Original article

Effect of prostaglandin D2 on VEGF release by nasal polyp fibroblasts

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A B S T R A C T

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.

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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) [1-6]. In NP, VEGF is mainly expressed in vascular endothelial cells, basal membranes, perivascular spaces, and epithelial cells [7]. 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 [4,5]. 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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D-prostanoid (DP) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) are the two major receptors reported for PGD2 to date. 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. On the other hand, the CRTH2 receptor couples to the Gi protein, and signaling through CRTH2 mainly shows a pro-inflammatory effect. 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 NPDE, 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 (MK0524) were purchased from Cayman (Ann Arbor, MI, USA). A CRTH2 receptor-selective antagonist (OC000459) was purchased from ChemScence 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 $2 \times 10^{-2}$ M (PGD2) or $2 \times 10^{-3}$ M (others), filtered through a 0.2 µm filter, and stored at $-80\degree C$ 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 in patients 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 5% CO2 for 10 days, and then non-adherent cells were removed by changing the culture medium. After reaching confluence, NPDE were harvested by treatment with 0.25% trypsin and 0.02% EDTA, and were then divided into new dishes. The cellularity of NPDE was characterized using immunohistochemistry; more than 95% of confluent cells were found to be vimentin-positive. NPDE were used after 6 to 8 passages.

**Generation of NPDE**

NPDE were generated as previously reported. 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 5% CO2 for 10 days, and then non-adherent cells were removed by changing the culture medium. After reaching confluence, NPDE were harvested by treatment with 0.25% trypsin and 0.02% EDTA, and were then divided into new dishes. The cellularity of NPDE was characterized using immunohistochemistry; more than 95% of confluent cells were found to be vimentin-positive. NPDE were used after 6 to 8 passages.

**Cell culture**

NPDE 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, PK-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 NPDE 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 NPDE was performed as described previously.11 Levels of VEGF mRNA in NPDE 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'-TGCAGATTATGCGGATCAAACC-3' and reverse 5'-TCCATTCCACATTGCTGCCTGCTAC-3' (81 bp). 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).
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 \(\mu M\) 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 \(\mu M\) 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 \(\mu M\) 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 \(\mu M\) \((P = 0.018, n = 7)\). 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 \(\mu M\) (Fig. 4). On the other hand, treatment with the CRTH2 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 \((P = 0.006)\) \(\mu M\) (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 \(\mu M\) 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-\(\alpha\), 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-\(\alpha\), LPS and PGE2. Together with the
finding that a substantial amount of PGD2 is detected in the middle meatus of patients with CRSwNP.\textsuperscript{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 sinusoidal tissues 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.\textsuperscript{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.\textsuperscript{11} We also have found that the mRNA levels of DP did not correlate with

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**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 \( \mu\)M 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 \( \mu\)M 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.
shown (n = 5). M, molecular weight marker.

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 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. Although unbalanced expression of PGE2 and PGE2 is associated with the pathogenesis of CRSwNP, both PGD2 and PGE2 can promote VEGF release by NPDF. This finding is not surprising since both EP and EP2/EP4 receptors couple to the Gs protein, which activates adenylyl cyclase. It is known that signaling through adenylyl cyclase/protein kinase A enhances VEGF release by fibroblasts. Thus our results support previous findings and may suggest that activation of adenylyl 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 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 receptors. Indeed, signaling through the TP receptor promotes VEGF release in human lung cancer cell lines. However, MK-0524 can act as a TP antagonist, and therefore an effect of TP signaling on the observed partial inhibition is unlikely. It is also unlikely that EP3 mediates VEGF release by NPDF since a previous report showed that sulprostone, an EP3 agonist, did not induce VEGF release by NPDF. In addition, EP3 couples to the Gi protein and inhibits adenylyl cyclase. The second possible explanation of the observed partial inhibition is that there might be an effect of 15-deoxy-Δ12,14-PGJ2, which is a derivative of PGD2 that is known to function as a ligand of peroxisome proliferator-activated receptor (PPAR)-γ. Although little is known regarding whether 15-deoxy-Δ12,14-PGJ2 promotes VEGF release by fibroblasts, vascular endothelial cells and cardiac myofibroblasts can release VEGF in response to 15-deoxy-Δ12,14-PGJ2. The effects of 15-deoxy-Δ12,14-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. For example, treatment with DP receptor-selective agonists inhibited CRTH2 receptor-induced chemotaxis of human eosinophils. 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 of 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.

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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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