Surfactant Sodium Lauryl Sulfate Enhances Skin Vaccination

MOLECULAR CHARACTERIZATION VIA A NOVEL TECHNIQUE USING ULTRAFILTRATION CAPILLARIES AND MASS SPECTROMETRIC PROTEOMICS

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The skin is a highly accessible organ and thus provides an attractive immune environment for cost-effective, simple, and needle-free delivery of vaccines and immunomodulators. In this study, we pretreated mouse skin with an anionic surfactant, sodium lauryl sulfate (SLS), for a short period of time (10 min) followed by epicutaneous vaccination with hen egg lysozyme antigen. We demonstrated for the first time that pretreatment of skin with surfactant SLS significantly enhances the production of antibody to hen egg lysozyme. Short term pretreatment with SLS disorganized the stratum corneum, extracted partial lamellar lipids, induced the maturation of Langerhans cells, and did not result in epidermis thickening. To reveal the mechanism underlying these changes, particularly at the molecular level, we used a novel proteomic technique using ultrafiltration capillaries and mass spectrometry to identify in vivo proteins/peptides secreted in the SLS-pretreated skin. Two secretory proteins, named as calcium-binding protein S100A9 and thymosin β4, were identified by this novel technique. These two proteins thus may provide new insight into the enhancing effect of surfactants on skin vaccination. Molecular & Cellular Proteomics 5:523–532, 2006.

The skin provides an attractive immune environment for vaccine delivery. It contains many immunocompetent cells, including keratinocytes, γδ T cells, and Langerhans cells, which may initiate potent immunity when skin is exposed to various antigens (1). One mechanism that has been proposed is that these antigens activate Langerhans cells in the skin that then migrate to draining lymph nodes to orchestrate robust systemic immune responses (2). The presence of both significant associated lymphoid tissue and immunocompetent cells suggests that skin might be an effective non-invasive route for vaccination. Simple access to and durability of the skin are additional advantages of applying vaccines to the external tissue. Moreover both humoral and cellular immunity are induced after immunization via the skin (3).

Production of an immune response against a foreign antigen usually requires needle injections by medical personnel. The development of a needle-free non-invasive method for the inoculation of vaccines via the skin may reduce medical costs by allowing personnel with a lower level of medical training to administer the vaccine. Techniques for skin vaccination can be categorized into three groups depending on which stratum of the skin is targeted (4). The first group encompasses epidermal immunization, which includes stripping, chemical modification, and transepidermal immunization (5–8). The second group covers epidermal and dermal immunization, which includes gene gun technology and electroporation (9). The third group targets dermal immunization, which includes intradermal immunization and microseeding (10). Although application of the above techniques can efficiently elicit immune responses at certain levels, they normally utilize xenogenic as adjuvants or severely damage skin by altering the epidermal structure. An alternative approach is to brush the skin before antigen application, presumably stimulating skin immunocompetent cells, with little or no harm to the epidermis (5, 6). However, brushing is difficult, if not impossible, to standardize for future clinical use. To address this concern, we pretreated skin with various concentrations of surfactant SLS,1 washed away and followed by topical application of antigens. Surfactants are found in numerous product categories from makeup to skin care as well as being the main constituents of shower gels, bath foam, and shampoos (11). Surfactants have been used for skin drug delivery and serve as penetration enhancers that are absorbed into skin to reversibly decrease barrier resistance (12, 13).

More recently, Vyas et al. (14) and Gupta et al. (15) used non-ionic surfactant-based vesicles (niosomes) for topical delivery of encapsulated DNA encoding hepatitis B surface antigen (14) and tetanus toxoid (15). A detectable antibody re-
sponse was evoked in mice after niosome-mediated topical immunization (14, 15). One potential drawback to the above technique is that surfactant-based vesicles remain in the host following vaccination, possibly resulting in adverse effects.

The question of whether pretreatment of skin with surfactants prior to epicutaneous application of antigens evokes sufficient antibody responses has not been explored. To test the efficiency of surfactant pretreatment on skin vaccination, we pretreated mouse skin with the surfactant SLS followed by epicutaneous application of hen egg lysozyme (HEL) antigen. Our results demonstrated for the first time that pretreatment of skin with surfactant SLS significantly enhances the production of antibody to HEL. In an attempt to understand the mechanism of this response, particularly at the molecular level, we applied a novel proteomic technique using ultrafiltration capillaries and mass spectrometry to detect in vivo the proteins/peptides secreted in the pretreated skin. The novel capillary ultrafiltration technique will allow us to obtain pure, low abundance, and in vivo secretory proteins/peptides. Secretory proteins such as S100A9 and thymosin β4 identified by this novel technique will provide new insight into the enhancement of skin vaccination by surfactants.

EXPERIMENTAL PROCEDURES

Skin Vaccination and ELISA—Female ICR mice at 3–6 months old (Jackson Laboratory, Bar Harbor, ME) were anesthetized by administering 10 mg of ketamine and 1.5 mg of xylazine/100 g of body weight. The abdominal skin of ICR mice was depilated with an electric trimmer. 100 μl of SLS (0.1, 1, and 5%, v/v) or PBS was applied to the depilated skin for 10 min and then rinsed with water to remove unabsorbed SLS from the skin immediately followed by epicutaneous vaccination with HEL (100 μg/100 μl; HEL/PBS) (Sigma). For epicutaneous vaccination, the HEL was spread as a thin film over preshaved skin followed by the application of a Tagaderm patch (3M, St. Paul, MN) for 1 h. Unabsorbed HEL on the skin was washed away 1 h following patch removal. Each animal was vaccinated with HEL for 1 month without booster. Naïve groups of mice were prepared by pipetting 100 μl of PBS onto the preshaved skin. Each group contained five mice. Three independent experiments were performed. Mice were maintained in the University of Alabama at Birmingham animal care facility in accordance with the animal protocol approved by the Institutional Animal Care and Use Committee. Serum samples were assayed for anti-HEL antibodies 1 month after vaccination with HEL. Titers of anti-HEL immunoglobulin G (IgG) were determined by ELISA as described previously (5, 6). The HEL (0.5 μg/well) serves as the capture antigen to coat a 96-well ELISA plate (Corning Inc., Corning, NY). Serum samples and peroxidase-conjugated goat anti-mouse IgG (1:5,000 dilution) (Promega, Madison, WI) were incubated sequentially on the ELISA plates for 1 h at room temperature with extensive washing between each incubation. The end point was calculated as the dilution of serum producing the same A492 as a 1:100 dilution of preimmune serum. Sera negative at the lowest dilution tested were assigned end point titers of 100. The data were presented as geometric mean end point ELISA titers.

Electron Microscopy—The areas of the skin treated with PBS or SLS were fixed overnight at 4 °C in modified Karnovsky’s fixative, containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.06% CaCl2, in 0.1 M cacodylate buffer, pH 7.4. Skins were then washed in 0.1 M cacodylate buffer, postfixed in 0.25% ruthenium tetroxide (RuO4) (Polyscience, Warrington, PA) in 0.1 M cacodylate for 45 min in the dark at room temperature. Lastly the specimens were rinsed in buffer, dehydrated in graded ethanol solutions, and embedded in an Epox-epoxy resin mixture. Ultrathin 60–80-nm sections were examined under a Hitachi 7000 transmission electron microscope. Images were captured at 75 kV after staining with uranyl acetate/lead citrate.

Histology—For histological observation, the skins treated with PBS or SLS were cross-sectioned, stained with hematoxylin and eosin (H&E) (Sigma) (16), and viewed on a Zeiss Axioskop2 Plus microscope (San Marcos, CA). Images were collected using an Axiocam digital camera in conjunction with Axiovision 3.1 software.

Immunohistochemical Staining of Epidermal Langerhans Cells—Mice were exposed to PBS or SLS on the dorsal side of ears for 10 min and killed for staining of epidermal Langerhans cells. The preparation and staining of epidermal Langerhans cells were performed as published with minor modifications (17). Briefly after removal of ear, the ear tissue was incubated in 0.25% trypsin (Sigma) for 60 min at 37 °C, and the epidermis sheets were separated from dermis, dispersed in Hank’s balanced salt solution containing 0.025% DNase and 10% fetal bovine serum, and then fixed immediately in cold acetone for 10 min. The epidermal sheets were rehydrated in PBS for 60 min and incubated in the blocking buffer (0.5% BSA in PBS) for 30 min at room temperature. After one wash, the epidermal sheets were incubated with biotinylated anti-major histocompatibility complex (MHC) class II antibody (BD Biosciences) overnight on a shaker at 4 °C. The sheets were then washed three times in PBS at room temperature. Streptavidin-TRITC (BD Biosciences) (1:100) was added, and the epidermal sheets were incubated on a shaker at room temperature for 2 h. For double staining, MHC class II-stained epidermal sheets were further incubated with FITC-conjugated rat anti-mouse CD86 monoclonal antibody (BD Biosciences) (1:100) at room temperature for 2 h. After washings as described above, epidermal sheets were mounted on slides with 90% glycerol. Three photographs were taken from each epidermal sheet using a Leitz microscope connected to a digital camera and saved in a computer with the IP Spectrum software. The result was evaluated with Photoshop. The number of MHC class II-stained cells was counted on each photograph, and the mean stained cell number/mm² was calculated for each experimental group with at least five mice per group.

Design and Preparation of Capillary Ultrafiltration (CUF) Probes—A real CUF probe is pictured in Fig. 5A. The probe consists of a semipermeable hollow membrane fiber (Fig. 5, A and B, a) joined to a polytetrafluoroethylene (PTFE) tube (Fig. 5, A and B, c). The semipermeable hollow membrane fiber, with a molecular mass cutoff of 50 kDa, was made with polyacrylonitrile and obtained from a kidney dialyzer (AN69-HF, Hospal-Gambro, Inc.). The AN69-HF was reported to have a mean pore size of 290 nm and a maximum pore size of 550 nm (18). One end of the semipermeable hollow membrane fiber was attached to the PTFE tubing (inner diameter/outter diameter, 0.102/0.406 mm, Cole-Parmer Instrument Co.) through a small section of fused silica capillary (Fig. 5, A and B, b) and connected to a vacuum line (Fig. 5, C and D, f) with negative pressure that will drive the ultrafiltration process to collect interstitial fluids. The negative pressure was created by vacuuming the Vacutainer with a syringe (Fig. 5D, h).

Continuous Sampling in Vivo with CUF Probes—The dorsal ear skin of 2–3-month-old female ICR mice (Jackson Laboratory) was cleaned with water before implantation with CUF probes. The CUF probe was then inserted near the head. The mice were anesthetized with 10 mg
of ketamine and 1.5 mg of xylazine/100 g of body weight during implantation. The semipermeable membrane fiber in the front end of the CUF probe was entirely covered by ear skin. Implantation of the CUF probe involved subcutaneously inserting a 22-gauge needle into the ear skin as a guide and then feeding the probe through the needle. The needle was then removed. After implantation of CUF probes, 50 µl of PBS or SLS (1.0%, v/v) was epicutanously applied to the ear skin for 10 min and then rinsed with water to remove unabsorbed PBS or SLS from the skin. The sample collection was conducted for 24 h after implantation. During the 24-h collection, mice wore elastomer saddle tethers (Instech Solomon, Plymouth Meeting, PA) and were placed in a balanced level arm (Instech Solomon). The Vacutainer was replaced every hour, and collected samples were maintained at −86 °C to avoid degradation. After the collection, −15–20 µl of sample was obtained from both PBS- and SLS-treated skins. The protein concentrations of collected samples were ∼0.3 mg/ml. The protein concentration was determined using the BCA™ protein assay kit (Pierce). The samples collected from PBS- and SLS-treated skin were adjusted to the same concentration by adding distilled water. They were digested with 0.04 µg/µl trypsin for 15 h. The trypic digests of collected samples were then subjected to MALDI-TOF MS and Q-TOF MS/MS analyses.

MALDI-TOF MS—Peptides in the trypic digests of CUF probe-collected samples were eluted from ZipTips with 75% acetonitrile, 0.1% trifluoroacetic acid and air-dried. Peptide fragments were then reconstituted in matrix solution containing α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% trifluoroacetic acid and analyzed with a PerSeptive Voyager-DE MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) (20). Peptides were laser-evaporated at 337 nm, and each spectrum was the cumulative average of 50–100 laser pulses. All peptides were measured as monoisotopic masses, and a trypsin autolytic peak at 2,164.1 m/z was selected for internal calibration. Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. This procedure resulted in mass accuracies of 100 ppm. For the detection of intact proteins, samples collected by CUF probes were mixed with matrix solution containing α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% trifluoroacetic acid. The proteins with m/z ranging from 12,400 to 13,700 and 4,500 to 5,500 were observed in the MALDI-TOF MS spectra.

Q-TOF MS/MS Sequencing and Database Searching—Samples digested with or without trypsin were eluted from ZipTips (21) and were introduced into a nano reverse-phase column (75 µm × 15 cm, with Jupiter 4-μm Proteo beads packed in our laboratory) and gradient-eluted into a Q-TOF 2 quadrupole-time-of-flight tandem mass spectrometer (Micromass, Manchester, UK) through an electrospray interface for tandem mass spectral analyses. Liquid chromatography was performed using an LC Packings Ultimate LC system, Switchos microcolumn switching unit, and Famos autosampler (LC Packings, San Francisco, CA). Spectral analyses were performed in automatic switching mode whereby multiply charged ions were subjected to MS/MS if their intensities rose above six counts. The MassLynx 3.5 software (Micromass, Manchester, UK) (22) was used for instrument operation, data acquisition, and analysis. The search for amino acid sequence similarity was performed using BLAST and/or Scansp available from the ExPASy internet server at www.expasy.ch. Accession numbers of proteins were taken from the SWISS-PROT database.

RESULTS

Elicitation of Antibody to HEL by Skin Vaccination—In an attempt to investigate whether mice immunized epicutanously with HEL could elicit antibody, we spread HEL (100 µg/µl) over preshaved abdominal skin of ICR mice with a piece of the 3M Tegaderm patch for 1 h. After washing away the unabsorbed HEL, mice were maintained in their cages for 1 month. An equal volume of PBS was applied to the non-vaccinated mice in the naïve group (Fig. 1). Mice epicutanously administered HEL had a detectable specific IgG response (geometric mean titer = 1,613 for HEL-vaccinated mice compared with geometric mean titer = 120 for non-vaccinated naïve mice).

To test the effect of surfactants on skin vaccination, we applied three different doses (0.1, 1.0, and 5%) of SLS to the surface of preshaved skins for 10 min prior to vaccination with HEL. Pretreatment of skin with SLS significantly enhanced the HEL-specific IgG response in the immunized mice (Fig. 1). Pretreatment with 0.1, 1.0, and 5.0% solutions of SLS resulted in a 2.2-, 4.8-, and 1.6-fold higher HEL antibody titer over the HEL alone. In addition, a mixture of 100 µg of HEL with 0.1, 1.0, and 5% SLS was prepared. Mice did not evoke significant HEL-specific IgG response when they were immunized simultaneously with HEL and SLS (data not shown).

Simultaneous immunization was accomplished through epicutaneous application of the three HEL/SLS mixtures onto abdominal skins. The geometric mean of HEL antibody titer was 100, 110, 110, 132, and 152 for non-vaccinated, HEL-, HEL/0.1% SLS-, HEL/1.0% SLS-, and HEL/5.0% SLS-vaccinated mice, respectively. These results suggest that pretreatment with SLS yields better immunologic potency than simultaneous administration with the antigen. Moreover the strongest enhancement of the HEL-specific IgG response was obtained with the 1.0% SLS strength.

Effect of Surfactant SLS on Skin Architecture—Previous...
Surfactant Enhances Skin Vaccination: A Novel Technique

Fig. 2. Epicutaneous treatment of SLS for 10 min does not result in inflammation. The ear skins of ICR mice were cross-sectioned and stained with H&E after epicutaneous exposure to PBS (A and B) or SLS (C–F) at the indicated concentrations (v/v) for 10 min (A, C, D, and E) or 24 h (B and F). Although 24-h treatment with 5% (v/v) SLS causes significant epidermal (epi) thickening (F), short term exposure (10 min) has no effect as compared with a PBS control. Bar, 25 μm.

These studies indicate that SLS application resulted in disruption of stratum corneum, skin irritation, and activation of signaling transduction in the epidermal cells utilized relatively long application periods (>24 h) (23, 24). The effects of short term SLS application on skin architecture remain unexplored. We thus epicutaneously applied SLS (0.1, 1.0, and 5%, v/v) onto the skin for 10 min and investigated the ultrastructure of stratum corneum barrier, epidermal inflammation, and Langerhans cell morphology. Our findings are in agreement with previous evidence (24) that epicutaneous application of 5% (v/v) SLS for 24 h significantly removed the lamellar lipids in stratum corneum (data not shown) and induced a thickening of epidermis (Fig. 2F). In contrast to previous studies, our data indicated that epicutaneous application of 1% (v/v) SLS for 10 min followed by washing with water only partially depleted lamellar lipids (Fig. 3, C and D). Application of the 1% (v/v) SLS solution also resulted in disorganization of the structure and widening of the intracellular spaces of the stratum corneum (Fig. 3, A and B). Application of 0.1 and 5.0% SLS solutions resulted in similar disorganization of stratum corneum structure and similar widening of intracellular spaces. More importantly, epicutaneous application of SLS (0.1, 1.0, and 5%, v/v) (Fig. 2, B–E) or PBS (Fig. 2A) onto the skin for 10 min did not change the thickness of epidermis or induce detectable skin inflammation.

The skin is an immunological organ. Langerhans cells form a contiguous network in the epidermis where they serve as sentinels of skin immunity by capturing and processing antigens that get through the stratum corneum and responding to cell signals activated by surrounding keratinocytes (1). It has been shown that disruption of the stratum corneum alters the maturational status of Langerhans cells (26). It has been shown that using ear skin is an ideal model to investigate the Langerhans cell maturation (17). Epidermal sheets were prepared from ear skin, and Langerhans cell maturation was examined by double staining the sheets with Langerhans cell surface marker (MHC class II) and maturation marker (CD86). Spreading 1.0% (v/v) SLS on the dorsal surface of ear skin for 10 min did not change the number of MHC class II-positive Langerhans cells residing in the epidermis (Fig. 4, A and B). These results indicate that SLS did not induce a migration of Langerhans cells from the epidermis. Intriguingly in the PBS-treated skin (Fig. 4C), the expression of CD86 was undetectable. In contrast, after spreading of 1.0% (v/v) SLS on the dorsal surface of ear skin for 10 min, the expression of CD86 on the MHC class II-positive Langerhans cells was dramatically enhanced (Fig. 4D), indicating that epicutaneous application of SLS induced Langerhans cell maturation. Application of 0.1 and 5% (v/v) SLS for 10 min produces effects similar to those of 1.0% (v/v) SLS on the number and maturation of epidermal Langerhans cells (data not shown).

In Vivo Sampling of Secreted Proteins Using CUF Probes—A novel proteomic approach was used to characterize the molecular nature of surfactant SLS-induced skin changes. Traditional proteomic approaches, primarily using two-dimensional gel electrophoresis (2-DE), are often successful in identifying the proteins/peptides released from cells (27). However, most of the secreted proteins/peptides identified via 2-DE gels are of high abundance. The 2-DE gel approaches generally are not sufficiently sensitive for the detection of low abundance proteins/peptides including secretory proteins. Moreover it remains a significant challenge.

Langerhans cell maturation (17).
to obtain pure secretory proteins/peptides in vivo. To obtain low abundance and/or secretory proteins/peptides in vivo, our laboratory designed novel CUF probes (Fig. 5, A and B). Capillary ultrafiltration using CUF probes involves applying a vacuum to semipermeable membrane fibers to extract fluid containing the desired secretory molecules from the interstitial space. The CUF probe (Figs. 5 and 6) was implanted into the mouse ears of ICR mice to collect skin-secreted proteins. A semipermeable membrane hollow fiber with molecular mass cutoff of 50 kDa was connected to the front end of the CUF probes to avoid collection of cell debris or contaminated proteins leaking from lysed cells (Fig. 5, A and B). The semipermeable membrane hollow fiber was entirely covered by ear skin following implantation (Fig. 6A). After implantation, a 1% (v/v) solution of SLS was applied to the dorsal surface of the ear skin for 10 min. The ear skin was then washed extensively with distilled water to remove SLS from the skin surface. Skin-secreted proteins were collected using CUF probes for 24 h following SLS application. During the 24-h collection, mice wore an elastomer saddle tether and were placed in a balanced level arm to avoid relocation of the CUF probes (Fig. 5, C and D). Mouse ears treated with PBS served as a control group. Histological analysis clearly demonstrated that the CUF probe was implanted into the subcutaneous space (Fig. 6B). At 24 h postimplantation, a collection of 15–20 μl of fluid was obtained from each mouse. After digestion with trypsin, the fluid containing a complex mixture of peptides was subjected to MALDI-TOF MS and Q-TOF MS/MS analyses.

Mass Spectrometric Identification of the CUF Probe-collected Proteins—Comparing the MALDI-TOF MS spectra of samples collected from PBS- and SLS-treated mouse ears (Fig. 7), we found that at least four peptide peaks (1,399.6, 1,428.6, 1,609.8, and 1,681.8 m/z) were exclusively present in the sample collected from the PBS-treated group (Fig. 7A). Six peptide peaks (1,179.6, 1,308.6, 1,694.8, 1,708.8, 2,395.0, and 2,706.2 m/z) were exclusively detectable in SLS-treated mouse ears (Fig. 7B). Two peptide peaks (1,475.7 and 1,796.8 m/z) were present in samples both from PBS- and SLS-
To confirm that S100A9 was exclusively present in the PBS-treated skin and thymosin β4 was solely found in the SLS-treated skin, we measured the intact proteins in the samples from PBS- and SLS-treated skin via mass spectrometry (Fig. 9). The samples were collected continuously with CUF probes for 24 h. Collected samples without trypsin digest were subjected to MALDI-TOF MS (Fig. 9, A and B) and Q-TOF MS/MS (Fig. 9, C–F) analysis. The spectra of MALDI-TOF MS illustrated that one major protein peak detected between 12,400 and 13,700 m/z was present in the PBS- but not in the SLS-treated skin (Fig. 9A), and another major protein peak detected between 4,500 and 5,500 m/z was present in the SLS- but not in the PBS-treated skin (Fig. 9B). The observed mass of S100A9 in mass spectrometry has been reported as 12,791 m/z (28). This protein was detected in PBS- (Fig. 9, C and E) but not in SLS-treated skin (data not shown). On the other hand, although the mass of thymosin β4 has been reported to be 5,679 Da (29), we found an observed mass of 4,963 m/z (Fig. 9A) in the CUF-collected samples. This protein with an observed mass of 4,963 m/z was calculated as acetylated thymosin β4 (29). In addition, this protein was exclusively detected in SLS- (Fig. 9, D and F) but not in PBS-treated skin (data not shown). These results indicate that S100A9 and acetylated thymosin β4 were exclusively present in the PBS- and SLS-treated skin, respectively.

**DISCUSSION**

Routine use of cleansers, shampoos, cosmetics, and other skin care products may result in daily exposure of our skin to surfactants (30). In addition, it is known that surfactants efficiently enhance transdermal delivery of various drugs such as testosterone (31) and antidepressant agents (32). Scientifically it has been reported that the anionic surfactant SLS can enhance skin permeability by extracting lamellar lipids from stratum corneum (25). Moreover higher concentrations of SLS can form micelles and penetrate into the epidermis (33). It thus seems likely that SLS application may be beneficial in skin vaccination. Surfactants have been designed as vesicles to encapsulate the antigens (14, 15). Mice exhibit a detectable antibody response after epicutaneous application of surfactant-based vesicles (niosomes) encapsulated with antigens. The use of niosomes, however, is likely to have unexpected side effects because the surfactants cannot be removed. We thus pretreated mice with anionic surfactant SLS and epicutaneously applied HEL antigen after washing. Our results demonstrated that pretreatment of mouse skin with 0.1, 1.0, and 5% (v/v) anionic surfactant SLS for 10 min significantly enhanced production of antibody to HEL (Fig. 1). Surprisingly, however, the 5% (v/v) solution exhibited the lowest enhancement. Additionally vaccinating mice simultaneously using the mixture of SLS with HEL did not elicit detectable antibody to HEL. One possible explanation is that higher concentrations of SLS may be absorbed into epidermis and cannot be removed completely after washing. Another possibility is that...
higher concentrations of SLS may impair the skin immuno-
competent cells such as keratinocytes (34) leading to lower
immune responses. Alternatively mixing the anionic SLS with
HEL may biochemically decrease the immunogenicity of HEL.
It is worth investigating the efficiency of various surfactants at
different concentrations and electric charges on the enhance-
ment of skin vaccination.

A strategy involving pretreatment of skin with surfactant
prior to antigen application has significant clinical promise. It
is possible that the best approach with the least side effects
may involve short term exposure followed by washing. It is
also worth investigating whether antigens need to actually
penetrate surfactant-treated skin to stimulate the desired im-
mune response. Recent evidence has shown that intestinal
dendritic cells can reach invasive pathogens by either entering
or extending dendrites into the epithelium and intestinal lumen
(35). It is thus possible that skin dendritic cells might extend
dendrites into the surfactant-induced disorganized stratum
corneum, eliminating the requirement for skin penetration.

It has been reported that skin exposed to anionic SLS for

Fig. 8. Identification of S100A9 and thymosin β4 by Q-TOF MS/MS se-
quencing. An internal peptide of S100A9 with m/z value at 1,681.8 ana-
lyzed by MALDI-TOF MS (Fig. 7) was sequenced by Q-TOF MS/MS as SITTI-
IDTFHQYSR (A). An internal peptide of thymosin β4 with m/z value at 1,694.8
analyzed by MALDI-TOF MS (Fig. 7) was sequenced by Q-TOF MS/MS as acety-
lated Ac-SDKPDMAEIEKFDK (B).
subjected to the MALDI-TOF MS (24 h). Collected samples containing a mixture of intact proteins were SLS-treated skins were collected continuously with CUF probes for 24 h. Previous studies indicated that maturation and migration of Langerhans cells can be independently regulated events (26, 39). Thus perturbation of the skin barrier by SLS may signal Langerhans cells to maintain cutaneous homeostasis against increasing exposure to external substances including antigens.

Very little is known about the molecular mechanisms mediating the effects of SLS on skin. The expression of some proteins such as vascular endothelial growth factor (40, 41) and heat shock protein 27 (42) was found to be altered in SLS-treated keratinocytes. We applied a novel proteomic technique using ultrafiltration capillaries and mass spectrometry to detect in vivo secreted proteins/peptides in the PBS- and SLS-treated skins. Comparing the MALDI-TOF MS spectra of sample collected from PBS- and SLS-treated skins (Fig. 6), we found that at least four and six peptide peaks were exclusively present in the sample collected from PBS- and SLS-treated skins, respectively. S100A9 was detected exclusively in the PBS-treated skin, whereas thymosin β4 was present exclusively in the MALDI-TOF MS spectrum of samples collected from SLS-treated skin. Thymosin β4 is a 5-kDa peptide originally recognized as thymic hormone. Thymosin β4 possesses an actin-binding domain and serves as an actin buffer by binding the monomeric actin (1:1 ratio) to prevent polymerization into actin filaments. Although thymosin β4 is expressed ubiquitously in the cytoplasm of various cell types including skin cells, it has also been detected outside of cells in blood plasma or in wound fluid. However, nothing is known about the molecular mechanisms mediating the effects attributed to extracellular thymosin β4. Interestingly thymosin β4 enhanced the antigen-presenting capacity of macrophages when macrophage monolayers were cultured in its presence (43). Data from the detection of intact proteins via mass spectrometry indicated that an acetylated form of thymosin β4 is present in SLS-treated skin. It is thus worth investigating the possible role of extracellular (acylated) thymosin β4 on the enhancement of SLS-mediated skin vaccination. S100A9, a calcium-binding protein, exists in extracellular fluids and is abundantly detectable in the cytosolic fraction of keratinocytes of normal epidermis (44) and neutrophils (45). S100A9 is frequently co-expressed with S100A8 and forms an S100A8-S100A9 complex, which was recognized as calprotectin (45).

The complex is robustly secreted from cells in response to tissue injury, inflammation, and disease. The binding of calcium induces conformational changes in S100A8-S100A9, the calcium-saturated status, which allows the binding of other proteins (46). Conformational changes in S100A9 and/or binding with other proteins may lead to the failures of collection by CUF probes and/or detection via mass spectrometry. Quantitative mass spectrometric analysis using ICAT may help in qualifying the relative amounts of secreted S100A9 in the PBS- and SLS-treated skins (47). To further validate the differential expression of S100A9 and (acylated) thymosin β4 in the skin treated with or without SLS, other techniques such as ELISA and immunohistochemistry will be conducted in the future when antibodies are available.

Because of differences in the ionization process between MALDI-TOF MS and Q-TOF MS/MS, eight peptides with high

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**Fig. 9. Measurements of the intact proteins (S100A9 and thymosin β4) by mass spectrometry.** The samples from PBS- and SLS-treated skins were collected continuously with CUF probes for 24 h. Collected samples containing a mixture of intact proteins were subjected to the MALDI-TOF MS (A and B) and Q-TOF MS/MS (C–F) analysis. A major protein peak with m/z between 12,400 and 13,700 was present in the PBS-treated skin but not in the SLS-treated skin (A). Conversely a major protein peak with m/z between 4,500 and 5,500 was present in the SLS-treated skin and absent in the PBS-treated skin (B). A Q-TOF MS spectrum of a protein peak (12,400–13,700 m/z) is shown (C). The protein with an observed mass of 12,971 (E) derived from multiple charges (12+ to 15+) (C) matched with the mass of S100A9. A Q-TOF MS spectrum of a protein peak (4,500–5,500 m/z) is shown (D). The protein with an observed mass of 4,963 (F) derived from multiple charges (5+ to 7+) (D) matched with the mass of acetylated thymosin β4.

24 h impaired the skin barrier and caused inflammation as well as irritation such as allergic contact dermatitis (36, 37). However, our results demonstrated that treatment of skin with SLS for 10 min followed by extensive washing only caused a partial impairment of the integrity of lipid lamellar structure and did not result in epidermal thickening (Figs. 2 and 3). More importantly, the partial impairment of lipid lamellar structure caused by SLS is sufficient to enhance skin vaccination induced by HEL antigen. Thus short term exposure to surfactants may be an efficient and relatively safe means to enhance skin vaccination.

Although 10 min of SLS exposure was not sufficient to cause migration of Langerhans cells from the epidermis, significant maturation of Langerhans cells was observed (Fig. 4). Acute cutaneous barrier perturbation by acetone treatment or tape stripping induces maturation of Langerhans cells in hairless mice (26, 38). Previous studies indicated that maturation and migration of Langerhans cells can be independently regulated events (26, 39). Thus perturbation of the skin barrier by SLS may signal Langerhans cells to maintain cutaneous

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**Fig. 9.** Measurements of the intact proteins (S100A9 and thymosin β4) by mass spectrometry. The samples from PBS- and SLS-treated skins were collected continuously with CUF probes for 24 h. Collected samples containing a mixture of intact proteins were subjected to the MALDI-TOF MS (A and B) and Q-TOF MS/MS (C–F) analysis. A major protein peak with m/z between 12,400 and 13,700 was present in the PBS-treated skin but not in the SLS-treated skin (A). Conversely a major protein peak with m/z between 4,500 and 5,500 was present in the SLS-treated skin and absent in the PBS-treated skin (B). A Q-TOF MS spectrum of a protein peak (12,400–13,700 m/z) is shown (C). The protein with an observed mass of 12,971 (E) derived from multiple charges (12+ to 15+) (C) matched with the mass of S100A9. A Q-TOF MS spectrum of a protein peak (4,500–5,500 m/z) is shown (D). The protein with an observed mass of 4,963 (F) derived from multiple charges (5+ to 7+) (D) matched with the mass of acetylated thymosin β4.

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24 h impaired the skin barrier and caused inflammation as well as irritation such as allergic contact dermatitis (36, 37). However, our results demonstrated that treatment of skin with SLS for 10 min followed by extensive washing only caused a partial impairment of the integrity of lipid lamellar structure and did not result in epidermal thickening (Figs. 2 and 3). More importantly, the partial impairment of lipid lamellar structure caused by SLS is sufficient to enhance skin vaccination induced by HEL antigen. Thus short term exposure to surfactants may be an efficient and relatively safe means to enhance skin vaccination.

Although 10 min of SLS exposure was not sufficient to cause migration of Langerhans cells from the epidermis, significant maturation of Langerhans cells was observed (Fig. 4). Acute cutaneous barrier perturbation by acetone treatment or tape stripping induces maturation of Langerhans cells in hairless mice (26, 38). Previous studies indicated that maturation and migration of Langerhans cells can be independently regulated events (26, 39). Thus perturbation of the skin barrier by SLS may signal Langerhans cells to maintain cutaneous homeostasis against increasing exposure to external substances including antigens.

Very little is known about the molecular mechanisms mediating the effects of SLS on skin. The expression of some proteins such as vascular endothelial growth factor (40, 41) and heat shock protein 27 (42) was found to be altered in SLS-treated keratinocytes. Here we applied a novel proteomic technique using ultrafiltration capillaries and mass spectrometry to detect in vivo secreted proteins/peptides in the PBS- and SLS-treated skins. Comparing the MALDI-TOF MS spectra of sample collected from PBS- and SLS-treated skins (Fig. 6), we found that at least four and six peptide peaks were exclusively present in the sample collected from PBS- and SLS-treated skins, respectively. S100A9 was detected exclusively in the PBS-treated skin, whereas thymosin β4 was present exclusively in the MALDI-TOF MS spectrum of samples collected from SLS-treated skin. Thymosin β4 is a 5-kDa peptide originally recognized as thymic hormone. Thymosin β4 possesses an actin-binding domain and serves as an actin buffer by binding the monomeric actin (1:1 ratio) to prevent polymerization into actin filaments. Although thymosin β4 is expressed ubiquitously in the cytoplasm of various cell types including skin cells, it has also been detected outside of cells in blood plasma or in wound fluid. However, nothing is known about the molecular mechanisms mediating the effects attributed to extracellular thymosin β4. Interestingly thymosin β4 enhanced the antigen-presenting capacity of macrophages when macrophage monolayers were cultured in its presence (43). Data from the detection of intact proteins via mass spectrometry indicated that an acetylated form of thymosin β4 is present in SLS-treated skin. It is thus worth investigating the possible role of extracellular (acylated) thymosin β4 on the enhancement of SLS-mediated skin vaccination. S100A9, a calcium-binding protein, exists in extracellular fluids and is abundantly detectable in the cytosolic fraction of keratinocytes of normal epidermis (44) and neutrophils (45). S100A9 is frequently co-expressed with S100A8 and forms an S100A8-S100A9 complex, which was recognized as calprotectin (45).

The complex is robustly secreted from cells in response to tissue injury, inflammation, and disease. The binding of calcium induces conformational changes in S100A8-S100A9, the calcium-saturated status, which allows the binding of other proteins (46). Conformational changes in S100A9 and/or binding with other proteins may lead to the failures of collection by CUF probes and/or detection via mass spectrometry. Quantitative mass spectrometric analysis using ICAT may help in qualifying the relative amounts of secreted S100A9 in the PBS- and SLS-treated skins (47). To further validate the differential expression of S100A9 and (acylated) thymosin β4 in the skin treated with or without SLS, other techniques such as ELISA and immunohistochemistry will be conducted in the future when antibodies are available.

Because of differences in the ionization process between MALDI-TOF MS and Q-TOF MS/MS, eight peptides with high
intensities (1,399.6, 1,479.8, 1,609.8, 1,179.6, 1,308.6, 1,708.8, 2,385.0, and 2,706.2 m/z) (Fig. 7) in the MALDI-TOF MS spectrum do not appear in the Q-TOF MS spectrum (data not shown), resulting in a failure to identify these proteins. The MALDI-TOF-TOF instrument is able to obtain a fragmentation spectrum on the same ions detected in the peptide mass fingerprint of MALDI-TOF MS (48). Thus use of MALDI-TOF-TOF may be an alternative method of sequencing unidentified proteins demonstrable in the MALDI-TOF MS spectrum. Identification of S100A9 and thymosin β4 as in vivo secreted proteins was made possible by a newly designed technique of CUF probes using semipermeable membrane hollow fibers to capture secreted proteins/peptides in PBS- and SLS-treated skins. Secreted proteins/peptides were identified by MALDI-TOF MS in combination with Q-TOF MS/MS without prior 2-DE gel separation. Our data demonstrate for the first time that in vivo secreted proteins collected by CUF probes can be identified directly by mass spectrometry.

In summary, we demonstrated that short term pretreatment of skin with SLS is a simple method to enhance skin vaccination. The selection and standardization of various adjuvants or immunomodulators are still obstacles to the development of non-invasive transdermal vaccines. Our method using short term application of surfactants may offset these challenges. In parallel, we present a novel technique using ultrafiltration capillaries to capture in vivo secreted proteins. When combined with mass spectrometry, two proteins were identified and sequenced as S100A9 and thymosin β4. These proteins will be potentially selected as targets for understanding how surfactants enhance skin vaccination. We also believe this technique has numerous potential clinical applications involving in the detection of in vivo biomarkers.

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