Original article

PGD_2 induces eotaxin-3 via PPARγ from sebocytes: A possible pathogenesis of eosinophilic pustular folliculitis

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Background: Eosinophilic pustular folliculitis (EPF) is a chronic intractable pruritic dermatosis characterized by massive eosinophil infiltrates involving the pilosebaceous units. Recently, EPF has been regarded as an important clinical marker of HIV infection, and its prevalence is increasing in number. The precise mechanism by which eosinophils infiltrate into the pilosebaceous units remains largely unknown. Given that indomethacin, a COX inhibitor, can be successfully used to treat patients with EPF, we can assume that COX metabolites such as prostaglandins (PGs) are involved in the etiology of EPF.
Objective: To determine the involvement of PGs in the pathogenesis of EPF.
Methods: We performed immunostaining for PG synthases in EPF skin lesions. We examined the effect of PGD_2 on induction of eotaxin, a chemoattractant for eosinophils, in human keratinocytes, fibroblasts, and sebocytes and sought to identify its responsible receptor.
Results: Hematopoietic PGD synthase was detected mainly in infiltrating inflammatory cells in EPF lesions, implying that PGD_2 was produced in the lesions. In addition, PGD_2 and its immediate metabolite 15-deoxy-Δ 12,14-PGJ_2 (15d-PGJ_2) induced sebocytes to produce eotaxin-3 via peroxisome proliferator–activated receptor gamma. Consistent with the above findings, eotaxin-3 expression was immunohistochemically intensified in sebaceous glands of the EPF lesions.
Conclusion: The PGD_2/PGJ_2-peroxisome proliferator–activated receptor gamma pathway induces eotaxin production from sebocytes, which may explain the massive eosinophil infiltrates observed around pilosebaceous units in EPF. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: *Prostaglandin D_2, hematopoietic prostaglandin D synthase, eotaxin-3/CCL26, sebocyte, peroxisome proliferator–activated receptor gamma*

Abbreviations used

CRT_H2:	Chemoattractant receptor–homologous molecule expressed on T_H2 cells
EPF:	Eosinophilic pustular folliculitis
GAPDH:	Glyceraldehyde 3-phospate dehydrogenase
H-PGDS:	Hematopoietic prostaglandin D synthase
L-PGDS:	Lipocalin-type prostaglandin D synthase
PG:	Prostaglandin
PPARγ:	Peroxisome proliferator–activated receptor gamma
siRNA:	Small-interfering RNA

Eosinophilic pustular folliculitis (EPF) is a chronic intractable pruritic dermatosis characterized by massive eosinophil infiltrates involving the pilosebaceous units.[1] The evidence accumulated to date indicates that T_H2-mediated immunologic mechanisms are involved in the pathogenesis of EPF.[2,3] Recently, EPF has been regarded as an important clinical marker of HIV infection, and its prevalence is increasing in number.[4] An immunohistochemical study has demonstrated the expression of intercellular adhesion molecules for inflammatory cells including eosinophils around hair follicles.[5] Other studies have reported that IL-5 level, which induces proliferation and differentiation of eosinophils, is elevated in the blood and skin lesions of patients with EPF, but it can be decreased by treatment with IFN-γ.[6,7] Three members of the eotaxin family—eotaxin-1/CCL11, eotaxin-2/CCL24, and eotaxin-3/CCL26—are known to promote the growth and recruitment of eosinophils and skin inflammation.[8] T_H2 cytokines, such as IL-4, -5, and -13, enhance the production of eotaxins by skin component cells, such as lymphocytes, macrophages, endothelial cells, fibroblasts, and keratinocytes.[9-11] These findings suggest that the pathogenesis of EPF consists of a T_H2-type immune response; intriguingly, however, EPF is usually resistant to topical or systemic corticosteroids that suppress the functions of T cells. Therefore, the pathogenesis of EPF might not be explained solely by T_H2 immunity. Since EPF can be successfully treated with indomethacin, a COX inhibitor,[12] we hypothesize that the prostaglandin (PG) family known as the prostanoids, which occur downstream of COX, might be involved in the etiology of EPF.

Prostanoids are released from cells immediately after their formation. Because they are chemically and metabolically unstable, they usually function only locally through membrane receptors on target cells.[13] Recently, individual prostanoid receptor gene–deficient mice have been used as models to dissect the respective roles of each receptor in combination with the use of compounds that selectively bind to prostanoid receptors as

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agonists or antagonists.[14,15] The prostanoids PGD_2 and PGE_2 are 2 of the major COX metabolites in the skin. PGE_2 has been reported to have an inhibitory effect on eosinophil trafficking and activation.[16] PGD_2, on the other hand, is known to be involved in chronic allergic inflammation.[17] Two types of PGD synthase (PGDS), which catalyzes the isomerization of PGH_2, a common precursor of various prostanoids that catalyze PGD_2, have been identified: one is the lipocalin-type PGDS (L-PGDS), and the other is the hematopoietic PGDS (H-PGDS).[18] L-PGDS is localized in the central nervous system, the male genital organs, the heart, and melanocytes in skin.[18,19] H-PGDS is widely distributed in the peripheral tissues and localized in antigen-presenting cells, mast cells, megakaryocytes, T_H2 lymphocytes, and dendritic cells.[18,20-22]

The aim of this study was to verify the hypothesis that prostanoids are involved in the development of eosinophil infiltration in the pilosebaceous units of the EPF skin lesions. We found that inflammatory cells in EPF lesions were positively immunostained for H-PGDS, suggesting that PGD_2 production was increased in EPF lesions. Moreover, we found that PGD_2 increased eotaxin-3 mRNA expression in sebocytes via peroxisome proliferator–activated receptor gamma (PPARγ) and that eotaxin-3 was detected around sebaceous glands in EPF lesions. Our data suggest that PGD_2 is involved in the pathogenesis of EPF lesions by inducing eotaxin-3 from sebocytes via PPARγ.

METHODS

Human subjects

We obtained biopsy specimens from 5 patients with EPF, 6 patients with folliculitis, and 4 healthy subjects. Informed consent was obtained from all subjects involved in this study. The Ethics Committee of Kyoto University approved the study.

Histologic examination

Paraffin-embedded sections were stained with hematoxilin-eosin and immunostained with H-PGDS, a monoclonal mouse antihuman antibody (dilution 1:500), L-PGDS, a polyclonal rabbit antihuman antibody (dilution 1:1000) (both were established at the Osaka Bioscience Institute, Osaka, Japan), and eotaxin-3/CCL26, a polyclonal goat antihuman antibody (dilution 1:100, R&D Systems, Minneapolis, Minn). As negative controls for H-PGDS and L-PGDS antibodies, we used isotype-matched control antibody and rabbit serum, respectively. Antigen retrieval was achieved by pepsin treatment for L-PGDS and preincubation with proteinase K for eotaxin-3. Nonspecific binding was blocked by addition of 10% goat serum for 30 minutes at room temperature. Afterward, sections were incubated for 1 hour at room temperature with the primary antibody followed by incubation with a species-specific biotinylated immunoglobulin (Vector, Burlingame, Calif) for 30 minutes at room temperature. Thereafter, they were incubated for 30 minutes with the avidine-biotin-peroxidase complex kit (Vector) and visualized with 3,3′-diaminobenzidine. They were lightly counterstained with hematoxylin. The number of immunoreactive cells per high power field was enumerated at 3 locations (original magnification ×200) per sample, and data were expressed as the number of H-PGDS– and L-PGDS–positive cells per high power field.

Preparation of human eosinophils and flow cytometry

Peripheral blood was obtained from 3 patients with EPF and 3 healthy donors. Polynuclear cells were separated by centrifugation of whole blood over Mono-Poly Resolving Medium (DS Pharma Biomedical, Osaka, Japan), followed by removal of remaining red cells by ACK lysing buffer (Lonza Walkersville, Inc, Walkersville, Md). They were stained with the antibodies against surface markers of eosinophils: antihuman CCR3-phycoerythrin (dilution 1:100, R&D Systems) and antihuman CD16-fluorescein isothiocyanate (dilution 1:100, Becton Drive Biosciences, Franklin Lakes, NJ). Eosinophils were identified with CCR3 positive and CD16 negative by flow cytometric analysis. With the use of an IntraStain kit (Becton Drive Biosciences), intracellular H-PGDS was detected by staining with polyclonal rabbit antihuman H-PGDS antibody (dilution 1:50, Cayman Biochemical) followed by antirabbit Alexa Fluor 647 (dilution 1:200, Life Technologies, Tokyo, Japan). The expression of H-PGDS was analyzed for mean fluorescence intensity.

For purification of eosinophils, the peripheral blood of patients with mild allergic rhinitis was collected by negative selection by using Eosinophil Isolation Kit (Miltenyl Biotec, Bergisch Gladbach, Germany). Both the purity and the viability of eosinophils were confirmed to exceed 95%.

Cell culture

Normal human epidermal keratinocytes (Kurabo, Osaka, Japan) were grown in Humedia-KG2 medium (Kurabo) with human epidermal growth factor (0.1 ng/mL), insulin (10 μg/mL), hydrocortisone (0.5 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 ng/mL), and bovine brain pituitary extract (0.4%, v/v). Primary skin fibroblasts were isolated by standard methods[23] from healthy human skin and were cultured grown in Dulbecco modified Eagle medium (Gibco, Karlsrude, Germany) with 10% FBS (Gibco).

The immortalized human sebaceous gland cell lines SZ95 (a kind gift from Dr Christos C. Zouboulis) were cultured in sebomed basal medium (Biochrom AG, Berlin, Germany) with 10% FBS and recombinant human epidermal growth factor (Sigma Chemical, St Louis, Mo).

As for normal human epidermal keratinocytes and fibroblasts, the cells grew to 80% to 90% confluent and were starved for 3 hours, followed by treatment with PGD_2 (10 μM) (Cayman Biochemical) for 24 hours at 37°C in 5% CO_2.

Agonists used were the DP agonist BW245c (Cayman Biochemical), the chemoattractant–homologous receptor expressed on T_H2 cells (CRT_H2) agonist 15-keto-PGD_2 (DK-PGD_2) (Cayman Biochemical), and the PPARγ agonist 15-deoxy-Δ 12,14-PGJ_2 (15d-PGJ_2) (Cayman Biochemical). Antagonists used were the DP antagonist BWA868c (Cayman Biochemical), the CRT_H2 antagonist CAY10471 (Cayman Biochemical), and the PPARγ antagonist GW9662 (Cayman Biochemical). Sebocytes were starved for 3 hours and treated with PGD_2 (1-20 μM), BW245c (1-10 μM), DK-PGD_2 (1-10 μM), and 15d-PGJ_2 (1-7 μM) for 21 hours at the confluency of 30% to 40%. For treatment with antagonists, BWA868c (1-10 μM), CAY10471 (1-10 μM), and GW9662 (1-3 μM) (Cayman Biochemical) were preadded at 30 minutes.

SZ95 cells were transfected with PPARγ small-interfering RNA (siRNA) or nontargeting siRNA (Dharmacon, Lafayette, Colo) at 20% confluence by using Lipofectamine 2000 (Life Technologies). At 48 hours after transfection, the cells were starved for 3 hours and treated with or without PGD_2 (7.5 μM) for an additional 21 hours.

For detection of PGD_2, purified eosinophils (1×10^6 cells per well) were incubated in 50 μL of RPMI 1640 with 10% FBS in the presence and absence of 10^{-6} mol/L phorbol 12-myristate 13-acetate (Sigma-Aldrich, St Louis, Mo) and 10^{-5} mol/L calcium ionophore A23187 (Sigma-Aldrich). The concentration of PGD_2 in the supernatant was detected by the use of PGD_2-MOX Enzyme Immunoassay Kit (Cayman Biochemical).

Quantitative RT-PCR

Total RNA was isolated with RNeasy kits and digested with DNase I (Qiagen, Hilden, Germany). The cDNA was reverse transcribed from total RNA samples by using the Prime Script RT reagent kit (Takara Bio, Otsu, Japan). Quantitative RT-PCR was performed by using Light Cycler 480 SYBR Green I Master (Roche, Mannheim, Germany) and the Light Cycler real-time PCR apparatus (Roche) according to the manufacturer's instructions. The primers used for PCR had the following sequences: eotaxin-1, 5′-CTC CGCAGCACTTCTGTGGC-3′ (forward) and 5′- GGTCGGCACAGA-TATCCTTG-3′ (reverse); eotaxin-2, 5′-GCCTTCTGTTCCTGGGTGTC-3′ (forward) and 5′- CCTCCTGAGTCTCCACCTTG-3′ (reverse); eotaxin-3,

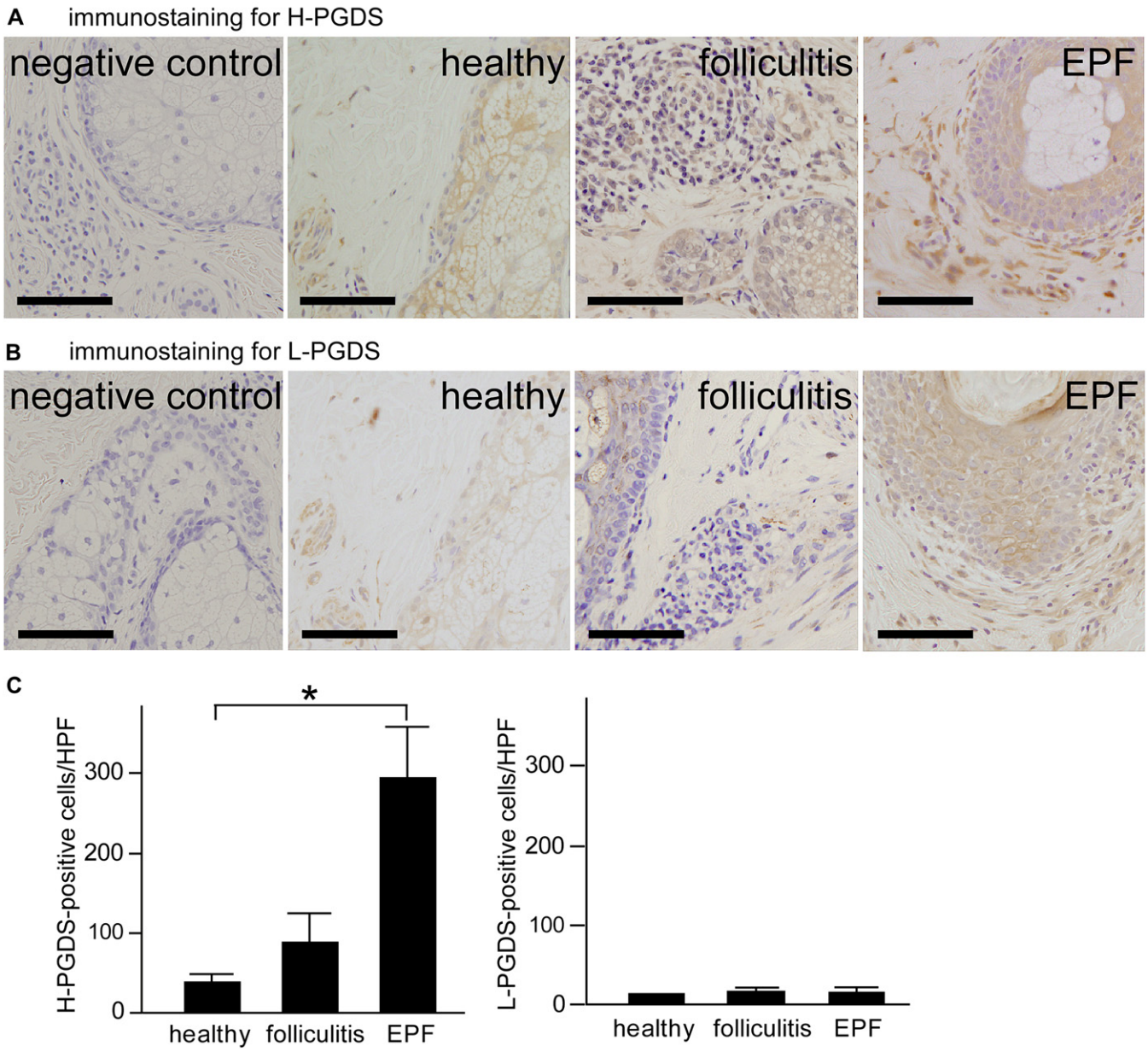

FIG 1. Immunohistochemistry of PGDS. Skin specimens taken from healthy subjects (n = 4), patients with folliculitis (n = 6), and patients with EPF (n = 5) were immunostained for H-PGDS **(A)** and L-PGDS **(B)** and respective negative controls. The infiltrating inflammatory cells around the pilosebaceous gland in EPF were stained with anti–H-PGDS antibody. **C,** The numbers of H-PGDS– and L-PGDS–positive cells were counted. Bar = 100 μm. *$P < .05$. *HPF,* High power field.

5′-CCTCCTGAGTCTCCACCTTG-3′ (forward) and 5′-AAGGGGCTTGT GGCTGTATT-3′ (reverse); PPARγ, 5′-ACAGACAAATCACCATTCGT-3′ (forward) and 5′-CTCTTTGCTCTGCTCCTG-3′ (reverse); and Glyceraldehyde 3-phospate dehydrogenase (GAPDH), 5′-AATGTCACCGTTGTC CAGTTG-3′ (forward) and 5′- GTGGCTGGGGCTCTACTTC-3′ (reverse). The results were normalized to those of the housekeeping GAPDH mRNA.

Statistical analysis

Unless otherwise indicated, data are presented as means ± SD and are a representative of 3 independent experiments. *P* values were calculated with the 2-tailed Student *t* test. *P* values less than .05 are considered to be significantly different between the indicated groups and are shown as asterisk in the figures.

RESULTS

Increased H-PGDS expression in EPF lesions

To verify PGDS expression in EPF lesions, we performed immunostaining with anti–H-PGDS and anti–L-PGDS antibodies. We found that the infiltrating inflammatory cells around pilosebaceous units were strongly positive for H-PGDS in lesions from patients with EPF, but not in healthy subjects (Fig 1, *A*). There were a few cells stained for L-PGDS (Fig 1, *B*). The number of H-PGDS–positive cells was significantly greater in EPF skin lesions than in normal healthy skin samples or in folliculitis lesions (Fig 1, *C*).

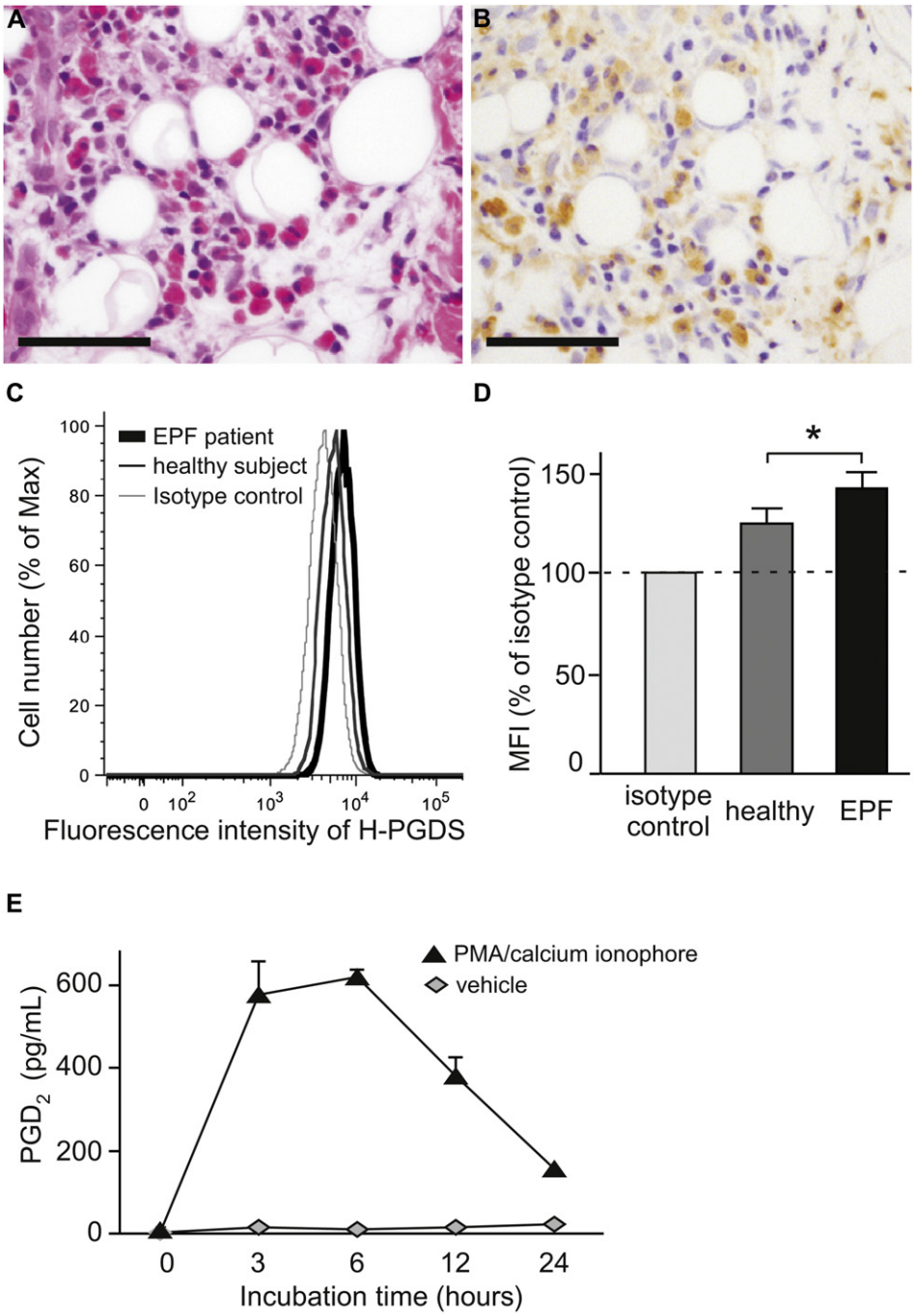

FIG 2. H-PGDS expression and PGD_2 production in eosinophils. Skin specimens of patients with EPF were stained with hematoxylin-eosin **(A)** and anti–H-PGDS antibody **(B)**. Bar = 100 μm. H-PGDS expression in eosinophils was determined by flow cytometry. **C,** Representative flow cytometry results. **D,** The MFI of isotype control was set as 100%, and the MFI of H-PGDS was calculated accordingly (n = 3). $*P < .05$. **E,** PGD_2 levels in eosinophil culture supernatants with or without phorbol 12-myristate 13-acetate and calcium ionophore. *MFI*, Mean fluorescence intensity.

H-PGDS expression and PGD_2 production in eosinophils

Numerous infiltrating eosinophils were stained with anti–H-PGDS antibody (Fig 2, *A* and *B*), suggesting that eosinophils may express H-PGDS. In fact, flow cytometric analysis showed that H-PGDS was detected in eosinophils, and its expression level was higher in patients with EPF than in healthy subjects (Fig 2, *C* and *D*). In addition, we examined the production of PGD_2 from the supernatant of eosinophil culture at 0, 3, 6, 12, and 24 hours after incubation with or without phorbol 12-myristate 13-acetate and calcium ionophore. We found that a significant amount of PGD_2 was produced by eosinophils activated with phorbol 12-myristate 13-acetate and calcium ionophore (Fig 2, *E*).

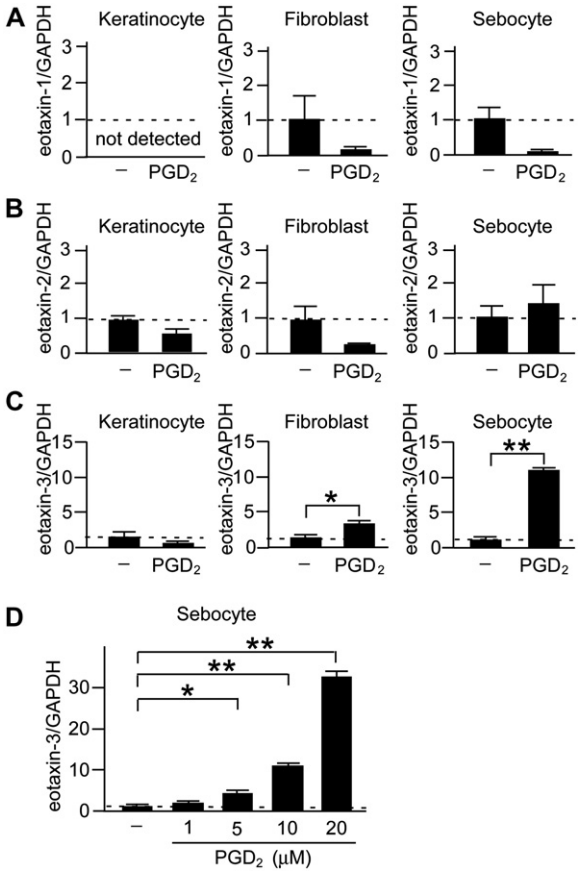

FIG 3. Effect of PGD_2 on eotaxin mRNA expression in human keratinocytes, fibroblasts, and the sebaceous gland cell line SZ95. Cells were incubated with PGD_2 (**A-C**; 10 μM). The mRNA expression levels of eotaxin-1 (Fig 3, *A)*, eotaxin-2 (Fig 3, *B)*, and eotaxin-3 (Fig 3, *C*, and *D)* were evaluated by means of quantitative RT-PCR and normalized according to that of GAPDH. Data are shown as arbitrary units where the value for an unstimulated sample is set at 1 (n = 4). $*P < .05$, $**P < .01$.

PGD_2 increased eotaxin-3 mRNA expression in human sebocytes

We next asked whether PGD_2 could affect the production of chemokines for eosinophil migration. Since the diagnostic hallmark of EPF is the accumulation of eosinophils around the pilosebaceous units, we focused on sebocytes, the cells that constitute the pilosebaceous glands, as well as on keratinocytes and fibroblasts. We found that PGD_2 did not affect the expression of eotaxin-1 or -2 in human keratinocytes, fibroblasts, or sebocytes (Fig 3, *A* and *B*). It did induce eotaxin-3 expression, only slightly in fibroblasts but markedly in the human sebaceous gland cell line SZ95 (Fig 3, *C*). Moreover, we observed that PGD_2 increased eotaxin-3 mRNA expression in sebocytes in a dose-dependent manner (Fig 3, *D*). These findings suggest that PGD_2 induces eotaxin-3 production abundantly in sebocytes and that sebocytes might play a key role in eosinophil trafficking to the pilosebaceous units in EPF.

Dispensable role of the DP1 and CRT_H2 receptors in PGD_2-induced eotaxin-3 expression

Two receptors for PGD_2 have been identified: one is DP1, and the other is CRTH2, also known as DP2, both of which are G protein–coupled receptors.[24,25] We next undertook to determine which receptor mediates eotaxin-3 upregulation by PGD_2. Neither the DP1 agonist BW245c nor the CRT_H2 agonist DK-PGD_2 induced eotaxin-3 in the human sebaceous gland cell lines SZ95 (Fig 4, *A*). In addition, eotaxin-3 upregulation induced by PGD_2 was not suppressed by either the DP1 antagonist BW868c or the CRT_H2 antagonist CAY10471 (Fig 4, *B*).

Involvement of PPARγ in PGD_2-induced eotaxin-3 expression in human sebocytes

PGD_2 spontaneously converts into the cyclopentenone PGs of the J series, such as PGJ_2, Δ12-PGJ_2,[12] and 15d-PGJ_2.[26] We found that 15d-PGJ_2 dose dependently increased eotaxin-3 expression in sebocytes (Fig 5, *A*). PGJ_2 elicits its function through PPARγ, and the PPARγ antagonist GW9662 suppressed 15d-PGJ_2–induced eotaxin-3 increase in a dose-dependent manner (Fig 5, *B*). We also observed that PGD_2-induced eotaxin-3 increase was suppressed by GW9662 in a dose-dependent manner (Fig 5, *C*). In addition, we examined the effect of PPARγ knockdown by RNA interference in order to confirm the role of PPARγ in PGD_2-induced eotaxin-3 expression. We observed that PPARγ mRNA expression was inhibited by PPARγ siRNA and that PGD2-induced eotaxin-3 increase in sebocytes was suppressed by siRNA knockdown of PPARγ (Fig 5, *D*). In addition, we compared PPARγ expression among keratinocytes, fibroblasts, and sebocytes and found that it was higher in sebocytes than in keratinocytes and fibroblasts (Fig 5, *E*). These data suggest that PGD_2 induces eotaxin-3 expression in sebocytes, through PPARγ, which is highly expressed in sebocytes. Consistently, eotaxin-3 expression tended to be greater in sebocytes of EPF lesions than in those of normal skin samples (Fig 5, *F*).

DISCUSSION

In our current study, H-PGDS was detected in eosinophils by means of flow cytometric analysis, and these H-PGDS–positive cells were accumulated around the pilosebaceous areas in EPF, implying that PGD_2 is abundantly produced in this condition. In addition, eotaxin-3, which is produced by sebocytes via PPARγ upon stimulation by PGD_2, was highly expressed in the sebaceous glands in EPF lesions, likewise implying an abundance of PGD_2 in EPF. These findings may provide an explanation of the massive eosinophil infiltration that occurs around the pilosebaceous units in EPF.

Since indomethacin is generally effective against EPF, COX metabolites are presumed to be involved in the pathomechanism of EPF. Among these COX metabolites, the prostanoid PGD_2 has previously been reported to directly attract inflammatory cells such as T_H2 cells, eosinophils, and basophils and to be involved in chronic allergic inflammation.[17,27] This partly explains how the prostanoids are involved in the mechanism of EPF. Yet it remains unclear how eosinophils infiltrate the pilosebaceous units. In this study, we found that PGD_2 induces eotaxin-3 upregulation in sebocytes. PGD_2 enhances eotaxin-3 expression even in fibroblasts. Since the dermal papilla is a discrete population of specialized fibroblasts, PGD_2 may indirectly attract eosinophils via eotaxin produced by sebocytes and dermal papilla cells.

The underlying mechanism of controlling EPF by indomethacin has been reported to be attributed to the downregulation of CRT_H2 expression.[28] This is an intriguing observation; however,

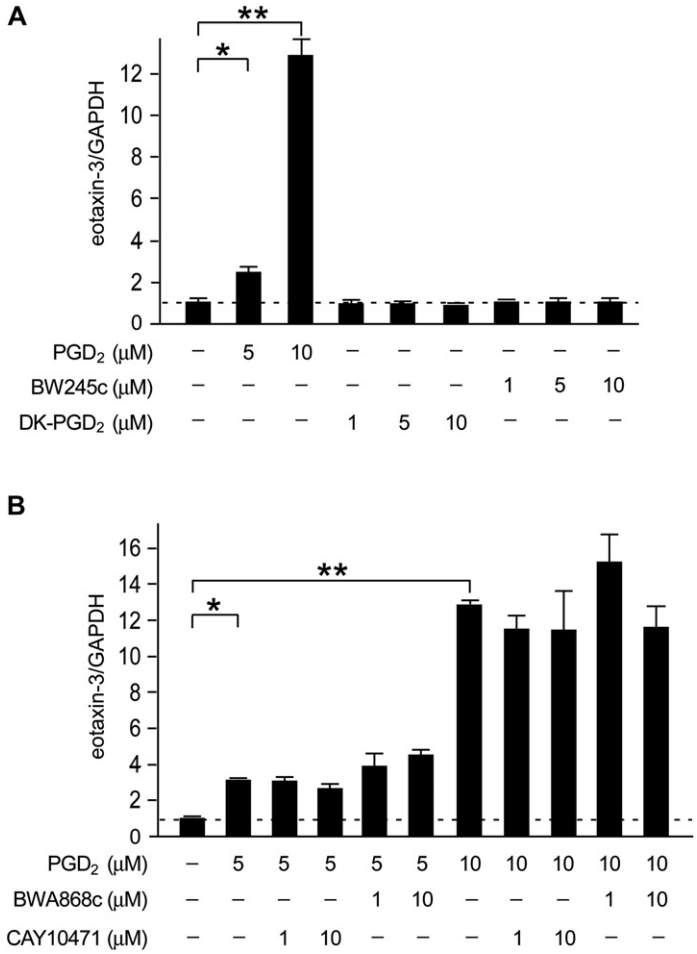

FIG 4. Role of DP1 and CRT_H2 in eotaxin-3 mRNA expression in human sebocytes. SZ95 cells were incubated with PGD_2 in the presence or absence of the DP1 agonist BW245c and the CRT_H2 agonist DK-PGD_2 **(A)** or the DP1 antagonist BW868c and the CRT_H2 antagonist CAY10471 **(B)**. Eotaxin mRNA levels were evaluated by quantitative RT-PCR, and data are shown as arbitrary units where the value for an unstimulated sample is set at 1 (n = 4). $*P < .05$, $**P < .01$.

it remains unclear how indomethacin is specifically effective against EPF. Only a few reports have addressed the efficacy of indomethacin on the other eosinophil-infiltrating skin disorders.[29,30] Our findings indicate that H-PGDS is expressed in peripheral eosinophils of patients with EPF, whereas it is only marginally expressed in those of healthy subjects; nevertheless, it remains uncertain how this difference between patients with EPF and healthy subjects arises. This unique expression profile of H-PGDS in EPF may explain the initiation and/or maintenance of the disease. H-PGDS expression is evident in T cells, and it has recently been reported that CCR8+ T_H2 cells are essential to attract eosinophils to the skin.[31] We detected some T-cell infiltration around the pilosebaceous units in EPF (data not shown), suggesting that CCR8+ T cells and eosinophils jointly initiate and maintain eosinophil infiltration into EPF skin lesions.

It has been demonstrated that sebocytes are capable of producing the neutrophil chemoattractant CXCL8, which may play a role in the pathogenesis of acne,[32] but it remains unknown whether and how sebocytes produce eosinophil chemoattractants. Here we have demonstrated for the first time that eotaxin-3 mRNA expression in sebocytes was enhanced by incubation with PGD_2 and 15d-PGJ_2 and mediated by PPARγ but not by DP1 or CRT_H2. It has been reported that 15d-PGJ_2 binds to PPARγ where it promotes adipocyte differentiation[33,34] and that PPARγ is detected in sebocytes where it is involved in lipid synthesis.[35-37] In our study, larger quantities of PPARγ were detected in sebocytes than in keratinocytes or fibroblasts. Therefore, sebocytes may play an important role in attracting eosinophils into the skin under certain conditions.

Conclusions

We found that H-PGDS is expressed in EPF lesions and that PGD_2 and its metabolite 15d-PGJ_2 induce marked upregulation of eotaxin-3 via PPARγ in sebocytes. These results may explain how EPF shows a massive eosinophil infiltration around pilosebaceous units.

Clinical implications: Inhibition of the PGD_2/PGJ_2-PPARγ pathway may be a therapeutic target for EPF and other diseases involving eosinophil infiltration.

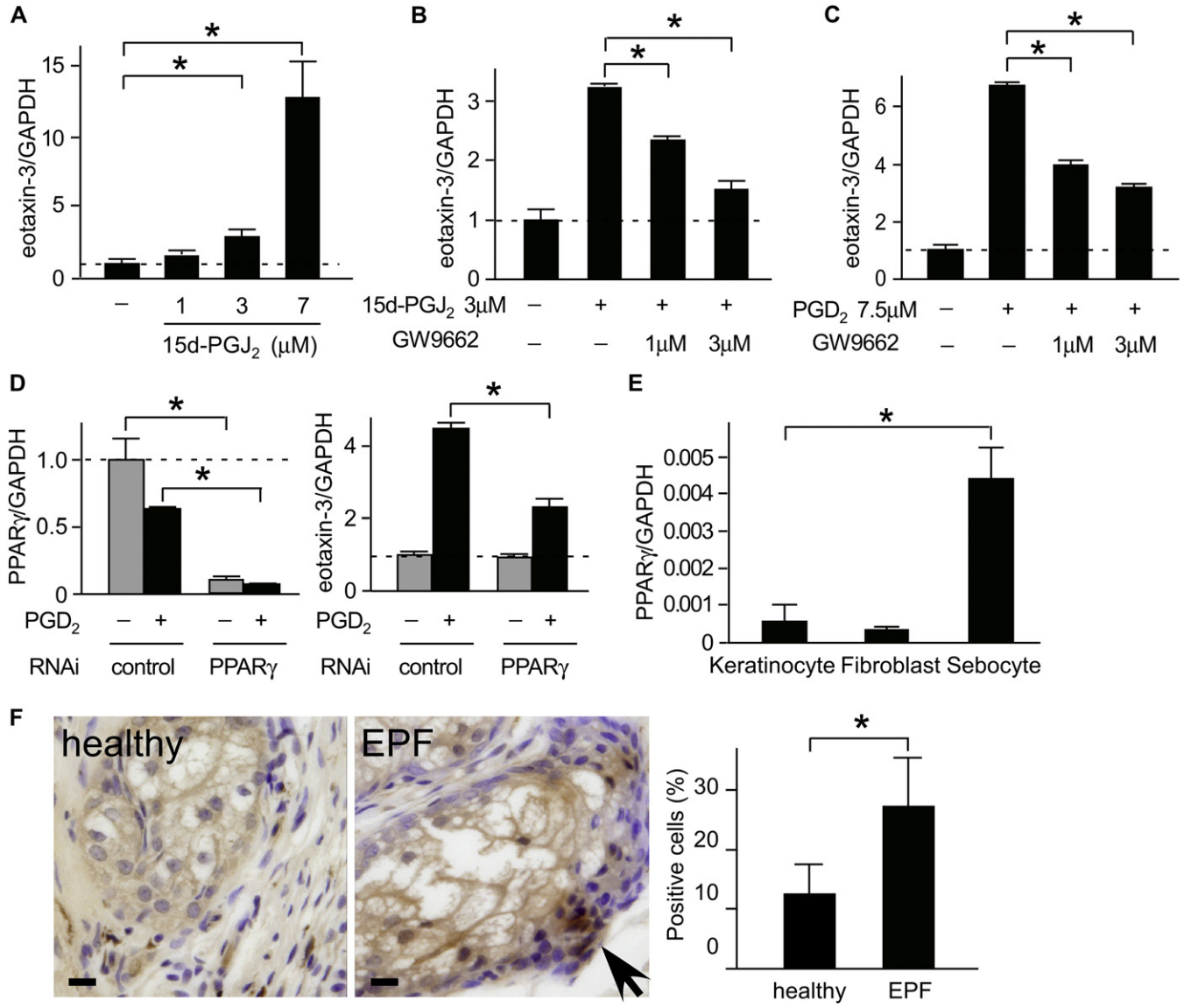

FIG 5. Involvement of PPARγ in eotaxin-3 mRNA expression in sebocytes. SZ95 cells were incubated with 15d-PGJ_2 **(A** and **B)** or PGD_2 **(C)** in the presence or absence of the PPARγ antagonist GW9662 *(Fig 5, B* and *C)*. Eotaxin mRNA levels were evaluated by means of quantitative RT-PCR. **D,** The effect of transfection with PPARγ siRNA on mRNA expressions for PPARγ and eotaxin-3. **E,** PPARγ mRNA expression in normal human epidermal keratinocytes, fibroblasts, and SZ95 cells. **F,** Immunostaining for eotaxin-3. Sebocytes strongly positive for eotaxin-3 are indicated by an arrow (right panel). The percentage positive for eotaxin-3 among sebocytes was examined (n = 3 each, right panel). Bar = 10 μm. $*P < .05$.

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