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Original article

Effect of prostaglandin D2 on VEGF release by nasal polyp fibroblasts

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ARTICLE INFO

Article history:

Received 14 December 2015

Received in revised form

4 March 2016

Accepted 16 March 2016

Available online 16 April 2016

Keywords:

CRTH2

DP

Nasal polyp fibroblast

PGD2

VEGF

Abbreviations:

CRSwNP, chronic rhinosinusitis with nasal polyps; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DP, D-prostanoid; PG, prostaglandin; NPDF, nasal polyp-derived fibroblasts; VEGF, vascular endothelial growth factor

ABSTRACT

Background: Vascular endothelial growth factor (VEGF) is known to be associated with the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP). VEGF is produced by a variety of cells including fibroblasts. It was recently reported that prostaglandin (PG) E₂ induces VEGF release by nasal polyp fibroblasts. However, little is known regarding possible regulation of VEGF by other PGs. We have reported that molecules that regulate PGD₂ metabolism play roles in the pathogenesis of CRS including in local eosinophilia and type 2 cytokine production. In the present study, we sought to determine whether PGD₂ regulates VEGF release by nasal polyp fibroblasts.**Methods:** Nasal polyp fibroblasts were established from nasal polyps. These fibroblasts were stimulated with serial dilutions of PGD₂ or PGD₂ receptor (DP/CRTH2)-selective agonists in the presence or absence of receptor-selective antagonists. The concentration of VEGF in the culture supernatants was determined using ELISA.**Results:** 5 μM of PGD₂ significantly induced VEGF release by nasal polyp fibroblasts. VEGF release was also obtained by stimulation with a DP receptor-selective, but not with a CRTH2 receptor-selective agonist. In addition, PGD₂-induced VEGF release was significantly inhibited by pre-treatment with DP receptor-selective antagonists. In contrast, pre-treatment with a CRTH2 receptor-selective antagonist significantly enhanced PGD₂-induced VEGF release.**Conclusions:** PGD₂ stimulates VEGF production via DP but not CRTH2 receptors in nasal polyp fibroblasts.Copyright © 2016, Japanese Society of Allergology. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Vascular endothelial growth factor (VEGF) induces a variety of functions including angiogenesis and vascular hyper-permeability, and it is thought to be associated with the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP).^{1–3} In NP, VEGF is mainly expressed in vascular endothelial cells, basal membranes, perivascular spaces, and epithelial cells.¹ VEGF drives the proliferation and survival of NP epithelial cells.² The level of VEGF protein in nasal lavage is significantly higher in patients with CRSwNP as compared with control subjects, and the serum level of VEGF is significantly increased during acute exacerbation in those patients.^{2,3}

In addition to VEGF expression in NP epithelial cells, NP-derived fibroblasts (NPDF) produce VEGF in response to various stimuli such as hypoxia, TNF- α , LPS, and rhinovirus.^{4–6} Macrolides and corticosteroids inhibit such VEGF production.^{4,6} More recently, it was reported that prostaglandin (PG) E₂, one of the major PGs detected in NP, promotes VEGF release by NPDF via EP2 and EP4 receptors.^{7–9} However, little is known regarding whether other PGs might also regulate VEGF release.

PGD₂ is another major PG associated with the pathogenesis of CRSwNP.^{7,8,10} For example, we have shown that the level of PGD₂ in middle meatus secretions is significantly higher in CRS patients with NPs than in those without NPs.¹¹ The mRNA level of haemopoietic-type PGD₂ synthase in NP was also significantly and positively correlated with the degree of NP eosinophilia and the radiological severity of CRS.¹⁰ In addition, we have observed that this synthase was expressed in infiltrating c-kit⁺ and vimentin⁺ cells in NP, suggesting that mast cells and fibroblasts are the dominant sources of PGD₂ release in CRS (unpublished data).

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Peer review under responsibility of Japanese Society of Allergology.

D-prostanoid (DP) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) are the two major receptors reported for PGD₂ to date.¹² The DP receptor couples to the Gs protein, and signaling through the DP receptor is known to result in both pro-inflammatory and anti-inflammatory effects on airway inflammation.^{10,13–16} On the other hand, the CRTH2 receptor couples to the Gi protein, and signaling through CRTH2 mainly shows a pro-inflammatory effect.^{17–19} In terms of the relationship between PGD₂ receptor expression and the pathogenesis of CRSwNP, we have previously shown that the amount of mRNA for eotaxin, which selectively induces eosinophil chemotaxis, significantly and positively correlated with the amount of the mRNA for the DP receptor but not for the CRTH2 receptor in sinonasal tissues.¹¹

In the present study, we sought to determine whether PGD₂ regulates VEGF release by NPDP, and if so, which PGD₂ receptor might mediate such release.

Methods

Reagents

We purchased the following study materials: RPMI-1640, L-glutamine-penicillin-streptomycin solution, protease, collagenase, hyaluronidase, DNase I, FCS, trypsin, EDTA (all from Sigma, St. Louis, MO, USA), and red blood cell lysis buffer (Roche, Indianapolis, IN, USA). PGD₂, receptor-selective agonists for DP (BW245C) and CRTH2 (DK-PGD₂), and a DP receptor-selective antagonist (MK-0524) were purchased from Cayman (Ann Arbor, MI, USA). A CRTH2 receptor-selective antagonist (OC000459) was purchased from ChemScene LLC (Monmouth Junction, NJ, USA). Another DP receptor-selective antagonist, ONO-4053, was provided by Ono Pharmaceuticals (Osaka, Japan). PGD₂ and receptor-selective agonists/antagonists were dissolved in DMSO (Sigma) to a stock concentration of 2×10^{-2} M (PGD₂) or 2×10^{-3} M (others), filtered through a 0.2 μm filter, and stored at -80°C until use.

Subjects

The study involved 28 Japanese CRSwNP patients (Table 1). The presence of CRSwNP was determined based upon diagnostic criteria reported in a European position paper on rhinosinusitis and nasal polyps.²⁰ All patients failed to respond to medical treatment, including macrolide therapy, and thus received endoscopic sinus surgery. Among these, 7 and 21 patients were diagnosed into non-eosinophilic CRS (non-ECRS) and eosinophilic CRS (ECRS), respectively, based on the JESREC (Japanese Survey of Refractory Eosinophilic Chronic Rhinosinusitis) criterion.²¹ For at least four weeks prior to surgery none of the participants received systemic glucocorticoids, and for at least two weeks prior to

surgery none of the participants received pharmacotherapy for rhinosinusitis, such as macrolide antibiotics or intranasal steroids. Informed consent for participation in the study was obtained from each patient, and the study was approved by the Human Research Committee of the Okayama University Graduate School of Medicine and Dentistry.

Generation of NPDP

NPDP were generated as previously reported.²² Briefly, NPs were dispersed by enzymatic digestion using protease, collagenase, hyaluronidase, and DNase. The dispersed NP cells were suspended with culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) in a culture dish (Sumitomo Bakelite, Tokyo, Japan) at 37°C ad 5% CO₂ for 10 days, and then non-adherent cells were removed by changing the culture medium. After reaching confluence, NPDP were harvested by treatment with 0.25% trypsin and 0.02% EDTA, and were then divided into new dishes. The cellularity of NPDP was characterized using immunohistochemistry; more than 95% of confluent cells were found to be vimentin-positive. NPDP were used after 6 to 8 passages.

Cell culture

NPDP were seeded into 48-well culture plates (Asahi Techno Glass, Tokyo, Japan). When the cells reached confluence, they were rested in medium containing 0.5% FCS for 4 days. The cells were then stimulated with serial dilutions (0.5, 5.0, or 50 μM) of PGD₂ or control buffer (DMSO) for 24 or 72 h. In order to determine receptor specificity, serial dilutions (0.1, 1.0, or 10 μM) of BW245C, DK-PGD₂, or control buffer were added for 72 h. Alternatively, serial dilutions (0.1, 1.0, or 10 μM) of DP receptor-selective antagonists (MK0524 or ONO-4053) or a CRTH2 receptor-selective antagonist (OC000459) were added to NPDP treated with 5 μM of PGD₂ for 72 h.

Measurement of VEGF

The levels of VEGF were determined using a DuoSet™ ELISA development kit (R&D Systems, Minneapolis, MN, USA). The detection limit was 8 pg/mL.

Real-time quantitative PCR

Real-time quantitative PCR for analysis of the mRNA level of DP and CRTH2 receptors in NPDP was performed as described previously.¹¹ Levels of VEGF mRNA in NPDP following exposure to 5 μM PGD₂ or control buffer (0.25% DMSO) for 2 h were also determined. The primers designed for VEGF determination had the following sequences: forward 5'-TGAGATTATGCGGATCAAACC-3' and reverse 5'-TGCATTCACATTTGTTGTGCTGTAG-3' (81 bp).²³ The absolute copy number for each sample was calculated, and samples are reported as copy number relative to GAPDH mRNA that was used as an internal control. RT-PCR for GAPDH, DP and CRTH2 were also performed, and gel images using 2% agarose were developed.

Statistical analysis

Values are given as the median. The nonparametric Wilcoxon's signed rank test was used to analyze data. P-values less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS software (version 11.0, Chicago, IL, USA).

Table 1
Subjects background.

| | |
|--|-------------|
| Number | 28 |
| Sex (male:female) | 18:10 |
| Age (y) | 56.1 ± 14.7 |
| Age range (y) | 27–80 |
| Patients with bronchial asthma | 12 |
| Patients with allergic rhinitis | 9 |
| Serum IgE (IU/ml) | 325 ± 438 |
| Blood eosinophilia (% of white blood cell) | 6.7 ± 4.7 |
| CT grading score (Lund–Mackay system) | 15.9 ± 5.2 |
| FEV1/FVC ratio | 74.7 ± 11.5 |

Results are shown as mean ± standard deviation.
FEV1/FVC, forced expiratory volume in one second/forced vital capacity.

Results

Effect of PGD₂ on VEGF release by NPDF

As compared to control (0.25% DMSO), a significant increase in the production of VEGF protein was seen following both 24-h ($P = 0.038$, $n = 9$) and 72-h ($P = 0.008$) stimulation of NPDF with 5 μM of PGD₂ (Fig. 1). The levels of VEGF were significantly higher after 72-h stimulation as compared with after 24-h stimulation ($P = 0.011$). Therefore, for further investigation, we stimulated NPDF with PGD₂ or receptor-selective agonists for 72 h. No significant difference in 5 μM PGD₂-induced VEGF production by NPDF ($n = 25$) was seen between ECRS ($n = 7$) and non-ECRS ($n = 18$) patients ($P = 0.856$). Moreover, the presence of asthma ($P = 0.542$) or allergic rhinitis ($P = 0.533$) had no impact on VEGF release. Similar to the protein level, mRNA expression of VEGF was also increased following exposure to PGD₂ ($P = 0.043$, $n = 5$) (Fig. 2).

Effect of PGD₂ receptor-selective agonists on VEGF release by NPDF

To evaluate the receptor specificity of PGD₂ stimulation of VEGF release, we stimulated NPDF with PGD₂ receptor-selective agonists. A significant release of VEGF was obtained by stimulation with the DP receptor-selective agonist, BW245C, in a dose-dependent manner ($P = 0.011$, $n = 8$) (Fig. 3A). In contrast, no significant VEGF release was seen following stimulation with the CRTH2 receptor-selective agonist, DK-PGD₂ (Fig. 3B). Instead, a trend of inhibition of VEGF release was seen in response to 10 μM of DK-PGD₂ ($P = 0.093$).

Effect of receptor-selective antagonists on PGD₂-induced VEGF release by NPDF

To confirm the above receptor specificity of PGD₂ in VEGF stimulation, PGD₂-stimulated NPDF were treated with receptor-selective antagonists. As compared with control (0.25% DMSO), MK-0524 and ONO-4053, both of which are DP receptor-selective antagonists, inhibited PGD₂-induced release of VEGF in a dose-dependent manner. The maximum inhibition (34.9% and 46.6% for MK-0524 and ONO-4053, respectively) was seen at an antagonist concentration of 10 μM ($P = 0.018$, $n = 7$). In addition, significant inhibition was seen following treatment with ONO-4053 ($P = 0.028$) but not with MK-0524 ($P = 0.091$) at a concentration of 0.1 μM (Fig. 4). On the other hand, treatment with the CRTH2

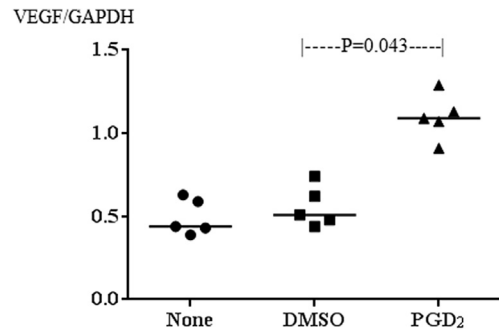


Fig. 2. PGD₂-induced VEGF mRNA expression by NPDF. NPDF were stimulated with 5 μM PGD₂ or control buffer (0.25% DMSO) for 2 h. Relative amounts of VEGF mRNA in NPDF were determined by real-time quantitative PCR. Bar represents median. P -values were determined using the Wilcoxon signed-rank test.

receptor antagonist, OC000459, did not inhibit the VEGF release induced by PGD₂. Instead, this treatment significantly augmented VEGF release at concentrations of 1.0 ($P = 0.004$, $n = 11$) and 10 ($P = 0.006$) μM (Fig. 5).

Quantification of DP/CRTH2 mRNA in NPDF

Real-time PCR revealed that the level of DP receptor mRNA in NPDF was significantly higher than that of CRTH2 mRNA ($P = 0.008$, $n = 9$) (Fig. 6A). Gel images by RT-PCR confirmed dominant expression of DP mRNA in NPDF ($n = 5$) (Fig. 6B).

Discussion

In the present study, we examined the effect of PGD₂ on VEGF release by NPDF. We found for the first time that NPDF produced VEGF in response to 5 μM of PGD₂. Little is known regarding whether PGD₂ induces VEGF production in humans. One report showed that PGD₂ induced VEGF mRNA expression in human retinal capillary pericytes through activation of adenylate cyclase.²⁴ Our results were consistent with this report, and suggest that not only pericytes but also fibroblasts produce VEGF in humans in response to PGD₂. Several factors such as hypoxia, TNF- α , LPS, rhinovirus and PGE₂ can induce VEGF release by NPDF.^{4–6,9} The amount of VEGF protein in response to PGD₂ is comparable to that in response to hypoxia, TNF- α , LPS and PGE₂. Together with the

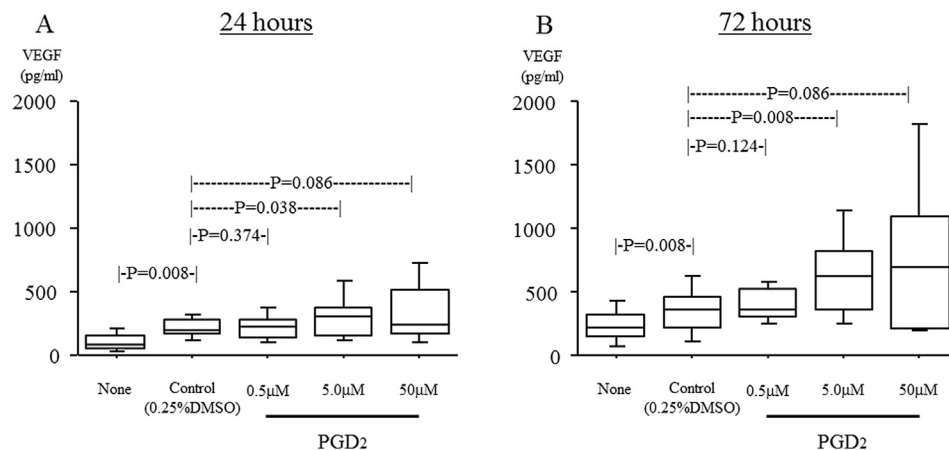


Fig. 1. PGD₂-induced VEGF protein production by NPDF. NPDF were stimulated with a serial concentration of PGD₂ or control buffer (0.25% DMSO) for 24 h (A) or 72 h (B). The rectangle includes the range from the 25th to 75th percentiles; the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. P -values were determined using the Wilcoxon signed-rank test.

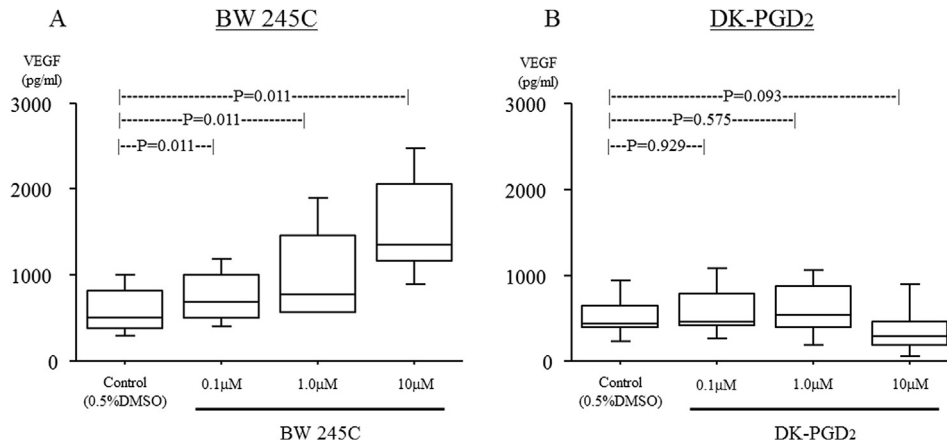


Fig. 3. Effect of PGD₂ receptor-selective agonists on VEGF release by NPDF. NPDF were stimulated with a serial concentration of BW245C (A) or DK-PGD₂ (B) for 72 h. The rectangle includes the range from the 25th to 75th percentiles; the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. *P*-values were determined using the Wilcoxon signed-rank test.

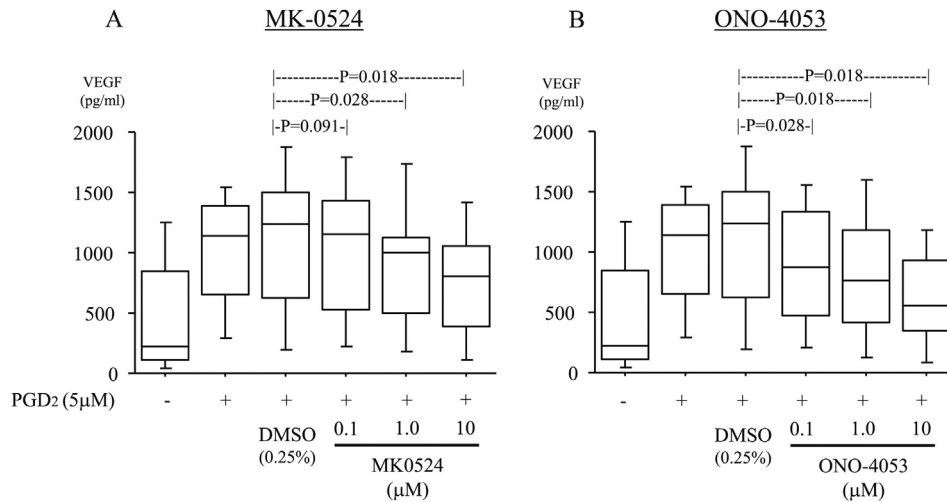


Fig. 4. Effect of DP receptor-selective antagonists on PGD₂-induced VEGF release by NPDF. NPDF were stimulated with 5 μM PGD₂ in the presence or absence of a serial concentration of MK-0524 (A) or ONO-4053 (B) for 72 h. The rectangle includes the range from the 25th to 75th percentiles; the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. *P*-values were determined using the Wilcoxon signed-rank test.

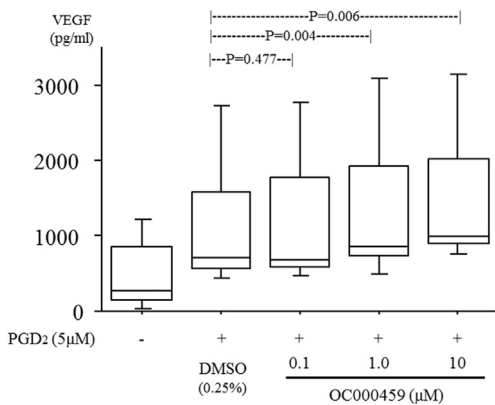


Fig. 5. Effect of CRTH2 receptor-selective antagonist on PGD₂-induced VEGF release by NPDF. NPDF were stimulated with 5 μM PGD₂ in the presence or absence of a serial concentration of OC000459 for 72 h. The rectangle includes the range from the 25th to 75th percentiles; the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. *P*-values were determined using the Wilcoxon signed-rank test.

finding that a substantial amount of PGD₂ is detected in the middle meatus of patients with CRSwNP,¹¹ PGD₂ may be one of the dominant factors of increasing VEGF in CRSwNP.

Stimulation with BW245C but not with DK-PGD₂ mimicked the effect of PGD₂ on VEGF release by NPDF. In addition, treatment with MK0524 and ONO-4053 but not with OC000459 significantly abrogated the effect of PGD₂. These findings suggest that PGD₂ induced VEGF release via the DP receptor and not via the CRTH2 receptor in NPDF. This selective release through the DP receptor may be due to the predominant expression of the DP receptor over that of the CRTH2 receptor in NPDF as shown using real-time PCR. The predominant expression of the DP receptor is consistent with our previous report showing that the DP receptor is expressed by a variety of cells including constitutive cells and infiltrating inflammatory cells, whereas the CRTH2 receptor is mainly expressed by infiltrated inflammatory cells in NP.¹¹

No significant difference in PGD₂-induced VEGF production by NPDF was seen between ECRS and non-ECRS patients. We previously reported that mRNA level of DP in sinonasal tissues significantly correlated with that of eotaxin but not IL-5 or RANTES.¹¹ We also have found that the mRNA levels of DP did not correlate with

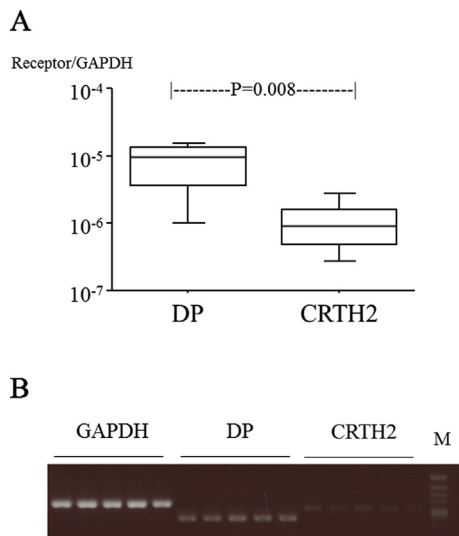


Fig. 6. Relative amounts of DP and CRTH2 receptor mRNA in NPDF. **(A)** Real-time quantitative PCR. The absolute copy number for each sample was calculated, and samples were reported copy number relative to GAPDH used to be an internal control. The rectangle includes the range from the 25th to 75th percentiles; the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. P-values were determined using the Wilcoxon signed-rank test. **(B)** RT-PCR. Gel images of mRNA expressions of GAPDH, DP and CRTH2 in NPDF were shown (n = 5). M, molecular weight marker.

the degree of NP eosinophilia (unpublished data). These results suggest that VEGF has more activities than eosinophilic inflammation in the pathogenesis of CRSwNP.

It is known that PGE₂ promotes VEGF release by NPDF via EP2 and EP4 receptors.⁹ Although unbalanced expression of PGD₂ and PGE₂ is associated with the pathogenesis of CRSwNP, both PGD₂ and PGE₂ can promote VEGF release by NPDF.^{8,10} This finding is not surprising since both DP and EP2/EP4 receptors couple to the Gs protein, which activates adenylate cyclase.^{9,12} It is known that signaling through adenylate cyclase/protein kinase A enhances VEGF release by fibroblasts.^{25,26} Thus our results support previous findings and may suggest that activation of adenylate cyclase by PGD₂ also promotes VEGF release by fibroblasts.

Both MK-0524 and ONO-4053 abrogated PGD₂-induced VEGF release by NPDF in a dose-dependent manner. ONO-4053 but not MK-0524 showed significant abrogation at a concentration of 0.1 μM, suggesting that PGD₂ antagonism of ONO-4053 is more potent than that of MK-0524. On the other hand, it should be noted that both of these antagonist showed partial inhibition. There are two possible explanations of this partial inhibition. First, PGD₂ is known to couple not only to DP and CRTH2 receptors but also to TP and EP3 receptors.¹² Indeed, signaling through the TP receptor promotes VEGF release in human lung cancer cell lines.²⁷ However, MK-0524 can act as a TP antagonist, and therefore an effect of TP signaling on the observed partial inhibition is unlikely.²⁸ It is also unlikely that EP3 mediates VEGF release by NPDF since a previous report showed that sulprostone, an EP1/EP3 receptor agonist, did not induce VEGF release by NPDF.⁹ In addition, EP3 couples to the Gi protein and inhibits adenylate cyclase.²⁹ The second possible explanation of the observed partial inhibition is that there might be an effect of 15-deoxy-Δ^{12,14}-PGJ₂, which is a derivative of PGD₂ that is known to function as a ligand of peroxisome proliferator-activated receptor (PPAR)-γ.¹² Although little is known regarding whether 15-deoxy-Δ^{12,14}-PGJ₂ promotes VEGF release by fibroblasts, vascular endothelial cells and cardiac myofibroblasts can release VEGF in response to 15-deoxy-Δ^{12,14}-PGJ₂.^{30,31} The effects of

15-deoxy-Δ^{12,14}-PGJ₂ and/or PPAR-γ on PGD₂-induced VEGF release by NPDF should be investigated in the future.

Interestingly, treatment with OC00045 significantly augmented PGD₂-induced VEGF release by NPDF. An opposing role of DP and CRTH2 receptors can be seen in various conditions such as skin inflammation and ulcerative colitis.^{32–34} For example, treatment with DP receptor-selective agonists inhibited CRTH2 receptor-induced chemotaxis of human eosinophils.³² In the present study, although not significant, treatment with 10 μM of DK-PGD₂ tended to inhibit VEGF release by NPDF. These results suggest that signals through the CRTH2 receptor regulate the effect of DP receptor signaling on VEGF release, and that blockage of CRTH2 receptor signaling augments DP receptor signaling, which then enhances VEGF release in NPDF. The lack of dose-dependent effect by PGD₂ but not DP receptor-selective agonist on VEGF release may be due to the stimulation of CRTH2 receptor by high concentration of PGD₂.

NPDF may produce many other cytokines and extracellular matrix involved in the pathogenesis of CRS. In fact, our preliminary result suggests that PGD₂ significantly induces IL-6 and IL-8 but not TGF-β by NPDF. Determining how PGD₂ promotes the release cytokines and extracellular matrix other than VEGF by NPDF should be investigated in future studies.

In conclusion, the present study shows that PGD₂ promotes VEGF release from NPDF via the DP receptor. This result suggests the therapeutic potential of DP receptor-selective antagonists for prevention of edema formation and/or angiogenesis mediated by VEGF in CRSwNP.

Acknowledgments

The authors would like to thank Yuko Okano for her editorial assistance. This work was supported in part by grants from Ministry of Education, Culture, Sports, Science, and Technology of Japan (15H01987 and 26670742).

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

MO and KN designed the study and wrote the manuscript. TF, SK, KK, SM and YH contributed to data collection. TH and RO performed the statistical analysis and interpretation of the results.

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