Iontophoresis and Sonophoresis Stimulate Epidermal Cytokine Expression at Energies That Do Not Provoke a Barrier Abnormality: Lamellar Body Secretion and Cytokine Expression Are Linked to Altered Epidermal Calcium Levels

Eung Ho Choi, Min Jung Kim, Byung-Il Yeh,* Sung Ku Ahn, and Seung Hun Lee†

Department of Dermatology and *Department of Biochemistry and Institute of Basic Medical Science, Yonsei University Wonju College of Medicine, Wonju, Korea; †Department of Dermatology, Yonsei University College of Medicine, Seoul, Korea

We performed this study to identify whether the expression of epidermal cytokines is altered by changes in epidermal calcium content, independent of skin barrier disruption. Iontophoresis and sonophoresis with the energies that do not disrupt the skin barrier, but induce changes in the epidermal calcium gradient, were applied to the skin of hairless mice. Immediately after iontophoresis and sonophoresis, immersion in a solution containing calcium was carried out, and iontophoresis in either high- or low-calcium solutions was performed. The biopsy specimens were taken for real-time quantitative RT-PCR to detect changes in mRNA level of interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α), and transforming growth factor-β in the epidermis and for immunohistochemical stain with primary antibodies to IL-1α and TNF-α. The expression of each cytokine mRNA increased in the epidermis treated with iontophoresis and sonophoresis compared to a nontreated control as well as in tape-stripped skin used as a positive control and was lower after immersion in a high-calcium solution than in low-calcium solution. IL-1α and TNF-α immunohistochemical protein staining increased with iontophoresis at low calcium. These studies suggest that changes in epidermal calcium can directly signal expression of epidermal cytokines *in vivo*, independent of changes in barrier function. ***Key words: calcium ion/skin barrier. J Invest Dermatol 121:1138–1144, 2003***

The skin barrier is composed of corneocytes and intercorneocyte lipid lamellae (Elias, 1983). Acute disruption of the skin barrier with either solvents or tape stripping produces a homeostatic response in the subjacent nucleated layers of the epidermis, resulting in rapid restoration of normal barrier function (Feingold, 1991). This response includes: (1) increased lamellar body (LB) secretion from the outer stratum granulosum (SG) (Menon *et al*, 1992b); (2) increased lipid synthesis in all epidermal layers (Menon *et al*, 1985; Grubauer *et al*, 1987; Feingold *et al*, 1990; Holleran *et al*, 1991a, b); (3) the formation of new LB in SG cells followed by further LB secretion; and (4) increased DNA synthesis in the basal layer of the epidermis (Proksch *et al*, 1991).

Acute disruption of the skin barrier can also increase cytokines expression in the epidermis. It is known that the calcium ion plays an important role in the proliferation and differentiation of keratinocytes *in vitro.* Nevertheless, there have been few *in vivo* studies owing to the paucity of adequate experimental models. Recent *in vivo* studies have shown, however, that manipulation of epidermal calcium *in vivo* directly regulate epidermal differentiation (Choi *et al*, 2002; Elias *et al*, 2002b). Iontophoresis using proper electric current (Lee *et al*, 1998) and sonophoresis using proper ultrasound (Menon *et al*, 1994) can induce changes in the epidermal calcium that increase LB secretion without increasing transepidermal water loss (TEWL). From these results we hypothesized that iontophoresis and sonophoresis, if their density or intensity did not disrupt the skin barrier, could be used for *in vivo* studies to define the expression of cytokines in response to changes in epidermal calcium *in vivo.*

The purpose of this study was to elucidate whether changes in the epidermal calcium gradient can stimulate epidermal cytokine expression. We used iontophoresis and sonophoresis as methods at energy levels that induce changes in the epidermal calcium gradient without altering TEWL and then applied real-time, quantitative RT-PCR and immunohistochemical staining to demonstrate changes in expression of epidermal cytokines. Our results suggest that reduction in epidermal calcium directly stimulate generation of primary cytokines in the epidermis.

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Reprint requests to: Eung Ho Choi, MD, Department of Dermatology, Yonsei University Wonju College of Medicine, 162 Ilsan-dong, Wonju, 220-701 Korea. Email: choieh@wonju.yonsei.ac.kr

Abbreviations: IL, interleukin; Ion 1 h, iontophoresis for 1 h; Ion 2 h, iontophoresis for 2 h; LB, lamellar body; NC, nontreated control; PBS, phosphate-buffered saline; SC, stratum corneum; SG, stratum granulosum; TGF, transforming growth factor; TNF-α, tumor necrosis factor-α; TEWL, transepidermal water loss; US, sonophoreis for 10 min.

MATERIALS AND METHODS

Animals Adult hairless mice were purchased from the animal laboratory of Yonsei University and were 8–12-wk-old females at the time of study.

The use of mice was approved by the institutional review board in Yonsei University Wonju College of Medicine.

Iontophoresis treatment After attachment of the patches (3.46 cm^2) on the flank of hairless mice, the iontophoresis was performed with direct

current (6 V, 0.6 mA/3.46 cm^2) of on:off duty ratio (2:1) for 1 and 2 h, respectively, the same as described in the previous report, in which TEWL was not increased after treatment (Lee *et al*, 1998). The biopsy specimens were taken at 1 and 2 h after patch removal for real-time quantitative RT-PCR and at 1, 3, and 6 h for immunohistochemical stains. Specimens from disrupted skin after tape stripping were used as a positive control, and specimens from skin with a patch but no current, as a normal control.

Sonophoresis treatment The back of hairless mice was divided into two sections to be used as the treatment site and control site. After anesthesia with chloral hydrate, the treatment sites of the back received 10 min of 300 mW per cm^2 1-MHz continuous wave ultrasound (Skin joy, Sonic tech, Korea) treatment as in our previous study (Lee and Choi, 2001) in which TEWL was not increased after treatment, and the negative control sites were only dabbed with the transmission gel (Biosonic, Amite) which is generally applied before ultrasound treatment. At 1 and 2 h after ultrasound treatment, skin biopsy specimens were taken.

Immersion in solution containing calcium ion After the treatment with 1 h of iontophoresis (Ion 1 h) and 10 min of sonophoresis (US), the hairless mice were immediately immersed with one flank submerged in phosphate-buffered saline (PBS) solution containing 0.1 mM calcium ($PBS + Ca^{2+}$) and PBS solution without calcium ion (PBS) for 2 h as described in the previous report (Lee *et al*, 1992), and then biopsy specimens were taken. The biopsy skin was used for real-time quantitative RT-PCR and immunohistochemical stain. Nile red stain and OsO_4 postfixation for electron microscope were performed for the skin treated with sonophoresis. Nile red, a fluorescence probe for lipids, was used to demonstrate the distribution and content of lipids in the stratum corneum (SC) (Grubauer *et al*, 1987). The digital photographs of epidermis ($\times 200$) obtained at random from the cross-sections of each group were compared with the scores defined by intensity and extent of fluorescence in the outermost epidermis. Electron microscope examination after OsO_4 postfixation was used to observe the secretion of LB between the SC–SG junction as described previously (Kim *et al*, 2001).

Iontophoresis using high- and low-calcium solution Iontophoresis using 1.0 mM $CaCl_2$ (high-calcium) solution and 0.03 mM $CaCl_2$ (low-calcium) solution on hairless mice was carried out for 1 h. The skin specimens were taken at 3 h after iontophoresis for real-time quantitative RT-PCR to compare mRNA of interleukin (IL)-1α and tumor necrosis factor-α (TNF-α).

Calcium-capture cytochemistry The skin specimens were taken after iontophoresis and sonophoresis and performed calcium-capture cytochemistry to observe the epidermal calcium under electron microscope as our previous report (Lee *et al*, 1998).

Separation of epidermis After cervical dislocation, the full-thickness skin specimens were excised immediately and put on a petri dish with epidermal surface facing downward. Subcutaneous fat was removed with a scalpel and floated dermis side down on 10 mL of 10 mM EDTA in Ca-, Mg-free PBS at 37°C for 35 min. The epidermis was then isolated, soaked in Trizol reagent (Gibco BRL, NY), and stored in a −70°C freezer.

Real-time quantitative RT-PCR Total RNA was isolated from each skin obtained after iontophoresis and sonophoresis treatment using Trizol reagent. One microgram of total RNA was reverse-transcribed with AMV reverse transcriptase (Promega, Madison, WI). Pairs of primers for amplification of IL-1α, TNF-α, and transforming growth factor (TGF)-α 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. Probe and primer sequences used were as follows: IL-1α probe, CTGTGTAATGAAAGACGGCACACCCACC; IL-1α forward primer, CAACCAACAAGTGATATTCTCCATG; IL-1α reverse primer, GATCCACACTCTCCAGCTGCA; TNF-α probe, CACGTCGTAGCAAACCACCAAGTGGA; TNF-α forward primer, CATCTTCTCAAAATTCGAGTGACAA; TNF-α reverse primer, TGGGAGTAGACAAGGTACAACCC; TGF-α probe, TTCAGCGCTCACTGCTCTTGTGACAG; TGF-α forward primer, TGACGTCACTGGAGTTGTACGG; TGF-α reverse primer, GGTTCATGTCATGGATGGTGC; GAPDH probe, TGCATCCTACACCACCAACTGCTTAG; GAPDH forward primer, TTCACCACCATGGAGAAGGC; and GAPDH reverse primer, GGCATGGACTGTGGTCATGA (Overbergh *et al*, 1999). Real-time quantitative PCR was performed using the ABI Prism 7700 sequence detector (TaqMan, Perkin Elmer/Applied Biosystems, Foster City, CA). The TaqMan PCR conditions were as follows; 15 s at 94°C and 1 min at 60°C with a total of 40 to 45 cycles. Data were analyzed with the software provided with the TaqMan. TaqMan C_t values were followed by GAPDH normalization. To avoid contamination, all assays were performed using the universal thermal cycling parameters (Applied Biosystems) with AmpErase UNG. All experiments were performed in duplicate.

Immunohistochemical stain For immunohistochemical stains, the skin biopsy was taken at 1, 3, and 6 h after iontophoresis for 1 and 2 h. Tissue samples, quick-frozen and fixated in liquid nitrogen, were placed in a cryomold filled with OCT compound and quick-frozen with isopentane. The frozen tissue samples were then sliced into sections 6 to 8 μm thick with a cryostat, fixed on gelatin-coated slides, and fixated for 5 min with acetone chilled at 20°C. After being rinsed with TBS buffer solution, the tissue sections were cultured in 3% H_2O_2 for 30 min and rinsed once more with TBS buffer solution. Horse serum (0.5%) and sheep serum (2%) were administered to the sample slides and 30 min was allowed for reactions to proceed. A total of 100 to 150 μL of primary antibodies for IL-1α (Endogen, Woburn, MA) and TNF-α (Endogen, Woburn, MA) was dropped on the slides placed inside a wet box. The slides were left to sit through the reaction for 1 h at room temperature and rinsed with TBS buffer. The samples then received administration of secondary antibodies (EnVision, Dako, Carpinteria, CA), sat for 30 min, and were rinsed with TBS buffer solution. Two drops of diaminobenzidine solutions (Dako) were dropped onto each sample slide and allowed 5 min to stain. The slides were then mounted and observed under the optical microscope with 10× objective lens and with the intensity profile of the image analyzer. Background coloring was not carried out as part of the diaminobenzidine staining process and the unstained sections were used for analysis. Intensity profile refers to the method used to determine the degree of staining in dyed tissue. The images of specimen observed through the optical microscope were converted into a gray scale and the intensity of stain in the desired area was used to calculate the average, which was used as a reference point or an indicator.

RESULTS

Calcium ion controls the secretion of LB after sonophoresis *in vivo* Iontophoresis and sonophoresis induced a marked decrease in calcium content in the epidermis, especially the top part, similar to as in our previous report (Lee *et al*, 1998). We used nile red staining and quantitative electron microscopy to assess changes in LB secretion and lipid deposition in the SC. The fluorescence in the SC after US and nontreated controls (NC) decreased with immersion in PBS containing calcium compared to PBS alone (**Fig 1**). After sonophoresis treatment, secretion of LB increased at the SC–SG junction in comparison to NC (**Fig 2**). In contrast, LB secretion was inhibited by immersion in PBS containing calcium compared to PBS only as in the previous report (Menon *et al*, 1994) (**Fig 2**).

Expression of primary cytokines increases after the treatment with iontophoresis and sonophoresis We used iontophoresis and sonophoresis, with the energies that did not disrupt the skin barrier but induce changes in the epidermal calcium gradient (Lee *et al*, 1998; Choi *et al*, 2002). In this trial, distilled water for iontophoresis and transmission gel for sonophoresis us that did not contain calcium ion were used. The expression of mRNA of IL-1α, TNF-α, and TGF-β increased in murine epidermis at 1 and 2 h after iontophoresis and sonophoresis on skin surface. Results were compared to mRNA levels of NC. Expression of all three cytokines increased in the epidermis after treatment with iontophoresis for 1h and iontophoresis for 2 h (Ion 2 h) and with US compared to the NC as well as the tape-stripped skin used as a positive control (**Figs 3, 4**). The expression of all three cytokines in Ion 2 h was increased more than Ion 1 h and US at both 1 and 2 h after treatment. Immunohistochemical stains for IL-1α and TNF-α in epidermis treated with iontophoresis showed increased expression compared to NC. The increased expression was observed from 1 h

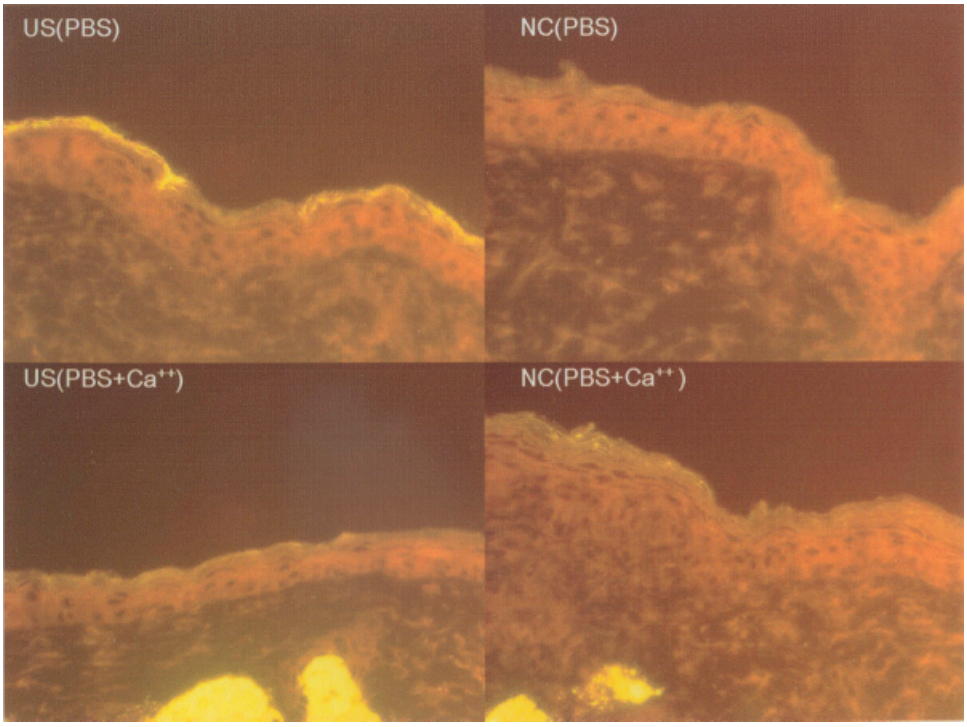

Figure 1. Nile red stain after US. Nile red stain was used to observe neutral lipids in SC. After US, the fluorescence in the SC was decreased with the immersion in PBS containing calcium compared to PBS only and in NC. *US*(*PBS*), immersion in PBS after ultrasound treatment; *US*($PBS + Ca^{2+}$), immersion in PBS containing calcium after ultrasound treatment; *NC*(*PBS*), immersion in PBS without treatment; *NC*($PBS + Ca^{2+}$), immersion in PBS containing calcium without treatment.

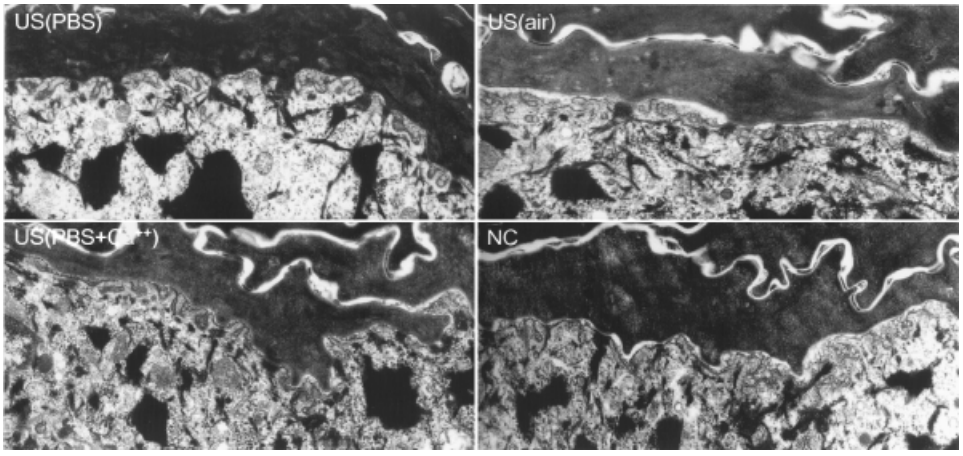

Figure 2. Secretion of LB in the SC–SG junction after US was increased compared to NC. It was inhibited by immersion in PBS containing calcium compared to PBS only. *US*(*PBS*), immersion in PBS after ultrasound treatment; *US*($PBS + Ca^{2+}$), immersion in PBS containing calcium after ultrasound treatment; *US*(*air*), air exposure after ultrasound treatment.

after iontophoresis and continued to increase at 3 and at 6 h (**Figs 5–7**).

Calcium ion controls the expression of cytokines mRNA in the epidermis To assess whether the expression of mRNA of IL-1α, TNF-α, and TGF-β was stimulated directly by changes in epidermal calcium, we used two unrelated models. First, we employed the immersion technique, with solutions containing calcium, described previously (Lee *et al*, 1992; Lee *et al*, 1994). The expression of mRNA of all three cytokines decreased in murine epidermis after 2 h of immersion in PBS containing high calcium versus PBS only (control) after Ion 1 h and US on the skin surface. Results are illustrated as the reduction rate (percentage) on the control (**Fig 8*A***). In the second experiment, we compared cytokine expression after iontophoresis of high versus low calcium. mRNA of IL-1α was 21% lower than following iontophoresis of low calcium, and TNF-α levels also were decreased by 66% in low versus high calcium (**Fig 8*B***). Together, these results suggest that change in calcium ion alone and specifically a reduction in epidermal calcium ion stimulate cytokine generation *in vivo*.

DISCUSSION

For some time it has been suggested that reduction in calcium levels comprise a signal that initiates the barrier repair response after barrier disruption. Disruption of the epidermal permeability barrier causes an immediate loss of the calcium gradient, and barrier recovery is parallel with the restoration of the calcium gradient in the epidermis (Menon *et al*, 1992a). Artificial restoration of the barrier function by occlusion with a water vapor-impermeable membrane abrogates the expected increase in lipid synthesis and retards the barrier recovery, as well as block the normalization of the epidermal calcium gradient (Menon *et al*, 1992b, 1994). Prolonged occlusion of tape-stripped epidermis induced transitional cells and delayed the restoration of epidermal calcium gradient until 48 h (Ahn *et al*, 1999). Therefore, the state of calcium deprivation after acute barrier perturbation by occlusion is maintained for a very long time. Furthermore, in chronic forms of barrier abnormality such as in essential fatty acid-deficient mice and topical lovastatin-treated mice, the epidermal calcium gradient is abnormal (Menon *et al*, 1994). In essential fatty acid-deficient mice, occlusion normalizes epidermal calcium gradient

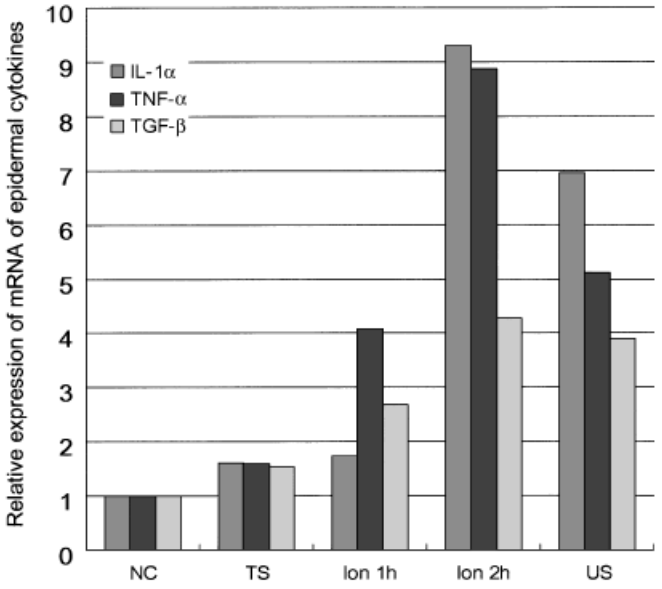

Figure 3. IL-1α, TNF-α, and TGF-β expression are increased in murine epidermis at 1 h after iontophoresis and ultrasound on skin surface. Total RNA was extracted, reverse-transcribed, and analyzed by real-time quantitative RT-PCR using GAPDH as an endogenous control. Results are illustrated relative to the mRNA levels of NC. All three cytokines showed increased expression in iontophoresis treated skin for 1 h (Ion 1 h) and 2 h (Ion 2 h) and the ultrasound-treated skin (US) compared to the NC as well as tape-stripped skin (TS) used as a positive control.

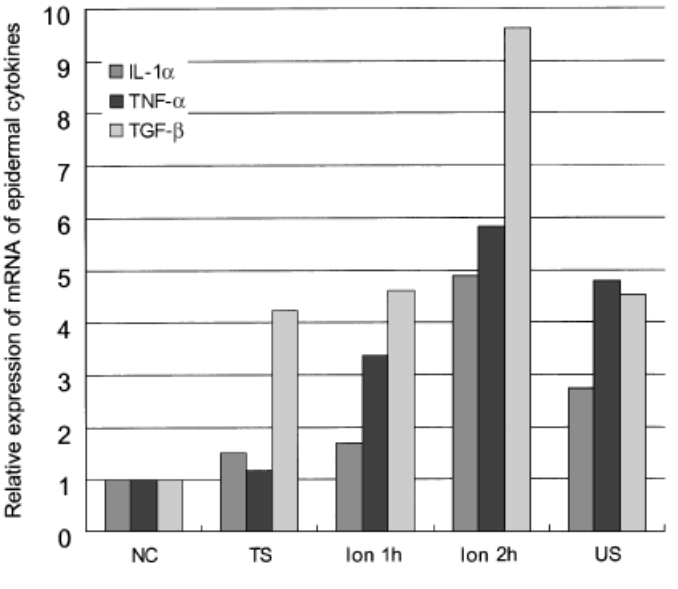

Figure 4. IL-1α, TNF-α, and TGF-β expression are increased in murine epidermis at 2 h after iontophoresis and ultrasound on skin surface. Total RNA was extracted, reverse-transcribed, and analyzed by real-time quantitative RT-PCR using GAPDH as an endogenous control. Results are illustrated relative to the mRNA levels of the NC. All three cytokines showed increased expression in iontophoresis treated skin for 1 h (Ion 1 h) and 2 h (Ion 2 h) and ultrasound-treated skin (US) compared to the NC as well as tape-stripped skin (TS) used as a positive control.

within 48 h. Recently, Elias *et al* (2002b) showed direct evidence that permeability barrier status regulates the formation of the epidermal calcium gradient and that passive diffusion rather than active mechanisms appears to suffice to explain gradient formation.

Disruption of the epidermal permeability barrier in murine skin stimulates the production of epidermal cytokines including TNF-α, IL-1α, IL-1β, and IL-1ra (Wood *et al*, 1992). An increase of these cytokines was observed in acute barrier disruption by either topical acetone treatment or tape stripping and in essential fatty acid-deficient epidermis (Wood *et al*, 1992; Wood *et al*, 1994a, b). A similar result was observed in human skin following tape stripping (Nickoloff and Naidu, 1994). Therefore, the increased level of these cytokines in the epidermis and dermis

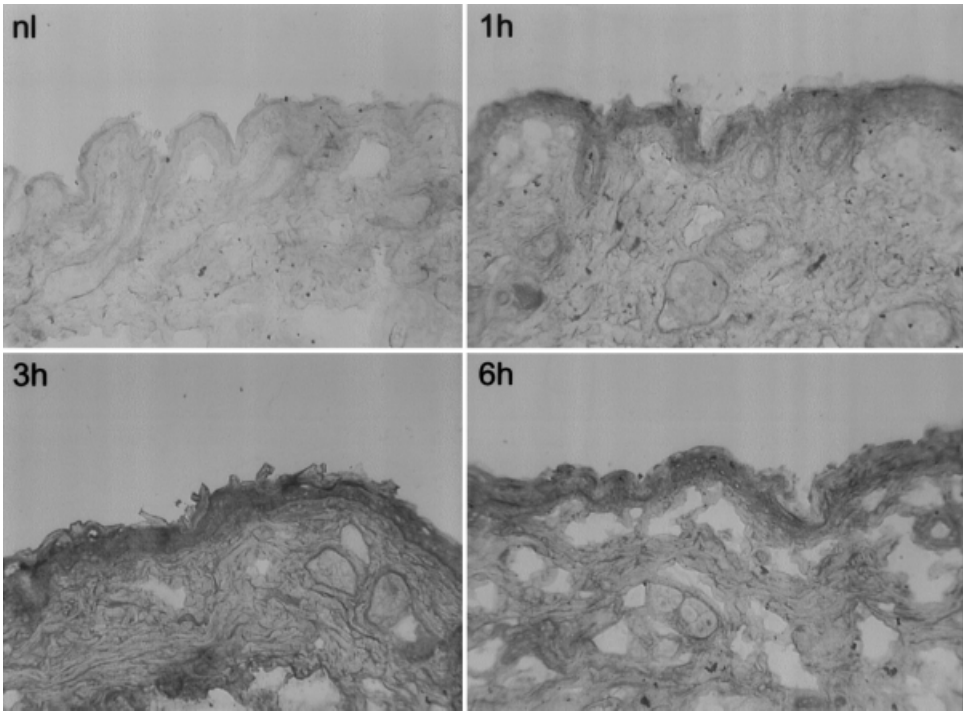

Figure 5. Immunohistochemical stain of TNF-α after 2 h positive iontophoresis. *nl*, control; *1h*, 1 h after iontophoresis; *3h*, 3 h after iontophoresis; *6h*, 6 h after iontophoresis.

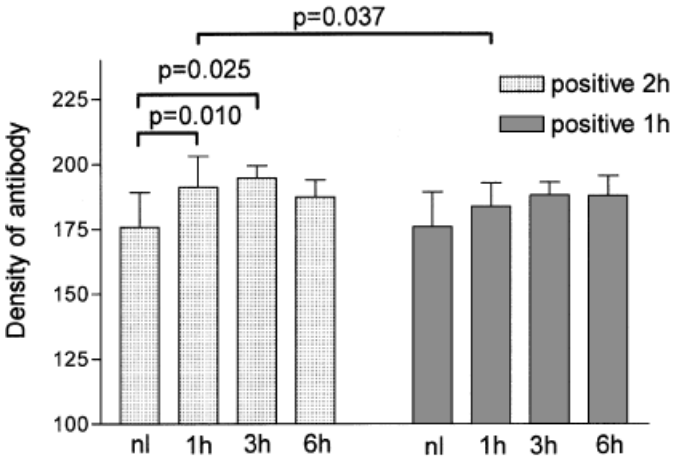

Figure 6. IL-1α expression after iontophoresis. *nl*, control; *1h*, 1 h after iontophoresis; *3h*, 3 h after iontophoresis; *6h*, 6 h after iontophoresis.

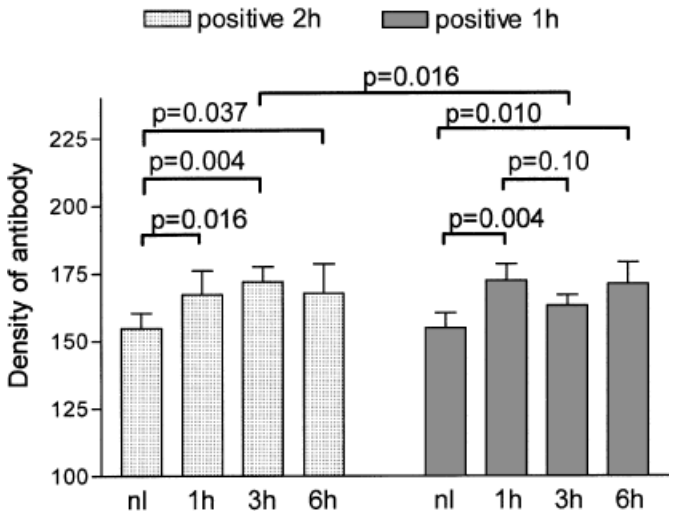

Figure 7. TNF-α expression after iontophoresis. *nl*, control; *1h*, 1 h after iontophoresis; *3h*, 3 h after iontophoresis; *6h*, 6 h after iontophoresis.

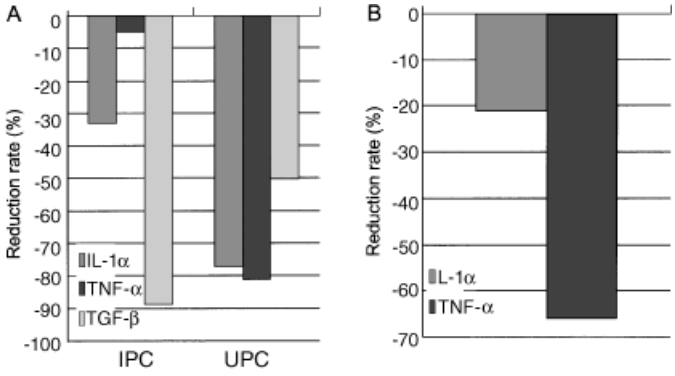

Figure 8. IL-1α, TNF-α, and TGF-β expression are controlled by the calcium ion. (*A*) The expression of mRNA of each cytokines was decreased in murine epidermis by 2 h of immersion in PBS including calcium ion compared to PBS only (control) after iontophoresis (1 h) and ultrasound on skin surface. All three cytokines showed less expression by addition of the calcium ion. (*B*) The expression of mRNA of IL-1α and TNF-α was decreased in high-calcium iontophoresis compared to low calcium. Total RNA was extracted, reverse-transcribed, and analyzed by real-time quantitative RT-PCR using GAPDH as an endogenous control. Results are illustrated as the reduction rate (percentage) on the control. *IPC*, 2 h of immersion in PBS including calcium ion after 1 h iontophoresis; *UPC*, 2 h of immersion in PBS including calcium ion after ultrasound treatment.

in response to cutaneous injury has been suggested as an initiator of a homeostatic repair response or an immune/inflammatory reaction in the past (Wood *et al*, 1996). Latex occlusion of essential fatty acid-deficient mice for 24 to 48 h lowered the mRNA levels of epidermal TNF-α, IL-1α, or IL-1ra to nearly control values. In contrast, latex occlusion of mice immediately after acute barrier disruption blocked neither a stimulation of epidermal mRNA for TNF-α, IL-1α, and IL-1ra, nor an increase of epidermal TNF-α protein (Wood *et al*, 1994a, b). Therefore, it was speculated that the generation of cytokines was linked to epidermal injury as a by-product of barrier disruption, rather than as homeostatic signaling mechanisms in the epidermis (Feingold, 1997).

Through many studies it has become evident that the secretion of LB was induced by barrier perturbation that could remarkably induce the loss of calcium ion in the upper epidermis. The loss of calcium from the upper epidermis, following barrier disruption, signals the increased secretion and synthesis of LB, a response that facilitates normal barrier recovery (Lee *et al*, 1992). Nevertheless, some reports using sonophoresis and iontophoresis showed that changes in calcium ion in the outer epidermis directly regulate LB secretion, independent of barrier perturbation (Menon *et al*, 1994; Lee *et al*, 1998). Therefore, now it is believed that LB secretion does not mean barrier perturbation. The purpose of our study was to elucidate whether the change of calcium ion in epidermis independent of barrier disruption can induce the cytokine expression. Theoretically there are no doses or direction that can shift calcium ions without LB secretion because the shift of calcium ion in the upper epidermis induces LB secretion. Of course we believe that there may be a threshold to induce LB secretion by a shift of the calcium ion, but we could not find any definite threshold in spite of many trials. We do not insist that any current density of iontophoresis or any intensity of ultrasound will not disrupt the skin barrier. So we used the same electric current and ultrasound by same iontophoresis and sonophoresis devices that were already shown not to induce skin barrier disruption represented by an increase of TEWL and epidermal proliferation (Lee *et al*, 1998; Choi *et al*, 1999, 2001, 2002).

The epidermal expression of mRNA of IL-1α, TNF-α, and TGF-β increased at 1 and 2 h after iontophoresis and sonophoresis. All three cytokines showed increased expression in Ion 1 h, Ion 2 h, and US compared to NC as well as tape-stripped skin used as a positive control (**Figs 1, 2**). Tape stripping is known as a method increasing the expression of epidermal IL-1α and TNF-α mRNA from previous studies (Wood *et al*, 1992, 1994a, b, 1996). In the previous studies the expression of cytokines mRNA after tape stripping was remarkably increased, four- to sevenfold. Nevertheless, in our result it was not so high. We thought that it might be from the practical method using tape stripping. Because we only stripped five times in all mice without checking the TEWL, we might not have mice with the barrier enough disrupted. Nevertheless, the remarkable increase of all cytokines expression was observed in Ion 2 h, US, and some Ion 1 h. In our preliminary study using RT-PCR, we observed the same results after using iontophoresis and sonophoresis. Therefore, we decided to confirm the results with real-time quantitative RT-PCR. The expression of all three cytokines was increased in Ion 2 h compared to Ion 1 h and US at both 1 and 2 h after treatment. From our previous research using iontophoresis, we found that calcium

deprivation in the epidermis was increased with current time (Lee *et al*, 1998). Therefore, these results were more compatible with our hypothesis. TGF-β is known as an inhibitory cytokine to inflammation and related to cancer and wound healing (Massague, 1990). TGF-β mRNA is increased in the epidermis after perturbation of the skin barrier in humans and hairless mice (Nickoloff and Naidu, 1994; Lew *et al*, 1998). Also we observed an increase of TGF-β mRNA and immunoreactivity in the dermis after sonophoresis (Lee and Choi, 2001). Immunohistochemical stains for IL-1α and TNF-α in the epidermis showed an increased expression compared to NC. The increased expression was observed from 1 h after iontophoresis and increased at 3 and at 6 h (**Figs 3–5**). Overall an increased tendency was shown with the 2-h current compared to 1 h. These results were similar to previous results of the acute perturbation model. We used image analysis to compare epidermal IL-1α and TNF-α expression by treatment because their expression was not localized at any specific layer but diffuse in the epidermis and we needed a more objective method to exclude the subjective bias that can be present with immunohistochemical stains.

Because keratinocytes produce cytokines in response to exogenous stimuli such as ultraviolet B radiation, phorbol-12-myristate 13-acetate, and lipopolysaccharide (Wilmer *et al*, 1994), we question whether damage by the electric current of iontophoresis and heat produced by ultrasound of sonophoresis can induce cytokine production not by a change in epidermal calcium. Therefore, we introduced a classic immersion study using a solution containing calcium (Lee *et al*, 1992). The expression of all three cytokines was reduced from approximately 30% to 90% in both iontophoresis and sonophoresis. Although Ion 2 h has shown a remarkable increase of cytokines expression, we used Ion 1 h in this study because the long period of complete anesthesia over 4 h was needed to complete the study with Ion 2 h, which has technical difficulties. Also we performed iontophoresis using high and low calcium and observed that cytokines expression decreased in high calcium compared to low calcium. We believe that these results will support our hypothesis. Nevertheless, the issues of toxicity or irritancy of electric current were not completely solved because the reduction was not complete. Our results directly showed that changes in epidermal calcium regulate mRNA expression of epidermal cytokines and then protein *in vivo*. We did not employ another marker for cellular injury to prove that there was no damage to epidermal cells. Rather we adopted an indirect method measuring epidermal proliferation to evaluate repeated skin barrier damage. Epidermal proliferation indicated keratinocyte damage in a previous study (Choi *et al*, 2001). We did not find any epidermal proliferation after repeated iontophoresis and sonophoresis using the same intensity or current of the same devices. This indirect evidence supports the conclusion that there was no damage after iontophoresis and sonophoresis. Recently it was reported that modulations in epidermal calcium regulate the expression of differentiation specific markers (Elias *et al*, 2002b). We also identified that iontophoresis-inducing changes in the epidermal calcium gradient without skin barrier disruption can increase sphingolipids and neutral lipids in the SC and change the expression of cornified cell envelop protein indicating terminal differentiation (Choi *et al*, 2002).

In this study, the increased fluorescence in the SC after sonophoresis indicates an increase of SC intercellular lipids by an increase of LB secretion owing to calcium deprivation from the epidermis after sonophoresis, which depends on epidermal calcium as in a previous study (Menon *et al*, 1994).

We believe that a key experiment to resolve the issue would be to confirm that a decrease in calcium ion in cultured keratinocytes stimulates cytokine production. That is our next experiment. From this study we conclude that the change of epidermal calcium may be a direct signal for inducing the expression of epidermal cytokines *in vivo*.

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REFERENCES

Ahn SK, Hwang SM, Jiang SJ, Choi EH, Lee SH: The changes of epidermal calcium gradient and transitional cells after prolonged occlusion following tape stripping in the murine epidermis. *J Invest Dermatol* 113:189–195, 1999

Choi EH, Ahn SK, Lee SH: The effect of repeated changes in the epidermal calcium gradient by repeated iontophoresis on the skin barrier. *J Skin Barrier Res (Korean)* 3:62–67, 2001

Choi EH, Lee SH, Ahn SK, Hwang SM: The pretreatment effect of chemical skin penetration enhancers in transdermal drug delivery using iontophoresis. *Skin Pharmacol Appl Skin Physiol* 12:326–335, 1999

Choi EH, Park WS, Son ED, Hwang SM, Kim MJ, Ahn SK, Lee SH: The effect of change in epidermal calcium gradient on stratum corneum lipid and epidermal differentiation. In: Mark R, Leveque JL, Voegeli R (eds). *The Essential Stratum Corneum*. London: Dunitz, 2002; p 77–80

Elias PM: Epidermal lipids, barrier function and desquamation. *J Invest Dermatol* 80 (Suppl.):44–49, 1983

Elias PM, Ahn SK, Brown BE, Crumrine D, Feingold KR: Origin of the epidermal calcium gradient: Regulation by barrier status and role of active vs passive mechanisms. *J Invest Dermatol* 119:1269–1274, 2002a

Elias PM, Ahn SK, Denda M, *et al*: Modulations in epidermal calcium regulate the expression of differentiation-specific markers. *J Invest Dermatol* 119:1128–1136, 2002b

Feingold KR: Permeability barrier homeostasis. Its biochemical basis and regulation. *Cosmet Toilet* 112:49–59, 1997

Feingold KR, Mao-Quiang M, Menon GK, Cho SS, Brown BE, Elias PM: Cholesterol synthesis is required for cutaneous barrier function in mice. *J Clin Invest* 86:1738–1745, 1990

Feingold KR: The regulation of epidermal lipid synthesis by permeability barrier requirements. In: *Critical Reviews in Therapeutic Drug Carrier Systems*. Boca Raton (FL): CRC Press, 1991; Vol 8, p 193–210

Grubauer G, Feingold KR, Elias PM: The relationship of epidermal lipogenesis to cutaneous barrier function. *J Lipid Res* 28:746–752, 1987

Holleran WM, Feingold KR, Mao-Quiang M, Gao WN, Lee JM, Elias PM: Regulation of epidermal sphingolipid synthesis by permeability barrier function. *J Lipid Res* 32:1151–1158, 1991a

Holleran WM, Mao-Quiang M, Gao WN, Menon GK, Elias PM, Feingold KR: Sphingolipids are required for mammalian barrier function. inhibition of sphingolipid synthesis delays barrier recovery after acute pertubation. *J Clin Invest* 88:1338–1345, 1991b

Kim TH, Choi EH, Kang YC, Lee SH, Ahn SK: The effects of topical α-hydroxyacids on the normal skin barrier of hairless mice. *Br J Dermatol* 144:267–273, 2001

Lee SW, Choi EH: The effect of ultrasound on the skin of aged and young mice. *Kor J Invest Dermatol* 8:209–218, 2001

Lee SH, Choi EH, Feingold KR, Jiang S, Ahn SK: Iontophoresis itself on hairless mouse skin induces the loss of the epidermal calcium gradient without skin barrier impairment. *J Invest Dermatol* 111:39–43, 1998

Lee SH, Elias PM, Feingold KR, Mauro T: A role for ions in barrier recovery after acute perturbation. *J Invest Dermatol* 102:976–979, 1994

Lee SH, Elias PM, Proksch E, Menon GK, Mao-Quiang M, Feingold KR: Calcium and potassium are important regulators of barrier homeostasis in murine epidermis. *J Clin Invest* 89:530–538, 1992

Lew W, Song DH, Lee SH: The effect of epidermal barrier disruption on the gene expression of transforming growth factor-β subtypes. *Kor J Invest Dermatol* 5:90–96, 1998

Massague J: The transforming growth factor-β family. *Annu Rev Cell Biol* 6:597–641, 1990

Menon GK, Elias PM, Lee SH, Feingold KR: Localization of calcium in murine epidermis following disruption and repair of the permeability barrier. *Cell Tissue Res* 270:503–512, 1992a

Menon GK, Feingold KR, Elias PM: The lamellar body secretory response to barrier disruption. *J Invest Dermatol* 98:279–289, 1992b

Menon GK, Feingold KR, Moser AH, Brown BE, Elias PM: De novo sterologenesis in the skin. II. Regulation by cutaneous barrier requirements. *J Lipid Res* 26:418–427, 1985

Menon GK, Price LF, Bommannan B, Elias PM, Feingold KR: Selective obliteration of the epidermal calcium gradient leads to enhanced lamellar body secretion. *J Invest Dermatol* 102:789–795, 1994

Nickoloff BJ, Naidu Y: Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. *J Am Acad Dermatol* 30:535–546, 1994

Overbergh L, Valckx D, Waer M, Mathieu C: Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine* 4:305–311, 1999

Proksch E, Feingold KR, Mao-Quiang M, Elias PM: Barrier function regulates epidermal DNA synthesis. *J Clin Invest* 87:1668–1673, 1991

Wilmer JL, Burleson FG, Kayama F, Kanno J, Luster MI: Cytokine induction in human epidermal keratinocytes exposed to contact irritants and its relation to chemical-induced inflammation in mouse skin. *J Invest Dermatol* 102:915–922, 1994

Wood LC, Elias PM, Calhoun C, Tsai JC, Grunfeld C, Feingold KR: Barrier disruption stimulates interleukin-1α expression and release from a pre-formed pool in murine epidermis. *J Invest Dermatol* 106:397–403, 1996

Wood LC, Elias PM, Sequeira-Martin SM, Grunfeld C, Feingold KR: Occlusion lowers cytokine mRNA levels in essential fatty acid deficient and normal mouse epidermis but not after acute barrier disruption. *J Invest Dermatol* 103:834–838, 1994b

Wood LC, Feingold KR, Sequeira-Martin SM, Elias PM, Grunfeld C: Barrier function coordinately regulates epidermal IL-1 and IL-1 receptor antagonist mRNA levels. *Exp Dermatol* 3:56–60, 1994a

Wood LC, Jackson SM, Elias PM, Grunfeld C, Feingold KR: Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *J Clin Invest* 90:482–487, 1992