Essential role of EP3 subtype in prostaglandin E2-induced adhesion of mouse cultured and peritoneal mast cells to the Arg-Gly-Asp-enriched matrix

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Sakanaka M, Tanaka S, Sugimoto Y, Ichikawa A. Essential role of EP3 subtype in prostaglandin E2-induced adhesion of mouse cultured and peritoneal mast cells to the Arg-Gly-Asp-enriched matrix. Am J Physiol Cell Physiol 295: C1427–C1433, 2008.—Accumulating evidence has indicated that mast cells can modulate a wide variety of immune responses. Migration and adhesion play a critical role in regulation of tissue mast cell function, in particular, under inflammatory conditions. We previously demonstrated that prostaglandin (PG) E2 stimulates adhesion of a mouse mastocytoma cell line, P-815, to the Arg-Gly-Asp (RGD)-enriched matrix through cooperation between two PGE2 receptor subtypes: EP3 and EP4 (Hatae N, Kita A, Tanaka S, Sugimoto Y, Ichikawa A. J Biol Chem 278: 17977–17981, 2003). We here investigated PGE2-induced adhesion of IL-3-dependent bone marrow-derived cultured mast cells (BMMCs). In contrast to the elevated cAMP-dependent adhesion of P-815 cells, EP3-mediated Ca2+ mobilization plays a pivotal role in PGE2-induced adhesion of BMMCs. Adhesion and Ca2+ mobilization induced by PGE2 were abolished in the Ptger3−/− BMMCs and were significantly suppressed by treatment with pertussis toxin, a phospholipase C inhibitor, U-73122, and a store-operated Ca2+ channel inhibitor, SKF 36965, indicating the involvement of Gαi-mediated Ca2+ influx. We then investigated PGE2-induced adhesion of peritoneal mast cells to the RGD-enriched matrix. EP3 subtype was found to be the dominant PGE receptor that expresses in mouse peritoneal mast cells. PGE2-induced adhesion of the peritoneal mast cells of the Ptger3−/− mice, but not that of the Ptger3+/− mice. In rat peritoneal mast cells, PGE2 or an EP3 agonist stimulated both Ca2+ mobilization and adhesion to the RGD-enriched matrix. These results suggested that the EP3 subtype plays a pivotal role in PGE2-induced adhesion of murine mast cells to the RGD-enriched matrix through Ca2+ mobilization.

Gαi; Ca2+ mobilization; store-operated Ca2+ channel; bone marrow-derived cultured mast cells

Prostaglandin E2 (PGE2) is one of the major eicosanoids produced during inflammatory responses. Although mast cells are not the major source of PGE2, a wide variety of cells that can generate PGE2 are found in close proximity of mast cells, such as smooth muscle cells, respiratory epithelial cell, fibroblasts, and macrophages (2, 4, 9, 13). Based on these findings, we hypothesized that PGE2 can affect migration and adhesion of tissue mast cells by acting on the specific receptors. Using a mouse mastocytoma, P-815, our laboratory previously demonstrated that PGE2 induces the adhesion to the Arg-Gly-Asp (RGD)-enriched matrix through elevation of cAMP levels, which results from cooperation between two PGE2 receptor subtypes: EP3 and EP4 (7). EP3 subtype is known to be coupled to multiple G proteins, such as Gαi, Gαs, and G12 (17, 23). In P-815 cells, augmentation of the EP4-mediated cAMP generation by the EP3 subtype was suppressed by treatment with pertussis toxin (PTX), indicating that the EP3 subtype is coupled to Gαi and liberation of βγ-subunit may be involved in augmented cAMP generation. Very recently, Weller et al. demonstrated that PGE2 can function as a chemotactic factor of mast cells by acting on the EP3 subtype (28). Although the EP4 subtype is expressed in bone marrow-derived cultured mast cells (BMMCs), PGE2-induced chemotaxis is mediated solely by the EP3 subtype; PGE2-induced chemotactic responses were suppressed by treatment with PTX and mimicked by an EP1/EP3 agonist. Since accumulating evidence suggests that the dominant receptor that is responsible for PGE2-induced responses in mast cells depends on the species and tissue localization (3), it is possible that the EP4-independent pathway is involved in PGE2-induced adhesion of BMMCs. We here characterized the PGE2-induced adhesion of BMMCs and peritoneal mast cells to the RGD-enriched matrix and found that the EP3 subtype plays a dominant role in adhesion of BMMCs and peritoneal cells.

MATERIALS AND METHODS

Animals. Specific-pathogen-free, 6-wk-old female C57BL/6 mice were obtained from Japan SLC (Hamamatsu, Japan). The Ptger3−/− mice (25) were back-crossed for at least eight generations to the C57BL/6 background. This study was approved by the Committee on Animal Experiments of Mukogawa Women’s University and conformed with the Guiding Principles in the Care and Use of Animals of the American Physiological Society.

Materials. Sulprostone was a generous gift from Dr. M. P. L. Caton of Rhone-Poulenc. ONO-AE-248 and ONO-AE-437 were gener-

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ously supplied by ONO Pharmaceuticals (Osaka, Japan). ProNectin F (ProF) and ProNectin L (ProL) were generously supplied by Sanyo Chemical Industries (Kyoto, Japan). The following materials were obtained from the sources indicated: GRGDS peptide from PEPTIDE Institute (Osaka, Japan); Cellmatrix (type I-P collagen) from Nitta Gelatin (Osaka, Japan); Histodenz, bovine serum albumin (BSA), thapsigargin, and an anti-dinitrophenyl IgE (clone SPE-7) from Sigma-Aldrich (St. Louis, MO); PTX from Seikagaku (Tokyo, Japan); PGE2 from Cayman Chemical (Ann Arbor, MI); 12-O-tetradecanoylphorbol 13-acetate (TPA) and Go6976 from Calbiochem (San Diego, CA); SKF 96365 from TOCRIS (Bristol, UK); thrombin from bovine plasma and fura-2 AM from NACALAI TESQUE, (Kyoto, Japan); Moloney murine leukemia virus (MMLV) reverse transcriptase from Invitrogen (Carlsbad, CA); and Taq DNA polymerase from TOYOBO (Osaka, Japan). All other chemicals were commercial products of reagent grade.

Preparation of BMMCs. BMMCs were prepared as described previously with a minor modification (24). Bone marrow cells were cultured in RPMI-1640 containing 10% heat-inactivated FBS, 50 μM β-mercaptoethanol, and 0.1 mM nonessential amino acids at 37°C in a fully humidified 5% CO2 atmosphere for 4–5 wk in the presence of 10 ng/ml IL-3 instead of WEHI-3-conditioned medium. Greater than 95% of the viable cells were confirmed to be immature mast cells, as assessed by staining with acidic toluidine blue (pH 2.5).

Measurement of cell adhesion. Twelve-well culture plates were coated with 10 μg/ml ProF, 10 μg/ml ProL, 0.3 mg/ml Cellmatrix, or 3% BSA. BMMCs were seeded on the well at a density of 1 × 10⁶ cells·mL⁻¹·well⁻¹ and incubated in the medium containing 10 μM indomethacin. The adherent cells were recovered by treatment with trypsin. Cell number of the adherent and nonadherent cells was counted using the COULTER Z2 cell counter (Beckman Coulter, Fullerton, CA). Percentages of the adherent cells were calculated according to the following formula: cell adhesion (%) = (the number of adherent cells + the number of nonadherent cells) / (the number of adherent cells × 100).

Measurement of cytosolic Ca²⁺ concentrations. Cytosolic Ca²⁺ concentration was measured with fura-2 AM, as previously described (24). Fluorescent intensities were measured, at an excitation wavelength of 340 or 380 nm and an emission wavelength of 510 nm, with a fluorescence spectrometer (CAF-110, Jasco, Tokyo, Japan).

Purification of peritoneal mast cells. Mouse peritoneal cells were recovered with Tyrode-HEPES buffer containing 100 mM sodium phosphate, pH 7.4, containing 3% sucrose, 2% paraformaldehyde, and 0.1% glutaraldehyde, and stained with the acidic toluidine blue. Percentages of mast cells in the total peritoneal cells were determined based on the count of the cells stained with 2% Giemsa solution.

RESULTS

PGE₂-induced adhesion of BMMCs to ProF. Our laboratory previously reported the PGE₂-induced adhesion of P-815 cells to an RGD-enriched matrix, ProF (7). In the present study, we investigated the effects of PGE₂ on adhesion activity of BMMCs. PGE₂ significantly augmented the adhesion of BMMCs to ProF or ProL, but not to type I collagen or BSA (Fig. 1A). The stimulated adhesion to ProF was suppressed in the presence of indomethacin. The adherent cells were fixed by 100 mM sodium phosphate, pH 7.4, containing 3% sucrose, 2% paraformaldehyde, and 0.1% glutaraldehyde, and stained with the acidic toluidine blue. Percentages of mast cells in the total peritoneal cells were determined based on the count of the cells stained with 2% Giemsa solution.
the GRGDS peptide (Fig. 1B), indicating that BMMCs adhere to ProF via the RGD sequence as well as P-815 cells did. The PGE2-induced adhesion to ProF was a saturable and concentration-dependent response (Fig. 1C).

**EP3 subtype is essential for PGE2-induced adhesion and Ca2+ mobilization.** We then investigated the time course of adhesion of BMMCs to ProF. PGE2-induced adhesion was transient, and the adhesion level returned to the base level 6 h after the stimulation (Fig. 2A). PGE2-induced adhesion was well mimicked by sulprostone (EP1 and EP3 agonist) and ONO-AE-248 (EP3 agonist), but not by ONO-AE-1-437 (EP4 agonist), indicating the involvement of the EP3 subtype. We, therefore, investigated the adhesion using BMMCs derived from the Ptger3−/− mice. PGE2-induced adhesion was abolished in the Ptger3−/− BMMCs (Fig. 2B). No significant difference between the Ptger3+/+ and Ptger3−/− BMMCs was found in adhesion to ProF stimulated with TPA or monomeric IgE (16), indicating that the machinery required for the adhesion to ProF is not impaired in the Ptger3−/− BMMCs (Fig. 2, C and D). BMMCs from the Ptger3+/+ mice were found to express both the EP3 and EP4 subtype mRNAs, as previously reported (Fig. 2E).

**Gq-mediated Ca2+ mobilization is one of the signaling events downstream of the EP3 subtype (10).** PGE2 induced a drastic Ca2+ mobilization in the Ptger3+/+ BMMCs, followed by a sustained phase with a moderate cytosolic Ca2+ level (Fig. 3). Similar responses were observed in the Ptger3+/+ BMMCs stimulated with sulprostone and ONO-AE-248, but not with ONO-AE-1-437. In the Ptger3−/− BMMCs, PGE2-induced Ca2+ mobilization was drastically attenuated, and no EP agonists tested could induce Ca2+ mobilization (Fig. 3 and data not shown). Treatment with thrombin (Fig. 3) or monomeric IgE (data not shown) induced similar levels of Ca2+ mobilization in BMMCs, followed by a sustained phase with a moderate cytosolic Ca2+ level (Fig. 3). Similar responses were observed in the Ptger3+/+ BMMCs stimulated with sulprostone and ONO-AE-248, but not with ONO-AE-1-437. In the Ptger3−/− BMMCs, PGE2-induced Ca2+ mobilization was drastically attenuated, and no EP agonists tested could induce Ca2+ mobilization (Fig. 3 and data not shown). Treatment with thrombin (Fig. 3) or monomeric IgE (data not shown) induced similar levels of Ca2+ mobilization in BMMCs, followed by a sustained phase with a moderate cytosolic Ca2+ level (Fig. 3).
Characterization of EP3-mediated adhesion and Ca\(^{2+}\) mobilization in BMMCs. Since the EP3 subtype is known to be coupled to multiple trimeric G proteins, we then investigated the signaling pathway downstream of the EP3 subtype. PGE2-induced adhesion and Ca\(^{2+}\) mobilization in BMMCs were strikingly suppressed by pretreatment with PTX, indicating the dominant role of G\(_i\)/G\(_o\) (Fig. 4, A and B). Pretreatment of BMMCs with a specific inhibitor of phospholipase C, which is known to be activated upon G\(_{\gamma}\) liberation from trimeric G\(_i\) complex, abolished the adhesion responses and Ca\(^{2+}\) mobilization induced by PGE2 (Fig. 4, C and D). It remains unknown which kinds of Ca\(^{2+}\) channels are involved in the Ca\(^{2+}\) influx induced by the EP3 subtype. We found that SKF 96365, a specific inhibitor of store-operated Ca\(^{2+}\) channels, has a potential to block the PGE2-induced Ca\(^{2+}\) mobilization and adhesion (Fig. 4, E and F). These results collectively indicate that the EP3 subtype is coupled to G\(_i\) and induces Ca\(^{2+}\) mobilization, which is maintained by persistent activation of the store-operated Ca\(^{2+}\) channels.

Deficiency in PGE2-induced adhesion in peritoneal mast cells of the Ptger3\(^{-/-}\) mice. Several functions specific to mature mast cells, such as cationic secretagogue-induced degranulation, are not reproduced in BMMCs, even though BMMCs can well mimic the functions of immature mast cells. It is of great interest to determine whether mature mast cells utilize the EP3 or EP4 subtype for adhesion. We then investigated the PGE2-induced adhesion to ProF in peritoneal mast cells, which are regarded as typical connective tissue-type mast cells. Pu-

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**Figure 4.** Characterization of EP3-mediated adhesion and Ca\(^{2+}\) mobilization. A: BMMCs were pretreated with pertussis toxin (PTX) or without PTX (control) (0.1 mg/ml) for 1 h and then incubated with (solid bars) or without (open bars) 0.1 \(\mu\)M PGE2 for 1 h. The values are presented as means ± SE (n = 3). The value **P < 0.01 is regarded as significant by the Student’s t-test (vs. none). B: BMMCs were pretreated as described above and then stimulated with 0.1 \(\mu\)M PGE2 (indicated by the arrows). The cytosolic Ca\(^{2+}\) levels were measured. Bars = 1 min. This is a representative figure of three independent experiments showing similar results. C: BMMCs were pretreated with (U-73343 and U-73122) or without (control) an inhibitor for phospholipase C (4 \(\mu\)M) for 7 min and then incubated with (solid bars) or without (open bars) 0.1 \(\mu\)M PGE2 for 1 h. The values are presented as means ± SE (n = 3). The value **P < 0.01 is regarded as significant by Student’s t-test (vs. none). D: BMMCs were pretreated as described above and then stimulated with 0.1 \(\mu\)M PGE2 (indicated by the arrows). The cytosolic Ca\(^{2+}\) levels were measured. Bars = 1 min. This is a representative figure of three independent experiments showing similar results. E: BMMCs were stimulated with (solid bars) or without (open bars) 0.1 \(\mu\)M PGE2 for 1 h in the presence (SKF 96365) or absence (control) of 50 \(\mu\)M SKF 96365. The values are presented as means ± SE (n = 3). The value **P < 0.01 is regarded as significant by Student’s t-test (vs. none). F: BMMCs were pretreated with or without 50 \(\mu\)M SKF 96365 for 1 min and then stimulated with 0.1 \(\mu\)M PGE2 (indicated by the arrows). The cytosolic Ca\(^{2+}\) levels were measured. Bars = 1 min. This is a representative figure of three independent experiments showing similar results.
rified peritoneal mast cells were found to exclusively express the EP3 subtype by RT-PCR analyses (Fig. 5A). PGE2-induced adhesion of peritoneal mast cells to ProF was concentration dependent, and the EC50 value of this response was similar to that in BMMCs (Figs. 5B and 1C). In the Ptger3+/+ peritoneal cells, PGE2-induced adhesion of mast cells was completely abolished, whereas a similar level of adhesion was observed in the presence of TPA compared with the Ptger3+/+ peritoneal cells (Fig. 5, C–E). No significant difference was found in the number of peritoneal mast cells between the Ptger3+/+ and Ptger3−/− mice (percentages of mast cells in total peritoneal cells, Ptger3+/+ 2.43 ± 0.380%, Ptger3−/− 2.44 ± 0.516%). PGE2-induced adhesion of the peritoneal mast cells was significantly suppressed by SKF 36965, indicating that the adhesion should require Ca2+ influx (Fig. 5F). In addition, the GRGDS peptide blocked the PGE2-induced adhesion of the peritoneal mast cells (Fig. 5G). These results indicate that the EP3 subtype mediates the PGE2-induced adhesion of mouse peritoneal mast cells in a similar manner to that of BMMCs.

**EP3-mediated Ca2+ mobilization and adhesion of rat peritoneal mast cells.** Although the inhibitory effects of SKF 36965 on the adhesion of peritoneal mast cells imply the requirement of PGE2-induced Ca2+ influx, it was found to be quite difficult to determine the cytosolic Ca2+ levels using purified mouse peritoneal mast cells in a reproducible manner. We then used rat peritoneal mast cells to investigate PGE2-induced signal transduction, since rat peritoneal mast cells can be readily purified and have been used as the good model for connective tissue-type mast cells. The adhesion of the peritoneal mast cells to ProF was significantly augmented by PGE2, in the presence of the EP3 agonist, but not by the EP4 agonist (Fig. 6A). The PGE2- or EP3 agonist-induced adhesion was abolished in the presence of the GRGDS peptide or SKF 36965, as well as in the mouse peritoneal mast cells (Fig. 6A). PGE2 and the EP3 agonist were found to induce Ca2+ mobilization in the rat peritoneal mast cells, which was completely suppressed by SKF 36965 (Fig. 6B). These results suggest that PGE2 can evoke Ca2+ mobilization and stimulate the adhesion to ProF in the rat peritoneal mast cells, which can be mimicked by the EP3 agonist.

**DISCUSSION**

In contrast to the adhesion through cAMP elevation by cooperation between the EP3 and EP4 subtypes in P-815 cells (7), the EP3 subtype was found to play an essential role in PGE2-induced adhesion of BMMCs and peritoneal mast cells through Ca2+ mobilization. The signaling pathways utilized in...
mast cells stimulated by PGE2 can be classified into two types: one is mediated through cAMP elevation by the Gs-coupled subtype, such as EP2 and EP4; and the other is through Ca2+ mobilization by the Gi-coupled subtype, EP3. PGE2-induced cAMP elevation suppressed the histamine release upon antigen stimulation in rat peritoneal and human lung mast cells (12, 22) and resulted in release of vascular endothelial growth factor in human cord blood-derived cultured mast cells through activation of the EP2 subtype (1). In these kinds of mast cells, cAMP elevation plays a key role in the PGE2-induced signal transduction. On the other hand, PGE2 or an EP1/EP3 agonist, sulprostone, was reported to stimulate IL-6 release and poten-
tiate degranulation upon antigen stimulation in a mouse IL-3-dependent mast cell line, MC9 (6). Nguyen et al. (21) demonstrated PGE2-induced Ca2+ mobilization and PGE1-mediated augmentation of antigen-induced degranulation in BMMC, both of which are abolished in BMMC derived from the EP3-deficient mice. They also showed that PGE2 failed to enhance cAMP formation in BMMC, although the expression of the EP4 subtype was detected. These results indicate that the action of PGE2 is mediated exclusively by the EP3 subtype in BMMC and MC9 cells. Considering the characteristics of mast cells involved in these PGE2-induced responses together, degrees of maturation, species difference, and/or malignant conversion may determine the available EP subtypes and their roles in the signal transduction. BMMC were found to express both the EP3 and EP4 subtype (21, 28, and this study), which is similar in the expression pattern to P-815 cells. Further studies are required to clarify how the signaling pathway downstream of the EP3 subtype is differentially regulated, since the EP3 subtype augments EP4-mediated cAMP production in P-815 cells but induces Ca2+ mobilization in BMMC and rat peritoneal mast cells.

Our laboratory characterized in detail the EP3-mediated Ca2+ mobilization in the present study. We found that PGE2-induced Ca2+ increase consists mainly of Ca2+ influx, which is suppressed by a specific inhibitor for store-operated Ca2+ channels, SKF 96365. Since the adhesion of BMMC and rat peritoneal mast cells was completely suppressed in the presence of the inhibitors that suppressed Ca2+ mobilization induced by PGE2, it is likely that Gi-mediated Ca2+ influx plays a pivotal role downstream of the EP3 subtype, at least in the adhesion response. PTX-sensitive adhesion of BMMC to fibronectin was also reported with the other mediators, such as thrombin and 5-hydroxytryptamine (15, 26). Thrombin was reported to induce the adhesion of BMMC by acting on protease-activated receptor-1, which was accompanied by degranulation and IL-6 production (26). Although the author did not measure the cytosolic Ca2+ levels, these responses should be mediated by Ca2+ mobilization, as well as in the present study.

It remained unknown which subtypes of the EP receptors are expressed in mature tissue mast cells. We demonstrated that the EP3 subtype is the only detectable EP subtype in mouse peritoneal mast cells and that PGE2-induced adhesion is abolished in the Ptger3−/− mast cells. Kunikata et al. (14) demonstrated that antigen-induced histamine release is significantly suppressed by PGE2 or ONO-AE-248 (EP3 agonist) in lung tissues, and such suppressive effect was abolished in the Ptger3−/− lung tissues. Since it remains controversial whether the EP3 subtype suppresses or potentiates degranulation upon antigen stimulation (5, 6, 14, 21), much attention should be paid to the heterogeneity of tissue mast cells. In regard to mouse peritoneal mast cells, we observed no detectable degranulation in the presence of PGE2, whereas high concentrations of TPA induced significant levels of degranulation (data not shown).

Recent studies demonstrated that mast cells can undergo chemotaxis in response to various chemotactants, such as histamine, 5-hydroxytryptamine, PGE2, leukotriene B4, and sphingosine-1-phosphate (8, 11, 15, 27, 28). These results indicate that mast cells have a tendency to accumulate at the inflamed site that is characterized by production of these
chemoattractants. We noticed that the adhesion of BMMCs in response to PGE2 is rapid and transient. Process of mast cell activation can be generally classified into two phases: mast cells release histamine and neutral proteases through degranulation and generate a series of lipid mediators in the immediate phase, whereas they produce chemokines and cytokines in the late phase (18). Considering this time course, it is likely that PGE2-induced transient adhesion to fibronectin restricts chemotaxis of mast cells during the immediate phase. PGE2 may regulate the localization of tissue mast cells under inflammatory conditions and potentiate the impact of proinflammatory mediators generated by mast cells.

We conclude that the EP3 subtype is essential for PGE2-induced adhesion of mouse BMMCs and rat peritoneal mast cells through Ca2+ mobilization. It is likely that EP3-mediated Ca2+ mobilization is mediated by the Gq-phospholipase C axis. This pathway may play a critical role in regulation of mast cell adhesion under inflammatory conditions.

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