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Induction of integrin β3 in PGE_2-stimulated adhesion of mastocytoma P-815 cells to the Arg-Gly-Asp-enriched fragment of fibronectin

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ABSTRACT

We previously demonstrated that prostaglandin (PG) E_2 stimulates adhesion of mastocytoma P-815 cells (P-815 cells) to the Arg-Gly-Asp (RGD)-enriched matrix via the PGE_2 receptor subtype EP4 [Hatae N, Kita A, Tanaka S, Sugimoto Y, Ichikawa A. Induction of adherent activity in mastocytoma P-815 cells by the cooperation of two prostaglandin E_2 receptor subtypes, EP3 and EP4. J Biol Chem 2003;278:17977–81]. Here we investigated the role of various integrin subtypes in the induction of adherent activity in PGE_2-stimulated P-815 cells. FACS analysis showed that P-815 cells express high levels of integrin α4, α5, β1 and β2 subunits and moderate levels of integrin αIIb, αv, β3 and β7 subunits. When treated with PGE_2, the EP4 agonist ONO-AE1-329 or the cell permeable cAMP analogue, 8-Br-cAMP, P-815 cells showed markedly increased cell surface expression of integrin αIIb, αv and β3 subunits, and these expressions were significantly reduced by addition of the protein synthesis inhibitor cycloheximide. Along with increased cell surface expression, mRNA and protein levels of the integrin β3 subunit, but not of integrin αIIb and αv subunits, were simultaneously elevated. On the other hand, adhesion of P-815 cells in response to PGE_2 or 8-Br-cAMP was abolished by antibodies specific for integrin αv and β3 subunits, but not by antibodies for integrin α4, α5, β1, β2 and β7 subunits. Moreover, treatment with tirofiban, an integrin αIIbβ3 antagonist, or eptifibatide, an integrin αvβ3/αIIbβ3 antagonist resulted in a decrease in adhesion of P-815 cells in response to PGE_2 or 8-Br-cAMP. These results suggest that *de novo* synthesis of the integrin β3 subunit plays a pivotal role in PGE_2-induced adhesion of P-815 cells to the RGD-enriched matrix through EP4-mediated cAMP signaling.

1. Introduction

Cell adhesion is of fundamental importance to many normal biological processes, including inflammation and the function of immune responses. Mast cells [1] are an active participant in the pathogenesis of local inflammation and modulation of immune responses. Mast cells, which originate from bone marrow stem cells, traffic throughout the circulation and adhere to the extracellular matrix (ECM) in various tissues. Therefore, it is important to understand the mechanisms underlying mast cell adherence. Adhesion of the cells to the ECM is mediated by members of the integrin superfamily of cell surface receptors. The interaction of mast cell integrin with ECM is involved in multiple physiological processes including recruitment of mast cell progenitors from the circulation into connective tissues [2–4], and mast cell functions such as β-hexosaminidase release [5], cytokine production [6], survival [7], growth [8] and migration [9–11].

Prostaglandin (PG) E_2 is one of the major eicosanoids produced during inflammatory responses. Although mast cells are not the major source of PGE_2, a variety of cells that can generate PGE_2 are found in close proximity to mast cells, such as smooth muscle cells, respiratory epithelial cells, fibroblasts and macrophages [12–14]. Based on these findings we hypothesized that PGE_2 can affect the adhesion of mast cells to the ECM components by acting on specific receptors. Using a mouse mastocytoma cell line, P-815 (P-815 cells), we previously demonstrated that PGE_2 induces the adhesion of the cells to the Arg-Gly-Asp (RGD)-enriched matrix through elevation of cAMP levels mediated by PGE_2-receptor EP4 subtype activity [15]. However, little is known about the types of integrins involved and how they bind upon PGE_2-stimulation in P-815 cells.

Integrins are heterodimeric cell surface receptors which are composed of α and β subunits in non-covalent association. There are at least 17 α and 9 β subunits, which can combine to generate 25 integrin subtypes. At least eight subtypes are known to bind the RGD triad present in ligands [16]. Integrin α5β1 is one of the preferential subtypes taking part in the attachment of mouse bone marrow-derived mast cells (BMMC), when activated by IgE and

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antigen [7,17], monomeric IgE [18], stem cell factor (SCF) [19,20] and thrombin [21]. In addition, Takahashi et al. reported that 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced adhesion of canine mastocytoma cells to fibronectin, laminin and collagen was mediated through integrin α5β1 [22]. In addition to integrin α5β1, there are some reports showing the participation of integrin αv or αIIb to vitronectin in the attachment of BMMC [8], and integrin αIIbβ3 to fibrinogen in BMMC [23] and human cord blood-derived mast cells [24]. These studies demonstrate the dominant types of integrins that are responsible for the attachment of mast cells to the ECM depending on the cell origins and their activation states.

We noticed that in the above studies, similar short periods of 1–3 h are used as the optimal incubation time required for BMMC adhesion [18–24]. However, at least 6 h is required for PGE_2-induced adhesion of P-815 cells to the RGD-enriched matrix to occur, suggesting the essential role of a lag time for this event [15]. Consequently we speculated that in the case of PGE_2-induced adhesion of P-815 cells, *de novo* synthesis of integrins might play an important role. The present study was performed to test this speculation. As a result, we found that the inducible integrin β3 is essential for the adhesion of PGE_2-stimulated P-815 cells.

2. Materials and methods

2.1. Materials

An EP4-specific agonist, ONO-AE1-329, was kindly provided by ONO Pharmaceuticals (Osaka, Japan). ProNectin-F^{TM} (ProF, a protein polymer containing multiple copies of the RGD sequence from human fibronectin; the RGD-enriched matrix fragment) was from Sanyo Chemical Industries (Kyoto, Japan). The following materials were obtained from the sources indicated; PGE_2 from Cayman Chemical (Michigan, USA), Fisher's medium from ICN Biomedicals (Irvine, USA), cycloheximide from Merck Calbiochem (Darmstadt, Germany), the fibronectin active fragment (GRGDS) from PEPTIDE institute (Osaka, Japan), tirofiban from Toronto Research Chemicals (Toronto, Canada) and eptifibatide from ProSpec-Tany TechnoGene (Rehovot, Israel). Integrin-function-blocking monoclonal antibodies (mAbs), anti-α4 mAb, anti-α5 mAb, anti-αv mAb, anti-β1 mAb, anti-β2 mAb, anti-β3 mAb and anti-β7 mAb, and all isotype-matched negative controls, were from Becton Dickinson Biosciences (San Jose, USA).

2.2. Cell culture and cytotoxicity

P-815 cells were maintained in suspension culture in Fisher's medium containing 10% FCS at 37 °C in a CO_2-humidified atmosphere [15]. Cell viability was determined by the trypan blue exclusion method.

2.3. Adhesion assay

Twenty four-well tissue culture plates were coated with 10 μg/ml of ProNectin-F^{TM}, as described previously [15]. For the adhesion assay, 0.5 ml of P-815 cells were seeded in each well at a density of 5×10^5 cells/ml, and incubated in Fisher's medium with 10% FCS in the presence or absence of test compounds for various times. After removing the floating cells by aspiration, the adherent cells were recovered by treatment with PBS containing 0.02% EDTA and 0.25% trypsin at 37 °C for 5 min, and the collected cells were suspended in PBS containing 0.02% EDTA and 2% FCS. The numbers of non-adherent and adherent cells were counted using a COULTER Z1 cell counter (Beckman Coulter, Brea, USA). The percentage of adherent cells were calculated according to the following formula: cell adhesion (%) = the number of adherent cells × 100/(the number of adherent cells + the number of non-adherent cells). At least three independent experiments were carried out for each condition.

2.4. FACS analysis

P-815 cells (5×10^5 cells) were washed twice with PBS containing 2% FCS (2% FCS/PBS) and incubated with rat anti-mouse CD16/CD32 mAb (1:30 in 2% FCS/PBS) in order to block nonspecific binding sites for 1 h. The cells were washed with 2% FCS/PBS and incubated with the appropriate anti-mouse integrin mAb (FITC or PE conjugated, 1:80 in 2% FBS/PBS) for 30 min. Anti-mouse integrin mAbs used were PE-conjugated anti-α2 mAb, FITC-conjugated anti-α4 mAb, PE-conjugated anti-α5 mAb, PE-conjugated anti-αIIb mAb, PE-conjugated anti-αv mAb, FITC-conjugated anti-β1 mAb, PE-conjugated anti-β2 mAb, PE-conjugated anti-β3 mAb and FITC-conjugated anti-β7 mAb. Appropriate isotype-matched negative controls were used. All Abs mentioned above were from Becton Dickinson Biosciences. The cells were then washed twice with 2% FCS/PBS, and the cell pellet was resuspended in 2% FCS/PBS. All procedures were performed at 4 °C. The samples were then analyzed using a FACScan flow cytometer (Nippon Becton Dickinson, Tokyo, Japan) with CellQuest Pro software (Becton Dickinson). Before the analysis, propidium iodide was added to the cell suspension to determine the number of dead cells and exclude them from the analysis. For determination of the fluorescence intensity, 10,000 cells/sample were analyzed. The percentage of positive cells and the geometric mean fluorescence intensity were recorded.

2.5. Quantitative RT-PCR analysis

Total RNAs were isolated from P-815 cells using TaqMan RNA to 4 kit (Applied Biosystems, Carlsbad, USA) according to the manufacture's instructions. Total RNAs were reversed transcribed using High Capacity RNA-to-cDNA Kit (Applied Biosystems) and amplified by PCR using primers for each integrins as follows; αIIb (Mm00439768_m1), αv (Mm00434506_m1), β3 (Mm00443980_m1), and GAPDH (Mm99999915_g1). Real time PCR was assayed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Normalization of samples was achieved by measurement of the endogenous reference gene, GAPDH. All reactions were run in triplicate, and the mean value was used to calculate the ratio of target gene/GAPDH expression in each sample.

2.6. Western blotting analysis

P-815 cells were collected, washed two times in PBS, and lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 1 M NaCl, 250 mM EDTA, 500 mM EGTA, 1% Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, 1 mM dithiothreitol, and protease inhibitor cocktail) for 30 min at 4 °C, and centrifuged at 15,000 × *g* for 30 min at 4 °C. The resultant supernatant (30 μg protein/lane) was subjected to SDS–PAGE on 7.5% polyacrylamide gels, and the separated proteins were transferred electrophoretically onto a PVDF membrane (Millipore, Tokyo, Japan). Immunoblot analysis was performed as described previously [25]. An anti-integrin αIIb mAb (1:20,000, R & D Systems, Minneapolis, USA), rabbit anti-integrin αv Ab (1:10,000, Millipore), and rabbit anti-integrin β3 Ab (1:10,000, Cell Signaling, Danvers, USA), and anti-β actin mAb (1:20,000, Sigma–Aldrich, St. Louis, USA) was used as primary Ab, and a horseradish peroxidase-conjugated anti-mouse IgG Ab (1:10,000, DakoCytomation, Glostrup, Denmark), -rabbit IgG Ab (1:2500, DakoCytomation), and -rabbit IgG Ab (1:5000), and -mouse IgG Ab (1:10,000), respectively, were used as secondary Abs. The membranes were stained using an ECL kit according to the manufacturer's

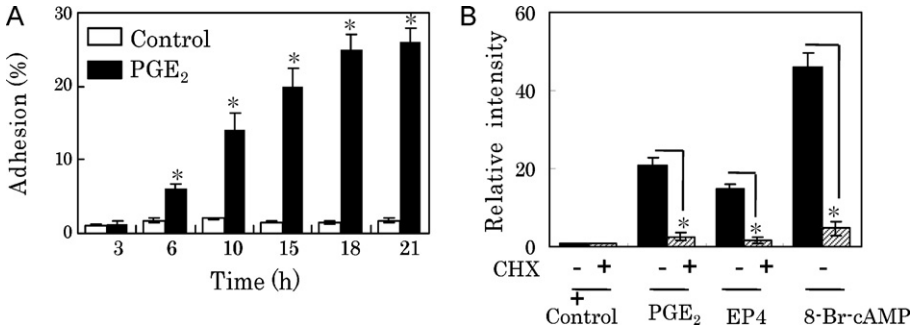

Fig. 1. Effects of PGE_2, an EP4 agonist (ONO-AE1-329) and 8-Br-cAMP on adhesion of P-815 cells to the RGD-enriched matrix. (A) P-815 cells (5×10^5 cells/ml) were incubated on plates coated with the RGD-enriched fragment of fibronectin (RGD matrix) in the presence or absence of 1 μM PGE_2 in Fisher's medium with 10% FCS for various times as indicated. (B) P-815 cells were incubated with or without 0.05 μg/ml cycloheximide (CHX) for 0.5 h, followed by 1 μM PGE_2, 20 nM EP4 agonist (ONO-AE1-329), or 1 mM 8-Br-cAMP for 18 h. In both (A) and (B) after incubation the number of adherent P-815 cells was examined as described in Section 2. Results represent means ± SD of three or more samples, and similar results were obtained in at least three experiments. $^*P < 0.01$ vs. each control. Since CHX retarded the growth rate of cells, the total cell number of control in CHX-treated group (3×10^5 cells) was lower than that in CHX-untreated group (6×10^5 cells), of which values were defined as 1 of "relative intensity" in the respective group.

instructions. Protein concentrations were determined by Bradford Ultra reagent (Novexin, Brussels, Belgium) using bovine serum albumin for the standards.

2.7. Statistical analysis

Data are expressed as mean ± SD. Statistical significance was assumed to be $p < 0.05$ using the Student's t test or one-way ANOVA.

3. Results

3.1. Characteristics of PGE_2-stimulated P-815 cells attachment to the RGD-enriched matrix

As shown in Fig. 1A, the number of P-815 cells attached to the RGD-enriched matrix began to increase at 6 h and reached to a plateau level at 18 h after the addition of PGE_2. Although P-815 cells express both the Gs-coupled EP4 receptor and the Gi-coupled EP3 receptor as cell-specific PGE_2-signal transduction pathway, we confirmed that PGE_2-induced adhesion was preferentially mediated by EP4-induced cAMP formation via Gs [15]. We also found that the increase of adherent cells in response to PGE_2, ONO-AE1-329, or 8-Br-cAMP was inhibited by addition of the protein synthesis inhibitor, cycloheximide (Fig. 1B). These results suggest that PGE_2-induced adhesion of P-815 cells is via a protein synthesis-dependent pathway accelerated by EP4-mediated cAMP signaling.

3.2. Integrins participating in PGE_2-stimulated adhesion of P-815 cells identified by FACS analysis

The expression of a variety of integrins on the surface of P-815 cells in the presence or absence of PGE_2 were determined using

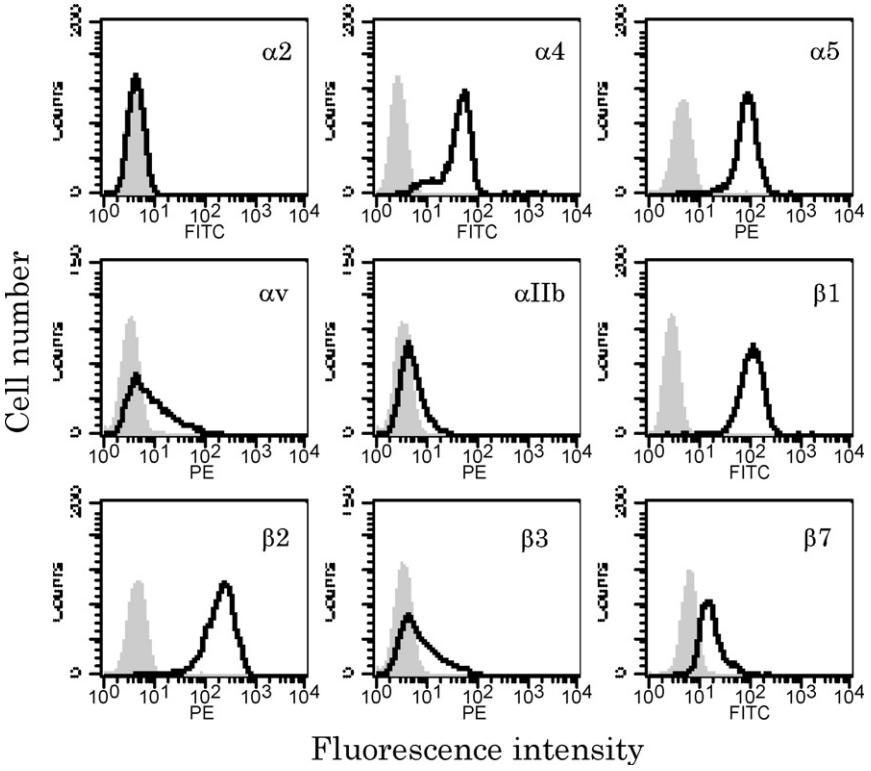

Fig. 2. FACS analysis of integrin subtypes expressed on the surface of P-815 cells. P-815 cells (5×10^5 cells) were incubated with a variety of anti-mouse integrin mAbs in PBS containing 2% FCS for 30 min, followed by FACS analysis. Open graphs indicate the fluorescence profile for integrin-positive cells after fluorescence staining. The gray areas show the profile observed for isotype-matched controls. Similar experimental results were obtained in three or more experiments, and representative results are shown.

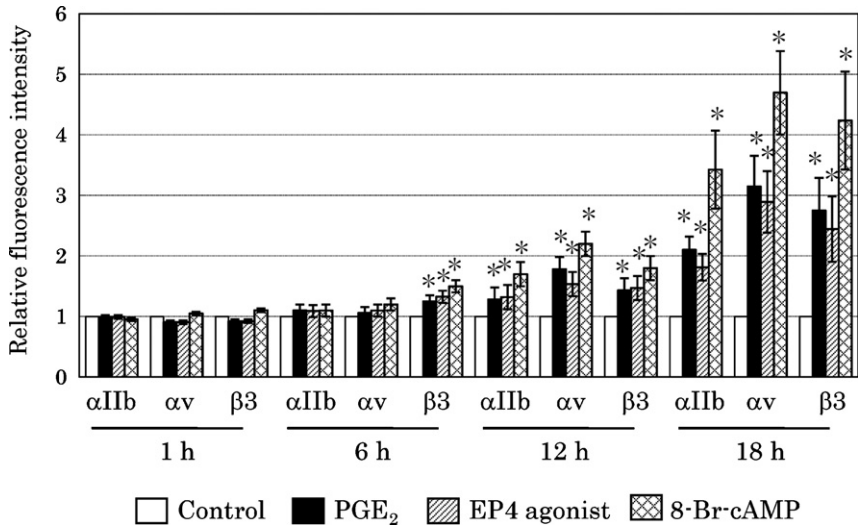

Fig. 3. Time-dependent changes of integrin αIIb, αv and β3 subunits expressed on P-815 cells treated with or without PGE_2, an EP4 agonist (ONO-AE1-329), or 8-Br-cAMP. P-815 cells (5×10^5 cells/ml) were incubated in the presence or absence of 1 μM PGE_2, 20 nM EP4 agonist (ONO-AE1-329), or 1 mM 8-Br-cAMP in Fisher's medium with 10% FCS for various times as indicated, followed by FACS analysis. Relative fluorescence intensities were calculated by dividing the geometric mean fluorescence intensity of the treatment cell sample by the geometric mean fluorescence intensity of the control cell sample. $^*P < 0.05$ vs. each control.

FACS analysis. Under normal conditions, P-815 cells were found to express high levels of integrin subunits α4, α5, β1 or β2, and moderate levels of integrin αIIb, αv, β3 and β7 subunits and no integrin α2 subunit (Fig. 2). Among these, integrin αIIb, αv and β3 subunits were increased in response to PGE_2, ONO-AE1-329, or 8-Br-cAMP, all in a time dependent manner (Fig. 3). Such increases were completely inhibited by the addition of cycloheximide (Fig. 4).

We next examined whether PGE_2 or 8-Br-cAMP could augment mRNA and protein expression levels of these integrin subunits. Both PGE_2- and 8-Br-cAMP treatment increased in integrin β3 subunit mRNA, but not integrin αIIb and αv subunit mRNAs (Fig. 5). The level of integrin β3 subunit mRNA of 8-Br-cAMP-stimulated P-815 cells was higher than that of PGE_2-stimulated P-815 cells. Furthermore, integrin β3 subunit protein levels but not levels of integrin αIIb and αv subunit proteins were similarly augmented in PGE_2- or 8-Br-cAMP-stimulated P-815 cells on the basis of western blotting analyses (Fig. 6). These results indicate that PGE_2 via cAMP-mediated pathway activates *de novo* synthesis of the integrin β3 subunit.

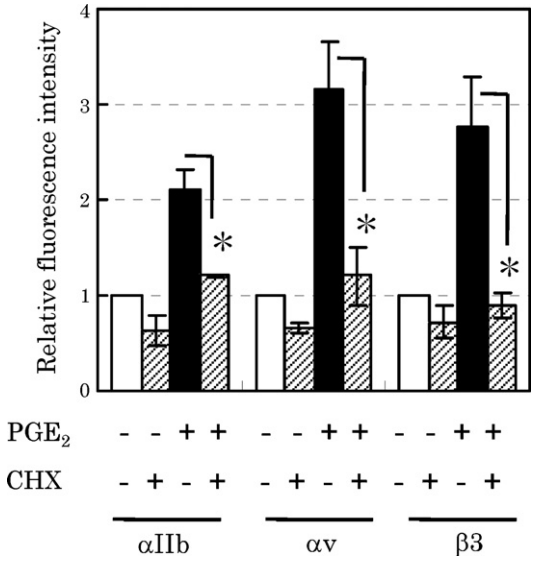

Fig. 4. Effect of cycloheximide on expression of integrin αIIb, αv and β3 subunits on the surface of PGE_2-stimulated P-815 cells. P-815 cells (5×10^5 cells/ml) were pretreated with or without 0.05 μg/ml cycloheximide (CHX) for 0.5 h and then incubated with 1 μM PGE_2 for 18 h, followed by FACS analysis. Relative fluorescence intensities were calculated as in Fig. 3. Experiments were performed at least three times and representative results are shown. $^*P < 0.01$ vs. PGE_2-stimulated cells.

3.3. *Functionally active integrin αIIb, αv and β3 subunits are expressed on the surface of PGE_2-stimulated P-815 cells*

To clarify whether integrin αIIb, αv and β3 subunits expressed on the surface of either PGE_2- or 8-Br-cAMP- stimulated P-815 cells is functionally active, specific function-blocking mAbs and receptor antagonists against various integrins were applied to PGE_2- or 8-Br-cAMP-stimulated P-815 cells. As a result, either PGE_2- or 8-Br-cAMP-induced adherent activity was mostly disappeared by the addition of the anti-αv and -β3 mAbs (Fig. 7A). Since no appropriate anti-αIIb mAb was commercially available, the functional ability of the integrin αIIb subunit remains unclear. On the other hand, anti-α5, -β1, -β2, and -β7 mAbs did not affect in the attachment of P-815 cells (data not shown). Of note, addition of anti-α4 mAb resulted in a significant increase of PGE_2- or 8-Br-cAMP-induced adherent activity of P-815 cells (Fig. 7B).

Next, we examined the effects of integrin receptor inhibitors, tirofiban and eptifibatide on the adhesion of cells to the RGD-enriched matrix. The former is a selective inhibitor for integrin αIIbβ3 and the latter is an inhibitor of high-affinity binding sites for both integrin αIIbβ3 and integrin αvβ3 [26–28]. P-815 cells were treated with PGE_2 for 17 h in the presence or absence of mAbs for anti-αv or anti-β3, followed by incubation with tirofiban and/or eptifibatide for 1 h (Fig. 8). Tirofiban inhibited PGE_2-induced adhesion by 50 ± 8%, which was further increased to 75 ± 8% in the presence of anti-αv mAb. In contrast, inhibition of PGE_2-induced adherent cells by eptifibatide was not further increased by tirofiban (Fig. 8), and also by anti-αv mAb in the preliminary experiment (data not shown). These results suggest that both integrin αIIbβ3 and integrin αvβ3 are functionally active in the process of PGE_2-stimulated P-815 cell adhesion to the RGD-enriched matrix.

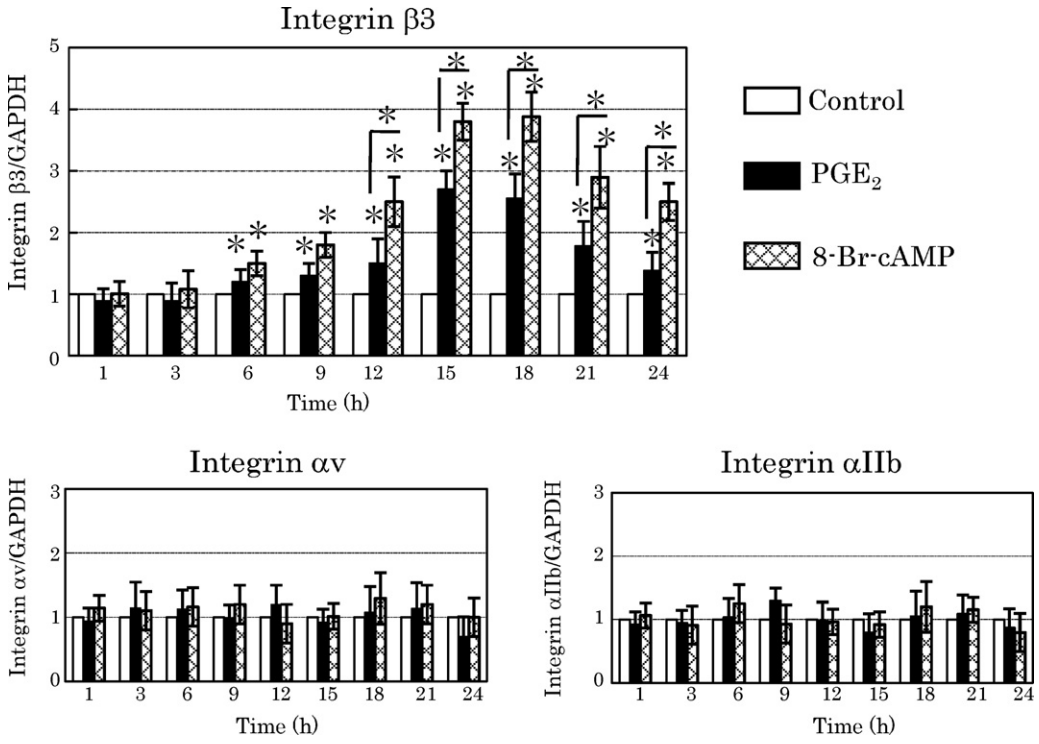

Fig. 5. Changes in mRNA levels of integrin αIIb, αv and β3 subunits in P-815 cells treated with or without PGE_2 or 8-Br-cAMP. P-815 cells were incubated for the various times with or without 1 μM PGE_2 or 1 mM 8-Br-cAMP, and the amount of integrin αIIb, αv and β3 mRNA was determined by quantitative RT-PCR. Results represent means ± SD of at least three experiments. $^*P < 0.01$ vs. each control or between bracketed results.

4. Discussion

Accumulating evidence has shown that adhesion molecules of the integrin family are implicated in the activation and migration of mast cells. However, the functional status of adhesion molecules expressed on mast cells has not been clearly addressed in previous studies. The present study was performed to elucidate the functional expression of integrin family subunits in the adhesion of mastocytoma P-815 cells to the RGD-enriched matrix, in response to stimulation by a local proinflammatory hormone, PGE_2. In particular, we investigated whether *de novo* synthesis of integrins is involved in PGE_2-induced adhesion of P-815 cells, since we found that adhesion activity by PGE_2 occurred at least 6 h after stimulation (Fig. 1A, [15]), and this response was sensitive to the protein synthesis inhibitor cycloheximide (Fig. 1B). Regarding the subtypes of integrins involving in adhesion to the RGD-enriched matrix, we found that the selective cell surface expression of integrin αIIb, αv and β3 subunits is critical in the induction of PGE_2-stimulated P-815 cell adhesion to the RGD-enriched matrix (Figs. 3, 4). These integrins are functional, because adherence was mostly inhibited by treatment with specific function-blocking mAbs against integrin αv and β3 subunits (Fig. 7A), and a selectivity inhibitor for integrin αIIbβ3, tirofiban, or an integrin αIIbβ3 and integrin αvβ3 receptor inhibitor, eptifibatide (Fig. 8). Although complex formation of integrin αIIbβ3 and integrin αvβ3 was not assessed in the present study, these complex are involved

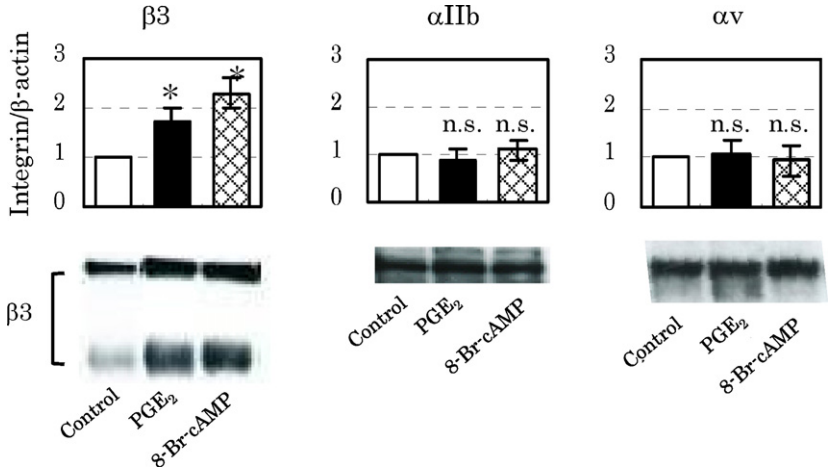

Fig. 6. Changes in protein levels of integrin αIIb, αv and β3 subunits in P-815 cells treated with or without PGE_2 or 8-Br-cAMP. P-815 cells were incubated with or without 1 μM PGE_2 or 1 mM 8-Br-cAMP for 18 h, and integrin αIIb (114 kDa), αv (130 kDa) and β3 (130 kDa and 97 kDa) protein expression in cell lysates was analyzed by Western blotting analysis. The intensities of immunoreactive bands were densitometrically determined and are expressed as the relative value using the mean intensity in the control cells as the standard. Relative band intensities are presented as means ± SD of at least three experiments. $^*P < 0.05$ vs. control. n.s., not significant.

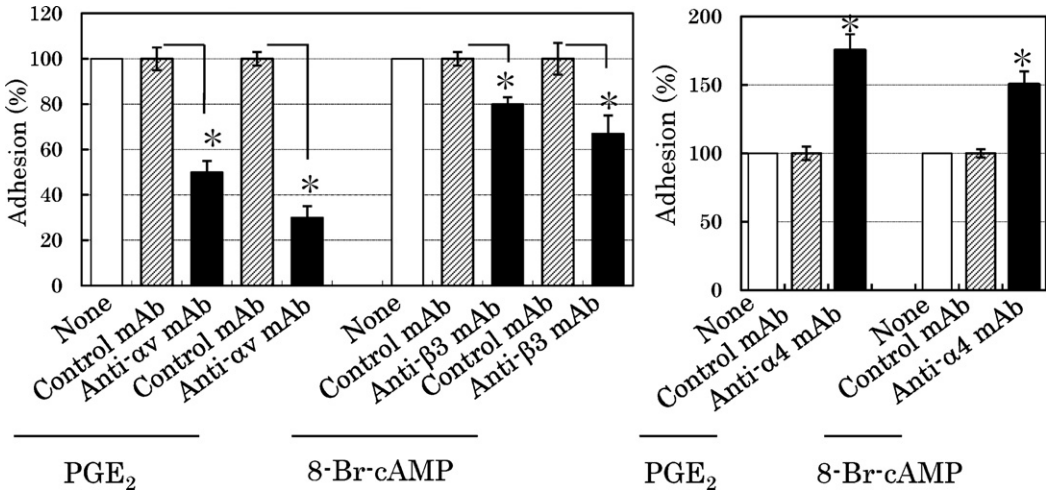

Fig. 7. Effects of anti-integrin mAbs on adhesion of P-815 cells to the RGD-enriched matrix. (A) P-815 cells (5×10^5 cells/ml) were incubated with specific function-blocking anti-αv or anti-β3 integrin mAbs (20 μg/ml of each) or the appropriate isotype-matched control mAbs (20 μg/ml of each) for 30 min, followed by an incubation with or without 1 μM PGE_2 or 1 mM 8-Br-cAMP for 18 h. (B) P-815 cells (5×10^5 cells/ml were incubated with specific function-blocking anti-α4 mAbs (20 μg/ml of each) for 30 min, followed by incubation with 1 μM PGE_2 or 1 mM 8-Br-cAMP for 18 h. After incubation the number of adherent P-815 cells was quantified. Results represent means ± SD of at least three experiments. $^*P < 0.01$ vs. each control mAb. Cell adhesion (%) without mAbs but with PGE_2 or 8-Br-cAMP is defined as representing 100% adhesion.

in adhesion of BMMC to fibrinogen and vitronectin [24] and human mast cells derived from fetal liver cells to vitronectin [29]. It is assumed that competitive association of the integrin β3 subunit with integrin αIIb and αv subunits determines the surface expression levels of integrin αIIbβ3 and integrin αvβ3 in mast cells [30]. Of note, P-815 cell adhesion is closely dependent on *de novo* synthesis of the integrin β3 subunit but not the integrin αIIb and αv subunits, although cell surface expression of integrin αIIb, αv and β3 subunits was similarly apparent. Accumulative evidence shows that integrin α and β subunits are synthesized and paired in the endoplasmic reticulum, and transported in vesicles and delivered to the plasma membranes by reactions of externalization and dimerization in several cultured cells [31,32]. However, there is little information about the surface expression of newly synthesized subunits such as β3 subunit in PGE_2-/EP4 agonist-/8-Br-cAMP-stimulated P-815 cells. Therefore, the present results provide the first evidence of a novel role of inducible integrin β3 subunit in cell-ECM interactions.

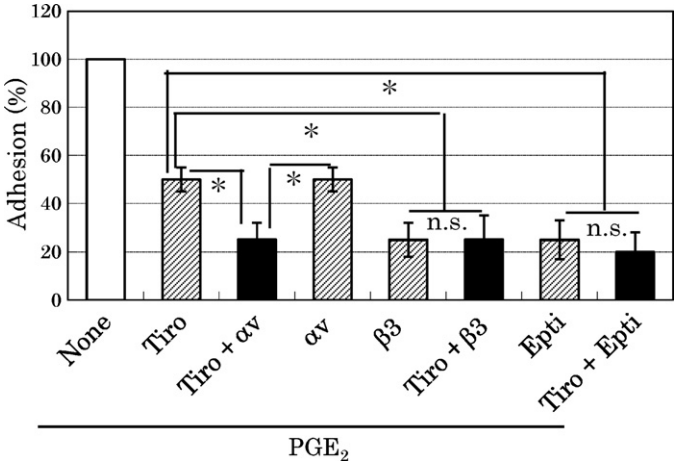

Fig. 8. Effects of αIIbβ3 and/or αvβ3 integrin antagonists on adhesion of PGE_2-stimulated P-815 cells to the RGD-enriched matrix. P-815 cells (5×10^5 cells/ml) were incubated with specific function-blocking anti-αv or anti-β3 integrins mAbs (20 μg/ml of each) for 30 min followed by incubation with 1 μM PGE_2 for 18 h. 50 μM tirofiban (αIIbβ3 integrin antagonist; Tiro) and/or 50 μM eptifibatide (both αIIbβ3 and αvβ3 integrin antagonist; Epti) were added 1 h prior to the end of incubation. After incubation the number of adherent P-815 cells was quantified and % inhibition calculated. Results represent means ± SD of at least three experiments. $^*P < 0.01$, n.s., not significant. Cell adhesion (%) without mAbs or antagonists with PGE_2 is defined as representing 100% adhesion.

In a previous paper [33], we reported that PGE_2-induced adhesion of BMMC to the RGD matrix was transient and reached a maximum at 1 h, and then the adhesion level returned to the basal level at 6 h. In this experiment, however, PGE_2-induced adhesion was mimicked by EP1 and EP3 agonist, sulprostone, and an EP3 agonist, ONO-AE-248, and these changes were abolished in *Ptger3*$^{-/-}$ BMMC, suggesting the involvement of the EP3 receptor but not the EP4 receptor [33]. Similarly, PGE_2-induced adhesion of BMMC was reduced by treatment with pertussis toxin, a phospholipase C inhibitor, U-73122, and a store-operated Ca^{2+}-channel inhibitor, SKF 36965, indicating the involvement of PGE_2/EP3-mediated Ca^{2+} mobilization in BMMC adhesion. In addition, BMMC and P-815 cells were demonstrated to express both EP3 and EP4 subtypes [15,33]. PGE_2-induced cAMP elevation suppressed histamine release upon antigen stimulation in rat peritoneal mast cells [34,35], while BMMC from EP3 deficient mice abolished both Ca^{2+}-mobilization and antigen-induced degranulation [36]. On the basis of these results we propose that mechanism of PGE_2-induced adhesion of BMMC can be classified into two types; cAMP elevation by the Gs-coupled EP4 subtype and Ca^{2+}-mobilization by the Gi-coupled EP3 subtype. Considering the characteristics of mast cells involved in these PGE_2-induced responses, degrees of maturation, species differences, and/or malignant conversion may determine the available EP subtypes and their roles in signal transduction. Further examinations are required to clarify how the signaling pathway of downstream is selected in different mast cell types with or without the stimuli.

cAMP is a common second messenger controlling cellular processes. Protein kinase A is a general receptor for cAMP, resulting in the phosphorylation of cellular targets. However, Rangarajan et al. reported that cAMP induced integrin-mediated ovarian carcinoma cell adhesion to fibronectin occurs upon stimulation of the β2-adrenergic receptor through Epac, a guanine nucleotide exchange factor for the small GTPase Rap1 [37]. Similarly, Fukuhara et al. reported that formation of cAMP potentiated vascular endothelial cadherin-mediated cell–cell contacts which enhance endotheliar barrier function, involve the Epac-Rap1 signaling pathway [38]. However, we previously showed that addition of the pharmacological inhibitor of PKA, H89, abolished

PGE_2-induced adhesion of P-815 cells [15]. Therefore, we suspect that Epac-Rap1 signaling is not involved in PGE_2-induced adhesion to the RGD-enriched matrix, although the pathway of downstream of cAMP signaling requires further investigation.

There is a lag time in the response of PGE_2-induced adhesion of P-815 cells, even though integrin α4, α5, β1 and β2 subunits are abundantly present on the cell surface. Availability of only the integrin β3 subunit was necessary in the case of PGE_2-induced adhesion of P-815 cells. How cells select particular integrins for adhesion from all of the integrins expressed on the cell surface is still unresolved. Accumulating evidence has shown that mutual functions of integrin subunits are regulated, at least in part, by a process termed as "integrin cross-talk" [39–41]. Very recently, Gonzalez et al. demonstrated that integrin β1 negatively modulates integrin αvβ3-ligand binding via activation of PKA in endothelial cells [42]. In the present study, using integrin-function-blocking mAbs (Fig. 7B), we found that the adhesion of PGE_2- or 8-Br-cAMP-stimulated P-815 cells increased approximately 1.5-fold only by the addition of the anti-integrin α4 subunit mAb among a variety of integrin mAbs. In addition, anti α4 mAb slightly increased the basal adhesion activity of P-815 cells. These suggest that the highly expressed integrin α4 subunit may act as a negative factor against P-815 cell adhesion, in spite of high surface expression of integrin αv, αIIb and β3 subunits. Further examination is necessary to fully elucidate this mechanism.

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