Lipid rafts regulate the lamellipodia formation of melanoma A375 cells via actin cytoskeleton-mediated recruitment of β1 and β3 integrin

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Abstract. Lipid rafts, distinct liquid-ordered plasma membrane microdomains, have been shown to regulate tumor cell migration by internalizing and recycling cell-surface proteins. The present study reports that lipid rafts are a prerequisite for lamellipodia formation, which is the first step in the processes of tumor cell migration. The results from the wound-healing assay and immunostaining indicated that lipid rafts were asymmetrically distributed to the leading edge of migrating melanoma A375 cells during lamellipodia formation. When the integrity of lipids rafts was disrupted, lamellipodia formation was inhibited. The investigation of possible molecular mechanisms indicated that lipid rafts recruited β1 and β3 integrins, two important adhesion proteins for cell migration, to the lamellipodia. However, the different distribution characteristics of β1 and β3 integrins implied disparate functions in lamellipodia formation. Further immunostaining experiments showed that the actin cytoskeleton was responsible for lipid raft-mediated β1 and β3 integrin distribution in the lamellipodia. Together, these findings provide novel insights into the regulation of lipid rafts in lamellipodia formation, and suggest that lipid rafts may be novel and attractive targets for cancer therapy.

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Abbreviations: ECM, extracellular matrix; GM1, monosialotetrahexosylganglioside; MβCD, methyl-β cyclodextrin; CD, cytochalasin D

Key words: lipid rafts, actin cytoskeleton, integrin, lamellipodia, melanoma

Introduction

Cell migration serves crucial roles in numerous biological and pathological processes, including embryonic morphogenesis, tissue repair and cancer metastasis (1-3). During the complicated migratory processes, one of the most important steps is the formation of lamellipodia, which are broad, flat protrusions at the leading edge of cells that have the ability to sense the surrounding environment, and drive and guide cell locomotion (4,5). Lamellipodia formation requires the assembly of the actin cytoskeleton and the motility of membranes (6). Lipid rafts, liquid-ordered plasma membrane microdomains, are in principle well suited to serve major roles in regulating membrane motility. Lipid rafts accumulate at the leading edges in migrating fibroblast-like cells and regulate cell motility by selectively excluding or including proteins (7-9). Furthermore, when the integrity of lipid rafts is disrupted, the migration of multiple cancer cells is inhibited (10-12). However, whether lipid rafts influence lamellipodia formation of cancer cells has not been described.

Beyond actin polymerization, the generally known basic mechanism of lamellipodia formation, the adhesion of membrane protrusions to the extracellular matrix (ECM) is also necessary for the formation of lamellipodia. It has been reported that lamellipodia that do not establish stable adhesions become retracted towards the cell body (13). Integrin, a major cell surface receptor, mediates the adhesion between cells and the ECM, and serves important roles in cell migration. Integrin transmits signals into cells and generates positive feedback to control lamellipodia formation (14-16). However, the core function of integrin is to nucleate the formation of focal adhesions at the lamellipodia, which physically link the actin cytoskeleton to the ECM and generate the traction to pull the cell body forwards (17). In addition, integrin recycling is believed to be linked to the migration of cells. Generally, integrins are internalized at the rear of the migrating cell and are recycled to the leading edge, thus resulting in high ras-related C3 botulinum toxin substrate activity and lamellipodia-like protrusions (18). Recently, integrins have been shown to be localized in lipid rafts, and the disruption of lipid rafts

inhibits the internalization and function of integrins (19-21). In our previous study, it was found that lipid rafts regulate the internalization of β3 integrin through sarcoma protein kinase-rhodopsin (Rho)-Rho-associated protein kinase (ROCK)-mediated actin cytoskeleton dynamics in migrating human melanoma A375 cells (22). In A375 cell spreading, lipid rafts control β1 integrin clustering via the recruitment and modification of certain adaptor proteins (23). However, the role and the association of lipid rafts and integrins in lamellipodia formation in human melanoma A375 cells remain unclear.

Based on our previous results (22,23), the present study aimed to investigate the association between lipid rafts and the lamellipodia formation of A375 cells and determine whether lipid rafts can control the lamellipodia formation of A375 cells by regulating β1 and β3 integrin distribution in the cell membrane.

Materials and methods

Cell culture. Human melanoma A375 cells were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Science (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37˚C in 5% CO_2.

Antibodies and regents. Antibodies to β1 integrin (clone TDM29; 1:200; cat. no. CBL481) and β3 integrin (clone LM609, 1:200; cat. no. MAB1976) were purchased from EMD Millipore (Billerica, MA, USA). Tetramethylrhodamine or fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (1:250; cat. nos. T5393 and F9006, respectively), methyl-β cyclodextrin (MβCD, cat. no. C4555), cytochalasin D (CD, cat. no. C2618) and cholesterol (cat. no. C8667) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Rhodamine-conjugated phalloidin (cat. no. R415) and Alexa Fluor® 488-conjugated cholera toxin subunit B (cat. no. C22841) were purchased from Molecular Probes; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). DMEM was purchased from Thermo Fisher Scientific, Inc. and FBS was obtained from Gibco; Thermo Fisher Scientific, Inc.

Analysis of lamellipodia formation at wound edges. A375 cells were grown to confluence on glass coverslips and scratch wounded with the narrow end of a 10-μl pipette tip. The cells were then washed twice with phosphate-buffered saline and incubated with fresh 2% FBS/DMEM with or without 5 mM MβCD at 37˚C. After 3 h, the MβCD was removed, and fresh medium containing 1 mM cholesterol was added for 6 h to allow the integrity of the lipid rafts to recover. The formation of lamellipodia at the wound edges was investigated with phase contrast microscopy (Nikon Corporation, Tokyo, Japan), and the percentage of cells displaying lamellipodia was calculated.

Immunofluorescence. A375 cells were grown to confluence on glass coverslips and scratch wounded. The cells were then treated with 5 mM MβCD for 0, 1, 2 and 3 h, or treated with 0.05 μg/ml CD for 1 h. Subsequent to being washed with phosphate-buffered saline, the cells were fixed with 10% formaldehyde for 10 min at 22˚C and permeabilized with 0.1% Triton X-100 for 3 min. The cells were then blocked in 3% bovine serum albumin for 1 h at 37˚C, incubated with the aforementioned primary antibodies for 1 h at 22˚C and subsequently incubated with fluorochrome-conjugated secondary antibody for 45 min at 22˚C. F-actin was labeled with rhodamine-conjugated phalloidin, and the lipid raft marker, ganglioside GM1, was labeled with Alexa Fluor 488-conjugated cholera toxin subunit B. The coverslips were mounted and observed under a confocal microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis. Data shown represent the mean ± standard deviation from three independent experiments. Statistical comparisons were performed using one-way analysis of variance followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS version 19.0 software (IBM Corp, Armonk, NY, USA).

Results

Lipid rafts regulate lamellipodia formation in A375 cells. To investigate the role of lipid rafts in lamellipodia formation in A375 cells, a cell migration model was established by scratch wound healing, and 5 mM MβCD was used to deplete cholesterol and disrupt the integrity of the lipid rafts (22). At 3 h post-wounding, 50% of cells without MβCD treatment extended broad lamellipodia towards the scratch area (Fig. 1A and B). However, 5 mM MβCD-treated A375 cells appeared to have lost the ability to form lamellipodia protrusions (Fig. 1A). The percentage of cells extending lamellipodia following MβCD treatment was decreased to <20% of cells (Fig. 1B). When cholesterol was added to the MβCD-treated A375 cells to rescue lipid raft integrity, the strongly suppressed lamellipodia reformed (Fig. 1A and B). These results indicated that intact lipid rafts are indispensable in lamellipodia formation in A375 cells.

Lipid rafts are asymmetrically distributed to the leading edge in migrating A375 cells. To further investigate how lipid rafts influence the lamellipodia formation of A375 cells, the distribution of lipid rafts in migrating cells was first detected. A375 cells were wounded and subjected to immunostaining for ganglioside GM1, which is raft-enriched and is regarded as a marker to identify lipid rafts. The results showed that lipid rafts were asymmetrically distributed in the cell membrane during the formation of the lamellipodia. When the cells were initially wounded (0 h; Fig. 2), GM1 was homogeneously distributed in the cytoplasm and at the cell periphery of A375 cells. At 1 h post-wounding, GM1 aggregated at the leading edge of A375 cells, and this was followed by the formation of small, scattered lamellipodia (2 h) (Fig. 2). At 3 h post-wounding, broad, flat lamellipodia formed towards the scratch area, and this was accompanied by increased GM1 at the leading edge of the lamellipodia (Fig. 2). However, when MβCD was added following wounding, GM1 remained evenly distributed and lamellipodia formation was inhibited (1-3 h; Fig. 2). These results indicated that the aggregation of lipid rafts at the leading edge in cell membranes contributes to lamellipodia formation.

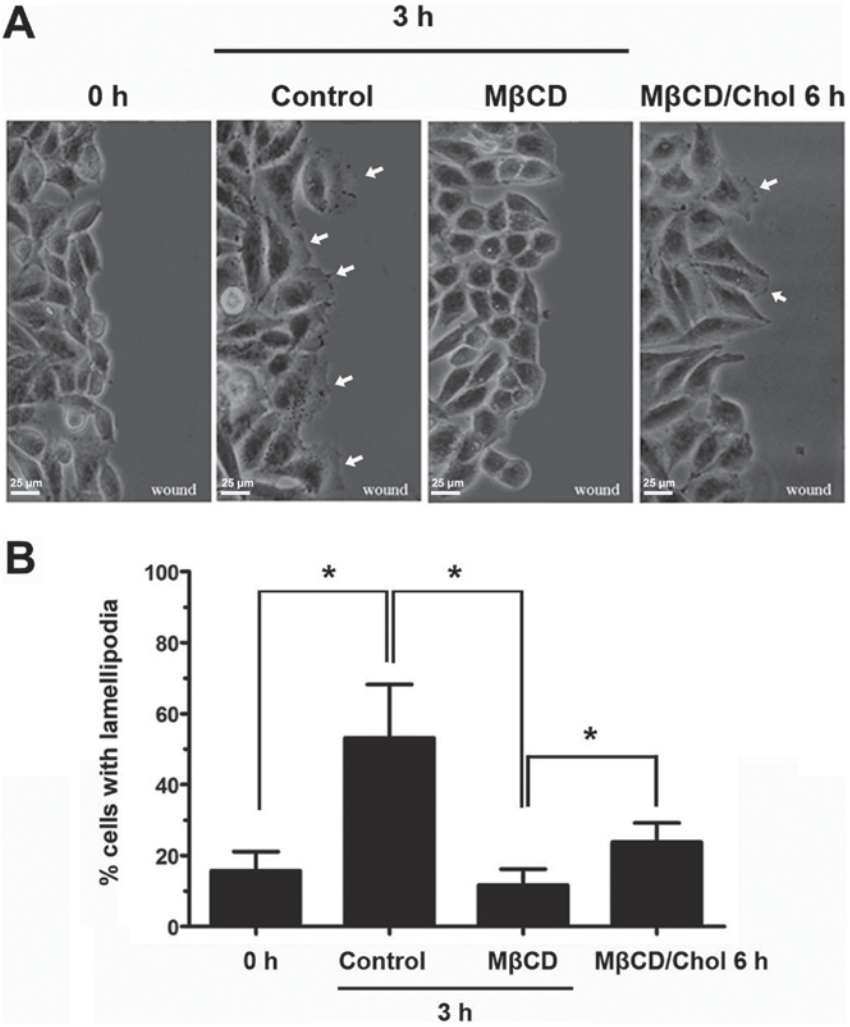

Figure 1. Disruption of lipid rafts inhibits lamellipodia formation in A375 cells. (A) Confluent monolayers of A375 cells were wounded and treated with 5 mM MβCD or left untreated for 3 h. MβCD was then removed, and 1 mM cholesterol was added for 6 h. Phase contrast microscopy was used to observe the formation of lamellipodia at the wound edges in cells treated with different drugs. (B) Percentages of A375 cells with lamellipodia formation following various treatments. Values are presented as the mean ± standard deviation from at least three independent experiments. *P<0.05. MβCD, methyl-β cyclodextrin; Chol, cholesterol.

Lipid rafts recruit β1 and β3 integrins to lamellipodia. Lipid rafts function as scaffold-like platforms for protein recruitment and signal transfer. Therefore, we speculated that lipid rafts may spatially concentrate the proteins closely associated with lamellipodia formation to the leading edges of cells. Integrins are believed to be crucial adhesion molecules in cell membranes that mediate lamellipodia formation (15). Our previous data have shown that β1 and β3 integrins are highly expressed in melanoma A375 cells and are closely associated with cell migration (23). Thus, the present study sought to investigate whether lipid rafts regulate lamellipodia formation by affecting the distribution of β1 and β3 integrin. Immunofluorescence staining revealed the different distributions of β1 and β3 integrins in the lamellipodia of A375 cells prior to and following MβCD treatment. In control migrating A375 cells, β1 integrin, which showed a dispersed distribution, colocalized with GM1 mainly at the front edges of the lamellipodia (Fig. 3A). However, β3 integrin, which had a spot-like distribution in the lamellipodia, colocalized with GM1 within the cell membrane surrounding the front edge of the lamellipodia (Fig. 3B). Following MβCD treatment for 3 h, the morphology of the cells became spindle shaped. Meanwhile, the asymmetrical distribution of β1 integrin disappeared and became an even distribution in the periphery of the cells (Fig. 3A). By contrast, β3 integrin was visible as larger foci following MβCD treatment and was distributed at the cell periphery (Fig. 3B). These results indicated that β1 and β3 integrins, two important proteins that may serve different roles in lamellipodia formation, are recruited to lamellipodia by lipid rafts.

Actin cytoskeleton is responsible for lipid raft-mediated β1 and β3 integrin distribution in lamellipodia. Given the aforementioned results, the mechanism of lipid raft-mediated β1 and β3 integrin recruitment in lamellipodia was further investigated. The actin cytoskeleton has been reported to associate with lipid rafts, and to regulate their structure and organization (24). In addition, integrins connect the ECM with the actin cytoskeleton inside the cell. Thus, we speculated that the actin cytoskeleton may be responsible for lipid raft-mediated β1 and β3 integrin distribution in lamellipodia. To verify this hypothesis, the colocalization between β1 integrin and actin,

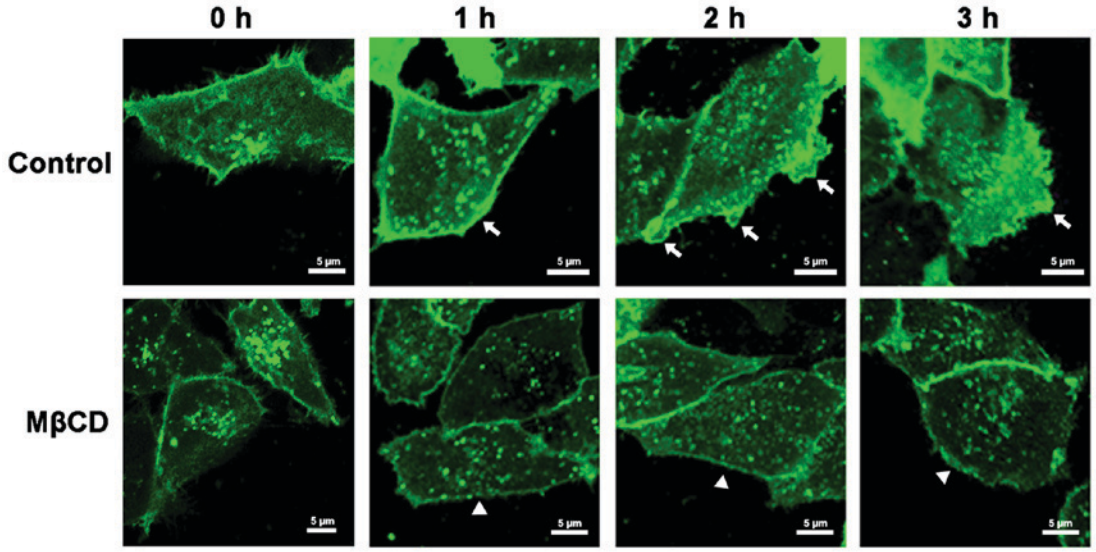

Figure 2. Effects of MβCD treatment on the distribution of lipid rafts in migrating A375 cells. Control and 5 mM MβCD-treated A375 cells were fixed at 0, 1, 2 and 3 h post-wounding, then stained for GM1 and imaged with confocal microscopy (x60 magnification). The arrows in the images indicate the asymmetrical distribution of GM1 in cell membranes and the lamellipodia formation. The arrowheads in the images indicate the cells without lamellipodia. Scale bar, 5 μm. MβCD, methyl-β cyclodextrin; GM1, monosialotetrahexosyl ganglioside.

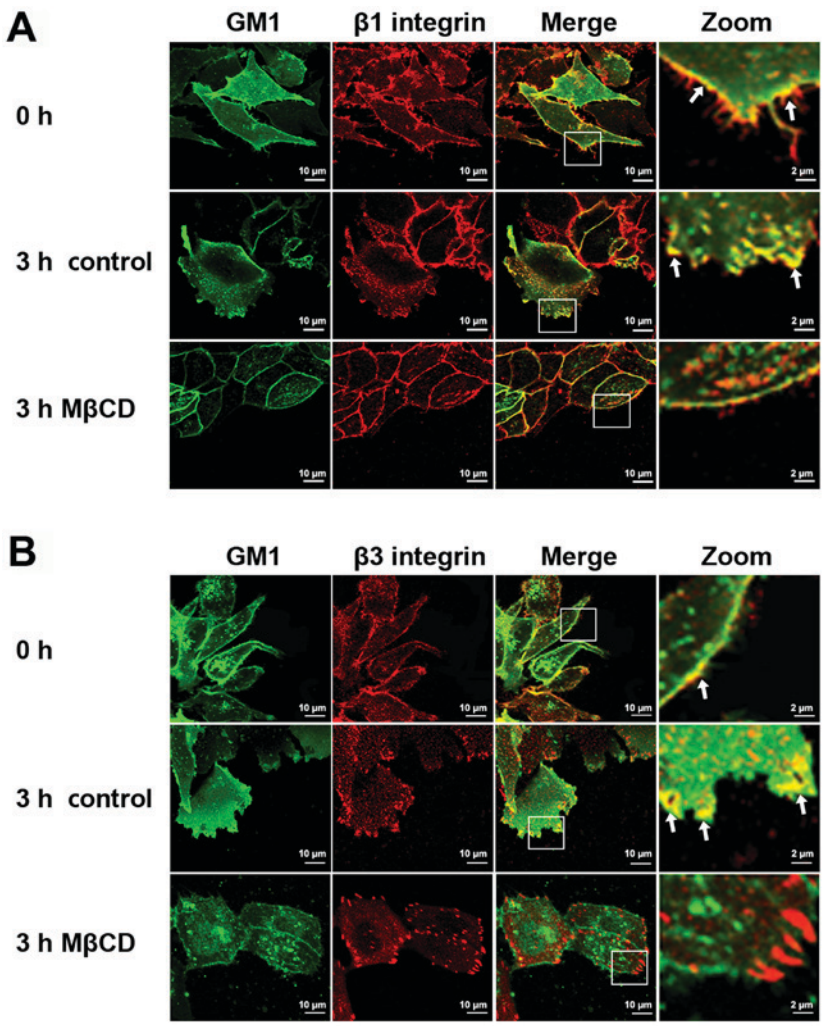

Figure 3. Lipid rafts recruit β1 and β3 integrins to lamellipodia. (A) A375 cells treated with MβCD for 0 and 3 h, or left untreated, were stained for GM1 (green) and β1 integrin (red). (B) A375 cells treated with MβCD for 0 and 3 h, or left untreated, were stained for GM1 (green) and β3 integrin (red). The images were obtained with confocal microscopy (x60 magnification). The right panel shows magnified views of the boxed area in the merged images. The arrows in the images indicate the colocalization between GM1 and β1 integrin, or GM1 and β3 integrin. Scale bar, 10 μm. MβCD, methyl-β cyclodextrin; GM1, monosialotetrahexosyl ganglioside.

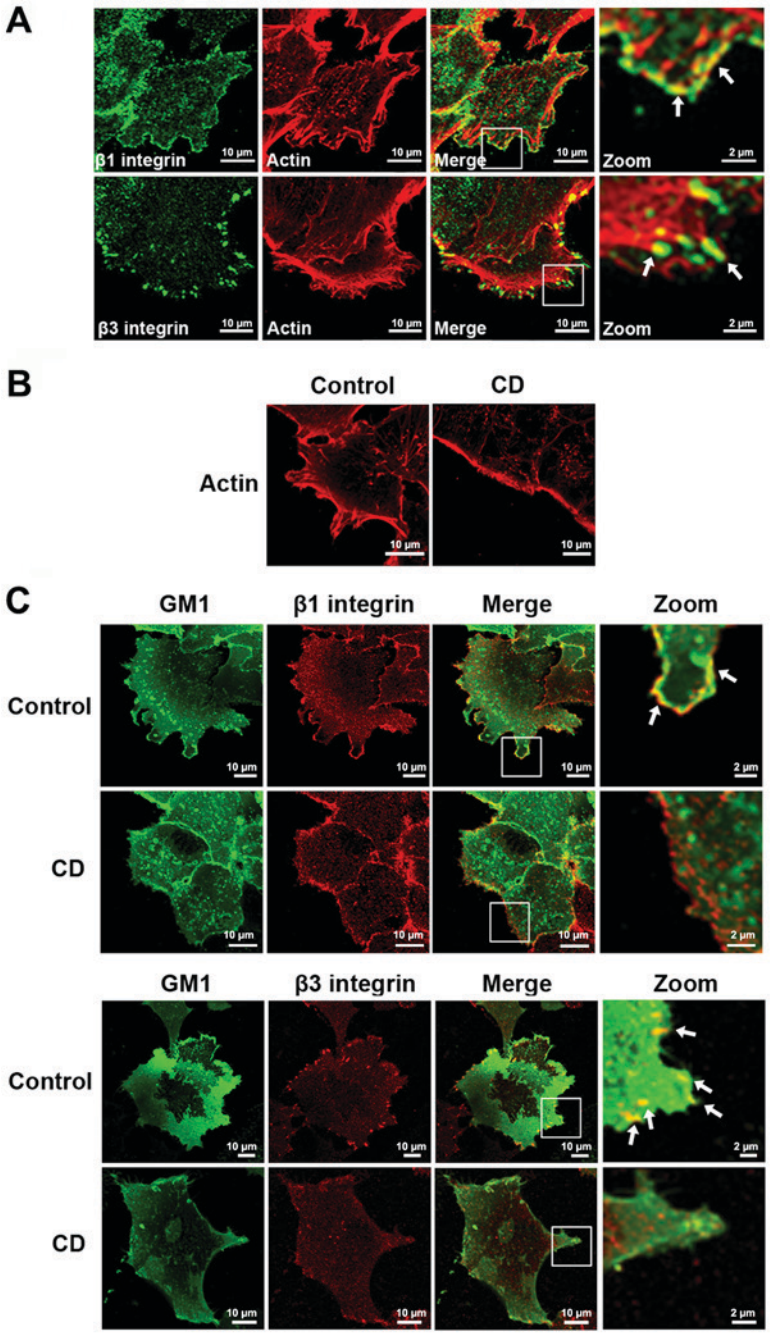

Figure 4. Lipid raft-mediated β1 and β3 integrin distribution in lamellipodia requires an intact actin cytoskeleton. (A) Migrating A375 cells were stained for F-actin (red), β1 integrin and β3 integrin (green), respectively. (B) A375 cells treated with 0.05 μg/ml CD for 1 h, or left untreated, were stained for F-actin. (C) A375 cells treated with 0.05 μg/ml CD for 1 h, or left untreated, were stained for GM1 (green), β1 integrin and β3 integrin (red) antibody, respectively. The images were obtained with confocal microscopy (x60 magnification). The right panel shows magnified views of the boxed area in the merged images. The arrows in the images indicate the colocalization. Scale bar, 10 μm. CD, cytochalasin D; GM1, monosialotetrahexosyl ganglioside.

and β3 integrin and actin was first detected. β1 integrin colocalized with the submembranous cortical actin cytoskeleton at the forefront of the lamellipodia. However, β3 integrin and actin colocalized at the ends of the stress fibers, which were inside the leading edge (Fig. 4A). These results indicated that the actin cytoskeleton was associated with the β1 and β3 integrins. Next, the cells were treated with CD to disrupt the actin cytoskeletal arrangement, and it was found that the colocalization between GM1 and β1 integrin, and GM1 and β3 integrin, in the lamellipodia disappeared following CD treatment (Fig. 4B and C). Together, the results suggest that the actin cytoskeleton may be responsible for lipid raft-mediated β1 and β3 integrin distribution in lamellipodia.

Discussion

Lipid rafts, detergent-resistant membrane domains enriched in cholesterol and sphingolipids, have been implicated in cancer progression, including the migration and invasion of cancer cells (25,26). However, the role of lipid rafts in lamellipodia formation remains obscure. In the present study, by observing the morphological changes in lipid raft-disrupted A375 cells,

it was determined that intact lipid rafts are indispensable in lamellipodia formation in melanoma cells.

One of the most critical functions of lipid rafts is to act as platforms for localizing signaling proteins and eliciting signal transduction (9). This characteristic has been extensively reported, particularly in T cells and other leukocytes (27-30). Several cell surface receptor proteins have been reported to be localized to lipid rafts and to execute their functions through association with lipid rafts (28,31,32). In resting cells, lipid rafts are evenly distributed over the entire cell surface. However, when the cells are stimulated, lipid rafts move laterally and coalesce into larger aggregated patches, thus resulting in the concentration and redistribution of raft-associated proteins, and efficient and sustained signal transduction (33). In the present study, using immunofluorescence assays, it was found that as A375 cells migrated to the scratches, lipid rafts aggregated at the leading edge of the cells with an asymmetrical distribution, which was critical for lamellipodia formation.

Given these results and the function of lipid rafts as platforms, we speculated that proteins associated with lipid rafts and lamellipodia formation may be recruited to the leading edges of A375 cells along with the asymmetrical distribution of lipid rafts. β1 and β3 integrins have been found to be membrane raft-associated proteins (23). As adhesion molecules, β1 and β3 integrins regulate the migration and lamellipodia formation of cancer cells. Thus, the present study examined whether the asymmetrical distribution of lipid rafts could aggregate β1 and β3 integrin into the lamellipodia of A375 cells. The results showed that in migrating A375 cells, β1 and β3 integrins colocalized with GM1 in the lamellipodia. However, MβCD treatment changed the distributions of β1 and β3 integrins in the cells and inhibited lamellipodia formation, thus suggesting that lipid rafts are involved in the recruitment of β1 and β3 integrin in the lamellipodia. In the process of these experiments, a notable phenomenon of β1 and β3 integrin being differently distributed in A375 cells became apparent. Subsequent research on the colocalization of β1 and β3 integrins with the actin cytoskeleton also confirmed that β1 integrin was distributed mainly along the leading edge of the lamellipodia, thereby determining the shape of the cell, whereas β3 integrin was distributed inside the leading edge of the lamellipodia and colocalized with actin at the ends of stress fibers. Despite belonging to the same family, the different distribution characteristics of β1 and β3 integrin suggested that they have different functions. In fact, distinct functions of β1 and β3 integrin in different cell types or even at different stages of one cell type have been reported. For example, β1 integrin is widely distributed on various cell types and is involved in the physiological processes of cell proliferation, survival and differentiation (34). However, β3 integrin is mainly expressed on the surface of cancer cells and platelets, and it participates in the migration and invasion of tumor cells, and the coagulation of platelets (35). In cell migration, β1 integrin promotes random migration, whereas β3 integrin promotes persistent migration in the same epithelial cell background (36). In the assembly of focal adhesion, β1 integrin has been shown to be the core component of focal adhesion in epithelial cells isolated from human breast tumors and in spreading human melanoma A375 cells (23,37), whereas in migrating A375 cells, focal adhesions largely consist of β3 integrin (22). However, to the best of our knowledge, this is the first study to describe the distinct distributions of the two integrins in the lamellipodia formation of A375 cells.

Next, the present study detected how lipid rafts recruit β1 and β3 integrin to the lamellipodia. Lipid rafts and the actin cytoskeleton have been reported to be closely associated. In our previous study, it was found that lipid rafts regulate the dynamics of the actin cytoskeleton. When the integrity of lipid rafts is disrupted, A375 cells form strong stress fibers, thus suggesting inhibition of the depolymerization of the actin cytoskeleton (22). By contrast, other studies have indicated that the organization, structure and function of lipid rafts requires an intact actin cytoskeleton (24). Thus, the present study investigated the role of the actin cytoskeleton in lipid raft-mediated β1 and β3 integrin distribution in the lamellipodia. Immunofluorescence assays showed that the actin cytoskeleton colocalized with β1 and β3 integrins. When the arrangement of the actin cytoskeleton was disrupted by CD, the distribution of β1 and β3 integrin in lamellipodia, and their colocalization with GM1 disappeared. These results demonstrated that lipid rafts recruit β1 and β3 integrin to lamellipodia via the actin cytoskeleton.

In summary, the present data indicated that lipid rafts recruit β1 and β3 integrin to the leading edge in melanoma A375 cells, thereby facilitating lamellipodia formation, in a manner dependent on the intact actin cytoskeleton. These findings provide novel insight into the association between lipid rafts and lamellipodia formation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ created the study concept and designed the experiments. JB performed the experiments and wrote the manuscript. RW analyzed the data and edited the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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