

Heparinoid suppresses Der p-induced IL-1 β production by inhibiting ERK and p38 MAPK pathways in keratinocytes

Ryo Utsunomiya¹  | Xiuju Dai¹  | Masamoto Murakami¹ | Kana Masuda¹ | Hidenori Okazaki² | Teruko Tsuda¹ | Hideki Mori¹ | Ken Shiraishi¹ | Mikiko Tohyama¹ | Koji Sayama¹

¹Department of Dermatology, Ehime University Graduate School of Medicine, Toon City, Japan

²Department of Dermatology, Ehime Prefectural Central Hospital, Matsuyama City, Japan

Correspondence

Ryo Utsunomiya, Department of Dermatology, Ehime University Graduate School of Medicine, Toon City, Japan.
Email: rutsun@m.ehime-u.ac.jp

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Abstract

Epidermal keratinocytes initiate skin inflammation by activating immune cells. The skin barrier is disrupted in atopic dermatitis (AD) and epidermal keratinocytes can be exposed to environmental stimuli, such as house dust mite (HDM) allergens. We showed previously that HDM allergens activate the NLRP3 inflammasome of keratinocytes, thereby releasing pro-inflammatory cytokines. Heparinoid is an effective moisturizer for atopic dry skin. However, a recent report showed that heparinoid treatment can improve inflammation of lichen planus. Therefore, we hypothesized that it acts on epidermal keratinocytes not only as a moisturizer, but also as a suppressant of the triggers of skin inflammation. We found that HDM allergen-induced interleukin (IL)-1 β release from keratinocytes was inhibited significantly by heparinoid pretreatment without affecting cell viability. However, heparinoid did not affect caspase-1 release, suggesting that heparinoid did not affect HDM allergen-induced inflammasome activation. Heparinoid treatment not only decreased intracellular levels of pro-IL-1 β , but also suppressed IL-1 β messenger RNA (mRNA) expression in keratinocytes. Among the intracellular signalling pathways, the activation of extracellular signal-regulated kinase and p38 pathways, which are required for IL-1 β expression in keratinocytes, was inhibited by heparinoid treatment. The inhibitory effect of heparinoid on IL-1 β mRNA expression was also confirmed with living skin equivalents. Our results demonstrated that heparinoid suppresses the initiation of keratinocyte-mediated skin inflammation.

KEYWORDS

atopic dermatitis, ERK, Heparinoid, IL-1 β , p38

1 | INTRODUCTION

Atopic dermatitis (AD) is a common inflammatory skin disease with a chronic course, characterized by severe itching and xerosis.^[1] Several

causes of AD have been proposed and it is likely that the condition results from complex interactions between genetic and environmental factors.^[2] One of the most important factors in AD pathogenesis is a defect in the skin barrier. House dust mite (HDM) allergens play

an important role in exacerbating AD, via penetration through the disrupted skin barrier.^[3] We previously reported that HDM allergens act as a danger signal for the skin, via activation of the NLRP3 inflammasome, in keratinocytes.^[4] Recently, skin barrier function has become a focus in skin inflammatory disorders, from the viewpoint of endogenous causes, because loss-of-function mutations in the human gene encoding profilaggrin and filaggrin have been identified as the cause of several skin disorders, including AD and ichthyosis vulgaris.^[5] Furthermore, a dry environment can sometimes cause several skin issues, especially in AD patients and the elderly.^[6] Skin barrier function tends to be disrupted due to fissuring, which can facilitate the induction of other skin disorders.^[7] In a dry environment, a damaged skin barrier readily allows several exogenous antigens, such as HDM allergens, to invade the skin, and this can cause subsequent induction of keratinocyte-mediated skin inflammation.^[3] Thus, several exogenous and endogenous factors may interact in skin barrier disruption, resulting in exacerbation of AD symptoms.

For AD patients, there is a continuing need to develop new therapies, focusing on protecting and recovering the skin barrier.^[5,8] One useful way to protect the skin is to use a moisturizer; there have been reports that topical use of emollients in neonates at high risk of AD prevents the development of eczema.^[9,10] There are several skin moisturizers available for daily dermatological use, such as petrolatum, urea-containing moisturizers, and heparinoid ointment. Heparinoid, a relatively new medication, was established as an analogue of heparin, which is a sulphated, linear polysaccharide derived from animal tissue.^[11] The active ingredient of heparinoid ointment is a mucopolysaccharide polysulphate, also derived from animal sources.^[12] The chemical structure of heparinoid permits considerable hydrogen bonding with adjacent water molecules, which leads to effective hydration of the surrounding tissue.^[13] Today, heparinoid ointment is regarded as an effective moisturizer for dry skin and is commonly used.^[14]

Interestingly, the effects of heparinoid include not only skin moisturizing but also an increase in blood flow and inhibition of fibrosis.^[15] Additionally, heparinoid has an inhibitory action on metalloproteinase-3 expression, which is upregulated in rheumatoid synovial fibroblasts in rheumatoid arthritic and osteoarthritic joints.^[16] Moreover, a recent report showed that heparinoid treatment can improve the regional inflammation of lichen planus.^[17] Oral chondroitin sulphate can reduce inflammatory cell infiltration in patients with psoriasis vulgaris.^[18] Taken together, it seems that heparinoid may be beneficial to human skin due to its numerous effects. However, the detailed mechanism of action of heparinoid on keratinocytes in skin inflammation remains unclear.

In this study, we evaluated the expression of interleukin (IL)-1 β in keratinocytes treated with heparinoid. The release of IL-1 β , and levels of IL-1 β messenger RNA (mRNA) were significantly suppressed by heparinoid treatment. To our knowledge, this is the first report showing a suppressive effect of heparinoid on keratinocyte-mediated inflammation. We suggest that heparinoid acts not only as a moisturizer but also as a reducer or suppressor of skin inflammation induced by exogenous causes, such as HDM allergens.

2 | MATERIALS AND METHODS

2.1 | Heparinoid powder

Heparinoid is an artificially polysulphated chondroitin sulphate that is derived from animal sources. Heparinoid powder was provided by Maruho Co., Ltd. (Osaka, Japan). The heparinoid powder was dissolved and diluted in MCDB (Molecular, Cellular, and Developmental Biology) 153 medium (Nissui, Tokyo, Japan).

2.2 | Cell culture and stimulation

Primary human keratinocytes were isolated from more than three individual infants' skin samples discarded after surgery. This study was conducted according to the principles of the Declaration of Helsinki. All procedures involving human subjects received approval from the Ethics Committee of Ehime University School of Medicine, Japan. We also received written consent from patients' guardians.

Keratinocytes were cultured in MCDB153 medium. Cells that had been passaged four times were used in the experiments. After keratinocytes were cultured subconfluently, the medium was changed to MCDB 153 without bovine pituitary extract (BPE). At 1 hour after the medium change, stimulation was conducted. To treat keratinocytes with affinity-chromatography-purified natural allergens of *Dermatophagoides pteronyssinus* (Der p), which were obtained from Cosmo Bio (Tokyo, Japan), we used a final concentration of 100 μ g/mL. Keratinocytes were pretreated with heparinoid for 1 hour before stimulation with Der p. The concentration of heparinoid was varied from 0.0001% to 0.3% in each examination. Keratinocytes were pretreated with 10 μ mol/L of U-0126 (Cell Signaling, Danvers, MA, USA), an MEK inhibitor, for 1 hour before stimulation. Keratinocytes were also pretreated with 10 μ mol/L of SB203580 (R&D Systems, Minneapolis, MN, USA), a p38 inhibitor, for 1 hour before stimulation. To treat keratinocytes with HMGB1 (R&D Systems), we used a final concentration of 100 ng/mL.

2.3 | Preparation and stimulation of human living skin equivalents and stimulation

The living skin equivalent (LSE) preparation method has been described previously.^[19,20] Briefly, a collagen gel was prepared by mixing six volumes of ice-cold porcine collagen type I solution (Nitta Gelatin, Osaka, Japan) with one volume of 8 $\times$ Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA). In total, 19 volumes of 1 $\times$ DMEM, supplemented with 20% foetal calf serum (FCS) and one volume of 0.1 N NaOH were prepared. Then, 1 mL of this solution was added to each culture insert (Transwell-COL, membrane pore-size: 3 μ m; Corning, New York, NY, USA) in a six-well culture plate (Corning). Following polymerization of the gel in the inserts at 37°C, two volumes of fibroblast suspension solution (5 $\times$ 10⁵ cells/mL in 1 $\times$ DMEM supplemented with 10% FCS) were added to eight volumes of the collagen solution (final collagen concentration, 0.8 mg/mL), and then 3.5 mL of the fibroblast-containing collagen solution

were added to each insert. When the fibroblast-containing gel polymerized, DMEM supplemented with 10% FCS and ascorbic acid (final concentration, 50 ng/mL) was added. The gel was kept in submerged culture for 5 days, until the fibroblasts contracted the gel. The keratinocytes were kept submerged in culture medium for 2 days; when they reached confluence, the LSE was lifted to form an air-liquid interface and cornification medium^[20] was added. This medium was changed every other day. Then, at 10 days after airlift, the LSEs were stimulated for 18 hours with several concentrations of heparinoid, diluted in the medium. LSEs were pretreated with 0%, 0.001%, 0.01%, or 0.1% (w/v) heparinoid for 1 hour and stimulated with Der p (100 µg/mL) for 24 hours.

2.4 | RNA preparation and real-time reverse transcription polymerase chain reaction

Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). NucleoSpin RNA (Macherey-Nagel, Düren, Germany) was used to isolate total RNA after the cells were stimulated with heparinoid. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) or an AB StepOnePlus Real-Time PCR System (Applied Biosystems). Primers and probes specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and IL-1β were obtained from Applied Biosystems. PCR analysis was performed using a TaqMan RT-PCR Master Mix Reagent Kit (Applied Biosystems) according to the manufacturer's protocol. Target gene expression was normalized to the GAPDH signal using delta-delta CT method. Levels of gene expression in allergen-treated cells were quantified relative to those in untreated cells. Reverse transcription (RT)-PCR was performed using RT-PCR High Plus (Toyobo, Osaka, Japan), according to the manufacturer's protocol. The products were visualized on 2% agarose gels containing ethidium bromide and were then sequenced to confirm the accuracy of amplification. The primer pairs used were: GAPDH: ACCACAGTCCATGCCATCAC (FW), TCCACCACCCTGTTGCTGTA (RE), TLR2: GGAATTCTGGCA AGTGGATCATTGACA (FW), CAAGCTTGACTTTATCGCAGCTCTC AG (RE) and TLR4: GGAATTCGAGGCATTTAGGCAGCTATA (FW), CAAGCTTCCAATGGGGAAGTTCTCTA (RE).

2.5 | Enzyme-linked immunosorbent assays

Cell culture supernatants were collected and stored at 30°C. The release of IL-1β, IL-8, tumor necrosis factor (TNF)-α and natural human caspase-1 was quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems).

2.6 | MTT assay

Viable cell numbers were assessed by MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye uptake. Keratinocytes were incubated with or without heparinoid stimulation for 72 hours. Then, 0.025 mL MTT solution (5 mg/mL in

phosphate-buffered saline [PBS]) was added to each well. After a 2-h incubation at 37°C, 0.1 mL extraction buffer (20% SDS, 50% dimethyl formamide) was added and incubated at 37°C overnight. Then, optical densities at 590 nm were measured using a 96-well multiscanner autoreader, with the extraction buffer as a blank. Percentage cytotoxicity was determined as follows: percentage cytotoxicity = $(1 - (A_{570} \text{ of test sample}) / (A_{570} \text{ of control sample})) \times 100\%$.

2.7 | Trypan blue exclusion test

Cell viability was assessed using the trypan blue exclusion test. Keratinocytes were incubated with or without heparinoid for 72 hours. After removal of the growth medium, the cells were washed with sterile PBS, 1-mL amounts of Tris-EDTA buffer were added, followed by incubation at 37°C for 5 minutes. The buffer was collected and the wells washed twice with sterile PBS, which was also collected. Fifty-microliter amounts of the combined buffer and washes were mixed with 50-µL amounts of 0.4% (w/v) trypan blue. After 1 minute at 37°C, cells were counted using a hemocytometer.

2.8 | Protein extraction and Western blot

Cell lysate and supernatant were obtained after cell stimulation and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and analysed using a Vistra ECF Kit (GE Healthcare, Tokyo, Japan). Membranes were scanned using a FluorImager (Molecular Dynamics, Sunnyvale, CA, USA). The following primary antibodies were used: anti-extracellular signal-regulated kinase (ERK), anti-phospho-ERK, anti-c-Jun NH2-terminal kinase (JNK), anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-caspase-1, anti-IL-1β, anti-phospho-c-Raf, anti-c-Raf, anti-phospho-B-Raf, anti-B-Raf, anti-phospho-epidermal growth factor (EGFR), anti-IκB-α, anti-phospho-IκBα (Cell Signaling), anti-EGFR (Santa Cruz, Dallas, TX, USA) and anti-βactin antibody (Abcam, Cambridge, UK).

2.9 | Statistical analysis

Statistical analyses were performed using SPSS for Windows software (SPSS Inc., Chicago, IL, USA). Representative data are shown in the figures. Data using the keratinocytes obtained from at least three individuals showed the same tendency. Relative mRNA expression and levels of secreted cytokines are presented as means ± standard error (SE). Statistical significance was determined using Student's *t* test. Among-group comparisons were performed via analysis of variance followed by the post hoc Tukey-Kramer test (ANOVA followed by *F*-test for contrasts). A *P* value < .05 was considered to indicate statistical significance.

2.10 | siRNA transfection

The siRNA for TLR2 and control siRNA were purchased from Dharmacon (Lafayette, CO, USA). Cells were seeded for 24 hours and transfected with siRNA for TLR2 or control at a concentration of

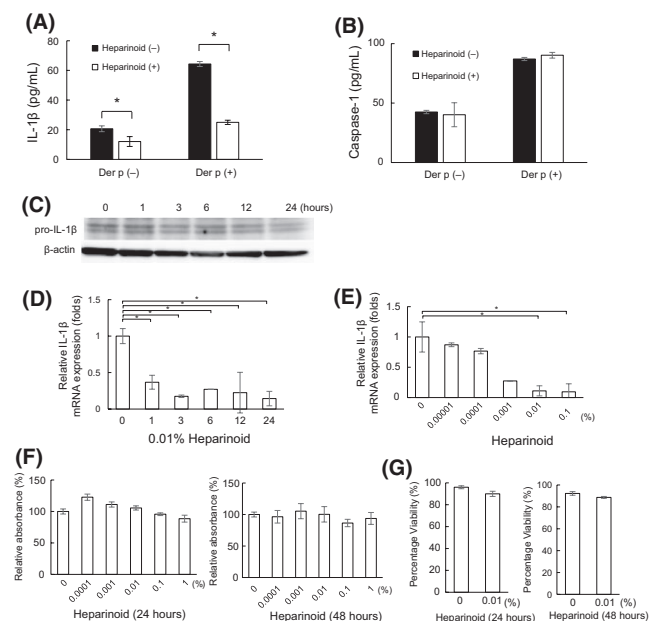


FIGURE 1 Heparinoid inhibits the release of interleukin (IL)-1 β from keratinocytes, reduces intracellular levels of pro-IL-1 β protein, and decreases the basal expression of IL-1 β messenger RNA (mRNA) in keratinocytes. The release of IL-1 β and caspase-1 protein in the supernatant of cell cultures with or without *Dermatophagoides pteronyssinus* (Der p) stimulation was evaluated by enzyme-linked immunosorbent assay (ELISA). As a pretreatment, 0.01% heparinoid was added to keratinocytes 1 h before Der p stimulation. Data represent the means $\pm$ SE of three replicates. * P < .05. (A) Protein levels of IL-1 β . (B) Protein levels of caspase-1. Intracellular levels of pro-IL-1 β in keratinocytes were evaluated using western blot analysis. (C) Pro-IL-1 β expression in keratinocytes treated with 0.01% heparinoid. β -actin was used as a loading control. IL-1 β mRNA expression was evaluated using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Data represent the means $\pm$ SE of three replicates. * P < .05. (D) IL-1 β mRNA expression over time in keratinocytes stimulated with 0.01% heparinoid. (E) IL-1 β mRNA expression in keratinocytes stimulated for 24 h with various concentrations of heparinoid. To assess whether heparinoid treatment caused cell death directly, viable cell numbers and cell viability after heparinoid treatment were evaluated. Viable cell numbers were assessed using the MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (F) and cell viability was assessed using the trypan blue exclusion test (G). Results were compared with the no-stimulation control. Data represent means $\pm$ SE of six replicates. * P < .05

100 nmol/L according to the manufacturer's instructions. Transfected cells (after 48 hours) were used in the following experiments.

3 | RESULTS

3.1 | Heparinoid inhibits Der p-induced IL-1 β release from keratinocytes by suppressing the expression of IL-1 β

IL-1 β was released from keratinocytes with Der p stimulation (Figure 1A). Pretreatment with heparinoid significantly reduced Der

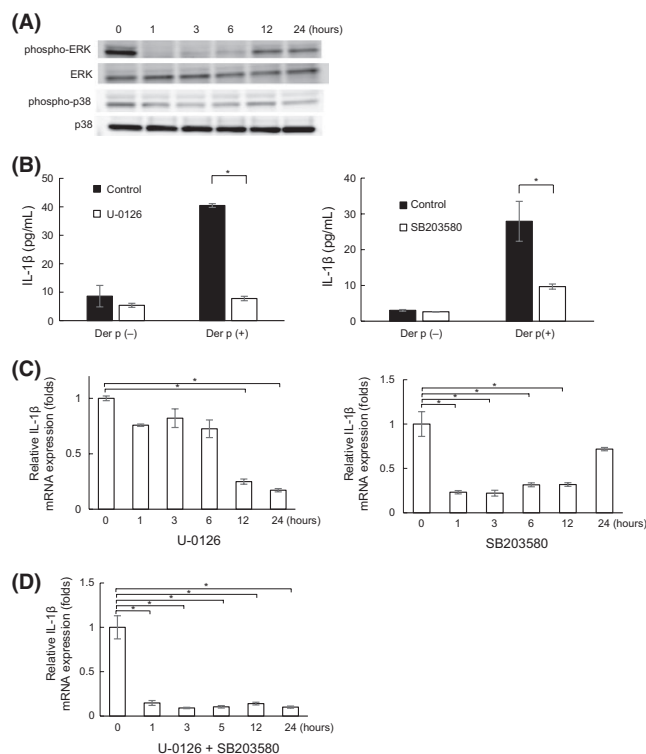


FIGURE 2 Heparinoid suppresses IL-1 β production by inhibiting the ERK and p38 signal pathways. To confirm the phosphorylation of ERK and p38, keratinocytes were treated over time with 0.01% heparinoid, and protein levels were evaluated with Western blotting (A). U-0126 or SB203580 were added to keratinocytes 1 h before Der p stimulation and then keratinocytes were stimulated with 100 μ g/mL of Der p for 24 h. Then, IL-1 β expression in the supernatant of cell cultures was measured using ELISA (B). Data represent the means $\pm$ SE of three replicates. * P < .05. IL-1 β mRNA expression in keratinocytes treated with U-0126 or SB203580 (C), and with both together (D) was measured using qRT-PCR over time. Data represent means $\pm$ SE of three replicates. * P < .05

p-induced release of IL-1 β (Figure 1A). The release of IL-1 β is caused by activation of the NLRP3 inflammasome in keratinocytes.^[4] However, the release of caspase-1 was not affected by heparinoid (Figure 1B). Thus, heparinoid did not affect the activation of the inflammasome but inhibited the release of IL-1 β .

IL-8 and TNF- α were also released from keratinocytes after Der p stimulation (Figure S1A,B). Pretreatment with heparinoid also significantly reduced Der p-induced release of IL-8, but not TNF- α (Figure S1A,B).

According to the previous data, we hypothesized that heparinoid would reduce the storage of pro-IL-1 β in keratinocytes by suppressing the expression of IL-1 β mRNA. Thus, we evaluated intracellular pro-IL-1 β using Western blot analysis. As shown in Figure 1C, 0.1% heparinoid treatment slightly decreased the protein level of pro-IL-1 β at 12 hours and 24 hours. Furthermore, the expression of IL-1 β mRNA was reduced significantly from 1 hour after the addition of 0.01% heparinoid (Figure 1D). This effect was sustained until 24 hours after heparinoid stimulation. Additionally, this inhibitory effect was seen in a dose-dependent manner at 3 hours after

heparinoid stimulation (Figure 1E) and was significant with stimulation by 0.001%, 0.01%, or 0.1% (w/v) heparinoid.

To evaluate the cell toxicity of heparinoid, we investigated viable cell numbers using the MTT assay and cell viability using the trypan blue exclusion test after heparinoid treatment. In the MTT assay, there was no significant difference in uptake between control cells and cells treated with 0.01% heparinoid for 24 or 48 hours (Figure 2F). The trypan blue exclusion test also showed no significant difference in the ratio of viable cells between the control and the 0.01% heparinoid treatment (Figure 2G). These results demonstrated that heparinoid was not toxic to the keratinocytes.

3.2 | Heparinoid suppresses IL-1 β production by inhibiting EKR and p38 MAPK pathways

We analysed the activation of MAPK-family pathways (because several reports have shown that production of IL-1 β is mediated by the MAPK family).^[21–23] As shown in Figure 2A, the phosphorylation of ERK at basal level was strongly suppressed by heparinoid at 1, 3, and 6 hours, and was slightly suppressed after 12 hours stimulation. However, the phosphorylation of p38 at basal level was reduced by heparinoid from 1 hours to 24 hours (Figure 2A). These results suggested that heparinoid blocks ERK, p38 pathways. To investigate whether the heparinoid-mediated inhibition of ERK and p38 pathways resulted in reduced IL-1 β release, we treated keratinocytes with U-0126 (MEK inhibitor) and SB203580 (p38 inhibitor). Both U-0126 and SB203580 significantly suppressed Der-p-induced IL-1 β release (Figure 2B). A significant suppressive effect on IL-1 β mRNA expression was found 12 hours and 24 hours after U-0126 treatment. SB203580 also suppressed mRNA expression, and the effect was stronger from 1 hour after stimulation than that of U-0126; however, the effect was not pronounced at 24 hours (Figure 2C). Furthermore, co-treatment with U-0126 and SB203580 strongly suppressed IL-1 β mRNA expression, similar to the effect of heparinoid (Figure 2D). Taken together, heparinoid-mediated inhibition of the ERK and p38 pathways reduced the expression of IL-1 β mRNA and suppressed Der p-induced IL-1 β release from keratinocytes.

3.3 | TLR2 and TLR4 are not involved in the heparinoid-induced reduction of IL-1 β

To elucidate the role of TLR2 and TLR4 in IL-1 β production, the expression of TLR2 and TLR4 in keratinocytes was analysed by RT-PCR. Figure 3A shows the mRNA expression of TLR2, TLR4, and GAPDH examined through RT-PCR. The expression of TLR4 mRNA was not detected. To clarify the involvement of TLR2 in the heparinoid-induced reduction of IL-1 β , keratinocytes were pretreated with TLR2 siRNA. Figure 3B shows the suppression of TLR2 mRNA expression by TLR2 siRNA. Treatment with the TLR2 siRNA did not reduce the release of IL-1 β from keratinocytes after Der p treatment (Figure 3C). High-mobility group protein 1 (HMGB1), which acts as a ligand for TLR2 and TLR4, was reported to be released from Der p-treated keratinocytes.^[4] To elucidate whether HMGB1 increased the release of

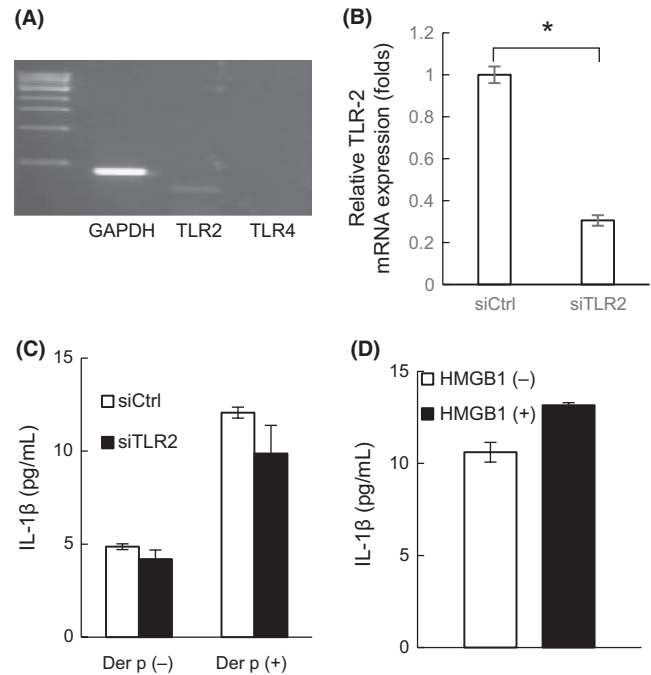


FIGURE 3 The Der p-induced release of IL-1 β is not related to the activation of Toll-like receptor 2 (TLR2) or the release of high-mobility group protein 1 (HMGB1). To clarify the involvement of TLR2, keratinocytes were pretreated with TLR2 siRNA. Induction of siRNA for TLR2 was evaluated by RT-PCR (A) and qRT-PCR (B). Data represent the means $\pm$ SE of three replicates. * P < .05. Keratinocytes pretreated with TLR2 siRNA were stimulated by Der p. The release of IL-1 β protein in the supernatant of cell cultures with or without Der p stimulation was evaluated by ELISA (C). Keratinocytes were also stimulated with HMGB1 to evaluate the involvement of HMGB1 in IL-1 β release (D). Data represent the means $\pm$ SE of three replicates. * P < .05

IL-1 β , keratinocytes were also treated with HMGB1, but no effect on IL-1 β secretion was observed (Figure 3D).

3.4 | Heparinoid treatment reduces the expression of IL-1 β mRNA in LSEs

To confirm whether this effect would occur in a more natural human epidermal system, we used LSEs, which were stimulated with 0.01%, 0.1%, or 0.3% heparinoid for 18 hours. The expression of IL-1 β mRNA was reduced significantly in a dose-dependent manner (Figure 4A). Der p stimulation also significantly increased IL-1 β mRNA expression in LSEs (Figure 4B), and this increase was significantly inhibited by 0.01 or 0.1% (w/v) heparinoid (Figure 4C).

4 | DISCUSSION

Previously, we showed that HDM allergens activate the NLRP3 inflammasome of keratinocytes to release pro-inflammatory cytokines.^[4] HDM allergens penetrate the disrupted skin barrier readily in AD patients and dry skin exacerbates the symptoms of AD.^[3] Heparinoid

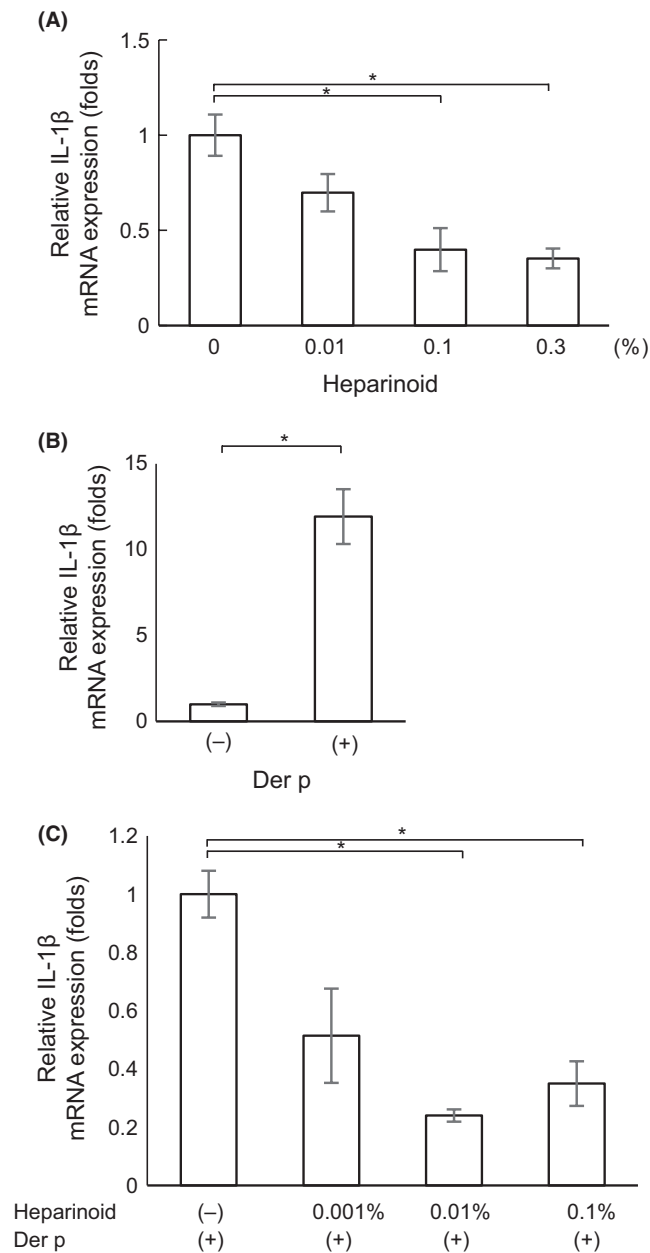


FIGURE 4 Heparinoid treatment reduces expression of IL-1β mRNA in living skin equivalents (LSEs). IL-1β mRNA expression levels in LSEs were evaluated by qRT-PCR. Data represent means $\pm$ SE of three replicates. * $P < .05$. (A) LSEs were treated with 0%, 0.01%, 0.1% or 0.3% heparinoid for 18 h. (B) LSEs were grown with or without 100 μ g/mL Der p for 24 h. (C) LSEs were pretreated with 0%, 0.001%, 0.01%, or 0.1% heparinoid for 1 h and stimulated with 100 μ g/mL Der p for 24 h

ointment is used widely as a moisturizer to treat AD and dry skin. Hyaluronic acid (HA) is a kind of heparinoid that is thought to play a role in homeostasis.^[24,25] Furthermore, HA treatment has been reported to suppress UVB-induced release of IL-6 and IL-8 from keratinocytes.^[26] Thus, in this study we investigated the inhibitory effect of heparinoids on keratinocytes. It has been reported that IL-1β is overexpressed in AD lesions.^[27] Moreover, epidermal keratinocytes form an interface to communicate with the body through the expression of receptors

that sense microorganisms and other environmental stress factors, via production of cytokines and chemokines, including members of the IL-1 family.^[28] Based on these reports, we focused on IL-1β, which is involved in the initiation of inflammation.^[29]

First, the release of IL-1β (Figure 1A) and IL-8 (Figure S1A) from keratinocytes activated by HDM allergens was decreased significantly after pretreatment with heparinoid. However, the release of caspase-1 was unaffected by heparinoid pretreatment. This suggests that heparinoid acts as an anti-inflammatory agent that suppresses pro-inflammatory responses, and the effect is not mediated by inhibition of the inflammasome. We previously reported that IL-1α and IL-1β in sweat increased the production of IL-8, which was abrogated by IL-1RA.^[30] Therefore, the reduction of IL-8 release by heparinoid in the present study may be due to the lowered IL-1β release. The results of the MTT assays and the trypan blue exclusion test showed no significant effects on viable cell counts or cell death. Thus, heparinoid had no general cytotoxic effect on keratinocytes and suppression was not due to general cell damage.

We next analysed intracellular pro-IL-1β in keratinocytes, which is an inactive 269-residue precursor of IL-1β. Pro-IL-1β requires processing by caspase-1 to generate the active, mature 153-residue cytokine.^[31] Intracellular pro-IL-1β levels were reduced by 0.1% heparinoid treatment. Thus, we suggest that heparinoid may reduce production of pro-IL-1β; the intracellular pro-IL-1β in keratinocytes diminished gradually.

Next, we examined expression of IL-1β mRNA in keratinocytes to analyse the cause of the pro-IL-1β reduction. Heparinoid pretreatment reduced expression of IL-1β mRNA significantly. This indicated that the reduction in pro-IL-1β level was likely due to inhibition of IL-1β transcription. Data using the keratinocytes obtained from at least three individuals showed the same tendency. (Figure S2). Since NF-κB activates IL-1β transcription, we analysed the phosphorylation of IκBα. However, IκBα phosphorylation was not detected when stimulated with heparinoid. MAPK pathways are involved in the transcriptional activation of IL-1β in keratinocytes^[32] and it has been reported that HA combined with glucocorticoid inhibits the p38 pathway in acute lung injury in rats.^[33] Heparinoid is an artificially polysulphated chondroitin sulphate that is derived from animal sources. In addition, chondroitin sulphate reduces ERK1/2 phosphorylation and abrogates p38MAPK phosphorylation in chondrocytes.^[34,35] Therefore, we next analysed whether heparinoid affected activation of the MAPK family and found that heparinoid blocked the phosphorylation of ERK and p38 in keratinocytes. Although treatment with U-0126, a MEK inhibitor, and SB203580, a p38 inhibitor, decreased IL-1β mRNA expression, their effects were not similar to those of heparinoid. The MEK inhibitor strongly suppressed IL-1β mRNA at a later phase. The p38 inhibitor suppressed IL-1β mRNA expression in the early phase, but not in later phases. However, co-treatment with U-0126 and SB203580 showed significant suppression of IL-1β mRNA expression, similar to that seen with heparinoid treatment. These results suggest that the inhibitory effect of heparinoid on IL-1β mRNA expression was exerted through the inhibition of phosphorylation of not only p38 but also ERK. This suppressive effect was also confirmed in LSEs, which better mimic the natural differentiated epidermis.

Since Raf proteins are essential in linking Ras and the MEK-ERK pathway,^[36] we explored the phosphorylation of B-Raf and c-Raf. c-Raf phosphorylation was suppressed by 0.01% (w/v) heparinoid (Figure S3). However, B-Raf phosphorylation was not affected by heparinoid (Figure S3), suggesting that the heparinoid target was likely a receptor tyrosine kinase or a G protein-coupled receptor. We hypothesized that the EGFR was a likely candidate because heparin-binding EGF-like growth factor (HB-EGF), which exhibits a high affinity for heparin, is an EGFR ligand.^[37] Cook et al found that mucopolysaccharides inhibited human keratinocyte proliferation.^[38] HB-EGF phosphorylated EGFR and promoted ERK phosphorylation in keratinocytes, as previously reported,^[39] but heparinoid did not affect the phosphorylation of either EGFR or c-Src, which is activated by EGFR (Figure S3). Hence, the EGFR signal is not involved. We currently cannot identify the heparinoid receptor. Further work is needed.

Keratinocytes are known as central skin sentinels that can recognize irritants or toxins through TLRs and inflammasome machinery.^[40] We previously reported that the inflammasome is stimulated by Der p. In addition, HMGB1 was released from Der p-treated keratinocytes.^[4] HMGB1 acts as a ligand for TLR2 and TLR4.^[41] In addition, Der p 2, which is a major allergen of Der p, can signal to cells via the activation of TLR2.^[42] To clarify the involvement of TLR2, keratinocytes were stimulated with HMGB1 or pretreated with siRNA for TLR2. HMGB1 stimulation did not increase the release of IL-1 β from keratinocytes. Moreover, knockdown of TLR2 did not affect the Der p-induced release of IL-1 β or IL-8 from keratinocytes. These results indicate that TLR2 is not involved in the Der p-induced release of IL-1 β and IL-8 from keratinocytes. However, contamination with endotoxin during Der p and heparinoid preparation is possible, so the involvement of TLR4 activation is of concern. However, we previously reported that endotoxin does not stimulate the secretion of IL-1 β in keratinocytes.^[36] In addition, heparinoid did not increase the expression or release of IL-1 β in this study. In addition, expression of TLR4 in the keratinocytes used in this study was not detected by qRT-PCR. Therefore, endotoxin is not involved in the production of IL-1 β from keratinocytes and TLR4 had no impact on the results of this study due to the lack of expression in the keratinocytes we used. The contamination of DNA or RNA in Der p and heparinoid is also of concern. Previously, we reported that Der p did not trigger the phosphorylation of I κ B α , while poly I:C, which is structurally similar to double-stranded RNA, did.^[4] In addition, TLR9 responds to single-stranded DNA.^[43] No phosphorylation of I κ B α was observed in keratinocytes treated with or without heparinoid in this study. This means that Der p and heparinoid were not contaminated with a large enough quantity of DNA or RNA to activate NF- κ B signaling. Previous studies have shown that dsDNA activates the AIM2 inflammasome.^[44] However, we reported that the Der p-induced release of IL-1 β was abrogated by knockdown of the NLRP3 inflammasome,^[4] indicating that activation of AIM2 inflammasome had little, if any, involvement in this study. Der p was extracted from Der p in this study, and it mainly contains Der p 1 and Der p 2. We previously reported that the Der p-induced release of IL-1 β was

abrogated by cysteine protease inhibitor. In addition, purified Der p 1, but not Der p 2, activated the NLRP3 inflammasome to elicit IL-1 β release from keratinocytes.^[4] These results indicate that the release of IL-1 β was mainly caused by the cysteine protease activity of Der p 1. Therefore, we assume that any possible contamination with DNA or RNA had little significance in this study.

Long-term use of topical steroids causes adverse effects, such as skin atrophy and infections, and there is a need to prevent inflammation in chronic inflammatory diseases, like AD.^[45] Heparinoid is known to have no serious side effects, probably because it cannot exert effects on an intact epidermis. The dermal absorption rate of heparinoid is extremely low in intact skin samples, but increases in dried skin samples.^[15] Once the skin barrier is disrupted, external stimulants invade the epidermis and exacerbate skin inflammation. Heparinoid could also be absorbed by the disrupted epidermis and then act on keratinocytes to prevent the production of IL-1 β and mitigate inflammation. Heparinoid may provide an effective option for preventing inflammation in chronic skin inflammatory diseases, which often result in a disrupted skin barrier. Thus, we are planning a clinical trial in human volunteers.

There is no previous report about the bioactivity of heparinoid in keratinocytes, and the anti-inflammatory effects of heparinoid have been reported here for the first time. This study showed that heparinoid acts not only as a moisturizer but also as a reducer/suppressor of skin inflammation.

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CONFLICT OF INTERESTS

Ryo Utsunomiya, Xiuju Dai, Masamoto Murakami, Hidenori Okazaki, Teruko Tsuda, Hideki Mori, Ken Shiraishi and Mikiko Tohyama have no conflicts of interest to declare. This work was supported by JSPS KAKENHI (Grant Number JP16K10128). Koji Sayama received an honorarium from Maruho Co., Ltd. and was funded by a scientific grant from Maruho Co., Ltd.

AUTHOR CONTRIBUTIONS

Ryo Utsunomiya, Hidenori Okazaki and Teruko Tsuda devised, performed, and oversaw the experiments, analysed the data and contributed to the writing of the manuscript; Ryo Utsunomiya, Xiuju Dai, Masamoto Murakami, Hideki Mori, Ken Shiraishi, Mikiko Tohyama and Koji Sayama performed the analysis and wrote the discussion.

ORCID

Ryo Utsunomiya  <http://orcid.org/0000-0001-7257-5784>

Xiuju Dai  <http://orcid.org/0000-0002-1487-7466>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

FIGURE S1 The release of IL-8 and tumor necrosis factor (TNF)- α protein in the supernatant of cell cultures with or without Der p stimulation was evaluated by ELISA. Data represent means $\pm$ SE of three replicates. * $p < 0.05$. As a pretreatment, 0.01% (w/v) heparinoid was added to keratinocytes 1 h before Der p stimulation. (A) IL-8 protein levels. (B) TNF- α protein levels

FIGURE S2 IL-1 β mRNA expression over time in keratinocytes stimulated with 0.01% heparinoid. Primary human keratinocytes were obtained from at least three individuals. Representative data from keratinocytes obtained from two individuals are shown. Data represent the means $\pm$ SE of three replicates. * $p < 0.05$

FIGURE S3 Keratinocytes were treated over time with 0.01% heparinoid and protein levels were evaluated with Western blotting. The phosphorylation levels of c-Raf, B-Raf, and epidermal growth factor (EGFR) expression are shown

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