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ORIGINAL ARTICLE

Hyaluronan Oligosaccharides Improve Rosacea-Like Phenotype through Anti-Inflammatory and Epidermal Barrier-Improving Effects

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Background: Rosacea is a common skin disease associated with increased expression of cathelicidin, kallikrein 5 (KLK5), toll-like receptor (TLR) 2, and abnormal barrier function. Recently, it was reported that hyaluronan (HA) could influence immune function via various receptors and HA oligosaccharides (oligo-HAs) could suppress TLR-dependent cytokine expression. **Objective:** We investigated if oligo-HAs could influence on inflammation and epidermal barrier induced by LL-37, which had a major role in rosacea. **Methods:** We cultured normal human keratinocytes and treated them with LL-37 and oligo-HAs or the LL-37 alone. A rosacea-like BALB/c mouse model injected with LL-37 was used to determine the role of oligo-HAs in rosacea *in vivo*. **Results:** Interleukin-8 (IL-8) and tumor necrosis factor (TNF)-α release was suppressed when keratinocytes were co-treated with oligo-HAs and LL-37 compared with keratinocytes treated with LL-37 only. Treatment with oligo-HAs resulted in decreased transepidermal water loss as well as improved redness. Decreased inflammatory cell infiltration, IL-17A and KLK5 expression and increased CD44 and filaggrin expression were also noted. **Conclusion:** Our findings suggest that oligo-HA improves rosacea-like phenotype through anti-inflammatory and epidermal barrier improving effect. **(Ann Dermatol 32(3) 189～196, 2020)**

-Keywords-
Hyaluronic acid, Rosacea

INTRODUCTION

Rosacea is a common, chronic inflammatory skin disease characterized by uncontrolled vasodilation, chronic inflammation, and later fibrosis[1]. Complex pathomechanisms involving dysregulation of the immune, vascular, and nervous systems as well as barrier function impairments have been suggested[2-4]. The understanding of rosacea immunology remains limited, and most studies have focused on the consistently abnormal innate immune system. Environmental factors (e.g., ultraviolet [UV], temperature changes, spicy food, exercise, stress) or an altered skin microbiome activates pattern recognition receptors such as toll-like receptors (TLRs), which stimulate the release of antimicrobial peptides such as LL-37[4]. In rosacea, increased expression of TLR2, LL-37, and kallikrein 5 (the protease that cleaves the cathelicidin precursor protein into LL-37) has been observed, and inhibition of kallikrein 5 (KLK5) has been linked to improvement in the erythema and inflammatory papules in rosacea[5,6].

Regarding abnormal barrier function in rosacea, a few studies showed that patients with rosacea had reduced epidermal hydration levels and different sebaceous fatty acid profiles compared to control subjects[7,8]. However, these observational studies did not investigate the causes of barrier function abnormalities.

Current rosacea therapeutics have a wide range of targets.

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Doxycycline has the broadest activity, downregulating a dozen or more mediators in the inflammatory rosacea cascade, followed by ivermectin and azelaic acid. In addition to anti-inflammatory medications, topical brimonidine has been approved for symptomatic relief of the erythema of rosacea. However, a single medication cannot control rosacea, and combined treatment is required in many cases. Therefore, new therapeutic tools are currently being developed.

Hyaluronan (HA) is the well-known predominant component of the extracellular matrix. It is abundant in many tissues including the skin, where it acts as a hydrating agent and organizer to form structural scaffolding[9]. Recent studies demonstrated that HA can actively regulate dynamic cellular processes, such as cell proliferation and migration during embryonic tissue development, wound repair, tumor invasion, and skin aging[10].

The predominant receptor for HA on the cell surface is the transmembrane glycoprotein CD44. HA signaling also occur through TLR2 and TLR4, and epithelial HA is reported to protect against apoptosis through nuclear factor-κB in a TLR-dependent manner[11]. The anti-inflammatory effect of HA oligosaccharides (oligo-HAs) has also been demonstrated. Oligo-HAs protected mice from the shock response and inhibit the release of cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α[12].

Previous studies of the role of HA and oligo-HAs suggested that HA can modulate important molecular pathways that are also involved in the development of rosacea, resulting in anti-inflammatory and barrier-improving effects. However, no studies have evaluated the effect of oligo-HAs on rosacea. Therefore, we investigated the effects of oligo-HAs on rosacea in cultured human keratinocytes and mouse models.

MATERIALS AND METHODS

Materials

A synthetic human cathelicidin LL-37 peptide with the amino acid sequence LLGDFFRKSKEKIGKEFKRIVQRIKDF LRNLVPRTES was commercially prepared and purified to >95% purity by high-performance liquid chromatography. Oligo-HA was purchased from Open Biosystems (Huntsville, AL, USA). The molecular weight of oligo-HA was 776.65 Da. The study was approved by the Animal Care and Use Committee of the Bundang CHA Medical Center (IACUC 170069).

Measurement of IL-8 and TNF-α release

Normal human keratinocytes (Cefobio, Seoul, Korea) were grown in EpiLife medium (Cascade Biologics, Portland, OR, USA) supplemented with 0.06 mM Ca^{2+}, 1% EpiLife defined growth supplement, and 1% penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. We cultured human keratinocytes to confluence and treated them with the cathelicidin peptides (3.2 μM) with or without oligo-HA (10 μg/ml) for 24 hours.

The supernatants were collected and placed in a sterile 96-well plate for enzyme-linked immunosorbent assay (ELISA) to detect the production of IL-8 and TNF-α using kits from R&D Systems (Minneapolis, MN, USA) in accordance with the manufacturer's instructions.

Mice

Six-week-old female BALB/c mice were purchased from Orient Bio (Seongnam, Korea) and acclimatized for 1 week before conducting the experiments. All animal experiments were performed under specific pathogen-free conditions and in accordance with the guidelines of the Animal Care Committee of the CHA University of Korea.

In vivo treatments

Rosacea-like skin lesion was induced using the LL-37 peptide as described previously[2]. The hair on the backs of the BALB/c mice was shaved using an electric shaver. After 24 hours, 40 μl of LL-37 in nanopure water (320 μM; InvivoGen, San Diego, CA, USA) was injected intradermally (i.d.) into the shaved area using a 0.5-ml insulin syringe (31 gauge), resulting in the formation of a dermal bleb. Control mice were injected i.d. with 40 μl of nanopure water alone. At 1 hour after i.d. injection, the oligo-HA groups were injected intraperitoneally (i.p.) or i.d. with oligo-HA (100 μg/mouse) in phosphate-buffered saline (PBS), respectively. These procedures were repeated twice per day for 2 days. At 48 hours after the initial injection, the dorsal skin was photographed. The severity of rosacea-like skin lesions was evaluated based on the redness index and area. The redness area was assessed by the Antera 3D® (Miravex, Dublin, Ireland) and redness index was assessed with a Mexameter® (Courage+Khazaka, Köln, Germany). The Vapometer® (Delfin Technologies Ltd., Kuopio, Finland) was used to evaluate transepidermal water loss (TEWL). After the final measurement, BALB/c mice were anaesthetized, and tissue samples of the dorsal skin were excised, fixed in 10% formalin, and embedded in paraffin. Paraffin-embedded skin samples were sliced and then stained with hematoxylin and eosin (H&E).

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded tissue sections were

deparaffinized and rehydrated in a graded alcohol series. Endogenous peroxidases were inactivated by hydrogen peroxide. For heat-induced epitope retrieval, the slides were autoclaved in citrate buffer (10 nM, pH 6) under standard operating conditions for 10 minutes. To detect CD4, IL-17A, KLK5, filaggrin, and CD44 in histological specimens from mice, primary antibodies including a monoclonal mouse anti-CD4 antibody (BD Biosciences, Franklin Lakes, NJ, USA), monoclonal rat anti-mouse IL-17A antibody (Covalab, Villeurbanne, France), polyclonal rabbit anti-KLK5 antibody (Abcam, Cambridge, UK), polyclonal mouse anti-profilaggrin antibody (Covance, Princeton, NJ, USA), and monoclonal rat anti-human/mice CD44 antibody (eBioscience, San Diego, CA, USA) were used. The H score and cell count were evaluated by the pathologist with blinded samples. The H score is obtained by the formula: 3× percentage of strongly staining nuclei + 2× percentage of moderately staining nuclei + percentage of weakly staining nuclei, giving a range of 0 to 300. Interpretation of H scores is as follows: 0, negative; 1～100, weak positive; 101～200, moderately positive; >200, strongly positive. Fluorescence intensity was also measured by Image J software. The CD4+ cell count was measured as the number of cells per high power field (HPF) at the most infiltrative sites.

Statistics

Statistical analysis was conducted using SPSS software ver. 12.0 (SPSS Inc., Chicago, IL, USA). Student's t-test and Mann–Whitney U-test were used. Differences between groups with $p < 0.05$ were considered as statistically significant.

RESULTS

Oligo-HAs suppressed proinflammatory cytokine expression in LL-37-treated human keratinocytes

To explore the effect of oligo-HAs on rosacea *in vitro*, we first analyzed the expression of IL-8 and TNF-α, which are proinflammatory cytokines well-known to be increased in skin with rosacea, using human keratinocytes cultured in the presence of the active cathelicidin-derived peptide LL-37. Oligo-HAs decreased the release of IL-8 and TNF-α which was induced by LL-37 ($p < 0.05$; Fig. 1).

Treatment with oligo-HAs improved the rosacea-like phenotype and decreased TEWL

To assess the functions of oligo-HAs on rosacea *in vivo*, we first induced rosacea-like mouse models by intradermal injection of LL-37. At 1 hour after injection, oligo-HAs were injected i.p. or i.d. into the mice twice daily for 2 days. At 48 hours after the initial injection, erythema was assessed by global photograph and Antera 3D® (Miravex; Fig. 2A). Oligo-HA-treated rosacea-like mice developed much smaller redness areas and erythema index compared to rosacea-like mice ($p < 0.05$; Fig. 2B, C). Oligo-HAs improved the rosacea like phenotype. There were no differences in the area and degree of erythema between injecting LL-37 i.p. and i.d. TEWL, as measured with a vapometer, was also decreased in the oligo-HA-treated rosacea-like mice compared to in rosacea-like mice ($p < 0.05$; Fig. 2D).

Treatment with oligo-HAs reduced inflammatory cell infiltration, IL-17 and KLK5 expressions

As shown in Fig. 3A, LL-37 markedly induced inflam-

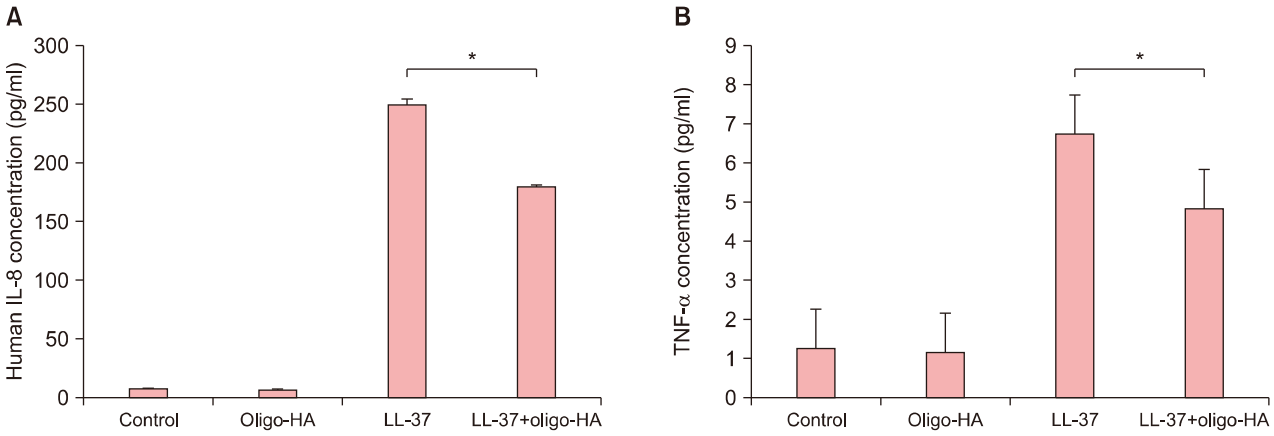

Fig. 1. The changes of proinflammatory cytokine expression in LL-37 treated human keratinocytes. LL-37 increased the production of Interleukin-8 (IL-8) and tumor necrosis factor (TNF)-α determined by enzyme-linked immunosorbent assay in human keratinocytes. Further treatments with hyaluronan oligosaccharides (oligo-HAs) decreased IL-8 and TNF-α release. (A) IL-8 concentration. (B) TNF-α concentration. *$p < 0.05$.

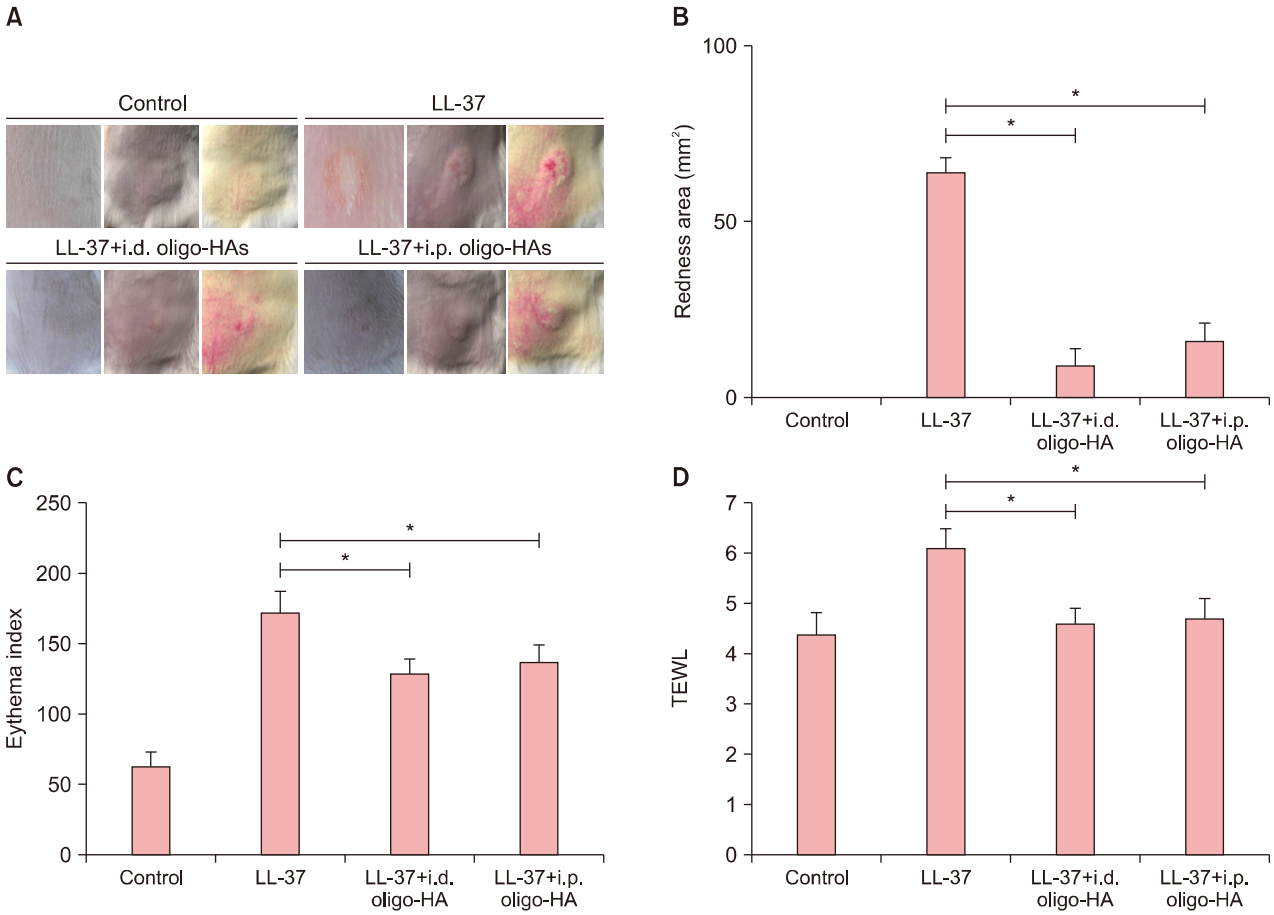

Fig. 2. Treatment with hyaluronan oligosaccharides (oligo-HAs) improved the rosacea-like phenotype and decreased transepidermal water loss (TEWL). (A) Intradermal (i.d.) injection of LL-37 induced the rosacea-like mouse models. After 48 hours of the initial injection, the dorsal skin was photographed. Erythema around injection site was shown through global photograph (left) and Antera 3D® (Miravex, Dublin, Ireland). Antera 3D® three-dimensional aspect (center), and hemoglobin distribution (right) are all recorded. After the injection of LL-37, oligo-HAs were further injected intraperitoneally (i.p.) or i.d. (B, C) The redness area and erythema index were assessed by the Antera 3D® and Mexameter® (Courage+Khazaka, Köln, Germany). Oligo-HAs-treated rosacea-like mice developed far less redness area and erythema index compared with rosacea-like mice. (B) Redness area. (C) Erythema index. (D) TEWL was evaluated by the Vapometer® (Delfin Technologies Ltd., Kuopio, Finland) and its value decreased in oligo-HAs-treated rosacea-like mice. All values were not significantly different according to the injection method; i.d. or i.p. $*p<0.05$.

matory cell infiltration. We investigated the effect of oligo-HAs on inflammatory cell infiltration. Oligo-HAs reduced cellular infiltration in the mouse skin regardless of the injection method. Immunohistochemical staining was performed to identify the types of inflammatory cells. The number of CD4+T cells and expression of IL-17A were decreased in oligo-HA-treated rosacea-like mice compared to in rosacea-like mice (Fig. 3B, C). The number of CD4+ T cells is as follows: control group, 20/HPF; LL-37 treated group, 42/HPF; LL-37+i.d. oligo-HA group, 16/HPF; LL-37+ i.p. oligo-HA group, 18/HPF. IL-17A expression is evaluated by H score. The data is as follows, control group, 5 (weak); LL-37 treated group, 140 (moderate); LL-37+i.d. oligo-HA group, 30 (weak); LL-37+i.p. oligo-HA group, 40 (weak).

KLK5 expression in the epidermis was increased in mice with the rosacea phenotype induced by LL-37, whereas treatment with oligo-HAs reduced the expression of KLK5 (Fig. 3D). Staining intensity was evaluated by Image J software, typically range from 0 to 255, where 0 corresponds to black and 255 corresponds to white. The lower the value, the stronger the stain and the data are as follows: control group, 162; LL-37 treated group, 144; LL-37+i.d. oligo-HA group, 190; LL-37+i.p. oligo-HA group, 188.

Treatment with oligo-HAs increased CD44 and filaggrin expression

The major receptor for HA on the cell surface of keratinocytes is CD44. To investigate the effect of exogenous injection of oligo-HAs on the expression pattern of CD44,

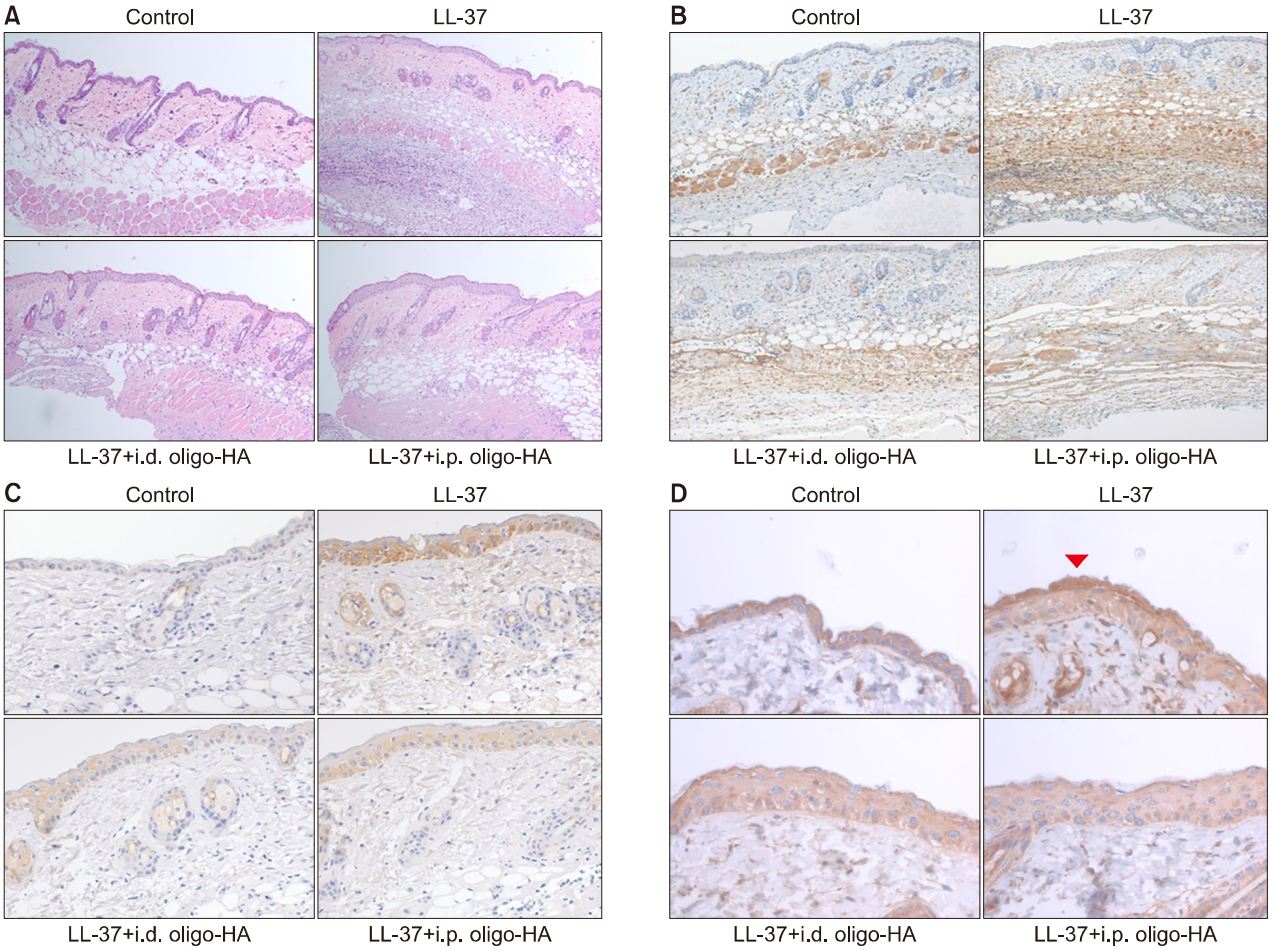

Fig. 3. Treatment with hyaluronan oligosaccharides (oligo-HAs) reduced inflammatory cell infiltration, interleukin-17 (IL-17), and kallikrein 5 (KLK5) expressions. (A) Hematoxylin and eosin-stained tissue sections of LL-37 induced rosacea-like mice showed marked inflammatory cellular infiltration. Further treatments with oligo-HAs reduced cellular infiltration (H&E, ×100). (B) Majority of infiltrated cells were CD4+ T cells and infiltration of CD4+ T cells decreased in oligo-HAs treated group (CD4, ×100). (C) IL-17A expression was markedly increased in the epidermis of LL-37 induced rosacea-like mice. Oligo-HAs decreased the IL-17A expression regardless of the injection method (IL-17A, ×200). (D) Expression of KLK5 was increased in LL-37 induced rosacea-like mice (arrow head). Further treatment with oligo-HAs reduced KLK5 expression (KLK5, ×400). i.d.: intradermally, i.p.: intraperitoneally.

we performed immunohistochemical staining with CD44. Expression of CD44 was markedly increased in oligo-HA-treated rosacea-like mice, regardless of the injection method (Fig. 4A). For quantification, H-score was evaluated and the data are as follows: control group, 40 (weak); LL-37 treated group, 80 (weak); LL-37+i.d. oligo-HA group, 120 (moderate); LL-37+i.p. oligo-HA group, 105 (moderate). To assess the effect of oligo-HAs on the skin barrier, filaggrin expression was also measured. We found that filaggrin expression was slightly decreased in rosacea-like mouse skin compared to in control mouse skin, whereas oligo-HAs increased filaggrin expression in rosacea-like mice (Fig. 4B). H score was evaluated and the data are as follows: control group, 220 (strong); LL-37 treated group, 120 (moderate); LL-37+i.d. oligo-HA group, 205 (strong); LL-37+i.p. oligo-HA group, 230 (strong).

DISCUSSION

Rosacea is a common chronic inflammatory skin disease that predominantly affects the central facial skin. The pathophysiology of rosacea is very complex, and dysregulation in the immune, vascular, and nervous systems, as well as barrier function impairments, are suggested[2-4]. The understanding of rosacea immunology remains limited, and most studies have focused on the consistently abnormal innate immune system, such as increased expression of cathelicidin, KLK5, and TLR2.

HA regulates many aspects of the tissue repair process, such as activation of inflammatory cells and regulation of

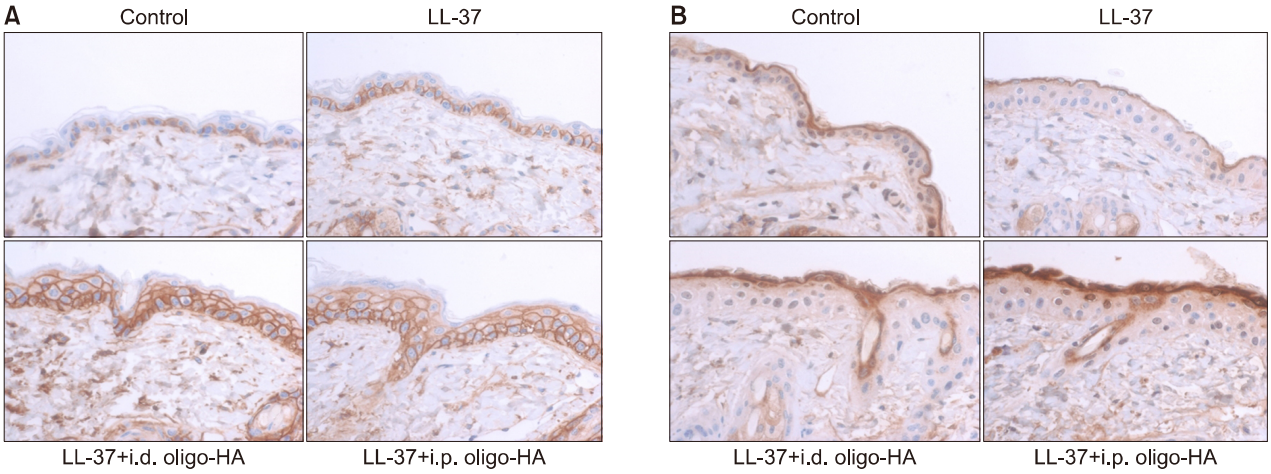

Fig. 4. Treatment with hyaluronan oligosaccharides (oligo-HAs) increased CD44 and filaggrin expression. (A) Immunohistochemical stain with CD44 was performed in controls and LL-37 induced rosacea-like mice. Oligo-HAs increased the expression of CD44 (CD44, ×400). (B) Oligo-HAs also increased the expression of filaggrin. The degree of expression was not related to the injection method of oligo-HAs (Filaggrin, ×400). i.d.: intradermally, i.p.: intraperitoneally.

epithelial cell and fibroblast behaviors[10]. HA plays different roles depending on its molecular weight. HA with mixed sizes of 150～500 kDa induced inflammatory responses, whereas larger molecular weight HA mixtures and oligo-HAs of different sizes appeared to inhibit inflammatory responses[12,13].

Several studies have indicated that interactions of HA with CD44 influence epidermal structure and function in the skin. CD44 knockout mice showed reduced epidermal HA staining, marked thinning of the epidermis, decreased expression of proliferation and differentiation markers, and defects in epidermal lipid synthesis and delayed barrier recovery[14]. These results indicate that interactions of HA with CD44 in the epidermis are important for proliferation, differentiation, and cholesterol synthesis in the epidermis, which ultimately influence homeostasis of the skin permeability barrier. Oligo-HAs also stimulate ceramide production by upregulating the mRNA expression of ceramide synthesis-associated enzymes[15].

Recently, it was reported that HA influences immune function via various receptors such as CD44, TLR2, and TLR4 and oligo-HAs suppresses TLR-dependent cytokine expression[12]. We confirmed that human keratinocytes treated with LL-37 and oligo-HAs produced much less IL-8 and TNF-α compared to LL-37-treated human keratinocytes without oligo-HAs, suggesting that oligo-Has have anti-inflammatory actions in rosacea.

To confirm the role of oligo-HAs *in vivo*, we used rosacea-like mouse models, which have been widely employed in studies of rosacea[2]. We observed prominent erythema and increased TEWL in LL-37-injected mice, which is consistent with the rosacea-like phenotype in human skin. Oligo-HA-treated mice developed a much smaller redness area, and decreased erythema index.

Next, we performed immunohistochemical analysis to explore immunological and skin barrier changes by oligo-HAs which resulted in decreased erythema in the rosacea-like mice model. A recent study suggested that papulopustular rosacea is characterized by an influx of T cells and changes in the inflammatory IL-17/interferon-γ cytokine profile[16]. In our study, mice injected with LL-37 showed significant CD4+ T cell infiltration and increased expression of IL-17, as shown in a previous study of rosacea, and treatment with oligo-HAs reduced IL-17 expression and CD4+ T cell infiltration. IL-17 activates keratinocytes to produce the chemokine ligands 1 and 8, facilitate neutrophil infiltration, and promote angiogenesis[17]. IL-17 is produced by not only Th17 cells but also keratinocytes[18]. In our study, IL-17A expression in the epidermis changed more dramatically than that in the dermis by LL-37 and oligo-HAs. Further studies of IL-17 expression in human rosacea skin and expression of other IL-17 family members are needed.

Several studies suggested a link between rosacea, sensitive skin, and disruption of the epidermal barrier function[19]. TEWL is a good indicator of the skin barrier function and is increased in many inflammatory skin disorders such as rosacea and atopic dermatitis. Studies demonstrated that oligo-HAs and HAs decrease TEWL[20]. To examine improvement effect of oligo-HAs on the epidermal barrier, we investigated TEWL and filaggrin expression in rosacea-like mouse model. We found that TEWL was mark-

edly decreased and filaggrin was significantly increased after oligo-HA injection in LL-37-treated rosacea-like mouse models, suggesting the epidermal barrier improving effect of oligo-HAs in the rosacea phenotype.

CD44 is known to be required to resolve pulmonary inflammation. CD44-deficient mice showed persistent inflammation following bleomycin-induced noninfectious lung injury, characterized by impaired clearance of apoptotic neutrophils and accumulation of HA fragments at the site of tissue injury[21]. The absence of CD44 also leads to suboptimal expression of IRAK-M, Tollip, and A20 in response to lipopolysaccharide *in vivo* and in macrophages *in vitro*. Inflammation is greater in CD44-knockout mice than in wild-type mice[22]. CD44 ligation on macrophages selectively promotes the uptake of neutrophils *in vitro*, and phagocytosis of apoptotic neutrophils provides an anti-inflammatory signal and promotes the release of transforming growth factor-β 1[23]. HA signaling can modify inflammatory reactions caused by microbial challenge[24]. *Demodex* mites and *Staphylococcus epidermidis* may also play a role as triggers of rosacea, and HA-CD44 interaction can protect against the inflammatory response mediated by these strains[25]. A recent study suggested that CD44 regulates tight junction assembly and barrier function. Upon barrier disruption, CD44 knockout mice showed delayed recovery of epidermal barrier functions[26]. In our study, oligo-HAs increased CD44 expression. The HA-CD44 interaction contributes to improving skin barrier functions, as confirmed by the increased filaggrin expression and reduction of TEWL.

There were some limitations to the present study. We did not investigate the molecular pathway of the anti-inflammatory and barrier repairing effect of oligo-HAs. Further research is needed on the molecular workup of cytokines such as IL-8 and TNF-α, which showed significant changes *in vitro*. The sample size was also small. However, our study revealed that oligo-HAs reduced inflammatory cell infiltration, IL-17 expression, and KLK5 expression, suggesting that oligo-HAs can modulate the expression of key pathologic molecules of rosacea. Oligo-HAs also increased CD44 and filaggrin expression, which may have contributed to improving the skin barrier functions. Collectively, oligo-HAs improved the rosacea-like phenotype via anti-inflammatory and epidermal barrier improving effect. Further studies of the specific molecular mechanism are needed.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

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