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Hypoxic glioblastoma release exosomal VEGF-A induce the permeability of blood-brain barrier

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ABSTRACT

Exosomes are nano-vesicles released by tumor cells to modulate extracellular environment. Accumulating evidence revealed that glioblastoma derived exosomes contain multiple pro-angiogenic factors to induce the proliferation of endothelial cells. Here, we investigated the role of GBM-derived exosomes in inducing the permeability of the blood-brain barrier. We found that VEGF-A was over-expressed in hypoxic GBM-derived exosomes, which enhance the permeability of a BBB *in vitro* model by interrupting the expression of claudin-5 and occludin. *In vivo* permeability assay showed hypoxic GBM-derived exosomes remained functional in the blood circulation and induced the permeability of BBB.

1. Introduction

Glioblastomas (GBM) are the most common and aggressive primary tumors arising in the central nervous system (CNS). Like other solid tumors, GBM has extensive areas of hypoxia, the hypoxic response involves in the regulation of multiple cytokines, growth factors and proteases, which induces major alterations in vessel structure as it stimulates the proliferation of endothelial cells (ECs) and leads to angiogenesis—the formation of new blood vessels from pre-existing vessels [1,2]. Studies have demonstrated that hypoxia is a major cause for the disruption of the blood-brain barrier (BBB), which is a complex structure formed by highly specialized brain microvascular endothelial cells (BMVECs) joined continuously by tight junction complexes, the function of the BBB is to protect the microenvironment of the CNS. Tight junction complexes are the major targets of hypoxic BBB disruption. In GBM, the tight junctions between BMVECs are damaged, resulting in the pathological fenestration and leakage of the BBB [3–5]. However, the pathogenesis of BBB breakdown in GBM is poorly understood. Exosomes are a class of extracellular vesicles (EVs) defined as 40–150 nm diameter membrane nano-vesicles that are released into extracellular environment by many cell types, including tumor cells [6]. It has been demonstrated that tumor cell-derived exosomes mirror the characteristics of the origin cell and act as multicomponent delivery vehicle for nucleic acids and proteins in the microenvironment. Accumulating evidence revealed that GBM-derived exosomes contain different pro-angiogenic factors which are required by ECs to proliferate and migrate [7]. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, has been proposed as the most studied pro-angiogenic factor and is reported to be transported in GBM-derived exosomes [7,8], but the role of VEGF as exosome cargo remains to be further elucidated.

In this study, we reported that exosomes derived from hypoxic glioblastoma cell line U87 promote the proliferation of BMVECs *in vitro*, and induce the permeability of the BBB through VEGF-A by reducing the expression of claudin-5 and occludin.

2. Materials and methods

2.1. Cell lines and culture conditions (hypoxic/normoxic)

Human glioma cell lines (U87, U251) and human embryonic kidney cells (HEK-293) were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS in the presence of 1% penicillin/streptomycin (all from Invitrogen, USA). Immortalized human brain microvascular endothelial cell line hCMEC/D3 was obtained from ATCC and were maintained in endothelial basal medium (EBM-2, Lonza, Switzerland)

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supplemented with 5% FBS, 1.4 μM hydrocortisone, 5 μg/ml ascorbic acid (Sangon Biotech, China), 1 ng/ml basic Fibroblast Growth Factor (bFGF, Genescript, China), 1% chemically defined lipid concentrate (Invitrogen), 10 mM HEPES (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). Routine culture was done in a humidified incubator (Thermo Fisher, USA) maintained at 37 °C with 5% CO2 and 95% air. For hypoxia experiments, cells were exposed to a mixture of 1% O_2, 94% N_2 and 5% CO_2, the hypoxic condition was generated in an oxygen-regulated incubator (Thermo Fisher).

2.2. Exosomes isolation

Cells at passage 3–15 were cultured in DMEM containing 10% exosome-depleted FBS (dFBS, prepared by ultracentrifugation at 120,000×g for 18 h to remove bovine exosomes [9]). The conditioned medium was harvested after 48 h. Exosomes were isolated by differential centrifugation [10,11]. Briefly, the medium was centrifuged at 4 °C for 10 min at 300×g to eliminate cell debris. Supernatants were further centrifuged at 16,500×g, 4 °C for 30 min and filtered through a 0.22 μm filter. Exosomes were then pelleted by ultracentrifugation at 110,000×g (L-80XP, Beckman Coulter, USA), 4 °C for 70 min. Exosome pellets were resuspended in PBS for further analysis.

2.3. Electron microscopy

Exosomes were visualized using transmission electron microscopy (TEM) as described [12]. Firstly, exosome suspensions were absorbed onto a 400-mesh carbon-coated copper grids, rinsed in filtered PBS, then stained with freshly prepared 2% phosphotungstic acid in aqueous suspension. Images for samples were acquired using a Tecnai 12 TEM (Philips, Holland), operated at an acceleration voltage of 80 kV.

2.4. Internalization of exosomes by hCMEC/D3

Exosomes were labeled with the green fluorescent dye PKH67 (Sigma-Aldrich, USA). Briefly, 10 μg of exosomes in 100 μl of PBS was suspended in 1 ml of diluent C, mixed with PKH67 solution and incubated for 5 min. The labeling was stopped by adding 1% BSA solution. The exosomes were rinsed in PBS and pelleted by ultracentrifugation. All the staining procedures were conducted at room temperature. The hCMEC/D3 cells were cultured for 12 h, after which the medium was replaced with fresh medium containing PKH67-labeled exosomes (5 μg/ml), after incubation at 37 °C for varying times, cells were washed with PBS, trypsinized and analyzed by flow cytometry on a MACSQuant instrument from Miltenyi Biotec (Bergisch Gladback, Germany), results were analyzed with FlowJo (Treestar, USA). Alternatively, hCMEC/D3 cells were seeded on chamber slides and incubated with PKH67-labeled exosomes (5 μg/ml) for 4 h, cells were rinsed with PBS, fixed with 4% paraformaldehyde, and incubated with DAPI (Beyotime, China), cells were visualized under a confocal scanning microscope (Zeiss LSM700).

2.5. Western-blot

Exosomes and cells were lysed in RIPA buffer containing 1% PMSF (Beyotime). The proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with CD63, TSG101, Calnexin (Bioworld, China), β-actin, GAPDH, claudin-5, occludin (Thermo Fisher), VEGF-A (Santa Cruz, USA) primary antibodies, then followed by secondary antibodies (Abcam, USA) and ECL (Thermo Fisher) were used to detect the protein bands.

2.6. siRNA transfection

Non-silencing negative control and the pre-designed siRNA oligonucleotides targeting human VEGF-A (GenePharma, Shanghai, China) were transfected in U87 cells using Lipofectamine 2000 (Thermo Fisher) at a final concentration of 100 nM. The conditioned medium from transfected cells was collected 48 h post-transfection and following the same protocol as with non-transfected U87 cells.

2.7. Endothelial cell viability assay

Cellular viability was carried out by MTT Assay. hCMEC/D3 (4000 cells/well) were seeded into 96-well plates and cultured overnight and treated with exosomes (20 μg/ml) for a total of 48 h. The medium was then replaced with fresh medium containing 0.5 mg/ml MTT for 4 h. After incubation, the medium was removed from the plate and DMSO was added. Absorbance was measured at 570 nm using a microplate reader (Thermo Fisher).

2.8. In vitro blood-brain barrier model

In vitro model was set up as described [13]. The membrane of transwell system (Corning, USA) was coated with rat tail collagen type I (5 μg/cm^2, Corning) before seeding the cells, hCMEC/D3 cells were seeded onto the inserts (pore size 0.4 μm) at a density of 4×10^4 cells/cm^2 and grown for 7 days to achieve confluence. Cell culture medium was changed every 2 days. At day 6, the medium was replaced with fresh EBM-2 medium containing 20 μg/ml exosomes or 50 ng/ml VEGF (Novoprotein, China) or control medium. After 10 h, the TEER values and the permeability of FITC-Dextran were measured.

2.9. Trans-endothelial resistance

The TEER values were measured every day using an endothelial voltammeter (EVOM, World Precision Instruments, USA) and were calculated as: Resistance × 1.12 cm^2 (insert surface area), expressed in Ω/cm^2. The final TEER value was obtained by subtracting the resistance of a collagen-coated control insert from a coated insert with cells. For each experiment, at least 3 replicates were measured.

2.10. FITC-dextran permeability assay

The paracellular of FITC-Dextran (FD, MW 70 kDa, Sigma-Aldrich) were measured at day 7. In brief, the monolayers were rinsed with HBSS, FD (1 mg/ml) was added on the luminal side. Passage through the monolayer was monitored by sampling from the lower chamber at 30 min' intervals throughout a 1.5-h time course, and quantification was assessed by fluorescence multi-well plate reader (Molecular Devices, USA) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. The apparent permeability (Papp) was calculated using Equation (1).

$$Papp = Q/(A\times C\times t) \quad (1)$$

where Papp represents the apparent permeability (cm/s), Q is the amount of FD transported per minute (μg/min), A is the surface area of the filter (cm^2), C is the initial concentration of the FD (μg/ml) and t is the time (s). For each experiment, at least 3 replicates were measured.

2.11. Immunostaining and confocal analysis

Cells grown on glass chamber slides were washed with PBS and

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