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Original Article

Prostaglandin D_2 and interleukin-5 reduce CRTH2 surface expression on human eosinophils

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Abstract

Background: Recently, a second prostaglandin D_2 (PGD_2) receptor, chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2), was identified. Because PGD_2 was reported to have chemotactic activity on eosinophils, CRTH2 expressed on eosinophils attracted interest as a receptor associated with eosinophil migration to, and accumulation at, inflammatory sites. To elucidate the mechanism regulating the expression of CRTH2 on eosinophils, the effects of PGD_2, interleukin (IL)-4, IL-5 and interferon (IFN)-γ on CRTH2 expression were investigated.

Methods: Blood eosinophils were purified using Percoll and anti-CD16 antibody coated magnetic beads. Eosinophils were incubated with PGD_2 and/or IL-4, IL-5 and IFN-γ. The expression of CRTH2 on eosinophils was measured using a FACScan cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Results: Prostaglandin D_2 and IL-5, but not IL-4 and IFN-γ, downregulated the expression of CRTH2 on eosinophils. Furthermore, PGD_2- and IL-5-induced downregulation of CRTH2 on human eosinophils was inhibited by phenylarsine oxide, a receptor internalization inhibitor.

Conclusions: These results suggest that PGD_2 and IL-5 regulate CRTH2 expression on eosinophils through CRTH2 internalization. The decreased expression of CRTH2 on tissue eosinophils may make these cells remain at the site of allergic inflammation.

Key words: CRTH2, eosinophils, interferon-γ, interleukin-4, interleukin-5, prostaglandin D_2.

Introduction

Allergic inflammation is characterized by the selective accumulation of inflammatory cells, such as activated Th2 lymphocytes and eosinophils. The Th2 cells, as well as eosinophils, play an essential role in the pathogenesis of allergic inflammation.[1,2] Phenotypic characterization of Th2 cells has become a focus of interest. Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) was recently cloned as a Th2-selective surface molecule distinguished from Th1 cells.[3] It was also found that CRTH2 was expressed on eosinophils in addition to Th2 cells.[4]

Interestingly, CRTH2 was recently identified as a second receptor for prostaglandin D_2 (PGD_2).[5] Prostaglandin D_2 is a major arachidonic acid metabolite released by antigen-activated mast cells[6] and Th2 cells.[7] Large amounts of PGD_2 are generated in the asthmatic lung.[8,9] Prostaglandin D_2 and CRTH2 may be implicated in the pathogenesis of allergic diseases.

The mechanism by which CRTH2 expression is regulated is unclear. Therefore, in the present study, the modulation of surface CRTH2 expression on human eosinophils by PGD_2, Th2 cytokines (such as interleukin (IL)-4 and IL-5)and Th1 cytokines (such as interferon (IFN)-γ), was investigated.

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METHODS

Eosinophil purification

Peripheral venous blood was obtained from subjects with mild to moderate eosinophilia. Eosinophil donors were either healthy or had mild allergic rhinitis and blood was obtained at a time when they were relatively asymptomatic and off medication. Eosinophils were isolated by sedimentation with 6% dextran followed by centrifugation on 1.088 Percoll (Pharmacia, Uppsala, Sweden) density gradients, as modified from the method of Hansel *et al*.[10,11] Cells were further purified by negative selection using anti-CD16 immunomagnetic beads and a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Eosinophils were then suspended in Hank's balanced salt solution (HBSS; Life Technologies, Grand Island, NY, USA) with 1% fetal calf serum (FCS) in tubes coated with 3% human serum albumin. The resulting eosinophil purity was more than 99%.

Flow cytometric analysis of surface CRTH2

This method was used for studying the effect of PGD_2 on CRTH2 surface expression. Eosinophils were resuspended at 1×10^6 cells/mL in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% FCS and incubated with various concentrations of PGD_2 and IL-4 (5 ng/mL) or IL-5 (5 ng/mL) or IFN-γ (100 U/mL) for 3 h at 37°C. Cells were washed twice with phosphate-buffered saline (PBS) and then stained with biotinylated antihuman CRTH2 antibody (Ab; BM-16; final concentration 10 μg/mL; a kind gift from Dr Kinya Nagata and Dr Hiroyuki Hirai, Bio Medical Laboratories, Saitama, Japan) and phycoerythrin (PE)-labeled streptavidin. BM16 is not a neutralizing anti-CRTH2 monoclonal antibody (mAb). Biotinylated rat IgG2a Ab (Becton Dickinson, San Jose, CA, USA) was used as an isotype-matched control. Stained cells were analyzed using a flow cytometer (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The expression of CRTH2 is given as change in mean fluorescence intensity (ΔMFI), that is, the difference in MFI between the sample and the isotype-matched control.

Blockade of CRTH2 internalization on eosinophils

Inhibition of internalization of surface CRTH2 was assayed by eosinophils pretreated with 8 μmol/L phenylarsine oxide (PAO; Sigma-Aldrich, St Louis, MO, USA) for 5 min and then treated with PGD_2 (100 nmol/L) or IL-5 (5 ng/mL) for 3 h at 37°C. The PAO (8 μmol/L) was non-toxic to cells (as assessed by annexin V–fluorescein isothiocyanate and propidium iodide (PI) staining).

Flow cytometric analysis of intracellular or whole (intracellular and surface) CRTH2 expression

The method used is a modification of a method described earlier.[12] Briefly, eosinophils were resuspended at 1×10^6 cells/mL in RPMI 1640 supplemented with 10% FCS and incubated with PGD_2 (100 nmol/L) for 3 h at 37°C. After being washed in PBS, cells were stained with non-biotinylated BM16 (for intracellular staining) or biotinylated BM16 (for whole staining) at a final concentration of 10 μg/mL in PBS for 30 min at 4°C. Then, 2 mL lysing solution (FACS™ Lysing Solution; Becton Dickinson) was added to each sample for 10 min in the dark at room temperature. After washing, permeabilizing solution (FACS™ Permeabilizing Solution; Becton Dickinson) was added for 10 min in the dark at room temperature. After being washed in PBS, cells were incubated for 30 min with BM16 or isotype-matched Ab (rat IgG2a) at a final concentration of 30 μg/mL in PBS for 30 min at 4°C. Cells were washed with PBS and then stained with PE-labeled streptavidin for 30 min at 4°C. Stained cells were analyzed using a flow cytometer.

Statistical analysis

Data are presented as the mean ± SD. A one-way ANOVA with repeated measures was used for comparison of more than two variables. When the initial *P*-value was less than 0.05, Scheffé's *F*-test was used to determine the significance between groups. Significance was established at $P < 0.05$.

RESULTS

Effect of PGD_2, IL-4, IL -5 and IFN-γ on CRTH2 surface expression

We conducted *in vitro* studies aimed at elucidating the mechanism controlling the expression of CRTH2 on eosinophils. We selected PGD_2, IL-4, IL-5 and IFN-γ as possible factors that control CRTH2 expression on eosinophils at allergic inflammatory sites. Prostaglandin D_2 reduced CRTH2 surface expression on human eosinophils in a dose-dependent manner. The surface

expression of CRTH2 decreased significantly to 74.7 ± 5.3, 47.5 ± 10.2 and 43.8 ± 11.4% (compared with medium alone) following stimulation with PGD_2 at concentrations of 10, 100 and 1000 nmol/L, respectively (Fig. 1a). Neither IL-4 nor IFN-γ was able to significantly modulate the surface expression of CRTH2. The surface expression of CRTH2 decreased significantly to 80.1 ± 10.8% (compared with medium alone) following stimulation with IL-5 (5 ng/mL; Fig. 1b). The dose of IL-5 used in the present study was chosen on the basis of results from previous studies showing that IL-5 specifically bound to eosinophils and reached saturation at an approximate concentration of 1–1.25 nmol/L.[13,14] Eosinophils were incubated with PGD_2 (100 nmol/L) and IL-4 (5 ng/mL) or IL-5 (5 ng/mL) or IFN-γ (100 U/mL) for 3 h. The reduction in the surface expression of CRTH2 was not enhanced by the addition of cytokines compared with eosinophils incubated with PGD_2 alone (Fig. 1c).

Effect of PAO pretreatment on PGD_2- and IL-5-induced CRTH2 downregulation on eosinophils

To elucidate the mechanism of CRTH2 downregulation on eosinophils, we pretreated eosinophils with PAO, an inhibitor of receptor internalization,[15] following PGD_2 or IL-5 treatment. In the present study, PAO inhibited PGD_2- and IL-5-induced CRTH2 downregulation on human eosinophils (Fig. 2a; Table 1).

Effect of PGD_2 on whole and intracellular CRTH2 expression in human eosinophils

Whole CRTH2 expression in PGD_2-treated eosinophils (43.1 ± 15.3 ΔMFI) was significantly lower than that in non-treated eosinophils (74.5 ± 14.3 ΔMFI). In contrast, there was no difference in the intracellular expression of CRTH2 before (20.1 ± 1.14 ΔMFI) and after (20.8 ± 4.96 ΔMFI) treatment with PGD_2 (Fig. 2b).

Discussion

Prostaglandin D_2, a ligand against CRTH2 expressed on Th2 cells and eosinophils, is produced in the asthmatic lung and is considered to be an important proinflammatory mediator in allergic disease.[16] Because PGD_2 was reported to have chemotactic activity on eosinophils,[5,17] CRTH2 expressed on eosinophils attracted interest as a receptor associated with eosinophil migration to, and accumulation at, inflammatory sites. CRTH2 is one of seven transmembrane G-protein-coupled receptors as well as a chemokine receptor.[5] One proposed regulatory mechanism for the expression of the seven

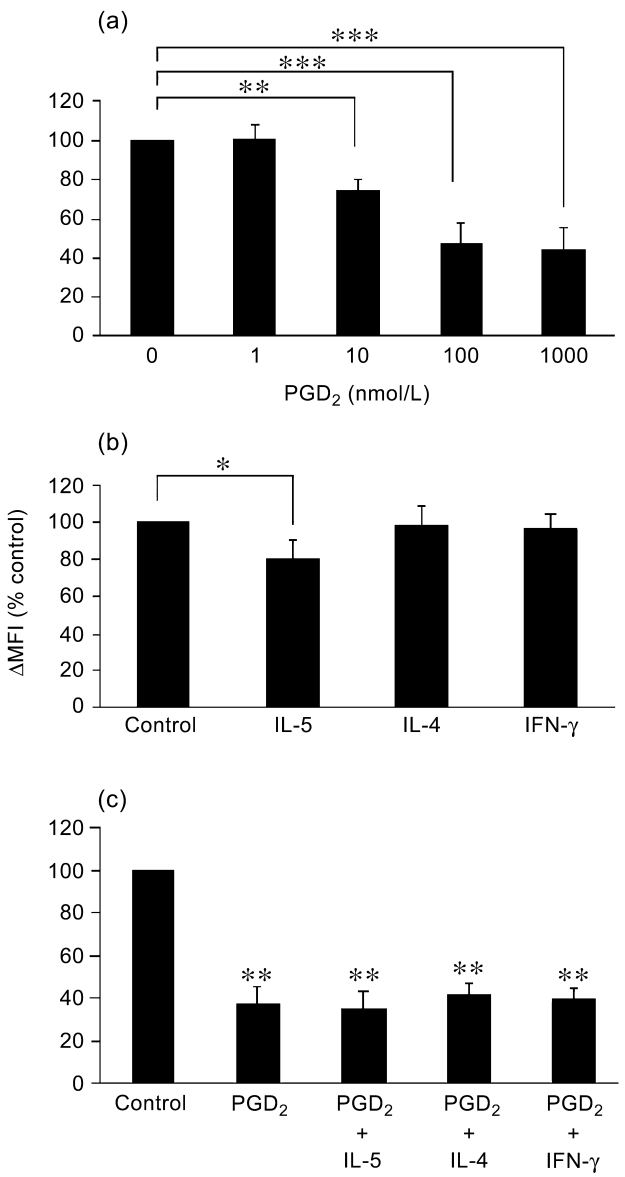

Fig. 1 Effect of prostaglandin (PG) D_2 and cytokines on the expression of CRTH2 on human eosinophils. (a) Prostaglandin D_2 induced a dose-dependent downregulation of CRTH2 on human eosinophils ($n = 5$; $^{**}P < 0.01$, $^{***}P < 0.001$). (b) Interleukin (IL)-5 induced a significant downregulation of CRTH2 on eosinophils; IL-4 and interferon (IFN)-γ did not change CRTH2 expression on human eosinophils under these conditions ($n = 5$; $^{*}P < 0.05$). **(c)** Interleukin-4, IL-5 and IFN-γ did not modify PGD_2-induced CRTH2 downregulation on eosinophils ($n = 4$; $^{**}P < 0.01$ compared with control). ΔMFI, change in mean fluorescence intensity.

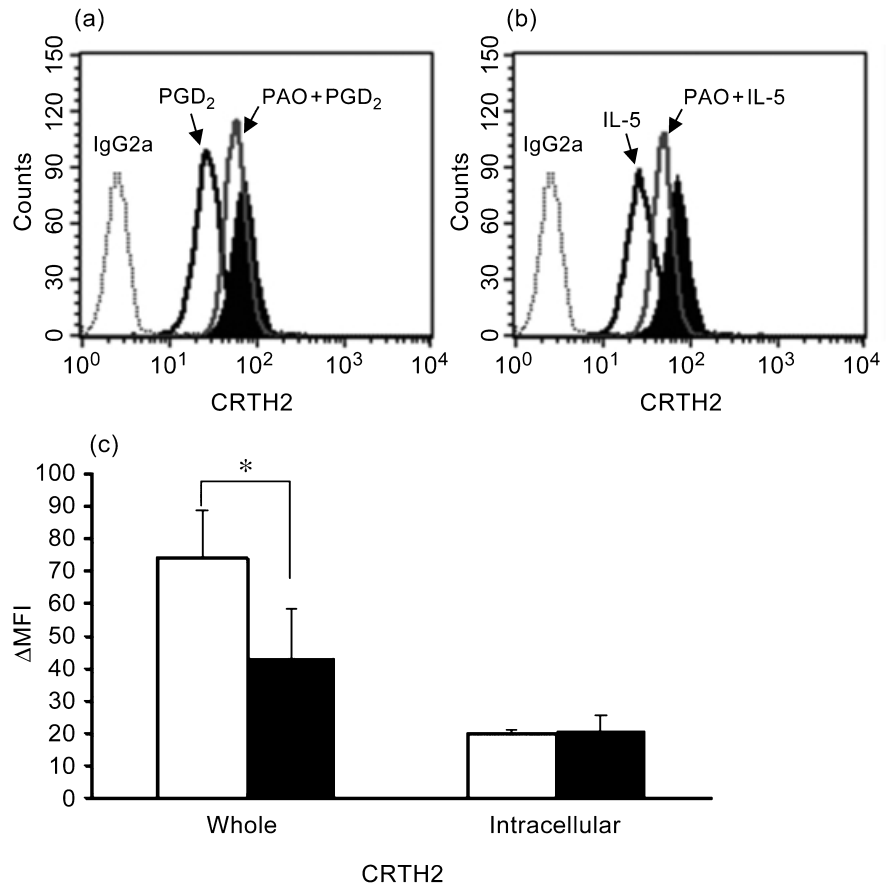

Fig. 2 Flowcytometric analysis suggesting internalization and degradation of CRTH2. Phenylarsine oxide (PAO) pretreatment inhibited (a) rostaglandin (PG) D_2- and (b) interleukin (IL)-5-induced CRTH2 downregulation. Three similar representative experiments are shown. (c) Whole CRTH2 expression in PGD_2-treated eosinophils (■) was significantly lower than that in non-treated eosinophils (□). In contrast, there was no difference in the intracellular expression of CRTH2 between eosinophils treated with (■) and without (□) PGD_2 ($n = 3$; $^*P < 0.05$).

Table 1 Effect of phenylarsine oxide pretreatment on prostaglandin D_2- and interleukin-5-induced CRTH2 down-regulation

	ΔMFI (% control)	*P*
Control	100.00	–
PGD_2	40.13 ± 6.57	0.002
PAO + PGD_2	68.93 ± 6.58	0.119
IL-5	55.10 ± 21.71	0.018
PAO + IL-5	73.36 ± 14.01	0.213

Data are the mean ± SD. Scheffé's *F*-test was used to determine the significance between groups ($n = 3$).

FACS analysis was performed to assess surface expression of CRTH2 on eosinophils.

PGD_2, prostaglandin D_2; PAO, phenylarsine oxide; IL-5, interleukin-5.

transmembrane G-protein-coupled chemokine receptors, such as CCR1, CCR2b, CCR3 and CXCR4, is ligand-induced internalization.[18–21] Cytokines have also been shown to modulate surface protein expression and mRNA expression for certain chemokine receptors. For example, IL-3, IL-5, granulocyte–macrophage colony stimulating factor (GM-CSF) and IL-4 attenuated the surface expression of CXCR4.[21] In contrast, the regulatory mechanism for the expression of CRTH2 is not well understood. We have observed, in a case of eosinophilic pneumonia, that the expression of CRTH2 on bronchoalveolar lavage fluid (BALF) eosinophils was much lower than that of eosinophils in peripheral blood (ΔMFI: 27.5 *vs* 132.3, respectively; K Hamada *et al.*, unpubl. obs.). This observation suggested the presence of unknown factors produced at the inflammatory site regulating the expression of CRTH2 on eosinophils. Moreover, the expression of CCR3 on eosinophils in BALF was also lower than that on peripheral eosinophils. Indeed, in the present study, we chose PGD_2, IL-4, IL-5 and IFN-γ as possible factors and found that PGD_2 and IL-5 downregulated the expression of CRTH2 on eosinophils. Hirai *et al.* reported the downregulatory effect of PGD_2 on the expression of CRTH2 on Th2 cells.[22] As has been reported for chemokine receptors, such as CCR1, CCR3 and CXCR4, although it is only one of several possible explanations, ligand-induced

internalization is the regulatory mechanism responsible for the downregulation of CRTH2. Indeed, the internalization inhibitor PAO inhibited PGD_2- and IL-5-induced CRTH2 downregulation on human eosinophils. Thus, the mechanism of CRTH2 downregulation may be receptor internalization. Interestingly, the intracellular expression of CRTH2, which was expected to increase as a result of internalization, was not changed by PGD_2. Unchanged intracellular expression of CRTH2 after PGD_2 stimulation suggested the degradation of CRTH2 following CRTH2 internalization. Ligand-induced downregulation of receptor expression may explain the diminished chemotactic migration after repetitive stimulation by a certain chemoattractant.[23] The decreased expression of CRTH2 on tissue eosinophils may make these cells remain at the site of allergic inflammation.

In summary, PGD_2 and IL-5 reduced CRTH2 expression on human eosinophils. Further studies are necessary to elucidate the mechanism and clinical relevance of PGD_2- and IL-5-induced CRTH2 downregulation.

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