The Effects of Sonophoresis on the Expression of Hyaluronic Acid and CD44 in Mouse Skin
- The Role of TNF-α, IL-1α, and Calcium Ion

Sang Eun Lee

Department of Medicine
The Graduate School, Yonsei University

The Effects of Sonophoresis on the Expression of Hyaluronic Acid and CD44 in Mouse Skin - The Role of TNF-α, IL-1α, and Calcium Ion

Directed by Professor Seung Hun Lee

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This certifies that the Master's Thesis of
Sang Eun Lee is approved.

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Thesis Supervisor : ***Prof. Seung Hun Lee***

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Thesis Committee Member : ***Prof. Kyung-Su Kim***

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Thesis Committee Member: ***Prof. Dong Kyun Rah***

The Graduate School
Yonsei University

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Finally, I would like to thank with all my heart to my entire family-my father, mother and brother- very much for their support and encouragement.

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<ABSTRACT>

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Hyaluronic acid (HA) is a major extracellular matrix component in the epidermis that plays a role in cellular migration, proliferation and differentiation through it's major cell surface receptor, CD44. HA has been demonstrated to be accumulated in the epidermis by permeability barrier disruption. Previously we demonstrated that sonophoresis can modulate the epidermal calcium gradient and stimulate epidermal cytokine expressions. We performed this study to identify whether sonophoresis could increase the expression of HA and CD44 in mouse epidermis without barrier disruption and to uncover the mechanisms involved in the upregulation of HA and CD44 expression following sonophoresis. Sonophoresis without transepidermal water loss change significantly increased the HA expression in mouse epidermis at 6 h after sonophoresis compared to untreated skin as well as in tape-stripped skin used as a positive control. The increased expression of HA was temporally and locally associated with increased expression of hyaluronic acid synthase (HAS)3 and CD44. To test whether TNF-α and IL-1α may have a functional role in sonophoresis-induced increase of HA and CD44, we used TNF-α and IL-1 specific inhibitors and the expression of HA, HAS3, and

CD44 showed significant inhibition at 6 h after sonophoresis in each cytokine inhibitor pretreated skin compared to the skin without pretreatment. To determine whether the epidermal calcium gradient changes may involve in the upregulation of HA and CD44 following sonophoresis, we compared HA, HAS3, and CD44 expression in epidermis treated with sonophoresis of Ca^{2+}-free gel vs. Ca^{2+}-containing gel. Ion capture cytochemistry revealed that sonophoresis of Ca^{2+}-containing gel prevented the epidermal calcium gradient change by excess calcium at all levels of the epidermis. The expression of HA, HAS3, and CD44 mRNA and immunohistochemical protein staining decreased in the epidermis after sonophoresis of Ca^{2+}-containing gel vs. Ca^{2+}-free gel, suggesting that the change in calcium ion can stimulate the expression of HA, HAS3, and CD44 in epidermis. To determine whether the upregulation of HA and CD44 is stimulated by TNF-α and IL-1α directly, three different concentrations of TNF-α (50ng, 100ng, 300ng in 0.1ml PBS) or IL-1α (50ng, 100ng, 300ng in 0.1ml PBS) were injected intradermally into the flanks of hairless mouse. The results showed a dose dependent stimulation of HA, HAS3, and CD44 expression by both cytokines. From these results we can suggest that epidermal calcium gradient change and sequentially induced TNF-α and IL-1α by sonophoresis could upregulate the HA expression through HAS3 induction and CD44 expression without barrier impairment.

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I. INTRODUCTION

Hyaluronic acid (HA), a high-molecular-weight linear polymer composed of repeating units of D-glucuronic acid and N-acetyl-D-glucosamine is a major extracellular matrix in skin which serves not only structural roles but also plays a dynamic roles in many biological process[1]. While HA is known to be expressed exclusively in the dermis, its actual concentration around the cells is higher in the epidermis than dermis[2,3]. In epidermis, HA is strongly expressed around the basal and spinous cells[4,5] and recently it has been demonstrated that HA exists in the normal stratum corneum[6].

HA is synthesized by hyaluronic acid synthase (HAS) at the inner surface of the plasma membranes[7,8] and among the three isoenzymes of HAS (HAS1, 2, 3), the expression of HAS2 and HAS3 play crucial roles in the regulation of HA synthesis in human skin fibroblasts and keratinocytes, respectively, but the precise regulatory mechanisms has still remained uncertain[9,10].

CD44 is a major cell surface receptor for HA and expressed in the whole

epidermis with stronger signals in the suprabasal layers, except for the upper granular cell layers[11]. HA binds to cells through CD44 and induces downstream signaling pathways leading to the onset of HA-dependent functions in various cell types and tissues,[12] but the functions of epidermal HA and CD44 interaction are not well known.

Previous study demonstrated that HA and CD44 interaction with Rac1-PKNγ plays a pivotal role in PLCγ1-regulated Ca^{2+} signaling and cortactin-cytoskeleton function required for keratinocyte cell-cell adhesion and differentiation[13]. In recent study about the role of HA and CD44 interaction in the epidermal permeability barrier, it has been demonstrated that CD44 deficiency in CD44 knockout mice skin lead to decreased proliferation and differentiation, delayed permeability barrier recovery and decreased cholesterol synthesis, lamellar body formation and secretion suggesting that HA and CD44 interaction regulates the normal epidermal permeability barrier functions[14].

It has been shown that HA is accumulated in the epidermis by epidermal injury or barrier disruption using acetone application and this HA response is associated with a strong induction of HAS2, HAS3 and CD44 expression, and with considerable epidermal hyperplasia[15,16]. The mechanisms are postulated that locally released growth factors, such as epidermal growth factor (EGF), keratinocyte growth factor (KGF) and cytokines which are activated during wound healing may contribute to stimulate HA synthesis in response to skin injury[17,18,19]. But, Several studies have reported that glycolic acid (GA) application increases HA content of human skin in both epidermis and dermis without epidermal permeability barrier impairment[20]. The mechanism of this HA response is not known, but from these results we can speculate that HA could be upregulated by not only skin injury but also other mechanisms. In our previous study, we reported that GA can modulate the epidermal calcium gradient through the chelating effects on the cationic ions without

permeability barrier disruption, resulting in stimulation of lamellar body secretion and epidermal cytokine expressions[21]. These effects of GA on the epidermal permeability barrier is very similar to the effect of permeability barrier disruption, thus we hypothesized that any procedures which can modulate the epidermal calcium gradient are able to upregulate the HA content in the epidermis without permeability barrier impairment. Recently, sonophoresis also has been reported to be able to decrease the calcium concentration in the outer epidermis without disrupting barrier function and then stimulate the generation of primary cytokines and lamellar body secretion[22,23].

We performed this study to identify whether sonophoresis could increase the expression of HA and CD44 in mouse epidermis without barrier disruption and to uncover the mechanisms involved in the upregulation of HA and CD44 expression following sonophoresis.

To determine whether TNF-α and IL-1α may have a functional role in sonophoresis-induced increase of HA and CD44, we used TNF-α and IL-1 inhibitor and compared the expression of HA, HAS3, and CD44 in epidermis after sonophoresis with cytokine inhibitor pretreatement vs. sonophoresis alone. To determine whether the expression of HA and CD44 are regulated by changes of epidermal calcium ion, we compared HA, HAS3, and CD44 expression in epidermis treated with sonophoresis of Ca^{2+}-free gel vs. 1.5mM Ca^{2+}-containing gel and treated with immersion in PBS containing 1.2mM Ca^{2+} after tape-stripping vs. tape-stripping alone. In addition, to determine whether the upregulation of HA and CD44 is stimulated by TNF-α and IL-1α directly, three different concentrations of TNF-α (50ng, 100 ng, 300ng in 0.1ml PBS) or IL-1α (50ng, 100 ng, 300ng in 0.1ml PBS) were injected intradermally into the flank of mouse.

II. MATERIALS AND METHODS

1. Animals

Adult female hairless mice were purchased from the animal laboratory of Yonsei University and were 8 to 12 weeks old at the time of study. The research protocols were approved by the institutional review board of the Yonsei University College of Medicine.

2. Sonophoresis treatment

After anesthesia with chloral hydrate, the treatment sites on the back received 5 min of 800 mW per ㎠, 3-MHz continuous wave ultrasound (Jung Hoon Corporation, Korea) as in our previous study[23] in which TEWL did not increase after treatment. The negative control sites were only dabbed with the transmission gel (Biosonic, Amite) generally applied before ultrasound treatment. The ultrasound probe was positioned at a distance of the applied transmission gel thickness from the stratum corneum surface. The positive control sites were achieved by sequential applications of cellophane tape (six to eight times). Samples for immunohistochemical stain and real-time quantitative RT-PCR studies were obtained 6 h following barrier disruption.

A. Soluble TNF receptor p75 fusion protein pretreatment

To examine the roles of TNF-α as one of the factors which may be involved in sonophoresis-induced upregulation of HA and CD44 expressions, a soluble TNF receptor p75 fusion protein (Etanercept, Amgen and Wyeth, Thousand Oak, CA) was used to neutralize the

effect of TNF-α. Mice were intraperitoneally given etanercept 10mg at 30 min before sonophoresis and tape-stripping.

B. IL-1α receptor antagonist pretreatment

To examine the roles of IL-1α as one of the factors which may be involved in sonophoresis-induced upregulation of HA and CD44 expressions, a recombinant mouse IL-1 receptor antagonist (IL-1ra, R&D systems, Abingdon, UK) was used as specific blocker for IL-1α. Mice were pretreated intradermally with IL-1ra 50μg in 0.1ml of PBS into the flank at 2 h before sonophoresis and tape-stripping.

C. Inhibition of epidermal calcium gradient changes

To determine whether HA and CD44 expressions are regulated by alterations in extracellular calcium, we experimentally altered the extracellular calcium gradient without disrupting the barrier, using high-frequency sonophoresis as described above. To prevent the epidermal calcium gradient change by sonophoresis using transmission gel without added calcium, sonophoresis using transmission gel with added calcium (1.5mM) was performed. To investigate whether HA upregulation following barrier disruption also regulated by epidermal calcium gradient change, we employed the immersion technique. Immediately after tape-stripping induced barrier disruption, the tape-stripped mouse was immersed in PBS containing calcium for 2 h. At 6 h after sonophoresis and tape-stripping, skin biopsy specimens were taken for immunohistochemical stain, real-time quantitative RT-PCR and calcium-capture cytochemistry.

3. TNF-α and IL-1α administration

To determine whether cytokine administration increases HA and

CD44 directly in mouse epidermis, TNF- α (50ng, 100 ng, 300ng in 0.1ml PBS), IL-1α (50ng, 100 ng, 300ng in 0.1ml PBS) was injected intradermally into the flanks of adult female hairless mice aged 8–12 week. Samples for immunohistochemical stain and real-time quantitative RT-PCR studies were taken 6 h after cytokine administration.

4. Calcium-capture cytochemistry

Biopsy specimens were immediately cut into small pieces (0.5 mm^3) in a drop of ice cold fixative containing 2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate, and 1.4% sucrose and fixed overnight on ice. The fixative was removed and the samples post-fixed in osmium/pyroantimonate for 2 h on ice and washed for 10 min in ice cold distilled water at pH 10. All post-fixed tissues were rinsed in 0.1 M cacodylate buffer for 10 min, dehydrated in a graded ethanol series, and embedded in epon - epoxy resin. Ultrathin sections (UCT, Leica, Bensheim, Germany) were cut, double stained with uranyl acetate and lead citrate, and examined with a transmission EM. Each section was incubated with ethylenediamine tetraacetic acid as a control.

5. Immunohistochemistry

Paraffin-embedded skin tissues were cut in 5 μm sections. After deparaffinization and rehydration, the sections were incubated with rat anti-CD44 and biotin-conjugated HA binding protein followed by adding ABC peroxidase reagent. Subsequently, peroxidase activity was localized with diaminobenzidine substrate (Vector Labs, Burlingame, CA). As controls, skin sections were incubated with preimmune serum followed by incubating with ABC peroxidase reagents.

A. CD44 staining

The deparaffinized sections were incubated for 30 min at 95°C in antigen retrieval solution (Dako, Carpinteria, California). After blocking the tissue peroxidase activity and unspecific binding, the sections were incubated with an anti-CD44 antibody (IM7, a kind gift from Dr Jayne Lesley, San Diego) overnight at 4°C and then with biotinylated anti-rat secondary antibody (Rabbit polyclonal CD44, Abcam, Cambridge, United Kingdom, 1:100) for 1 h at room temperature. Avidin–biotin peroxidase and DAB treatments were carried out.

B. Hyaluronic acid staining

For detection of HA, rehydrated specimens were blocked in 3% normal donkey serum, then overlaid with 10 μg/ml of the biotinylated probe (biotinylated HA-binding protein (bHABP), from Seikagaku Ltd, Tokyo, Japan) in PBS and 3% donkey serum overnight at 4°C in a humidified chamber. Cy3-conjugated streptavidin (1:500; Jackson Immunoresearch, West Grove, PA) was used to detect the bHABP. Slides were mounted in 30% glycerol.

C. Hyaluronic acid synthase staining

After preincubation with 1% bovine serum albumin, primary antibodies were applied for 60 min. HAS were detected with rabbit anti-mouse HAS2 and HAS3 primary antibodies (1:50; provided by J.A.M., Salt Lake City, Utah, USA) in combination with Cy2-coupled goat anti rabbit secondary antibody (1:50; Dianova, Hamburg, Germany).

6. Separation of epidermis

After cervical dislocation, skin excised from the treated area was placed epidermis side downward on plastic Petri dishes. Subcutaneous fat was removed with a scalpel, and skin was then floated epidermis upward on 10 mL of 10 mM ethylenediamine tetraacetic acid (EDTA) in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline(PBS). Incubations were continued at 37°C for 35 min in order to separate the epidermis from the dermis. Epidermis was scraped off the underlying dermis with a scalpel and kept at -70°C.

7. RT-PCR

Total RNA was isolated from each skin obtained after sonophoresis and tape-stripping using Trizol reagent. One microgram of total RNA was reverse-transcribed with AMV reverse transcriptase (Promega, Madison, WI). Pairs of primers for amplification of HABP4, HAS2, HAS3 and CD44 were designed using the Primer Express Software (Applied Biosystems, Foster City, CA). In all experiments, primer concentrations were first optimized to avoid unspecific binding of primers, and after running the PCR products, a dissociation curve analysis was performed to verify the specificity of the amplification products. The primers used for the RT-PCR of mouse HABP4, HAS2, HAS3, CD44 and GAPDH are shown in Table I.

Table I. Primers used in the RT-PCR reactions

mHAS2	5'-ACAGGCACCTTACCAACAGGGTGT-3'	24mer
	5'-GCATGCAT GATCAAAGTTCCCACG-3'	25mer
mHAS3	5'-ACTGCCTTCAAGCCCTTGG-3'	20mer
	5'-AATGTTCCAGATGCGGCCAC-3'	20mer
CD44	5'-CCCACCATGGACCAAATGA-3'	19mer
	5'-GGTGCTCCGGATAAAGAAGGA-3'	21mer
mHABP4	5'-TTTGACCAAAGAGGGAAACG-3'	20mer
	5'-GAACCTTTGGCTGCAGACTCC-3'	20mer
mGAPDH	5'-AATGGTGAAGGTCGGTGTGA-3'	20mer
	5'-CTGGAAGATGGTGATGGGC-3'	19mer

8. Statistical analyses

Data were expressed as mean ± SE. Statistical analyses were performed using paired and unpaired Student's t-tests.

III. RESULTS

1. Sonophoresis does not affect TEWL, but increases the hyaluronic acid, CD44 and hyaluronic acid synthase3 in mouse epidermis

Sonophoresis did not affect TEWL values, either immediately following, or at 3, 6, 24 h after sonophoresis (data not shown).

(1) Sonophoresis induces the accumulation of hyaluronic acid in mouse epidermis.

In untreated skin, high levels of HA were seen in the dermis, whereas HA levels between keratinocytes of the epidermis were barely detectable. After sonophoresis, however, epidermal HA accumulated to high levels within the basal and lower spinous layer in the intercellular spaces. The pattern of staining was similar to that of tape-stripped skin as a positive control (Figure 1a). The expression of mRNA of HA-binding protein (HABP)4 increased in murine epidermis at 6 h after sonophoresis on skin surface compared to mRNA levels of untreated skin as well as the tape-stripped skin used as a positive control (Figure 1b).

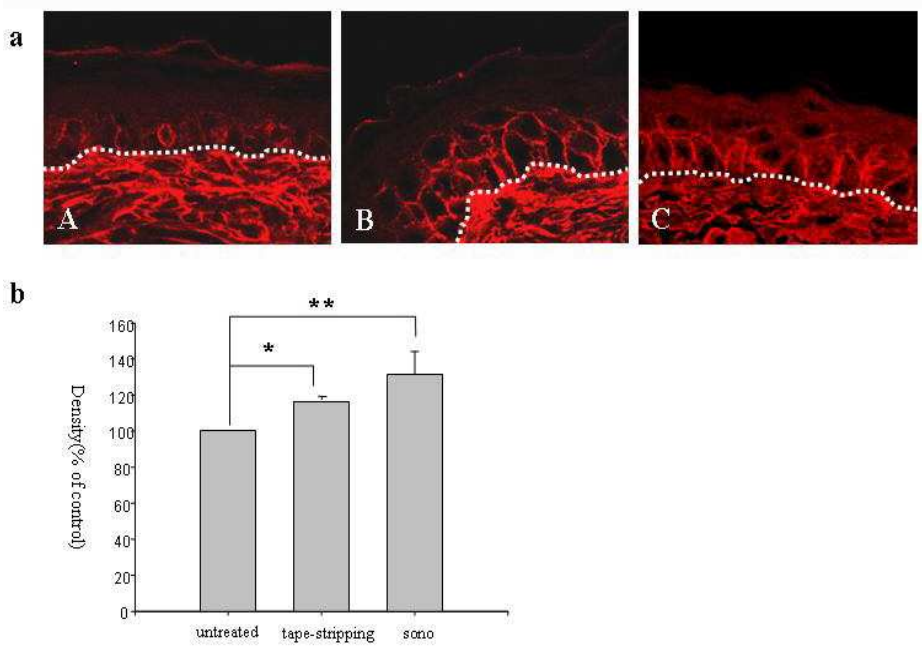

Figure 1. Hyaluronic acid (HA) expression in tape-stripped and sonophoresis-treated mouse epidermis. (a) Sections were examined by confocal microscopy to analyze the localization of HA in the epidermis after tape-stripping (B) and sonophoresis (C). In untreated skin (A), HA were barely detectable, however, 6 h after sonophoresis the HA-specific signal (Cy3, red), is markedly increased within the basal and spinous layers in the intercellular space similar to the tape stripped sites. Dashed lines, dermal-epidermal junction. (b) Epidermal HA content in untreated, tape-stripped and sonophoresis-treated skin quantified by RT-PCR. The expression of mRNA of HA-binding protein(HABP)4 was increased 6 h after sonophoresis compared to the untreated epidermis. *p<0.05; **p<0.001 (Student's t test). **sono**, sonophoresis.

(2) Sonophoresis increases the protein expression and mRNA levels of CD44 in mouse epidermis.

In untreated skin, CD44 was seen in suprabasal and spinous layer with a pattern of typical membrane staining. The staining intensity was weak in normal epidermis, however, after sonophoresis there was significant increase of CD44 expression in the epidermis. The pattern of staining was similar to that of tape-stripped skin as a positive control (Figure 2a). The expression of mRNA of CD44 increased in murine epidermis at 6 h after sonophoresis on skin surface compared to mRNA levels of untreated skin as well as the tape-stripped skin used as a positive control (Figure 2b).

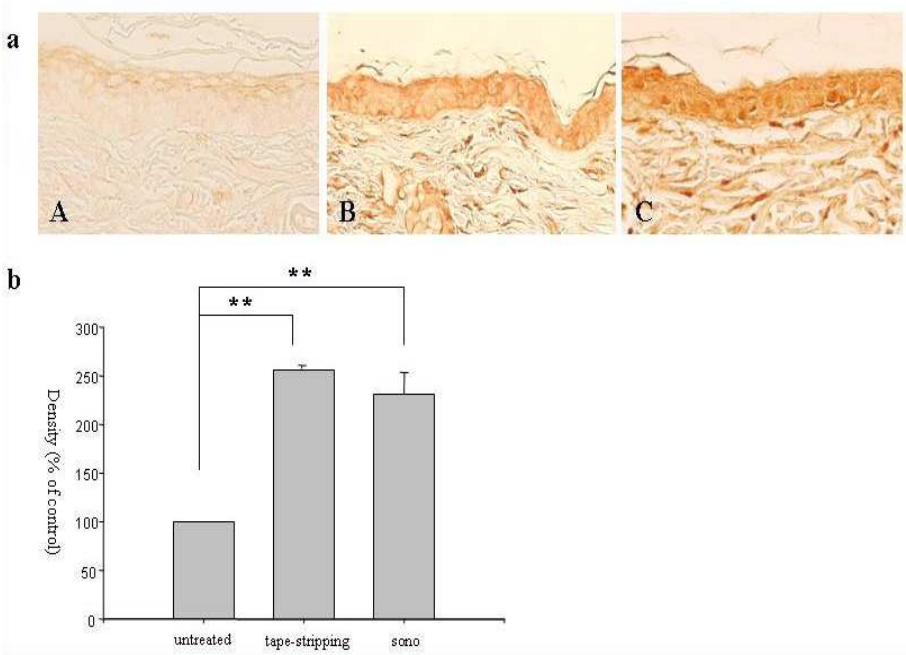

Figure 2. CD44 expression in tape-stripped and sonophoresis-treated mouse epidermis. (a) Immunohistochemical staining for CD44 of control (A), tape-stripped (B) and sonophoresis (C)-treated hairless mouse skin. The CD44 expression was increased 6 h after sonophoresis compared to the untreated

epidermis. (b) RNA was extracted from the epidermis and RT-PCR was performed. The expression of mRNA of CD44 was increased 6 h after sonophoresis compared to the untreated epidermis. $^{**}p<0.001$ (Student's *t* test). **sono**, sonophoresis.

(3) Sonophoresis increases the protein expression and mRNA levels of hyaluronic acid synthase3, but not hyaluronic acid synthase2 in mouse epidermis.

Immunofluorescent signals of hyaluronic acid synthase (HAS)3 were barely detectable in normal epidermis. However, after sonophoresis, there was significant increase of HAS3 expression in the epidermis as well as the tape-stripped skin used as a positive control. Immunofluorescent signals of HAS3 were distributed in the cytoplasm of keratinocytes (Figure 3a). The expression of mRNA of HAS3 increased in murine epidermis at 6 h after sonophoresis on skin surface compared to mRNA levels of untreated skin as well as the tape-stripped skin used as a positive control. However, HAS2 mRNA levels showed no significant increase in both sonophoresis-treated and tape-stripped skin compared to the untreated epidermis, suggesting that HAS3 expression is responsible for sonophoresis induced upregulation of HA in keratinocytes of mouse epidermis (Figure 3b).

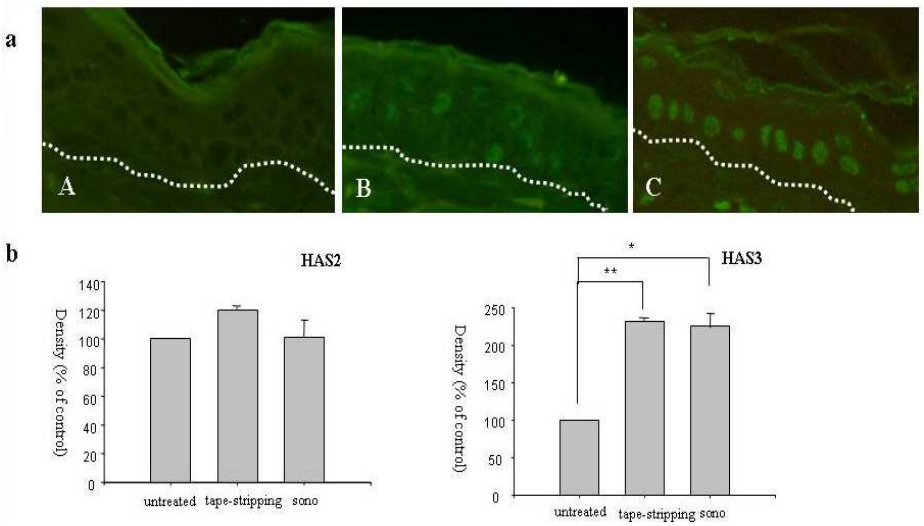

Figure 3. Hyaluronic acid synthase (HAS) expression in tape-stripped and sonophoresis-treated mouse epidermis. (a) Immunofluorescent staining for HAS3 of control(A), tape-stripped (B) and sonophoresis (C)-treated hairless mouse skin. In confocal image the HAS3 expression was increased 6 h after sonophoresis compared to the untreated epidermis. Dashed lines, dermal-epidermal junction. (b) Analysis of the mRNA levels of the two HAS isoforms in the epidermis using RT-PCR. The expression of mRNA of HAS3 was increased 6 h after sonophoresis compared to the untreated epidermis, whereas the expression of mRNA of HAS2 showed no significant increase in both sonophoresis-treated and tape-stripped skin compared to the untreated epidermis. *p<0.05; **p<0.001 (Student's t test). **sono**, sonophoresis.

2. Sonophoresis-induced hyaluronic acid and CD44 expression in mouse epidermis are regulated by TNF-α.

To investigate the role of TNF-α in the upregulation of HA, HAS3 and CD44 expressions following sonophoresis treatment, mouse were pretreated with etanercept intraperitoneally and treated with sonophoresis as described above. CD44 and HA expressions showed significant inhibition at 6 h after sonophoresis in etanercept pretreated skin compared to only sonophresis- treated skin. Pretreatment with etanercept before sonophoresis significantly decreased the mRNA levels of HA-binding protein (HABP)4, CD44, HAS3 (Figure 4). These results suggest that TNF-α may play a role, at least in part, in sonophoresis-induced HA and CD44 expression.

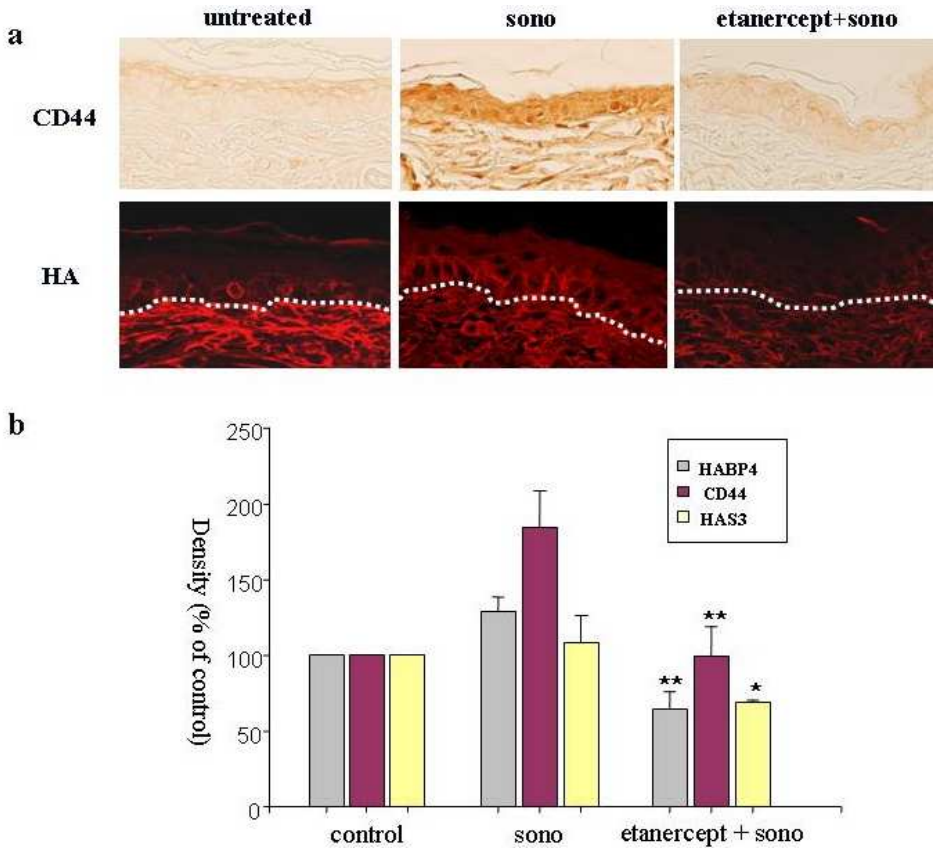

Figure 4. A pretreatment with TNF-α antagonist abolished sonophoresis - induced increase of hyaluronic acid (HA), CD44 and hyaluronic acid synthase (HAS)3 expression. (a) Immunohistochemical staining for HA and CD44 of sonophoresis-treated skin with and without etanercept pretreatment. The expression of HA and CD44 in the epidermis pretreated with etanercept followed by sonophoresis was significantly decreased compared to that of sonophoresis-treated epidermis. Dashed lines, dermal-epidermal junction. (b) Total RNA was extracted, reverse-transcribed, and analyzed by RT-PCR using GAPDH as an endogenous control. Results are illustrated relative to the mRNA levels of the control.

The mRNA expression of HA-binding protein (HABP)4, CD44 and HAS3 of the skin pretreated with etanercept 30 min before sonophoresis was significantly inhibited compared to the sonophoresis-treated skin. *p<0.05 vs. sonophoresis; **p<0.001 vs. sonophoresis (Student's t test). **sono**, sonophoresis.

3. Sonophoresis-induced hyaluronic acid and CD44 expression in mouse epidermis are regulated by IL-1α.

To investigate the role of IL-1α in the upregulation of HA, HAS3 and CD44 expressions following sonophoresis treatment, mouse were pretreated with IL-1 receptor antagonist (IL-1ra) intradermally and treated with sonophoresis as described above. CD44 and HA expression showed significant inhibition at 6 h after sonophoresis in IL-1ra pretreated skin compared to only sonophoresis-treated skin. Pretreatment with IL-1ra before sonophoresis significantly decreased the mRNA levels of HA-binding protein (HABP)4, CD44, HAS3 compared to only sonophoresis-treated skin (Figure 5). These results suggest that IL-1α may play a role, at least in part, in sonophoresis-induced HA and CD44 expression.

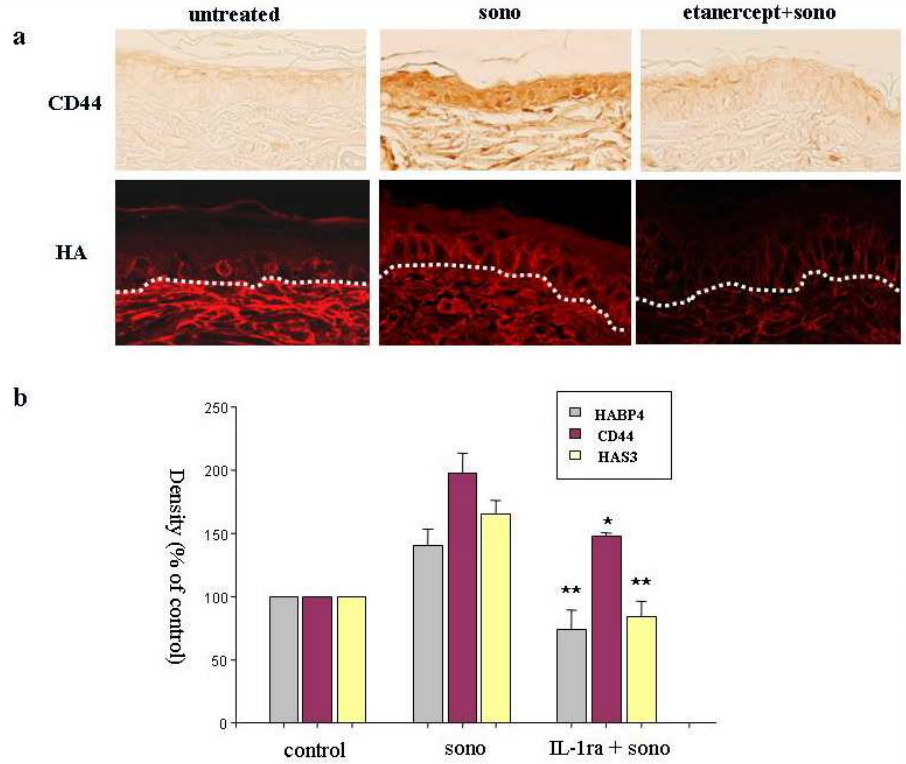

Figure 5. A pretreatment with IL-1 receptor antagonist (IL-1ra) abolished sonophoresis-induced increase of hyaluronic acid (HA), CD44 and hyaluronic acid synthase (HAS)3 expression. (a) Immunohistochemical staining for HA and CD44 of sonophoresis-treated skin with and without IL-1ra pretreatment. The expression of HA and CD44 in the epidermis pretreated with IL-1ra followed by sonophoresis was significantly decreased compared to that of sonophoresis- treated epidermis. Dashed lines, dermal-epidermal junction. (b) Total RNA was extracted, reverse-transcribed, and analyzed by RT-PCR using GAPDH as an endogenous control. Results are illustrated relative to the mRNA levels of the control. The mRNA expression of HA-binding protein (HABP)4, CD44 and HAS3 of the skin pretreated with IL-1ra 2 h before sonophoresis was significantly inhibited compared to the sonophoresis-treated skin. *p<0.05 vs. sonophoresis; **p<0.001 vs. sonophoresis (Student's t test). **sono**, sonophoresis.

4. Administration of intracutaneous TNF-α and IL-1α increases

the expression of hyaluronic acid, CD44 and hyaluronic acid synthase 3.

To determine whether the upregulation of HA and CD44 is stimulated by TNF-α and IL-1α directly, three different concentrations of TNF-α (50ng, 100 ng, 300ng in 0.1ml PBS) or IL-1α (50ng, 100 ng, 300ng in 0.1ml PBS) was injected intradermally into the flanks of adult hairless mice. Intradermal injection of TNF-α and IL-1α to the dorsal surface of mice showed increased expressions of HA and CD44 in epidermis at 6 h after administration compared to untreated skin (Figure 6, 7). Quantitative analysis by RT-PCR was performed. There was no significant increase in mRNA levels of HA-binding protein (HABP)4, HAS3 and CD44 with 50ng TNF-α /0.1ml PBS injection compared to untreated skin, however, there was a significant increase with injection of 100ng/0.1ml PBS and 300ng/0.1ml PBS of TNF-α (Figure 6). IL-1α injection also showed similar dose dependent increase of mRNA levels of HABP4, HAS3 and CD44 (Figure 7). These results suggest the dose dependent stimulation of HA and CD44 expressions by TNF-α and IL-1α in mouse epidermal keratinocytes.

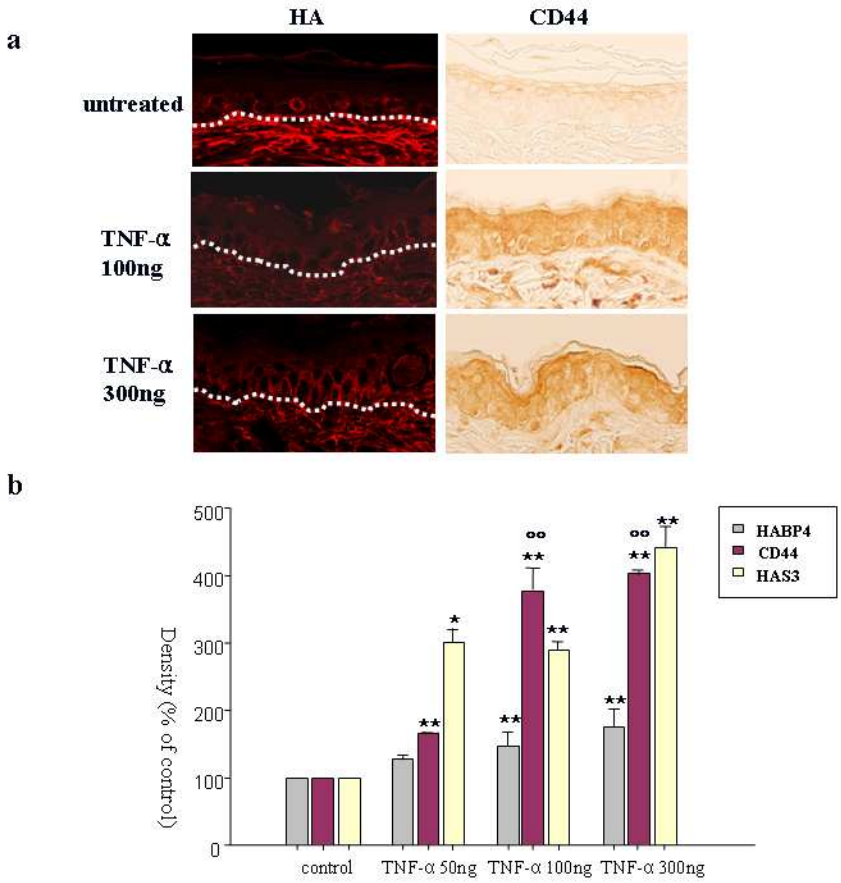

Figure 6. TNF-α increased the expression of hyaluronic acid (HA), CD44 and hyaluronic acid synthase (HAS)3 in mouse epidermis. (a) Immunohistochemical staining for HA and CD44 of TNF-α injected mouse skin. The HA and CD44 expression was increased in epidermis 6 h after TNF-α injection compared to control. TNF-α 300ng/0.1ml PBS injected skin showed more strong intensity of immunohistochemical stain of HA and CD44 compared to TNF-α 100ng/0.1ml PBS injected skin. (b) The mRNA expression of HA-binding protein (HABP)4, CD44 and HAS3 was quantitated by RT-PCR in epidermis 6 h after injection. The mRNA levels of HABP4, CD44 and HAS3 were increased in a dose-dependent manner according to the injected doses of TNF-α. **p*<0.05 vs. TNF-α 0ng/0.1ml injected epidermis; ***p*<0.001 vs. TNF-α

0ng/0.1ml injected epidermis; ˚˚p<0.001 vs. TNF-α 50ng/0.1ml injected epidermis (Student's t test).

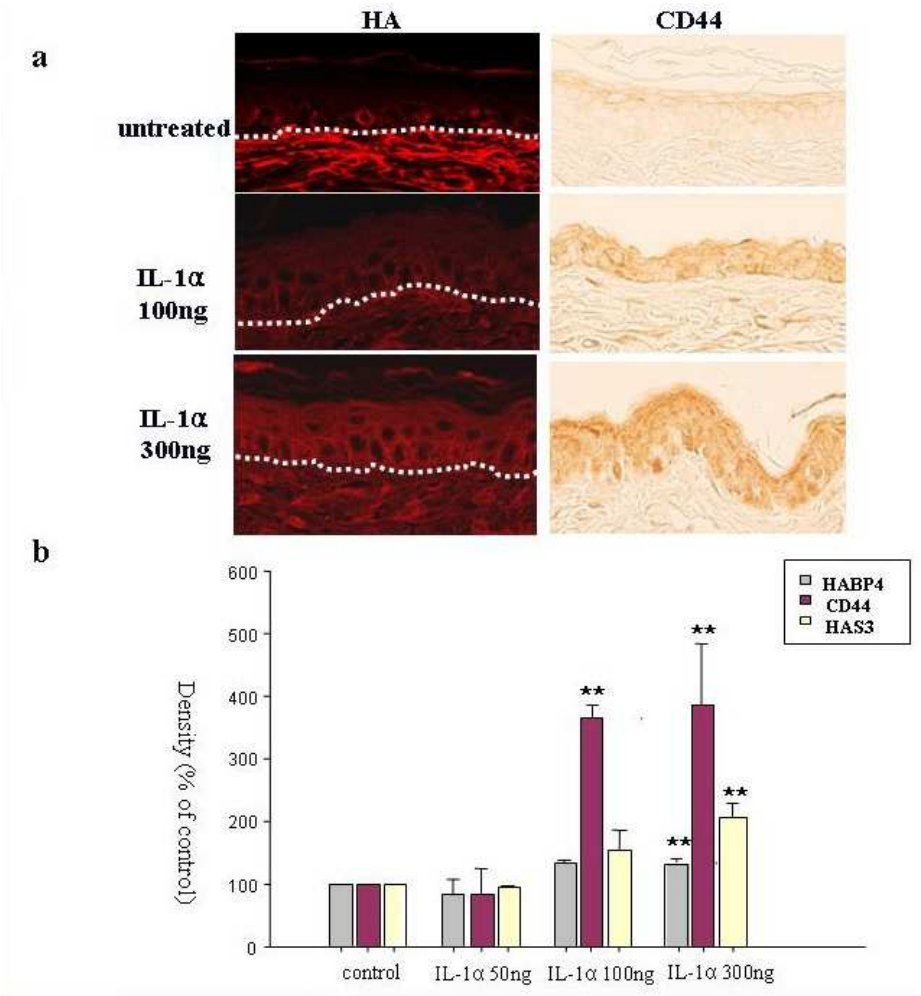

Figure 7. IL-1α increased the expression of hyaluronic acid (HA), CD44 and hyaluronic acid synthase (HAS)3 in mouse epidermis.
(a) Immunohistochemical staining for HA and CD44 of IL-1α injected mouse skin. The HA and CD44 expression was increased in epidermis 6 h after IL-1α injection compared to control. IL-1α 300ng/0.1ml PBS injected skin showed more strong intensity of immunohistochemical stain

of HA and CD44 compared to IL-1α 100ng/0.1ml PBS injected skin. (b) The mRNA expression of HA-binding protein (HABP)4, CD44 and HAS3 was quantitated by RT-PCR in epidermis 6 h after injection. The mRNA levels of HABP4, CD44 and HAS3 were increased in a dose-dependent manner according to the injected doses of IL-1α. $**p<0.001$ vs. IL-1α 0ng/0.1ml injected epidermis (Student's *t* test).

5. The inhibited depletion of calcium ion in the upper epidermis by sonophoresis with Ca^{2+}-containing gel and 2 h of immersion in PBS including calcium ion after tape-stripping abolished sonophoresis- and tape-stripping- induced increase of hyaluronic acid, CD44, and hyaluronic acid synthase3 mRNA expression.

To assess whether the upregulation of HA, HAS3 and CD44 expressions following sonophoresis was associated with the depletion of calcium ion at the upper epidermis, we used sonophoresis of 1.5mM Ca^{2+}- containing gel and compared the expression of HA and CD44 after sonophoresis of transmission gel with versus without added calcium ion. After sonophoresis of Ca^{2+}- containing gel, which elevated the calcium content of the upper epidermis (Figure 8), the expression of HA and CD44 protein and mRNA showed significant inhibition compared to that of Ca^{2+}- free gel sonophresed skin (Figure 9, 10). To assess whether the upregulation of HA, HAS3 and CD44 expressions following tape-stripping was also associated with the depletion of calcium ion at the upper epidermis, we used immersion technique, with solution containing calcium ion. After 2 h of immersion in PBS containing 1.2mM calcium ion immediately after tape-stripping, the mRNA expression of HA-binding protein (HABP)4, CD44 and HAS3 was significantly inhibited compared to the

upregulated expression after tape-stripping alone (Figure 10). These results suggest that depletion of calcium ion at the upper epidermis by sonophoresis and tape-stripping may play a role in sonophoresis- and tape-stripping- induced upregulation of HA and CD44 expression.

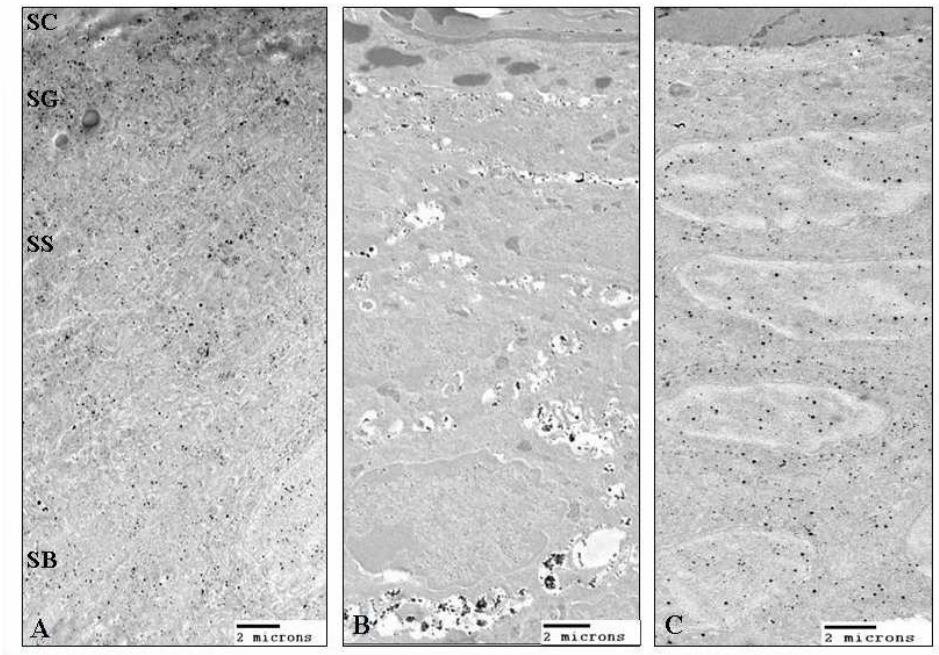

Figure 8. Calcium ion distribution in the epidermis after sonophoresis of a Ca^{2+}- free gel and 1.5mM Ca^{2+}- containing gel. Epidermal calcium gradient in control murine epidermis (A), following sonophoresis of a Ca^{2+}- free gel(B) and following sonophoresis of a Ca^{2+}- containing gel (C). These samples were obtained immediately after sonophoresis. While the control epidermis showed normal epidermal calcium gradient, with high concentration at the stratum granulosum and lower at the stratum spinosum and the stratum basale (A), the epidermis treated with sonophoresis of a Ca^{2+}- free gel showed decreased calcium precipitates in the upper epidermis and increased calcium precipitates in the lower epidermis (B). However, the epidermis treated with sonophoresis

of a Ca^{2+}- containing gel showed increased calcium precipitates in all nucleated layers of the epidermis (C). Scale bar, 2㎛. SC, strtum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale.

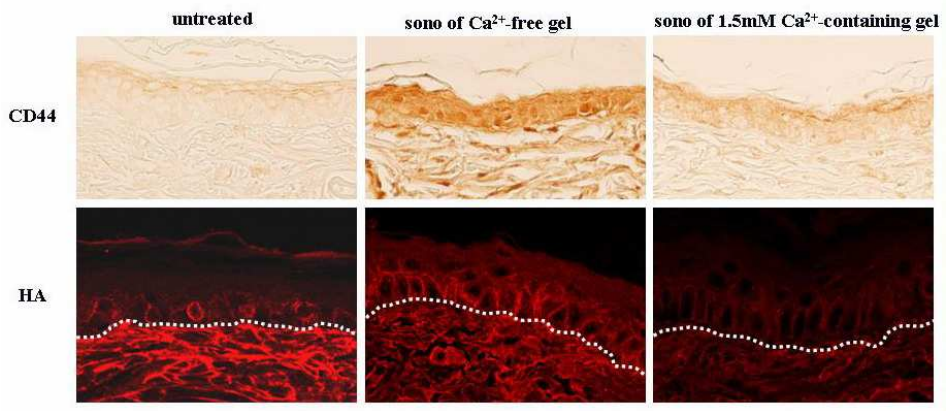

Figure 9. Immunohistochemical staining for HA and CD44 of sonophoresis treated skin with Ca^{2+}- free gel and 1.5mM Ca^{2+}- containing gel. The expression of HA and CD44 was significantly inhibited at 6 h after sonophoresis of Ca^{2+}- containing gel versus Ca^{2+}- free gel. Dashed lines, dermal-epidermal junction.

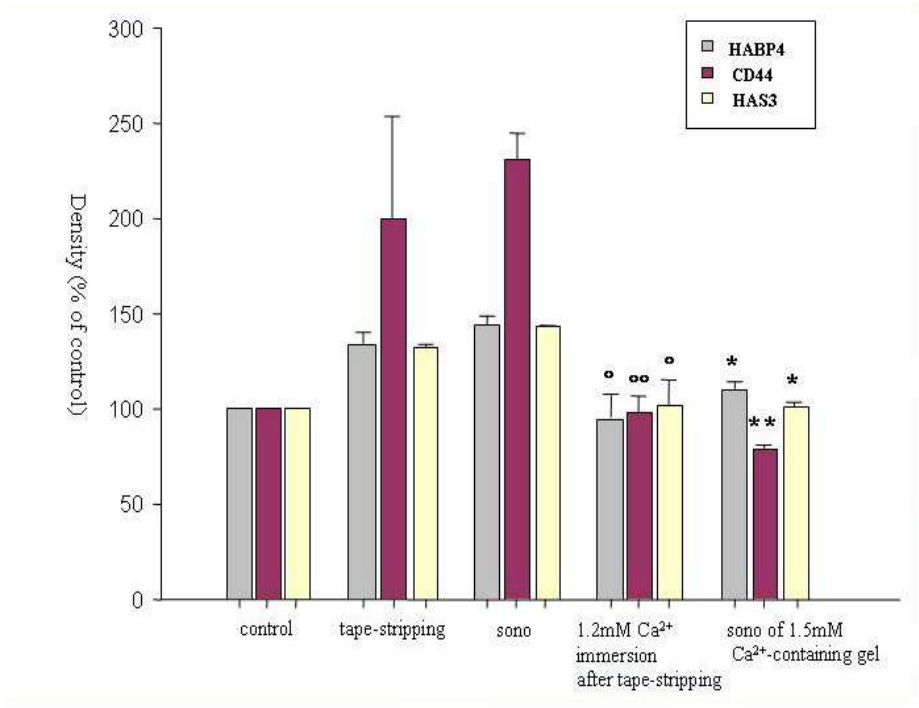

Figure 10. The inhibited depletion of calcium contents in the upper epidermis by sonophoresis with Ca^{2+}- containing gel and 2 h of immersion in PBS including calcium ion after tape-stripping abolished sonophoresis- and tape-stripping- induced increase of hyaluronic acid (HA), CD44 and HAS3 mRNA expression. Total RNA was extracted, reverse-transcribed, and analyzed by RT-PCR using GAPDH as an endogenous control. Results are illustrated relative to the mRNA levels of the control. The mRNA levels of HA-binding protein (HABP)4, CD44 and HAS3 were significantly decreased in epidermis treated with sonophoresis of 1.5mM Ca^{2+}- containing gel compared to Ca^{2+}-free gel and also decreased in epidermis by 2 h of immersion in PBS including 1.2mM calcium ion after tape-stripping compared to tape-stripping alone. *p<0.05 vs. sonophoresis; **p<0.001 vs. sonophoresis; °p<0.05 vs. tape-stripping; °°p<0.001 vs. tape-stripping (Student's t test). **sono**, sonophoresis.

IV. DISCUSSION

Recently, it has been shown that wound-induced upregulation of HA synthesis is not limited to dermal mesenchymal cells, but is very prominent also in the epidermis, suggesting that epidermal HA synthesis after tissue trauma is an epidermal wound healing response[16]. Another previous study demonstrated that a minor trauma which causes an epidermal barrier disruption such as an application of acetone on mouse skin also could increase epidermal HA content[15]. However, the present study demonstrates that sonophoresis without significant TEWL changes could upregulate epidermal HA expression in mouse skin at 6h after sonophoresis (Fig 1). The increased expression of HA was temporally and locally associated with increased expression of HAS3 and CD44 (Fig 2, 3). The exact mechanisms of wound-induced epidermal HA expression has not been fully elucidated, but was reported to be associated with a strong induction of HAS2 and HAS3 by locally released EGF and KGF. We hypothesized that other factors induced by sonophoresis may stimulate HAS induction and increase HA synthesis in epidermis.

Previously Menon et al[22] demonstrated that sonophoresis can altered the epidermal calcium gradient without disrupting permeability barrier and that changes in calcium gradient induced lamellar body secretion and our previous study demonstrated that changes of epidermal calcium gradient by sonophoresis directly induce the expression of epidermal cytokines, such as IL-1α and TNF-α, independent of barrier disruption similar to the acute barrier perturbation model[23]. Thus, we hypothesized that the epidermal calcium gradient change and calcium ion-induced cytokines, such as TNF-α and IL-1α may be involved in the upregulation of HA and CD44 in mouse epidermis following sonophoresis. By using a soluble TNF-α receptor fusion protein and IL-1 receptor antagonist, we have demonstrated

that TNF-α and IL-1α play a role, at least in part, in sonophoresis-induced upregulation of HA and CD44. The inhibited expression of HAS3 protein and mRNA levels by TNF-α and IL-1 inhibitor pretreatment showed similar tendency with that of HA levels, suggesting that HAS3 expression is regulated by TNF-α and IL-1α and is responsible for sonophoresis-induced upregulation of HA in mouse epidermis (Fig 4, 5). Elevated HA levels are associated with virtually all disease processes involving inflammation[24-28] and various proinflammatory cytokines have been reported to regulate the gene expression of HAS and influence HA expressions in various cell types[29-31]. IL-1α, IL-1β, and TNF-α stimulate HA expression in synovial fibroblasts[32] and IL-1β and TNF-α also stimulate HA expression in human dermal fibroblasts by inducing HAS[33-35]. In periodontal ligament cells HAS2 and HAS3 mRNA levels are upregulated by IL-1β and TNF-α via p38 and ERK1/2 MAPK transcription factor activation[36]. Previous studies on keratinocytes have indicated that HAS2 and HAS3 are regulated by a variety of growth factors and cytokines[16], however, there have been few reports about the involvement of IL-1α and TNF-α in HAS expression in keratinocytes. Our results showed that both TNF-α and IL-1α could modulate HAS3 and HA expression following sonophoresis. We also demonstrated that both TNF-α and IL-1α were able to induce HAS3 mRNA levels in a dose dependent manner in mouse epidermis directly by each cytokine injection, suggesting that TNF-α and IL-1α play a role in the regulation of HA synthesis following sonophoresis through the induction of HAS3 expression in mouse epidermis (Fig 6, 7). Present study showed that in normal untreated mouse skin, both HAS2 and HAS3 are expressed in epidermis. However, among the isoenzymes of HAS, HAS3 was increased by sonophoresis and tape-stripping, with corresponding increase of HA expression, whereas HAS2 showed no significant changes after either sonophoresis or tape-stripping

compared to basal status, in contrast to an earlier study that showed both HAS2 and HAS3 mRNA levels were upregulated in mouse epidermis by tape-stripping[16] (Fig 3). The expression patterns of HAS isoenzymes are different between species and cell-type and their expression is independently regulated[37]. In human keratinocytes AS1 and HAS3 are expressed, whereas HAS2 are expressed in human dermal-derived fibroblasts[9,10]. Mouse skin expresses HAS1 and HAS2, HAS1 being expressed more strongly in the epidermis and HAS2 in the dermis[38] and recent study demonstrated that mouse epidermal keratinocytes abundantly expressed HAS3 mRNA from the basal to the granular cell layers, suggesting that HAS3 gene expression plays a crucial role in the regulation of HA synthesis in the epidermis[10]. These previous studies support our results that HAS2 and HAS3 are expressed in normal mouse epidermis, however, in contrast to HAS2, HAS3 expression is regulated by TNF-α and IL-1α and mainly involved in the HA synthesis in mouse epidermis. In present study, the CD44 expression was also inhibited with TNF-α and IL-1α inhibitor pretreatment, suggesting that these cytokines can regulate CD44 expression in mouse epidermis (Fig 4, 5). And we also directly demonstrated that TNF-α and IL-1α are involved in the upregulation of CD44 in murine epidermal keratinocytes by both cytokines injection (Fig 6, 7). Recent evidences have shown that IL-10 and TNF-α enhance CD44 expression on the surface of normal monocytes and demonstrated the involvement, at least in part, of p38 MAPK in TNF-α-induced CD44 expression in monocytes[39].

We also hypothesized that epidermal calcium gradient changes induced by sonophoresis may involve in the upregulation of HA and CD44 in keratinocytes of mouse epidermis following sonophoresis.

By utilizing sonophoresis of transmission gel containing 1.5mM calcium, we interfered the depletion of calcium ion in the upper epidermis

by sonophoresis and showed that these inhibition of calcium gradient change prevent the upregulation of HA and CD44 after sonophoresis (Fig 8-10). To exclude the possible effects of sonophoresis, we employed the immersion technique. Immediately after tape-stripping induced barrier disruption, the tape-stripped flank of mouse was immersed in PBS containing 1.2mM calcium for 2 h. Six hours after tape-stripping, the immersed epidermis also showed inhibited expression of HAS3 and CD44 mRNA compared to tape-stripped epidermis (Fig 10). These results suggest that depletion of calcium ion from the upper nucleated cell layers may provide the signal for induced expressions of HAS3 and CD44 leading to increased HA synthesis in the mouse epidermis. From our results we concluded that changes of epidermal calcium ion by sonophoresis may be a signal for inducing the expression of TNF-α and IL-1α and these increased cytokines upregulate HA contents through HAS3 induction and CD44 expression (Fig 11). We also proposed that any procedures which can decrease calcium ion in the upper epidermis, such as sonophoresis, iontophoresis and GA application, can upregulate the HA content and CD44 in epidermis without significant barrier impairment through epidermal cytokines induction.

Further studies will be required to distinguish other factors which involved in the mechanisms contribute to the regulation of epidermal HA and CD44 synthesis.

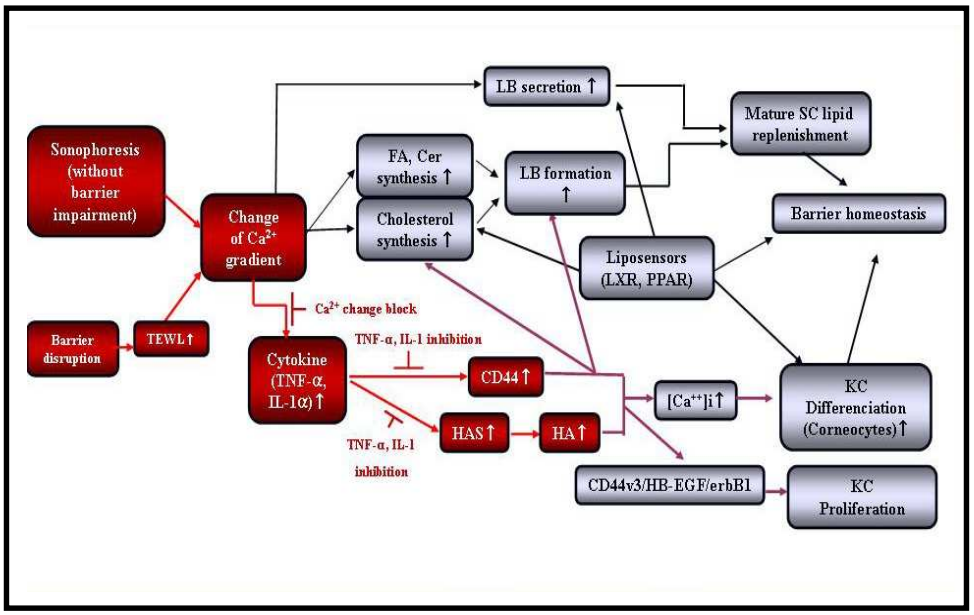

Figure 11. A proposed mechanism involved in sonophoresis-induced hyaluronic acid and CD44 expression.

V. CONCLUSION

The aim of this study is to identify whether sonophoresis could increase the expression of hyaluronic acid and CD44 in mouse epidermis without barrier disruption and to uncover the mechanisms involved in the upregulation of hyaluronic acid and CD44 expression following sonophoresis. The summary of the results are described below.

1. Sonophoresis does not affect TEWL, but increases the hyaluronic acid, CD44 and hyaluronic acid synthase3 in mouse epidermis
2. Sonophoresis-induced hyaluronic acid synthase3, hyaluronic acid and CD44 expression in mouse epidermis are regulated by TNF-α.
3. Sonophoresis-induced hyaluronic acid synthase3, hyaluronic acid and CD44 expression in mouse epidermis are regulated by IL-1α.
4. Sonophoresis-induced hyaluronic acid synthase3, hyaluronic acid and CD44 expression in mouse epidermis are regulated by the epidermal calcium gradient change.
5. Administration of intracutaneous TNF-α and IL-1α increases the expression of hyaluronic acid, CD44 and hyaluronic acid synthase3 in a dose dependent manner in mouse epidermal keratinocytes.

In conclusion, the epidermal calcium gradient change by sonophoresis can upregulate the HA content and CD44 in epidermis without significant barrier impairment through epidermal cytokine (TNF-α and IL-1α) induction. HAS3 is mainly involved in the HA synthesis in mouse epidermis following sonophoresis.

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< ABSTRACT(IN KOREAN)>

초음파 영동법이 표피 히알루론산 합성 및 CD44 발현에 미치는 영향

<지도교수 이승헌>

연세대학교 대학원 의학과

이상은

히알루론산은 세포외 기질의 중요한 성분으로 피부에서는 많은 양의 히알루론산이 진피의 결합조직에 존재하고 있다고 알려져 있으나 근래에 들어 표피에도 상당량의 히알루론산이 각질형성세포 사이의 기질에 존재하고 있음이 밝혀졌다. 히알루론산은 주요 세포표면수용체인 CD44에 결합하여 세포내 신호전달을 유발하고 다양한 생화학적 효과를 나타내며 표피에서의 히알루론산과 CD44의 상호작용은 각질형성세포의 분화와 콜레스테롤 합성의 증가, 층판소체의 형성과 분비를 조절함으로써 표피투과장벽의 항상성을 유지하는데 기여한다.

히알루론산은 급성 장벽 손상시에 표피에서 그 발현이 증가되며 손상을 받은 표피에서는 히알루론산 합성효소(hyaluronic acid synthase, HAS)2와 CD44가 동시간대에 기저층과 과립층에서 증가하는 것이 보고되었다. 이는 상처치유과정에서 분비가 증가하는 표피성장인자, 각질형성세포 성장인자, IL-1α, IFN-γ 등의 성장인자와 싸이토카인이 히알루론산 합성효소의 발현을 증가시켜 히알루론산의 합성을 증가시킨다고 생각된다.

그러나 최근 연구에 의하면 화학박피술에 사용되는 글리콜산(glycolic acid)의 국소 도포시 표피와 진피 모두에서 히알루론산의 증가가 보고되었는데, 그 작용기전은 정확히 알려져 있지 않으나 글리콜산은 피부장벽 기능의 손상 없이도 칼슘 이온의 변화와 층판소체의 분비 촉진을 유발하며 싸이토카인의 변화를 초래할 수 있음이 몇몇 연구에서 보고된 바 있어 이는 히알루론산의 증가가 피부장벽손상이 아닌 다른 기전을 통해 이루어질 수도 있을 것이라는 의구심을 가지게 한다. 최근 초음파 영동법이 피부 장벽에 미치는 영향이 글리콜산과 같은 기전, 즉 피부 장벽 기능의 손상 없이 칼슘 이온 변화와 싸이토카인의 변화, 층판 소체의 분비를 유도하여 피부장벽 회복 기전을 유발함이 보고되고 있어 본 연구에서는 표피장벽 기능의 손상 없이 초음파 영동법을 실시하였을 때 tape stripping을 이용하여 표피 손상을 유발한 경우와 마찬가지로 표피 히알루론산과 CD44의 발현이 증가하는지를 알아보고 히알루론산 합성의 조절기전을 알아보고자 하였다. 초음파 영동법을 무모쥐에 실시한 뒤 6시간 후 표피 히알루론산과 HAS3, CD44의 발현은 모두 정상대조군에 비해 증가된 양상을 보였으며 이는 tape stripping으로 표피손상을 일으킨 경우와 유사하였다. 또한 초음파 영동법 시행시 분비가 증가하는 대표적인 싸이토카인인 TNF-α, IL-1α 가 히알루론산과 CD44의 발현의 기전과 연관이 있는지를 알아보기 위하여 수용성 TNF 수용체 융합 단백과 IL-1 수용체 길항제를 사용하여 각 싸이토카인을 억제한 후 초음파 영동법을 시행한 결과 히알루론산과 HAS3, CD44의 발현이 모두 전처치 없이 초음파 영동법만 시행한 군에 비해 의미있게 억제된 양상을 보였다. TNF-α 또는 IL-1α의 증가가 초음파 영동법에 의한 표피 히알루론산과 CD44의 발현에 관여하는지를 직접적으로 증명하기 위하여 무모쥐에 TNF-α 또는

IL-1α를 다양한 농도로 직접 주입하고 6시간 후에 히알루론산과 HAS3, CD44의 발현이 각 싸이토카인의 투여 용량에 의존적으로 증가하는 양상을 보였다. 그리고 초음파 영동법에 의한 표피내 칼슘 이온 기울기의 변화가 히알루론산과 CD44의 발현의 기전과 연관이 있는지를 알아보기 위하여 1.5mM의 칼슘을 포함한 젤과 칼슘이 전혀 포함되지 않은 젤을 각각 도포하고 초음파 영동법을 시행하여 본 결과 1.5mM의 칼슘을 포함한 젤을 사용하여 초음파 영동법을 시행한 군에서는 초음파 영동법시에 관찰되는 칼슘 이온 기울기의 변화가 억제되는 양상이 관찰되었으며 히알루론산과 HAS3, CD44의 발현이 의미있는 감소를 보였다. 결론적으로 급성 장벽의 손상이 없이도 초음파 영동법에 의해서 표피에 히알루론산과 그 수용체인 CD44의 발현이 증가됨을 확인하였고, 그 기전으로 초음파 영동법에 의한 표피의 칼슘 이온 기울기의 변화와 이에 의한 TNF-α와 IL-1α의 증가가 HAS3의 발현을 증가시켜 표피의 히알루론산의 합성을 증가시키며 또한 그 수용체인 CD44 발현의 증가에도 관여한다고 추정해 볼 수 있다.