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Short communication

Intratumorally implanted mesenchymal stromal cells potentiate peripheral immunotherapy against malignant rat gliomas

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ABSTRACT

Bone marrow-derived mesenchymal stromal cells (MSCs) target glioma extensions and micro-satellites efficiently when implanted intratumorally. Here, we report that intratumoral implantation of MSCs and peripheral immunotherapy with interferon-gamma (IFNγ) producing tumor cells improve the survival of glioma-bearing rats (54% cure rate) compared to MSC alone (0% cure rate) or immunotherapy alone (21% cure rate) by enforcing an intratumoral $CD8^+$ T cell response. Further analysis revealed that the MSCs up-regulate MHC classes I and II in response to IFNγ treatment in vitro and secrete low amounts of immunosuppressive molecules prostaglandin E_2 and interleukin-10.

1. Introduction

The prognosis for patients with high-grade gliomas remains very poor despite extensive surgical resection and adjuvant chemo- and radiotherapy (Lamborn et al., 2008; Grossman et al., 2010). Treatment failure is mainly ascribed to the infiltrative capacity of the tumor cells, which form microsatellites deep within the normal brain.

Bone marrow-derived multipotent mesenchymal stromal cells (MSCs) display inherent tumor-tropic properties and constitute a novel treatment approach with the potential to target tumor microsatellites. Following intratumoral implantation, the MSCs migrate extensively throughout experimental brain tumors, whereas no infiltration of the normal brain has been detected (Bexell et al., 2009). This glioma-specific tropism has been exploited to deliver anti-neoplastic agents such as pro-drug converting enzymes, oncolytic viruses and pro-inflammatory cytokines (Yong et al., 2009; Gunnarsson et al., 2010; Matuskova et al., 2010).

Several reports show that MSCs can be polarized into an immunostimulatory/anti-tumoral phenotype when exposed to e.g. interferon-gamma (IFNγ) (Le Blanc et al., 2003; Stagg et al., 2006; Romieu-Mourez et al., 2007, 2009; Waterman et al., 2012). In this context, peripheral immunotherapy using cytokine-secreting tumor cells can eradicate experimental gliomas by inducing a pro-inflammatory tumor-microenvironment (Visse et al., 1999, 2000; Fritzell et al., 2013b) and in the present study we investigated the combinatorial effect of intratumoral MSCs and immunotherapy against rat gliomas.

2. Material and methods

2.1. Cells

The N32 rat glioma cell line is syngeneic with Fischer 344 rats and resembles anaplastic astrocytoma (Janelidze et al., 2009). The cells have been transduced to express IFNγ (N32-IFNγ) (Visse et al., 1999) and both cell lines were cultured as described elsewhere (Eberstal et al., 2012).

MSCs were isolated from the bone marrow of male Fischer 344 rats. Previously, the cells have been characterized and transduced to express enhanced green fluorescent protein (GFP; MSC-GFP) and were cultured as earlier described (Bexell et al., 2009).

All cells were maintained at 37 °C in the presence of 6.0% CO_2.

2.2. Flow cytometry and ELISA

100,000 MSC-GFP cells were cultured with recombinant IFNγ (rIFNγ; 0–10,000 U/ml, Miltenyi Biotec Norden AB, Lund, Sweden) for 24 h. Afterwards, cells were pre-incubated with anti-CD16/CD32 and stained using PE-RT1A (OX-18; MHC I), PE-RT1B (OX-6; MHC II) or

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PE-IgG$_1$ κ isotype control (BD Biosciences, Stockholm, Sweden). Fluorescence was analyzed using a C6 Flow Cytometer (Accuri Cytometers, Inc., Ann Arbor, USA).

Supernatants were assessed for the production of interleukin (IL)-10 (BD Biosciences) and prostaglandin E_2 (PGE_2) (Cayman Chemicals, Larodan Fine Chemicals AB, Malmö, Sweden) using ELISA.

2.3. Survival study

All animal procedures were performed according to the practices of the Swedish board of Animal Research and were approved by the Committee of Animal Ethics in Lund-Malmö, Sweden.

3000 N32 tumor cells were inoculated intracerebrally (i.c.) into the right striatum of male Fischer 344 rats (8–9 weeks old; NOVA-SCB AB, Sollentuna, Sweden), as previously described (Bexell et al., 2009). On days 1, 14 and 28, animals were immunized subcutaneously (s.c.) with 3,000,000 irradiated (80 Gy) N32-IFNγ cells into the right thigh. On days 7 and 17, rats received 250,000 MSC-GFP cells i.c.

Animals were euthanized when neurological symptoms appeared and post-mortem examinations confirmed i.c. tumors.

2.4. Tumor-infiltration study

3000 N32 tumor cells were inoculated i.c. into male Fischer 344 rats. On days 4 and 14, 3,000,000 irradiated N32-IFNγ tumor cells were injected intraperitoneally (i.p.) and on days 7 and 11, animals received 250,000 MSC-GFP cells i.c. Animals were euthanized on day 25 and brains were snap frozen and cut into 6 μm sections.

Sections were fixed in acetone (10 min) and endogenous peroxidase was blocked using peroxidase blocking solution (Dako, Glostrup, Denmark). 5% donkey serum was added for 20 min (Jackson ImmunoResearch Laboratories, West Grove, USA) and afterwards, sections were incubated 60 min with anti-rat TCRαβ (R73) or anti-rat CD8α (OX-8, 5 μg/ml; BD Biosciences). Later, sections were incubated for 30 min with donkey anti-mouse-biotin or donkey anti-rabbit-biotin (5 μg/ml), followed by streptavidin–peroxidase for 30 min (5 μg/ml; Jackson ImmunoResearch). Finally, sections were stained with AEC (Dako) for 5 min before Mayer's Hematoxylin (30 s). As negative controls, the primary antibodies were omitted.

Tumor sections were analyzed using a light microscope (BX-60, Olympus America Inc., Melville, USA) and images were taken at 10× magnification. T cell infiltration was calculated as percent stained cells/tumor area using analySIS® software (Olympus).

2.5. Statistics

In vitro statistical analyses were performed using paired samples *t*-test. Log-rank test was used for calculating differences between groups in the survival curve and the Mann–Whitney *U*-test was used for comparison between two unpaired groups in vivo. Statistical analyses were performed using GraphPad Prism Software (GraphPad Software, San Diego, USA), where $p < 0.05$ was considered statistically significant.

3. Results

3.1. The MSC-GFP cells acquire an immunostimulatory phenotype in response to IFNγ

First, we assessed whether treatment with IFNγ could induce MSC-GFP cells with an immunostimulatory phenotype and addition of rIFNγ (0–10,000 U/ml) significantly up-regulated major histocompatibility complex (MHC) classes I and II ($p < 0.01$; Fig. 1A–B). Moreover, the MSC-GFP cells secreted low levels of the immunosuppressive factors PGE_2 (mean: 81 pg/ml) and IL-10 (mean: 2 pg/ml), however neither factor was significantly reduced upon treatment with rIFNγ (Fig. 1C–D).

3.2. Improved survival by intratumoral MSCs and peripheral IFNγ-immunotherapy

Next, we investigated the impact of intratumorally grafted MSC-GFP cells against rat gliomas either as monotherapy or in combination with peripheral IFNγ-immunotherapy. As shown in Fig. 2, MSC-GFP and immunotherapy significantly increased the cure rate of glioma-bearing rats (54%) compared with MSC-GFP alone (0%; $p < 0.001$) or immunotherapy only (21%; $p < 0.01$).

3.3. The combination therapy increases tumor-infiltrating TCR^+ and $CD8^+$ cells

The degree of tumor-infiltrating T cells in glioblastoma patients correlates positively with survival (Lohr et al., 2011; Kmiecik et al., 2013) and here we found that the amounts of tumor-infiltrating $TCR\alpha\beta^+$ cells were significantly elevated in animals receiving the combination therapy (mean: 9.0%) compared with MSC-GFP alone (mean: 0.5%; $p < 0.001$) or immunotherapy alone (mean: 2.1%; $p < 0.01$) (Fig. 3B–C). The levels of $CD8\alpha^+$ cells were also elevated in animals receiving the combination therapy (mean: 8.0%) compared with MSC-GFP alone (mean: 1.6%; $p < 0.001$) or immunotherapy alone (mean: 3.5%; $p < 0.05$) (Fig. 3B, D).

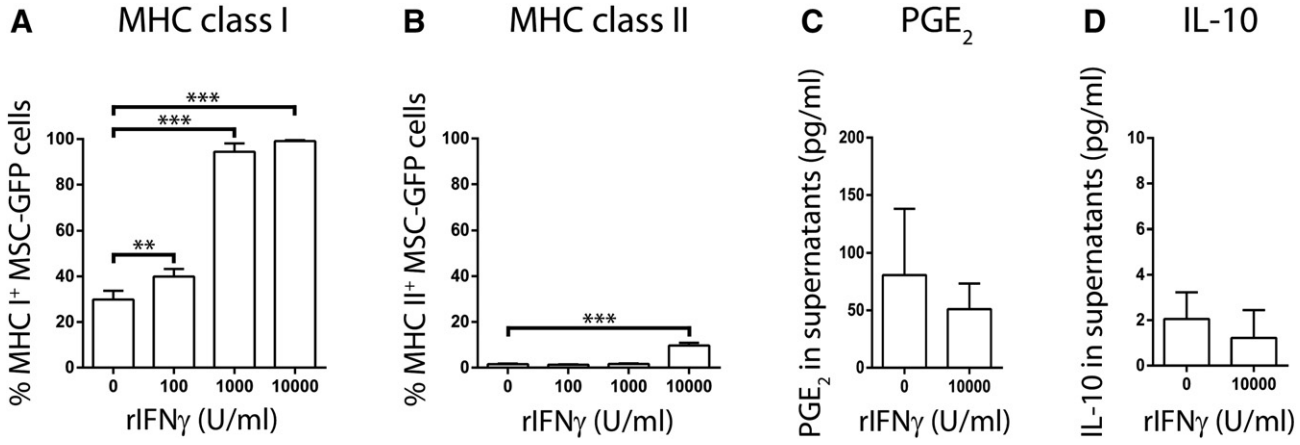

Fig. 1. MSC-GFP cells up-regulate MHC I and II upon IFNγ treatment and secrete low levels of PGE_2 and IL-10. 100,000 MSC-GFP cells were cultured for 24 h in the presence of rIFNγ (0–10,000 U/ml). The expression of (A) MHC class I and (B) MHC class II were determined in duplicates in three separate experiments using flow cytometry. The levels of (C) PGE_2 and (D) IL-10 were determined in supernatants in duplicates in three separate experiments using ELISA. Differences between groups were determined using paired samples *t*-test.