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Brain slice invasion model reveals genes differentially regulated in glioma invasion ☆

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Abstract

Invasion of tumor cells into adjacent brain areas is one of the major problems in treatment of glioma patients. To identify genes that might contribute to invasion, fluorescent F98 glioma cells were allowed to invade an organotypic brain slice. Gene expression analysis revealed 5 up-regulated and 14 down-regulated genes in invasive glioma cells as compared to non-invasive glioma cells. Two gene products, ferritin and cyclin B1, were verified in human gliomas by immunohistochemistry. Ferritin exhibited high mRNA levels in migratory F98 cells and also showed higher protein expression in the infiltrating edge of human gliomas. Cyclin B1 with high mRNA expression levels in stationary F98 cells showed marked protein expression in the central portions of gliomas. These findings are compatible with the concept of tumor cells either proliferating or migrating. Our study is the first to apply brain slice cultures for the identification of differentially regulated genes in glioma invasion.
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Diffuse infiltration of brain is the major problem in treatment of gliomas. Invasive tumor cells may be detected centimeters away from the center of gliomas. Therefore, these tumors cannot be completely resected and recurrences occur in nearly all gliomas [1]. In addition, gliomas often show resistance to radio- and chemotherapy. This observation might be explained by decreased proliferation and reduced sensitivity to apoptosis of the invading glioma cells [2].

Several studies have addressed gene expression of glioma cells in relation to migration or infiltration. Differential display or microarray analysis of glioma cells migrating on coated surfaces revealed differentially expressed genes [3,4]. Common to these studies is a motility-promoting matrix used for cell migration based on components derived from glioma cell cultures. The majority of candidate genes with differential expression still require confirmation and individual analysis. Other approaches focused on the study of genes known to promote invasion, such as urokinase plasminogen activator and matrix metalloproteinases [5–7]. However, the role of these proteins in invading cells has not been compared with stationary tumor cells.

Another study utilized laser capture microdissection to isolate human glioma cells [8]. This approach revealed overexpression of P311 in glioma cells at the invasive edge. In vitro experiments provided evidence for a role of P311 in glioma cell migration.

Brain structures strongly influence speed and direction of invasion. Most evident is infiltration of malignant gliomas along major white matter tracts. Thus, experiments targeting infiltration of malignant gliomas provide data of special interest if conducted in a system closely resembling normal brain. Therefore, we established an organotypic brain slice model based on previous reports [9,10].

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Brain slices were kept in culture for up to 2 weeks allowing invasion of glioma cells. Employing fluorescent F98 rat glioma cells enabled us to separate invaded tumor cells from normal brain cells.

This approach represents a model closer to glioma infiltration in vivo than in vitro based experiments employing coated surfaces.

Materials and methods

Generation of F98 cells expressing the enhanced green fluorescent protein (EGFP). The rat glioma cell line F98 was transfected with the pEGFP-N1-plasmid (Clontech, Palo Alto, USA) by using SuperFect Transfection Reagent (Qiagen, Hilden, Germany). Stably transfected cells were enriched by G418 selection (Invitrogen, Karlsruhe, Germany) and subsequent fluorescent activated cell sorting (FACS) analysis as reported previously [11].

Invasion-assay. Twenty-four hours prior to the preparation of the brain slices F98-EGFP were seeded on membrane-containing inserts covering 6-well plates (Greiner, Solingen, Germany). A pore size of 0.4 μm prevented the glioma cells from migrating through the membrane. The wells were filled with Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% horse serum, 10% fetal calf serum (FCS), 100 U/ml penicillin, and 10 μg/ml streptomycin to cover cells totally. Eighteen 500 μm thick brain slices were generated from 16-day-old male NMRI mice using a vibratome. The slices were then placed on membranes carrying the F98-EGFP. The cultures were maintained for 14 days in an incubator with 5% CO_2 and 95% humidity to allow F98-EGFP migration into the brain slice. Next, slices were lifted from the membranes with the glioma cells. The cells adhered more strongly to the membranes than to the slices which allowed proper separation of the invasive F98-EGFP population in the brain slice from stationary cells. Slices were washed several times with HBBS (Invitrogen, Karlsruhe, Germany) and digested with 0.25% trypsin/DNAse solution for 10 min at 37 °C. After centrifugation at 4 °C, the pellet was resolved in PBS and the fluorescent glioma cells were separated from brain cells and counted by FACS. To guarantee equal treatment, the non-invasive F98-EGFP cells that remained on the membrane were sorted as well. Total RNA of 1.4×10^6 invaded and 6×10^6 stationary F98-EGFP cells was extracted using TRIzol reagent (Life Technologies, Hilden, Germany). Flask cultured, non-sorted F98-EGFP cells served as control for RNA quality. RNA of sorted and non-sorted cells was checked by gel electrophoresis and revealed intact RNA in all cases.

Cell culture. The rat glioma cell line F98-EGFP and the human glioblastoma cell lines U-373MG, T98G, SNB-19, and U-87MG were cultured in DMEM Glutamax-I (Invitrogen, Karlsruhe, Germany) with 10% FCS and 50 μg/ml gentamycin (Invitrogen, Karlsruhe, Germany)

Smart cDNA amplification. The SMART PCR cDNA Synthesis Kit (Clontech, Heidelberg, Germany) was utilized for RT-PCR of 1 μg total RNA of each F98-EGFP population and linear PCR-amplification of the cDNA pools from the stationary-, invaded-, and flask-cultured F98-EGFP cells.

Suppression subtractive hybridization and cDNA library construction. Suppression subtractive hybridization [12] was performed with both, cDNA of stationary F98-EGFP cells as tester (genes up-regulated in stationary F98-EGFP are enriched) and cDNA of invaded F98-EGFP cells as tester (genes up-regulated in invaded glioma cells are enriched). We used the PCR-Select cDNA Subtraction Kit (Clontech, Heidelberg, Germany) according to the manufacturer's protocol with 2 μg SMART cDNA of each F98-EGFP population. The subtracted cDNA sequences were inserted into the T/A cloning vector pCR2.1 (Invitrogen, Karlsruhe, Germany). After electroporation of the TOP10 *Escherichia coli* cells, 384 individual clones (192 of each library) were isolated from the X-gal agar plates and amplified in 96-well plates. PCR amplification of the cDNA inserts was carried out with the PCR-Select adaptor-specific primers using bacteria lysates as template (30 s 94 °C, 30 s 60 °C, and 60 s 72 °C, 32 cycles).

Screening procedures. We employed Dot blot, reverse Northern blot, and virtual Northern blot analysis to validate individual clones for differential expression [13]. All probes were labeled with digoxigenin using the DIG-Chem-Link Labeling and Detection Set (Roche, Mannheim, Germany). A first screening was performed using the Dot blot technique. Candidates with differential expression were retested by the more sensitive reverse Northern blot. Both methods were performed utilizing labeled SMART cDNA of both cell populations as probe. Twenty-four clones over-represented in invaded and 19 over-represented in stationary F98-EGFP were detected and identified by sequencing. Several clones revealed redundant genes. To further substantiate differential expression, we performed virtual Northern blotting. This technique is performed if the available RNA amounts will not suffice to perform classical Northern blots. Virtual Northern blot technique uses linear-amplified cDNA (SMART cDNA) instead of RNA. The original transcript ratios are maintained during this procedure. Four hundred nanograms SMART cDNA of each population was fractionated by agarose gel electrophoresis in a minigel chamber and transferred overnight to a nylon membrane (Roche, Mannheim, Germany). Membranes were then hybridized with gene specific probes overnight. To ensure reproducible amplification of SMART cDNA, we hybridized gene probes on independent batches of SMART cDNA with similar results. During SMART cDNA amplification, transcript length and expression patterns are preserved [14].

Sequence analysis. Sequencing reactions were performed with the adaptor-specific primers using the BigDye sequencing kit (Perkin-Elmer, Weiterstadt, Germany). Sequences were determined on an ABI377 sequencer (Perkin-Elmer, Weiterstadt, Germany). Sequence homology searches were performed against GenBank database using the blastn program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Homology to database sequences was defined as sequence identity >95% over a region of 50–400 bp depending on cDNA insert length.

Immunohistochemistry and immunocytochemistry. We investigated four gliomas, two cases with hippocampal sclerosis, and four autopsy cases (brain) without neuropathological findings. The tumor samples (two astrocytomas WHO grade II and III, and two glioblastomas) showed infiltration and core areas on the same section. All samples were collected from patients of the Charité University Hospital (Berlin, Germany). Infiltration zone was defined as area with detectable tumor cells and resident cells of normal brain. Tissue samples were routinely formalin-fixed and paraffin-embedded. Following deparaffination, antigen presentation was enhanced by heating in a microwave oven. The antibodies to ferritin (A0133, dilution 1:200), glial fibrillary acidic protein (GFAP, M0761, dilution 1:50), and cyclin B1 (M3530, dilution 1:200) from Dako (Hamburg, Germany) were employed. For immunofluorescence a 1:100 dilution of Cy2- and Cy3-conjugated secondary antibodies (Dianova, Hamburg, Germany) was used. For light microscopy we utilized the streptavidin–biotin HRP/DAB staining system from Dako (Hamburg, Germany). Positive cells were visualized with diaminobenzidine (DAB). Nuclei were counterstained with hematoxylin. For immunocytochemistry 2×10^4 cells per well were seeded on Permanox Chamberslides (Nunc, Wiesbaden, Germany). Cells were fixed with methanol after incubation overnight. Negative controls without primary antibodies were performed.

Results

The organotypic brain slice culture system as invasion model

We established an invasion model that enabled us to study gene expression differences in stationary and invasive glioma cells. Glioma cells attached to a porous membrane were confronted with a brain slice. We found that a portion of the cells had invaded the brain slice while a second population remained on the membrane. Since glioma cells were transfected with the fluorescent protein EGFP, we were able to separate the invading glioma cells from the brain

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