Dental Mesenchymal Stem Cells’ Effect on Glioma Cells

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ABSTRACT
Glioma is a malignant tumor with a high mortality rate within few years of diagnosis. Due to limited effect of currently available cancer therapies, alternative therapies are investigated. Studies have shown that mesenchymal stem cells (MSCs) may have a therapeutic potential in glioma treatment. The objective of this in-vitro study was to examine the effect of three different human dental MSCs (D-MSC) on rat glioma cells. We utilized three experimental set ups in order to study the effect of D-MSC on glioma cell survival: unstimulated conditioned medium, stimulated conditioned medium, and direct co-culture. Unstimulated conditioned medium showed an inhibitory effect of 10-30 % on cell survival. Stimulated conditioned medium showed no statistically significant inhibition of glioma cell survival. Direct co-culture immunofluorescence microscopy revealed propidium iodide absorption, indicating cell death, in glioma. Results indicate that D-MSC has a negative influence on glioma proliferation but secreted/expressed factors mediating this effect are unidentified. This is a screening of D-MSCs potential therapeutic effect in treating glioma.
INTRODUCTION

Gliomas are aggressive tumors in the central nervous system with poor prognosis (70% mortality in 2 years and 90% in 5 years), even when treated (Ostrom et al., 2015). Gliomas have unconstrained growth, invasive growth pattern, angiogenetic and immunosuppressive effects, making resection complicated (Van Meir et al., 2010) with high risk of recurrence (Kamiya-Matsuoka and Gilbert, 2015). Radiotherapy exposes not only the tumor but also surrounding brain tissue to radiation, causing several side effects. Combined with chemotherapy the median life expectancy is prolonged by 2.5 months in comparison to radiotherapy alone. However, this strategy lacks definite dose-dense regiments and the long-term adverse effects are not yet known (Norden and Wen, 2006).

Mesenchymal stem cells (MSC) are multipotent precursor cells found in adult stromal tissues with the ability to proliferate, form colonies, self-renew and differentiate into bone, cartilage and connective tissue for tissue-maintenance and repair. Studies have been focused mainly on its immunomodulatory and regenerative features of MSCs (Coulson-Thomas et al., 2016), while their potential in the field of oncology is being investigated (Uccelli et al., 2008). It has been shown that different types of MSCs have a potential inhibitory effect on glioma growth and therefore it may exist a therapeutic potential in treating gliomas with MSC (Nakamura et al., 2004; Gu et al., 2010; Jiao et al., 2011). MSCs also possess properties to home to tumor sites and can be utilized as drug delivery-vectors, which increase their anti-tumor therapeutic potential (Kosaka et al., 2012; Lee et al., 2009).

MSCs used in studies investigating its effect on glioma are most often derived from bone marrow tissue (BM-MSC) or adipose tissue (ASC). To our best knowledge, there are no studies where dental-derived MSCs have been used for targeting gliomas. Several human dental-MSCs (D-MSC) has been discovered from both the deciduous and permanent dentition: dental pulp stem cells (DPSC) (Gronthos et al., 2000), stem cells from exfoliated deciduous teeth (SHED) (Miura et al., 2003), periodontal ligament stem cells (PDLSC) (Seo et al., 2004), stem cells from apical papilla (SCAP) (Sonoyama et al., 2006), and dental follicle precursor cells (DFPCs) (Morsczeck et al.,
In this *in-vitro* study we used human SCAP, DPSC and PDLSC from three matched donors in direct or indirect co-culture with glioma to study the induced effect on glioma survival.

**MATERIAL & METHODS**

**Literature selection**

To find articles regarding glioma we used the term "glioma mortality". The search was narrowed to English full texts, review articles, human subjects and articles published between 2006/02/22 and 2016/02/19 (10 years).

To find articles concerning MSC therapy on glioma the following MeSH terms were applied: "Mesenchymal stromal cells", "therapy", "glioma" and "rats". The search was limited to English full texts with abstracts.

In addition, relevant original studies and review articles regarding D-MSCs were selected by the tutor.

**Ethical consideration**

In this study we used already collected human dental mesenchymal stem cells. The donors had given their written consent and had been informed regarding the usage of isolated cells for research projects. The isolated cells from donors were coded (numeric) and no personal information was accessible, except age and gender. The key to decode the information about the donors was only available for the tutor.

The (glioma) C6 cell line (CCL-107™) was purchased from ATCC.

Collection and usage of all human cells used in this study were ethically approved by the Regional Ethical Review Board in Umeå (DNR: 2013-276-31M).

**Dental MSC isolation and classification**
Surgically removed impacted third molars were collected from seven donors (15-25 year of age) at the Maxillofacial Surgery Section at University hospital in Umeå. Isolated tissues were digested by L collagenase type I (Collagenase type 1, Worthington Biochemicals Corp., Lakewood, NJ), and 4 mg/mL dispase II (Dispase®II, Roche Diagnostics, Indianapolis, IN) and incubated for 60 minutes. All incubations of cells in this study were performed at 37 °C and 5% CO₂ if not mentioned otherwise. Single cell suspensions of SCAP, DPSC and PDLSC were obtained by passing the cells through a 70 mm strainer (Falcon, BD Labware, Franklin Lakes, NJ), seeded at 10⁴ cells/T25 flask (Costar, Cambridge, MA), cultured with α-MEM: Minimum essential medium eagle – alpha modification + GlutaMax (Gibco/Invitrogen, Carlsbad, CA) supplemented with 15 % FBS (Fetal Bovine Serum, Gibco), 100 mM L-ascorbic acid 2-phosphate (L-ascorbic acid 2-phosphate, WAKO Pure Chemical Industries ltd., Tokyo, Japan), and 1 % PEST (Penicillin-Streptomycin Solution, Gibco). On reaching ~90% confluence, the cells were passed to T75 flasks (Costar) by using TE (Trypsin/EDTA solution, Gibco) and plated at a density of 5000 cells/cm². Cell cultures with any signs of contamination were discarded and finally three out of seven donors, where all 3 types of dental MSCs did grow well, were chosen for further experiments.

D-MSCs were separated by cell-type and patient number. D-MSCs and glioma cells were kept in ultra-low temperature freezers at -80 °C in a solution consisting of 90% FBS and 10% DMSO (Dimethylsulfoxide, Sigma-Aldrich, Saint Louis, Missouri). Cultured in T75 flasks (Nunc, Thermo Fischer Scientific, Waltham, Massachusetts) with growth medium consisting of 1% PEST, 10% FBS and 89% α-MEM (Gibco) and incubated. Change of medium were performed every 2-3 days. On reaching ~90% confluence, cells were washed by HBSS (Hanks’ Balanced Salt Solution, Gibco) and detached by TE for passage to 3-4 new flasks.

Flow cytometry analysis
D-MSCs were collected (10⁶ of each type) for characterization of positive MSC associated surface markers CD73, CD90, and CD105 (BD Bioscience, San Jose, CA). The cells were also analyzed for a negative cocktail of HLA-DR, CD45, CD34, CD19, and CD11b (BD Bioscience), for a complete MCS-marker characterization. All
antibodies used for FACS analysis were PE-conjugated. Briefly, optimal concentrations of all antibodies were calculated (1:25-1:30) and \(10^4\) cells for each analysis was chosen. As negative control a corresponding isotype control was used for each sample (mouse IgG1, \(\kappa\)). After following manufacturer’s protocol, the data were acquired using FACScalibur (BD Bioscience).

**GFP-marking**
Poly-D-lysine coated 6-well plates (Corning, Corning, NY) were rinsed twice in PBS (Phosphate-buffer saline, Gibco) followed by plating HEK293T cells in HEK293T medium (KO-DMEM (KnockOut DMEM, Gibco), 10 % FCS (Fetal Calf Serum, Gibco), 1 % PEST, 1 % L-glutamine) and incubated overnight. On reaching 78-80 % confluency, transfection reaction consisting of KO-DMEM and EmGFP vector (Gibco), was prepared and incubated for 15 minutes at room temperature. Media replaced with HEK293T Low Serum Medium (KO-DMEM, 1 % FCS, 1 % PEST, 1 % L-glutamine) and cells were transfected and incubated for 48 hr. Media was extracted, passed through a steriflip filter unit (Millipore, Darmstadt, Denmark) and concentrated using an Amicon 100 kDa cutoff filter unit (Millipore). Centrifugation step were performed at 3000 rpm for 30 minutes at 4 °C. The supernatant was concentrated down to approximately 250 mL and transferred into Eppendorf tube and stored on ice.

D-MSC from patient #12 were seeded at a density of 200,000 cells per well of a 6 well plate in 2 mL of medium and incubated overnight. Cells were then transduced. Medium and polybrene (Millipore) solution was made (ratio 1:5) in falcon tubes with lentivirus. D-MSC media was replaced with lentivirus in separate wells and incubated overnight. 1 mL medium was added to each infected well and incubated overnight. All media containing Lentivirus were removed and replaced with 3 mL medium to each well and incubated for 48 hrs.

**Exposure of glioma cells to conditioned media**

**Conditioned medium**
On reaching \(\sim\)70-90 % confluence of D-MSCs from donor #12-14, growth medium was collected in sampling tubes and centrifuged at 1200 rpm for 6 minutes. Media were
filtered from dead cells and transferred to new tubes and stored in freezer at -20 °C. Control medium was made by keeping growth medium incubated for 2 days in culture condition before freezing at -20 °C.

Glioma cells were quantified using Bürker's chamber (Neubauer improved, Hirschmannlab, Eberstadt, Germany) and plated in 96-well plates (Costar), at a density of 4 000 cells/well in either 200 μL unstimulated conditioned medium or control medium. Outermost wells were kept empty to prevent evaporation. 15 μL viability reagent (alamarBlue®, Thermo Fischer Scientific) was added to each well and incubated for 9 hr. To acquire data, 150 μL medium was extracted from each well to new 96-well plate and measured using microplate spectrophotometer (Synergy HT, Biotek, Winooski, VT) at 570 and 600 nm, day 1, 6 and 12.

**Stimulated conditioned medium**

MSCs and gliomas were cultured in T25 flasks (Nunc) at a ratio of 1:1. On reaching ~80-100 % confluence, the stimulated conditioned media were collected, as described above for unstimulated conditioned medium. Control medium was acquired from glioma with matched cell quantity and volume of media. The alamar blue cell viability assay was used as described above but with lower cell density (1000 cells/well). Readings were made at day 1, 4 and 6.

**Direct co-culture experiment**

Glioma cells were labeled with Hoechst stain solution (Sigma-Aldrich) at 1:1000 dilution and incubated for 30 minutes and centrifuged at 1100 rpm for 5 minutes. Medium was removed and cells were washed with HBSS and centrifuged at 1100 rpm for 5 minutes. HBSS was removed and replaced with growth medium. GFP-transfected D-MSCs from patient #12 were diluted to the same cell concentration as Hoechst-stained glioma. Each cell type of D-MSCs and glioma were cultured alone or together (ratio 1:1) in sliding chambers (Nunc) and incubated for 48 hrs.

For PI staining (Propidium Iodide, Life technologies, Eugene, Oregon), buffer and PI solution were prepared beforehand and stored on ice. Growth medium from samples
was removed and the cells were rinsed in PBS. 2 mL of PI-buffer staining solution was added and the chambers were incubated at room temperature in dark for 15 minutes. Cells were rinsed in PBS and fixed with 2 mL 4 % PFA (Paraformaldehyde, Sigma-Aldrich) at room temperature in dark for 30 minutes. PFA solution was carefully extracted and samples rinsed twice in PBS. Walls of sliding chambers were removed and the slides were cover slipped with Prolong mounting medium. The cells were observed by ECLIPSE 90i immunofluorescence microscope and images captured with Nikon elements2 software (Nikon, Burgerweeshuispad, Amsterdam) at magnification 20-40x.

**Statistical Analysis**

Data are presented as mean ±SD of the mean values. To determine statistical significance between experimental groups two-way analysis of variance was performed using Prism (Prism, GraphPad Software, San Diego, California). A value of p<0.05 was considered statistically significant. p<0.05 expressed as *, p<0.01 ** and p<0.001 ***.

**RESULTS**

The objective was to determine if D-MSCs had an effect on glioma regarding growth (inhibitory or stimulatory) and cell survival. There was no statistical difference between cell types and donors (data not shown), thus data for matched donors was combined for further analysis.

**Isolation of SCAP, DPSC and PDLSC**

All D-MSCs expressed the positive markers CD73, CD90 and CD105 and lacked the expression of negative markers. The expression profiles of the MSC markers were inter- and intra-individually similar among the various D-MSCs (Figure 1).

**Effect of unstimulated conditioned medium on glioma cells**

Conditioned medium from unstimulated SCAP, DPSC, PDLSC (in comparison with control 100 %) reduced the number of viable glioma cells by 10-30 % after 1-12 days, p<0.05. This effect was seen in all groups and time points, except in glioma cells co-
cultured with unstimulated conditioned media from SCAP at day 6. Further, there were no statistically significant differences between various types of D-MSCs (Figure 2).

**Effect of stimulated conditioned medium on glioma cells**
Stimulated conditioned medium from SCAP, DPSC and PDLSC had limited effect on the number of viable glioma cells compared to the control. Glioma cells co-cultured with stimulated conditioned media from SCAP and DPSC revealed an inhibitory effect on day 4, p<0.05. There were no statistically significant differences between various types of D-MSC (Figure 3).

**Direct co-culture immunofluorescence microscopy**
Glioma, SCAP, DPSC and PDLSC cultured separately, acted as control groups. Imaging of glioma, SCAP, DPSC and PDLSC, all showed no uptake of PI. Thus cells were not undergoing cell death in mono-cultures (data not shown).

Glioma co-cultured with SCAP, DPSC and PDLSC revealed an uptake of PI, mainly, in glioma, indicating D-MSC may promote cell death in glioma (Figure 4).

**DISCUSSION**
In this study we aimed to explore how various D-MSC could affect glioma cell survival. We utilized three various experimental set-ups: glioma cultured in unstimulated conditioned D-MSC-medium, glioma cultured in stimulated conditioned D-MSC-medium, and direct co-culture of glioma and D-MSCs.

Although a crucial step for characterization of MSCs, is their specificity regarding expression of certain CD-markers and lack of expression of negative markers, this is not sufficient for complete characterization. MSCs must also possess the ability to differentiate to various cell types. All the isolated D-MSCs from the three matched donors were previously proven to be able to differentiate towards bone and fat tissue (data not shown).

Our findings revealed a decreased cell survival of glioma cells when cultured in
unstimulated conditioned medium from D-MSCs. However, alamar blue does not distinguish if the decreased cell numbers are due to elevated cell death or/and inhibited cell proliferation. On the other hand, our data indicates that D-MSCs might secrete factors that mediate negative effect on glioma viability and proliferation. Similar results can be seen in studies utilizing conditioned medium from ASC and umbilical cord-derived mesenchymal stem cells (UC-MSC). Conditioned medium from AT-MSC and UC-MSC had an inhibitory effect on glioma cell survival by 35 % and 40-50 % respectively (Yang et al., 2014; Ma et al., 2014). However, the level of discrepancy between our study and the studies with ASC and UC-MSC can, except the various MSC sources, be due to differences in experimental set up.

Glioma cultured in stimulated conditioned medium had little to no statistically significant inhibition of glioma proliferation/cell viability, indicating that stimulation of D-MSCs did not have any additive effect on glioma cells. Nevertheless, immunofluorescence microscopy analysis of direct co-culture experiments showed that glioma in close proximity of D-MSCs absorbed PI, revealing that our observed data are most probably due to increased cell death rather than inhibition of cell proliferation of glioma cells.

In the present study, cells from three matched donors were used in the unstimulated and stimulated conditioned medium experiments. Our results showed no statistical difference between donors, indicating that the observed effect on glioma are most probably donor independent.

In order to quantify the observed data from direct co-cultures of D-MSCs and glioma cells (Figure 4), selection of non-interfering staining markers for cells types, cell survival, and cell death should be selected for future flow cytometric analysis.

The discovery of MSC’s anti-tumor properties are encouraging, but their long-term effects and tumorigenic risks remain to be evaluated. In contrast to MSC’s anti-tumor effect seen in other studies such as the present one, some other studies show the opposite desired effect. In fact, MSC has been shown to support glioma outgrowth in
one study (Yu et al., 2008). In addition, a sub-population of glioma cells have been shown to mimic characteristics of MSCs, such as local immunosuppressive capacities (Ochs et al., 2013).

**Considerations**
The timeframes for alamar blue assays were limited by logistics and were therefore selected at days 1, 4, 6, and 12.

The unstimulated and stimulated conditioned medium experimental series had different cell quantities and time points for read-outs.

It might have been more suitable to GFP-label the target cells (glioma). However, since labelled D-MSC were going to be utilized in other projects, these cells (rather than glioma) were selected to be marked with GFP.

FACS analysis was performed for quantitative analysis of the co-culture experiments. However, data could not be used as the different dyes interfered with each other. Another way to get quantitative data would have been to manually count the cells, which would have been extremely time consuming. Therefore, only observational (qualitative) data was shown (Figure 4).

**Conclusion**
The presented results in our study indicate that D-MSCs have a general but limited inhibitory effect on glioma cell survival, independent of D-MSC and donor. Further quantitative analysis is needed to verify the effect of direct co-cultures of D-MSC and glioma. In addition, further examination should focus whether the effect is mainly due to induced cell death in glioma by D-MSCs or/and involvement of inhibition of cell proliferation.

**ACKNOWLEDGEMENTS**
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REFERENCES


Figure 1: Characterization of dental mesenchymal stem cells (D-MSCs). D-MSC analyzed for positive markers CD73, CD90 and CD105 and negative markers cocktail (HLA-DR, CD45, CD34, CD19, and CD11b).
Figure 2: Cell survival inhibition of glioma cultured in unstimulated conditioned medium from D-MSCs. Glioma cells were cultured in unstimulated conditioned media from D-MSCs for different time points. The alamar blue uptake of glioma cells exposed to unstimulated conditioned media was measured on day 1, 6 and 12. The absorbance level in control cultures (glioma) was set to 100%. * = p<0.05, ** = p<0.01, *** = p<0.001.
Figure 3: Cell survival inhibition of glioma cultured in stimulated conditioned medium from D-MSCs. Glioma cells and various D-MSCs were co-cultured for 48hrs and the conditioned media was selected from each groups (termed as stimulated conditioned media). New glioma cells (not previously exposed to D-MSCs) were cultured in collected stimulated conditioned media from D-MSCs for different time points. The alamar blue uptake of glioma was measured on day 1, 4 and 6. The absorbance level in control cultures (glioma) was set to 100 %. * = p<0.05, *** = p<0.001.
Figure 4: PI-uptake after co-culture of glioma cells and D-MSCs. Glioma cells were labeled with Hoescht (blue), while D-MSCs were labeled with GFP (green). After 42 hrs of co-culture the cells were stained by PI solution (red) to visualize dead or late stage dying cells. (A) Glioma, (B) glioma + SCAP, (C) glioma + DPSC, and (D) glioma + PDLSC. Scale bar 50 μm.