Biochemical Pharmacology 79 (2010) 982–989

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

# Prostaglandin $D_2$ induces the production of human β-defensin-3 in human keratinocytes

Naoko Kanda *, Takeko Ishikawa, Shinichi Watanabe

*Department of Dermatology, Teikyo University School of Medicine, 11-1, Kaga-2, Itabashi-Ku, Tokyo 173-8605, Japan*

ARTICLE INFO

*Article history:*
Received 2 October 2009
Accepted 10 November 2009

*Keywords:*
Prostaglandin $D_2$
CRTH2
Human β-defensin-3
Keratinocyte
c-Fos

ABSTRACT

The antimicrobial peptide human β-defensin-3 (hBD-3) is produced by epidermal keratinocytes and protects the skin from infections. This peptide induces the release of a lipid mediator, prostaglandin $D_2$ from dermal mast cells. Prostaglandin $D_2$ binds to cell-surface G protein-coupled receptors, D prostanoid receptor, and chemoattractant receptor-homologous molecule expressed on T helper cell type 2 (CRTH2). Both receptors are detected on epidermal keratinocytes. It is reported that prostaglandin $D_2$ is involved in cutaneous allergy, however, its role in antimicrobial defense is unknown. We examined the *in vitro* effects of prostaglandin $D_2$ on hBD-3 production in normal human keratinocytes. Prostaglandin $D_2$ enhanced hBD-3 secretion and mRNA expression in human keratinocytes. Prostaglandin $D_2$-induced hBD-3 production was suppressed by the CRTH2 antagonist ramatroban and by antisense oligonucleotides against c-Jun and c-Fos, components of a transcription factor, activator protein-1 (AP-1). Prostaglandin $D_2$ enhanced the transcriptional activity and DNA binding of AP-1, expression, phosphorylation, and DNA binding of c-Fos proteins in keratinocytes. Prostaglandin $D_2$-induced hBD-3 production, AP-1 activity, and c-Fos expression and phosphorylation were suppressed by U0126, PP2, and pertussis toxin, which are inhibitors of mitogen-activated protein kinase kinase (MEK), *src*, and $G_i$ proteins, respectively. The phosphorylation of extracellular signal-regulated kinase (ERK), downstream kinase of MEK, was induced by prostaglandin $D_2$, and suppressed by ramatroban, pertussis toxin, PP2, and U0126. These results suggest that prostaglandin $D_2$ induces hBD-3 production in human keratinocytes by activating AP-1 through the expression and phosphorylation of c-Fos via the CRTH2/$G_i$/*src*/MEK/ERK pathway. Prostaglandin $D_2$ may promote cutaneous antimicrobial activity via hBD-3.



## 1. Introduction

The antimicrobial peptide human β-defensin-3 (hBD-3) is produced by epidermal keratinocytes, and its production is enhanced by infections [1]. Further, hBD-3 exhibits broad-spectrum activity and acts against gram-positive and gram-negative bacteria and fungi in a salt-insensitive manner [1]. This peptide forms lytic pores in the lipid bilayers of microbes, particularly *Staphylococcus aureus*, and destroys these organisms [2]. It also induces the production of interleukin-8 (IL-8) and IL-18 in epidermal keratinocytes [3]. IL-8 induces chemotaxis of neutrophils, which phagocytose bacteria and fungi. IL-18 enhances interferon-γ production in T helper cell type 1 (Th1) cells, which protect against intracellular pathogens such as mycobacteria [4]. Furthermore, hBD-3 binds CC chemokine receptor 6 (CCR6) and acts as a chemoattractant for CCR6-positive cells [5], especially T helper cell type 17 (Th17) cells. Th17 cells produce IL-17 and IL-22, which protect the skin from extracellular bacteria and fungi by promoting neutrophil recruitment via the induction of IL-8 production in keratinocytes [6].

The number of dermal mast cells increases at sites of infection, and these cells prevent the dissemination of pathogens by inducing innate and adaptive immune responses [7]. Mast cells identify pathogens through toll-like receptors or complement receptors and release a variety of cytokines or mediators such as tumor necrosis factor-α, IL-6, leukotriene $C_4$, and histamine [8]. The lipid mediator prostaglandin $D_2$ is one such mediator and is released via the action of toll-like receptor 2 on mast cells in response to lipoteichoic acid derived from gram-positive bacteria [8]. Prostaglandin $D_2$ binds G protein-coupled receptors, D prostanoid receptor (DP), and chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2) [9,10]. DP stimulation activates $G_s$ and the cyclic AMP/protein kinase A (PKA) pathway [9], while CRTH2 stimulation activates $G_i$ and suppresses cyclic AMP signals [10] or induces $Ca^{2+}$ signals [11]. Prostaglandin $D_2$ plays important roles in cutaneous allergy; it acts as a chemoattractant for eosinophils, basophils, and

* Corresponding author. Tel.: +81 3 3964 2473; fax: +81 3 5375 5314.
*E-mail addresses:* nmk@med.teikyo-u.ac.jp (N. Kanda), takeko@med.teikyo-u.ac.jp (T. Ishikawa), watanabe@med.teikyo-u.ac.jp (S. Watanabe).

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doi:10.1016/j.bcp.2009.11.012

Th2 cells through the action of CRTH2 [12]. DP and CRTH2 receptors have been detected in epidermal keratinocytes [13]. In a mouse model of ovalbumin-induced atopic dermatitis, CRTH2 activation exacerbated epidermal thickening and dermal infiltration by mast cells and eosinophils [14]. In contrast, DP activation suppressed such inflammation through the induction of the immunoregulatory cytokine IL-10 [15] and reduced pruritus [16]. However, the role of prostaglandin $D_2$ in cutaneous infection has not been fully elucidated. It is reported that prostaglandin $D_2$ enhances the clearance of *Pseudomonas aeruginosa* from the mouse lung [17]; however, the precise mechanism underlying this clearance is unknown.

In a recent study, it was reported that hBD-3 acts on mast cells and enhances their chemotaxis and degranulation, which results in the release of prostaglandin $D_2$ [18]. These results indicate a relationship between hBD-3 and prostaglandin $D_2$; however, it has not been determined whether prostaglandin $D_2$ alters hBD-3 production in epidermal keratinocytes. In the present study, we investigated the *in vitro* effects of prostaglandin $D_2$ on hBD-3 production in normal human keratinocytes. We found that prostaglandin $D_2$ enhanced hBD-3 production in these cells. To our knowledge, this is the first study to reveal the antimicrobial role of prostaglandin $D_2$ in the skin.

## 2. Materials and methods

### 2.1. Reagents

The following chemicals were purchased from Calbiochem (La Jolla, CA): SB202190, prostaglandin $D_2$, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), GF109203X, 8-(2-aminoethylamino)adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS), and pertussis toxin. U0126 and 1,2-bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM) were purchased from Sigma Chemical Co. (St. Louis, MO). SP600125 and U46619 were purchased from Biomol (Plymouth Meeting, PA). Ramatroban [(+)-(3R)-3-(4-fluorobenzenesulfonamido)-1,2,3,4-tetra-hydrocarbazole-9-propionic acid] was donated by Bayer Yakuhin Ltd. (Osaka, Japan). BW245C, 13,14-dihydro-15-keto-prostaglandin $D_2$ (DK-$PGD_2$), MK0524, and rosiglitazone were purchased from Cayman Chemical (Ann Arbor, MI).

### 2.2. Culture of keratinocytes

Human neonatal foreskin keratinocytes were purchased from Clonetics (Walkersville, MD). The keratinocytes were cultured in serum-free keratinocyte growth medium (KGM; Clonetics, Walkersville, MD) containing keratinocyte basal medium (KBM) supplemented with 0.5 μg/ml hydrocortisone, 5 ng/ml epidermal growth factor, 5 μg/ml insulin, and 0.5% bovine pituitary extract. Cells in the third passage were used. Each experiment described below was performed four times using the same lot of keratinocytes.

### 2.3. Secretion of hBD-3

Keratinocytes ($5 \times 10^4$ cells/well) were seeded into 24-well plates containing 0.4 ml KGM, adhered overnight, washed, and incubated with KBM for 24 h. The cells were washed and treated for 48 h with the indicated concentrations of prostaglandin $D_2$ in KBM. Each treatment was performed in triplicate. The hBD-3 concentration in the culture supernatants was measured using ELISA (Phoenix Pharmaceuticals Inc., Burlingame, CA) according to the manufacturer's instruction. The cell number was counted at the end of the incubation, and the amounts of hBD-3 were standardized to the cell number and shown as ng per one million cells.

### 2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

In order to measure the mRNA levels of hBD-3, the keratinocytes seeded at $5 \times 10^4$ cells/well in 24-well plates were incubated with prostaglandin $D_2$ for 12 h as described above. Total cellular RNA was extracted and reverse transcribed to produce cDNA. Real time PCR was performed in a fluorescence temperature cycler (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany), using specific forward and reverse primers as described [19]. A SYBR Green I system was utilized in the reaction; 20 μl of the PCR mixture containing 2 μl of cDNA, 3 mM $MgCl_2$, 0.5 μM of each primer, and 2 μl of the reagent from light cycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics GmbH, Mannheim, Germany). Initial denaturation at 95 °C for 10 min was followed by 45 cycles, each cycle consisting of denaturation at 95 °C for 15 s, annealing at 62 °C for 5 s and elongation at 72 °C for 10 s. Cycle-to-cycle fluorescence emission readings were monitored and analyzed using LightCycler software (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instruction. The hBD-3 mRNA expression was normalized to β-actin and was shown as a fold induction relative to control keratinocytes treated with medium alone.

### 2.5. Plasmids and transfection

The plasmid pAP-1-luc contains seven copies of activator protein-1 (AP-1)-like sequences (5′-TGACTTCA-3′) in the hBD-3 promoter at position-1258 [20] in front of the TATA box, upstream of the firefly luciferase reporter gene. Transient transfection was performed using Fugene 6 (Roche Diagnostics GmbH, Mannheim, Germany) as previously described [21]. Keratinocytes were seeded in 24-well plates and grown to approximately 60% confluence. The plasmids pAP-1-luc and herpes simplex virus thymidine kinase promoter-linked *Renilla* luciferase vector (pRL-tk) were premixed with Fugene 6 and added to the keratinocytes. After 6 h, the cells were washed and incubated in KBM for 24 h and treated with the indicated concentrations of prostaglandin $D_2$. After 18 h, the activities of the firefly luciferase and *Renilla* luciferase in the cell extracts were measured using a dual-luciferase assay system (Promega, Madison, WI). The transcriptional activity of AP-1 was expressed as the ratio of the firefly/*Renilla* luciferase activity.

### 2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a digoxigenin gel shift kit, second generation (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. An oligonucleotide containing AP-1 site in the hBD-3 promoter at position-1258 (5′-CATCACGGTGACTTCAGCTCCCAATTG-3′) [20] was labeled with digoxigenin and used as a probe. The keratinocytes were incubated with medium alone or 100 nM prostaglandin $D_2$ for 30 min, and nuclear proteins were obtained using a Nuclear Extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. The nuclear extract was incubated with the digoxigenin-labeled probe at 4 °C for 2 h. The DNA-protein complexes were resolved by electrophoresis on a 6% polyacrylamide gel. The gel was transferred to a nylon membrane, and the digoxigenin signal was detected according to the kit protocol, using alkaline phosphatase-conjugated anti-digoxigenin antibody. For supershift assays, antibodies to c-Fos/c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with nuclear extract on ice for 30 min prior to the incubation with labeled probe.

### 2.7. Western blotting

Western blotting was performed as described previously [21,22]. In brief, the keratinocytes were incubated with 100 nM prostaglandin $D_2$ for the indicated periods and lysed in a lysis buffer; the resultant lysates were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were then blocked, probed with primary antibodies (anti-pan extracellular signal-regulated kinase (ERK)1/2, anti-pan c-Fos, anti-pan c-Jun, anti-S63/73-phosphorylated c-Jun, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies from Santa Cruz Biotechnology, Santa Cruz, CA; anti-S374-phosphorylated c-Fos antibody from Assay Designs Inc., Ann Arbor, MI; anti-phospho ERK1(pT202/pY204)/ERK2(pT185/pY187) antibody from Novus Biologicals, Inc., Littleton, CO) and then with the appropriate secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA), and visualized using an ECL detection system (Amersham Biosciences, Arlington Heights, IL) according to the manufacturer's instructions. Protein bands were analyzed using densitometry.

### 2.8. Treatment with antisense oligonucleotides

Antisense oligonucleotides against signal transducer and activator of transcription 1 (STAT1), STAT3, c-Fos, c-Jun, and a random control oligonucleotide were synthesized using phosphoramidite chemistry, as previously described [22,23]. Keratinocytes were transfected with the indicated oligonucleotides (0.2 μM each) premixed with Fugene 6 in KGM for 24 h. The cells were incubated in KBM for 24 h and then incubated with 100 nM prostaglandin $D_2$.

### 2.9. Measurement of cyclic AMP amount

Keratinocytes were seeded at $10^4$ cells/well in 96-well plates and cultured for 24 h in KBM, treated for 15 min with 1 mM 3-isobutyl-L-methylamine (Sigma Chemical Co., St Louis, MO), then incubated with 100 nM prostaglandin $D_2$, BW245C, or DK-$PGD_2$ for 5 min, and were analyzed for intracellular cyclic AMP levels using ELISA (R&D Systems, Minneapolis, MN).

### 2.10. Measurement of intracellular $Ca^{2+}$ concentration

Intracellular $Ca^{2+}$ concentration was measured by spectrofluorometry as described [11,24]. Keratinocytes were cultured with KBM for 16 h either in the presence or absence of pertussis toxin (100 ng/ml). Keratinocytes were loaded with 3 μM Fura 2-AM (Calbiochem, La Jolla, CA) for 30 min, then put on the stage of the inverted fluorescence microscope, and prostaglandin $D_2$, DK-$PGD_2$, or BW245C were applied to the cells at 100 nM in 1 ml Tyrode's salt solution.

### 2.11. Statistical analyzes

Statistical analyzes were performed using one-way ANOVA with Dunnett's multiple comparison test (Fig. 1A and B), and one-way ANOVA with Tukey–Kramer multiple comparison test (Figs. 2B, 2C, 3A, 3B, 4A, 4B, 5A, 7A, 7B, and 7C). A $P$ value of $<0.05$ was considered significant.

## 3. Results

### 3.1. Prostaglandin $D_2$ enhances hBD-3 production via CRTH2 receptors in keratinocytes

We examined the effects of prostaglandin $D_2$ on hBD-3 production in human keratinocytes. Prostaglandin $D_2$ dose-dependently increased hBD-3 secretion (Fig. 1A). The stimulatory effect of prostaglandin $D_2$ was manifested at 1 nM and maximized at 100 nM, at which concentration the hBD-3 secretion was 10.4-fold the secretion in the controls. The addition of prostaglandin $D_2$ at this concentration did not increase the number of keratinocytes relative to the number in the controls ($n = 4$; mean ± S.E.M. 93 ± 9%). In parallel with protein secretion, prostaglandin $D_2$ increased the hBD-3 mRNA levels (Fig. 1B).

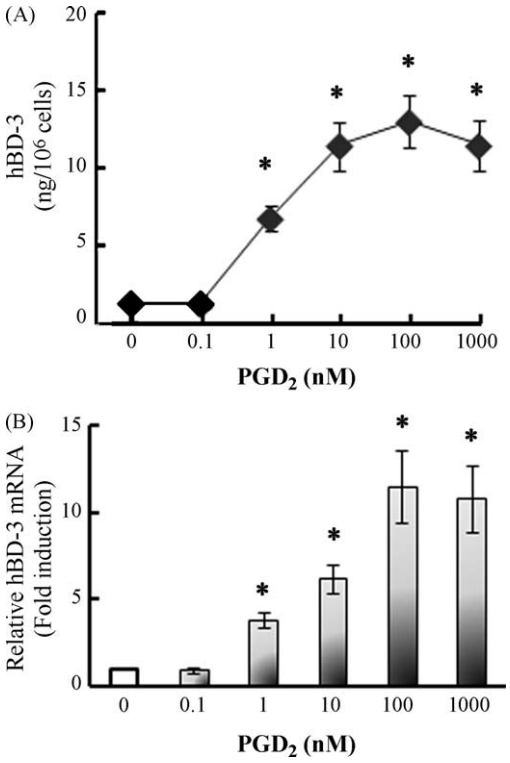


**Fig. 1.** Stimulatory effects of prostaglandin $D_2$ on hBD-3 secretion (A) and mRNA expression (B). Keratinocytes were treated with medium alone or with the indicated concentrations of prostaglandin $D_2$ ($PGD_2$). At 48 h, hBD-3 secretion was analyzed (A), and at 12 h, the mRNA levels were measured (B). Data are represented as mean ± S.E.M. of four independent experiments with each treatment per one experiment performed in triplicate. $^*P < 0.05$ vs. controls, by one-way ANOVA and Dunnett's multiple comparison test. The hBD-3 mRNA levels were normalized against those of β-actin and are represented as fold inductions relative to control keratinocytes treated with medium alone.

It is reported that human keratinocytes express DP and CRTH2 [13]. Prostaglandin $D_2$ and CRTH2 agonist DK-$PGD_2$ did, but DP agonist BW245C did not, increase intracellular calcium concentration in human keratinocytes (Fig. 2A and B), and the increase was suppressed by pertussis toxin, indicating that CRTH2 may be functional and that Gi/o proteins may be involved in the generation of calcium signal. On the other hand, DP agonist BW245C moderately increased intracellular cyclic AMP concentration, however, prostaglandin $D_2$ and DK-$PGD_2$ did not (Fig. 2C), indicating that DP on keratinocytes may functionally induce cyclic AMP signal, however, the signal may be possibly counteracted by the inhibitory effect by CRTH2 coupled to Gi/o proteins [10]. Although DP and CRTH2 are specific prostaglandin $D_2$ receptors, prostaglandin $D_2$ also binds to thromboxane $A_2$ receptor, TP with low affinity or is non-enzymatically converted to 15-deoxy-Δ12,14-prostaglandin $J_2$, which binds to the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) [25]. For this reason, we examined the receptor subtypes involved in prostaglandin $D_2$-induced hBD-3 production. The CRTH2 agonist DK-$PGD_2$ enhanced hBD-3 secretion (Fig. 3A) and hBD-3 mRNA expression (Fig. 3B) to an extent similar to that observed with prostaglandin $D_2$, while the DP agonist BW245C, the PPAR γ

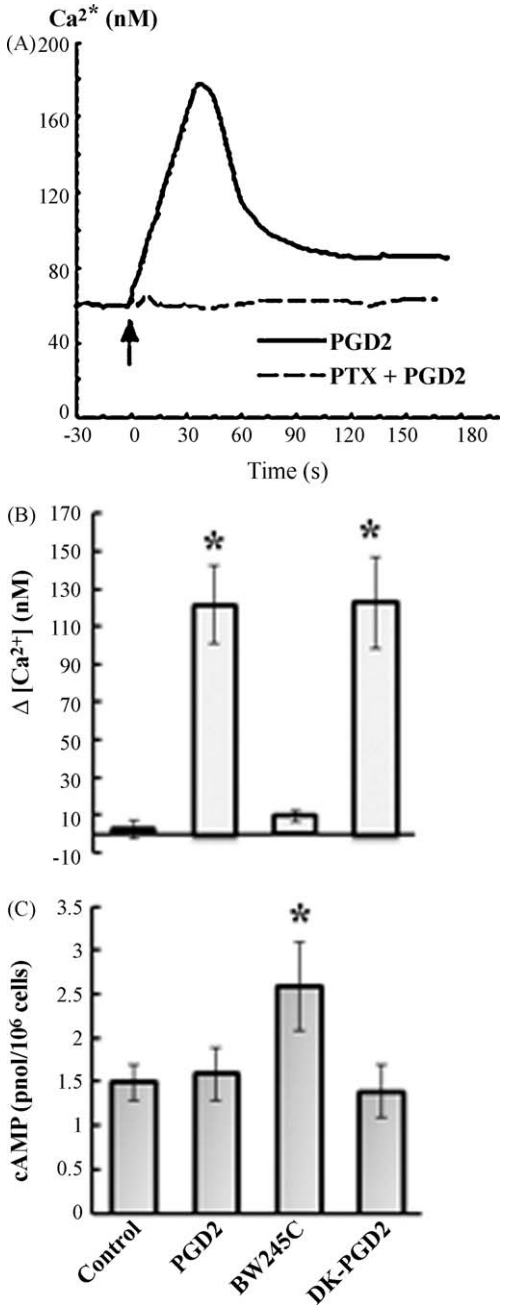


**Fig. 2.** Effects of prostaglandin $D_2$ receptor agonists on intracellular calcium (A and B) or cyclic AMP concentrations (C). (A) Keratinocytes were preincubated with 100 ng/ml pertussis toxin (PTX) or medium alone for 16 h, loaded with Fura-2/AM, and treated with 100 nM prostaglandin $D_2$ ($PGD_2$), and intracellular calcium concentration was monitored. The arrow indicates the moment when prostaglandin $D_2$ was added. (B) Keratinocytes were incubated with prostaglandin $D_2$, DP agonist BW245C, or CRTH2 agonist DK-$PGD_2$ (100 nM each), or buffer alone, and the maximal increase of intracellular calcium concentration ($\Delta[Ca^{2+}]$) of each group is shown. (C) Keratinocytes were preincubated with 1 mM 3-isobutyl-L-methylamine and incubated with the reagents above for 5 min, and intracellular cyclic AMP concentration was measured. In (B) and (C), data are represented as mean ± S.E.M. of four independent experiments. $^*P < 0.05$ vs. controls, by one-way ANOVA and the Tukey–Kramer multiple comparison test. The results in (A) represent the data of four separate experiments.

agonist rosiglitazone, and the TP agonist U46619 did not. BW245C at higher concentrations (1 or 10 μM) did not increase the hBD-3 secretion or mRNA expression (data not shown). The CRTH2 antagonist ramatroban, but not the DP antagonist MK0524, suppressed prostaglandin $D_2$-induced hBD-3 secretion (Fig. 3A)

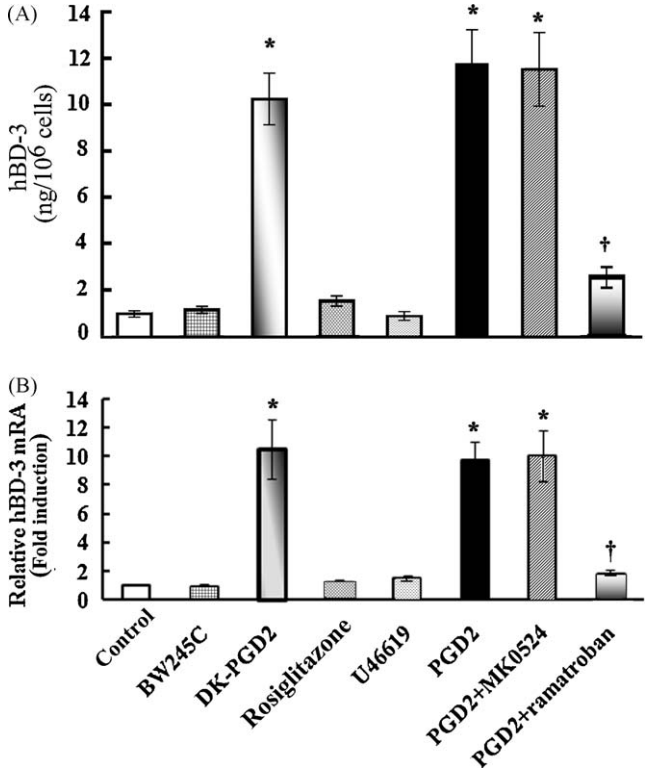


**Fig. 3.** Effects of prostaglandin $D_2$ receptor agonists or antagonists on hBD-3 production. Keratinocytes were treated with 100 nM prostaglandin $D_2$ ($PGD_2$), CRTH2 agonist DK-$PGD_2$, DP agonist BW245C, PPARγ agonist rosiglitazone, or TP agonist U46619. At 48 h, hBD-3 secretion was analyzed (A), and at 12 h, the mRNA levels were measured (B). In some experiments, the keratinocytes were pre-treated with 500 nM ramatroban (CRTH2 antagonist) or MK0524 (DP antagonist) for 30 min prior to the treatment with prostaglandin $D_2$. $^*P < 0.05$ vs. controls; $\dagger P < 0.05$ vs. prostaglandin $D_2$ alone, by one-way ANOVA and the Tukey–Kramer multiple comparison test. Data are represented as mean ± S.E.M. of four independent experiments with each treatment per one experiment performed in triplicate. The hBD-3 mRNA levels were normalized against those of β-actin and are represented as fold inductions.

and hBD-3 mRNA expression (Fig. 3B). These results indicate that the CRTH2 receptors are involved in prostaglandin $D_2$-induced hBD-3 production.

### 3.2. *Prostaglandin $D_2$ may induce hBD-3 production by activating AP-1 via the expression and phosphorylation of c-Fos*

The hBD-3 promoter contains STAT- or AP-1-binding sequences, and these transcription factors may induce the expression of the *hBD-3* gene [26]. Therefore, we determined whether these transcription factors were involved in prostaglandin $D_2$-induced hBD-3 production by transfecting keratinocytes with antisense oligonucleotides against these factors. We have previously confirmed that antisense oligonucleotides against STAT1, STAT3, and the AP-1 components, c-Fos and c-Jun, selectively reduce the protein levels of the respective transcription factors [21,23]. The transfection of these antisense oligonucleotides did not reduce the viability of keratinocytes; >92% cells were viable after the transfection. Antisense oligonucleotides against c-Fos and c-Jun remarkably reduced prostaglandin $D_2$-induced hBD-3 secretion (Fig. 4A, lanes 4 and 5) and hBD3 mRNA expression (Fig. 4B). In contrast, antisense oligonucleotides against STAT1 or STAT3 did not alter prostaglandin $D_2$-induced hBD-3 production (Fig. 4A and B, lanes 6 and 7, respectively). These results suggest that AP-1 but not STAT1 or STAT3 is required for the hBD-3 production induced by prostaglandin $D_2$.

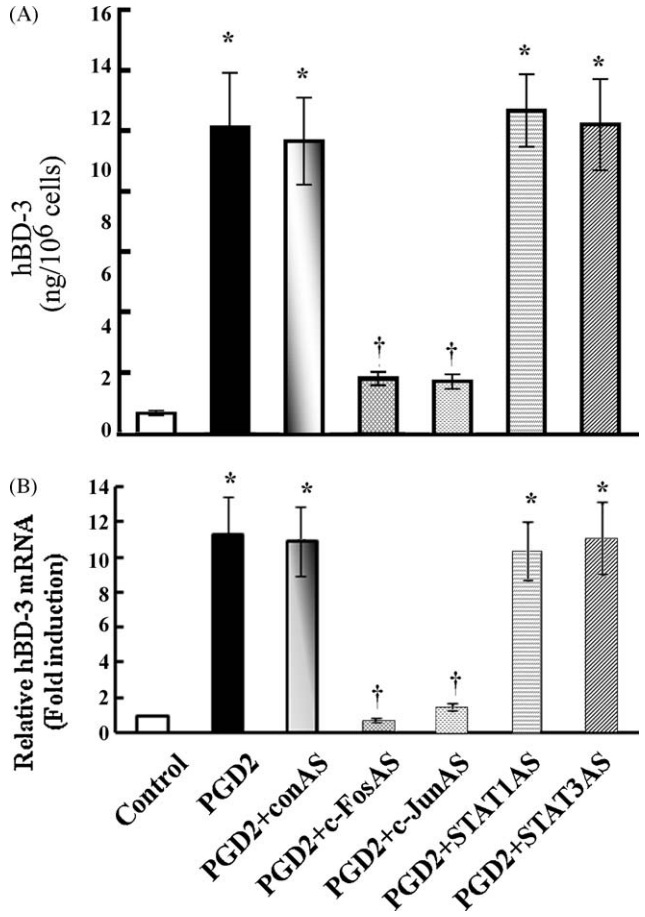


**Fig. 4.** Effects of antisense oligonucleotides on prostaglandin $D_2$-induced hBD-3 production. Keratinocytes transfected with antisense oligonucleotides (AS) (0.2 μM each) were treated with 100 nM prostaglandin $D_2$ ($PGD_2$). At 48 h, hBD-3 secretion was analyzed (A), and at 12 h, the mRNA levels were measured (B). $^*P < 0.05$ vs. controls; $\dagger P < 0.05$ vs. prostaglandin $D_2$ alone, by one-way ANOVA and the Tukey–Kramer multiple comparison test. Data are represented as mean ± S.E.M. of four independent experiments with each treatment per one experiment performed in triplicate. The hBD-3 mRNA levels were normalized against those of β-actin and are represented as fold inductions.

We subsequently examined whether prostaglandin $D_2$ enhanced AP-1 transcriptional activity or c-Fos and c-Jun expression. Prostaglandin $D_2$ enhanced AP-1 transcriptional activity (Fig. 5A) and DNA binding (Fig. 5B, lane 2) in the keratinocytes. The supershift assays revealed that the complexes from the keratinocytes without prostaglandin $D_2$ were supershifted only by anti-c-Jun antibody, and not by anti-c-Fos antibody (Fig. 5B, lanes 5 and 6) while those from prostaglandin $D_2$-treated cells were supershifted both by anti-c-Fos and anti-c-Jun antibodies (Fig. 5B, lanes 7 and 8). The results suggest that AP-1 elements may be constitutively bound by c-Jun but not by c-Fos proteins in keratinocytes while in prostaglandin $D_2$-treated cells, the elements may be bound both by c-Jun and c-Fos, indicating the change of AP-1 composition by prostaglandin $D_2$. The protein levels of total c-Fos (Fig. 5C, first panel) and its phosphorylated form (Fig. 5C, second panel) were constitutively low, and these were drastically increased by prostaglandin $D_2$; however, the protein levels of total c-Jun (Fig. 5C, third panel) and its phosphorylated form (Fig. 5C, fourth panel) were constitutively much higher than those of c-Fos and were not altered by prostaglandin $D_2$. The increase of c-Fos protein levels by prostaglandin $D_2$ appeared at 15 min, peaked at 30 min, then reduced and returned to the basal level at 4 h while the levels of c-Jun were not altered by prostaglandin $D_2$ at any time points until 24 h (Fig. 6). These results suggest that prostaglandin $D_2$ may activate AP-1 by increasing the levels of total and phosphorylated c-Fos proteins. AP-1 transcriptional activity (Fig. 5A) and DNA binding (Fig. 5B), total and phosphorylated c-Fos levels (Fig. 5C) increased by prostaglandin $D_2$ (lane 2 of Fig. 5A–C) were suppressed by the CRTH2 antagonist ramatroban (lane 4 of Fig. 5A–C), but not by

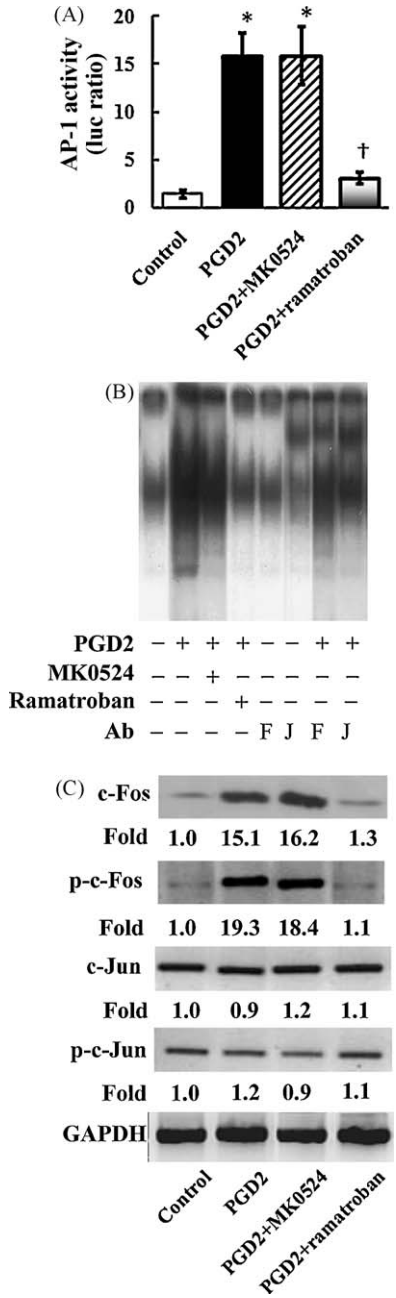


**Fig. 5.** Stimulatory effects of prostaglandin $D_2$ on the AP-1 transcriptional activity (A) and DNA binding (B), c-Fos expression and phosphorylation (C). Keratinocytes transfected with pRL-tk and pAP-1-luc (A) or untransfected keratinocytes (B and C) were pre-treated with 500 nM ramatroban (CRTH2 antagonist) or MK0524 (DP antagonist) for 30 min and treated with 100 nM prostaglandin $D_2$ ($PGD_2$). At 18 h, the transcriptional activity of AP-1 was analyzed (A), and at 30 min, nuclear AP-1 DNA binding was analyzed by EMSA (B) and the levels of total c-Fos, c-Jun, and phosphorylated c-Fos (p-c-Fos), c-Jun (p-c-Jun) were analyzed by western blotting (C). In (B), EMSA was performed with nuclear protein extracts and labeled AP-1 probe in the presence or absence of the antibodies (Abs) against c-Fos (F) or c-Jun (J). $^*P < 0.05$ vs. controls; $\dagger P < 0.05$ vs. prostaglandin $D_2$ alone, by one-way ANOVA and the Tukey–Kramer multiple comparison test. Data in (A) are represented as mean ± S.E.M. of four independent experiments with each treatment per one experiment performed in triplicate. The levels of c-Fos, c-Jun, p-c-Fos, and p-c-Jun were normalized against those of GAPDH and are represented as fold inductions. The results in (B) and (C) represent the data of four separate experiments.

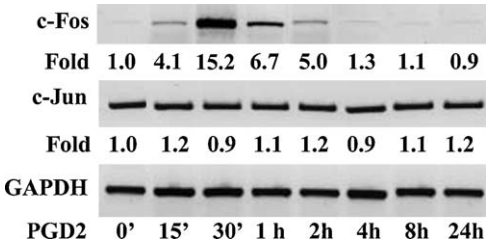


**Fig. 6.** The time course of c-Fos and c-Jun levels after the incubation with prostaglandin $D_2$. Keratinocytes were incubated with 100 nM prostaglandin $D_2$ ($PGD_2$), and the levels of c-Fos and c-Jun proteins were analyzed at the indicated time-points. The levels of c-Fos and c-Jun were normalized against those of GAPDH and are represented as fold inductions. The results represent the data of four separate experiments.

the DP antagonist MK0524 (lane 3 of Fig. 5A–C). These results suggest that prostaglandin $D_2$ may activate AP-1 by increasing total and phosphorylated c-Fos proteins through CRTH2 receptors.

### 3.3. $G_i$, MEK, and src are involved in prostaglandin $D_2$-induced expression and phosphorylation of c-Fos, activation of AP-1 and production of hBD-3

Prostaglandin $D_2$ activates PKA, protein kinase C (PKC), c-Jun N-terminal kinase (JNK), ERK, and p38 mitogen-activated protein kinase (MAPK) [27]. We therefore examined the signaling pathways involved in prostaglandin $D_2$-induced hBD-3 production and AP-1 activity by using specific inhibitors of these pathways. U0126, PP2, and pertussis toxin, which are inhibitors of MAPK kinase (MEK), *src*, and $G_i$ proteins, respectively, suppressed prostaglandin $D_2$-induced hBD-3 secretion (Fig. 7A) and mRNA expression (Fig. 7B), but GF109203X, Rp-cAMPS, SP600125, and SB202190, which are inhibitors of PKC, PKA, JNK, and p38 MAPK, respectively, did not alter hBD-3 secretion and mRNA expression. In parallel with hBD-3 production, prostaglandin $D_2$-induced increases of AP-1 transcriptional activity (Fig. 7C), total and phosphorylated c-Fos levels (Fig. 7D) were suppressed by U0126, PP2, and pertussis toxin but not by GF109203X, Rp-cAMPS, SP600125, or SB202190. These signal inhibitors did not reduce the viability of keratinocytes at the concentrations used; >93% cells were viable after the incubation. These results suggest that $G_i$ proteins, MEK, and *src* are involved in prostaglandin $D_2$-induced c-Fos expression and phosphorylation, AP-1 activation, and the resultant hBD-3 production.

### 3.4. Prostaglandin $D_2$-induced ERK phosphorylation depends on CRTH2, $G_i$ proteins, src, and MEK

ERK is a downstream kinase of MEK and is activated by the dual phosphorylation of tyrosine/threonine residues [22]. We determined whether prostaglandin $D_2$ induces the dual phosphorylation of ERK in keratinocytes. Prostaglandin $D_2$-induced the dual phosphorylation of ERK1 and ERK2, and this phosphorylation was suppressed by U0126, PP2, pertussis toxin, and ramatroban, but not by GF109203X or Rp-cAMPS, or intracellular calcium chelator BAPTA/AM (Fig. 8). CRTH2 agonist DK-$PGD_2$ induced ERK1/2 phosphorylation while DP agonist BW245C did not (Fig. 8). These results suggest that the activation of ERK by prostaglandin $D_2$ depends on CRTH2, $G_i$ proteins, *src*, and MEK.

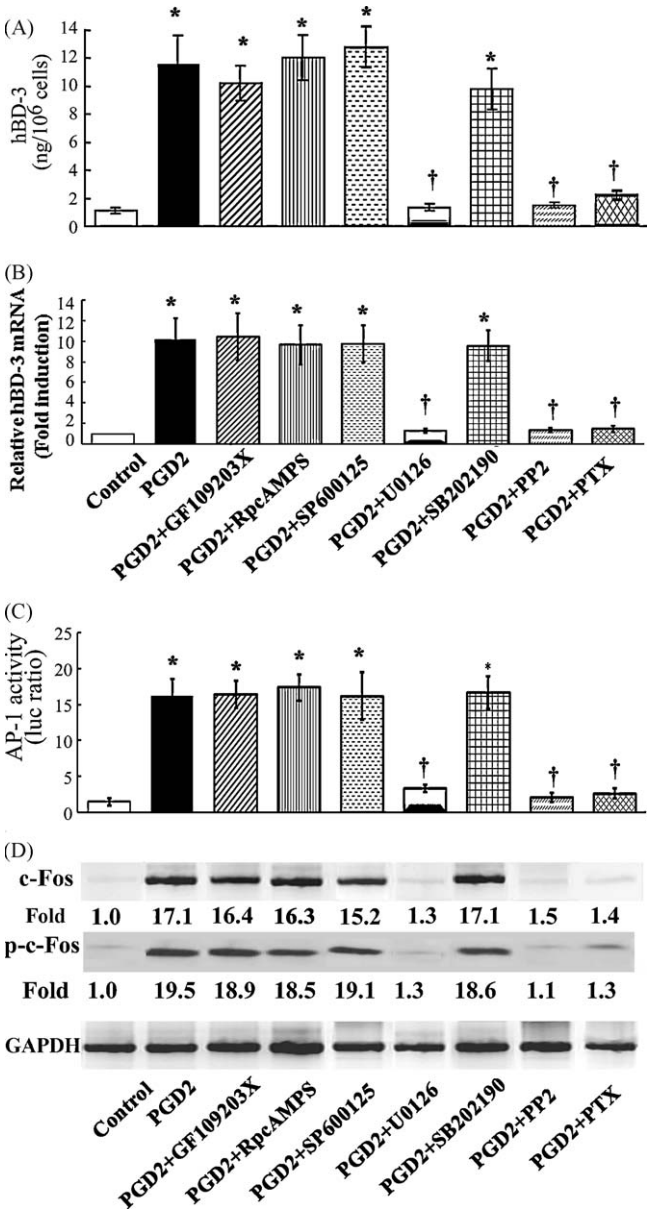


**Fig. 7.** Effects of signal inhibitors on prostaglandin $D_2$-induced hBD-3 secretion (A), mRNA expression (B), AP-1 activity (C), and c-Fos expression and phosphorylation (D). Keratinocytes transfected with pRL-tk and pAP-1-luc (C) or untransfected keratinocytes (A, B and D) were pre-treated for 16 h with 100 ng/ml pertussis toxin (PTX) or for 30 min with 1 μM GF109203X, 20 μM Rp-cAMPS, 10 μM SP600125, 10 μM U0126, 1 μM SB202190, or 0.1 μM PP2, and treated with 100 nM prostaglandin $D_2$ ($PGD_2$). The incubation was carried out for 48 h to analyze hBD-3 secretion (A), for 12 h to analyze the hBD-3 mRNA levels (B), for 18 h to analyze the transcriptional activity of AP-1 (C), and for 30 min to analyze the levels of total and phosphorylated c-Fos (p-c-Fos) (D). *$P < 0.05$ vs. controls; †$P < 0.05$ vs. prostaglandin $D_2$ alone, by one-way ANOVA and the Tukey–Kramer multiple comparison test. In (A)–(C), data are represented as mean ± S.E.M. of four independent experiments with each treatment per one experiment performed in triplicate. The levels of total c-Fos and p-c-Fos (D) were normalized against those of GAPDH and are represented as fold inductions. The results in (D) represent the data of four separate experiments.

## 4. Discussion

This study shows that prostaglandin $D_2$ induces hBD-3 production in human keratinocytes by activating the transcription factor AP-1. It has been reported that *S. aureus*-induced hBD-3 expression in human keratinocytes also depends on AP-1 [20]. Prostaglandin $D_2$ increased the transcriptional activity of AP-1 by inducing the expression, phosphorylation and DNA binding of the AP-1 component c-Fos; in contrast, c-Jun expression, phosphorylation and its DNA binding were constitutive and were not altered by prostaglandin $D_2$. This indicates that constitutive AP-1 activity may be mainly mediated by c-Jun homodimers, while prostaglandin $D_2$-induced AP-1 activity may be mediated, at least partially, by c-Fos/c-Jun heterodimers. It has been reported that the

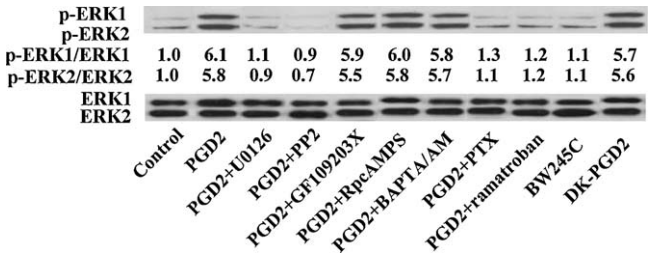


**Fig. 8.** Prostaglandin $D_2$-induced activation of ERK. Keratinocytes were pre-treated for 16 h with 100 ng/ml pertussis toxin (PTX) or for 30 min with 10 μM U0126, 0.1 μM PP2, 1 μM GF109203X, 20 μM Rp-cAMPS, 10 μM BAPTA/AM or 500 nM ramatroban (CRTH2 antagonist), and incubated with prostaglandin $D_2$ ($PGD_2$), BW245C (DP agonist), or DK-$PGD_2$ (CRTH2 agonist) (100 nM each) for 10 min to analyze ERK phosphorylation. The ratios of phosphorylated ERK1 or ERK2/total ERK1 or ERK2 are represented as fold inductions. The results represent the data of four separate experiments.

transcriptional capacities of c-Fos/c-Jun heterodimers are much higher than those of c-Jun homodimers [28]. Prostaglandin $D_2$ may thus promote the AP-1-mediated expression of *hBD-3* gene by altering the AP-1 composition from c-Jun/c-Jun homodimers to c-Fos/c-Jun heterodimers.

Prostaglandin $D_2$-induced c-Fos expression and phosphorylation depended on ERK. It is reported that the ERK-mediated phosphorylation of ternary complex factors such as Elk-1 or SAP1 promotes *c-fos* transcription by these transcription factors [29]. It is also reported that ERK phosphorylates C-terminal transactivation domain of c-Fos (aa209–380) and thus potentiates its transcriptional activity by favoring the interaction of c-Fos with other transcription factors, co-activators, or components of transcriptional machinery [30]. CRTH2, $G_i$ protein, *src*, and MEK were involved in the activation of ERK by prostaglandin $D_2$ (Fig. 8). It is reported that CRTH2 stimulation activates $G_i$ proteins [10,11]. The activation of $G_{\alpha i\beta\gamma}$, an inactive $G_i$ trimer, may release $G_{\alpha i}$ and $G_{\beta\gamma}$; the latter may activate *src* family tyrosine kinases [31]. Activated *src* may phosphorylate Shc, an adapter protein, and thus induce the association of Shc with Grb2. This may possibly trigger the membrane translocation of Sos, a guanine nucleotide exchange factor for ras, thereby activating ras and the downstream ras-raf1-MEK-ERK pathway [31]. It is also reported that prostaglandin $D_2$ activates phospholipase C, which produces diacylglycerol and activates PKC in human osteoblasts [27]. PKC can activate ERK by directly phosphorylating raf1 kinase, upstream of MEK/ERK [27,31]. However, PKC was not required for the prostaglandin $D_2$-induced activation of ERK in human keratinocytes (Fig. 8). It is also reported that the stimulation of other $G_i$-coupled receptors activates ERK independently from PKC in a cell type-specific manner (e.g., angiotensin II receptor AT1R in rat cardiac fibroblasts [31] and prostaglandin $E_2$ receptor EP4 in mouse colon carcinoma CT26 cells [32]). Prostaglandin $D_2$also induced calcium signal via CRTH2 in keratinocytes (Fig. 2A and B), however, the calcium signal may be dispensable for the activation of ERK by CRTH (Fig. 8). In addition to CRTH2, DP exists on keratinocytes, and DP agonist BW245C induced moderate cyclic AMP signal (Fig. 2C), however, the cyclic AMP signal may not contribute to the activation of ERK or resultant induction of hBD-3 in keratinocytes since neither was induced by BW245C (Figs. 3 and 8). It is reported that ERK is stimulated by exchange protein activated by cyclic AMP (Epac) [33], or either stimulated or suppressed by PKA dependently on cell types or co-stimuli [34]. However, cyclic AMP signal induced by DP may not be effectively linked to the MEK/ERK pathway in keratinocytes, related to the activities or subcellular localization of PKA and/or Epacs.

It is reported that the activation of ERK is necessary for antimicrobial killing since the disruption of ERK resulted in the deficiency of killing *Candida albicans* filaments by human neutrophils [35]. Similarly in keratinocytes, the inhibition of MEK/ERK pathway resulted in the loss of hBD-3 induction (Fig. 7), which may reduce the bactericidal activities of these cells since the contact of *S. aureus* with hBD-3 on the surface of keratinocytes is important for their effective killing [2].

In the present study, prostaglandin $D_2$-induced hBD-3 production in human keratinocytes; this indicates that prostaglandin $D_2$ helps defend against microbes in the skin. This is the first study to clarify the antimicrobial role of prostaglandin $D_2$ in the skin. The production of hBD-3 by epidermal keratinocytes is increased at sites of infection [1], where the number of mast cells is increased and their degranulation is enhanced [7,8]. Our results indicate that prostaglandin $D_2$ released from mast cells stimulates hBD-3 production in skin lesions caused by infections and thus prevents the dissemination of pathogens. The released hBD-3 may in turn act on keratinocytes and enhance the production of cytokines or chemokines related to innate and adaptive immunity in an autocrine manner [4–6]. In addition, hBD-3 may induce mast cell degranulation and the release of prostaglandin $D_2$; this indicates a positive feedback loop. We recently found that histamine, another mediator released by mast cells, induces the production of hBD-2 and hBD-3 in keratinocytes [23,36]. We have also obtained the results that prostaglandin $D_2$ increases hBD-2 production in keratinocytes as well as hBD-3 (data not shown) though the results are too preliminary and the precise mechanisms are now under study. Thus, prostaglandin $D_2$ and histamine may cooperatively act as mediators to link mast cells and keratinocytes and contribute to defense against pathogens. We should further examine if these mediators induce the other antimicrobial peptides such as cathelicidin or psoriasin in keratinocytes.

## Acknowledgements

We thank Ms. Hiroko Sato for the maintenance of keratinocytes and Bayer Yakuhin Ltd., for donating ramatroban. This work was supported by a grant from the Japan Society for the Promotion of Science (18244120).

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