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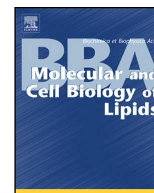

A bidirectional crosstalk between glioblastoma and brain endothelial cells potentiates the angiogenic and proliferative signaling of sphingosine-1-phosphate in the glioblastoma microenvironment

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ABSTRACT

Glioblastoma is one of the most malignant, angiogenic, and incurable tumors in humans. The aberrant communication between glioblastoma cells and tumor microenvironment represents one of the major factors regulating glioblastoma malignancy and angiogenic properties. Emerging evidence implicates sphingosine-1-phosphate signaling in the pathobiology of glioblastoma and angiogenesis, but its role in glioblastoma-endothelial crosstalk remains largely unknown. In this study, we sought to determine whether the crosstalk between glioblastoma cells and brain endothelial cells regulates sphingosine-1-phosphate signaling in the tumor microenvironment. Using human glioblastoma and brain endothelial cell lines, as well as primary brain endothelial cells derived from human glioblastoma, we report that glioblastoma-co-culture promotes the expression, activity, and plasma membrane enrichment of sphingosine kinase 2 in brain endothelial cells, leading to increased cellular level of sphingosine-1-phosphate, and significant potentiation of its secretion. In turn, extracellular sphingosine-1-phosphate stimulates glioblastoma cell proliferation, and brain endothelial cells migration and angiogenesis. We also show that, after co-culture, glioblastoma cells exhibit enhanced expression of $S1P_1$ and $S1P_3$, the sphingosine-1-phosphate receptors that are of paramount importance for cell growth and invasivity. Collectively, our results envision glioblastoma-endothelial crosstalk as a multi-compartmental strategy to enforce pro-tumoral sphingosine-1-phosphate signaling in the glioblastoma microenvironment.

1. Introduction

Glioblastoma (GBM) is the most common and devastating primary brain neoplasm, and is among the most angiogenic tumors in humans; its microvascular proliferation represents a key neuropathological feature [1]. Numerous studies indicate that GBM exhibits extremely high vascularity and angiogenesis; sustained endothelial activation significantly contributes to the GBM microenvironment, emerging as a critical regulator of GBM progression [2]. Indeed, excessive angiogenesis in GBM results in abnormal vessels, and is associated with uncontrolled cell growth and spread, as well as resistance to chemo/radiotherapy, and thus high mortality [3,4]. Furthermore, the perivascular niche serves as a reservoir for tumor-initiating cells within the brain, which supports tumor outgrowth and aggressive behavior [5–7]. As such, disrupting the GBM vasculature and/or its microenvironment is of interest for clinical management of this tumor.

Abbreviations: bECs, brain-derived endothelial cells; BSA, bovine serum albumin; DMEM, Dulbecco modified Eagle's medium; ERK, extracellular signal-regulated kinase; EC, endothelial cells; FBS, fetal bovine serum; FFA-BSA, fatty acid-free BSA; GEC, GBM-derived endothelial cells; GBM, glioblastoma; HPTLC, high-performance thin layer chromatography; S1P, sphingosine-1-phosphate; S1PRs, S1P-specific receptors; Sph, sphingosine; SphK, sphingosine kinase; VEGF, vascular endothelial growth factor

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GBM angiogenesis is thought to be linked to the expression of different angiogenic factors, with a key role of vascular endothelial growth factor A (VEGF) [8–10]. Notwithstanding promising results from preclinical studies, recent trials with the anti-VEGF drug bevacizumab failed to provide positive effects on GBM patient survival [11,12], suggesting that effective targeting of the multifaceted angiogenesis in GBM necessitates a multi-targeted approach [4,13,14]. Thus, a significant need exists in clinical practice for a deeper understanding of the molecular pathways orchestrating vascular development and pathogenesis necessary for the survival and expansion of GBM.

In the complexity of angiogenic signaling, sphingosine-1-phosphate (S1P) emerged as a key molecule [15,16]. S1P is formed intracellularly from ATP and sphingosine (Sph), in a reaction catalyzed by the two isoenzymes of sphingosine kinase (SphK1 and SphK2), and most of its effects are mediated by five S1P-specific receptors (S1PRs) ($S1P_{1–5}$) of [17,18]. S1P is a pleiotropic sphingoid mediator involved in proliferation, migration and invasion of a broad range of cells in healthy and pathological conditions [19,20]. S1P signaling encompasses pathways that promote the continuous acquisition of malignant biological features by tumor cells, and the dysregulation of S1P signaling has been linked to many cancers, including GBM [21,22].

S1P is capable to act as a potent proliferative signal, and multifunctional angiogenic factor, regulating both early and later stages of angiogenesis [15,16]. Indeed S1P is able to promote endothelial cell (EC) migration and proliferation, to stimulate endothelial cell entubulation, and to stabilize newly formed vessels [23–27]. Notwithstanding, whether and how brain-derived endothelial cells (bECs), and their biochemical crosstalk with GBM cells contribute to S1P metabolism and adaptation to the GBM microenvironment remain largely unknown.

The aim of this study was to investigate the role of the GBM-endothelial crosstalk in the origin and functional roles of extracellular S1P in the GBM vascular microenvironment. To this purpose, and in order to delineate S1P flow, we performed metabolic flux analysis using isotopically labeled sphingosine.

We show that bECs, and primary GBM-derived endothelial cells (GECs), constitutively release newly synthesized S1P in the extracellular environment, and, when co-cultured with GBM cells, exhibit increased SphK2 expression and activity, followed by enhanced S1P secretion. On their turn, after co-culture, GBM cells exhibit enhanced expression of $S1P_1$ and $S1P_3$, the S1PRs that are of paramount importance for cell growth and invasivity. Additionally, extracellular S1P stimulates not only GBM cell growth, but also GEC migration and tubule formation, in a $S1P_1$/$S1P_3$-dependent fashion.

2. Materials and methods

2.1. Materials

All reagents were of highest available analytical grade. Dulbecco modified Eagle's medium (DMEM), bovine serum albumin (BSA), fatty acid-free BSA (FFA-BSA), Sph, VEGF, non-essential amino acids, alkaline phosphatase (from bovine intestinal mucosa), and mitomycin c (from Streptomyces caespitosus), were purchased from Sigma-Aldrich (Saint Louis, MO, USA). DMEM Nutrient Mixture F-12, and bovine brain extract were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was from EuroClone (Pero, Milano, Italy). High performance TLC silica gel (HPTLC) plates, SphKI-178-Calbiochem®, PF-543, and (R)-FTY720-OMe were from Merck (Darmstadt, Germany); ABC294640, SEW2871 and TY-52156 were from Cayman Chemical (Ann Arbor, MI, USA). Collagen type I, and Matrigel were from BD Bioscience (San Diego, CA, USA). Bevacizumab was obtained from Roche (Grenzach-Wyhlen, Germany). S1P was purchased from Enzo Life Sciences (NY, USA), and VPC 23019 was from Avanti Polar Lipids (Alabaster, AL). D-*erythro*-sphingosine, isotopically tritiated at the C-3 ([^{3}H]-Sph) (21.0 Ci/mmol), [methyl-[^{3}H]-thymidine] (20 Ci/mmol) and [^{32}P]ATP (3000 Ci/mmol) were from PerkinElmer Life Science (Boston, MA, USA.

2.2. Cell cultures

The human glioblastoma cell lines T98G (ATCC Cat# CRL-1690, RRID:CVCL_0556), U87-MG (CLS Cat# 300367/p658_U-87_MG, RRID:CVCL_0022), CCF-STTG (CLS Cat# 300388/p721_CCF-STTG1, RRID:CVCL_1118), the rat C6 glioma cells (CLS Cat# 500142/p672_C6, RRID:CVCL_0194), and the brain-derived endothelial cell bEnd5 line (ECACC Cat# 96091930, RRID:CVCL_2252) were purchased from the American Type Culture Collection (Manassas, VA, USA). The brain microvascular endothelial cell MBEC line was kindly provided by Prof. Marco Presta (University of Brescia, Italy).

GBM cells were cultured in DMEM supplemented with 10% heath-inactivated FBS, 100 units/ml penicillin, 100 ng/ml streptomycin, 25 μg/ml amphoterin B, 1 mM sodium pyruvate, 2 mM L-glutamine. bECs were cultured in the same supplemented DMEM, with additional 1% MEM non-essential amino acids. The murine N9 microglial cells line was cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 4 mM glutamine, 25 mM HEPES, 1% penicillin/streptomycin, and 5% heath-inactivated FBS [28].

GECs were isolated from post-surgery specimens of four primary human GBMs, after informed consent and Institutional Review board approval (Fondazione IRCCs Cà Granda, Ospedale Policlinico, Milan, Italy). GECs were prepared, cultured in supplemented endothelial proliferation medium (EndoPM), and characterized as we previously reported [29]. In all GECs, immunofluorescence and FACS analyses demonstrated high expression of different endothelial markers.

All cultures were grown in the incubator at 37 °C and 5% CO_2, the medium being replaced with a fresh one every 2–3 days.

2.3. Endothelial and GBM cells co-cultures and conditioning

bECs and GECs were plated in the Falcon™ cell culture plate inserts (growth area: 4.2 cm^2; pore size, 0.4 μm), and the GBM cells were added to the lower chamber. When described, the N9 microglial cells were added to the lower chamber. Parallel cultures, consisting of a single GBM, N9, bEC, or GEC population, in the same transwell system were used as control cultures. All cultures and co-cultures were maintained in complete endothelial cell growth medium for 7 days, the medium being replaced with fresh one every 2 days. If applicable, cells were stimulated as indicated. In some experiments, bEC conditioning was performed by culturing bEnd5 cells in T98-conditioned medium. To this purpose, bEnd5 cells were cultured alone in the absence or presence of the medium from conditioned T98G-cells in the lower chamber for 7 days, the medium being replaced with the corresponding conditioned one every 2 days. Thereafter, the cells were used for analyses.

2.4. S1P metabolism and extracellular release

To evaluate S1P metabolism and extracellular release, pulse studies with [^{3}H]-Sph were performed, as previously described [30,31]. Since the incorporation of exogenous Sph into cells is dependent on both the relative amount of extracellular Sph per cell number (or cell protein), as well as the medium volume [32], we performed pulse studies by administrating the same amount of [^{3}H]-Sph/mg protein, in the same volume to all cell types. To do this, before pulse experiments, the amount of cell protein per dish was assessed using parallel dishes. Both prior and after co-culture, reproducible amounts of cell protein were obtained in the different experiments, and similar amounts were measured prior and after co-culture in the same cell type. The range of protein concentration was 250–900 μg/dish, depending on both the cell type, and the surface of the culture chamber (different in the upper and lower one). Cells were pulsed from the top with 1 ml of complete DMEM, with additional 1% MEM non-essential amino acids, 10 mM

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