Staphylococcus aureus peptidoglycan stimulates granulocyte macrophage colony-stimulating factor production from human epidermal keratinocytes via mitogen-activated protein kinases

Masahiro Matsubara*, Daisuke Harada, Haruhiko Manabe, Kazuhide Hasegawa

Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd., 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731, Japan

Received 20 March 2004; revised 7 April 2004; accepted 14 April 2004

Available online 27 April 2004

Edited by Masayuki Miyasaka

Abstract Epidermal keratinocytes with atopic dermatitis (AD) overproduce mediators such as granulocyte macrophage colony-stimulating factor (GM-CSF), which are associated with pathology of AD. We found that peptidoglycan (PGN) of *Staphylococcus aureus*, which is frequently observed in lesion with AD, induced the production of numerous mediators such as GM-CSF and regulated on activation, normal T-cell expressed and secreted. Moreover, PGN phosphorylated extracellular-signal-regulated kinases and p38 mitogen-activated protein kinase, which were involved in the induction of GM-CSF expression. These results suggested that PGN of *S. aureus* directly exacerbates inflammation of inflammatory skin disease.

Keywords: Atopic dermatitis; Keratinocyte; Granulocyte macrophage colony-stimulating factor; Mitogen-activated protein kinase; *Staphylococcus aureus*

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with a tendency for exacerbation and remission over time [1]. Patients with AD show higher levels and synthesis of cytokines, such as interleukin (IL)-4, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF), in their skin lesions [1]. IL-4 and IL-5, which are derived from the infiltrated Th2 cells and mast cells, are well known to induce the IgE class switching in B cells and eosinophilia, respectively [1]. IL-4 plays important roles in the induction of acute response whereas IL-5 and GM-CSF are involved in chronic inflammation of AD [1]. GM-CSF is locally critical in development and function of antigen-presenting dendritic cells (DC) and Langerhans cells (LC) [2–5]. GM-CSF also inhibits the production of Th-1-inducing cytokine IL-12 from LC [6]. Increase in GM-CSF production correlates with accumulation and activation of DC and LC in lesional skin with AD [1]. Therefore, excessive GM-CSF production is suggested to cause an imbalance in association with Th2-dominant immune response [6].

Although GM-CSF is especially produced from epidermal keratinocytes with AD [2], the mechanisms for enhanced GM-CSF production remains fully understood. Keratinocytes not only constitute the major epidermal cell population as a physical barrier but also synthesize several cytokines and chemokines, such as GM-CSF and regulated on activation, normal T-cell expressed and secreted (RANTES), which are responsible for the skin immune response [2,7–10]. However, abnormal production of cytokines and chemokines including GM-CSF is believed to participate in inflammation of skin disorders [2,7].

Increased numbers of *Staphylococcus aureus* are found in over 90% of AD skin lesions [1]. *S. aureus* thus is suggested to amplify the skin inflammation by superantigens such as staphylococcal entertoxins A and B, which target on several types cells such as keratinocytes to continue T-cell activation and release of pro-inflammatory mediators [11]. Recently, peptidoglycan (PGN), which predominates the cell wall of *S. aureus*, has been demonstrated to activate nuclear factor-κB (NF-κB) and induce abundantly IL-8 production from keratinocytes via functionally toll-like receptor 2 (TLR2) [12–14]. Activation of pathways of NF-κB and mitogen-activated protein kinases (MAPKs) is involved in several biological responses of keratinocytes [12,13,15–17]. These findings suggest that PGN play major roles in the production of cytokines and chemokines from keratinocytes.

In the present study, we investigated whether or not PGN induces the release of GM-CSF and activate MAPKs using human epidermal keratinocytes. Moreover, we also examined effects of PGN on the expression of other keratinocyte-derived mediators, as described below. Several chemokines, such as RANTES, monocyte chemoattractant protein-1 (MCP-1) and IL-8, are highly expressed in lesion site with AD or psoriasis and stimulate the migration of many types of cell. Intracellular adhesion molecule-1 (ICAM-1), a potent adherent molecule between T lymphocyte and keratinocyte, acts as a co-stimulant of the activation of T lymphocyte [20]. Tumor necrosis factor (TNF)-α and IL-6 are involved in migration of LC in skin inflammation [21]. Substance P, a product of preprotachykinin A gene, is discussed the relevance with skin disorder because it degranulates mast cell, causing itchy feelings [10].

* Corresponding author. Fax: +81-559-86-7430.
E-mail address: masahiro.matsubara@kyowa.co.jp (M. Matsubara).

0014-5793/$22.00
doi:10.1016/j.febslet.2004.04.028

2. Materials and methods

2.1. Materials

Olopatadine hydrochloride was synthesized in our laboratories. The following materials were purchased from the indicated commercial source: PD98059 (Cell Signaling); SB203580 (Calbiochem); *S. aureus*-derived PGN (Sigma–Aldrich); histamine (Sigma); recombinant human TNF-α (Strathmann Biotic); recombinant human IL-4 (Chemi-Con); and recombinant human IFN-γ (Strathmann Biotec). Olopatadine hydrochloride, PD98059 and SB203580 were dissolved in dimethyl sulfoxide at a concentration of 0.1 mol/L and stored at −20 °C before use. The final concentration of dimethyl sulfoxide in the assay system described below was 0.1 v/v%.

2.2. Cell culture

Primary human epidermal keratinocytes were purchased from Biowhittaker and grown in KGM-2 with the addition of supplement containing bovine pituitary extract, human epidermal growth factor, insulin, epinephrine, transferrin, hydrocortisone, gentamycin and amphotericin B according to the manufacturer's instruction (Clonetics). Cells were cultured at confluent state. After the cells were rapidly washed with Dulbecco's modified Eagle's medium (Nissui), they were resuspended in KGM-2 medium without hydrocortisone the day before assay. Cells between passages 3 and 6 were used.

2.3. Semi-quantification of mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Confluent cells in 6-well plates were treated with the indicated concentrations of agents for 0.5 h in KGM-2 without hydrocortisone and then stimulated with 30 μg/mL PGN for a given length of time. Total RNA was isolated according to the manufacturer's instruction (RNeasy, Qiagen). First strand-cDNA synthesis was performed from 1.5 μg of total RNA in 20 μL volumes with oligo(dT) priming using the Superscript First-strand Synthesis System (invitrogen). PCR was performed from the generated cDNA using ExTaq polymerase (Takara). Based on previous reports [22–25], the following primer pairs (including PCR product sizes in parentheses) were synthesized by Invitrogen: TLR2, 5′-GCC AAA GTC TTG ATT GAT TGG-3′ and 5′-TTG AAG TTC TCC AGC TCC TG-3′ (347 bp); TLR4, 5′-TGG ATA CGT TTC CTT ATA AG-3′ and 5′-GAA ATG GAG GCA CCC CTT C-3′ (507 bp); GM-CSF, 5′-GGC GTC TCC TGA ACC TGA GTA G-3′ and 5′-GTC GGC TCC TGG AGG TCA AA-3′ (91 bp); RANTES, 5′-TCC CCA TAT TCC TCG GAC-3′ and 5′-GAT GTA CTC CCG AAC CCA-3′ (186 bp); TNF-α, 5′-AAG ACC TCA CCT AGA AAT TGA CAC-3′ and 5′-TAG GCC GAT TAC AGA CAC AAC-3′ (419 bp); IL-6, 5′-TGA CAA CTC ATC TCA TTC TGC-3′ and 5′-AAG TTA GCC ATT TAT TTG AGG TAA GC-3′ (411 bp); IL-8, 5′-CTA GCC AGG ATC CAC AAG TCC-3′ and 5′-AGC ACT ACC AAC ACA GCT GGC-3′ (503 bp); MCP-1, 5′-CAG CCA GAT GCA ATC AAT GC-3′ and 5′-GTG GTC CAT GGA ATC CTG AA-3′ (198 bp); ICAM-1, 5′-TAT GGC AAC GAC TCC TTC T-3′ and 5′-CAT TCA GCG TCA CCT TGG-3′ (238 bp); PPTA, 5′-GAC AGC GAC CAG ATC AAG GAG GAA-3′ and 5′-CAG CAT CCC GTT TGC C-3′ (115 bp); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCC ATC ACC ATC TTC CAG GAG-3′ and 5′-TTC ACC ACC TTC TTG ATG TCA-3′ (572 bp). The denaturation, annealing, and extension conditions for TLR2, TLR4, GM-CSF, RANTES, IL-8, MCP-1, ICAM-1, PPTA and GAPDH were 94 °C for 60 s, 60 °C for 45 s and 72 °C for 45 s, respectively, and that for TNF-α and IL-6 were 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s, respectively. The PCR cycle conditions were 30, 35, 27, 27, 32, 33, 22, 29, 25, 33 and 19 cycles for TLR2, TLR4, GM-CSF, RANTES, TNF-α, IL-6, IL-8, MCP-1, ICAM-1, PPTA and GAPDH, respectively. These conditions are the optimal settings for each gene. PCR was performed with a GeneAmp PCR system 9700 (Applied Biosystems). The PCR products were electrophoresed in 2% w/v agarose gels and stained with SYBR Green I (Molecular Probes). The detection and densitometric analysis of band were performed with a Lumino Image Analyzer LAS-1000 plus (Fujifilm).

2.4. Western blot analysis

Confluent cells in 6-well plates were treated with the indicated concentrations of agents for 0.5 h in KGM-2 without hydrocortisone, and then stimulated with 30 μg/mL PGN for a given length of time. The cells were lysed with ice-cold lysis buffer (150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1 v/v% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, 10 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L Tris–HCl, pH 7.5) for 30 min on ice. Insoluble material was removed by centrifugation and the supernatant was boiled with Laemmli's buffer containing dithiothreitol for 3 min. Equivalent contents of protein in each sample (10 μg/lane) were separated by 7.5 or 10% SDS–PAGE and blotted onto a PVDF membrane (Immobilon, Millipore). Proteins were detected using an immunoblotting technique with the following antibodies: anti-TLR2 antibody (Santa Cruz); anti-actin antibody (Santa Cruz); anti-ERK-1/2 antibody (Cell Signaling); anti-phospho-ERK-1/2 antibody (Cell Signaling); anti-phospho-p38 MAP kinase antibody (Cell Signaling); anti-phospho-JNK-1/2 antibody (Cell Signaling); and anti-phospho-ATF-2 antibody (Cell Signaling). Horseradish peroxidase-linked whole antibody (Amersham Bioscience) was used as a secondary antibody. The antibodies were detected with Super Signal West Pico Chemiluminescent Substrate (Pierce) and analyzed using a Lumino Image Analyzer LAS-1000 Plus (Fujifilm).

2.5. Measurement of mediator production

Confluent cells in 24-well plates were treated with the indicated concentrations of agents for 0.5 h in KGM-2 without hydrocortisone and then stimulated with 30 μg/mL PGN for a given length of time. GM-CSF, RANTES, TNF-α, IL-6, or IL-8 levels in the supernatant were determined using commercially available ELISA according to the manufacturer's instructions. The applied ELISA kits were human GM-CSF AN'ALYZA (Genzyme/Teche), human RANTES Duo Set (R&D Systems), Human TNF-α US Kit (BioSource), Endogen Human IL-6 (PIERCE) and human IL-8 Duo Set (R&D Systems).

2.6. Cell surface ELISA for determination of ICAM-1

Confluent cells were cultured in 96-well plates. After stimulation, the medium was removed and the cells were fixed with 3.7% formaldehyde on ice for 30 min. The plates were then washed with phosphate-buffered saline and blocked with 1% w/v BSA on ice for 1 h. A primary anti-ICAM-1 antibody (Cymbus Biotechnology) was added and incubated on ice for 1 h. The plates were then washed and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody on ice for 1 h. After incubation, the plates were washed again. The antibodies were detected with Super Signal West Pico Chemiluminescent Substrate using Microplate Scintillation & Luminescence Counter TopCount NXT (Packard).

2.7. Statistical analysis

Statistical analysis was performed using the Dunnett test. P values $<$ 0.05 were considered significant.

3. Results

3.1. Effects of PGN on mRNA expression and protein production of GM-CSF

We confirmed that mRNA and protein of TLR2 was expressed in cultured human epidermal keratinocytes whereas mRNA of TLR4 was not (Fig. 1A and B), in agreement with previous study [12]. Treatment with *S. aureus*-derived PGN-induced mRNA expression of GM-CSF (Fig. 1C). The expression was temporary and peaked at 1 or 2 h after stimulation. PGN increased in GM-CSF mRNA levels more potently than TNF-α, IL-4 and IFN-γ (Fig. 1D). Furthermore, PGN increased in GM-CSF production in a concentration- and time-dependent manner (Fig. 1E and F). Elevation of GM-CSF production as well as mRNA expression induced by PGN was more significant than that by TNF-α, IL-4 and IFN-γ (Fig. 1F).

3.2. Effects of PGN on mRNA expression and protein production of RANTES

Treatment with PGN also induced mRNA expression of RANTES (Fig. 2A). The increase in mRNA levels was

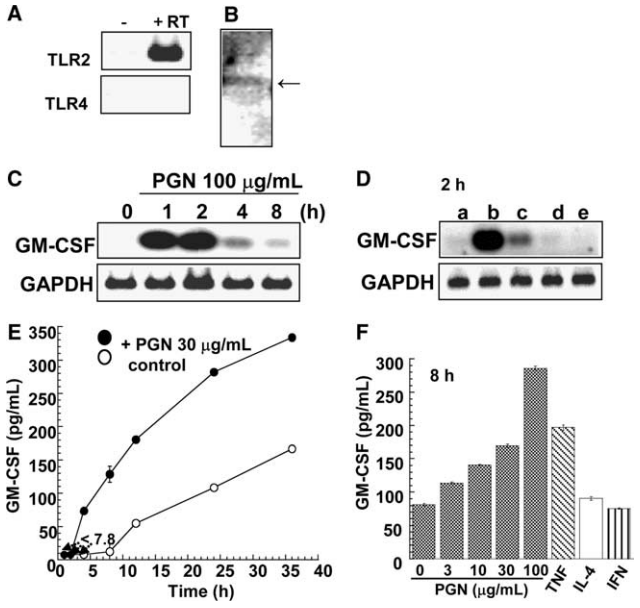

Fig. 1. PGN-induced GM-CSF expression and production in cultured keratinocytes. (A) Expression of TLR2 and TLR4 mRNA was analyzed by RT-PCR with specific primer sets. RT negative samples were included as control for the genomic contamination. (B) Total cell lysate was analyzed by Western blotting with an anti-human TLR2 antibody. The arrow shows apparent molecular mass 90 kDa for TLR2. (C) Keratinocytes were stimulated with 100 μg/mL PGN. Time course of GM-CSF mRNA levels was analyzed with the semi-quantitative RT-PCR method. One representative result of three independent experiments is shown. (D) Keratinocytes were stimulated with 100 μg/mL PGN (b), 100 ng/mL TNF-α (c), 100 ng/mL IL-4 (d) or 100 ng/mL IFN-γ (e) for 2 h. An unstimulated sample is (a). GM-CSF mRNA level in each sample was analyzed. One representative result of two independent experiments is shown. (E) Keratinocytes were stimulated with 30 μg/mL PGN. Time course of GM-CSF released into the supernatant was measured by ELISA. Values represent means ± S.E. of three determinations. (F) Keratinocyte were stimulated with the indicated concentrations of PGN, 100 ng/mL TNF-α, 100 ng/mL IL-4 or 100 ng/mL IFN-γ for 8 h. Amount of GM-CSF in each sample was measured. Values represent means ± S.E. of three determinations.

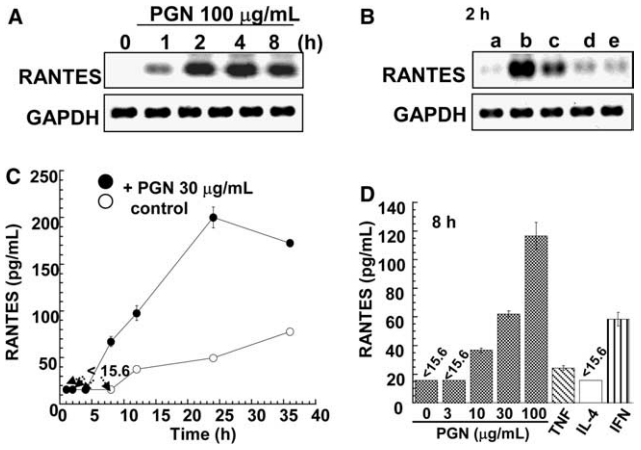

Fig. 2. PGN-induced RANTES expression and production in cultured keratinocytes. (A) Keratinocytes were stimulated with 100 μg/mL PGN. Time course of RANTES mRNA levels was analyzed with the semi-quantitative RT-PCR method. One representative result of two independent experiments is shown. (B) Keratinocytes were stimulated with 100 μg/mL PGN (b), 100 ng/mL TNF-α (c), 100 ng/mL IL-4 (d) or 100 ng/mL IFN-γ (e) for 2 h. An unstimulated sample is (a). RANTES mRNA level in each sample was analyzed. One representative result of two independent experiments is shown. (C) Keratinocytes were stimulated with 30 μg/mL PGN. Time course of RANTES released into the supernatant was measured by ELISA. Values represent means ± S.E. of three determinations. (D) Keratinocyte were stimulated with the indicated concentrations of PGN, 100 ng/mL TNF-α, 100 ng/mL IL-4 or 100 ng/mL IFN-γ for 8 h. Amount of RANTES in each sample was measured. Values represent means ± S.E. of three determinations.

relatively sustained, which was distinct from GM-CSF. PGN increased in mRNA levels and production of RANTES more potently than TNF-α, IL-4 and IFN-γ (Fig. 2B–D).

3.3. Effects of PGN on expression and production of various keratinocyte-derived mediators

mRNA expression of TNF-α, IL-6, IL-8, MCP-1, ICAM-1 and PPTA other than GM-CSF and RANTES was also upregulated by PGN (Fig. 3A). Time course of maximal induction differed among these mediators. Moreover, PGN also stimulated protein production of TNF-α, IL-6, IL-8 and ICAM-1 (Fig. 3B).

3.4. Involvement of ERK and p38 MAPK in PGN-induced GM-CSF production

Since MAPKs are involved in several biological responses [15–17], we examined whether or not PGN phosphorylates MAPKs, such as ERK-1/2, p38 MAPK and JNK-1/2. ERK-1/2, p38 MAPK and JNK-1/2 were weakly phosphorylated by control medium itself and then addition of PGN to control medium enhanced the phosphorylation of these MAPKs (Fig. 4A). PGN increased in the phosphorylated MAPKs more potently than TNF-α, IL-4 and IFN-γ (Fig. 4B). Moreover, PGN and TNF-α also phosphorylated activating transcriptional factor 2 (ATF2), which is downstream of p38 MAPK (Fig. 4B). We examined the roles of ERK and p38 MAPK pathways on PGN-induced GM-CSF production by pharmacological study using the selective inhibitor of MEK-1 and p38 MAPK, PD98059 and SB203580, respectively. PD98059 suppressed phosphorylation of ERK whereas SB203580 reduced phosphorylation of p38 MAPK and its downstream ATF2 (Fig. 5A). Pretreatment with PD98059 or SB203580 inhibited the increase in GM-CSF mRNA levels 2 h after stimulation (Fig. 5B). In addition, both inhibitors also concentration-dependently inhibited GM-CSF production (Fig. 5C). Suppression by SB203580 of expression and production of GM-CSF was more effective than that by PD98059.

3.5. Coordinative effects on PGN and histamine on GM-CSF production

Since number of mast cell is increased in lesion of AD [1] and histamine has been reported to activate ERK via histamine H_1 receptor in epidermal keratinocytes [8], effects of histamine on mRNA expression of GM-CSF were also examined. As expected, histamine-induced GM-CSF expression (Fig. 6A), and then its expression was prevented by the application of olopatadine hydrochloride, an anti-allergic drug with selective histamine H_1 receptor antagonism [26] (Fig. 6B). This drug did not affect PGN-induced GM-CSF expression (data not shown). Histamine concentration-dependently stimulated GM-CSF production (Fig. 6C). Moreover, coadministration with histamine and PGN augmented GM-CSF production compare with sole stimulation (Fig. 6D).

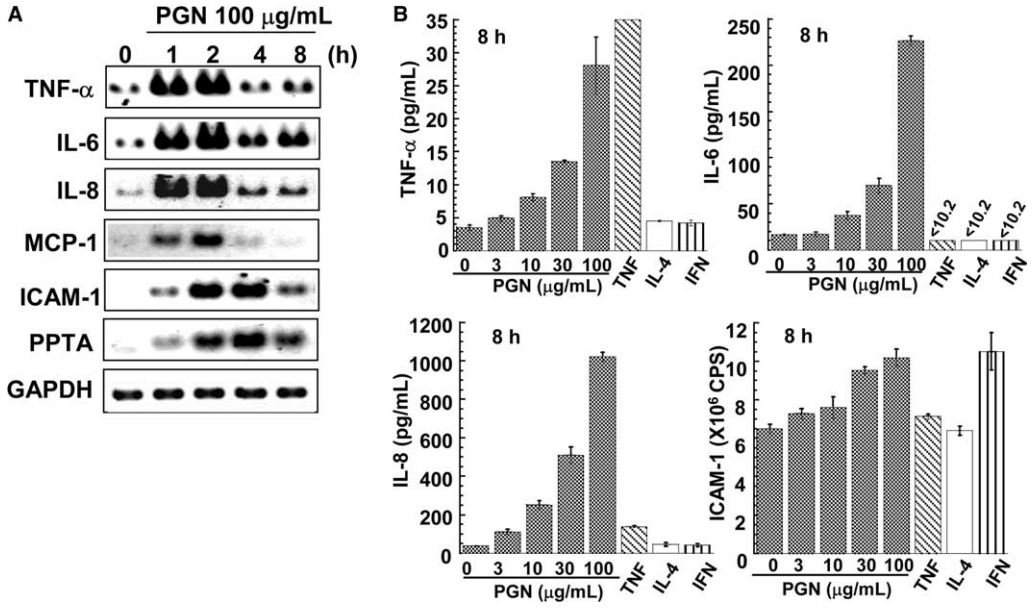

Fig. 3. PGN-induced expression and production of various mediators in cultured keratinocytes. (A) Keratinocytes were stimulated with 100 μg/mL PGN. Time course of the indicated mediator mRNA levels was analyzed with the semi-quantitative RT-PCR method. One representative result of two independent experiments is shown. (B) Keratinocyte were stimulated with the indicated concentrations of PGN, 100 ng/mL TNF-α, 100 ng/mL IL-4 or 100 ng/mL IFN-γ for 8 h. Amount of the indicated mediator in each sample was measured by ELISA. Values represent means ± S.E. of three determinations.

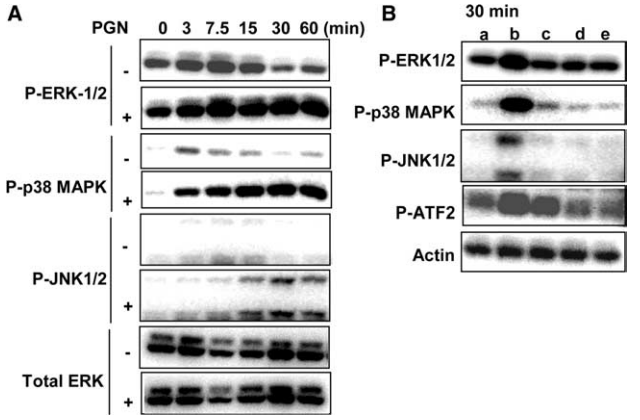

Fig. 4. PGN-induced phosphorylation of MAPKs in cultured keratinocytes. (A) Keratinocytes were stimulated with 100 μg/mL PGN. Phosphorylation of ERK1/2, p38 MAPK and JNK1/2 was detected by Western blotting using phospho-protein specific antibodies. Time course of phosphorylation levels is shown. (B) Keratinocytes were stimulated with 30 μg/mL PGN (b), 100 ng/mL TNF-α (c), 100 ng/mL IL-4 (d) or 100 ng/mL IFN-γ (e) for 30 min. An unstimulated sample is (a). The indicated phospho-protein levels in each sample were analyzed.

4. Discussion

In the present study, we demonstrated that PGN, a main constituent of outer membrane of *S. aureus*, induced expression and production of GM-CSF from human epidermal keratinocytes. Elevation of expression and production of GM-CSF by PGN was the most potent among stimulants (e.g., TNF-α). Furthermore, PGN also stimulated the expression or production of numerous keratinocyte-derived mediators such as RANTES, TNF-α, IL-6, IL-8, MCP-1, ICAM-1 and PPTA, which are believed to contribute to skin disorders. Since keratinocytes express TLR2, which is essential recognition of

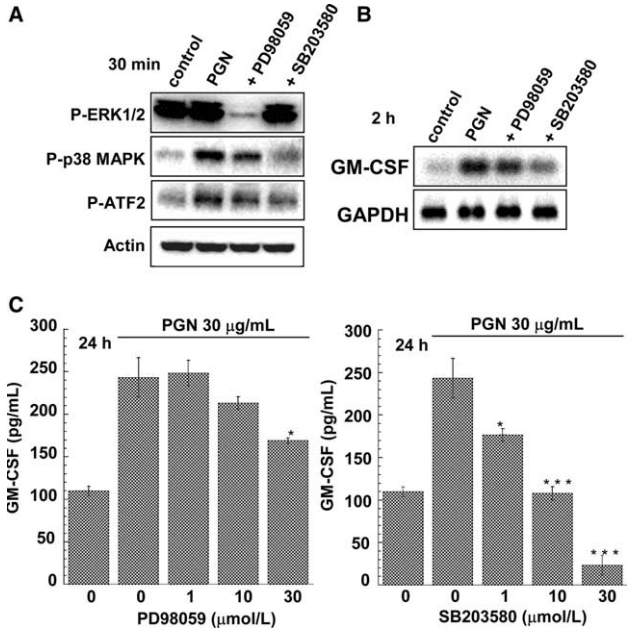

Fig. 5. Involvement of ERK and p38 MAPK in PGN-induced GM-CSF production. (A) Keratinocytes were pretreated with 10 μmol/L PD98059 and 10 μmol/L SB203580 for 30 min prior to stimulation with 30 μg/mL PGN for 30 min (A), 2 h (B) or 24 h (C). (A) Phosphorylation of ERK1/2, p38 MAPK and ATF-2 was detected by Western blotting using phospho-protein specific antibodies. (B) The GM-CSF mRNA level in each sample was analyzed with the semi-quantitative RT-PCR method. (C) Amount of GM-CSF in supernatant was measured. Values represent means ± S.E. of three determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group (Dunnett).

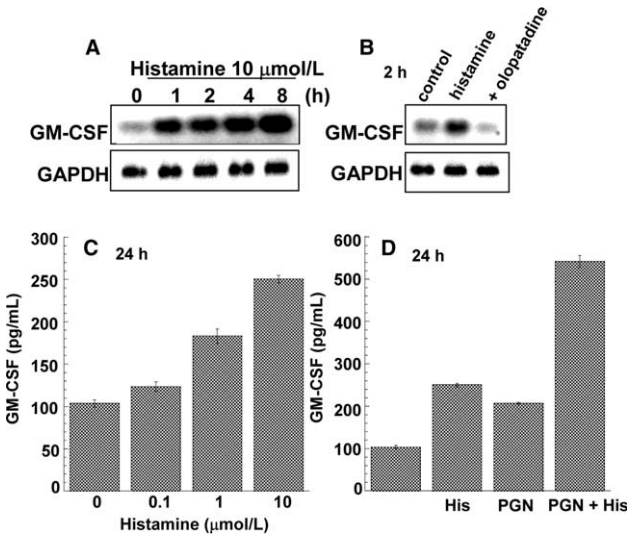

Fig. 6. Histamine- and PGN-induced GM-CSF production in cultured keratinocytes. (A) Keratinocytes were stimulated with 10 μmol/L histamine. Time course of GM-CSF mRNA levels was analyzed with the semi-quantitative RT-PCR method. One representative result of two independent experiments is shown. (B) Keratinocytes were pretreated with 1 μmol/L of a selective histamine H_1 receptor antagonist, olopatadine hydrochloride, for 30 min prior to stimulation with 10 μmol/L histamine for 2 h. GM-CSF mRNA level in each sample was analyzed. One representative result of two independent experiments is shown. (C) Keratinocytes were stimulated with the indicated concentration of histamine for 24 h. Amount of GM-CSF in supernatant histamine and 10 μg/mL PGN for 24 h. Amount of GM-CSF in each sample was measured. Values represent means ± S.E. of three determinations. (D) Keratinocytes were co-stimulated with both 10 μmol/L histamine and 10 μg/mL PGN for 24 h. Amount of GM-CSF in each sample was measured. Values represent means ± S.E. of three determinations.

PGN, PGN-induced production of above mediators may be responsible for TLR2 signaling. However, based only on the present studies, we cannot exclude the existence of target molecule for PGN other than TLR2 on keratinocytes. At least, we confirmed that PGN, which was used in this study, actually stimulated NF-κB-inducible luciferase reporter activity using HEK293 cells expressing TLR2 (data not shown).

MAPK pathways mediate survival, apoptosis, differentiation and cytokine synthesis in keratinocytes [15–17]. In this study, we found that PGN phosphorylated the three types of MAPK. p38 MAPK is especially phosphorylated among three MAPKs, and its downstream ATF2 was also phospholylated by PGN, in consistent with the previous study using HEK 293 cells stably expressing TLR2 [27]. The potency of stimulants for the induction for GM-CSF expression correlated with that for the phosphorylation of MAPKs, suggesting that MAPK pathways are involved in GM-CSF expression. Pharmacological analysis using the selective inhibitors of ERK and p38 MAPK pathways showed that both activation of ERK and p38 MAPK play roles in PGN-induced GM-CSF expression. In human monocytes, ERK and p38 MAPK co-operate in the LPS-induced GM-CSF production through TLR4 [28]. Probably, the signaling systems leading to GM-CSF generation is the common mechanism between TLR2 and TLR4. JNK was also stimulated by PGN. JNK stimulates production of several cytokines in various cell types [29], suggested that JNK may be involved in GM-CSF expression. Moreover, NF-κB pathway critically regulates IL-8 expression in keratinocytes via TLR2 [12–14,30]. Putative binding site of NF-κB locates on the promoter of GM-CSF gene [31]. Thus, MAPKs and NF-κB might contribute to an efficient GM-CSF gene expression in an interdependent or a synergistic fashion. There is a possibility that these three MAPKs and NF-κB participate in the induction of cytokines and chemokines other than GM-CSF, and the precise mechanism of expression of each mediators call for further research.

Since activation of ERK and p38 MAPK is required for induction of GM-CSF synthesis in keratinocyte, a biologically active substance, which activates either ERK or p38 MAPK, appears to induce expression of GM-CSF. Histamine is reported to activate ERK pathway and induce NGF production via a histamine H_1 receptor in keratinocytes [8]. Mast cells, which degranulate abundant histamine, infiltrate into the dermis in chronic AD [1]. As expected, histamine increased in GM-CSF production from keratinocytes.

GM-CSF, which is overproduced from keratinocytes AD [2], is essential for survival, differentiation and maturation of DC and LC [2–6] and has also mitogenic effects on keratinocytes itself [32]. Effects of GM-CSF on the antigen presenting cells shift the immune response to Th2-type, and effects of it on keratinocytes make epidermal hypertrophy, which is characteristic in chronic inflammation. Furthermore, the numerous mediators induced by PGN are believed to contribute to the development of skin disorders [7,10,18–21]. Immunobiochemical causes of inflammatory skin disease, such as AD and psoriasis, are believed to be very intricate. It is quite likely that there are numerous inducers for production of cytokines and chemokines from keratinocytes. Indeed, we found histamine as the candidate other than PGN. Moreover, PGN augmented the histamine-induced GM-CSF production significantly. Therefore, we speculate that this synergistic stimulation of keratinocytes may develop a more serious form of the disease under pathologic condition. More detailed examination of interaction keratinocyte with PGN and histamine should provide clues as to the underlying cause of inflammatory skin diseases.

In conclusion, our findings suggested that PGN of infected *S. aureus* stimulates directly cytokine production from epidermal keratinocytes in concert with biologically active substances like histamine, leading to the chronic inflammation with inflammatory skin disease. A further direction of this study will clinically be to clarify the correlation between the severity of *S. aureus* infection and amount of keratinocyte-derived cytokines.

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