Biochemical Pharmacology 88 (2014) 364–371

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Role of lipid raft components and actin cytoskeleton in fibronectin-binding, surface expression, and *de novo* synthesis of integrin subunits in PGE_2- or 8-Br-cAMP-stimulated mastocytoma P-815 cells☆

Yasuyo Okada, Jyun-ichi Nishikawa, Masanori Semma, Atsushi Ichikawa *

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Institute for Biosciences, Mukogawa Women's University, 11-68 Koshien-Kyubancho, Nishinomiya-shi, Hyogo 663-8179, Japan

ARTICLE INFO

Article history:
Received 2 November 2013
Received in revised form 27 January 2014
Accepted 28 January 2014
Available online 8 February 2014

Keywords:
Lipid raft components
Integrin
Cell adhesion
Fibronectin
Prostaglandin E_2

ABSTRACT

Integrins are heterodimeric adhesion receptors essential for adhesion of non-adherent cells to extracellular ligands such as extracellular matrix components. The affinity of integrins for ligands is regulated through a process termed integrin activation and *de novo* synthesis. Integrin activation is regulated by lipid raft components and the actin structure. However, there is little information on the relationship between integrin activation and its *de novo* synthesis. Cancerous mouse mast cells, mastocytoma P-815 cells (P-815 cells) are known to bind to fibronectin through *de novo* synthesis of integrin subtypes by prostaglandin (PG) E_2 stimulation. The purpose of this study was to clarify the relationship between lipid raft components and the actin cytoskeleton, and PGE_2-induced P-815 cells adhesion to fibronectin and the increase in surface expression and mRNA and protein levels of αvβ3 and αIIbβ3 integrins. Cholesterol inhibitor 6-*O*-α-maltosyl-β cyclodextrin, glycosylphosphatidylinositol-anchored proteins inhibitor phosphatidylinositol-specific phospholipase C and actin inhibitor cytochalasin D inhibited PGE_2-induced cell adhesion to fibronectin, but did not regulate the surface expression and mRNA and protein levels of αv and αIIb, and β3 integrin subunits. In addition, inhibitor of integrin modulate protein CD47 had no effect on PGE_2- and 8-Br-cAMP-induced cell adhesion. These results suggest that lipid raft components and the actin cytoskeleton are directly involved in increasing of adhesion activity of integrin αIIb, αv and β3 subunits to fibronectin but not in stimulating of *de novo* synthesis of them in PGE_2-stimulated P-815 cells. The modulation of lipid rafts and the actin structure is essential for P-815 cells adhesion to fibronectin.

1. Introduction

Plasma membranes generally contain several types of liquid-ordered domains with high concentrations of cholesterol and lipids, which are often termed lipid rafts. These domains are also enriched in sphingolipids, and glycosylphosphatidylinositol-anchored proteins (GPI-Aps) and other lipid-linked proteins [1–3], and they can modulate signaling pathways in diverse biological processes such as cell division, shape modulation, adhesion and motility [1,2,4–6].

On the other hand, integrins are a family of α and β heterodimeric adhesion metalloprotein receptors that facilitate the adhesion of cells to the extracellular matrix (ECM), as well as play a role in cellular functions such as survival, migration, proliferation and differentiation [7,8]. Integrin-mediated cell adhesion is highly dependent on and regulated by lipid raft components such as cholesterol, phospholipids, sphingolipids, and GPI-Aps, and the cytoskeleton [6,9], as well as divalent cations such as Ca^{2+} and Mn^{2+} which act via integrin metal ion-binding sites [10]. Indeed, depletion and sequestration of cholesterol with β-cyclodextrin derivatives was shown to reduce integrin-mediated cell adhesion to the ECM, which was reversed by cholesterol reconstitution [11–13]. Furthermore, modification of phospholipids, membrane fluidity and cholesterol content in rat fibroblasts was reported to reduce α5β1 integrin binding activity to fibronectin and focal adhesions [14]. The cell adhesion activity of integrin is mediated via multiple contact sites, by conformational changes in integrins and their cluster formation by lateral association on the membrane [15–17].

* Corresponding author.
E-mail address: aichikaw@mukogawa-u.ac.jp (A. Ichikawa).

http://dx.doi.org/10.1016/j.bcp.2014.01.039
0006-2952/

However, the mechanism and regulation of the *de novo* expression of integrins, and their interaction with lipid raft components to promote cell adhesion have not been clarified to date [18–21]. Studies on the interaction of lipid raft components to integrins in response of cell adhesion have mostly analyzed the role of individual integrins that are present on the cell surface, but not the role of integrins that are newly expressed on the cell surface as a response to various stimuli.

Mast cells are inflammatory and immunoregulatory cells resident in tissues. They develop from bone-derived progenitor cells that enter the tissues through the blood circulation. The specific localization and migration of mast cells in tissues is dependent on their interaction with the ECM [22]. The adhesion of mast cells is critical for their function such as maturation of precursor mast cells in response to stem cell factor and interleukin-4, and IgE-dependent degranulation [23]. However, we know very little about what regulatory mechanisms are involved in mast cell attachment to the ECM. Mouse mastocytoma P-815 cells (P-815 cells) are established as suspension cultured cells, which originated from mouse skin-derived cancerous mast cells with mucosal mast cell-like phenotypes [24]. Therefore, P-815 cells are regarded as a suitable model cells for examination of mast cell attachment to the ECM. Because, we previously demonstrated that prostaglandin (PG) E_2 stimulates the adhesion of P-815 cells to the Arg-Gly-Asp (RGD)-enriched peptide of fibronectin (RGD matrix) via PGE_2 receptor subtype EP4-mediated cAMP signaling, which stimulates the *de novo* synthesis and cell surface expression of integrin αIIb, αv and β3 subunits [19,25]. These results led us to examine whether PGE_2-induced synthesis and cell surface expression of integrin subunits, and P-815 cell adhesion to the RGD matrix are affected by the modification of lipid raft components and the actin cytoskeleton. Integrin receptor activation has been extensively characterized to illustrate how a low-affinity binding site is transformed into a high affinity form, an event that can modify cell adhesion [26]. Involvement of lipid rafts such as cholesterol was determined in the regulation integrin function [27]. Integrin associated protein CD47 is a transmembrane protein belonging to the immunoglobulin superfamily and interacts with integrins, G-proteins and GPI-Aps, forming a complex localize in lipid rafts [28]. CD47 has been previously shown to associated with integrin β subunits, mainly β3 and slightly β1 and β2 [29] In our previous paper, we showed that P-815 cells express β1, β2, β3 and β7, but among them integrin β3 specifically involved in adhesion of PGE_2- or 8-Br-cAMP-stimulated P-815 cells to the RGD matrix [19]. These data indicate that CD47 may have a role in integrin β3-mediated P-815 cell adhesion to the RGD matrix.

In the present paper, we show that cholesterol, GPI-Aps, and the actin cytoskeleton modulate the binding activity of integrin αIIb, αv and β3 subunits to the RGD matrix, but do not modulate integrin mRNA and protein levels in PGE_2- or 8-Br-cAMP-stimulated P-815 cells. In addition, CD47 has no effect on PGE_2-induced P-815 cell adhesion.

2. Materials and methods

2.1. Cell culture

P-815 cells were maintained in suspension culture in Fisher's medium containing 10% fetal calf serum (FCS) at 37 °C in a CO_2-humidified atmosphere [25]. Cell viability was determined by the trypan blue exclusion method.

2.2. Adhesion assay

P-815 cells were seeded in wells coated with 10 μg/mL of ProNectin-F^{TM} at a density of 5×10^5 cells/mL, and incubated in 0.5 mL Fisher's medium with 10% FCS in the presence or absence of the test compounds for various times [19]. The adherent cells were collected by treatment with phosphate buffered saline (PBS) containing 0.02% EDTA and 0.25% trypsin at 37 °C for 5 min, and the numbers of adherent cells and non-adherent cells were counted using a COULTER Z1 cell counter (Beckman Coulter, Brea, CA, USA). The percentage of adherent cells was calculated according to the following formula: cell adhesion (%) = number of adherent cells × 100/(number of adherent cells + number of non-adherent cells). At least three independent experiments were carried out for each condition.

2.3. Measurement of cAMP formation

P-815 cells (1×10^6 cells) were incubated in 0.5 mL HEPES-buffered saline containing 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, and 15 mM HEPES (pH 7.4) in the presence or absence of test compounds for 10 min at 37 °C. After incubation, cells were collected and their cAMP levels were determined by a radioimmunoassay as reported previously [25].

2.4. Preparation of 6-O-α-maltosyl-β cyclodextrin and cholesterol loading of 6-O-α-maltosyl-β cyclodextrin complexes

To deprive cells of cholesterol, P-815 cells were treated with 6-*O*-α-maltosyl-β cyclodextrin (Mal-βCD), which was previously developed by our group as a compound having much less cytotoxicity compared with the commercially available methyl-βCD derivatives tested [30–32]. Mal-βCD forms cholesterol-inclusion complexes (CLMs) when it is mixed with cholesterol powder in FCS-free Fisher's medium. The CLMs are separated from free cholesterol by filtration through a 0.2 μm Millipore filter (Merk Millipore, Tokyo, Japan). To load cholesterol, CLMs were applied to the Mal-βCD-treated cholesterol-deprived cells.

2.5. Quantification of the surface expression level of integrin by fluorescence activated cell sorter analysis

Fluorescence activated cell sorter (FACS) analysis was performed according to a previously described method [19]. Briefly, P-815 cells (5×10^5 cells) were suspended in PBS containing 2% FCS (2% FCS/PBS) and incubated with a rat anti-mouse CD16/CD32 mAb (1:30 in 2% FCS/PBS) for 1 h. The washed cells were incubated with the appropriate anti-mouse integrin mAb (1:80 in 2% FBS/PBS) for 30 min. Anti-mouse integrin mAbs used were PE-conjugated anti-αIIb mAb, PE-conjugated anti-αv mAb and PE-conjugated anti-β3 mAb. The appropriate isotype-matched negative controls were used. All Abs mentioned above were from Becton Dickinson Biosciences (San Jose, CA, USA). The cells were then washed and suspended in 2% FCS/PBS. All procedures were performed at 4 °C. The cell samples were then analyzed using a FACScan flow cytometer (Nippon Becton Dickinson, Tokyo, Japan) with CellQuest Pro software (Nippon Becton Dickinson). Propidium iodide was used to determine the number of dead cells. For determination of the fluorescence intensity, 10,000 cells/sample were analyzed.

2.6. Quantitative RT-PCR analyses

Quantitative RT-PCR analysis was performed basically as described previously [19]. Briefly, total RNAs were isolated from P-815 cells using the TaqMan RNA to four kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNAs were reverse transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) and