Prostaglandin D\(_2\) Augments Low-dose Antigen-induced Th2 Type Airway Inflammation in Mice

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SUMMARY

Prostaglandin D\(_2\) (PGD\(_2\)) is a mast cell-derived lipid mediator detected in large amounts in airways of asthmatics, but its role is largely unknown. To clarify the role of PGD\(_2\) in Th2-type airway inflammation which characterizes asthma, we studied the effects of aerosolized PGD\(_2\) on the inflammatory response to a low-dose antigen challenge in airways of mice. Mice sensitized with ovalbumin (OVA) were challenged with a conventional low-dose (1%) or a low-dose (0.1%) aerosolized OVA. Mice received low-dose OVA challenge were pretreated with aerosolized PGD\(_2\) (10\(^{-3}\) M) (PGD\(_2\) plus low-dose OVA mice) or saline (low-dose OVA alone mice) 24 hrs before the OVA challenge. Some mice were pretreated with PGD\(_2\) but challenged with saline (PGD\(_2\) alone mice). Airway inflammation was evaluated by the numbers of eosinophils, lymphocytes, and macrophages in bronchoalveolar lavage fluid. The degree of airway inflammation in the PGD\(_2\) alone mice and the low-dose OVA alone mice were only marginal. However, the PGD\(_2\) plus low-dose OVA mice displayed a similar degree of airway inflammation with mice received conventional low-dose OVA challenge. Levels of interleukin (IL)-4 and IL-5 were significantly increased in the PGD\(_2\) plus low-dose OVA mice than the low-dose OVA alone mice. PGD\(_2\) (10\(^{-7}\) - 10\(^{-5}\) M) did not affect the Th2-type cytokine production by OVA specific T cells in response to OVA stimulation in vitro. Immunohistochemical analysis of lung tissue revealed that airway epithelium of the PGD\(_2\) plus low-dose OVA alone mice were strongly stained with monoclonal antibody against macrophage-derived chemokine (MDC), a Th2 cell-specific chemokine. These results suggest that PGD\(_2\) augments Th2 cell-type airway inflammation via epithelial expression of MDC.

Key Words: Th2, prostaglandin D\(_2\), MDC, bronchial asthma

INTRODUCTION

Mast cells play an important part in asthma. Activated mast cells contribute to asthmatic airway inflammation by producing a variety of chemical mediators and cytokines. Prostaglandin D\(_2\) (PGD\(_2\)) is one of the major cyclooxygenase metabolites of arachidonic acid produced by mast cells\(^{1-3}\). PGD\(_2\) is released in large amounts during asthmatic attacks in humans and has been proposed as a marker of mast cell activation in asthma\(^{4,5}\). Recent studies showed that mice lacking a G protein-coupled receptors were resistant to experimentally-induced allergic asthma, suggesting a role for PGD receptor-mediated PGD\(_2\) action in the development of allergic asthma. The results in this experimental model predict that PGD\(_2\) is involved in Th2 type airway inflammation characterized by recruitment of eosinophils and Th2 cells\(^6\). Further, Fujitani et al. have recently reported that the levels of Th2 cytokines were elevated, accompanied by the enhanced
accumulation of eosinophils and lymphocytes in the lung of Prostaglandin D synthase transgenic mice. However, it remains unclear how PGD₂ participate in airway inflammation.

CD4⁺ T cells, especially Th2 cells, are accepted to play an important role in the pathogenesis of bronchial asthma. Th2 cells produce cytokines such as interleukin (IL) -4, IL -5, IL -9, and IL -13, which induce IgE production as well as mast cell and eosinophil activation. Recent studies of mice lacking T1/ST2, a Th2-specific cell surface molecule, strongly supported an importance of role of Th2 cells in the development of bronchial asthma. Th2 cells are recruited into the airway mucosa and are found in the bronchoalveolar lavage (BAL) fluid of patients with asthma. However, it remains unclear how Th2 cells are recruited into the lung and how Th2 cells regulate the development of airway hyperresponsiveness (AHR).

Over the past few years, the ability of chemokines (chemotactic cytokines) to attract inflammatory cells to the lung in patients with asthma has received considerable attention. Recent studies have suggested that bronchial epithelial cells may directly perpetuate Th2 type airway inflammation by producing certain chemokines such as thymus and activation-related chemokine (TARC) and macrophage-derived chemokine (MDC). TARC has been found to induce chemotaxis of T cells, especially Th2 cells. MDC has been shown to be a potent chemoattractant for Th2 cells, as well as for eosinophils and monocytes. Both TARC and MDC, CCR4 ligands, have been implicated in Th2 type inflammation associated with the development of AHR. We have previously reported that IL-9, a Th2 type cytokine, is essentially involved in Th2 type airway inflammation associated with AHR in a murine model of asthma. We have also suggested that IL-9 induces expression of MDC in bronchial epithelial cells that mediate antigen-induced Th2 type inflammation.

We have developed a low-dose antigen model for inducible allergic asthma, which allowed us to examine the effect of inhaled PGD₂ directly. Using this models, we showed here that PGD₂ enhanced the recruitment of eosinophils and CD4⁺ T cells (especially Th2 cells) into the lung. It was also indicated that MDC induced in airway epithelial cells by inhaled PGD₂ mediated the asthma-promoting activity of PGD₂. Our current study thus suggested the mechanism for the action of PGD₂ in the asthma.

MATERIAL AND METHODS

Animals
Specific pathogen-free male BALB/c mice (6 to 8 weeks old, SLC, Shizuoka, Japan) were used in all experiments. The study protocol was reviewed and approved by the Dokkyo University School of Medicine Committee on Animal Care and complies with National Institutes of Health guidelines for animal care.

Sensitization and antigen challenge of mice
Experimental protocol was designed so as to assess the effect of PGD₂ on the Th2-type airway inflammation induced by a low-dose antigen challenge. Sensitization and antigen challenges of mice were performed as described previously. Briefly, BALB/c mice were sensitized on day 0 and 5 by intraperitoneal injections of OVA (Sigma, St. Louis, MO) (8 μg/mouse) adsorbed to aluminium hydroxide (Wako Pure Chemical Industries, Osaka, Japan). Sensitized mice were challenged with a conventional dose or a low-dose aerosolized OVA. The conventional-dose OVA exposure group mice were challenged with 1% of aerosolized OVA for 60 min twice, separated by an interval of 4 h, on day 17, and functioned as a positive control (Fig. 1, Group 1). The low-dose OVA exposure group mice were challenged with 0.1% of OVA for 30 min on day 17 (Fig. 1, Group 3). In preliminary experiments, we confirmed that this dose of aerosolized OVA produced only marginal Th2-type airway inflammation. To determine whether PGD₂ augments low-dose OVA induced Th2-type airway inflammation, the low-dose OVA exposure mice received aerosolized PGD₂ (10⁻³ M) or saline on day 16. In preliminary experiments this concentration of PGD₂ itself were confirmed to induce no obvious histological changes such as inflammatory cell recruitment and tissue edema. Some mice pretreated with aerosolized PGD₂ or saline on day 16 were challenged with aerosolized saline instead of low-dose OVA, and functioned as a PGD₂ alone control (Fig. 1, Group 2) or a negative control (Fig. 1, Group 3).

Bronchoalveolar lavage
Bronchoalveolar lavage (BAL) was performed immediately before and 3 and 24 h after the last aerosol chal-
Fig. 1 Experimental protocols.

Group (1) : a conventional asthma model, Group (2) a nonconventional asthma model for analysis of effects of PGD$_2$-pretreatment low-dose antigen challenge. Group (3) a low-dose OVA alone mice and control mice. OVA : ovalumin. i.p : intraperitoneal injection. BAL : bronchoalveolar lavage.

Histological examination of lung

The lungs were taken from the mice, fixed in neutralized buffered formalin, and embedded in paraffin. Sections 3 mm thick were stained with Luna solution for eosinophils and toluidine blue for mast cells.

Immunohistochemistry

Paraffin sections of lung tissue were deparaffinized and hydrated by submersion in xylene followed by reagent-grade alcohol. The sections were rinsed for 5 min and incubated with 0.3% H$_2$O$_2$ for 30 min to quench endogenous activity. After washing three times in TBS for 15 min, the sections were incubated with goat-anti-mouse MDC or TARC Ab or an isotype control Ab overnight. Then, the sections were washed three times in TBS for 15 min, and a rabbit anti-goat IgG secondary Ab was applied for 30 min. After washing, the sections were incubated with streptavidin peroxidase reagent for 30 min. The sections were washed again and stained with peroxidase substrate solution until the desired intensity was reached. After rinsing in running water, the sections were counterstained with hematoxin. The used reagents were derived from commercially available DAKO labeled streptavidin-biotin kits.

Culture of primary human bronchial epithelial cells

In these studies, we used primary human bronchial epithelial cells (Normal Human Cell System Sanko). Cells were cultured in supplemented SABM medium in 25-cm$^2$ tissue plates at 37 ºC in 5% CO$_2$. Only cells that had reached 80% to 90% confluence were used for experiments.

Preparation of mRNA and RT-PCR

Total RNA was extracted from the primary human
bronchial epithelial cells by Trisol, a modified guanidine thiocyanate-phenol-chloroform method, as recommended by the manufacturer (Biotec Laboratories, Houston, TX). RT-PCR was performed to determine the relative quantities of TARC and MDC mRNA, using a modified method as described elsewhere. Briefly, 1 μg of RNA was reverse transcribed using oligo (dT) primers; the cDNA underwent 30, 30, or 22 cycles of amplification with primers specific for TARC, MDC, and β-actin. The sequences of primers from the coding regions of human genes were as follows: MDC: 5’-TACGACTGACTG CTGTGGTCC -3’ and 5’-TTCTGCCGGGAGGAC TATAATG -3’; TARC: 5’-CACCCAGCTCGAGGGAC CAAATGTG -3’ and 5’-TCAAGACCTCTCAAGGCTTGC AGG -3’; β-actin: 5’-TGAGGGGTCAACCCACTTG TGCCCATCTA -3’ and 5’-CTAGAACATTGCGGTG ACGATGGAGG -3’. PCR products in agarose gel were detected by ethidium bromide staining.

Stimulation of primary human bronchial epithelial cells with PGD2

To assess the effects of PGD2 on MDC and TARC gene expression, primary human bronchial epithelial cells were stimulated with various concentrations of PGD2 (10^-9 - 10^-6 M). Cultures were harvested after stimulation for 48 h and analyzed by RT-PCR.

Measurement of cytokines

Concentrations of cytokines (IL-2, IL-4, IL-5, IL-13, IFN-γ) in culture supernatants and BAL fluid supernatants were measured by ELISA kits (R & D, Abingdon, UK) according to the manufacturer’s instructions.

OVA-specific T cell and bone marrow mast cell (BMMC) response in vitro

Ten days after intraperitoneal immunization with OVA as described above, mice spleen cells were isolated and cultured with PGD2 (10^-9 - 10^-6 M) during stimulation with OVA (10 μg/ml) for 24 h^{40,40}. For BMMC culture, intact femurs and tibias were removed from mice, and bone marrow cells were harvested by repeated flushing with MEM. A cell culture was established at a density of 3 x 10^6 cells/ml in IMDM, supplemented with 10% FCS (inactivated at 56°C), 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, 20 U/ml mIL-3, and 50 Um/ml mIL-4. Nonadherent cells were transferred to fresh culture plates every 2 to 3 days for a total of at least 21 day to remove adherent macrophages and fibroblasts^{40,40}. Toluidine blue staining revealed that the resulting cell population consisted of > 99% BMMCs. These cells were cultured with 0.25 mM ionomycin during stimulation with PGD2 (10^-9 - 10^-5 M).

Statistical analysis

Data are expressed as means ± SEM. The statistical significance of differences between groups was assessed by analysis of variance. P values less than 0.05 were considered to indicate statistical significance.

RESULTS

Effect of PGD2 on infiltration of inflammatory cells in BAL fluid

The total cell number and cell populations in the BAL fluid of the sensitized mice were similar 24 h after provocation with PGD2 alone (Fig. 2, A: 0 h before OVA challenge) and 24 h after provocation with saline control. This finding indicated that PGD2 alone had no significant chemoattractive effect in the lung. Three hours after low-dose antigen (0.1% OVA) inhalation, the total cell number in BAL fluid was significantly higher in PGD2-pre-treated mice than in saline-pretreated control mice (Fig. 2, B). The infiltrated cells consisted predominantly of eosinophils, lymphocytes, and macrophages, with few neutrophils. Further increases in each cell type were seen in BAL fluid 24 h after low-dose antigen challenge, as compared with control (Fig. 2, C). However, the numbers of eosinophils and lymphocytes did not differ significantly between the conventional asthma model challenged with 1% OVA and the PGD2-pretreated mice challenged with 0.1% OVA 24 h after antigen exposure. In contrast, the number of macrophages in the standard asthma model was significantly greater than that in the PGD2-pretreated mice (Fig. 2, C).

Effect of PGD2 on cytokine concentrations in BALF

We analyzed Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-5) cytokines in BAL fluid. No Th1 cytokines were detected, and there was no significant difference in the levels of Th2 cytokines in BALF between 24 h (Fig. 3, A: 0 h before OVA challenge) and 48 h (Fig. 3, B: 24 h...
after OVA challenge) after PGD₃ pretreatment. This result indicated that PGD₃ alone had no significant effect on induction of Th2 cytokines or on cell populations in the airway. Three hours after low-dose antigen (0.1% OVA) inhalation, Th2 cytokine levels were significantly higher in PGD₃-pretreated mice than in the saline-pretreated control mice (Fig. 3, A). There was no significant difference in IL-5 levels between PGD₃-treated mice and conventional asthma models 24 h after OVA challenge, whereas the IL-4 level was lower in the former (Fig. 3, B). However, the levels of Th2 cytokines increased further 24 h after antigen challenge, as compared with control (Fig. 3, B).

Effects of PGD₃ on expression on MDC mRNA in vitro

We further studied the role of MDC- or TARC-mediated Th2 cell recruitment in the action of PGD₃ in the lung. First, we observed that MDC mRNA was expressed in primary human bronchial epithelial cells in a dose-dependent manner to PGD₃ (10⁻⁹ - 10⁻⁵ M) after 48-h incubation, while TARC mRNA was constitutively expressed and not influenced by PGD₃ (Fig. 4).
Fig. 3 Effects of PGD₂ on Th 1 (IL-2 and IFN-γ) and Th 2 (IL-4 and IL-5) cytokine production in BAL fluid. The levels of cytokines in BAL fluid were determined 0 h, 3 h (A), and 24 h (B) after the last challenge, as described in methods. There was no significant change in IL-5 or IL-4 levels in BAL fluid in the sensitized mice 24 h after provocation with PGD₂ alone, as compared with saline provocation (A). Three hours after inhalation of low-dose antigen (0.1% OVA), Th 2 cytokine levels in PGD₂-pretreated mice were significantly higher than the corresponding levels in saline-pretreated control mice (p<0.05). There was no significant difference in IL-5 level between PGD₂-pretreated and asthma models. However, further increases in Th 2 cytokine levels were noted 24 h after OVA challenge, as compared with the corresponding levels in the saline-pretreated mice (Fig. 3, B). Th 1 cytokines were not detected in BAL fluid in the PGD₂- or saline-pretreated and low-dose OVA (0.1%) challenged groups. (A) *p<0.05, between PGD₂ and saline-pretreated group. (B) *p<0.05, between PGD₂-pretreated low-dose OVA challenged groups and saline-pretreated and low-dose OVA challenged groups or PGD₂-pretreated saline challenged groups. N.S. : non significant difference. N.D. : non detectable.
Effects of PGD₂ on cytokine production by OVA-specific T cells and BMMC in vitro

To determine whether PGD₂ stimulation influences systemic immune responses, we analyzed the OVA-specific T cell response in vitro. PGD₂ stimulation did not affect OVA-induced Th2 cytokine production by mice spleen T cells (Fig. 5). We also analyzed the BMMC response in vitro. PGD₂ stimulation did not affect ionomycin-induced Th2 cytokine production by mice mast cells (data not shown).

Effects of PGD₂ on expression of MDC protein in vivo

We further studied the role of MDC- or TARC-mediated Th2 cell recruitment in the action of PGD₂ in the lung. We studied MDC expression by immunohistochemical analysis 3 and 24 h after OVA challenge. The PGD₂-pretreated mice showed strong staining for MDC in bronchial cells from 3 h (Fig. 6 A) to 24 h (data not shown) after Ag (0.1% OVA) challenge. In the saline-pretreated mice, no MDC was detected after Ag (0.1% OVA) challenge (Fig. 6 B). When PGD₂-pretreated mice inhaled saline, MDC staining was weak as compared with that after Ag challenge (Fig. 6 C). No MDC staining was observed in saline-pretreated and saline-challenged control mice (Fig. 6 D). MDC expression in the lung of positive control was not different from that in the PGD₂-pretreated mice (data not shown).

DISCUSSION

Here we presented a new mouse model for asthma in which exogenous administration of PGD₂ with low-dose antigen-induced marginal airway response led to the development of marked airway inflammation. Such an inflammation was the Th2 type as characterized by eosinophilia and lymphocytosis with elevated production of IL-4 and IL-5. PGD₂ is a major prostanooid produced by mast cells; however, its role in the pathogenesis of asthma remains unclear. Vasodilatation and increased permeability are established effects of PGD₂. In allergic situations, released PGD₂ may facilitate transendothelial migration of inflammatory cells, such as eosinophils, mast cells, lymphocytes, and monocytes, into loci via DP-mediated vasodilation-extravasation. On the other hand, evidence has recently been provided that PGD₂ attracts Th2 cells into the lung. In addition, a Gi-protein-coupled, seven-transmembrane-type receptor, CKTH2, which is preferentially expressed in Th2 cells, eosinophils, and basophils, has also been shown to contribute to the PGD₂-mediated chemotaxis of these
cells. Consistently with these previous studies, we found in this study increased numbers of eosinophils, lymphocytes, and macrophages in BAL fluid from PGD$_2$-pretreated, OVA-challenged mice. Furthermore, the Th2-type nature of the infiltrated T cells was supported by the observation that the levels of IL-$4$ and IL-$5$ in BAL fluid were significantly higher in PGD$_2$-pretreated, OVA-challenged mice than in PGD$_2$ or OVA alone–administered mice, while the levels of IFN-$\gamma$ and IL-$2$ in BAL fluid below the detection limits in the three groups. This phenomenon of skewed Th2 type inflammation might result from a direct chemotactic effect of PGD$_2$ on Th2 cells and eosinophils via DP receptor, CTH2 receptor, or both. However, we found no obvious inflammatory changes or increases in Th2 cytokines after treatment with PGD$_2$ ($10^{-5}$ M) alone, without immunization. These results prompted us to consider that PGD$_2$ per se has no chemotactic activity but rather PGD$_2$ induces chemotacticants for antigen–activated cells.

A CC chemokine MDC was a plausible candidate it acts as a chemotactic agent for activated Th2 cells and monocytes/macrophages via CCR4 receptor and for eosinophils via an unknown receptor. We observed that PGD$_2$ directly induced MDC, but not TARC, mRNA in bronchial epithelial cells in vitro. It is highly likely that bronchial epithelial cell–derived MDC mediates the effect of PGD$_2$ on the development of antigen–induced Th2 type inflammation. These results together indicated that the disease–promoting activity of PGD$_2$ in our model was mediated by MDC, likely produced by bronchial cells. In line with our conclusion, the importance of MDC has been pointed out by Gonzalo et al., who reported that anti–MDC antibody potently inhibited the development of asthma in another murine model. We have also report-
ed that MDC expression by airway epithelial cell was induced by TNF-α and IL-9 stimulation. PGD₂ induced MDC expression may be enhanced by inflammatory cytokine stimulation that released after OVA challenge. The critical mechanism of this synergistic effect of PGD₂ with OVA on MDC production in the pathogenesis of asthma needed to be clarified in the future.

Rather than conventional asthma models, we used a novel asthma model in this study, in which sensitized mice were pretreated with PGD₂, followed by low-dose antigen challenge. Our model can be modified into a chronic asthma models. Such a model might permit analysis of the effect of PGD₂ during allergic response on the development of asthmatic response to a subsequent antigen challenge. Using this model, we showed here that PGD₂ could induce an airway response with low-dose antigen challenge which otherwise dose not cause any inflammation in the lung. This finding suggests that the role of endogenous PGD₂ is to facilitate and sustain allergic responses in patients with frequent asthmatic symptoms. PGD₂ may therefore provide us with a novel target for the management of the disease. In this regard, our animal model for asthma will be useful to evaluate the efficacy of therapeutics for asthma directed against the action of PGD₂.

REFERENCES


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