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Mesenchymal stem cells and glioma cells form a structural as well as a functional syncytium *in vitro*

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ABSTRACT

The interaction of human mesenchymal stem cells (hMSCs) and tumor cells has been investigated in various contexts. HMSCs are considered as cellular treatment vectors based on their capacity to migrate towards a malignant lesion. However, concerns about unpredictable behavior of transplanted hMSCs are accumulating. In malignant gliomas, the recruitment mechanism is driven by glioma-secreted factors which lead to accumulation of both, tissue specific stem cells as well as bone marrow derived hMSCs within the tumor. The aim of the present work was to study specific cellular interactions between hMSCs and glioma cells *in vitro*. We show, that glioma cells as well as hMSCs differentially express connexins, and that they interact via gap-junctional coupling. Besides this so-called functional syncytium formation, we also provide evidence of cell fusion events (structural syncytium). These complex cellular interactions led to an enhanced migration and altered proliferation of both, tumor and mesenchymal stem cell types *in vitro*. The presented work shows that glioma cells display signs of functional as well as structural syncytium formation with hMSCs *in vitro*. The described cellular phenomena provide new insight into the complexity of interaction patterns between tumor cells and host cells. Based on these findings, further studies are warranted to define the impact of a functional or structural syncytium formation on malignant tumors and cell based therapies *in vivo*.

Introduction

Patients harboring malignant gliomas still have a dismal prognosis despite multimodal treatment strategies. Glioblastoma multiforme is an aggressive and highly vascularized tumor of the brain, which is known to interact extensively with the host environment. Malignant gliomas are supposed to recruit stem cells both from the entire brain itself as well as systemically from the circulation (Kim et al., 2011; Schichor et al., 2006). In human gliomas, bone marrow derived hMSCs have not been detected, yet. This might be due to the fact, that hMSCs cannot be determined easily by immunohistochemistry. They are not defined by a single, specific surface marker, but are characterized by the presence of a typical marker profile including strong expression of e.g. CD105, CD90, CD29, CD73 and others (Keating, 2006). However, the definite role of host stem cells in tumor growth, progression and recurrence is still not completely understood. In experimental systems it has been shown that various stem cells including hMSCs might inhibit tumor growth (Pisati et al., 2007; Staflin et al., 2004). In contrast, other studies indicate that they may contribute to tumor formation (Djouad et al., 2003; Liu et al., 2011; Ramasamy et al., 2007; Ricci-Vitiani et al., 2008). Despite these findings, hMSC-based cell-therapies aim at utilizing their affinity for diseased areas as cellular vectors (Choi et al., 2012; Ricci-Vitiani et al., 2008; Sonabend et al., 2008). The putative advantage of this approach would be the administration of a motile multipotent cell type in order to target single, infiltrative glioma cells within functional brain parenchyma. Both, endogenously recruited or exogenously administered hMSCs get into close and intense interaction with malignant glioma cells. The predictability of this interaction remains unclear, when concerns about adverse side-effects are addressed. Several other publications already described the intense intercellular crosstalk and unwanted cell fate in non-malignant brain diseases (Jaderstad et al., 2010; Snyder, 2011). A reasonable approach to identify detailed mechanisms of cross-talk between hMSCs and glioma cells would be a simple co-culture setting. Surprisingly, we found unexpected, complex interaction patterns between glioma cells and hMSCs that

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are distinct from paracrine interactions. A special focus of the present study is syncytium formation, which can be seen in two ways: communication between two different cell types via gap junctional coupling (functional syncytium) or fusion of two different cells (structural syncytium). Gap junctions are plasma membrane protein channels connecting cells and allowing communication through electrical coupling and transfer of small molecules (<1 kD, functional syncytium). Structural syncytium formation of hMSCs has gained attention due to their transdifferentiation potential into lineage restricted cells of cardiogenic or neurogenic tissues (Alvarez-Dolado et al., 2003). In fact, there is growing evidence that fusion of hMSCs with host cells serves as an alternative mechanism of transdifferentiation (Kamijo et al., 2006; Wurmser and Gage, 2002). In this study, we describe how adult hMSCs intensively interact with glioma cells via functional as well as structural syncytium formation, altering migratory potential and proliferative behavior. These phenomena give an impression of the complex interaction modes between different cell types and should be taken into account in designing *in vitro* studies or in interpreting results of *in vivo* cell therapeutic studies.

Materials and methods

Cell types

Isolation of primary hMSCs from bone marrow of healthy donors

HMSCs were generated out of bone marrow aspirates from healthy donors after written informed consent. Isolation was performed as described elsewhere (Otsu et al., 2009). In brief, mononuclear cell fraction was isolated by Ficoll density gradient centrifugation (1.077 g/mL, SIGMA-Aldrich) at 400 *g* for 25 min. The cells were cultured in DMEM (FG0415, Biochrom) supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco) and 20% fetal bovine serum (Biochrom) at 37 °C. Only hMSCs of passages 2–4 were used for experiments. Cells were checked by flow cytometry and resulted positive for CD105, CD90, CD166, CD29 and CD40, and negative for typical hematopoietic and endothelial markers (CD45, CD34).

Cell lines

The human glioma cells lines U87 and U373 (ATCC) as well as green fluorescent protein (GFP) transfected U373 cells (U373-GFP) were used. Cells were grown in complete growth medium consisting of DMEM, supplemented with 10% FBS, non-essential amino acids, 4 mM L-glutamin, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco). The U373-GFP cells were routinely propagated under the same conditions with addition of 1.2 mg/ml G-418 (SIGMA-Aldrich) to the medium. As control cells, we used an astrocyte cell line SVG p12 (LGC Promochem) cultured in MEM Earle's (FG0325, Biochrom) with above mentioned supplements.

Differentially regulated expression of connexins

Gene expression data

In order to identify differentially regulated expression of connexins, we analyzed microarray data as follows:

Gene expression data were generated from the following samples in 3 biological replicates each: hMSC monoculture (MSC), U87 glioma cells (U87), hMSC from indirect co-culture with U87 (hMSC grown on the bottom-plate of the Boyden chamber, MSC-IC) and U87 from indirect co-culture with hMSC (U87 grown above in the insert of the Boyden chamber, U87-IC) on Illumina HumanWG-6 v3.0 expression beadchips. These chips contain probes that detect the expression of 16 out of the 20 connexins annotated with protein-coding transcripts in Ensembl (Table S2). The expression of the remaining four connexins (Cx30.2/Cx31.9, Cx36, Cx40.1, Cx62) cannot be measured by this chip. All protein-coding transcripts of 10 connexins (Cx26, Cx30, Cx30.2, Cx45, Cx47/46.6, Cx50) can be analyzed by these chips while only some of the annotated transcripts can be measured for 6 connexins (Cx25, Cx32, Cx37, Cx40, Cx43, Cx59/Cx58). Analysis of the expression data was performed with R (http://www.r-project.org). Data was quantile normalized. Sequence information for the Illumina probes was received from the corresponding Illumina annotation file (http://www.illumina.com/support/annotation_files.ilmn) and data concerning connexins was extracted. The sequences were blasted against a database containing all available transcript sequences for connexins based on Ensembl Release 61 (1 February 2011, GRCh37, http://www.ensembl.org). The results of this analysis are summarized in the Supplementary Material (Table S1). Alternative connexin names were received from the HUGO Gene Nomenclature Committee (HGNC, http://www.genenames.org/) and Genecards (http://www.genecards.org/). In addition, all available Ensembl transcripts for connexin genes were tested for their potential to be recognized by probes on the Illumina HumanWG6 v3.0 array (Table S2). Mean group values for the expression of connexin probes and genes are given in Supplementary Material (Table S3).

All microarray data were deposited to the NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), a public functional genomics data repository (GEO series Accession Number: GSE26283).

Co-culture and conditioned medium culture of hMSC and glioma cell lines

U87 and hMSC lines were chosen for analysis. The co-culture experiments were set up in duplicates using 6-well transwell inserts (Costar) with 0.4 μm pore size. For indirect co-cultures, hMSCs were plated on the bottom of the 6-well plate at a density of 1×10^5 cells/2 ml, whereas the U87 cells were plated in the transwell at a density of 8×10^4 cells/ml. Cells were left to adhere for 3 h before being put together for 72 h. Conditioned media was harvested after 24 h from subconfluent cells just prior to trypsinization. For conditioned media culture, cells of both types were plated as for indirect co-culture, then conditioned media (diluted 1:1 with fresh medium) was added to the cells. For direct co-cultures both types of cells were mixed at the same ratio (1:1). Cells were left to grow for 72 h before RNA isolation.

RNA isolation and quantitative Real-time PCR for Connexin 43 expression

Total RNA was isolated from U87 cells and hMSCs either from monoculture, indirect co-culture, conditioned media culture or direct co-culture (1:1) using Trizol reagent (Invitrogen) following manufacturers' instructions. cDNA was generated from 1 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 50 μl final reaction volume, according to the manufacturers' protocol. Gene expression of Gap junction protein alpha 1/Connexin43 (GJA/Cx43) was quantified using real-time quantitative PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems). Real-time PCR reactions were performed using 1:10 dilution (1 μl/well) of each cDNA, TaqMan Universa PCR Master Mix (Applied Biosystems) and the TaqMan Gene Expression Assay for GJA/Cx43, Hs00748445_s1. Amplification of GAPDH was performed as internal control. The SDS v2.2 software was used to analyze data obtained from TaqMan Gene Expression Assays with the Comparative Ct Method (ΔΔCt algorithm). Independent experiments were performed in duplicates and repeated at least three times. Statistical significance was determined by two tailed student's *t*-test and p-value<0.05 was considered significant.

Protein extraction from cultured cells

The culture medium was removed and the cell monolayers were rinsed twice with cold PBS and then scrapped in cold lysis buffer (10 mM Tris, 0.5% NP-40, 150 mM NaCl, 10% Glycerol, mM EDTA, 1 mM DTT, 0.5 mM PMSF, pH 8.0) supplemented with protease inhibitor cocktail (P2714 and P8340, SIGMA-Aldrich). Cell lysates were passed through a 26 G needle several times and cellular debris was removed by centrifugation at 15,000×*g* for 10 min at 4 °C. Protein