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Originals

Prostaglandin D_2 Augments Low-dose Antigen-induced Th2 Type Airway Inflammation in Mice

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SUMMARY

Prostaglandin D_2 (PGD_2), a mast cell-derived lipid mediator is detected in lage amounts in airways of asthmatics, but its role of largely unkown. 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 antien challenge in airways of mice. Mice sensitized with ovalbumin (OVA) were challenged with a conventional-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-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^{-9} – 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 experssion 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 cycloxygenase 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

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accumulation of eosinophils and lymphocytes in the lung of Prostaglandin D synthase transgenic mice[7]. However, it remains unclear how PGD_2 participate in airway inflammation.

$CD4^+$ T cells, especially Th2 cells, are accepted to play an important role in the pathogenesis of bronchial asthma[8~13]. 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[14,15]. 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[16]. Th2 cells are recruited into the airway mucosa[8,17~22] and are found in the bronchoalveolar lavage (BAL) fluid of patients with asthma[13,23~30]. 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-regulated chemokine (TARC)and macrophage-derived chemokine(MDC)[31~33]. TARC has been found to induce chemotaxis of T cells, especially Th2 cells[34~36]. MDC has been shown to be a potent chemoattractant for Th2 cells[36,37], as well as for eosinophils and monocytes[38,39]. Both TARC and MDC, CCR4 ligands[40], have been implicated in Th2 type inflammation associated with the development of AHR[31,41~44]. 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[45].

We have developed a low-dose antigen model for inducible allergic asthma, which allowed us to examine the effect of inhaled PGD_2 directly. Using this models, we showed here that PGD_2 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 PGD_2 mediated the asthma-promoting activity of PGD_2. Our current study thus suggested the mechanism for the action of PGD_2 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 PGD_2 on the Th2-type airway inflammation induced by a low-dose antigen challenge. Sensitization and antigen challenges of mice were performed as described previously[40,49]. Briefly, BALB/c mice were sensitized on day 0 and 5 by intraperitoneal injections of OVA (Sigma, St. Louis, MO) (8 μg/mouse) adosorbed 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 PGD_2 augments low-dose OVA induced Th-2 type airway inflammation, the low-dose OVA exposure mice received aerosolized PGD_2 (10^{-3} M) or saline on day 16. In preliminary experiments this concentration of PGD_2 itself were confirmed to induce no obvious histological changes such as inflammatory cell recuitment and tissue edema. Some mice pretreated with aerosolized PGD_2 or saline on day 16 were challenged with aerosolized saline instead of low-dose OVA, and functioned as a PGD_2 alone control (Fig. 1, Group 2) or a negative control (Fig. 1, Group 3).

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed immediately befor and 3 and 24 h after the last aerosol chal-

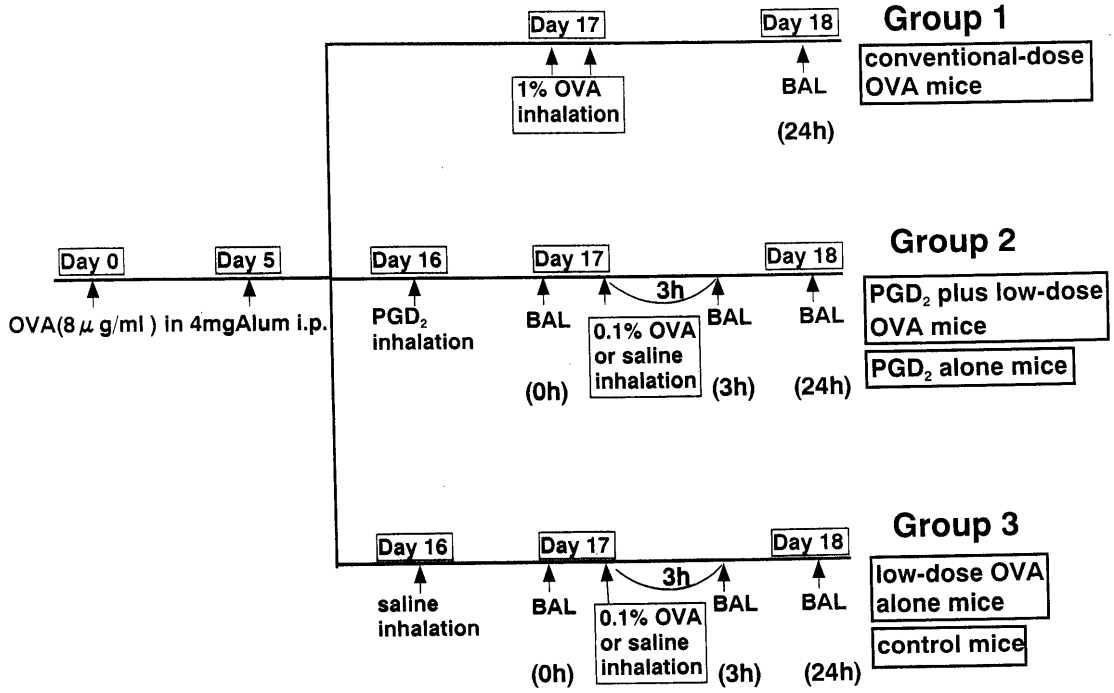

Fig. 1 Experimental protocols.
Group (1) : a conventional asthma model, Group (2) a nonconventional asthma model for analysis of effects of PGD_2-pretreatment low-dose antigen challenge. Group (3) a low-dose OVA alone mice and control mice. OVA ; ovalumin. i. p ; intraperitoneal injection. BAL ; bronchoalveolar lavege.

lenge. At the time of lavage, the mice were killed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg body weight). The trachea was exposed and cannulated with polyethylene tubing. The lungs were lavaged with three 0.5-ml aliquots of saline. The supernatant was stored at −70℃. Total cell numbers were counted with a hemocytometer. Cytospin preparations of BAL cell were stained with Diff-Quik solution (Osaka, Japan) to determine the differential cell count, evaluated on the basis of at least 500 leukocytes.

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.

Immunohistchemistry

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_2O_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 rising in running wtaer, the sections were counterstained with hematoxlin. 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℃ 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 Trisolv, a modified guanidine thiocyanate-phenol-chloroform method, as recommended by the manufacturer (Biotec Laboratories, Houston, TX). RT-PCR was performe to determine the relative quantities of TARC and MDC mRNA, using a modified method as described elsewhere[41]. 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′-TACAGACTGCACTC CTGGTTGTCC-3′ and 5′-TTCTGGCGGGGAGCAGC TATAATG-3′ ; TARC : 5-CACGCAGCTCGAGGGAC CAATGTG-3′ and 5′-TCAAGACCTCTCAAGGCTTTGC AGG-3′ ; β-action : 5′-TGACGGGGTCACCCACACTG TGCCCATCTA-3′ and 5′-CTAGAAGCATTGCGGTGG ACGATGGAGGG-3′. PCR products in agarose gel were detected by ethidium bromide staining.

Stimulation of primary human bronchial epithelial cells with PGD_2

To assess the effects of PGD_2 on MDC and TARC gene expression, primary human bronchial epithelial cells were stimulated with various concentrations of PGD_2 (10^{-9} – 10^{-5} 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 PGD_2 (10^{-9} – 10^{-5} M) during stimulation with OVA (10 μg/ml) for 24 h[40,50]. 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 3×10^6 cells/ml in IMDM, supplemented with 10% FCS (inactivated at 56℃), 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[40,50]. Toluidine blue staining revealed that the resulting cell population consisted of >99% BMMCs. These cells were cultured with 0.25 mM ionomycine during stimulation with PGD_2 (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 PGD_2 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 PGD_2 alone (Fig. 2, A ; 0 h before OVA challenge) and 24 h after provocation with saline control. This finding indicated that PGD_2 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 signficantly higher in PGD_2-pretreated 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 eosinphils and lymphocytes did not differ significantly between the conventional asthma model challenged with 1% OVA and the PGD_2-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 PGD_2-pretreated mice (Fig. 2, C).

Effect of PGD_2 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 detedted, 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

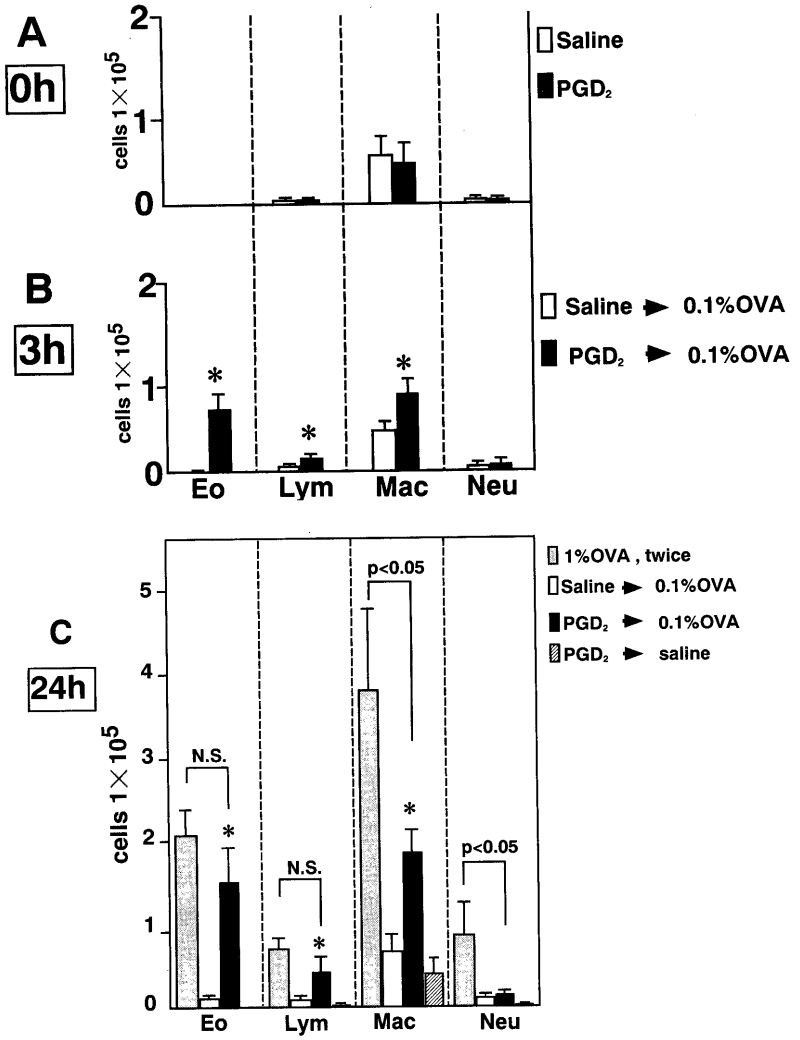

Fig. 2 Effects of PGD_2 on infiltration of inflammatory cells in BAL fluid. The number of inflammatory cells in BAL fluid was determined 0 h (A), 3 h (B), and 24 h (C) after the last challenge, as described in methods. Data represent means ± SEM (n = 6). Differences were considered significant if $p<0.05$. (B) * $p<0.05$, between PGD_2 and saline-pretreated group. (C) * $p<0.05$, between PGD_2-pretreated low-dose OVA challenged group and saline-pretreated and low-dose OVA challenged groups. N.S. : non signficant difference.

after OVA challenge) after PGD_2-pretreatment. This result indicated that PGD_2 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 PGD_2-pretreated mice than in the saline-pretreated control mice (Fig. 3, A). There was no significant difference in IL-5 levels between PGD_2-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 PGD_2 on expression on MDC mRNA in vitro

We further studied the role of MDC-or TARC-mediated Th2 cell recruitment in the action of PGD_2 in the lung. First, we observed that MDC mRNA was expressed in primary human bronchial epithelial cells in a dose-dependent manner to PGD_2 ($10^{-9}-10^{-5}$ M) after 48-h incubation, while TARC mRNA was constitutively experssed and not influenced by PGD_2 (Fig. 4).

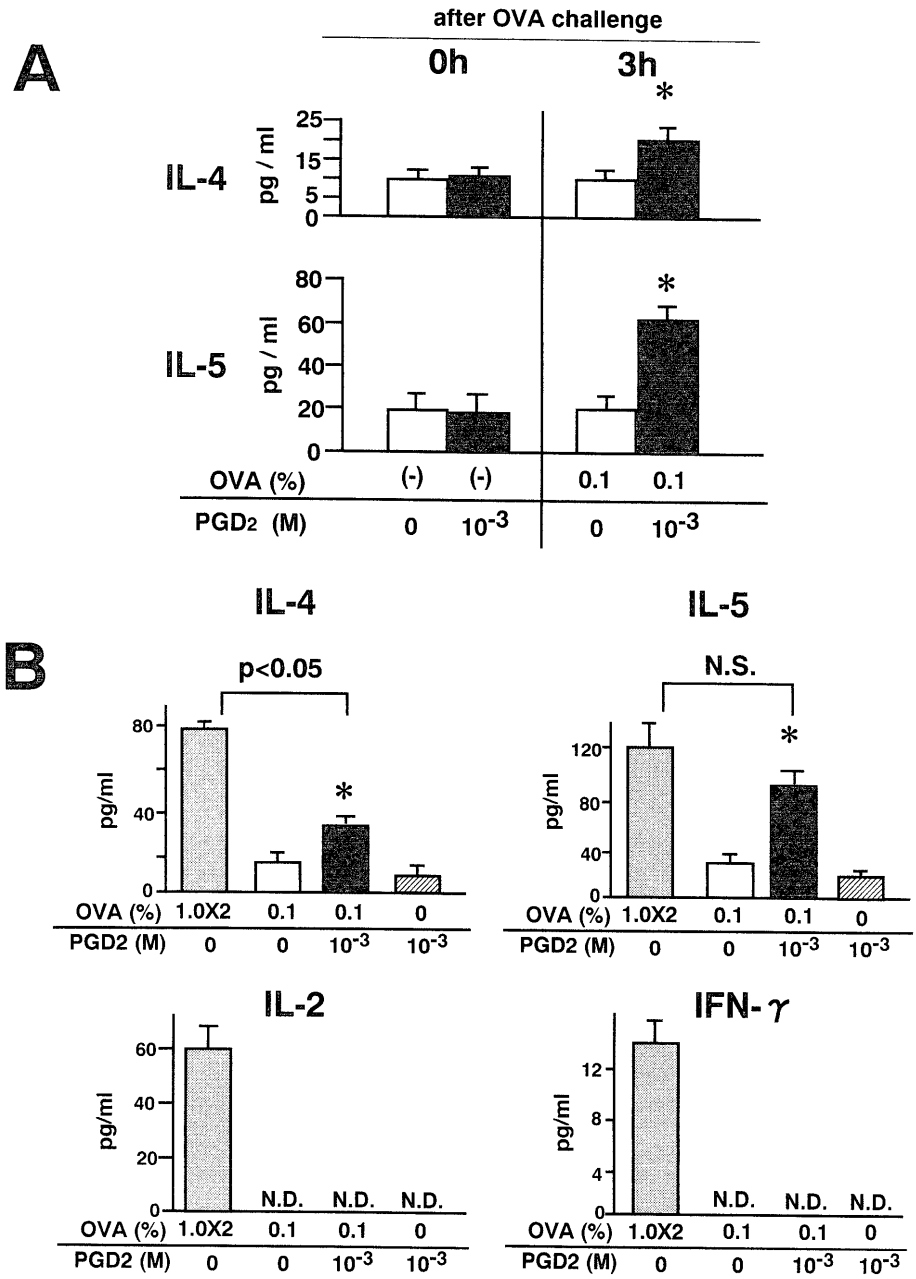

Fig. 3 Effects of PGD_2 on Th 1 (IL-2 and IFN-γ) and Th 2 (IL-4 and IL-5) cytokine production in BAL fluid. The levels of cytokine in BAL fluid were determined 0 h, 3 h (A), and 24 h (B) after the last challenge, as described in methods. There was no signficant change in IL-5 or IL-4 levels in BAL fluid in the sensitized mice 24 h after provocation with PGD_2 alone, as compared with saline provocation (A). Three hours after inhalation of low-dose antigen (0.1% OVA), Th 2 cytokine levels in PGD_2-pretreated mice were signficantly higher than the corresponding levels in saline-pretreated control mice ($p<0.05$). There was no signficant difference in IL-5 level between PGD_2-treated 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 PGD_2- or saline-pretreated and low-dose OVA (0.1%) challenged groups. (A) $^*p<0.05$, between PGD_2 and saline-pretreated group. (B) $^*p<0.05$, between PGD_2-pretreated low-dose OVA challenged groups and saline-pretreated and low-dose OVA challenged groups or PGD_2-pretreated saline challenged groups. N.S. : non significant difference. N.D. : non detectable.

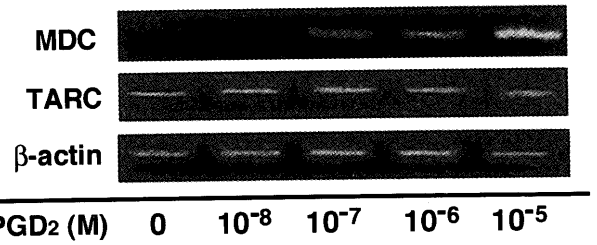

Fig. 4 Effects of PGD_2 on expression of MDC mRNA in vitro. MDC mRNA was expressed in primary human bronchial epithelial cells in a dose-dependent manner to PGD_2 ($10^{-8}-10^{-5}$ M) after 48-h incubation, while TARC mRNA was consitutively expressed and not influenced by PGD_2.

Effects of PGD_2 on cytokine production by OVA-specific T cells and BMMC in vitro

To determine whether PGD_2 stimulation influences systemic immune responses, we analyzed the OVA-specific T cell response in vitro. PGD_2 stimulation did not affect OVA-induced Th2 cytokine production by mice spleen T cells (Fig. 5). We also analysed the BMMC response in vitro. PGD_2 stimulation did not affect ionomycin-induced Th2 cytokine production by mice mast cells (data not shown).

Effects of PGD_2 on expression of MDC protein in vivo

We further studied the role of MDC- or TARC-mediated Th2 cell recruitment in the action of PGD_2 in the lung. We studied MDC expression by immunohistochemical analysis 3 and 24 h after OVA challenge. The PGD_2-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 PGD_2-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 PGD_2-pretreated mice (data not shown).

DISCUSSION

Here we presented a new mouse model for asthma in which exogenous adminstration of PGD_2 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. PGD_2 is a major prostanoid produced by mast cells ; however, its role in the pathogenesis of asthma remains unclear. Vasodilataion and increased permeability are established effects of PGD_2. In allergic situations, released PGD_2 may facilitate transendothelial migration of inflammatory cells, such as eosinophils, mast cells, lymphocytes, and monocytes, into loci via DP-medeated vasodilation-extravasation[51~54]. On the other hand, evidence has recently been provided that-PGD_2 attracts Th2 cells into the lung. In addition, a Gi-protein-coupled, seven-transmembrane-type receptor, CRTH2, which is preferentially expressed in Th2 cells, eosinophils, and basophils[55], has also been shown to contribute to the PGD_2-mediated chemotaxis of these

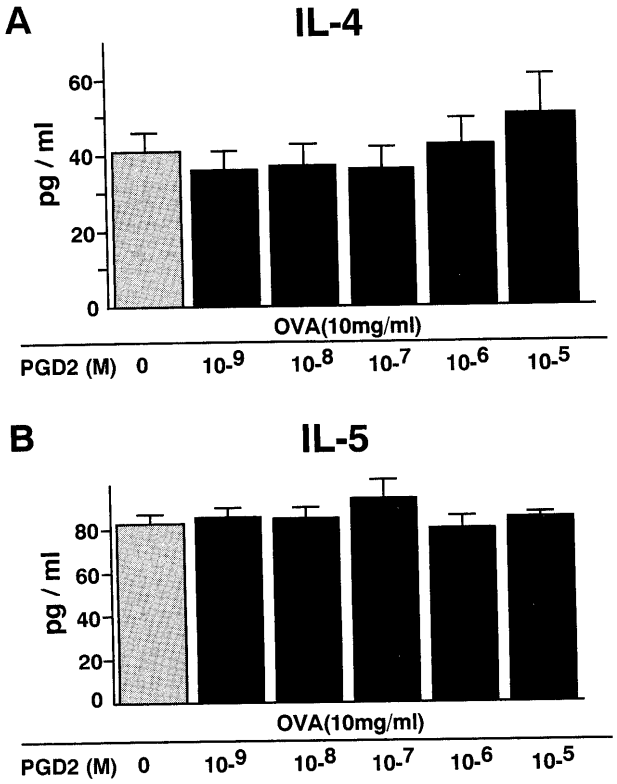

Fig. 5 Effect of PGD_2 on cytokine production by OVA-specific T cells in vitro. Spleen cells from sensitized and challenged mice were cultured for 24 h with PGD_2 ($10^{-9}-10^{-5}$ M) during OVA (10 μg/ml) stimulation. PGD_2 stimulation did not affect OVA-induced IL-4 (A)-or IL-5 (B) production by spleen cells. A representative data was shown out of triplicate experiments.

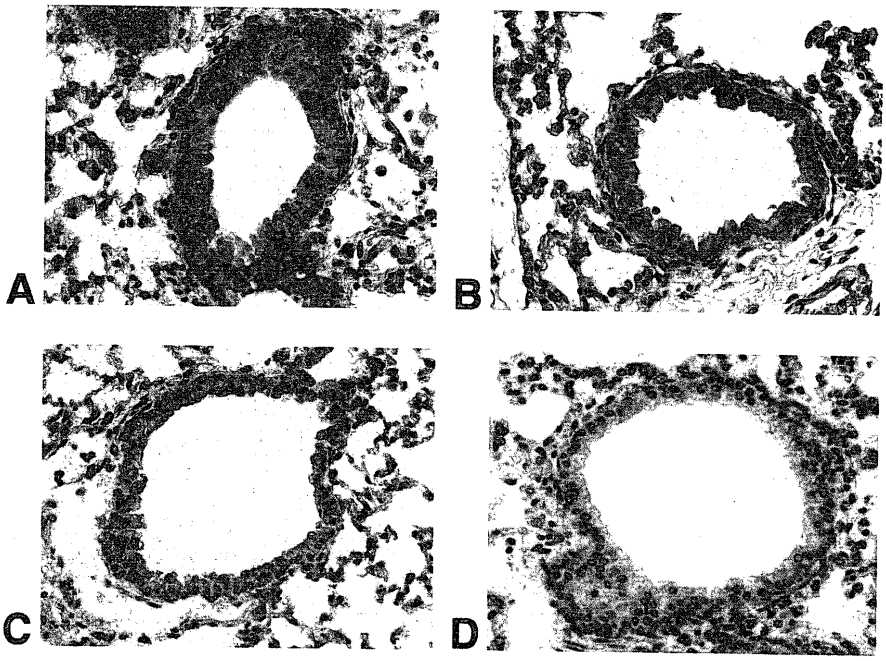

Fig. 6 Effects of PGD_2 on exression of MDC protein in lung tissue.
Lung sections were obtained from PGD_2-pretreated and low-dose OVA (0.1%) challenged group after 3 h (A), saline-pretreated and low-dose OVA (0.1%) challenged after 3 h (B), PGD_2-pretreated and saline-inhalation group after 3 h (C), and saline-pretreated and saline-inhalation group after 3 h (D). The sections were stained with ploYclonal antibodies against mouse MDC. Positive staining was detected with an avidin-biotin peroxidase staining system that results in a brown reaction product. Secions were counterstained with hematoxylin (blue) for contrast. Protein expression was strongly detected in the airway epithelium from PGD_2-pretreated and low-dose OVA (0.1%) challenged mice (A). In the saline-pretreated mice, MDC was not detected after antigen challenge (B). When PGD_2-pretreated mice inhaled saline, MDC staining showed signals (C) as compared those after low-dose OVA challenge (original magnification : ×200).

cells[56]. 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-γ 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, CRTH2 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 rether PGD_2 induces chemoattractants for antigen-activated cells.

A CC chemokine MDC was a plausible candidate it acts as a chemoattractant 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[57]. We have also report-

ed that MDC expression by airway epithelial cell was induced by TNF-α and IL-9 stimulation[45]. PGD_2 induced MDC expression may be enhanced by inflammatory cytokine stimulation that released after OVA challenge. The critical mechanism of this synergistic effect of PGD_2 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 wihch sensitized mice were pretreated with PGD_2, 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 PGD_2 during allergic responce on the development of asthmatic response to a subsequent antigen challenge. Using this model, we showed here that PGD_2 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 PGD_2 is to facilitate and sustain allergic responses in patients with frequent asthmatic symptoms. PGD_2 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 PGD_2.

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