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Immature mesenchymal stem cell-like pericytes as mediators of immunosuppression in human malignant glioma

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ABSTRACT

Malignant gliomas are primary brain tumors characterized by profound local immunosuppression. While the remarkable plasticity of perivascular cells – resembling mesenchymal stem cells (MSC) – in malignant gliomas and their contribution to angiogenesis is increasingly recognized, their role as potential mediators of immunosuppression is unknown. Here we demonstrate that FACS-sorted malignant glioma-derived pericytes (HMGP) were characterized by the expression of CD90, CD248, and platelet-derived growth factor receptor-β (PDGFR-β). HMGP shared this expression profile with human brain vascular pericytes (HBVP) and human MSC (HMSC) but not human cerebral microvascular endothelial cells (HCMEC). CD90 + PDGFR-β + perivascular cells distinct from CD31+ endothelial cells accumulated in human gliomas with increasing degree of malignancy and negatively correlated with the presence of blood vessel-associated leukocytes and CD8+ T cells. Cultured CD90 + PDGFR-β + HBVP were equally capable of suppressing allogeneic or mitogen-activated T cell responses as human MSC. HMGP, HBVP and HMSC expressed prostaglandin E synthase (PGES), inducible nitric oxide synthase (iNOS), human leukocyte antigen-G (HLA-G), hepatocyte growth factor (HGF) and transforming growth factor-β (TGF-β). These factors but not indoleamine 2,3-dioxygenase-mediated conversion of tryptophan to kynurenine functionally contributed to immunosuppression of immature pericytes. Our data provide evidence that human cerebral CD90+ perivascular cells possess T cell inhibitory capability comparable to human MSC and suggest that these cells, besides their critical role in tumor vascularization, also promote local immunosuppression in malignant gliomas and possibly other brain diseases.

1. Introduction

Malignant gliomas are aggressive primary brain tumors with a poor prognosis despite multimodal therapy including surgery and radiochemotherapy (Wen and Kesari, 2008; Wick et al., 2011). Besides their typical infiltrative growth pattern complicating tumor resection and radiotherapy, gliomas are characterized by a profound local and systemic immunosuppression. Mechanisms of tumor immune escape include glioma cell-derived immunosuppressive factors such as transforming growth factor-β (TGF-β) (Platten et al., 2001a), prostaglandin E_2 (PGE_2) (Fujita et al., 2011) and Fas ligand (Jansen et al., 2010) as well as secretion of interleukin-10 (IL-10) as a result of tumor-immune cell interaction (Segal et al., 2002; Yang et al., 2003). These mechanisms cooperate to hinder lymphocyte activation and effector function while promoting glioma proliferation and migration (Wick et al., 2001). Conversely, approaches targeting effector molecules such as TGF-β are therapeutic in animal models of malignant gliomas (Platten et al., 2001b; Uhl et al., 2004) and are used clinically in patients with malignant glioma (Bogdahn et al., 2011; Wick and Weller, 2011). While tumor cells are certainly capable of producing the aforementioned immunosuppressive mediators themselves, there is increasing evidence that the host microenvironment equally shapes the immunoregulation in glioma tissue (Charles et al., 2011).

In this context, the tumor vasculature ought to play a major role as infiltrating immune cells have to permeate the vessel wall. Malignant gliomas are characterized by extensive neoangiogenesis including the recruitment of endothelial cells and pericytes (Louis et al., 2007). Beside

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their role in angiogenesis (Abramsson et al., 2007; Dore-Duffy and LaManna, 2007), pericytes have been identified as a source of undifferentiated mesenchymal stem cell (MSC)-like cells (Crisan et al., 2008) as pericytes isolated from various tissues have a mesodermal differentiation capacity, show migratory function and constitutively express MSC markers (Covas et al., 2005; Crisan et al., 2008; Zannettino et al., 2008). Human bone marrow-derived MSC have been shown to interact with both the innate and the adaptive immune system guiding immune responses towards immune tolerance (Uccelli et al., 2008). Specifically, MSC have been shown to suppress proliferation of both CD8+ cytotoxic T cells and CD4+ helper T cells (Di Nicola et al., 2002; Aggarwal and Pittenger, 2005). As a consequence MSC have been demonstrated to be instrumental in treating immune-mediated diseases such as organ transplantation, graft-versus-host disease or multiple sclerosis (Uccelli et al., 2011). Whether human vascular pericytes share these immunomodulatory properties with MSC is unclear.

In the present study, we analyzed whether human brain pericytes share immunoregulatory properties and mechanisms with human MSC and examined the relevance of our findings in glioma immune escape.

2. Materials and methods

2.1. Cell culture and reagents

Human MSC were obtained from bone marrow from total hip replacement surgeries of nine different patients following informed consent (Opitz et al., 2009). After density gradient centrifugation, MSC isolated by plastic adherence were grown in Amniomax Basal Medium (AM) with 10% stimulatory supplement (Invitrogen Life Science, Carlsbad, USA). Passages 5–20 were used for experiments.

Human brain vascular pericytes (HBVP) were purchased from ScienCell Research Laboratories (Carlsbad, USA) and cultured in poly-L-lysine coated flasks in basal medium for human vascular pericytes (PM) containing 2% FBS, 1% pericyte growth supplement and 1% penicillin/streptomycin solution (all reagents from ScienCell Research Laboratories). Passages 3-15 were used for experiments.

Immortalized human cerebral microvascular endothelial cells (HCMEC) (Weksler et al., 2005) were cultured in rat tail collagen type-1 (Sigma-Aldrich, Taufkirchen, Germany) coated dishes in Clonetics EBM-2 Endothelial Cell Basal Medium-2 containing 5% FBS, 1% penicillin/streptomycin, 1.4 μM hydrocortisone, 5 μg/ml acid asorbic, 1% chemically defined lipid concentrate, 10 mM HEPES and 1 ng/ml bFGF (all reagents from Lonza, Basel, Switzerland). Passages 25–35 were used for experiments.

Peripheral blood mononuclear cells (PBMC) were isolated from unrelated healthy blood-donors by density gradient centrifugation using lymphocyte separation medium LSA 1077 (PAA Laboratories GmbH, Pasching, Austria) and plated in RPMI (Cambrex, Verviers, Belgium) containing 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (PAA Laboratories).

All cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Flow cytometry analysis

Flow cytometry was performed using a BD-FACS Canto II flow cytometer (BD-Biosciences, Heidelberg, Germany). Cells were detached, washed and re-suspended at 10^5 per 100 μl in PBS (PAA Laboratories) containing 1% BSA (Sigma-Aldrich). After incubation with the specific antibody at 4 °C cells were washed and analyzed by flow cytometry. For surfacemarker analyses, antibodies against human CD29-FITC, CD44-FITC, CD105-PE, CD34-PE (eBioscience, San Diego, CA), CD14-FITC (Acris Antibodies GmbH, Hiddenhausen, Germany), CD80-PE-Cy5, CD86-Pacific Blue, human lymphocyte antigen HLA-APC-PE-Cy7, HLA-DR-PE-Cy5 (BioLegend, San Diego, CA) and CD90 (Abcam, Cambridge, UK) with the secondary antibody anti-mouse (AlexaFluor from Invitrogen) were used. Data were analyzed using FlowJo flow cytometry analysis software (Tree Star, Ashland, OR, USA).

2.3. Isolation of human malignant glioma pericytes

CD90-positive human malignant glioma pericytes (HMGP) were isolated from freshly resected glioblastoma tissue after material for diagnostic procedures was separated by the Department of Neuropathology, Institute of Pathology, Heidelberg according to local ethical approvals. Tissue was dissected and washed with PBS. After centrifuging for 5 min at 300 *g*, cells were treated with accutase (PAA Laboratories) for 15 min at 37 °C, washed and mechanically dissociated using a 100 μm cell strainer. Subsequently, cells were re-suspended in PBS containing 1% BSA and flow cytometry sorting was performed as described above after co-staining with APC-conjugated CD90 (R&D Systems, Wiesbaden, Germany) and PE-conjugated CD31 antibodies (eBioscience). CD90-positive/CD31-negative cells were sorted and plated at 10^5 per 1 ml in high-glucose Dulbecco's Modified Eagle Medium (DMEM) containing 20% FBS and 1% P/S (PAA Laboratories).

2.4. RT-PCR

MSC or HBVP were either untreated, stimulated with IFNγ (20 ng/ml, ImmunoTools, Friesoythe, Germany) for 24 h or co-cultured with PBMC using Transwell-6 permeable inserts (Corning Incorporated, NY, USA) as described below. Cells were harvested and total RNA was isolated using the Qiagen RNAeasy RNA isolation kit (Qiagen, Hilden, Germany). cDNA was synthesized with the SuperscriptTM Choice System (Invitrogen Life Science) using random hexamers. For RT-PCR analysis of HMGP, adherent cells were harvested, total RNA was isolated using the Qiagen RNeasy Micro RNA isolation kit and cDNA was synthesized with the Applied Biosystems high capacity cDNA reverse transcription kit (Foster City, CA, USA). Primers were designed across exon boundaries and provided by Sigma-Aldrich. RT-PCR was performed using an ABI 7000 thermal cycler with SYBR Green PCR Mastermix (Applied Biosystems, CA, USA) according to standard protocols. Samples were normalized to GAPDH, which varied neither with IFNγ stimulation nor after PBMC co-culture and relative quantification of gene expression was determined by comparison of threshold values. PCR reactions were checked by including no-RT-controls and by both melting curve and agarose gel analysis.

Primer sequences were (5′-3′ forward, reverse):

AACAGTGTTGACATGAAGAGCC, TGTAAAACAGCACGTCATCCTT (CD31);
GCGCTTTGCTTGCTGAGTTT, TCCAAGGGTACTAGGTGTTGTAG (CD34);
CACAGAGTTCACTGAAACGGAA, AACCCCTGTAGCAATCTGCTT (CD40);
TCGCTCTCCTGTAACAGTCT, CTCGTACTGGATGGGTGAACT (CD90);
ATCGCAGCCAACTATCCAGAT, TTCCAGGCAAATGAGTGGTGG (CD248);
TCCCGTAGATGACTGCCC, ATGGGTGAAGTGCTGGGCAAA (Cyclooxygenase-2, COX2);
CTCTCTGCTCCTCCTGTTCGAC, TGAGCGATGTGGCTCGGCT (GAPDH);
TACAGGGGCACTGTCAATACC, CAGTAGCCAACTCGGATGTTT (hepatocyte growth factor, HGF);
GAGGAGACACGGAACACCAAG, GTCGCAGCCAATCATCCACT (human leukocyte antigen-G, HLA-G);
GATGTCCGTAAGGTCTTGCCA, TGCAGTCTCCATCACGAAATG (indoleamine 2,3-dioxygenase-1, IDO1);
TGCTTCATGCCTTTGATGAG, GAAGGCCTTATGGGAAGGAG (indoleamine 2,3-dioxygenase-2, IDO2),
TCCGCTATGCTGGCTACCA, CACTCGTATTTGGGATGTTCCA (inducible nitricoxid-synthase, iNOS);