



Original article

Cyto/chemokine profile of in vitro scratched keratinocyte model: Implications of significant upregulation of CCL20, CXCL8 and IL36G in Koebner phenomenon



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ABSTRACT

Background: Scratch injury induces Koebner phenomenon in psoriasis. Smoking is also a risk factor for psoriasis. Keratinocytes can produce various psoriasis-related molecules including TNF, IL1 A, IL1B, IL6, IL12B, IL17C, IL23 A, IL36 A, IL36 B, IL36 G, CXCL1, CXCL2, CXCL8, CXCL9, CXCL10, CCL20, IFNB, and CAMP. However, the scratch-induced molecular profiling remains elusive.

Objective: To profile the induction pattern of above-mentioned psoriasis-related and keratinocyte-derived molecules by scratch injury in the presence or absence of anti-psoriatic drugs or benzo[a]pyrene, a major environmental pollutant of tobacco smoke.

Methods: Confluent normal human keratinocytes were scratched and molecules were assayed by qRT-PCR, ELISA and Western blotting with or without drugs and benzo[a]pyrene.

Results: Among the 18 molecules, the scratch injury on a confluent keratinocyte sheet significantly and selectively upregulated the mRNA expression of four cyto/chemokines, CXCL8, CCL20, IL36G, and TNF, in a scratch-line-number-dependent manner under either low- or high-calcium condition. However, significant protein secretion was only demonstrated for CXCL8 and CCL20. The IL36 G protein was not secreted, but its intracellular level was significantly upregulated by scratch injury, whereas neither the secretion nor the intracellular level of TNF protein was affected by scratch injury. Dexamethasone, but not maxacalcitol nor the phosphodiesterase 4 inhibitor apremilast, partially inhibited the CXCL8 and CCL20 secretion. Benzo[a]pyrene significantly and synergistically enhanced the scratch-induced CCL20 secretion that may explain why smoking is a risk factor for psoriasis.

Conclusion: CCL20 and to a less extent CXCL8 may play a key role in triggering the Koebner phenomenon after scratch injury to keratinocytes.

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1. Introduction

Psoriasis is an immune-mediated inflammatory skin disease with a prevalence of approximately 0.1% to 3% in the general population [1,2]. Histopathologically, it is characterized by epidermal hyperproliferation and intraepidermal neutrophilic infiltration [3,4]. The infiltrating dermal immune cells include dendritic cells and T cells [2,5]. The TNF- α (TNF)/IL23/IL17A axis

appears to be a major driver in the pathogenesis of psoriasis because this disease exhibits excellent responses to biologics targeting TNF, IL23, and IL17A [1,2,6]. Environmental factors such as smoking are also associated with its pathogenesis [7]. Along with the canonical TNF/IL23/IL17A pathway, a plethora of molecules are upregulated in the lesional skin of psoriasis and its pustular variant, including TNF, IL1A, IL1B, IL6, IL12B, IL17C, IL23A, IL36A, IL36B, IL36G, CXCL1, CXCL2, CXCL8, CXCL9, CXCL10, CCL20, interferon- β (IFNB), and cathelicidin (CAMP) [2,5,8–16]. These cyto/chemokines and anti-microbial peptide play crucial roles in the development and perpetuation of psoriasis by amplifying keratinocyte proliferation and recruiting neutrophils and pathogenic T cells [2,5,8–16].

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In psoriasis, scratch injury *in vivo* is frequently associated with the development of a new lesion, which is called the Koebner phenomenon [17,18]. However, the molecular mechanisms by which scratch injury leads to the Koebner phenomenon remain elusive. Keratinocytes respond, as sensors, to various external stimulants such as contact with chemicals [19–21], microbial products [8,16,22], and ultraviolet irradiation [23,24]. These external stimuli also activate keratinocytes to produce the above-mentioned cyto/chemokines and anti-microbial peptides, generally in a stimulant-specific manner [1,16,19–25]. Therefore, it is conceivable that scratch injury may stimulate keratinocytes to produce a selective set of these psoriasis-related molecules. Although *in vitro* scratch injury of a keratinocyte sheet has been historically used as a good model for wound closure by reflecting the migratory and proliferative capacity of keratinocytes [26,27], comprehensive profiling of cyto/chemokines in the scratch model has not been reported.

In this study, we scratched a confluent keratinocyte sheet *in vitro* and examined the associated cyto/chemokine expression profile. We found that, in the *in vitro* scratched Koebner model, there was consistent and selective upregulation of the mRNA expression of four cyto/chemokines, *CXCL8*, *CCL20*, *IL36G*, and *TNF*, in keratinocytes among the 18 molecules mentioned above. However, significant protein secretion was confined only to *CXCL8* and *CCL20*. The *IL36G* protein was not secreted, but its intracellular level was significantly upregulated by scratch injury. Despite significant upregulation of gene expression, neither the secretion nor the intracellular level of *TNF* protein was affected by scratch injury. We also examined the inhibitory effects of anti-psoriatic therapeutic agents, dexamethasone, maxacalcitol, and the phosphodiesterase 4 inhibitor apremilast, on the scratch-induced protein responses of these four cyto/chemokines. In addition, we investigated whether the scratch-induced responses were augmented by the simultaneous presence of benzo[a]pyrene, one of the major environmental pollutants in tobacco smoke [28].

2. Materials and methods

2.1. Reagents and antibodies

CaCl₂ (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) was dissolved in UltraPure™ distilled water (Invitrogen, Carlsbad, CA) and added to culture medium at a final concentration of 1.6 mM. Dexamethasone (Cayman Chemical, Ann Arbor, MI) and apremilast (ChemScene LLC, Deerpark Dr, NJ) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) and added to culture medium at a final concentration of 1 μM. Maxacalcitol (ChemScene) was dissolved in DMSO and added to culture medium at a final concentration of 1 nM. Benzo[a]pyrene (Sigma-Aldrich) was dissolved in DMSO and added to the culture medium at a final concentration of 1 μM. Control cultures contained comparable amounts of DMSO (0.01%) and UltraPure™ distilled water (1.6%). The antibodies used were as follows: mouse anti-human *IL36G* (ab156783; Abcam, Cambridge, United Kingdom), rabbit anti-human β-actin (4970S), rabbit anti-human *TNF* (3703S), horseradish peroxidase-conjugated anti-rabbit (7074), and anti-mouse secondary antibodies (7076) (all from Cell Signaling Technology, Danvers, MA).

2.2. Cell culture

Normal human epidermal keratinocytes (NHEKs) from neonatal foreskin (Lonza, Basel, Switzerland) were cultured in KBM-GOLD medium (Lonza) supplemented with KGM-GOLD™ SingleQuots™ (Lonza), containing bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, GA-1000, and epinephrine, and maintained at 37 °C in 5% CO₂. The culture

medium was replaced every day and the cells were serially passaged at 70%–80% confluence. Cells were used at the third passage in all experiments. Cells (3.5 × 10⁵ cells/well) were seeded in six-well culture plates (Corning, Corning, NY), the culture medium was replaced every day, and then cells were treated with a low (= culture medium) or high level of CaCl₂ (low Ca²⁺: 0.1 mM, high Ca²⁺: 1.6 mM) when they reached 100% confluence.

2.3. Cell viability

The viability of NHEKs was determined using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), in accordance with the manufacturer's instructions.

2.4. *In vitro* scratched keratinocyte model

To establish the *in vitro* scratched keratinocyte model, NHEKs at 100% confluence were incubated for 24 h under low/high-Ca²⁺ conditions at 37 °C in 5% CO₂. Then, the cell sheets were scratched with a 250-μL Long Tip (Watson, Tokyo, Japan).

2.4.1. Time course assay

For the time course assay, the cell sheets were scratched (10 lines). After washing twice with the culture medium, the cells were incubated for 0, 3, 6, or 24 h under low/high-Ca²⁺ conditions, and the cells and supernatants were harvested at each time point.

2.4.2. Scratch-number-dependence assay

For the scratch-number-dependence assay, the cell sheets were scratched (0, 7, 10, or 14 lines). After two washes with the culture medium, the cells were incubated for 3 h under low/high-Ca²⁺ conditions and harvested.

2.4.3. Drug treatments

The cell sheets were scratched (10 lines). After two washes with the culture medium, they were treated with DMSO (0.01%, control), dexamethasone (1 μM) [29], maxacalcitol (1 nM) [30], dexamethasone (1 μM) plus maxacalcitol (1 nM), or apremilast (1 μM) [31] for 24 h under high-Ca²⁺ conditions, and then the cells and supernatants were harvested.

2.4.4. Benzo[a]pyrene treatment

The cell sheets were scratched (10 lines). After two washes with the culture medium, they were treated with DMSO (0.01%, control) or benzo[a]pyrene (1 μM) [32] for 24 h under high-Ca²⁺ conditions, and then the cells and supernatants were harvested.

2.5. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the cells using a RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) and reverse-transcribed using a PrimeScript™ RT reagent Kit (TaKaRa Bio Inc., Kusatsu, Japan). qRT-PCR was performed with TB Green™ Premix Ex Taq™ II (TaKaRa Bio Inc.), in accordance with the manufacturer's instructions. The qRT-PCR amplification schedule was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The mRNA expression levels of each target gene were measured in triplicate and were normalized against the cycle threshold of β-actin (ACTB; internal control). The primers were purchased from Invitrogen, the sequences of which are listed in Table 1.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Culture supernatants were collected and the concentrations of *CXCL8*, *CCL20*, and *TNF* were measured using Quantikine® Human

Table 1
Primer sequences for quantitative reverse-transcription polymerase chain reaction.

Primer sequences for qRT-PCR.		
Gene symbol		Sequence
<i>TNF</i>	sense	5'-GAGGCCAAGCCCTGGTATG-3'
	antisense	5'-CGGGCCGATTGATCTCAGC-3'
<i>IL1A</i>	sense	5'-AGATGCCTGAGATACCCAAAACC-3'
	antisense	5'-CCAAGCACACCCAGTAGTCT-3'
<i>IL1B</i>	sense	5'-ATGATGGCTTATTACAGTGCCAA-3'
	antisense	5'-GTCGGAGATTCTGTAGCTGGA-3'
<i>IL6</i>	sense	5'-ACTCACCTCTCAGAACAATG-3'
	antisense	5'-CCATCTTTGGAAGTTCAGGTTG-3'
<i>IL17C</i>	sense	5'-CCTCAGCTACGACCCAGTG-3'
	antisense	5'-GTCCTCATCCGTGTCACA-3'
<i>IL12B</i>	sense	5'-TCTCCCTGACATTCTGCGTT-3'
	antisense	5'-CTATAGTAGCGTCTCGGGC-3'
<i>IL23A</i>	sense	5'-GACAACAGTCAGTTCTGCTTGC-3'
	antisense	5'-AGAGAAGGCTCCCTGTGAA-3'
<i>IL36A</i>	sense	5'-TGGGTTCTCAGGACCAGAC-3'
	antisense	5'-GATGGGGTTCCTCTGTCTT-3'
<i>IL36B</i>	sense	5'-TTCAGGGCAAGCCTACTTTG-3'
	antisense	5'-TTCCATGAAGCAGCTCTCT-3'
<i>IL36G</i>	sense	5'-GAAACCTTCCTTTTCTACCGTG-3'
	antisense	5'-GCTGGTCTCTTTGAGGAG-3'
<i>CXCL1</i>	sense	5'-CCACTGCGCCAAACCGAAG-3'
	antisense	5'-GGATGCAGGATTGAGGCAAGC-3'
<i>CXCL2</i>	sense	5'-GAAAGCTTGTCTCAACCCCG-3'
	antisense	5'-GTTGGATTGCCAITTTTTCAGCA-3'
<i>CXCL8</i>	sense	5'-CTGGCCGTGGCTCTCTTG-3'
	antisense	5'-CCTTGGCAAACTGCACCTT-3'
<i>CXCL9</i>	sense	5'-TTCCTCTTGGGCATCATCTTGTGG-3'
	antisense	5'-AGTCCCTGGTGGTGTGATGACAG-3'
<i>CXCL10</i>	sense	5'-CAAACCTGCAGTCTGATTTGCTGCC-3'
	antisense	5'-TGCTGATGCAGGTACAGCGTACGGT-3'
<i>CAMP</i>	sense	5'-CTAACCTCTACCCCTCTCTG-3'
	antisense	5'-AATCCTCTGGTACTGTCTGT-3'
<i>IFNB</i>	sense	5'-TGCTCTCTCTGTGTGCTTCT-3'
	antisense	5'-AAGCCTCCCAATCAATTGCC-3'
<i>CCL20</i>	sense	5'-TTGCTCTGGGCTGCTTTGAT-3'
	antisense	5'-GCCGTGTGAAGCCACAATA-3'
<i>FLG</i>	sense	5'-TGAAGCCTATGACACCACTGA-3'
	antisense	5'-TCCCTACGCTTTCTGTCTCT-3'
<i>KRT10</i>	sense	5'-TGAAGCCTATGACACCACTGA-3'
	antisense	5'-TGTCGATCTGAAGCAGGATG-3'
<i>MKI67</i>	sense	5'-TTGGAGAATGACTCGTGAGC-3'
	antisense	5'-CGAAGCTTTCATGACAGGA-3'

CXCL8, CCL20, and TNF ELISA Kits (R&D Systems, Minneapolis, MN), in accordance with the manufacturer's instructions. The amount of IL36G protein was measured with the human IL36G ELISA Kit (Invitrogen), in accordance with the manufacturer's instructions. Absorbance was measured with an iMark microplate reader (Bio-Rad, Hercules, CA), and the concentrations of the cyto/chemokines were determined in each sample by comparison to a standard curve.

2.7. Western blotting

Protein lysates of NHEKs were isolated from the cells with 100 μ L/well of lysis buffer (25 mM HEPES, 10 mM Na₄P₂O₇/10H₂O, 100 mM NaF, 5 mM EDTA, 2 mM Na₃VO₄, and 1% Triton X-100), supplemented with 10 μ L/well of proteinase inhibitor cocktail (Sigma-Aldrich). The lysates were then centrifuged at 14,000 rpm for 30 min and the obtained supernatants were used for analysis. The protein concentration of each lysate was measured with a BCA protein assay kit (ThermoFisherScientific, Waltham, MA). Equal amounts of protein (20 μ g for β -actin; 85 μ g for the other proteins) were mixed with 2 \times sample buffer (Nacalai Tesque, Inc., Kyoto, Japan), boiled at 95 $^{\circ}$ C for 5 min, loaded onto BoltTM 4%–12% Bis-Tris Plus Gels (Invitrogen), and electrophoresed at 200V and 160mA for 22 min. The proteins were then transferred to PVDF membrane (Invitrogen) using Power Blotter (Invitrogen).

Membranes were blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich) in 0.1% TBS-T. Membranes were probed with anti-mouse human IL36G, anti-rabbit human TNF, or anti-rabbit human β -actin antibodies overnight at 4 $^{\circ}$ C. After incubation with horseradish-peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies at room temperature for 1 h, protein bands were visualized with SuperSignalTM West Pico Chemiluminescent Substrate (ThermoFisherScientific) using the ChemiDocTM Touch Imaging System (Bio-Rad).

2.8. Statistical analysis

All data are presented as mean \pm standard deviation (SD). The significance of differences between groups was assessed using Student's unpaired two-tailed *t*-test (two groups) or one-way ANOVA, followed by Tukey's multiple comparison test (multiple groups) using GraphPad PRISM 7.0 software (GraphPad Software, La Jolla, CA). A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Selective upregulation of TNF, CXCL8, IL36G, and CCL20 after scratch injury

We first examined the effects of scratching (10 scratch lines in a six-well culture plate) on the gene expression of 18 psoriasis-related molecules in a confluent NHEK sheet under low- or high-Ca²⁺ condition. Keratinocytes alter their phenotype from a proliferative state under low-Ca²⁺ conditions to a differentiating state under high-Ca²⁺ ones [33–35]. Here, we confirmed that the high-Ca²⁺ conditions downregulated *MKI67* expression (proliferation) but upregulated *KRT10* and *FLG* expression (differentiation) (Supplementary Fig. S1). Scratch injury did not affect the levels of *MKI67*, *KRT10*, and *FLG* expression (data not shown). In the scratch(+) sheet, the expression levels of *CXCL8* (7.8-fold maximum), *CCL20* (6.7-fold maximum), *IL36G* (10.2-fold maximum), and *TNF* genes (7.3-fold maximum) were significantly upregulated compared with those in the scratch (–) sheet under both low- (Fig. 1A) and high-Ca²⁺ conditions (Fig. 1B). The upregulation of these cyto/chemokine genes was induced 3 or 6 h after scratching and returned to the baseline levels at 24 h (Fig. 1A and 1B). The scratch injury resulted in rather selective induction of gene expression because it induced no or negligible alteration of the expression of the *IL1A*, *IL1B*, *IL6*, *IL17C*, *IL12B*, *IL23A*, *IL36A*, *IL36B*, *CAMP*, *IFNB*, *CXCL2*, and *CXCL9* genes (Supplementary Fig. S2 and S3). Scratch injury also slightly but inconsistently upregulated the expression of *CXCL1* (1.4-fold maximum) and *CXCL10* (3.6-fold maximum) compared with the levels in the scratch (–) control (Supplementary Fig. S3).

We next examined the gene expression of scratch(+) sheets 3 h after injury with 7, 10, or 14 scratch lines (Fig. 2A and B). The scratch-responsive genes (*CXCL8*, *CCL20*, *IL36G*, and *TNF*) were significantly (or tended to be) upregulated in a scratch-number-dependent manner (Fig. 2A and 2B). The scratch-number-dependent upregulation was observed under both low-Ca²⁺ (Fig. 2A) and high-Ca²⁺ conditions (Fig. 2B), but it seemed to be more apparent under the high-Ca²⁺ differentiated conditions (Fig. 2B). Dependence on scratch number was not observed in the expression of scratch-unresponsive genes: *IL1A*, *IL1B*, *IL6*, *IL17C*, *IL12B*, *IL23A*, *IL36A*, *IL36B*, *CAMP*, *IFNB*, and *CXCL9* (Supplementary Fig. S4 and S5). However, dependence on scratch number tended to be shown, albeit inconsistently, in the *CXCL1*, *CXCL2*, and *CXCL10* expression (Supplementary Fig. S5). These results suggested that the scratch injury triggered an early and burden-dependent induction of the expression of selected genes, *CXCL8*, *CCL20*, *IL36G*, and *TNF*. The inconsistent upregulation of *CXCL1*, *CXCL2*, and *CXCL10* was potentially interesting, but we did not focus on it further here.

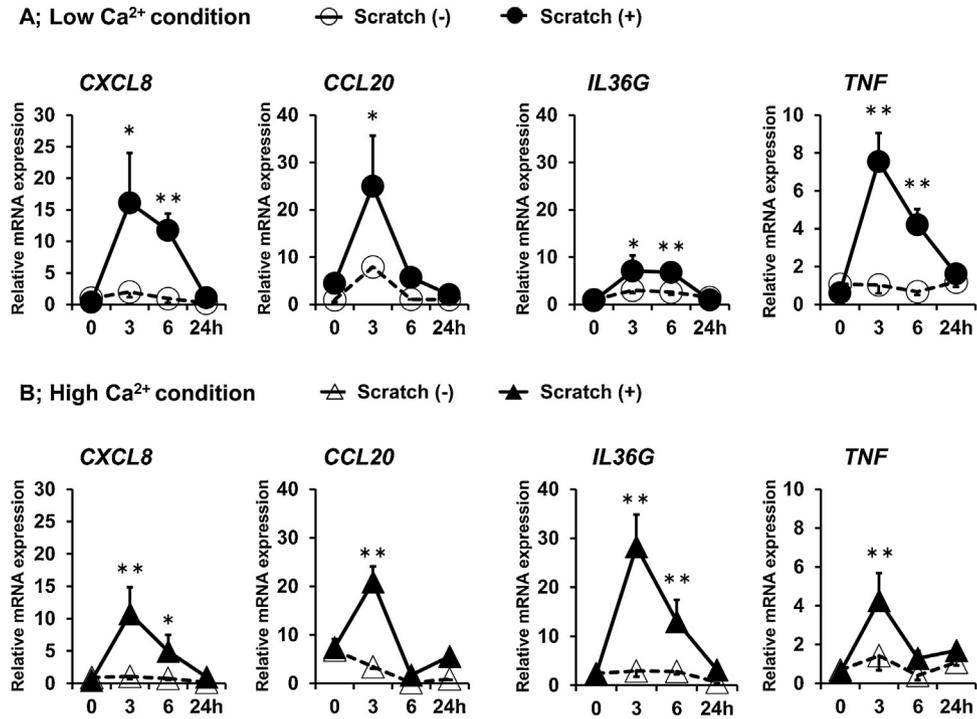


Fig. 1. Gene expression of *TNF*, *IL36G*, *CXCL8*, and *CCL20* was significantly upregulated in scratch(+) keratinocyte sheet (closed circle and closed triangle) compared with scratch(-) control sheet (open circle and open triangle). A: Low-Ca²⁺ conditions. B: High-Ca²⁺ conditions. Each experiment was performed in triplicate. (N = 5. Normalized against *ACTB* expression).

*: P < 0.05 and **: P < 0.01 compared with scratch(-) control.

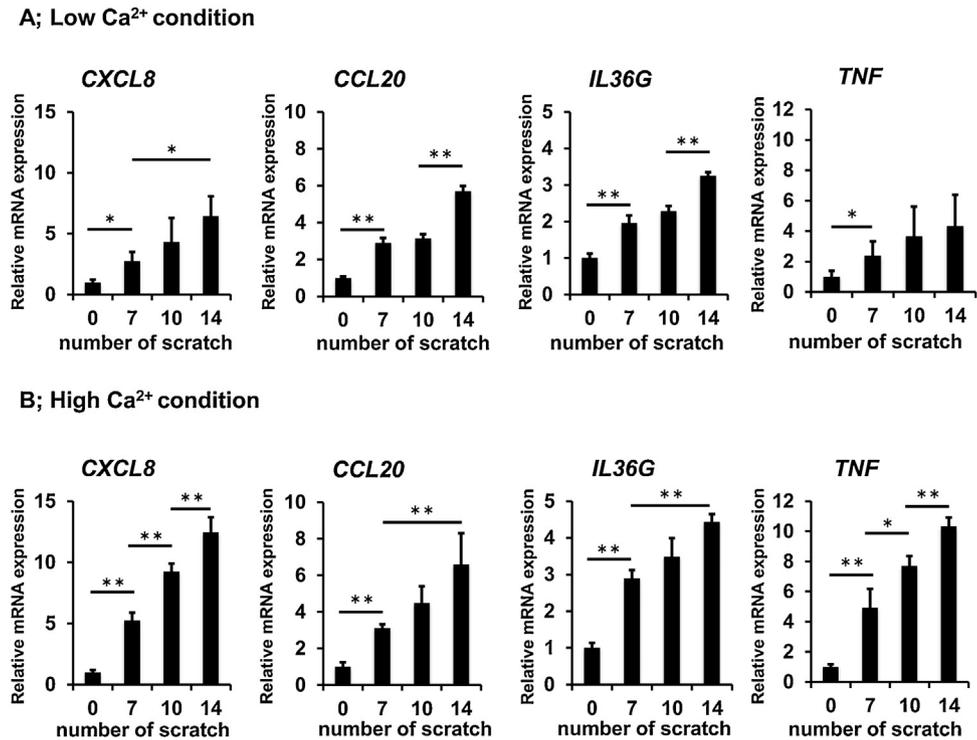


Fig. 2. The gene expression of *TNF*, *IL36G*, *CXCL8*, and *CCL20* was significantly (or tended to be) upregulated in a scratch-number-dependent manner. A: Low-Ca²⁺ conditions. B: High-Ca²⁺ conditions. Each experiment was performed in triplicate. (N = 5. Normalized against *ACTB* expression). *: P < 0.05 and **: P < 0.01 compared with scratch(-) counterparts.

3.2. Selective protein secretion of CXCL8 and CCL20, but not IL36 G and TNF, after scratch injury

In contrast to the gene expression, scratch injury significantly increased the protein secretion of only CXCL8 (Fig. 3A) and CCL20 (Fig. 3B) in the supernatant of keratinocyte culture, as assessed by ELISA. However, the secretion of IL36 G and TNF proteins was not detected in either scratch(+) or scratch(-) keratinocyte culture supernatants (data not shown). We then checked the intracellular protein levels of IL36 G and TNF by Western blotting (Fig. 3C and 3D). The scratch injury significantly upregulated the intracellular protein level of IL36 G (Fig. 3C). The intracellular level of TNF was not altered by scratch injury (Fig. 3D). The robust protein secretion of CXCL8 and CCL20 suggested that these two chemokines may exert more meaningful biological roles in keratinocytes injured by scratching than the increased intracellular level of IL36 G protein and the mere transcription of the *TNF* gene.

3.3. Effects of dexamethasone, maxacalcitol, and apremilast on scratch-induced CXCL8 and CCL20 upregulation

Conventional treatments for psoriasis include topical corticosteroid, vitamin D3 analogs, and their use in combination [3]. Systemic apremilast, a cAMP phosphodiesterase 4 inhibitor, is also a useful treatment modality in psoriasis [36]. We then examined the effects of dexamethasone, maxacalcitol, and apremilast on scratch-induced CXCL8 and CCL20 secretion. The drug treatment did not affect the viability of keratinocytes (data not shown). As shown in Fig. 4, the scratch-induced CXCL8 and CCL20 secretion was partially inhibited by dexamethasone, but not by maxacalcitol nor apremilast. Simultaneous addition of maxacalcitol to dexamethasone did not significantly enhance the inhibitory action of dexamethasone (Fig. 4). These results suggested that the scratch-induced CXCL8 and CCL20 release was susceptible, but only partially, to inhibitory action by steroid. In contrast, the scratch-induced intracellular IL36 G protein upregulation was inhibited by dexamethasone, maxacalcitol, and apremilast (Supplementary

Fig. S6A). The addition of maxacalcitol to dexamethasone further inhibited the scratch-induced intracellular IL36 G protein level compared with that upon dexamethasone monotreatment (Supplementary Fig. S6A). Notably, the baseline intracellular TNF protein level was inhibited by maxacalcitol and apremilast, but not by dexamethasone (Supplementary Fig. S6B).

3.4. Benzo[a]pyrene accelerates the scratch-induced secretion of CCL20, but not CXCL8

Benzo[a]pyrene is one of the major environmental contaminants contained in tobacco smoke [28]. As smoking is associated with a risk of psoriasis (7), we finally examined whether benzo[a]pyrene accelerates the scratch-induced responses. As shown in Fig. 5, benzo[a]pyrene upregulated the baseline protein secretion of CXCL8 and CCL20. The scratch injury induced a significantly higher level of CXCL8 secretion than the benzo[a]pyrene monotreatment (Fig. 5A). However, the simultaneous addition of benzo[a]pyrene was unable to further enhance the scratch-induced CXCL8 secretion (Fig. 5A). In sharp contrast, CCL20 secretion was enhanced by either benzo[a]pyrene (1.4-fold maximum) or scratch injury (3.2-fold maximum) (Fig. 5B). Notably, cotreatment with benzo[a]pyrene and scratch injury synergistically enhanced the CCL20 secretion (7.0-fold maximum) (Fig. 5B). Regarding IL36 G and TNF, their secretion was again not detected upon the monotreatment or cotreatment with benzo[a]pyrene and scratch injury (data not shown). These results further stress the importance of CCL20 as a molecule that senses mechanical and chemical stresses.

4. Discussion

Mechanical injury or scratching is an external stimulus to which the skin surface is frequently subjected. Mechanical trauma exacerbates and/or triggers inflammatory skin diseases such as psoriasis, which is known as the Koebner phenomenon [17,18]. Epidermal keratinocytes are the first cell population disrupted by

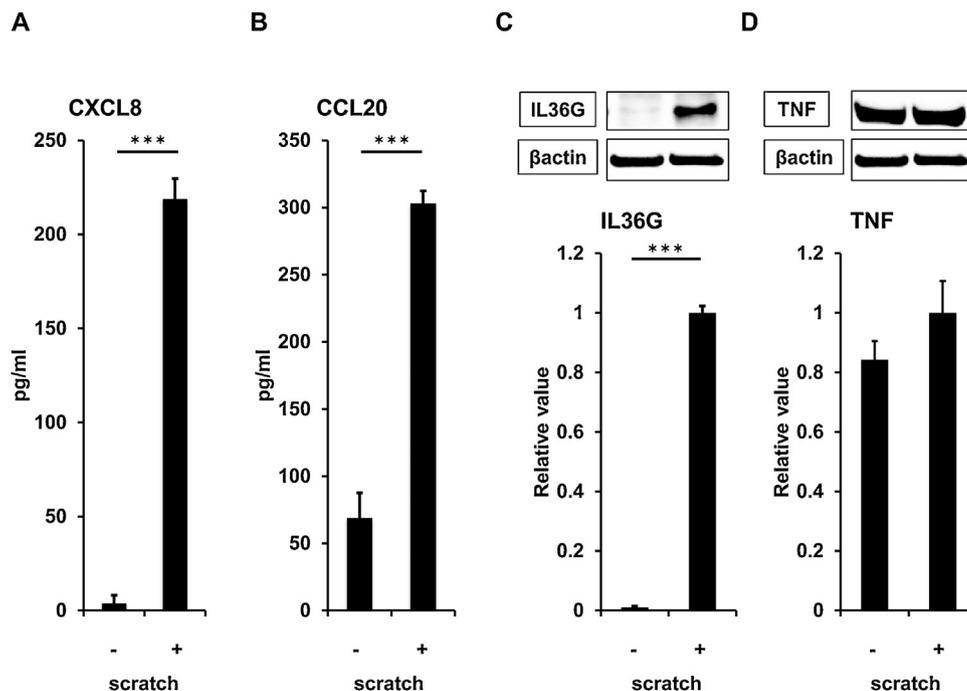


Fig. 3. The protein secretion of CXCL8 (A) and CCL20 (B) in the supernatant was significantly increased by scratch injury compared with non-scratched control. Intracellular protein levels of IL36 G (C) and TNF (D) were assessed by Western blotting. Each experiment was performed in triplicate under high- Ca^{2+} condition. $N = 3$. Representative data was shown. ***: $P < 0.001$ compared with scratch(-) control.

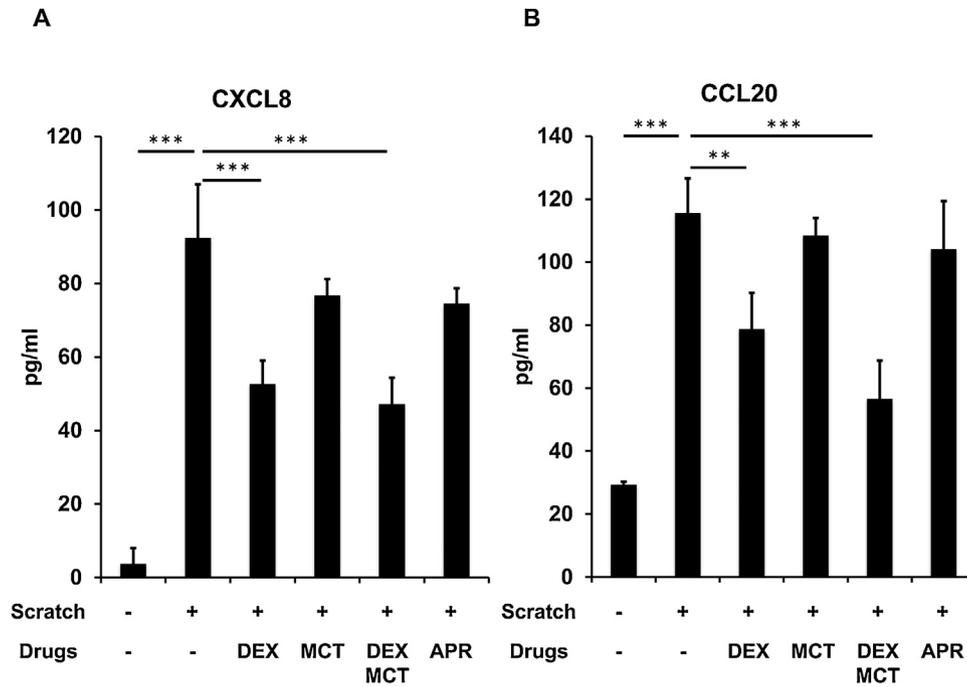


Fig. 4. Effects of dexamethasone (DEX), maxacalcitol (MCT), and apremilast (APR) on scratch-induced CXCL8 (A) and CCL20 (B) protein secretion in keratinocytes with or without scratch injury. Each experiment was performed in triplicate under high- Ca^{2+} condition. $N=3$. Representative data was shown. **: $P < 0.01$. ***: $P < 0.001$.

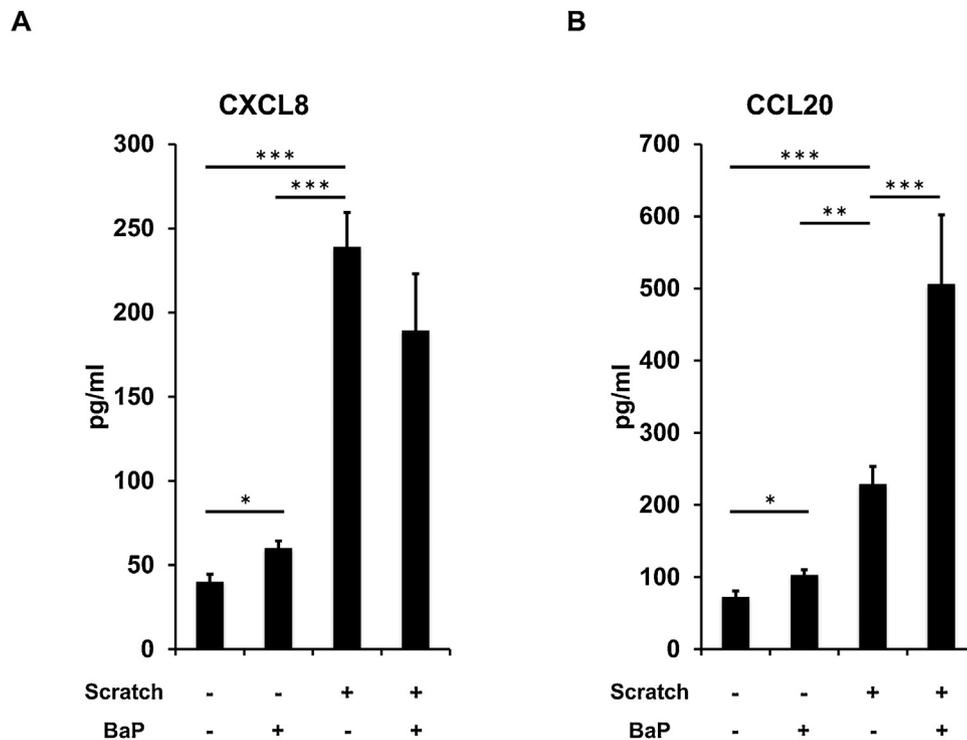


Fig. 5. Effects of benzo[a]pyrene on the scratch-induced CXCL8 (A) and CCL20 (B) secretion. Each experiment was performed in triplicate under high- Ca^{2+} condition. $N=3$. Representative data was shown. *: $P < 0.05$. ***: $P < 0.001$.

mechanical injury. However, the molecular events that occur in keratinocytes upon mechanical injury remain elusive.

We scratched confluent keratinocyte sheets under low- and high- Ca^{2+} conditions. Using this in vitro Koebner model, we demonstrated that the gene expression of a relatively selective set of cyto/chemokines (CXCL8, CCL20, IL36G, and TNF) was upregulated 3 to 6 h after scratching in a scratch-line-number-dependent

manner under low- Ca^{2+} and more consistently under high- Ca^{2+} conditions. Such upregulated gene expression levels returned to the baseline at 24 h. However, the secretion of proteins from cells was only detected for CXCL8 and CCL20, in a scratch-dependent manner. Regarding IL36G, scratch injury upregulated the intracellular protein level, whereas it did not induce its protein release. Notably, the intracellular protein level of TNF was stable despite its

significant mRNA upregulation by scratch injury in keratinocytes. In addition, TNF secretion was never observed upon scratch injury per se.

The scratch-responsive CXCL8 and IL36G are both neutrophil-attractant cyto/chemokines and their mRNA expression has been reported to be elevated in psoriatic lesional skin [1,12,37,38]. CCL20 is responsible for the chemoattraction of T (including IL17A-producing T) cells and other immunocytes [39,40]. Our results suggest that the keratinocyte sheet could be programmed to produce and release at least two chemokines upon its mechanical disintegration; one is the neutrophil-chemotactic CXCL8 and the other is the T-cell-chemotactic CCL20. As psoriatic lesional skin expresses increased levels of CXCL8 and CCL20 [2,5], it is conceivable that the release of CXCL8 and CCL20 from scratch-injured keratinocytes may trigger the Koebner phenomenon in psoriasis. In parallel with this, biologic treatments improve skin lesions in association with rapid downregulation of the lesional expression of CXCL8 and CCL20 in patients with psoriasis [41,42]. The scratch-induced upregulation of intracellular IL36G protein is potentially meaningful because its extracellular release may be achieved when keratinocytes are in close contact with recruited neutrophils [1,43]. Previous immunohistological studies revealed a preferential expression of IL36G in the upper epidermal layers in normal and psoriatic epidermis [12,22,44]. From these pieces of evidence, we decipher that CXCL8 recruits neutrophils into the scratched epidermis; Neutrophils may help to activate and release of intracellular IL36G; and IL36G may further recruit neutrophils in upper epidermis leading to a subcorneal pustular formation. Surprisingly, scratch-induced TNF upregulation did not induce the production of its protein. These results suggest that some additional stimuli are needed for TNF protein secretion.

The scratch-induced CXCL8 and CCL20 secretion was partially inhibited by dexamethasone but not by maxacalcitol nor apremilast. However, the intracellular level of TNF was inhibited by maxacalcitol and apremilast but not by dexamethasone. The intracellular level of IL36G was inhibited by all three anti-psoriatic agents. Few studies have examined the drug effects on these cyto/chemokines in keratinocytes. Takei-Taniguchi et al. demonstrated that protease-activated receptor 2-mediated CXCL8 was not affected by steroid, vitamin D nor cyclosporine in keratinocytes [45]. Further analysis is necessary to define the signaling cascade responsive to scratch injury and the mechanism through which these anti-psoriatic drugs interfere with this.

The crucial participation of CCL20 in external sensing was further demonstrated by benzo[a]pyrene experiments. We examined the effects of benzo[a]pyrene as a representative component of tobacco and environmental pollutant [28,46]. The scratch-induced secretion of CCL20, but not of CXCL8, was significantly and synergistically augmented in the presence of benzo[a]pyrene. This result is quite intriguing because we expected that benzo[a]pyrene might enhance the secretion of both neutrophil-attracting CXCL8 and T-cell-attracting CCL20 in the scratched keratinocytes. Although the reason why benzo[a]pyrene upregulated only the scratch-induced CCL20 secretion remains elusive, this fact may explain, at least in part, why smoking increases the risk of developing psoriasis, for which the recruitment of IL17A-producing T cells is crucially responsible [2,5,6].

In conclusion, the significant and consistent secretion of CCL20 and to a less extent CXCL8 in an in vitro scratch Koebner model may be useful to investigate the early events in mechanical injury leading to full-blown psoriasis and other inflammatory cutaneous disorders.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jdermsci.2019.04.002>.

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