations. Residual cells could be distinguished from GBM center cells in every case investigated here, by virtue of their distinct molecular marker profiles and by their distinct responses to in vitro drug and irradiation challenges. Our findings may be interpreted as representative for phenotypic tumor cell diversity, as recently described for cells separated

from different areas of the routinely resected GBM tissue.¹⁸ However, in contrast to the latter study, we used residual GBM cells derived via experimental biopsy and enriched in vitro for controlled comparisons with cells from the routinely resected tumor tissue. On this basis, we were able to demonstrate functional similarities among residual GBM cells as well as an overall topographic distribution of specific cellular markers and putative therapeutic targets. Presently, it remains to be elucidated how these dissimilarities arise. One might argue that distinct, topographically diverse environmental cues in situ could lead to a diversification of some of the cellular and molecular properties in GBM. On the other hand, the consistency of dissimilarities we observed between residual and center GBM cells at early and late stages of our controlled in vitro conditions might indicate the presence of distinct malignant subentities. Nevertheless, although origin and heritance of GBM center and residual cells can currently only be speculated on, it seems important to dissect and catalogue their properties, because postsurgical treatment regimens routinely target residual GBM cells.

Our study was performed on a limited number of cases; larger scale studies are warranted before profiling of GBM residual cells can be translated into routine clinical application. Currently, the analysis of center versus residual GBM cells cannot immediately be used to predict clinical patient courses or treatment responses. However, our comparative analysis of function, molecular markers, and in vitro responses to drug and irradiation challenges suggested that profiling of resected GBM tissue has little value for predicting an intraindividual profile of residual cells. Thus, the revealing of distinct cellular and molecular properties in residual cells may be required for future diagnosis and treatment and should lead to a more comprehensive understanding of GBM pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Characterization of tumor tissue and primary cells. (A) Representative fragments of tissue biopsies were allocated equally for histological analysis and for in vitro derivation of primary cultures. (B–F) Patient 023 results are shown. (B) Vascular proliferation and characteristic necrosis (hematoxylin and eosin [H&E]), pleomorphic glial tumor cells (glial fibrillary acidic protein [GFAP], microtubule-associated protein 2c [Map2c]), and an abundance of mitotic/proliferative activity (Ki67) are characteristics of a glioblastoma (GBM) (upper panel). In contrast, increased cellular density, abnormal grouping of cells

with occasional mitotic figures (H&E), reactive gliosis (GFAP), and few actively proliferating (Ki67) tumor cells (Map2c) are characteristics of infiltrated surrounding parenchyma (lower panel). Note that for immunohistochemistry (GFAP, Map2c, Ki67), antigens were detected using standard diaminobenzidine reaction (brown). (C) Single nucleotide polymorphism (SNP)-based genotyping demonstrated largely overlapping profiles of GBM-typical alterations¹⁹ in paired cultures. In the presented case, gain of chromosome 7 (*upward arrowheads*), loss of heterozygosity (LOH) of chromosome 10 (*downward arrowheads*), and focal amplifications of CDK4 and MDM2 on chromosome 12 (*boxed area*) were noted. Always, however, minor genomic alterations distinguished paired cultures from each other. In the presented case, the detected chromosome 10 LOH was copy-neutral only in cells from the routinely resected tissue (*asterisk in upper panel*). (D) Phase contrast of the #023 primary cultures at passage 7 (left). SNP-genotyping data of chromosome 12 highlight the amplifications of the CDK4 and MDM2 loci used in this case to determine the frequency of patient-specific GBM cells in vitro and in vivo. (E) Fluorescence in situ hybridization (*insets*, specific gene probe in red; centromer probe chromosome 12 in green) revealed the presence and enrichment of patient-specific cells in vitro (top graph, center biopsy; bottom graph, periphery biopsy). Both alterations were present in 100 (± 0)% of the cells from center and periphery cultures. (F) In contrast, the parental tissue revealed a frequency of only 73 (± 1.3)% (center) versus 12 (± 1.6)% (periphery) MDM2-/CDK4-amplified tumor nuclei, respectively. Scale bars: (B) H&E, 200 μ m; GFAP/Map2c/Ki67, 50 μ m; (D) 30 μ m; (E) 15 μ m.

FIGURE 2.

Regional distribution of stem/progenitor cells. (A) In vitro analysis of proliferation kinetics revealed linear expansion rates between culture passages 5 and 10 for all primary cultures investigated. The inset specifies the performance of the paired #023 samples. Slope analysis, however, demonstrated a significant difference between slower proliferating center cells and more rapidly expanding residual cells derived from periphery biopsies. Mean values of center (0.06 ± 0.02) and periphery-derived residual cell culture (0.15 ± 0.03) slopes indicated $>2\times$ faster proliferating residual cells ($n = 8$; $*p < 0.05$). (B) The Matrigel assay

showed a significantly higher invasion ratio for the periphery biopsy-derived residual cells compared to their paired center-derived samples. The mean invasion rates for center and residual cell cultures were $14\% \pm 3\%$ versus $34\% \pm 7\%$, respectively ($n = 3$; $*p < 0.05$). The photographs depict DAPI (4',6-diamidino-2-phenylindole)-stained cell nuclei on the bottoms of the membranes (case 023). (C) The neurosphere (NS) assay was applied to cells from periphery-derived residual cells and center biopsies at culture passage 5. The appearance of representative primary neurospheres at 3 weeks in culture is shown (*insets*, left). Individual ratios of neurosphere-forming cells are presented in the graph. With the exception of periphery sample #046, cases shown to generate 2° neurospheres continued to form 3° and higher degree neurospheres (not shown). Mean data analysis revealed among glioblastoma center cells a significantly higher ratio of continuously selfrenewing (stem) cells (*inset*, right, $*p < 0.05$). (D) Plating and differentiation of cells from 2°/3° neurospheres always resulted in glial fibrillary acidic protein (GFAP)-expressing astrocytes, β III tubulin-expressing neurons, and 2'3'cyclic nucleotide 3' phosphohydrolase (CNPase)⁺ oligodendrocytes. Note that many neurosphere-derived cells demonstrated coexpression of GFAP and β III tubulin. (E) Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) data demonstrate the levels of neural stem/progenitor-typical transcripts Sox2, Nestin, and Musashi-1 in a series of paired culture samples (upper graphs). Lower graphs represent mean data of the respective markers ($*p < 0.05$). For presentation in (E), original data were multiplied by a factor of 10^4 . Scale bars: (C) 200 μ m; (D) 50 μ m. PD = population doublings of propagated cultures; dic = days in culture (for further details please refer to the Supplemental Methods).

FIGURE 3.

Molecular marker analysis. (A) Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) data demonstrate levels (mean normalized) of a selection of currently investigated molecular targets in the field of neuro-oncology from a set of 6 paired culture samples (at culture passage 5). In 23/36 comparative measurements, mRNA expression levels varied >50% between center and periphery biopsy-derived cells of the same patient (indicated in red). Note the regional distribution (topography) of vascular endothelial growth factor receptor (VEGFR)-2 and platelet-derived growth factor receptor (PDGFR)-B (* p <

0.05). (B) qRT-PCR data show individual levels (mean normalized) of cell surface markers that have been implied as significant for the study of glioblastoma (GBM) initiation, maintenance, and/or spread.^{15,16} In 20/24 comparative measurements, mRNA expression levels varied >50% between center and periphery biopsy-derived residual cells of the same patient (indicated in red). Note the regional distribution (topography) of CD133 and urokinase plasminogen activator receptor (uPAR) (* $p < 0.05$). The insets show representative immunofluorescence photographs of CD133⁺ (red) and uPAR⁺ (green) cells. uPAR has been shown in several types of human cancer, including glioma, to be correlated with increased cell proliferation, invasion, and resistance to chemotherapy.²⁰ CD133 is an epitope that can be present on cancerous (glioma) stem cells and that can indicate radiotherapy-resistant cell populations of GBM.^{5,6,13} Coherence of qRT-PCR data was verified with flow cytometry analysis (see *inset*, CD133 graph). (A, B) For presentation, original data were multiplied by a factor of $10^3/10^5/10^6$ as indicated on the y axis. (B) Scale bars: 20 μ m. TGFBR = transforming growth factor-beta receptor.