Characterization of Transcutaneous Protein Delivery by a Hydrogel Patch in Animal, Human, and Tissue-Engineered Skin Models

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The development of a simple, easy-to-use, and noninvasive vaccination system is in high demand. For transcutaneous immunization (TCI), we previously reported that a hydrogel patch was an effective TCI device that accelerates antigen penetration through the stratum corneum in mouse and rat models. The present study was performed to characterize the transcutaneous protein delivery induced by the hydrogel patch in mouse, guinea pig, LWD pig, human, or tissue-engineered skin models, and to assess the activity of proteins delivered into the skin. The hydrogel patch promoted protein penetration through the stratum corneum in all skin models, indicating that our original hydrogel patch might have practical application for use in humans. In addition, proteins delivered into the skin by the hydrogel patch retained their activity, suggesting that the hydrogel patch is applicable for the delivery of therapies for other diseases as well. On the basis of these results, translational research in human is now in progress.

Key words hydrogel patch; transcutaneous protein delivery; vaccine device

The skin acts not only as a physical barrier, but also as an immunologic barrier, and is enriched with various immunocompetent cells such as Langerhans cells (LCs), keratinocytes, and mast cells.1–3) Thus, the skin is a convenient access site for transcutaneous immunization (TCI)4) or therapies for atopic dermatitis.5) For example, in TCI, LCs have a particularly major role as potent antigen-presenting cells, suggesting that direct antigenic protein (Ag) delivery to LCs might elicit an effective immune response. Ags painted on the bare skin, however, do not access LCs because the stratum corneum acts as a physical barrier to substance penetration.6) We previously developed a novel hydrogel patch that promotes Ag penetration through the stratum corneum. TCI using a hydrogel patch could deliver Ags to LCs and induce effective immune responses in mouse and rat.7,8)

For clinical application of the hydrogel patch, it is crucial to evaluate whether the hydrogel patch formulation enhances protein penetration through the stratum corneum of human skin. Because human skin has a thicker stratum corneum compared with mice or rats, human skin is less permeable to substances than rodent skin models. In the present study, we attempted to assess the characteristics of protein penetration by the hydrogel patch in various skin models with a stratum corneum of different thicknesses.

Further, we have little information about the retention of the activity of proteins delivered into the skin by a hydrogel patch. If the hydrogel patch can deliver proteins into the skin without reducing the activity of the proteins due to conformational changes, then it would be an applicable device for various dermatologic therapies. In the present study, we examined the activity of proteins delivered into skin by the hydrogel patch.

On the basis of our findings, we are now planning translational research of TCI using a hydrogel patch in humans and attempting to evaluate its efficacy as a transcutaneous delivery device for atopic dermatitis therapy.

MATERIALS AND METHODS

Animals Male ddY mice (6—8 weeks old) and female guinea pigs (3 weeks old) were purchased from SLC Inc. (Hamamatsu, Japan). Female triple-cross breed pigs (LWD; 35 kg weight) were purchased from IVTeC Inc. (Tokyo, Japan), and reared at the Kobe IVTeC laboratory in the Kobe Medical Device Development Center. Animals were handled in accordance with the Osaka University guidelines for experimental animal welfare. The research protocols described in this report were reviewed and approved by the Animal Care and Use Committee of Osaka University.

Hydrogel Patch Formulation The hydrogel patch formulation was prepared as previously described.7,8) In this study, the hydrogel patch formulation comprised the cross-linked HiPASTM acrylate medical adhesives (10%; CosMED Pharmaceutical Co., Ltd., Kyoto, Japan), octyldodecyl lactate (4.5%), glycerin (0.3%), and sodium hyaluronan (0.02%).

Analysis of Protein Activity after Penetration of the Stratum Corneum To evaluate the activity of proteins delivered into skin by the hydrogel patch, we performed two experiments according to the following methods.

(i) Hydrogel patch (2 cm2) containing 100 μg β-galactosidase (β-gal; Sigma-Aldrich, St. Louis, MO, U.S.A.) or phosphate-buffered saline (PBS) was applied to mouse auricular skin. Twenty-four hours later, the auricles were harvested from the mice, and epidermal sheets were prepared according to the method of Mackenzie and Squier. Epidermal sheets were fixed in 1.5% glutaraldehyde for 10 min at room temperature, washed with PBS, and stained with X-gal solution at 37 °C for 3 h. The samples were mounted with Perma Fluor (Thermo Shandon, Pittsburgh, PA, U.S.A.), and photographed using an optical microscope.

(ii) Hydrogel patch containing diphtheria toxin (100 μg/ 2 cm2) was applied to guinea pig auricular skin. Twenty-four hours later, the auricles were harvested from the guinea pigs, fixed in 10% neutral-buffered formalin at pH 7.4 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and embedded in...
paraffin. Sections (5-μm thick) were prepared for hematoxylin and eosin staining. Histopathologic examinations were performed at the Applied Medical Research Laboratory (Osaka, Japan).

**In Vivo Antigen Penetration Assay Using a Miniature Pig**  A hydrogel patch (4 cm²) containing 200 μg fluorescein isothiocyanate-labeled ovalbumin (FITC-OVA; Invitrogen, Carlsbad, CA, U.S.A.) was applied to the back skin of LWD pigs for 24 h. Harvested auricles were embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen in liquid nitrogen. Frozen sections (6-μm thick) were mounted with Prolong Gold antifade reagent (Invitrogen), and then photographed under fluorescence microscopy (BZ-8000; Keyence Corporation, Osaka, Japan).

**In Vitro Human Skin Penetration Assay** Ethical approval for using human skin was granted by the International Institute for the Advancement of Medicine (Jessup, PA, U.S.A.). Skin penetration analysis of Ag following application of the hydrogel patch was studied using Franz diffusion cells. Briefly, the skin was mounted between the donor and receptor chambers of the diffusion chamber. The receptor chamber was filled with a known volume of PBS. An FITC-OVA-containing hydrogel patch (50 μg/cm²) or FITC-OVA solution (50 μg) was applied to the human skin in the donor chamber. After 3, 6, 9, or 12 h incubation, aliquots from the receptor chamber were withdrawn and the penetration levels were analyzed by measuring the FITC-OVA fluorescence intensity. At the end of the permeation experiment, the hydrogel patch was removed and the skin surface was washed with PBS. The skin that had been exposed to the patch was harvested from the Franz diffusion chamber and epidermal sheets were prepared. The epidermal sheets were mounted with Prolong Gold antifade reagent with 4',6'-diamino-2-phenylindole (Invitrogen), and then photographed under fluorescence microscopy (BZ-8000).

**Skin Penetration Assay Using TEST-SKIN LSE-High** TEST-SKIN LSE-high, an artificial skin model that has no pores, was obtained from Toyobo, Osaka, Japan. The dermal portion of LSE-high was placed on a polycarbonate membrane in contact with 1 ml assay medium in a 6-well plate. A polyethylene ring was affixed to the surface of the LSE-high with silicone sealant, and a hydrogel patch containing FITC-OVA (50 μg/cm²) or FITC-OVA solution (50 μg) was applied to the LSE-high and covered with wound management film (BIOCLUSIVE; Johnson & Johnson Medical, Ltd., Tokyo, Japan). After 12 or 24 h-incubation, aliquots from the receptor compartment were withdrawn and the penetration levels were analyzed by measuring FITC-OVA fluorescence intensity.

**RESULTS AND DISCUSSION**

**Activity of Protein Delivered into the Skin through the Stratum Corneum by the Hydrogel Patch** We previously reported that the use of our hydrogel patch accelerates the penetration of proteins through the stratum corneum in mouse and rat. To investigate whether proteins delivered into a living epidermis by the hydrogel patch retained their activity, we used two types of proteins; β-gal and diphtheria toxin. X-Gal staining of epidermal sheets obtained from mice treated with a hydrogel patch containing β-gal showed β-gal activity (Fig. 1A). X-Gal staining images suggested that the proteins penetrated through the stratum corneum via paracellular pathways. We confirmed that β-gal was delivered into the epidermal layer in which LCs exist (unpublished data). We then evaluated the activity of diphtheria toxin in guinea pig, whose stratum corneum is thicker than that in mouse (Fig. 1B). Skin treated with a hydrogel patch containing diphtheria toxin showed marked injury. Moreover, in histopathologic sections of auricular skin, the diphtheria toxin induced tissue damage in both living epidermis and dermis, indicating that in guinea pig, a hydrogel patch promoted protein penetration and the proteins retained their activity. These results suggested that proteins delivered by the hydrogel patch maintain their molecular structure in the skin. Therefore, the hydrogel patch may be a suitable transcutaneous therapeutic delivery device for biotechnology- or nucleic acid-based medicines, such as therapeutics using nu-
clear factor-κB decoy oligonucleotides for atopic dermatitis. For application of these hydrogel patches for other therapeutics, we are planning to investigate how the activity of proteins applied to the hydrogel patch is maintained.

Transdermal Protein Delivery in Human Skin and a Tissue-Engineered Skin Model In mouse and guinea pig skin models, our TCI system using a hydrogel patch promoted protein penetration through the stratum corneum. Human skin, however, is less permeable to substances than mouse, rat, and guinea pig skin. To assess the feasibility of clinical application of the hydrogel patch, we evaluated transcutaneous protein penetration in skin models with a thicker stratum corneum.

We first investigated in vivo protein penetration through the stratum corneum by application of a hydrogel patch using LWD pigs, which have skin similar to that in humans. Analysis of the localization of proteins in pig skin treated with the hydrogel patch containing FITC-OVA indicated marked penetration of proteins into the epidermal layer (Fig. 2). This finding indicated that our TCI system using a hydrogel patch promoted the penetration of proteins through the stratum corneum in skin as thick as that of human skin. We then investigated skin penetration using an in vitro human skin model (Fig. 3A). Proteins applied directly to the skin did not penetrate the stratum corneum, whereas application of the hydrogel patch induced proteins to penetrate the skin. In epidermal sheets of human skin that had been treated with a hydrogel patch, FITC-OVA was detected in the intracellular gaps (Fig. 3B). Moreover, we found that detected fluorescence intensity was derived from FITC-OVA not free FITC in gel filtration chromatography analysis of aliquots from the receptor chamber (data not shown). These results suggested that hydrogel patch application promoted the penetration of proteins through the stratum corneum in human skin, and that the proteins penetrated the paracellular pathways, consistent with the findings in mouse. Another pathway of protein penetration is thought to be the pores. To investigate the involvement of pores in skin penetration, we examined protein delivery by the hydrogel patch using a tissue-engineered skin model without pores. The amount of protein that penetrated the skin by application of the hydrogel patch containing FITC-OVA suggested that the proteins were delivered through intercellular gaps rather than pores (Fig. 4).

Based on these findings, the hydrogel patch promotes the

![Fig. 2. Localization of FITC-OVA in Skin Sections from LWD Pigs Vaccinated Transcutaneously](image)

A hydrogel patch containing FITC-OVA (200 μg/4 cm²) was placed on the back skin of LWD pigs. Twenty-four hours later, auricles were harvested and frozen. Frozen sections (6-μm thick) were photographed using a fluorescence microscope. Arrowheads indicate FITC-OVA that penetrated the stratum corneum.

![Fig. 3. Penetration Profiles of FITC-OVA through Human Skin after Application of a Hydrogel Patch Containing FITC-OVA or FITC-OVA Solution](image)

Human skin was mounted in Franz diffusion cells. A hydrogel patch containing FITC-OVA (50 μg/cm²; ○, ■, ▲) or FITC-OVA solution (50 μg; ○, ▲) was applied to the donor chamber, and then incubated for 3, 6, 9, or 12 h at 37 °C. (A) The penetration levels at the indicated periods were analyzed by measuring the fluorescence intensity of the FITC-OVA penetrating through the human skin to the receptor chamber. Each value represents the mean and S.E. of 3—4 experiments. (B) Twenty-four hours later, human skin applied with an FITC-OVA-containing hydrogel patch was removed from the Franz cells, and epidermal sheets were prepared. The epidermal sheets were photographed under fluorescence microscopy.

![Fig. 4. Penetration Profiles of FITC-OVA through TESTSKIN LSE-High after Application of a Hydrogel Patch Containing FITC-OVA or FITC-OVA Solution](image)

Hydrogel patches containing FITC-OVA (50 μg/cm²; ○) or FITC-OVA solution (50 μg; ○, ▲) were applied to skin in the donor chamber and then incubated for 12 or 24 h at 37 °C. The penetration levels were analyzed by measuring the fluorescence intensity of FITC-OVA that penetrated through the TESTSKIN LSE-high to the receptor chamber. Mean values are presented in the bar graph. Circles represent individual data points.
distribution of proteins to stratum corneum and enhances their penetration through the stratum corneum in various skin models, suggesting that TCI using a hydrogel patch can be effective in clinical studies.

CONCLUSION

The present study demonstrated that the hydrogel patch enhanced protein penetration through the stratum corneum in various skin models with a stratum corneum of different thicknesses, and that proteins applied via a hydrogel patch maintained their activity. On the basis of these results, the hydrogel patch is an effective transcutaneous delivery device for clinical use.

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