Biomaterials 57 (2015) 50–58

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Clinical study and stability assessment of a novel transcutaneous influenza vaccination using a dissolving microneedle patch

Sachiko Hirobe [a], Hiroaki Azukizawa [b], Takaaki Hanafusa [b], Kazuhiko Matsuo [a], Ying-Shu Quan [c], Fumio Kamiyama [c], Ichiro Katayama [b], Naoki Okada [a, **], Shinsaku Nakagawa [a, *]

[a] Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Department of Dermatology, Course of Integrated Medicine, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] CosMED Pharmaceutical Co. Ltd., 32 Higashikujokawanishi-cho, Minami-ku, Kyoto 601-8014, Japan

ARTICLE INFO

Article history:
Received 6 January 2015
Received in revised form
1 April 2015
Accepted 3 April 2015
Available online 24 April 2015

Keywords:
Drug delivery
Hyaluronic acid
Immune response
ELISA

ABSTRACT

Transcutaneous immunization (TCI) is an attractive vaccination method compared with conventional injectable vaccines because it is easier to administer without pain. We developed a dissolving microneedle patch (MicroHyala, MH) made of hyaluronic acid and showed that transcutaneous vaccination using MH induced a strong immune response against various antigens in mice. In the present study, we investigated the clinical safety and efficacy of a novel transcutaneous influenza vaccine using MH (flu-MH), which contains trivalent influenza hemagglutinins (15 μg each). Subjects of the TCI group were treated transcutaneously with flu-MH, and were compared with subjects who received subcutaneous injections of a solution containing 15 μg of each influenza antigen (SCI group). No severe local or systemic adverse events were detected in either group and immune responses against A/H1N1 and A/H3N2 strains were induced equally in the TCI and SCI groups. Moreover, the efficacy of the vaccine against the B strain in the TCI group was stronger than that in the SCI group. Influenza vaccination using MH is promising for practical use as an easy and effective method to replace conventional injections systems.

1. Introduction

Infectious diseases are the leading cause of death worldwide. Therefore, the development of safe and effective vaccines is of paramount importance. For example, the H1N1 swine influenza outbreak in Mexico in 2009 [1] and the reemergence of tuberculosis [2] warrant increasing emphasis on vaccine development and indicate the need for global mass vaccination to avoid the risk of pandemic. However, conventional vaccinations are administrated with subcutaneous or intramuscular injections, which require medical personnel with technical skills and is accompanied with the risk of needle-related diseases and injuries. Moreover, antigen solutions require cold chain storage and transportation systems. Therefore, the development of vaccines, which are easy-to-use in administration and superior in stability of formulations, is critically important.

We have developed a transcutaneous microneedle delivery device, which meets the requirements of “the only application” vaccination system. This technique is readily adaptable for widespread practical use, particularly in developing nations with inadequate public health resources. Many nonclinical studies describe the efficacy of a transcutaneous vaccination using microneedles fabricated with silicon or metal and soluble polymers [3,4], but few reports demonstrate the safety and efficacy of these vaccines in humans. Only one transcutaneous influenza vaccine using silicon microneedles induced immune responses in humans [5]. However, microneedles made of metal, stainless steel, or silicon have risks of fractures, leaving fragments in the skin. Therefore, we developed a

Abbreviations: APC, antigen-presenting cell; EMA, European Medicines Agency; FCS, fetal calf serum; GMT, geometric mean titer; HA, hemagglutinin; HI, hemagglutination inhibition; MH, MicroHyala; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PPS, per protocol set; RBC, red blood cell; SCI, subcutaneous immunization; TBS-T, Tris–HCl-buffered saline containing 0.1% Tween-20; TCI, transcutaneous immunization.

* Corresponding author. Tel.: +81 6 6879 8175; fax: +81 6 6879 8179.
** Corresponding author. Tel.: +81 6 6879 8176; fax: +81 6 6879 8179.
E-mail addresses: okada@phs.osaka-u.ac.jp (N. Okada), nakagawa@phs.osaka-u.ac.jp (S. Nakagawa).

http://dx.doi.org/10.1016/j.biomaterials.2015.04.007
0142-9612/

dissolving microneedle patch (MicroHyala; MH) fabricated from hyaluronic acid which is a component of skin tissue [6]. Microneedles on MH penetrate the stratum corneum, which acts as a physical barrier on the outermost layer of the skin, and are dissolved by water in the skin. Subsequently, MH efficiently delivers various materials that can be loaded into the microneedles to abundant immunocompetent cells such as Langerhans cells, dermal dendritic cells, and keratinocytes in the epidermis and dermis below the stratum corneum [7]. Previously, we have reported that the delivery of a transcutaneous vaccine using MH induced an antigen-specific primary immune response against various antigens such as ovalbumin, tetanus–diphtheria toxoid, influenza HA antigens, and recombinant malaria SE36 antigen [8].

In order to lead the fundamental researches, which showed that microneedle formulations are safe and effective in animals, to the practical use, we conducted a clinical study of transcutaneous influenza vaccinations using MH and made compared them with conventional vaccine systems. Moreover, we performed the stability testing of formulation for the development of cold chain-free vaccine.

2. Material and methods

2.1. Preparation of flu-MH

MHs were fabricated using micromolding technologies with sodium hyaluronate as the base material in a clean room [6]. Sodium hyaluronate (JP grade, Kikkoman Biochemifa Company, Tokyo, Japan), dextran 70 (JP grade, Meito Sangyo, Nagoya, Aichi), and povidone (JPE grade, BASF Japan, Tokyo, Japan) were dissolved in distilled water at a weight ratio of 11:8:1 and were mixed with trivalent seasonal influenza hemagglutinin (HA) antigens [The Research Foundation for Microbial Diseases of Osaka University, Suita, Japan; 2011–2012 season strain: A/California/07/2009 (H1N1), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008]. The aqueous solution was cast into micromolds and was dried in a desiccator at room temperature, and MHs containing influenza HA antigens (flu-MHs) were then separated from micromolds. Some samples of flu-MHs were subjected to microorganism test. The resulting flu-MH contained trivalent seasonal influenza HA antigen (15 μg each) in 200 microneedles, which are 800 μm long. The flu-MH system comprised of patches with an area of 0.8 cm^2 that were fixed onto 2.3 cm^2 adhesive films. Subsequently, flu-MHs were packed in aluminum laminated polyethylene terephthalate films and were stored at 4 °C until clinical use or at 4 °C, 25 °C, or 40 °C for 6 month for stability testing.

2.2. Clinical study

2.2.1. Vaccination

Forty healthy men (20–49 years of age) volunteered and were enrolled in the study. Written informed consent was received before enrollment. Subjects were randomly divided into two groups of 20 and were treated with transcutaneous immunization (TCI; TCI group) or subcutaneous immunization (SCI; SCI group). One subject (TCI-16) in the TCI group was eliminated from the study because of a vasovagal syncope when his blood was drawn during the first vaccination. Trivalent influenza HA antigens were administered twice and the second vaccination was administered 3 weeks after the first. The study protocol is summarized in Table 1. The TCI group received flu-MHs containing 15 μg of each influenza HA antigen and were applied to the skin of the left upper arm for 6 h using a handheld applicator. The SCI group received subcutaneous injections in each left upper arm of 0.5 mL of Influenza HA Vaccine "BIKEN" (The Research Foundation for Microbial Diseases of Osaka University, Suita, Japan), which contained >15 μg of each influenza antigen. All clinical procedures were approved by Institutional Review Board for Clinical Research at Osaka University Hospital.

Table 1
Clinical protocol for assessing the safety and efficacy of the flu-MH.

Day	0	2	7	21	23	28	42
Vaccination	1st			2nd			
	●			●			
Assessment of local reactions		●	●	●	●	●	●
Blood test	●	●	●	●	●	●	●
Antibody measurement	●		●	●		●	●
Cytokine production	●			●			●

2.2.2. Sample collection

Blood samples were collected before and at 2, 7, and 21 days after vaccination. Sera were obtained by centrifugation of blood samples at 5000 rpm for 15 min. Peripheral blood mononuclear cells (PBMCs) were separated from blood samples taken before and 21 days after vaccination using a BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate (BD, Franklin Lakes, USA). Nasal wash samples were obtained according to a modified protocol of Ainai et al. [9]. Subjects washed their nasal cavities using a nose irrigation solution and a device (Hananoa; Kobayashi Pharmaceutical, Osaka, Japan), and the liquid from nasal washes was collected. Dental cotton was immersed in nasal washes to remove crude materials, and the collected liquid and dental cotton were placed on 0.45 μm filter units (Nalgene, Thermo Fisher Scientific). Subsequently, filtrates were concentrated to 1.0 mg/mL total protein using Vivaspin centrifugal concentrators (Vivaspin 20, MWCO = 30,000; Sartorium Stedim Biotech, Aubagne, France).

2.2.3. Confirmation of microneedle skin insertion

Microneedles with flu-MH were observed after application using a stereo microscope (VHX-1000; KEYENCE, Osaka, Japan). Because microneedles dissolve after insertion into skin, the remaining microneedles were counted and the ratio of dissolved microneedles was calculated.

2.2.4. Assessment of local and systemic adverse reactions

The presence and diameter of epidermal erythematous lesions were evaluated, and the presence of purpura was observed by applying pressure to erythematous areas using a glass plate and observing temporary disappearance of erythema. Subsequently, pigmentation, induration, pressure-induced pain, fever, and the presence of water blisters were assessed, and standard peripheral blood tests and biochemical tests of liver and renal function were performed.

2.2.5. Hemagglutination inhibition (HI) test

HI tests of sera and nasal washes were performed by SRL, Inc. A/California/7/2009 (H1N1) and B/Brisbane/60/2008 strains were detected in HI tests using chicken red blood cells (RBCs), and the A/Victoria/210/2009 (H3N2) strain was detected in HI tests using turkey RBCs. In these experiments, sera and nasal washes were treated with receptor-destroying enzyme [RDE (II); Denka Seiken Co., Ltd., Tokyo, Japan] and RBCs to remove nonspecific hemagglutination inhibitors. Sera and nasal washes were then diluted 1:10 and 1:2, respectively, and samples were then serially diluted half-fold and incubated with each HA antigen for 1 h at 37 °C. One hour after adding 0.5% RBCs, HI titers were determined as the reciprocal of the highest serum dilution that inhibited hemagglutination.

2.2.6. Measurement of HA antigen-specific antibody titer

HA antigen-specific antibody titers were determined using a sandwich ELISA. ELISA plates were coated with mouse anti-influenza A H1N1 HA (RayBiotech, Inc., Georgia, USA), mouse anti-influenza A H3 (Meridian Life Science, Inc., Tennessee, USA), or mouse anti-influenza B (RayBiotech, Inc.) antibodies overnight at 4 °C. Plates were then blocked with 10% fetal calf serum (FCS; Life Technologies, California, USA)/phosphate-buffered saline (PBS). Influenza HA antigens (10 μg HA/mL) in 10% FCS/PBS were then added and incubated for 2 h at 25 °C. The plates were then washed five times with Tris–HCl-buffered saline containing 0.1% Tween-20 (TBS-T), and half-fold serial dilutions of sera or nasal wash samples were added. After incubation at 25 °C for 2 h, the plates were washed five times with TBS-T, and HRP-rabbit anti-human IgG (Southern Biotech), HRP-goat anti-human IgA (Sigma–Aldrich), or HRP-goat anti-human IgM (Sigma–Aldrich) antibodies were added. Two hours later, the plates were washed five times with TBS-T and then once with distilled water. TMB-sensitive substrate solution (Moss Inc.) was added and the plates were incubated for 20 min at 25 °C in the dark. After the termination of color development by the addition of 2 N H_2SO_4, optical densities were measured at 450 and 650 nm. Antibody titers were expressed as the reciprocal $\log_2$ titer of the highest dilution that generated 0.1 absorbance units after subtracting the absorbance of preimmune sera.

2.2.7. ELISPOT assay of interferon (IFN)-γ producing cells

PBMCs were stimulated *in vitro* with each influenza HA antigen (10 μg/mL) for 24 h. HA-specific IFN-γ producing cells were measured using an IFN-γ ELISPOT assay kit (BD Biosciences).

2.3. Stability of the flu-MH

2.3.1. Mechanical failure force for microneedle

The force necessary for mechanical microneedle fracture was measured using a TA-XT plus texture analyzer (StableMicro Systems, Surrey, UK). Each flu-MH was attached to a test station using double-sided adhesive tape. Axial force was then applied using a flathead 5 mm diameter stainless steel cylinder, which was moved at a rate of 1.1 mm/min with a trigger force of 0.049 N.

2.3.2. HA antigen loading in flu-MH

Each HA antigen solution was prepared by dissolving flu-MH in PBS before and after 6-month storage at various temperatures. The protein concentrations of HA antigen solutions were measured using the Lowry method (DC Protein Assay Kit; BioRad) after adding equivalent volumes of 4% sodium dodecyl sulfate solution. A standard sample was HA antigen solution, which was used for preparation of the flu-MH. The hyaluronic acid concentrations in HA antigen solutions were measured using ELISA (QnE Hyaluronic Acid ELISA Assay; Biotech Trading Partners) and quantities of HA antigen (μg) were calculated relative to 1 mg of hyaluronic acid.

2.3.3. HA titer by hemagglutination assay

HA antigen solutions were prepared from flu-MH before and after 6-month storage at various temperatures, and were serially diluted half-fold from 1 μg HA/mL in PBS and were added to 96-well plates at 50 μL/well. Subsequently, 50 μL aliquots of 0.5% chicken RBCs suspension were added, stirred at 700 rpm for 1 min, and then incubated for 1 h at room temperature. HA titers were defined as the reciprocal of the lowest HA antigen dilution that caused hemagglutination.

2.3.4. Immunogenicity of HA antigen

HA antigen solutions were prepared from flu-MH before and after 6-month storage at various temperatures and were diluted to 100 ng HA/mL. Subsequently, female BALB/c mice (6-week old; SLC Inc, Hamamatsu, Japan) were subcutaneously immunized with 100 μL of antigen solutions (10 ng HA). This procedure was repeated twice at 4-week intervals, and sera were collected to determine HA-specific IgG titers at 2 weeks after the second immunization. Animals were maintained in the experimental animal facility at Osaka University and experiments were conducted in accordance with the guidelines provided by the Animal Care and Use Committee of Osaka University.

2.3.5. Measurement of HA antigen-specific antibody titers

HA antigen-specific antibody titers were determined using a sandwich ELISA. ELISA plates were coated with rabbit anti-influenza HA antibodies (clone 376 for A/H1N1, clone 2 for A/H3N2, clone 004 for B; Sino Biological Inc., China) overnight at 4 °C. The plates were then blocked with 8% skim milk (MEGMILK SNOW BRAND Co., Ltd., Japan)/PBS. Influenza HA antigens (10 μg HA/mL) in 8% skim milk/PBS were then added and incubated for 2 h at 25 °C. Plates were then washed five times with TBS-T and half-fold serial dilutions of sera were added. After incubation at 25 °C for 2 h, the plates were washed five times with TBS-T and HRP-goat anti-mouse IgG (Southern Biotech) was added. Two hours later, the plates were washed seven times with TBS-T and then once with distilled water. TMB-sensitive substrate solution (Moss Inc.) was added and the plates were incubated for 20 min at 25 °C in the dark. After the termination of color development by the addition of 2 N H_2SO_4, optical densities were measured at 450 and 655 nm. Antibody titers were expressed as the reciprocal $\log_2$ titer of the highest dilution that generated 0.1 absorbance units after subtracting the absorbance of preimmune sera.

3. Results

3.1. Analysis of microneedle dissolution

Among subjects of the TCI group, 19 received flu-MH and the delivery of influenza HA antigens into the skin was assessed by observing dissolution of flu-MH microneedles after application. In a pilot study and first vaccination of prospective study, microneedles did not completely dissolve after 6 h (Supplemental Fig. S1), reflecting the poor insertion of microneedles into skin using a spring-type applicator. Thus, a new applicator, whose spring was more strong, was used for the second vaccinations of prospective study and flu-MH microneedles completely dissolved in all subjects. These results indicated that the improvement of components of microneedle and applicator are important for practical use of a novel transcutaneous delivery device.

In this study, we evaluated the utility of TCI using MH and compared it with the efficacy of SCI. However, antigen delivery may have been deficient in TCI group. Thus, we assumed that solubility correlated with the amount of influenza HA antigen delivered to the skin and calculated the ratio of microneedle dissolution for all flu-MHs after application (Table 2). In seven patients, microneedles were >50% dissolved after both first and second vaccinations. Therefore, we established a “per protocol set” (PPS) of seven subjects in the TCI group to determine its safety and efficacy in subjects who did not show deviations from the protocol.

3.2. Safety assessment of transcutaneous influenza vaccination in humans

The primary aim of this study was to demonstrate that flu-MHs are safe for human use. The adverse effects at application sites of flu-MHs and at subcutaneous injection sites are listed in Table 3. Temporary erythema was observed in all subjects in the TCI group,

Table 2
Confirmation of microneedle skin insertion by microneedle dissolution.

Spring of handheld applicator used in application			Weak	Weak	Strong
Study	ID	Age	Microneedle dissolution (%)		
Pilot	**TCI-01**[a]	**49**	**86**	**100**	–
	TCI-02[a]	**27**	**100**	**57**	–
	TCI-03[a]	**29**	**100**	**96**	–
Prospective	TCI-04	22	–	42	100
	TCI-05	21	–	16	100
	TCI-06[a]	**22**	–	**70**	**100**
	TCI-07[a]	**23**	–	**94**	**100**
	TCI-08	22	–	13	100
	TCI-09	24	–	3	100
	TCI-10	23	–	26	100
	TCI-11[a]	**22**	–	**51**	**100**
	TCI-12	22	–	0	100
	TCI-13	25	–	41	100
	TCI-14	24	–	29	100
	TCI-15	22	–	0	100
	TCI-17	21	–	0	100
	TCI-18	20	–	27	100
	TCI-19[a]	**24**	–	**59**	**100**
	TCI-20	22	–	16	100

[a] PPS (microneedles dissolved by more than 50% at both the first and second vaccinations).

and images from subjects with the most remarkable erythema are shown in Fig. 1. Two days after vaccination, erythema was larger than the area of flu-MH application, and by day 21, the area had decreased in size and erythema was diminished. In the SCI group, erythema was observed in five subjects on day 2, and the area of erythema in one subject reached a diameter of 9 cm (Fig. 1). Twenty-one days after vaccination, eight subjects of the SCI group still had injection marks on their arms. The frequency of erythema in the TCI group was higher than that in the SCI group. This difference may reflect the easy recognition of reactions such as inflammation following the delivery of influenza HA antigens to the outer layer of skin compared with those present in the subcutaneous layer. After the second vaccination, erythema appeared in all subjects of the TCI group and in 13 subjects of the SCI group, and persisted for longer times in both the groups. It was assumed that these inflammatory reactions were caused by induction of immune responses at the first vaccination.

In TCI group, purpura, which is caused by capillary damage, was observed in more than half of the subjects two days after vaccination, but was undetectable by day 21. Pigmentation was observed in approximately half of the subjects in the TCI group and first appeared 7 days after vaccination. No obvious differences in the appearance and frequency of purpura and pigmentation were observed after the first and second vaccinations, in which the puncture of the skin with a dense set of microneedles (200 microneedles/patch) caused slight capillaropathy and induced purpura with the deposition of hemoglobin in the tissue. Moreover, pigmentation was likely generated by the hemosiderin produced by the decomposition of RBCs by phagocytes. However, purpura and pigmentation were eventually undetectable and the skin recovered its normal appearance. In the SCI group, purpura was observed in more than half of the subjects 2 days after the second vaccination (day 23). Induration was observed in some members of TCI and SCI groups two days after vaccination, but was undetectable after 7 days. Pressure-induced pain and fever tended to appear at higher frequencies in the SCI group than that in the TCI group. No subjects of either group experienced water blisters.

The administration of flu-MHs did not induce detectable adverse systemic effects, according to the results of standard peripheral blood tests (Supplemental Tables 1 and 2). Taken together, the results of safety testing of TCI using flu-MHs were acceptable, with no observed severe local and systemic adverse events.

3.3. Efficacy of transcutaneous influenza vaccination using MH

The efficacy of flu-MHs was determined as a secondary endpoint by measuring anti-HI antibody titers in sera (Fig. 2A), seroconversion and seroprotection rates, and relative increases in geometric mean titers (GMT), according to the guidelines of the European Medicines Agency (EMA; Table 4). In the TCI group, the first vaccination increase anti-HI antibody titers against all trivalent HA antigens and met the EMA criteria. The EMA criteria were also met by a single vaccination of the SCI group against A/H1N1 and A/H3N2 strains and by two vaccinations with the B strain. Therefore, the efficacy of vaccinating against A/H1N1 and A/H3N2 strains in

Table 3
Local adverse events caused by immunization.

Vaccination	Day	Erythema				Purpura		Pigmentation	
		Diameter (mm)		No. of positive subjects		No. of positive subjects		No. of positive subjects	
		TCI (n = 7)	SCI (n = 20)	TCI (n = 7)	SCI (n = 20)	TCI (n = 7)	SCI (n = 20)	TCI (n = 7)	SCI (n = 20)
1st	2	15.4 ± 4.9	12.2 ± 24.3	7 (100%)	5 (25%)	4 (57%)	1 (5%)	0 (0%)	0 (0%)
	7	12.7 ± 1.3	1.0 ± 1.3	7 (100%)	8 (40%)	6 (86%)	1 (5%)	2 (29%)	0 (0%)
	21	2.9 ± 5.0	1.0 ± 1.3	2 (29%)	8 (40%)	0 (0%)	1 (5%)	3 (43%)	0 (0%)
2nd	23	17.0 ± 9.5	1.7 ± 1.5	7 (100%)	13 (65%)	5 (71%)	13 (65%)	0 (0%)	0 (0%)
	28	13.0 ± 5.5	1.3 ± 1.2	7 (100%)	12 (60%)	3 (43%)	1 (5%)	0 (0%)	1 (5%)
	42	7.6 ± 5.2	1.1 ± 1.0	5 (71%)	13 (65%)	0 (0%)	0 (0%)	4 (57%)	0 (0%)
Vaccination	Day	Induration		Pressure-induced pain		Fever		Water blisters	
		No. of positive subjects		No. of positive subjects		No. of positive subjects		No. of positive subjects	
		TCI (n = 7)	SCI (n = 20)	TCI (n = 7)	SCI (n = 20)	TCI (n = 7)	SCI (n = 20)	TCI (n = 7)	SCI (n = 20)
1st	2	5 (71%)	1 (5%)	1 (14%)	5 (25%)	0 (0%)	5 (25%)	0 (0%)	0 (0%)
	7	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	21	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2nd	23	2 (29%)	1 (5%)	0 (0%)	1 (5%)	1 (14%)	1 (5%)	0 (0%)	0 (0%)
	28	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	42	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

The values parentheses indicate the percentage of positive subjects.

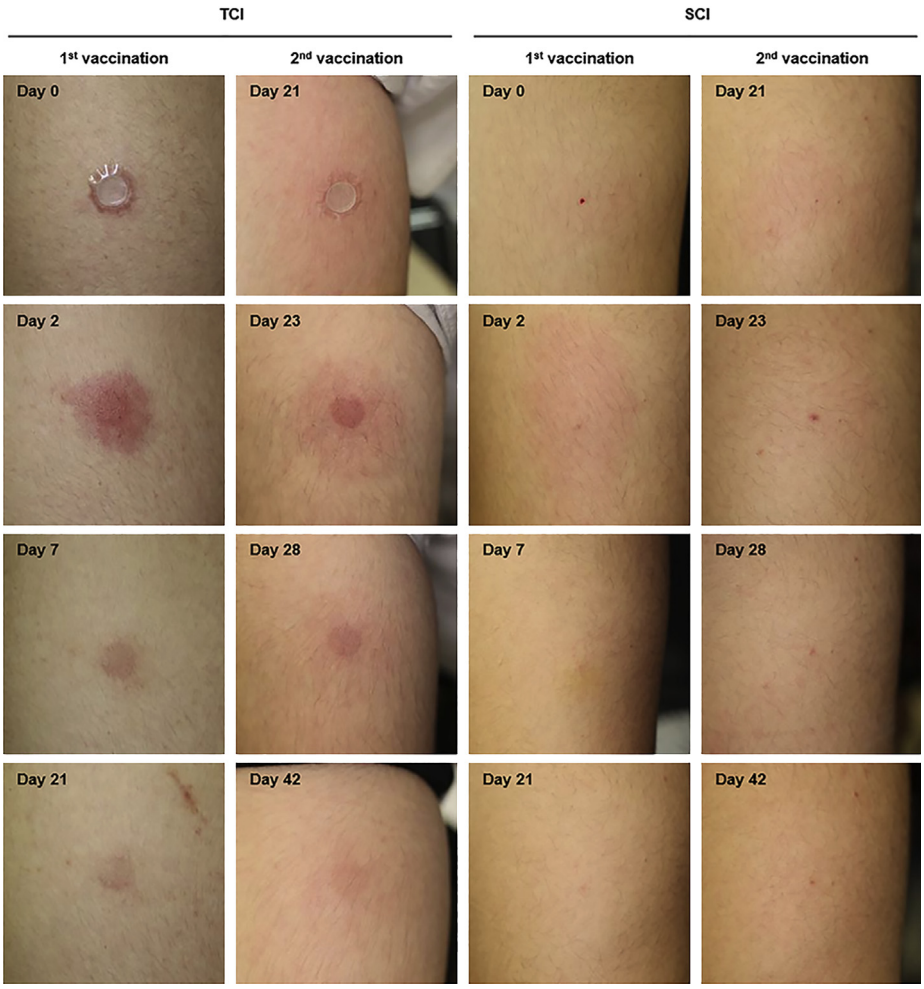

Fig. 1. Local skin images after TCI using flu-MH and after SCI using an injectable HA vaccine.

the TCI group was equivalent to that in the SCI group, although the TCI group was more effectively vaccinated against the B strain. IgG, IgA, and IgM antibodies were induced by each HA antigen in both groups, and there were no significant differences among trivalent HA antigens (Fig. 2B).

Because influenza viruses enter through mucosal surfaces, they may activate systemic and mucosal immunity and induce the production of secretory IgA. Therefore, we collected nasal washes and calculated seroconversions and relative increases in GMT of anti-HI antibody titers and estimated mucosal immunogenicity following TCI. Unlike anti-HI antibody titers in sera, those in nasal washes of both groups increased against the B strain (Fig. 3A). However, no marked increases in IgA antibodies were observed in nasal washes (Fig. 3B). Although it remains unclear why HI titers against the B strain were greatly increased, mucosal immune responses were barely detectable after treatment with the TCI system using MH. Thus, more effective vaccines against influenza may require the development of transcutaneous vaccine adjuvants that induce mucosal immune responses.

To assess T-cell dependency of immune responses, HA-specific IFN-γ-producing cells in the PBMC compartment were counted (Table 5). The numbers of IFN-γ-producing cells tended to increase following vaccination in both groups, although the ratio of subjects with higher levels of IFN-γ-producing cells before vaccination was higher in the SCI group. TCI using MH activated T cells and induced HA-specific IFN-γ-producing cells, indicating that TCI is protective against the influenza virus.

According to these efficacy assessments, the production of antibody against the B strain was greater in the TCI group than that in the SCI group, indicating an equal or a superior vaccine efficacy of TCI using MH to that of conventional injectable vaccination.

3.4. Stability of flu-MH at various temperatures

The present transcutaneous vaccine formulation was superior to conventional injectable vaccine formulations and has the potential to greatly reduce transportation, storage, and stock costs if the dry preparation can be dispensed without low temperature control (cold chain). Thus, we evaluated the stability of flu-MH before and after 6-month storage at 4 °C, 25 °C, or 40 °C. Although the failure force of microneedles decreased by 20%–50% after long term storage, the ability to puncture the skin was sufficiently maintained (Fig. 4A). Moreover, no association was observed between decreased microneedle failure forces and storage temperatures.

In subsequent experiments, we examined the stability of influenza HA antigen loaded in the flu-MH after 6-month storage and confirmed the maintenance of HA antigen contents after storage for 6 months at various temperatures (Fig. 4B). Although HA titers after 6-month storage at 4 °C were equivalent to those immediately after preparation, HA titers clearly decreased with higher storage temperatures (Fig. 4C). Hence, we evaluated the influence of decreased HA titers on the efficacy of the vaccine by measuring antibody production in sera after immunization with HA antigen solutions that were prepared from flu-MH before and after 6-months storage

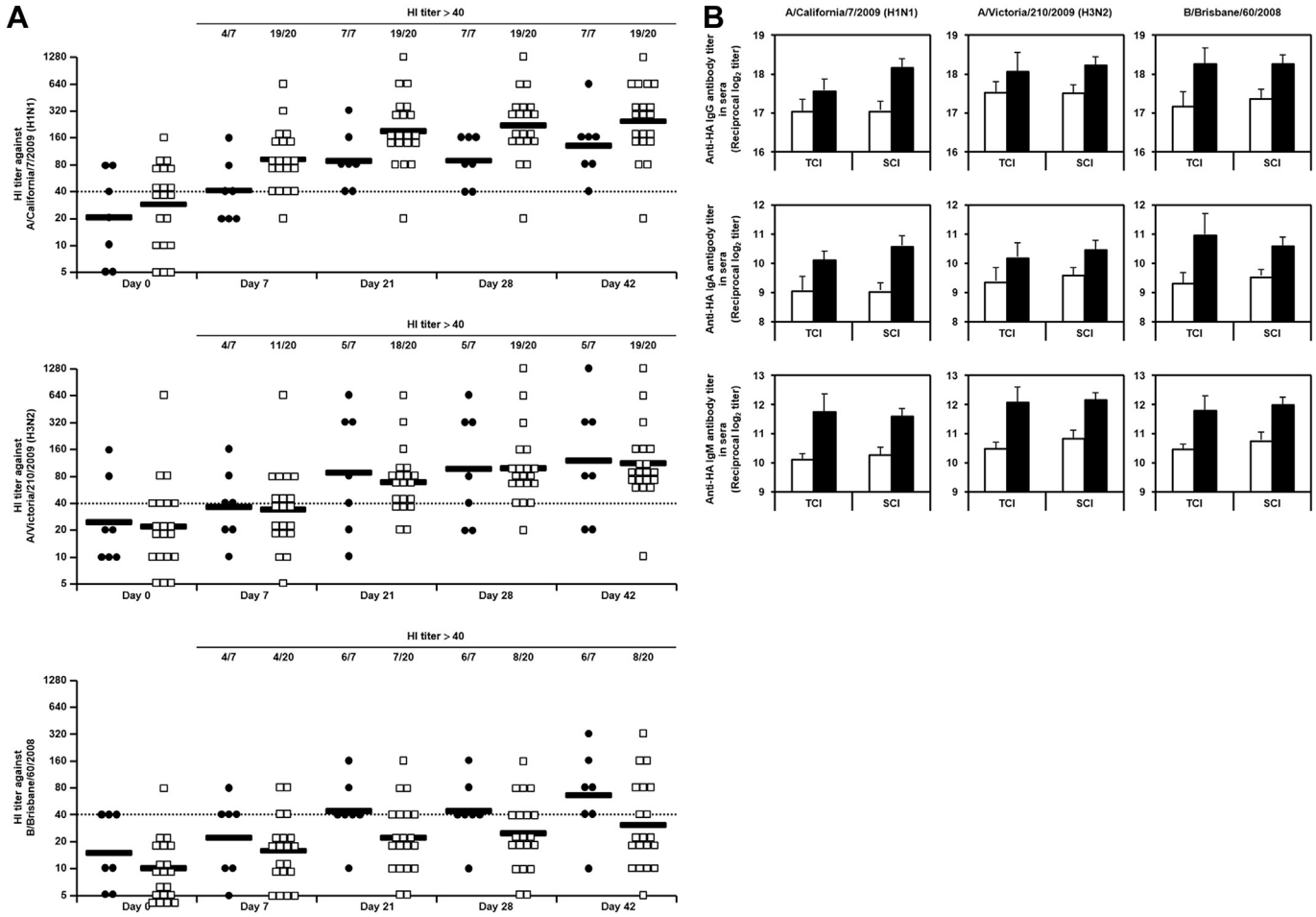

Fig. 2. HI serum titers and Ig isotypes of serum antibodies against influenza HA antigens. (A) HI titers against influenza HA antigens; Symbols indicate mean HI titers of treatment groups (●, TCI and □, SCI); GMT are indicated by bars. (B) IgG, IgA, and IgM ELISA titers; Data are expressed as the mean ± S.D. of results from seven (TCI) or twenty (SCI) subjects.

at various temperatures (Fig. 4D–F). Antibody production against the A/H1N1 strain tended to decrease with higher storage temperatures of flu-MH. However, the induction of antibody production was retained by HA antigens of the A/H3N2 and B strains and did not change immediately after preparation, even with increased storage temperatures. Taken together, the vaccine activity of the influenza HA antigen administered using flu-MH varied between strains and with stability in long-term storage.

4. Discussion

Vaccinations against influenza viruses are recommended yearly because epidemic strains differ every season. Therefore, worldwide mass vaccination is required to minimize the risk of influenza pandemics. Currently, influenza hemagglutinin (HA) antigens, which are prepared by inactivating influenza viruses with ether, are widely used as fundamental prophylaxis against influenza [10].

Table 4
Analysis of vaccine efficacy (serum HI titers).

Vaccination	Day	Parameters	A/California/7/2009 (H1N1)		A/Victoria/210/2009 (H3N2)		B/Brisbane/60/2008	
			TCI (n = 7)	SCI (n = 20)	TCI (n = 7)	SCI (n = 20)	TCI (n = 7)	SCI (n = 20)
1st	7	Seroconversion	29%	35%	29%	15%	14%	10%
		Seroprotection	57%	**95%**	57%	55%	57%	20%
		The relative increase in GMT	**2.8**	**7.0**	2.2	**3.1**	1.6	2.3
	21	Seroconversion	**57%**	**60%**	29%	40%	**43%**	25%
		Seroprotection	**100%**	**95%**	**71%**	**90%**	**86%**	35%
		The relative increase in GMT	**4.4**	**7.0**	**3.6**	**3.1**	**3.0**	2.3
2nd	28	Seroconversion	**57%**	**65%**	29%	**50%**	**43%**	30%
		Seroprotection	**100%**	**95%**	**71%**	**95%**	**86%**	40%
		The relative increase in GMT	**4.4**	**7.7**	**4.0**	**4.4**	**3.0**	2.5
	42	Seroconversion	**71%**	**65%**	**43%**	**60%**	**43%**	35%
		Seroprotection	**100%**	**95%**	**71%**	**95%**	**86%**	40%
		The relative increase in GMT	**6.6**	**8.6**	**4.9**	**5.1**	**4.4**	**3.1**

The values written in bold and underlined satisfy EMA criteria (seroconversion >40%, seroprotection >70%, and GMT fold increase>2.5).

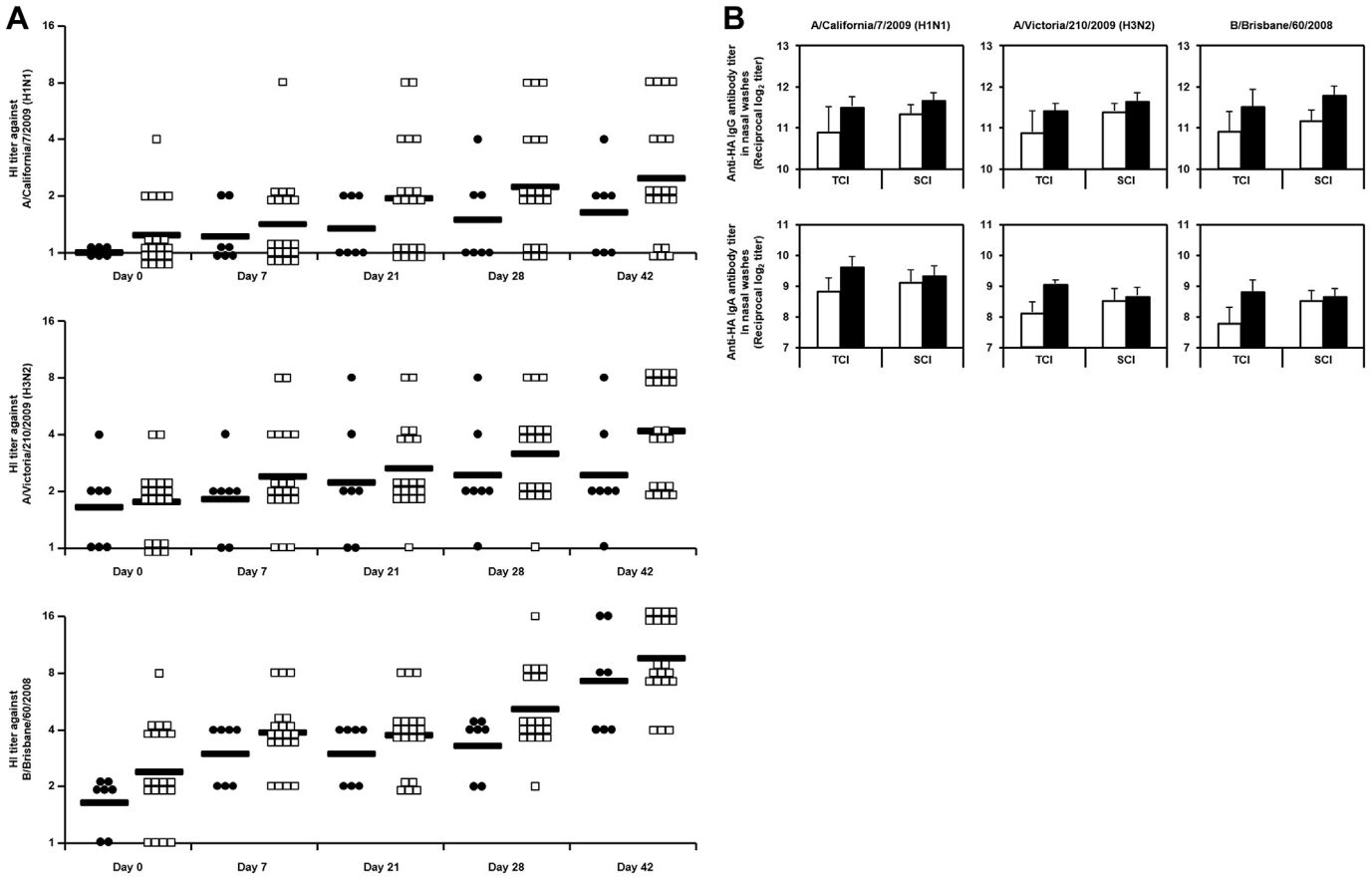

Fig. 3. HI titers and Ig isotypes of antibodies in nasal washes against influenza HA antigens. (A) HI nasal wash titers against each influenza HA antigen; Symbols indicate mean HI titers of treatment groups (●, TCI and □, SCI); GMT are indicated by bars. (B) IgG, IgA, and IgM ELISA titers; Data are expressed as the mean ± S.D. of results from seven (TCI) or twenty (SCI) subjects.

Table 5
IFN-γ-producing cells specific for each HA antigen (ELISPOT assay).

Vaccination	Day	A/California/7/2009 (H1N1)					
		Spots/10^5 cells					
		TCI (n = 7)			SCI (n = 20)		
		≤10	10–30	30 <	≤10	10–30	30 <
Base line	0	6	1	0	9	6	5
1st	21	3	4	0	2	9	9
2nd	42	3	2	1	2	14	4
Vaccination	Day	A/Victoria/210/2009 (H3N2)					
		Spots/10^5 cells					
		TCI (n = 7)			SCI (n = 20)		
		≤10	10–30	30 <	≤10	10–30	30 <
Base line	0	6	0	1	3	10	7
1st	21	3	2	2	1	13	6
2nd	42	2	3	1	2	14	4
Vaccination	Day	B/Brisbane/60/2008					
		Spots/10^5 cells					
		TCI (n = 7)			SCI (n = 20)		
		≤10	10–30	30 <	≤10	10–30	30 <
Base line	0	6	0	1	8	5	7
1st	21	2	2	3	3	9	8
2nd	42	3	1	2	1	13	6

However, these vaccines only decrease serious symptoms and mortality and do not prevent infection. Therefore, influenza vaccines that induce more robust and long-term immunity are under development using whole virus-particle inactivated vaccines and live vaccines [11,12]. However, the large-scale production of current influenza HA antigens remains difficult because influenza viruses must be propagated through a costly and time-consuming process using fertilized eggs. To overcome this problem, alternative techniques are being explored that involve the propagation of influenza virus in cultured cells and the expression of recombinant antigens in insect cells [13].

Because epidermal tissues mount robust immune responses, we have focused on the development of a novel vaccination system using a transcutaneous device that targets the skin [14,15]. This technique is readily adaptable for widespread clinical use, particularly in developing nations with inadequate public health resources. Previously, it was demonstrated that such vaccines are much easier to administer and are as effective as conventional intramuscular injections, even with reduced doses of antigen [5]. However, the use of hollow silicon microneedles is problematic for the transport and storage of antigen solutions at cold temperatures and may cause secondary infections because hollow microneedles differ from conventional vaccines that are delivered using hypodermic needles and syringes. In contrast, MH is safer because the microneedles dissolve in the skin, eliminating the requirement for cold vaccine preparations. This reduces the costs of transport and storage because MH is a dry solid, which will facilitate its

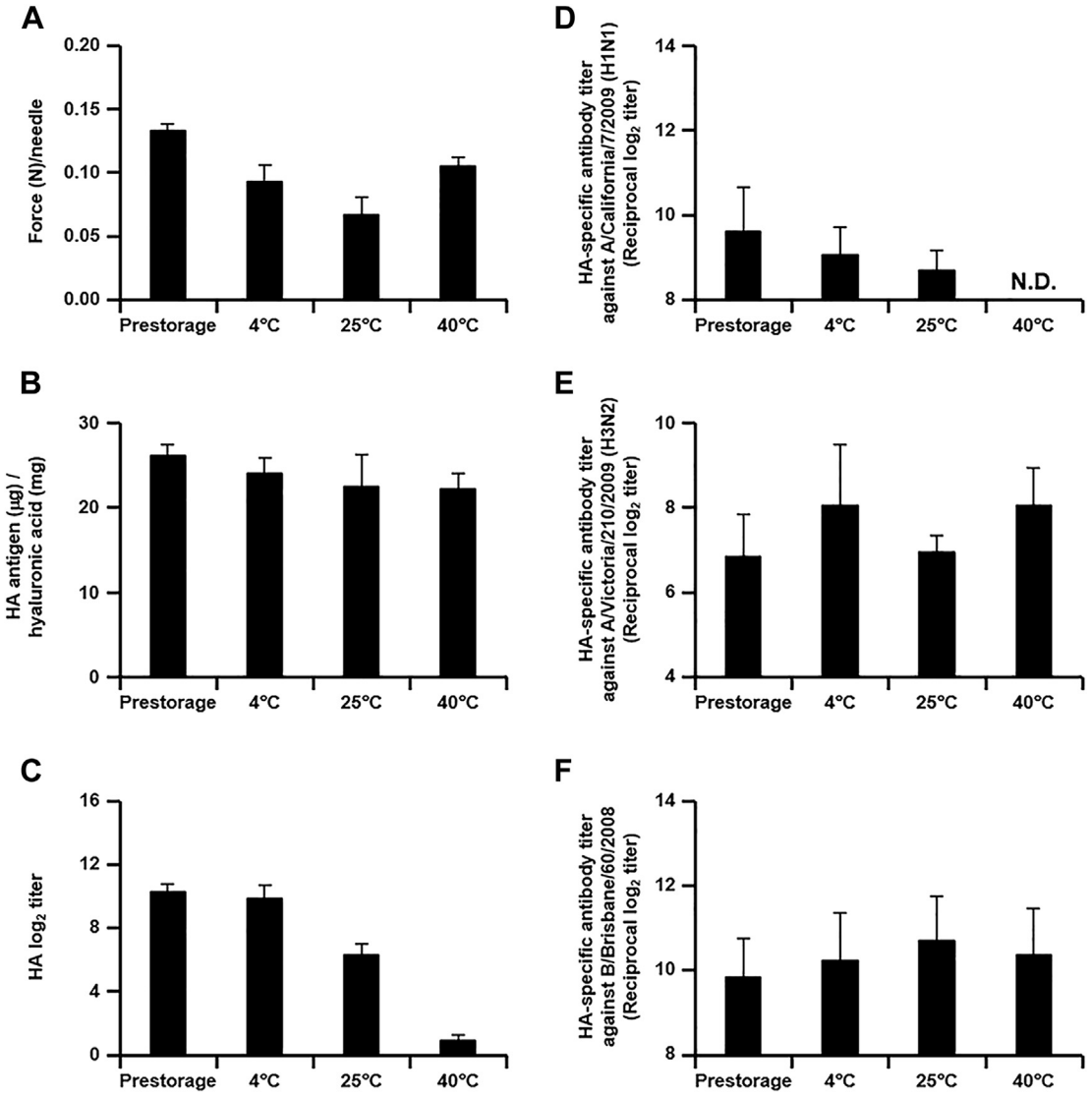

Fig. 4. The flu-MH before and after 6-month storage at various temperatures. (A) Mechanical failure of the flu-MH; The force required to fracture 55 microneedles was measured using a texture analyzer. Data represent the mean ± SD of three measurements. (B, C) Amounts and titers of HA antigen loaded in the flu-MH; HA antigen and hyaluronic acid contents were determined using the Lowry method and ELISA, respectively (B). HA titers in HA antigen solutions prepared from each flu-MH were determined using hemagglutination assays (C). Data represent the mean ± SD of five measurements. (D–F) HA-specific antibody titers in mice immunized subcutaneously with HA antigen solution prepared from each flu-MH; HA antigen solutions were administrated twice to back skins of BALB/c mice at 4-week intervals. Two weeks after the second vaccination, sera were collected and assayed for IgG titers specific for A/H1N1 (D), A/H3N2 (E), or B (F) using ELISA. Data are expressed as the mean ± SE of results from five mice.

widespread practical use. In addition, our MHs are already manufactured and sold as cosmetics, thus making the mass production of MH systems for use in medical supplies both possible and practical.

In this study, the production of antibody against the B strain by the TCI group was superior to that by the SCI group. Because most people are infected with influenza A virus during epidemics, many of the present subjects may have also been infected. In contrast, influenza B virus epidemics occur at low frequency, suggesting that TCI and SCI induced high antibody titers to the influenza A strain by boosting existing immunity. A strong primary immune response is required to protect against the influenza B strain, which may be accomplished only using TCI targeted near the surface of the skin, which is abundantly populated with antigen-presenting cells (APCs) such as Langerhans and dermal dendritic cells. Therefore, it is important to understand the function of immunocytes such as APCs, T cells, macrophages, and keratinocytes in the skin. Analysis of the immunological characteristics of each cell type (e.g., surface marker expression and cytokine production) can help elucidate the molecular and/or cellular mechanisms that underlie the immunity of the skin. In addition, recent studies using two-photon confocal microscopy or genetically modified mice have enabled direct observation of the kinetics and distribution of immune cells in the skin. These studies have also enabled evaluation of the interaction between APCs and T cells in draining lymph nodes and have led to a greater understanding of skin immunity in vivo [16,17]. Such studies are useful for the development/improvement of transcutaneous vaccination formulations and can guarantee the safety and efficacy of TCI systems.

In the present experiments, TCI elicited an effective immune response at only 50% of the required SCI dose, indicating that TCI may reduce the required doses of antigen. But, our study showed that it is necessary that MH components are optimized and applicators are improved to deliver vaccines more efficiently. Moreover, the selection of excipients is important in order to improve the

stability of antigens loaded in MH. Subsequently, this novel vaccination system using MH may be applied to various vaccines, including whole-particle and live vaccines.

There are some problems with the practical use of the MH system. One problem is the cost because the production of antigens for use in vaccines is currently very expensive. This problem is important to address in order to facilitate the widespread use of vaccination, particularly in developing nations. Adjuvants for transcutaneous vaccination also need to be developed to reduce the antigen dose and/or number of administrations. Another issue is the MH applicator, where improvements are needed to help the microneedles puncture the skin. The ideal applicator needs to be inexpensive, disposable, and self-administrable. Finally, the most essential problem is that satisfactory international regulatory guidelines regarding the standard for formulation and evaluation of safety and efficacy have not yet been established because of the novelty of microneedle formulations. Additional details about the characteristics of skin as a target for vaccination and of the fundamental properties of microneedles may lead to the establishment and refinement of guidelines for microneedle formulation. Such guidelines will help encourage researchers and pharmaceutical companies to develop practical microneedle systems.

5. Conclusion

The present data demonstrate that the administration of influenza vaccines to human subjects via the transcutaneous route using the MH system induces high levels of immunity. Thus, flu-MH may replace conventional injectable vaccinations following further improvements of applicators and microneedle formulations.

Acknowledgments

This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) is “08-17”, by a Health Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare is “KH23AC0040”, and by JSPS KAKENHI Grant Number 24390041 and 23659079 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.04.007.

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