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Fibronectin on extracellular vesicles from microvascular endothelial cells is involved in the vesicle uptake into oligodendrocyte precursor cells

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ABSTRACT

We previously reported transplantation of brain microvascular endothelial cells (MVECs) into cerebral white matter infarction model improved the animal's behavioral outcome by increasing the number of oligodendrocyte precursor cells (OPCs). We also revealed extracellular vesicles (EVs) derived from MVECs promoted survival and proliferation of OPCs *in vitro*. In this study, we investigated the mechanism how EVs derived from MVECs contribute to OPC survival and proliferation. Protein mass spectrometry and enzyme-linked immunosorbent assay revealed fibronectin was abundant on the surface of EVs from MVECs. As fibronectin has been reported to promote OPC survival and proliferation via integrin signaling pathway, we blocked the binding between fibronectin and integrins using RGD sequence mimics. Blocking the binding, however, did not attenuate the survival and proliferation promoting effect of EVs on OPCs. Flow cytometric and imaging analyses revealed fibronectin on EVs mediates their internalization into OPCs by its binding to heparan sulfate proteoglycan on OPCs. OPC survival and proliferation promoted by EVs were attenuated by blocking the internalization of EVs into OPCs. These lines of evidence suggest that fibronectin on EVs mediates their internalization into OPCs, and the cargo of EVs promotes survival and proliferation of OPCs, independent of integrin signaling pathway.

1. Introduction

Demyelination induced by subcortical ischemia contributes to motor and cognitive deficit [1,2]. There is no established treatment against white matter demyelination. We previously reported transplantation of brain microvascular endothelial cells (MVECs) into the white matter infarction reduced infarction area and improved motor deficit [3]. We also reported transplanted MVECs promoted survival of oligodendrocyte precursor cells (OPCs) [4]. Our *in vitro* analysis revealed extracellular vesicles (EVs) derived from MVECs promoted OPC survival and proliferation [5]. EVs are small vesicles containing proteins, lipids and nucleic acids, and are involved in intercellular communication [6]. We suppose some of these molecules contribute to promotion of OPC survival and proliferation and elucidation of the mechanism may lead to establishment of the novel therapeutics against white matter infarction.

In this study, we analyzed the molecular mechanism how EVs derived from MVECs (MVEC-EVs) affect OPC behavior, focusing on proteins. Protein mass spectrometry and enzyme-linked immunosorbent assay (ELISA) revealed fibronectin is abundant on the surface of MVEC-EVs. Although fibronectin is reported to promote survival and proliferation of OPCs via integrin signaling pathway [7], we couldn't attenuate the effects of EVs on OPCs by blocking the binding between fibronectin and integrins. Recently, heparan sulfate proteoglycan (HSPG) is reported to be the endocytosis receptor for EVs [8,9]. We found EV uptake was decreased by interfering the interaction between fibronectin on EVs and HSPG on OPCs. The

Abbreviations used: MVEC, microvascular endothelial cell; OPC, oligodendrocyte precursor cell; EV, extracellular vesicle; ELISA, enzyme-linked immunosorbent assay; HSPG, heparan sulfate proteoglycan; DMEM, Dulbecco's Modified Eagle Medium; BSA, bovine serum albumin; PDL, poly-D-lysine; EGM-2, endothelial cell growth medium-2; FBS, fetal bovine serum; PBS, phosphate buffered saline; BrdU, Bromodeoxyuridine.

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decrease in the EV uptake attenuated their survival/proliferation promoting effect on OPCs. These results indicate fibronectin on the surface of EVs contributes to their internalization into OPCs and thereby promotes OPC survival and proliferation.

2. Materials and methods

2.1. Ethics statement

All experiments were performed in accordance with the guidelines for Animal Experimentation at Gunma University Graduate School of Medicine and were approved by Gunma University Ethics Committee (Permit Number:16-009).

2.2. Animals

We used Sprague-Dawley rats (SLC, Japan) in this study (postnatal day 2 pups for OPC culture, and eight-week-old male rats for MVEC culture). Rats were sacrificed by decapitation under deep anesthesia to obtain the brains.

2.3. Cell culture

OPCs were prepared from postnatal day 2 rat brain cortices by immunopanning as previously described [5,10]. OPCs were plated into poly-D-lysine (PDL, Sigma)-coated dish, supplemented with forskolin (Sigma), ciliary neurotrophic factor, neurotrophin-3 and platelet-derived growth factor-AA (all obtained from PeproTech). After 3 days, OPCs were plated on PDL-coated 8-well slide glasses for survival and proliferation assays or on PDL-coated 35 mm culture dishes for flow cytometry.

MVECs were prepared from adult rat cerebra as previously described [5]. We used MVECs to collect EVs when the cells reached >95% confluent. The contaminating cells were below 10%.

Rat fibroblast-like cell line (Rat-1) was cultured in DMEM (Wako) containing 5% fetal bovine serum (FBS).

2.4. Isolation of EVs from culture media

For EV isolation, cells were cultured in medium containing exosome-depleted FBS (System Biosciences) (MVECs: EGM-2 containing 2% exosome-depleted FBS, Rat-1: DMEM containing 5% exosome-depleted FBS) for 72 h. To isolate EVs, except for mass spectrometry, we used the exosome precipitation solution, ExoQuick-TC (System Biosciences), according to the manufacture's protocol. EV pellet was suspended in serum-free medium or phosphate buffered saline (PBS). EV isolation for mass spectrometry was performed using MagCapture Exosome Isolation Kit PS (Wako) according to the manufacture's protocol. Protein concentration was measured using Protein Quantification Assay Kit (Takara Bio) according to the manufacturer's protocol.

2.5. Protein mass spectrometry

The surface protein peptide library of isolated MVEC-EVs was prepared using XPEP Exosome Mass Spec Kit (System Biosciences) according to the manufacture's protocol. Mass spectrometry was performed with Eksgent Ekspert NanoLC 425 (AB Sciex) coupled to TripleTOF 6600 mass spectrometer (AB Sciex). Peptide mixture was separated by ODS column (Eksigent ChromXP-C18-CL, 3 μm, 120 Å, 0.075 mm I.D. × 150 mm L, AB Sciex) with 2–30% acetonitrile gradient containing 0.1% formic acid for 60 min. Proteins were identified using Mascot sequence matching software (Matrix Science) [11] with SwissProt database.

2.6. ELISA

MVEC-EVs were treated with 5 mIU/ml heparitinase (heparin lyase III, HL) (Sigma) for 3 h at 37 °C or 0.25% trypsin (TR) (Sigma) for 10 min at 37 °C. Treated EVs were suspended in PBS, and pelleted using ExoQuick-TC. The pellets were resuspended in PBS. Quantification of fibronectin on the surface of EVs was performed using rat fibronectin ELISA kit (abcam) according to the manufacturer's protocol. Samples (50 μl/well) containing 6 μg of EV proteins were added to each well.

2.7. Analysis using integrin function inhibitory peptides

To assess the interaction between fibronectin on EVs and integrins on OPCs, we used peptide mimics (RGD peptides) of fibronectin binding: an antagonist peptide (Gly-Arg-Gly-Asp-Ser [GRGDS]) of integrin function, an inhibitory peptide (Gly-Arg-Gly-Asp-Thr-Pro [GRGDTP]) of fibronection, type-1 collagen and vitronectin, and a negative control peptide (Gly-Arg-Ala-Asp-Ser-Pro-Lys [GRADSPK]) that does not block integrin binding (all obtained from Sigma). OPCs cultured on 8-well slide glass were washed with PBS, and incubate with serum-free medium containing 300 μg/ml peptides for 30 min. After incubation, MVEC-EVs were added to OPC culture at 50 μg/ml of EV proteins. After 1 day's culture, OPC survival and proliferation were assayed.

2.8. Cell survival assay

Cell death with the typical morphological features of apoptosis including pyknotic nuclei was assessed by staining cell nuclei with Hoechst 33342 (Sigma, 2.5 μ M) for 40 min in 5% CO_2 at 37 °C. Samples were observed using a fluorescent microscope (Axioplan2, Zeiss) with cooled CCD camera (DP73, Olympus), and the number of pyknotic cells was counted.

2.9. BrdU incorporation assay

To assess cell proliferation, bromodeoxyuridine (BrdU; 10 μ M) (Roche Diagnostics) was added to the cultures for the last 4 h of culture, followed by fixation and staining with rat monoclonal BrdU antibody (abcam; 1:1000). Propidium iodide (PI, Sigma, 2 μg/ml) was used for nuclear staining. Samples were observed using a fluorescent microscope with cooled CCD camera, and the number of BrdU-positive cells in each sample was counted.

2.10. Treatments of the surface of EVs and OPCs

To investigate the involvement of fibronectin and glycosaminoglycans (heparan sulfate and heparin) in the uptake of EVs by OPCs, EVs and OPCs were treated with heparitinase, heparin, fibronectin, or RGD peptides (All obtained from Sigma) as follows;

Treatment 1) To remove heparan sulfate on EVs, EVs were treated with 5 mIU/ml heparitinase at 37 °C for 3 h, and suspended in PBS.
Treatment 2) To remove heparan sulfate on the surface of OPCs, OPCs were treated with 0.5 mIU/ml heparitinase at 37 °C for 3 h prior to addition of EVs.
Treatment 3) To block heparin-binding domain of fibronectin on EVs, EVs were incubated with 50 μg/ml heparin for 30 min. The heparin-bound EVs were collected using ExoQuick-TC.
Treatment 4) To block fibronectin-binding sites on OPCs, the cells were preincubated with 200 μg/ml human plasma fibronectin (Sigma) for 1 h prior to addition of EVs.