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The relationship between brain tumor cell invasion of engineered neural tissues and *in vivo* features of glioblastoma

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Glioblastoma is an aggressive brain tumor characterized by its high propensity for local invasion, formation of secondary foci within the brain, as well as areas of necrosis. This study aims to (i) provide a technical approach to reproduce features of the disease *in vitro* and (ii) characterize the tumor/host brain tissue interaction at the molecular level. Human engineered neural tissue (ENT) obtained from pluripotent stem cells was generated and co-cultured with human glioblastoma-initiating cells. Within two weeks, glioblastoma cells invaded the nervous tissue. This invasion displayed features of the disease *in vivo*: a primary tumor mass, diffuse migration of invading single cells into the nervous tissue, secondary foci, as well as peritumoral cell death. Through comparative molecular analyses, this model allowed the identification of more than 100 genes that are specifically induced and up-regulated by the nervous tissue/tumor interaction. Notably the type I interferon response, extracellular matrix-related genes were most highly represented and showed a significant correlation with patient survival. In conclusion, glioblastoma development within a nervous tissue can be engineered *in vitro*, providing a relevant model to study the disease and allows the identification of clinically-relevant genes induced by the tumor/host tissue interaction.

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1. Introduction

Human gliomas account for more than 70% of all brain tumors and of these, glioblastoma is the most frequent and malignant histological type. The prognosis of glioblastoma is poor and fewer than 3% of glioblastoma patients are still alive 5 years after diagnosis. Glioblastoma is characterized by infiltrative growth into the surrounding healthy brain, leading to secondary foci formation. The dispersed glioblastoma cells are out of reach of surgery, chemotherapy and radiation. Thus, the tumor cannot be completely removed and these remaining invading tumor cells are thought to be responsible for the poor prognosis of glioblastoma [1].

In order to understand the biological aspects of glioblastoma development, several experimental models have been developed including derivation of glioblastoma cell lines, organotypic slices and animal models [2]. A major breakthrough in glioblastoma research was the isolation of a subpopulation of cells with stem cell features, the glioblastoma initiating cells (GIC), harboring long term self-renewal ability and the multipotency capacity to differentiate into other cell types and form heterogeneous spherical structures in suspension called gliomaspheres [3]. These spheres could be differentiated in culture into cells that phenotypically resemble the tumor from the patient [4,5] are used to study glioblastoma/brain

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interaction *in vitro* when co-cultured with rodent or human organotypic brain slices *in vitro* [6,7] or after injection into the brain of immunocompromised mice *in vivo* [8]. Although significant progress has been achieved by foregoing experimental models, the vast majority do not mimic the pathophysiological microenvironment of human glioblastoma.

In this study, we have developed and characterized a three dimensional approach to imitate *in vitro* features of tumor/host nervous tissue interaction in an exclusively human environment. To achieve this goal, we used a nervous tissue engineering method developed previously [9] which allows the differentiation of human pluripotent stem cell into nervous tissue by using air–liquid interface culture. By co-culturing glioblastoma stem-like cells on the engineered nervous tissue, we establish a human tissue sharing similarities with glioblastoma development in the brain environment. This includes diffuse invasion, formation of secondary foci and necrosis areas. Using expression profile analysis, this model allowed us to identify general alterations in gene expression upon interaction of tumor cells with non-tumoral brain tissue.

2. Material and methods

2.1. Culture of undifferentiated ESC

The ESC cell line H1 was from WiCell Research Institute (Madison, WI, http://www.wicell.org). H1 was maintained in 80% Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (F12), 20% knockout serum replacement, 2 mM L-glutamine, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, and 10 ng/ml bFGF. Mouse embryonic fibroblasts were used as feeders and isolated from embryos of pregnant CF-1 mice (Charles River Laboratories, Wilmington, MA, http://www.criver.com). Fibroblasts were cultured in DMEM, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Feeders were mitotically inactivated by irradiation (45–50 Gy) before being seeded on a 0.1% gelatin-coated plate.

2.2. ESC-derived engineered neural tissue

It was performed as previously described [9], including some minor modifications. Briefly, ESC colonies were detached with type IV collagenase (1 mg/ml) and cultured in suspension in ultra-low attachment plates (Corning Costar, Acton, MA, http://www.corning.com/lifesciences) for 1 week in neural induction medium (DMEM/F12, N2 supplement (Gibco)) supplemented with 10 μM phenazopyridine. Approximately 5–10 ESC-derived clusters were plated on a hydrophilic polytetrafluoroethylene (PTFE) membrane (6 mm diameter, 0.4 lm; BioCell-Interface, La Chaux-de-Fonds, Switzerland, http://www.biocell-interface.com). This membrane was then deposited on a Millicell-CM (0.4 μm) culture plate insert designed for 6-well plates (Millipore, Billerica, MA, http://www.millipore.com). One milliliter of neural induction medium supplemented with 10 μM phenazopyridine [10] was added to each well under the membrane insert for differentiation.

2.3. Isolation and cultivation of gliomaspheres

Viable fragments of high-grade human glioblastoma were transferred to a beaker containing 0.25% trypsin in 0.1 mM EDTA solution containing and slowly stirred at 37 °C for 30–60 min. In some isolation processes, 0.6 mg/ml papaine and 10 mg/ml DNase were used instead of trypsin/EDTA. Dissociated cells were plated in 75-cm^2 tissue culture flasks plated at 2500–5000 cells per cm^2 in DMEM/F-12 medium (1:1) containing the N2, G5, and B27 supplements (all from Invitrogen, Carlsbad, CA, http://www.invitrogen.com). After several passage clusters of adherent cells detach and form spheres in different size in low attachment flasks (Corning Costar, Acton, MA, http://www.corning.com/lifesciences).

2.4. Immunohistochemistry

Immunohistochemistry analyses were carried out according to standard protocols. Briefly, samples were fixed in 4% paraformaldehyde in Dulbecco's BPS for 30 min at room temperature. The tissue was then fixed into selected agar gelatine for easier handling and embedded into paraffin. Sections (10 μm) were prepared with microtome deparaffinised and rehydrated. Slides were then incubated in citrate buffer (620-W microwave) for 15 min (0.01 M; pH 6.0) followed by three washes with PBS and incubated with appropriate dilutions of primary antibodies in PBS containing 0.3% Triton X-100 overnight at 4 °C. After several washes in PBS, the sections were incubated with a secondary biotinylated antibodies and ABC reagent solution for 1 h, separately (Vector laboratories, http://www.vectorlabs.com). Color was developed with 3,3′-diaminobenzidine (DAB, Sigma–Aldrich) incubation for 10–20 min.

2.5. Antibodies

The following primary antibodies against human antigens were used: rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com), rabbit anti-Musashi-1, rabbit anti-nestin, mouse anti-vimentin, rabbit anti-Sox-2 (all from Chemicon, Temecula, CA, http://www.chemicon.com), rabbit anti-glial fibrillary acidic protein (anti-GFAP) (all from Dako, Glostrup, Denmark, http://www.dako.com), mouse anti-βIII-tubulin (Sigma–Aldrich, St. Louis, http://www.sigmaaldrich.com), rabbit anti-α crystallin B (from Abcam, http:www.abcam.com).

2.6. RNA isolation

Isolation of total RNA was performed by using RNeasy mini kit from Qiagene following the manufacturer's protocol. RNA quality was verified by 2100 Bioanalyzer (Agilent) and the concentration was determined by the ND-1000 Spectrophotometer (NanoDrop).

2.7. Microarray

For each condition, 255 ng of total RNA was used to synthesize cRNA using the Illumina TotalPrep RNA amplification kit (Ambion). cRNA concentration was measured with a spectrophotometer and cRNA quality was determined by 2100 Bioanalyzer (Agilent). For microarray 750 ng of amplified cRNA product was hybridized to human HT-12 v4.0 Illumina microarrays (Illumina) according to the manufacturer's instructions. Microarray preprocessing was done using R (R Development Core Team, 2011) and BioConductor packages. Microarrays were normalized and differentially expressed genes were identified using Limma package. Probes having FDR corrected *p*-values less than 0.05 and fold change larger than 2.5 between compared conditions were considered significantly differentially expressed. Each such gene set ordered by fold change was characterized by Gene Ontology terms, KEGG and Reactome pathways, TRANSFAC binding predictions, MicroCosm miRNA sites, BIOGRID interaction data, human disease genes from Human Phenotype Ontology using g:Profiler webtool (default settings).

2.8. Q-PCR

Isolation of total RNA was performed by using RNeasy mini kit from Qiagene following the manufacturer's protocol. RNA concentration was assessed by nanodrop and 500 ng of total RNA was used to synthesize cDNA by using TAKARA kit following the manufactures protocol. Primer sequences were designed by Invitrogen primer design tools and are summarized in Sup. Table 2. RT-PCR was carried out in optical 384-well plates and labeled by using the SYBR green master mix (Applied Biosystems), and the fluorescence was quantified with a Prism 7900 HT sequence detection system (Applied Biosystems). The expression of MX1, OAS1-3, IFIT-3, ISG15, C1QB, DHX58 genes was examined in a total volume of 10 _l containing 1_ SYBR green reagent, 0.86 M of each gene-specific primer pair, and 1 ng of cDNA. For amplification the program recommended by Applied Biosystems was used (50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min). The relative level of each RNA was normalized to the corresponding GAPDH RNA levels. All TaqMan RT-PCR reactions were carried out in technical and biological triplicates, and the average cycle threshold (CT) values were determined.

2.9. Survival analysis

We performed survival analysis as a function of gene expression data. We used 518 normalized glioblastoma samples from The Cancer Genome Atlas (TCGA, downloaded 11.02.2013) for 10,113 genes that overlap between the TCGA and our Illumina dataset. We created single gene models using Cox Proportional Hazards regression models with coxph routine from Survival package in R. Genes were considered affecting survival if the coxph Wald test FDR corrected *p*-value was smaller than 0.05. Out of the 10,113 genes 349 have an effect with a *p*-value smaller than the threshold (1807 before FDR correction). We concentrated our analysis on genes that are up-expressed in ENT + GIC compared to ENT. We studied further genes that had fold change above 2.5. There were 132 such Illumina probes that corresponded to 115 unique gene identifiers, out of which 97 are present in TCGA dataset.

3. Results

The initial goal of this study was the *in vitro* generation of a human glioblastoma-like tissue. Glioblastoma initiating cells (GIC) were isolated from surgically removed glioblastoma and maintained in culture as described in Material and methods. These cells were able to form gliomaspheres in suspension when maintained in low attachment plates (Fig. 1A). We first addressed if a differentiation program into a tumor-like tissue could be reproduced

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