FEBS Letters 588 (2014) 4573–4582

journal homepage: www.FEBSLetters.org

CD44 variant, but not standard CD44 isoforms, mediate disassembly of endothelial VE-cadherin junction on metastatic melanoma cells

Pu Zhang [a,b], Changliang Fu [b], Huiyuan Bai [a], Erqun Song [a], Yang Song [a,*]

[a] Key Laboratory of Luminescence and Real-Time Analytical Chemistry (Southwest University), Ministry of Education, College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, China
[b] Department of Bioengineering, Pennsylvania State University, University Park, PA 16801, USA

ARTICLE INFO

Article history:
Received 15 August 2014
Revised 8 October 2014
Accepted 21 October 2014
Available online 4 November 2014

Edited by Michael R. Bubb

Keywords:
CD44 variant isoform
Tumor metastasis
VE-cadherin phosphorylation
Short interfering RNA
Adherens junction

ABSTRACT

Loss of endothelial adherens junctions is involved in tumor metastasis. Here, we demonstrate that, in the metastatic Lu1205 melanoma cells, expression of the CD44 variant CD44v8-v10 induced junction disassembly and vascular endothelial (VE)-cadherin phosphorylation at Y658 and Y731. Short interfering RNA (siRNA)-mediated CD44 knockdown or sialic acid cleavage reversed these effects. Moreover, microspheres coated with recombinant CD44v8-v10 promoted endothelial junction disruption. Overexpression of CD44v8-v10 but not of standard CD44 (CD44s) promoted gap formation in the non-metastatic WM35 melanoma cells, whereas CD44 knockdown or neuraminidase treatment dramatically diminished melanoma transendothelial migration. Endothelial cells transfected with the phosphomimetic VE-cadherin mutant Y658E supported transmigration of CD44-silenced Lu1205 cells. Our findings imply that CD44 variant isoform (CD44v) but not CD44s regulates endothelial junction loss, promoting melanoma extravasation.

1. Introduction

Tumor metastasis is a multistep process requiring tumor detachment from the primary sites, dissemination through the circulation, lodging onto blood vessel wall and extravasation to distant organs [1–5]. Within the blood vessel, tumor cell (TC) initiates a complex cascade of adhesive interactions with endothelium. It requires specific interactions between endothelial adhesive molecules and their ligands expressed on TCs [5]. Previous studies have shown that colon carcinoma cell adhesion to ECs involves sialylated molecules and integrins [6]. These adhesive interactions initiate a variety of signal transductions in both cancer and endothelial cells (ECs) [6–8].

CD44 is an N- and O-glycosylated cell surface protein [9,10]. CD44s is composed of 341 amino acids with a molecular mass of 85–90 kD [11]. At least ten exons (v1–v10) can be alternatively spliced to give rise to multiple CD44 variant isoform (CD44v) with a variety of molecular weights [12,13]. The expression of CD44 has been found to be associated with tumor progression, tumor immune evasion, receptor tyrosine kinase (RTK)-induced activation of anti-apoptotic pathways, and cancer-initiating cell survival [10,14]. The expression of CD44v confers breast cancer and colon carcinoma metastatic potentials [4]. CD44 was originally identified as a leukocyte homing receptor, and its extracellular amino-terminal domain contains hyaluronic acid (HA)-binding motifs and several N-glycosylation sites which may mediate CD44-HA binding [15–18]. CD44 possesses distinct sialofucosylated modifications with different binding affinities for selectins under static and hydrodynamic conditions [3,5]. CD44s can bind to E-selectin but not L- or P-selectin, whereas CD44v is a ligand for all three, mediating initial tethering of carcinoma cells to platelets and ECs [12,13]. CD44-fibrin interactions are required for sustained TC adhesion, ultimately leading to tumor extravasation and establishment of secondary tumor loci [19].

Intact endothelial monolayer is maintained by vascular endothelial-cadherin (VE-cadherin) homophilic interactions in adherens junctions [20]. Endothelial junction integrity is disrupted by

Abbreviations: HUVEC, human umbilical vein endothelial cell; EC, endothelial cell; CD44v, CD44 variant isoform; CD44s, CD44 standard isoform; TC, tumor cell; VE-cadherin, vascular endothelial-cadherin; TCM, tumor conditioned medium; siRNA, short interfering RNA; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate buffered saline; EGFP, enhanced green fluorescence protein; RTK, receptor tyrosine kinase; DMJ, deoxymannojirimycin; benzyl-GalNAc, benzyl-2-acetamido-2-deoxy-a-D-galactopyranoside

* Corresponding author at: College of Pharmaceutical Sciences, Southwest University, Beibei, Chongqing 400715, China. Fax: +86 2368251225.
E-mail addresses: ysong@swu.edu.cn, songyangwenrong@hotmail.com (Y. Song).

http://dx.doi.org/10.1016/j.febslet.2014.10.027
0014-5793/

phosphorylation of VE-cadherin tyrosine residues that results in dissociation of the p120 and β-catenin complex from the cytoplasmic tails of VE-cadherin [21,22]. VE-cadherin dimer disassembly plays an essential role in angiogenesis, tumor metastasis, cellular mesenchymal state maintenance, and endothelial viability [9,10,23–25]. Previous studies indicated that disassembly of VE-cadherin homodimers is mediated by adhesion events [26,27]. Tumor cell adhesion initiates a series of signaling cascades leading to loss of endothelial junction integrity [28–30]. Studies also suggested that VE-cadherin phosphorylation was coupled with junction breakdown in response to external stimuli [31,32]. Upon phosphorylation of VE-cadherin at tyrosine residues, Y658 and Y731, p120 and β-catenin were dissociated from VE-cadherin cytoplasmic domains [21,23,32–34]. Although the loss of adherens junction integrity has been shown to promote melanoma-lung metastasis in mouse model, the molecular basis of the underlying mechanism remains elusive.

In the current study, we demonstrated that highly metastatic melanoma cell line Lu1205 but not non-metastatic cell line WM35 facilitated endothelial junction breakdown and VE-cadherin phosphorylation at tyrosine resides, Y658 and Y731. The differences in the capacities of these cells to regulate junction integrity were attributed to the presence of spliced variant CD44v8-v10 on Lu1205. Short interfering RNA (siRNA) silencing and enzymatic digestion assays suggested that CD44 plays a major role in VE-cadherin junction disassembly. Overexpressing CD44v8-v10 but not CD44s in WM35 elevated the ability of WM35 to induce EC junction breakdown. Our results further revealed that by initiating VE-cadherin phosphorylation and junction breakdown, CD44v8-v10 on Lu1205 mediates melanoma transendothelial migration.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in F12-K medium with 10% FBS, 30 μg/ml of endothelial cell growth supplement, 50 μg/ml heparin (Mallinckrodt Baker), and 100 U/ml of penicillin–streptomycin (Biofluids). The Lu1205 melanoma cell line and HL-60 human myeloid cells (obtained from ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) and Iscove's Modified Dulbecco's Medium (IMEM) supplemented with 10% FBS and 100 U/ml of penicillin–streptomycin. SK-Mel-25 (ATCC) and WM35 melanoma cells (provided by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA) were maintained in Roswell Memorial Park Institute (RPMI) supplemented with 10% FBS and 100 U/ml of penicillin–streptomycin. All cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

2.2. Extraction of CD44 and preparation of CD44-coated polystyrene beads

Cell membrane proteins were isolated by using a MEM-PER Membrane Protein Extraction Kit (Pierce, Rockford, IL) following the manufacturer's instructions. Briefly, cells were lysed with detergent in the presence of protease inhibitors and EDTA. Then, a second detergent was added to solubilize the membrane proteins. Following differential centrifugation and incubation at 37 °C, hydrophobic proteins were separated from the hydrophilic proteins through phase partition. Western blotting was used to determine the extraction efficiency. To immunoprecipitate CD44 isoforms, anti-human CD44H (clone 2C5, R&D, Minneapolis, MN) antibody was crosslinked to protein G coupled Dynabeads (Invitrogen) with Bis(sulfosuccinimidyl) substrate (BS3) (Thermo Scientific). Then, the hydrophobic phase was incubated with anti-CD44-Dynabeads overnight at 4 °C. To elute CD44 from the beads, Ab-Ag-beads were washed 6 times with 2% NP-40/1% bovine serum albumin (BSA), followed by 3 times with lysis buffer containing 2% NP-40. Immunoprecipitates were diluted with phosphate buffered saline (PBS)/0.02% sodium azide and heated to 95 °C to elute CD44. Immunoprecipitated CD44 was then coated onto 15 μm polystyrene beads (Polysciences Inc., Warrington, PA) with carbonate/bicarbonate buffer. After washing with PBS/BSA, the coating efficiency on the microspheres was determined by flow cytometry. A calibration curve was established with QuantiBRITE Quantitation Kit (BDBiosciences) to relate the mean fluorescence intensity to the number of molecules bound per particle. The CD44 isoform site densities on beads were comparable to those of Lu1205. Beads were resuspended (1×10^6 microspheres/ml) in PBS/0.1% BSA for use in co-culture assays.

2.3. Flow cytometry

Cells were detached with trypsin carefully and incubated with BSA to block non-specific binding sites. Cells were then incubated with saturating concentrations of primary mAbs directed against specific molecules in PBS containing 1% BSA for 20 min at 4 °C. Cells were then washed twice to remove unbound primary antibodies. After an additional 20 min incubation with Alexa-546 conjugated goat anti-mouse Fab_2 fragment (1 μg/10^6 cells; Invitrogen) at 37 °C, the cells were washed twice and fixed with 2% formaldehyde and analyzed using a GUAVA personal flow cytometry (GUAVA Technologies, Burlingame, CA).

2.4. Western blotting

Melanoma and HUVECs were collected and rinsed with PBS, and lysed with RIPA lysis buffer (20 mM Tris, 5 mM $MgCl_2$, 1 mM PMSF, 20 mg/ml aproptonin, 10 mg/ml leupeptin, 1 mM Na_3VO_3, and 20 mM β-glycerophosphate). The lysates were centrifuged at 14000 rpm for 15 min. The protein concentrations across samples were checked by Bradford method. The samples were denatured by adding SDS running buffer (0.2% bromophenol blue, 4% SDS, 100 mM Tris[pH 6.8], and 20% glycerol) and β-mercaptoethanol. The samples were analyzed by SDS–PAGE on 12% gels. After the proteins were transferred to nitrocellulose membrane, green fluorescence protein (GFP) (Abcam, Eugene, OR), CD44 (clone 2C5), CD44v7/8 (VFF-17, Serotec, Inc., Raleigh, NC), CD44v10 (Millipore, Billerica, MA), or CD44s (156-3C11, Serotec, Inc., Raleigh, NC), phosphorylated VE-cadherin (Y658) (Chemicon, Temecula, CA), phosphorylated VE-cadherin (Y685) (ECM Biosciences, Versailles, KY), and phosphorylated VE-cadherin (Y731) (Chemicon, Temecula, CA) were detected with corresponding primary monoclonal antibodies (1:1000 diluted in blocking buffer) followed by HRP conjugated secondary antibodies. The labeled proteins were visualized using a chemiluminescence kit. Thereafter, membranes were stripped with stripping buffer before being reprobed with anti-β-tubulin (Cell Signaling Technology, Massachusetts, MA) to ensure equal loadings.

2.5. Immunofluorescence staining

Before experiments, 25-mm cover slips were coated with fibronectin (1 μg/ml). Equal amounts of HUVECs were then grown to 95–99% confluency. After co-culture experiments, HUVECs were then washed two times with PBS and fixed with 5% formaldehyde in PBS for 10 min. Following fixation, cells were permeabilized