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Lipid rafts control human melanoma cell migration by regulating focal adhesion disassembly

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ABSTRACT

Tumor cell migration is a crucial step in the metastatic cascade, and interruption of this step is considered to be logically effective in preventing tumor metastasis. Lipid rafts, distinct liquid ordered plasma membrane microdomains, have been shown to influence cancer cell migration, but the underlying mechanisms are still not well understood. Here, we report that lipid rafts regulate the dynamics of actin cytoskeleton and focal adhesion in human melanoma cell migration. Disrupting the integrity of lipid rafts with methyl-β cyclodextrin enhances actin stress fiber formation and inhibits focal adhesion disassembly, accompanied with alterations in cell morphology. Furthermore, actin cytoskeleton, rather than microtubules, mediates the lipid raft-dependent focal adhesion disassembly by regulating the dephosphorylation of focal adhesion proteins and the internalization of β3 integrin. We also show that Src–RhoA–Rho kinase signaling pathway is responsible for lipid raft disruption-induced stress fiber formation. Taken together, these observations provide a new mechanism to further explain how lipid rafts regulate the migration of melanoma cell and suggest that lipid rafts may be novel and attractive targets for cancer therapy.

1. Introduction

Metastasis is the major cause of cancer mortality. To metastasize, cancer cells must use their intrinsic migratory ability to invade adjacent tissues and the vasculature [1]. Therefore, understanding the molecular mechanisms regulating the migration of cancer cells is very important for anti-metastasis therapy.

The migration of cancer cells on substrate is the sum of several temporally and spatially coordinated processes, which include protrusion of the leading edge, adhesion of the leading edge to the substrate, movement of cell body, and release from contact sites at the trailing edge [2]. During these processes, the dynamics of actin cytoskeleton and focal adhesion are generally thought to play pivotal roles. The remodeling of actin cytoskeleton provides a driving force to push membrane forward at the leading edge and a traction force to move the cell body [3]. The formation of focal adhesions at cell front anchors membrane protrusions and their disassembly releases adhesions at the rear of the cells, which are required for cell relocation and forward progression, respectively [4–6]. Furthermore, actin cytoskeleton shows a close relationship with focal adhesion dynamics in cell migration. On the one hand, the actin cytoskeleton forms the intracellular scaffold for focal adhesions and provides the tension for their growth [7,8]. On the other hand, dendritic actin depolymerization results in disassembly of focal adhesions in the lamellipodium [9]. Thus, the factors influencing the remodeling of actin cytoskeleton are also potential regulators of focal adhesion dynamics.

One of the probable factors concerns cholesterol and sphingolipid enriched membrane microdomains, so-called lipid rafts, which form compartmental platforms for cellular signaling and protein–protein or protein–lipid interaction. Previous reports showed that lipid rafts can concentrate membrane lipids and some proteins, such as phosphatidylinositol 4, 5-bisphosphate and Pyk2/Cbl, and regulate the dynamics of actin cytoskeleton in T cell activation and neurites growth [10,11]. Recently, lipid rafts have been reported to promote cell migration in non-small cell lung cancer by incorporating focal adhesion molecules, such as FAK and Src, into raft fractions [12]. Also, it is increasingly clear that lipid rafts are implicated in the dynamic processes of internalization and recycling of cell-surface integrin which is the structural and functional core of focal adhesion and bridges focal adhesion with actin cytoskeleton in cell migration [13–16]. Despite recent significant advancements, the question of whether lipid rafts regulate focal adhesion dynamics through modulating actin cytoskeleton in cancer cell migration and the underlying mechanisms have not been well characterized.

In the present study, we demonstrate that lipid rafts contribute to focal adhesion disassembly by Src–RhoA–Rho kinase (ROCK) signaling pathway-mediated actin cytoskeleton dynamics, which is crucial for

Abbreviations: CD, cytochalasin D; F-actin, filamentous actin; MβCD, methyl-β cyclodextrin; MES, 4-Morpholineethanesulfonic acid; PAK-PBD, p21 binding domain of p21-activated kinase 1; Rhotekin-RBD, Rho binding domain of Rhotekin; ROCK, Rho kinase; Tyrp, tyrosine phosphorylation

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human melanoma cell migration. Because targeting lipid rafts for cancer therapy has been suggested [17], the findings of this study provide a potential strategy for treating melanoma via modulating lipid rafts.

2. Materials and methods

2.1. Reagents and plasmids

Methyl-β cyclodextrin (MβCD), cholesterol, cytochalasin D (CD), 4-Morpholineethanesulfonic acid (MES), mAbs to phosphotyrosine (PY20), vinculin (V4505), tubulin (T4026) and actin (A5441) were purchased from Sigma-Aldrich. C3 exoenzyme was purchased from Cytoskeleton. Glutathione-Sepharose 4B beads were purchased from Amersham Biosciences. Polyclonal antibody to paxillin (Ab-88) was purchased from Signalway Antibody. Y27632, mAbs to β1 integrin (TDM29) and β3 integrin (LM609) were purchased from Millipore. mAbs to paxillin (D-9), RhoA (26C4), Src (H-12) and flotillin-2 (B-6) and polyclonal antibodies to vinculin (H-300), Rac1 (C-14) and Cdc42 (B-8) were purchased from Santa Cruz Biotechnology. Rhodamine-conjugated phalloidin was purchased from Molecular Probes. GFP–vinculin plasmid was generously provided by Dr. Bernd Hoffmann (Institute of Bio- and Nanosystems, Germany). The GST-tagged expression plasmid pGEX-Rho binding domain of Rhotekin (Rhotekin-RBD) and the plasmid encoding a constitutively active form of RhoA (pcDNA-RhoA-Q63L) were kindly provided by Dr. Cindy K Miranti (Van Andel Research Institute, USA). The GST-tagged expression plasmid pGEX-p21 binding domain of p21-activated kinase 1 (PAK1-PBD) was a kind gift from Dr. Gary Bokoch (The Scripps Research Institute, La Jolla, CA, USA).

2.2. Cell culture and transient transfection

Human melanoma A375 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai, China). Human melanoma M21 cells were from the School of Basic Medical Sciences, Jilin University of China. The cells were cultured in DMEM containing 10% FBS at 37 °C with 5% CO_2. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.3. Wound healing assay

Cells were grown in 24-well culture plates and wounded after reaching confluency using a 10 μl pipette tip. Cells were then washed twice with PBS, incubated with fresh 2% FBS/DMEM containing 5 mM MβCD or not at 37 °C. Wound closure was recorded at the indicated time points under phase-contrast microscope (Nikon, Japan) and the images were analyzed using T-Scratch software [18].

2.4. Cell morphology analysis

Cells were seeded on glass coverslips overnight, and then incubated with fresh 2% FBS/DMEM containing 5 mM MβCD or not for different time periods or with 1 mM cholesterol for different time intervals after removal of MβCD. The morphological alteration was investigated using phase-contrast microscope.

2.5. Immunofluorescence

Cells plated on glass coverslips overnight were fixed with 10% formaldehyde after treatment with different drugs. Then the cells were permeabilized with 0.1% Triton X-100, blocked in 3% BSA, and incubated with the indicated primary antibodies and fluorochrome-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). The coverslips were mounted and observed under a confocal microscope (Olympus, Japan).

2.6. Live cell imaging

Cells grown in 35 mm dishes with 14 mm glass bottom were placed on a 37 °C heated stage within a 5% CO_2 atmosphere chamber and sequential images were captured using an UltraVIEW Vox (PerkinElmer Inc., USA) spinning-disk confocal microscope with a Ti-E microscope (Nikon, Japan). Cell migration to scratch wound was monitored in differential interference contrast channel at 30 s intervals over a 6 h time course using a 20× objective. For the observation of focal adhesion dynamics, cells were transiently transfected with a GFP–vinculin vector and cultured for 24 h before imaging in fluorescence channel at 30 s intervals over a 30 min time course using a 40× objective. Kymographs were generated using ImageJ with the Multiple Kymograph function (J. Rietdorf and A. Seitz, European Molecular Biology Laboratory, Heidelberg, Germany).

2.7. Immunoprecipitation and immunoblotting

Overnight cultured cells were lysed for 30 min on ice in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 2.5 mM sodium pyrophosphate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 1 mM PMSF, and 20 μg/ml aprotinin/leupeptin) and centrifuged at 12,000 *g* for 30 min. The supernatant was incubated with the indicated antibodies at 4 °C for 3 h, prior to incubating with 30 μl of protein A/G-Sepharose beads for another 3 h. The immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked in 5% non-fat milk and probed with the appropriate antibody. Blots were then revealed by chemiluminescence (GE Healthcare) after incubation with the HRP-conjugated secondary antibodies. Band intensity was quantified by ImageJ.

2.8. Internalization assay

Cells were seeded on glass coverslips overnight, and then incubated with β3 integrin antibody for 1 h at 4 °C after different treatment. Internalization of antibody-β3 integrin complex was initiated by addition of prewarmed serum-free medium to the cells, followed by incubation at 37 °C for 1 h. Surface-bound noninternalized surface antibodies were removed prior to fixation by an acid rinse (0.5% acetic acid, 0.5 M NaCl, pH 3.0, for 5 min). Cells were then permeabilized, and the internalized antibodies were immunostained with fluorochrome-labeled secondary antibody.

2.9. Subcellular fractionation

The subcellular fractionation was processed as previously described [19]. Briefly, cells (1×10^7) treated with different drugs were resuspended in ice-cold hypotonic buffer (42 mM KCl, 10 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 20 μg/ml aprotinin/leupeptin) for about 20 min. Then, the cells were homogenized by repeated passage through a 22-gauge needle (30 times) and centrifuged at 200 *g* for 10 min. Cytosolic fractions were obtained by re-centrifuging the supernatants (total fractions) at 13,000 *g* for 60 min at 4 °C. Membrane fractions were obtained by resuspending the pellets in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1 mM PMSF, and 20 μg/ml aprotinin/leupeptin) and centrifuging at 13,000 *g* for 60 min at 4 °C. The pellets (detergent-insoluble fractions) were lysed in 1% SDS. The different fractions were boiled in Laemmli buffer.

2.10. Flow cytometry

Overnight cultured cells were treated with different drugs before harvest. The cells were then fixed with 10% formaldehyde and incubated with anti-β3 integrin antibody or isotype-matched control IgG at room temperature for 1 h. After thorough washes, the cells were stained