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Phenotypic and Expressional Heterogeneity in the Invasive Glioma Cells[1,2]

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Abstract

BACKGROUND: Tumor cell invasion is a hallmark of glioblastoma (GBM) and a major contributing factor for treatment failure, tumor recurrence, and the poor prognosis of GBM. Despite this, our understanding of the molecular machinery that drives invasion is limited. *METHODS:* Time-lapse imaging of patient-derived GBM cell invasion in a 3D collagen gel matrix, analysis of both the cellular invasive phenotype and single cell invasion pattern with microarray expression profiling. *RESULTS:* GBM invasion was maintained in a simplified 3D-milieue. Invasion was promoted by the presence of the tumorsphere graft. In the absence of this, the directed migration of cells subsided. The strength of the pro-invasive repulsive signaling was specific for a given patient-derived culture. In the highly invasive GBM cultures, the majority of cells had a neural progenitor-like phenotype, while the less invasive cultures had a higher diversity in cellular phenotypes. Microarray expression analysis of the non-invasive cells from the tumor core displayed a higher GFAP expression and a signature of genes containing VEGFA, hypoxia and chemo-repulsive signals. Cells of the invasive front expressed higher levels of CTGF, TNFRSF12A and genes involved in cell survival, migration and cell cycle pathways. A mesenchymal gene signature was associated with increased invasion. *CONCLUSION:* The GBM tumorsphere core promoted invasion, and the invasive front was dominated by a phenotypically defined cell population expressing genes regulating traits found in aggressive cancers. The detected cellular heterogeneity and transcriptional differences between the highly invasive and core cells identifies potential targets for manipulation of GBM invasion.

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Introduction

Glioblastoma (GBM) is the most frequent and malignant brain cancer. Standard treatment only extends the life of patients with months, and the median survival in unselected patient populations is less than a year [1]. The tumors' ability to invade into the surrounding brain parenchyma is a major challenge as it makes complete resection unachievable. The invasive cells left in the brain after tumor resection are resistant to chemo- and radiotherapy and are thus responsible for the inevitable tumor recurrence [2,3].

GBM cells have the ability to move through the highly packed neuropil, but rarely enter into the circulation [4]. Thus, the invasion of glioma cells is different from the metastatic spread of other cancer cells and is likely dependent on a unique set of molecular pathways [5]. Moreover, GBMs display high levels of inter- and intratumoral

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heterogeneity, where only a subset of the tumor cells is invasive [5]. To understand the glioma-specific properties of invasion, models must recapitulate the heterogeneous cellular phenotype seen in patients while being simple enough to allow for interpretation.

To experimentally decipher the ability of glioma tumor cells to migrate and invade into the brain, it is essential that the model system retains this key characteristic of GBM. The traditional long term serum cultivated GBM cell lines express markers suggesting neural lineage, but display molecular characteristics more common to other cell lines than the tumor of origin [6]. Upon transplantation to the brain these cells establish rapidly growing tumors, but with sharply delineated borders to the brain parenchyma – more resembling brain metastases than glial tumors [7,8]. In contrast, the use of patient-derived GBM tissue allows for isolation of cells with invasive properties. These cells can be propagated as tumorspheres under serum-free, growth factor-enriched media and establish phenocopies of the parent tumor in serial xenotransplantation [7,9,10]. Importantly, these induced tumors are highly invasive, harboring cells that migrate widely throughout the brain [9,11–13].

The specific biological behavior of invasive GBM cells suggests the activation of certain genetic programs that distinguish them from cells in the tumor core [14]. While global expression profiles of glial tumors have been studied extensively, less is known about specific gene expression in the invasive cells [15,16]. The experimental studies exploring transcriptional differences associated with invasion in brain slices [17] or in vivo xenograft models [18] do not use real-time observations that allows for analysis of movement patterns. Thus, an approach that allows transcriptome analysis of invading GBM cells with identified differences in invasive capacity by real time observation has been called for [19].

We have previously described the phenotype and invasive characteristics of invading glioma cells in organotypic brain slices. Here we present studies on real-time quantification of human GBM cell invasion with comparative analysis of transcriptional profiles in non-invasive and invasive cells. Using a simple collagen 3D matrix system, we demonstrate how this system maintains the invasive characteristics found in more complex systems and how it allows for the detection of intra- and intertumoral heterogeneity to understand mechanisms of glial cell invasion.

Materials and Methods

Cell Culture

GBM biopsies were obtained from informed and consenting patients after approval by the Norwegian National Committee for Medical Research Ethics (07321b). The biopsies were dissociated into single cells and cultured in a serum-free medium supplemented with bFGF and EGF as previously described [20]. Seven primary cell cultures were established from brain tumor biopsies, all from IDH wild-type, treatment-naïve GBMs, of which one was classified as a giant cell GBM. Two of these cultures have previously been described (T0965, T1008) [12,21,22]. The tumorigenicity of all cultures was confirmed upon xenografting to SCID mice. The tumorspheres in the cultures displayed heterogeneous morphology and growth pattern characteristics, with population doubling times ranging from 2 to 8 days.

Grafting of GBM Cultures for Time-Lapse Microscopy

The plating of tumorspheres on fibronectin-coated plates and into rodent brain slices was performed as previously described [12,23]. For grafting tumorspheres into collagen gel rat collagen I protein (0.5 mg/ml) (Gibco) was prepared according to the manufacturer's recommendation and distributed as 30 µl drops in 24 well plates, before single tumor spheres of 150 to 250 µm were grafted into the gel by a 2 µl pipette. For cell-suspension grafting 2 µl containing approximately 500 cells was used. In experiments where tumorspheres were co-grafted with cell suspension verification of cell origin, whether single cell in suspension or sphere, was done by tracking cells by time-laps imaging starting immediately after grafting. After 30 to 45 min of gelation at 37 °C in cell incubator, grafts were supplemented with 200 µl of tumor sphere medium supplemented with 1% foetal bovine serum (FBS).The time-lapse imaging was performed on Olympus IX81 inverted fluorescence microscope with a temperature and environmental gas supply control. The time-lapse experiments lasted from one to 5 days with imaging every 20 min. Images were acquired using Olympus Soft Imaging Xcellence software.

Quantification of Invasive Parameters

For quantification of directionality and migratory velocity, post-processing of the images was performed using the ImageJ package Fiji with a manual cell tracking plug-in. The manual tracking was performed by two independent researches. Directionality is the ratio between the length of a straight line between the start and endpoint of migration to the total accumulated distance and was calculated by Ibidi Chemotaxis and Migration Tool. Total invasive increment is the sum of all distances that invasive cells have moved from the tumorsphere. It was identified by nuclear-stained grafts analyzed by ImageJ software with the "Find Maxima" option. The obtained total number of invasive cells and their X/Y coordinates were transferred to Microsoft Excel.

Immunochemistry

Gels were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min. Then, the samples were processed as described previously [6] with primary antibodies against nestin (mouse, 1:500, Abcam), β-III-tubulin (rabbit, 1:1000, Sigma) and GFAP (rabbit, 1:1000, Dako), MAP2 (mouse, 1:500, Millipore), Ki-67 (rabbit, 1:500, Santa Cruz), CTGF (goat, 1:100, Santa Cruz), synemin (rabbit, 1:200, Sigma Aldrich), TNFRSF12A (rabbit, 1:100, Sigma Aldrich), annexin A1 (goat, 1:200, R&D Systems) and anti-mouse AlexaFluor 488 (donkey, 1:500, Invitrogen) and anti-rabbit AlexaFluor 647 (donkey, 1;500, Invitrogen) secondary antibodies.

For immunostaining of membrane surface receptors the GBM spheres were incubated prior to grafting in 4 °C for 20 min with fluorescent-conjugated antibodies (1:20 dilution in 2% FBS in PBS) and washed twice. Antibodies used were anti- CD166 (PE, BD Pharmingen), CXCR4 (PE, Miltenyi Biotec), CD29 (FITC, Chemicon), CD133 (PE, Miltenyi Biotec), CD44 (APC, eBioscience), CD9 (FITC, eBioscience).

Grafting of Tumorspheres into Collagen Gel for the Isolation of the Invasive Cells

After 2 days in incubator, gels were treated with collagenase (10 mg/ml) (Sigma) in PBS, and visually confirmed for the separation of cores and invasive cells, before the mixture was passed through a 40 µm cell filter to separate the invasive cells from graft cores. The cells were spun down twice before the precipitates were further processed for western blot, qPCR or microarray.

Western Blot

Western blot and quantification of protein expression was performed as previously described [21].