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Facilitation of transcutaneous drug delivery and vaccine immunization by a safe laser technology

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Abstract

Full-surface laser ablation has been shown to efficiently disrupt stratum corneum and facilitate transcutaneous drug delivery, but it is frequently associated with skin damage that hampers its clinic use. We show here that a safer ablative fractional laser (AFL) can sufficiently deliver not only patch-coated hydrophilic drugs but also protein vaccines. AFL treatment generated an array of self renewable microchannels (MCs) in the skin surface, providing free paths for drug and vaccine delivery into the dermis while sustains integrity of the skin by quick healing of the MCs. AFL was superior to tape stripping in transcutaneous drug and vaccine delivery as a much higher amount of sulforhodamine B (SRB), methylene blue (MB) or a model vaccine ovalbumin (OVA) was recovered from AFL-treated skin than tape stripping-treated skin or control skin after patch application. Following entry into the MCs, the drugs or OVA diffused quickly to the entire dermal tissue via the lateral surface of conical-shaped MCs. In contrast, a majority of the drugs and OVA remained on the skin surface, unable to penetrate into the dermal tissue in untreated control skin or tape stripping-treated skin. Strikingly, OVA delivered through the MCs was efficiently taken up by epidermal Langerhans cells and dermal dendritic cells in the vicinity of the MCs or transported to the draining lymph nodes, leading to a robust immune response, in sharp contrast to a weak, though significant, immune response elicited in tape stripping group or a basal immune response

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in control groups. These data support strongly that AFL is safe and sufficient for transcutaneous delivery of drugs and vaccines.

Keywords

laser; transdermal patch; vaccine; drug; transcutaneous delivery; tape stripping

Introduction

Transdermal patch-based drug and vaccine delivery has great advantages over traditional needle injection by elimination of injection-related pain and biohazardous sharp wastes [1,2]. It can potentially be self-administration and thus it is convenient and cost-effective. The most commonly used patch has been nicotine patch in assisting nicotine replacement therapy for two decades in the USA [1]. Several other drug-coated patches are also available in the clinic, such as lidocaine and estrogen patches [1]. However, clinical application of transdermal patches has been limited to lipophilic drugs that can easily pass through stratum corneum (SC) layer and reach therapeutic levels in blood following topical delivery. There is only a limited success for transdermal patch delivery of hydrophilic drugs and vaccine antigens due to the impermeability of skin SC layer.

A variety of chemical enhancers (e.g., azone, DMSO, alcohols, fatty acids) and physical methods (e.g., iontophoresis, electroporation, ultrasound, and shock waves) have been tested extensively in the past two decades to increase SC permeability [1,3,4]. These chemical enhancers increase structures and are skin permeability mainly by disorganization of lipid insufficient to enhance transcutaneous delivery at a safe concentration because the majority of the SC layer remains intact after the treatment [3]. Iontophoresis and electroporation use electrical charges to force ionized drugs or vaccines across the SC layer [1,5,6], while ultrasound and shock waves temporarily reduce skin barrier property by generating cavitation or pressure waves to increase drug penetration [1,4,7]. Although these chemical and physical methods have been under investigation for many years, their clinical application is hampered due to their ineffectiveness or invasiveness.

Recently, mechanical strategies, like microneedles and tape stripping, have been developed to facilitate transcutaneous drug and vaccine delivery [8,9]. These strategies can effectively disrupt SC layer, and have been advanced to clinic trials. However, a low efficiency is concerned with tape stripping, whereas a risk of skin irritation is associated with microneedles [10,11]. Another system, called single-step Skin Prep System (SPS), was developed to overcome disadvantages of traditional tape stripping [12]. Unfortunately, the system failed to adequately boost immune responses of the travelers' diarrhea vaccine coated on patch in phase III clinical trials.

Laser technology has some unique advantages over the aforementioned chemical, physical, and mechanical methods in disruption of SC layer. First, laser acts precisely at a cellular level and thus an ablation area and the degree of the ablation are well controllable by adjustment of laser parameters, such as laser energy power, a percentage of skin coverage, patterns of laser ablation, and so on. Secondly, laser can illuminate the skin in a non-contact manner and the device can be used repeatedly without risking cross-contamination from person to person. Thirdly, few disposable biohazard materials are generated in the procedure unlike microneedles or tap stripping. In the past, laser beams were delivered to the whole skin surface evenly to disrupt the entire SC layer involved. This treatment frequently leads to delayed skin recovery, similar to full-surface laser skin rejuvenation [13]. Fractional photothermolysis was developed nearly a decade ago to replace full-surface skin resurfacing

by illuminating skin with multiple separated micro-laser beams that generate many tiny microchannels (MCs) in the skin [14]. The MCs span from skin surface to epidermal or dermal tissue depending on a laser power used [15]. These MCs provide free entry of drugs and vaccines into the skin. Because these MCs are surrounded by normal healthy skin, laser-affected area can gain a quick recovery, warranting the integrity of the skin, which is the key for the safety of this delivery technology.

Although ablative fractional lasers were evaluated for potential enhancement of transcutaneous delivery of several hydrophilic drugs (e.g., methyl 5-aminolevulinate, 5-aminolevulinic acid, imiquimod), polypeptides, and dextrans in the past 2 years [15-17], those studies were focused on laser-facilitated transcutaneous delivery in dissected skins. Investigation aimed at *in vivo* evaluation of a relative potency of AFL over other technologies in transdermal patch-based vaccine delivery and immunization is still lacking. In the current study, we corroborate safety of several AFL conditions and find that AFL is superior to tape stripping in facilitating transcutaneous delivery of hydrophilic model drug sulforhodamine B (SRB), methylene blue (MB), and a model vaccine antigen ovalbumin (OVA) in mice.

Materials and methods

Animals

Male BALB/c mice at 6~8 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). MHC II-EGFP mice expressing MHC class II molecule infused into enhanced green fluorescent protein were a kindly gift of Drs. Boes and Ploegh, Department of Pathology, Harvard Medical School conventional cages in the [18]. All mice were housed in animal facilities of Massachusetts General Hospital (MGH) in compliance with institutional guidelines. The study was reviewed and approved by the MGH Subcommittee of Research Animal Studies.

Laser device

The rear mouse skin was exposed to laser beams at 10.6 μ m wavelength emitted by an UltraPulse Fractional CO₂ Laser (Lumenis Inc., Santa Clara, CA) through a convenient moveable articulated arm. A laser power at 2.5 or 5.0mJ and skin coverage at 5% or 15% were used to illuminate dorsal mouse skin in a 6 $\times$ 6 mm² area unless otherwise specified.

Tape stripping

Tape stripping was performed by well established procedures [12,19,20]. Briefly, a sterile 3M adhesive tape with a size 1 $\times$ 1 cm² was applied onto the dorsal skin of hair-removed BALB/c mice and removed. The tape stripping was repeated for 15 times without a tape change, referred to as a mild stripping (1, 15). In a slightly invasive stripping, tape stripping was repeated for 6 times with a tape change every other stripping, referred to (3, 6).

Skin histological analysis

Full-thickness skin was excised at indicated times after laser illumination or tape stripping and subjected to paraffin cross or horizontal sections at 5 μ m thickness. Sections were stained with hematoxylin and eosin (H&E) and evaluated under a Zeiss Axiophot microscope. To determine an efficacy of drug or vaccine delivery, full-thickness skin was prepared as above and subjected to cryosectioning at 20 μ m thickness and fluorescence images were taken under a Zeiss Axiovert 100 TV fluorescence microscope or a Zeiss Axiophot bright-field microscope.

Patch preparation and transcutaneous delivery

Gauze patch was prepared by a published procedure with minor modifications [21]. In brief, sterile gauze pad was cut into small pieces ($1 \times 1 \text{ cm}^2$) and adhered to 3M TegadermTM adhesion, onto which 35 μl of SRB (4mg/ml, Invitrogen), Methylene Blue (MB, 5mg/ml, Sigma), or Texas Red-ovalbumin (TR-OVA, 10mg/ml, Invitrogen) was slowly dropped. An equal volume of PBS was used to prepare the sham patch. The resultant patches were immediately applied topically on AFL- or tape-treated skin or untreated control skin, overlaid with aluminum foil, and wrapped with self-adhesive bandage to shield from light and to ensure a close contact between the patch and the skin.

Quantification of transcutaneous delivery

The mice were euthanized at indicated times after patch application and gauze patches were gently removed. The skin involved was washed with tape water for 1 minute, excised, and homogenized in cold PBS buffer followed by centrifugation. The resultant supernatants were collected to measure fluorescence intensity of SRB and TR-OVA with excitation/emission wavelengths at 560/590 and 596/615nm, respectively, or optical absorbance of MB at 668nm. The amount of SRB, MB or OVA per square centimeter of the skin was calculated in the basis of SRB, MB or OVA standard curves assayed in parallel.

Intravital confocal imaging

Ears of MHC II-EGFP transgenic mice were subjected to intravital confocal imaging (FluoViewTM FV1000, Olympus) 6 hrs after patch application. Low magnification images ($4\times$ objective lens) were taken to explore transcutaneous delivery in a broad area, while high magnification images ($40\times$ objective lens) were taken to investigate antigen uptake by individual antigen presenting cells (APCs). Imaging was first focused on the epidermal layer at $10\mu\text{m}$ depth and then dermal layer at about $40\mu\text{m}$ depth to investigate antigen uptake by Langerhans (LCs) or dermal dendritic cells (dDCs), respectively.

Immunization and immune responses

The low dorsal skin of BALB/c mice was exposed to AFL, tape stripping, untreated followed by topical application of or left $700\mu\text{g}$ OVA-coated gauze patch. The patch was removed one day later and blood was collected one day before and 2 and 4 weeks after immunization to analyze serum OVA-specific antibody titer by enzyme-linked immunosorbent assay (ELISA) as previously described [22].

Statistical analysis

Values were expressed as Mean $\pm$ SEM (standard error of mean). Two-way ANOVA was used to compare time-dependent drug or vaccine delivery among multiple groups and Bonferroni post-test was used to analyze the difference between groups at each time point unless otherwise indicated. P value was calculated by PRISM software (GraphPad, San Diego, CA) and considered significant if it was less than 0.05.

Results

Skin histology

A clinical ablative fractional laser (AFL) with adjustable laser power and skin coverage was used to generate microchannel (MC) arrays in the skin. The laser illumination at 5% and 15% skin coverage generated a 9×9 or 14×14 MC array, respectively, in the low dorsal skin of BALB/c mice as illustrated in figure 1A. Four different laser conditions were tested, including a laser power of 2.5mJ or 5.0 mJ and 5% or 15% skin coverage, simplified as 2.5mJ 5%, 2.5mJ 15%, 5.0mJ 5% or 5.0mJ 15%, respectively. Skin recovery was evaluated

following each laser illumination. Among the four laser conditions, only the mildest AFL (2.5mJ 5%) gave rise to complete skin re-epithelialization within 1~2 days as shown by skin histological analysis (figure 1B). The conical-shaped MCs each spanned from the skin surface to the dermal tissue with $71\pm 7\ \mu\text{m}$ in diameter (d0, low panel, figure 1B) and $40\pm 3\ \mu\text{m}$ in depth (d0, upper panel, figure 1B). The microthermal zones (MTZs) were larger and clearly visible around MCs, outlined by a black dashed line (d0, figure 1B), each with $120\pm 5\ \mu\text{m}$ in diameter and $113\pm 17\ \mu\text{m}$ in depth as a result of heat dissipation. Yet, total laser-ablated area represented as little as 1.7% of the skin surface involved. In other words, the majority of the skin tissue in the array was spared from the laser-mediated ablation, which might be the reason for fast re-epithelialization and growth of new tissue into MCs in 24 hrs (d1, figure 1B). Indeed, most of MCs were healed and filled with newly synthesized tissue that was easily distinguished from the surrounding tissue (d1, figure 1B). In the following day, all MCs were completely restored to normal, indistinguishable from those surrounding tissues (d2, figure 1B). An increase in skin coverage to 15% generated MCs in a similar size, but resulted in delayed skin recovery, probably due to a reduced ratio of healthy to ablative tissues (data not shown). Likewise, a high laser power of 5.0mJ increased the size and depth of each MC, causing delayed skin recovery at both 5% and 15% skin coverage (data not shown). A further increase in laser energy to 10.0mJ or higher caused instant skin shrinkage and damage even at the smallest skin coverage (data not shown). The histological studies suggest that skin recovery ability is controlled by a ratio of ablative and non-ablative tissues and AFL 2.5mJ/0.5% can generate MCs with a quick skin recovery.

Similarly, while mild tape stripping (1, 15) effectively ablated SC layer with little damage on the epidermal layer underneath the SC layer, full restoration of SC layer was observed within 1 or 2 days (figure 1C). A slightly invasive tape stripping (3, 6) damaged not only SC layer but also the upper epidermal tissue (figure 1C), leaving only one-cell thickness of epidermal tissue, in contrast to several-cell thickness of epidermal tissue in control skin. This slightly invasive treatment delayed restoration of epidermal tissue (d1, low panel, figure 1C) and thickened epidermal tissue (d2, low panel, figure 1C), concurrent with dermal infiltration of large amounts of inflammatory cells (upper panel, figure 1C), indicative of incomplete skin recovery by day 2. More harsh tape stripping such as 10-12 strokes with a tape change every other stripping caused skin redness and damage instantly due to damage of the dermal tissue (data not shown). The studies corroborate that tape stripping (1, 15) was safe with a quick recovery, while tape stripping (3, 6) was slightly invasive with delayed skin recovery.

Transcutaneous delivery of SRB and MB

SRB, a hydrophilic model drug about 560Da in mass, was initially used to evaluate transcutaneous delivery in the skin treated with AFL or tape stripping. As shown in figure 2A, the compound entered laser-treated skin through the MCs 30 min after topical application of a SRB-coated gauze patch, as evidenced by the presence of strong pink-colored SRB only in AFL-treated skin, but not in control skin, with a highest density in laser-generated MCs. Even in the vicinity of each MC, SRB was higher than that in control skin, presumably resulting from drug dispersing from the MC. There was only a trace amount of SRB in the control and tape stripping-treated skin, with a slightly more SRB in the skin treated by slightly invasive tape stripping (3, 6) as compared to the mild tape stripping (1, 15) (figure 2A). Bright-field microscopy and fluorescence imaging of skin sections at 5 and 30 minutes corroborated that strong SRB signal was radically diffusing from laser-generated MCs into the surrounding dermal tissue in 5 minutes after patch application, and continuously spreading over the entire dermal tissue in 30 minutes (figure 2B&2C). In contrast, SRB was presented only on the skin surface in control or in the epidermis at these time points in tape stripping (1, 15) group. Although tape stripping (3, 6)

did facilitate penetration of the drug to the upper dermal tissue in 30 minutes, the efficacy was much lower than AFL.

A time-course study found that the amount of SRB in the skin increased linearly to 5.83 ± 0.44 , 15.17 ± 1.73 , 10.36 ± 0.72 or 31.37 ± 4.72 $\mu\text{g}/\text{cm}^2$ after 30 minutes of patch application to the skin treated with AFL 2.5mJ 5%, 2.5mJ 15%, 5.0mJ 5%, or 5.0mJ 15%, respectively. This represented a 12, 30, 21 or 63-fold increase compared to control group (0.50 ± 0.17 $\mu\text{g}/\text{cm}^2$, figure 2D, $p < 0.001$). The amount of SRB in tape stripping (1, 15) and (3, 6) groups was only 1.09 ± 0.24 or 3.09 ± 0.17 $\mu\text{g}/\text{cm}^2$, respectively, and was without statistic significance compared to the control group at this time point. The amount of SRB in the skin peaked at 9.62 ± 1.06 $\mu\text{g}/\text{cm}^2$ in 2.5mJ 5% group and 15.91 ± 1.10 in 5.0mJ 5% group after 6 hrs of patch application, while SRB amount was a highest level at 25.18 ± 2.90 $\mu\text{g}/\text{cm}^2$ in 2.5mJ 15% group and 31.37 ± 4.72 $\mu\text{g}/\text{cm}^2$ in 5.0mJ 15% group in 1 or 0.5 hr of patch application, respectively. Thus, a peaking time appeared to inversely correlate with MC density, while the efficiency might be controlled by a combination of MC density and laser power. SRB content in the skin diminished gradually after the peak in all laser groups (figure 2D), presumably due to entry of the compound into the circulation system through lymphatic or capillary networks in the dermis.

In comparison, tape stripping displayed much diminished efficacies and lacked an overt peak in which a high level of SRB in the skin was around 2.20 ± 0.23 $\mu\text{g}/\text{cm}^2$ in tape stripping (1, 15) group or 4.81 ± 1.23 $\mu\text{g}/\text{cm}^2$ in tape stripping (3, 6) group spanning from 3 to more than 9 hrs after patch application. There was no significant difference between tape stripping (1, 15) and control group or between the two tape stripping groups at each time point, although tape stripping (3, 6) increased SRB delivery significantly at 3 and 6 hrs as compared to control group ($p < 0.05$).

A similar, superior AFL-enhanced transcutaneous drug delivery was also demonstrated with methylene blue (MB), a hydrophilic photosensitizer with 320Da molecular weight. As shown in figure 3A, a highly intense blue-colored MB was seen in a pattern matching laser-generated MCs after 30 minutes of patch application. MB was diffusing from the lateral surface of the MCs and sufficiently penetrated deep into the dermal tissue in AFL group (figure 3B). In contrast, only a trace amount of MB was presented on skin surface of tape stripping (1, 15) and control groups, which was much less than MB deposited on skin surface treated with tape stripping (3, 6) or AFL (figure 3A). Consistent with this was good penetration of MB into the epidermis in tape stripping (3, 6) group, whereas no MB was able to enter the control skin and only a small amount of MB reached the epidermis after mild tape stripping (figure 3B). Quantification of skin MB at varying times showed that MB levels sharply rose in AFL-treated skin, peaking at $10 \sim 15$ $\mu\text{g}/\text{cm}^2$ under 2.5mJ/15%, 5mJ/5% and 5mJ/15% conditions or 8 $\mu\text{g}/\text{cm}^2$ under 2.5mJ/5% at 6 or 9 hrs after patch application, respectively (figure 3C). This high level of MB delivery was in marked contrast to a slow deposition of MB with a maximal level less than 1.0 $\mu\text{g}/\text{cm}^2$ in mild tape stripping and control skins ($p < 0.001$). Although more invasive tape stripping (3, 6) significantly enhanced MB delivery at and after 3 hrs as compared to tape stripping (1, 15) or control group ($p < 0.05$), the efficacy was far lower than AFL in all the conditions tested. These imaging and quantitative studies of two different drug systems suggest clearly that AFL is superior to tape stripping to facilitate transcutaneous drug delivery.

Transcutaneous delivery of OVA

We next addressed whether AFL could also enhance transcutaneous delivery of a model vaccine antigen, ovalbumin (OVA) that is much higher in mass (~ 45 kDa), impermeable to the skin. When Texas Red-conjugated OVA (TR-OVA) was coated onto a patch and topically applied to laser-treated skin for 0.5 or 3 hrs, TR-OVA was observed, after 0.5 hr of

patch application, in both the epidermis and in the upper dermal tissue surrounding MCs in laser-treated skin (figure 4A). It continued spreading horizontally and vertically into the entire dermal tissue in 3 hrs (figure 4A).

There was no dermis-penetration of TR-OVA in both tape stripping and control groups in the initial 0.5 hr of patch application, although some TR-OVA penetration of the upper dermis was detected in 3 hrs after patch application with more invasive tape stripping (3, 6) (figure 4A).

The amount of TR-OVA in the skin climbed precipitously in laser-treated skin following patch application and reached a peaking level in 3 hrs in all AFL-treated groups (figure 4B), which was significantly higher than that obtained with tape stripping or control group (figure 4B, $p < 0.001$). In comparison with the control group, laser treatment increased TR-OVA transcutaneous delivery by approximate 8~15 times within 3 hrs ($p < 0.001$), whereas slightly invasive tape stripping (3, 6), but not mild tape stripping (1, 15), increased TR-OVA delivery only by less than 4 times in the same period of time ($p < 0.05$). There was no significant difference between the two tape stripping groups or between each tape stripping and control groups at each time point (figure 4B). Thus, AFL is able to facilitate transcutaneous delivery of protein antigen OVA in a much more efficient manner than tape stripping.

Antigen uptake following OVA delivery

We went on to address whether the photothermal effect of AFL could compromise the capacity of skin APCs for processing and presentation of antigens in the vicinity of the MCs, in view of an importance of these processes in induction of a potent immune response [23]. To this end, Alexa Fluor 647-conjugated OVA (AF647-OVA) was coated onto a gauze patch and topically applied to one ear after AFL exposure at the mildest condition in MHC II-EGFP transgenic mice in which epidermal Langerhans cells (LCs) and dDCs were GFP-labeled [22]. Ear skin is relatively thin and less tolerable to photothermal effect and thus the mildest AFL was applied in this analysis. The other ear was left untreated before patch application as a control. As shown in figure 5A, AF647-OVA appeared to sufficiently enter laser-generated MCs as reflected by the same pattern of OVA distribution as AFL-generated MC arrays, in contrast to a background level of AF647-OVA seen in the control ear. With the facilitation of the MCs, OVA could travel radically into the vicinity of MCs. It is conceivable that LCs in the epidermis and DCs in the dermis both could capture OVA in AFL-treated skin should their functions not be adversely affected by AFL treatment. When the control ear was imaged by intravital confocal microscopy, focusing first on the epidermis (figure 5B, left) and then on the dermis (figure 5B, right), OVA-uptake by LCs or dDCs was hardly seen even with an extensive search (figure 5B, top panel). OVA fluorescence in the epidermal layer was mostly out of focus and probably not truly co-localized with LCs due to its poor penetration of epidermis as suggested by histology data (figure 4A). The dermis was largely devoid of OVA except for some non-specific fluorescence debris. In contrast, antigen (Ag) positive LCs and dDCs were readily visible in AFL-treated ear (middle panel) in the vicinity of the MCs. One enlarged antigen-captured single APC demonstrated the presence of OVA inside the cell as evidenced by merged yellow fluorescence of green (APCs) and red (OVA) (lower panel), in contrast to the red fluorescence of OVA outside APCs.

Besides local antigen uptake, transportation of free or antigen-captured APCs to the draining lymph node is equally important for stimulating a potent immune response [23]. We found a high amount of OVA in auricular lymph nodes draining AFL-treated ear and little OVA in those lymph nodes draining control ear (figure 5C). The data confirmed that functions of

afferent lymphatic vessels and APCs were well preserved in the vicinity of the MCs in AFL-treated skin.

AFL-enhanced transcutaneous immunization

In accordance with the sufficient local antigen uptake and transportation of antigen to the draining lymph nodes, AFL treatment augmented the production of OVA-specific antibody by 28-534 times at weeks and 53-545 times at 4 weeks after patch-based OVA immunization as compared to the control group (figure 6). In particular, AFL 5.0mJ 15% stimulated a highest level (1068 ± 445) of antibody production compared to other AFL conditions (124 ± 50 , 56 ± 14 , and 71 ± 29 in 2.5mJ 5%, 2.5mJ 15%, and 5.0mJ 5% conditions, respectively) (figure 6, $p < 0.001$). Tape stripping also increased OVA-specific antibody production by 5-8 times as compared to control group, but it was significantly lower than all AFL conditions tested ($p < 0.001$, figure 6), approximately 4-82 times lower at 2 weeks and 8-105 times lower at 4 weeks than AFL treatment groups. To our surprise, there was no significant difference between the two tape stripping groups. We conclude that AFL treatment followed by topical application of vaccine-coated patch can sufficiently deliver vaccines into the skin and greatly potentiate a vaccine-specific immune response.

Discussion

A safe and efficient technology is needed, more than ever, for transcutaneous delivery of hydrophilic drugs and vaccines. We show here that a clinical AFL even at a relatively low laser energy (2.5mJ) and covered area (5%) is superior to tape stripping in augmentation of transcutaneous delivery of hydrophilic drugs or a model vaccine OVA. While tape stripping is convenient, its efficacy is a concern. Although aggressive tape stripping can increase the efficiency, it causes delayed skin recovery as shown by histological analysis (figure 1C). In contrast, the laser-affected skin at a mildest condition was quickly recovered within 1-2 days after laser treatment, which ensured the integrity of the skin that is the key for the first line of our body's defense. Notably, a high density of granulocytes was present in and near the microthermal zone 1 and 2 days after AFL treatment (figure 1B). These cells could be proliferative fibroblasts that were necessary to synthesize new collagens as part of re-epithelialization and/or inflammatory cells that were recruited to clean damaged tissues and to release cytokines for tissue repair [24,25]. These cells are expected to return to the basal level after completion of their function, causing no potential risks. The AFL-based technology has been used for nearly a decade for cosmetic purpose in various human populations, and would hold great promise to safely and painlessly ablate SC layer in the clinic for transcutaneous delivery of hydrophilic drugs and vaccines in humans.

Apart from superior safety, AFL is efficient in transcutaneous delivery of small and large molecules. The colored SRB, MB and TR-OVA were readily seen in laser-generated MCs after gauze patch application. They apparently first enter the MCs and then radically diffuse into the surrounding tissue through the conical surface as illustrated in figure 7. The laser-generated MCs not only provide a 'fast lane' for drugs or vaccines to rapidly reach dermis (figure 7A), but also greatly increase the surface for drug or vaccine penetration into the dermis and achieve systemic distribution through dermal lymphatic and blood capillaries (figure 7B). In contrast, tape stripping affects primarily the outer layer of the skin, and the drug must cross multiple layers of cells and dense matrix when traveling from the outer epidermis through the germinativum to the dermis, thereby limiting its efficiency (figure 7).

Strikingly, while laser ablates a "tiny" tissue to create MCs, the surrounding cells remain functionally intact, capable of proliferation and re-epithelialization, consistent with a "sharp" attribute of laser. APCs in the vicinity of the MCs can also actively capture antigens as shown in figure 5B. This amazing attribute lays a foundation of its use in transdermal

patch-based vaccine delivery, an area attracting much of the attention lately. Most of current vaccines are administered into the muscular tissue where APCs are scarce [26,27]. On the contrary, the skin is an immunologically active tissue with an abundance of resident APCs. It also contains a dense network of capillary lymphatic vessels that direct the passage of antigens and antigen-captured DCs from the skin to the dLNs. The first vaccine, smallpox, was delivered by skin scarification nearly two centuries ago [28]. Intradermal vaccination has been shown to be superior to intramuscular vaccination in the clinic in many cases including influenza vaccine [29-31], rabies, hepatitis B virus (HBV) vaccines [32-35]. Unfortunately, current intradermal delivery techniques, such as the Mantoux method and bifurcated needles, are inconvenient and require specially trained personnel, hindering a broad clinic use of this route of vaccination [36]. The new technology under the current investigation can be a promising alternative because an AFL device at a fixed power and skin coverage can be fabricated as small as a handheld flashlight, which if integrates with drug or vaccine-coated patches, can be easily used for transcutaneous drug delivery and vaccine immunization in the future.

Our investigation demonstrated that OVA was actively taken up by both LCs and dDCs in the vicinity of MCs after 6 hrs of patch application (figure 5B). Meanwhile, a strong OVA signal was detected in the draining lymph nodes of AFL-treated mice, which may result from transportation of free OVA through afferent lymphatics because trafficking of Ag-captured APCs to the draining lymph nodes usually takes place 18 hrs after antigen delivery into the skin [23]. Accelerated transportation of OVA to lymph nodes would allow it to be sufficiently processed by resident DCs and presented to antigen-specific T and B cells, eliciting a vigorous immune response. In addition, the antigen in the MCs may serve as a stock, prolonging a release of the vaccine so as to continuously stimulate the immune system. It is thus not surprising in that a significantly higher level of OVA-specific antibody was produced in all AFL groups as compared to tape stripping groups (figure 6). However, slightly invasive tape stripping didn't induce a stronger humoral immune response than mild tape stripping although the former enhanced OVA delivery more efficiently than the latter (figure 4B), which is not anticipated. Tape stripping was often used to enhance transcutaneous immunization of patch-coated cholera toxin (CT) and heat-labile toxin (LT) that function as both antigen and adjuvant. Our study suggests that tape stripping followed by topical application of antigen-coated patch without adjuvant is less sufficient to induce a potent immune response, in agreement with previous investigations [19,20]. In addition to efficient delivery, AFL-generated microthermal zone may also produce and send "danger signals" that serve as endogenous adjuvant to enhance vaccine-induced immune response [25]. This may be particularly true when AFL (5.0mJ 15%) is used to treat the skin. Taken together, the study suggests the feasibility and potency of AFL as a novel means for safe, efficient transcutaneous vaccine delivery as well as adjuvantation of skin vaccination.

Conclusion

The goal of this study is to evaluate a safe, ablative fractional laser-based technology and to compare its relative efficiency with tape stripping in enhancing transcutaneous delivery of hydrophilic drugs and vaccine antigens coated onto gauze patches. We show for the first time that a clinical AFL at a relatively low energy and covered area is safe, potentially convenient, and its efficacy is superior to tape stripping for transdermal patch-based drug delivery and vaccine immunization. Strikingly, while the AFL gives rise to an array of MCs in the skin, it spares the majority of the skin involved from any damage. The cells surrounding the MCs proliferate and function normally to quickly heal the MCs. The lymphatic and blood capillaries draw the drugs and antigens into the circulation system sufficiently. Moreover, APCs in the vicinity of the MCs are competent in capturing the antigen released from the MCs and traveling into the dLNs where they elicit a vigorous

immune response. These results warrant further investigation of the potential of AFL-facilitated transdermal patch-based drug delivery and vaccine immunization in a preclinical study.

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Figure 1. Quick recovery of AFL-treated skin

A. Illustration of AFL-generated MCs in a $6 \times 6 \text{ mm}^2$ area at 5% and 15% skin coverage. **B.** Histology of AFL-treated skin. Skin samples were taken right after (d0), 1 day (d1), or 2 days (d2) after laser treatment at 2.5mJ and 5% skin coverage and compared to non-treated skin (Control). Cross- and horizon-sections at $50\mu\text{m}$ depth were shown in the upper and lower panel, respectively: arrow, a laser-ablated MC; dotted line, microthermal zones (MTZ); and scale bar, $100\mu\text{m}$. **C.** Skin histology following tape stripping. Skin was treated with mild tape stripping (1, 15) or slightly invasive tape stripping (3, 6) followed by sample collection and processing as **B**. The area outlined with a dotted rectangle in the upper panel was enlarged to show stratum corneum (SC) and epidermal layer (lower panel). Arrows in d2, the upper panel point to infiltrated inflammatory cells after slightly invasive tape stripping. Scale bar: $100\mu\text{m}$ (upper) and $25\mu\text{m}$ (lower).

Figure 2. AFL-assisted SRB delivery

SRB-coated patch was topically applied to AFL (2.5mJ 5%) or tape-treated or non-treated control skin for indicated times. The skin photos (A) were taken after mice were euthanized and flushed with tape water. Representative bright-field and fluorescence images of the skin cryosections are shown in B and C, respectively. Arrows in B and C indicate a laser-generated MC and a white dotted line in C outlines SRB signal. Scale bar, 1.5mm in A or 250µm in B and C. Quantitative analysis of *in vivo* SRB delivery over times is shown in D in which the dorsal skin of BALB/c mice was treated with four different AFL conditions, tape stripping (1, 15) and (3, 6), or left untreated followed by topical application of SRB-coated gauze patches. The data are presented as SRB amount $\pm$ SEM per centimeter square of the skin calculated in the basis of a SRB standard curve assayed in parallel. n=6.

Figure 3. AFL-facilitated MB delivery

MB-coated patch was topically applied to AFL (2.5mJ 5%) or tape-treated or non-treated control skin for 30 min. Skin photos (A) and representative bright-field images of skin cryosections (B) were obtained as figure 2B. Scale: 1.5mm in A or 200µm in B. The amount of MB delivery was analyzed in the skin at indicated times after various treatments and expressed as figure 2D except for determining the amount of MB in the basis of a MB standard curve assayed in parallel. n=6

Figure 4. AFL-facilitated TR-OVA delivery

Mouse dorsal skin was exposed to AFL (2.5mJ 5%), tape stripping, or left untreated followed by topical application of TR-OVA-coated patches. The skin was excised 0.5 and 3 hrs later and subjected to cryosectioning as figure 2C. Shown are representative fluorescence microscopic images (A) where arrow indicates a laser-generated MC and white dotted line outlines TR-OVA signal in the dermal tissue. Scale bar, 250μm. Mouse dorsal skin was treated as figure 2D followed by topical application of TR-OVA-coated gauze patch. Skin was excised at indicated times and TR-OVA amount $\pm$ SEM per centimeter square of the skin was quantified in the basis of an OVA standard curve assayed in parallel. n=6.

Figure 5. Antigen uptake and LN-drainage following OVA delivery

One ear of MHC II-EGFP transgenic mice was exposed to AFL (2.5mJ 5%) followed by topical application of AF647-OVA-coated patch (AFL). The other ear of the mouse was sham exposed and applied with AF647-OVA-coated patch (Control). Both ears were subjected to intravital confocal imaging 6 hrs later. Shown are low magnification images; scale bar, 750 μ m (A) and representative high magnification images (B) focusing on epidermis (10 μ m depth, left panel) or dermis (40 μ m depth, right panel), respectively. Asterisks (*) indicate AFL-generated MC containing a high amount of AF647-OVA. Arrows point to AF647-OVA positive LCs or dDCs. Scale bar, 50 μ m. Individual AF647-OVA positive LC and dDC in AFL-treated skin were selected and enlarged to show a merged yellow color of green LC/dDC and red AF647-OVA. C. Auricular draining lymph node. The lymph node was collected 6 hrs after topical application of AF647-OVA-coated patch. Similar results were obtained in three separate experiments.

Figure 6. Immune responses induced by AFL-facilitated transcutaneous delivery of OVA

The low dorsal skin of BALB/c mice was exposed to AFL treatment, tape stripping, or left untreated (control) followed by topical application of OVA-coated patch. OVA-specific antibody titers were measured in blood samples after 2 and 4 weeks of immunization. Student *t*-test was used to compare difference between groups at each time point. *, ** and ***, $p < 0.05$, 0.01 , and 0.001 , respectively, and $n = 6$.

Figure 7. Advantages of AFL in transcutaneous drug and vaccine delivery

AFL-generated MCs serve as 'fast lane' for quick delivery of patch-coated drugs and vaccines into epidermal and upper dermal tissues, by which drugs and vaccines radically diffuse into the entire epidermal and dermal tissues through the lateral surface of the MCs (**A**). In contrast, drugs and vaccines have to cross multiple cell layers when migrating from the skin surface to the epidermis then to the dermal tissue in tape stripping-treated skin (**A**). Conical-shaped MCs allow drugs and vaccines to diffuse along the conical side wall (pink arrows) instead of the basal surface. Thus, an area for drug and vaccine diffusion is increased based on the equation where R is the diameter and H is height of the MC (**B**).