Regular Article

Development of an Effective Psoriasis Treatment by Combining Tacrolimus-Encapsulated Liposomes and Iontophoresis

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Psoriasis is a chronic T-cell–mediated autoimmune skin disease. Tacrolimus (FK506) is commonly used treatment for psoriasis. However, since the molecular weight of FK506 is more than 500 Da, its skin penetration is limited, so that there is a need to improve the penetrability of FK506 to allow for more effective treatment. To this end, we employed iontophoresis (ItP), which is a physical, intradermal drug delivery technology that relies on the use of weak electric current. Previous findings suggest that activation of cell signaling by the weak electric current applied during ItP may affect the expression of inflammatory cytokines, leading to aggravation of psoriasis. In this study, we analyzed the effect of ItP on the expression of various inflammatory cytokines in the skin, and subsequently examined the therapeutic effect of ItP using negatively-charged liposomes encapsulating FK506 (FK-Lipo) in a rat psoriasis model induced by imiquimod. We found that ItP (0.34 mA/cm^2, 1 h) did not affect mRNA levels of inflammatory cytokines or epidermis thickness, indicating that ItP is a safe technology for psoriasis treatment. ItP of FK-Lipo suppressed the expression of inflammatory cytokines induced by imiquimod treatment to a greater extent than skin treated with FK506 ointment for 1 h. Furthermore, epidermis thickening was significantly suppressed only by ItP of FK-Lipo. Taken together, results of this study demonstrate the successful development of an efficient treatment for psoriasis by combining FK-Lipo and ItP, without disease aggravation associated with the weak electric current.

Key words psoriasis, iontophoresis, liposome formulation, inflammatory cytokine

INTRODUCTION

Psoriasis is one of the most common skin diseases in the world; it is a chronic T-cell–mediated autoimmune disease that results in cytokine overexpression and epithelial cell proliferation.[1,2] At present, treatment methods for psoriasis include topical therapy, phototherapy, and systemic therapy, with the most basic treatment method being topical therapy, which involves application of vitamin D analogues or corticosteroids.[3] However, the stratum corneum of the epidermis acts as a barrier[4] and limits the penetration of macromolecular drugs and polar hydrophilic drugs into the skin.[5] To overcome this limitation, various methods have been developed to promote intradermal drug delivery. These methods are broadly divided into chemical enhancement techniques, in which the skin is treated with absorption enhancers to improve drug permeability,[6] and physical enhancement techniques, in which physical stimuli such as electricity and ultrasonic waves are used to enhance the permeability of the skin.[7] Iontophoresis (ItP) is a physical enhancement technology[8] that facilitates intradermal delivery of charged substances by application of weak electric currents (0.3–0.5 mA/cm^2), with the advantages of controllable administration and good compliance.[9] Drugs that can be used with ItP application have long been thought to be limited to those that are hydrophobic and have a charge; however, in our previous research, we demonstrated the successful intradermal delivery of small interfering RNA (siRNA), a hydrophilic macromolecule, by ItP, and knocking down of specific genes.[10]

Tacrolimus (FK506) is an immunosuppressant that is widely used in the treatment of psoriasis[11] (Fig. 1). FK506 acts by inhibiting calcineurin, which is involved in T-cell activation and proliferation.[12] The FK506 binding protein forms a complex with calcineurin, inhibiting the cascade of cytokine gene transcription.[13] Therefore, we focused on FK506 for effective psoriasis treatment. However, a commercially available FK506 ointment has also been reported to exhibit low absorption, with topical application being inadequate to allow for delivery of the drug into deeper layers of skin.[14,15] Furthermore, as the molecular weight of FK506 is more than 500 Da, penetration

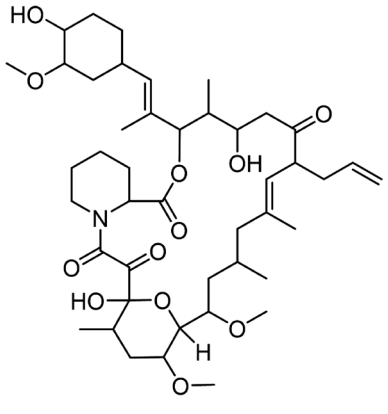

Fig. 1. Chemical Structure of FK506

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through the stratum corneum is difficult.[16] While intradermal delivery of FK506 might be possible using ItP, FK506 does not carry any charge, and therefore, requires a delivery vehicle to facilitate intradermal penetration. Our group previously demonstrated the successful intradermal delivery of insulin-encapsulated charged liposomes by ItP.[17] Therefore, we used FK506-encapsulated liposomes (FK-Lipo) in combination with ItP to provide enhanced intradermal delivery of FK506 as an effective psoriasis treatment.

We also previously found that ItP activates the cell signaling pathway *via* weak current treatment and cleaves intercellular spaces to permeate substances intradermally.[18] Activation of the cell signaling pathway may affect the expression of inflammatory cytokines and aggravate psoriasis. In this study, we therefore investigated whether ItP exacerbates the symptoms of psoriasis by first confirming the mRNA levels of cytokines in ItP-treated epidermis and their effects on the epidermal morphology. To investigate the therapeutic effect of transdermal delivery of FK-Lipo by ItP on psoriasis-induced skin, we evaluated the expression levels of inflammatory cytokines after ItP of FK-Lipo, as well as the effect on inflammation-induced epidermal thickening.

MATERIALS AND METHODS

Animals Male Wistar rats (7 weeks old, 180–220 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Isoflurane and trichloroacetaldehyde monohydrate (chloral hydrate) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The animal inhalation anesthesia machine (Small Animal Anesthetizer TK-40) was purchased from Biomachinery Co., Ltd. (Chiba, Japan). All animal protocols were evaluated and approved by the Animal and Ethics Review Committee of Tokushima University.

Materials Egg phosphatidylcholine (EPC) was purchased from NOF Corporation (Tokyo, Japan). Dicetylphosphate (DCP) was purchased from Sigma-Aldrich (Tokyo, Japan). 1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($DiIC_{18}$) was obtained from Invitrogen (CA, U.S.A.). HPLC-grade chloroform, methanol and acetonitrile were used for liposome preparation and HPLC analyses. FK506 was purchased from Cosmo Bio Corporation (Tokyo, Japan). Imiquimod cream (IMQ, Beselna Cream 5%) was obtained from Mochida Pharmaceuticals (Tokyo, Japan). Protopic ointment (Tacrolimus Hydrate 0.1%) was obtained from Maruho Pharmaceuticals (Osaka, Japan). OCT compound, Perma Fluor Aqueous Mounting Medium, and Entellan New® (hydrophobic mounting medium) were obtained from Sakura Finetek (Tokyo, Japan), Thermo Fisher Scientific (Tokyo, Japan), and Merck Millipore (Tokyo, Japan), respectively. QIAzol Lysis reagent and RNeasy Plus Universal Midi Kit were obtained from Qiagen (Hilden, Germany). PrimeScript RT Master Mix (Perfect Real Time) and TB GreenTM Premix Ex TaqTM II (Tli RNaseH Plus) were purchased from TaKaRa Bio (Shiga, Japan). All other reagents and solvents used in the experiments were of the highest grade commercially available, and purchased from local Japanese sources.

Preparation and Characterization of Liposomes The lipid composition of liposomes was EPC and DCP in a 6 : 4 M ratio, with a final lipid concentration of 10 mM. For fluorescence labeling, 0.1 mol% $DiIC_{18}$ was mixed in with the initial lipids. Blank liposomes were prepared by the lipid hydration method. Lipid solutions were dissolved in chloroform, mixed, and evaporated to obtain a thin lipid membrane, which was then hydrated with 1 mL of phosphate-buffered saline (PBS, pH 7.4) and freeze-thawed with liquid nitrogen (repeated three times). The size of the liposomes was then adjusted by ultrasound treatment for 10 min. To prepare FK506-encapsulated liposomes (FK-Lipo), FK506 (25 mg/mL) was dissolved in ethanol and added to the initial lipid mixture (final molar concentration: 10% of the lipids), followed by the same steps used to prepare blank liposomes described above. The resulting FK-Lipo suspension was then purified by a PD-10 desalination column (GE Healthcare, Tokyo, Japan). The FK-Lipo sample was introduced into the top of the column and balanced by PBS. Free (un-encapsulated) FK506 was adsorbed onto the column and eluted. Fractions comprising liposomes exhibited a pink color, and were collected and subjected to HPLC analysis to determine the concentration of FK-Lipo, as described in the next section. Both blank liposomes and FK-Lipo were diluted with PBS at a dilution of 1 : 100, and physicochemical properties, including mean particle sizes, polydispersity index (PDI), and ζ potential, were analyzed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.). Particle size, PDI and ζ potential of blank liposomes and FK-Lipo are summarized in Table 1.

Quantification of FK506 by HPLC The concentration of the encapsulated FK506 was determined by HPLC using previously reported conditions.[19] Analysis was carried out by automatic HPLC (Shimadzu, Kyoto, Japan) using an octadecylsilane (ODS) column (TSK gel ODS-80TM, 4.6 × 150 mm, Tosoh Corporation, Tokyo, Japan). A series of standard solutions were prepared at concentrations of 5, 10, 50, 100, and 500 μg/mL to generate a calibration curve. FK-Lipo was dissolved in tetrahydrofuran (THF) to break the lipid membranes, and 20 μL of the solution was applied into the injector. Experimental conditions included a mobile phase of acetonitrile/water (60/40, v/v), a flow rate of 1 mL/min, and UV detection at 214 nm. The entrapment efficiency (EE%) of FK506 was calculated by the following formula: EE% = (amount of drug entrapped/total amount of drug added) × 100%. The EE% of FK-Lipo prepared in this study was 45.2 ± 14.6%.

ItP ItP was performed in rats in accordance with our previous report.[10] Briefly, anesthesia was achieved by inhala-

Table 1. Physicochemical Properties of Liposomes

	Size (nm)	ζ-Potential (mV)	PDI	EE%
EPC/DCP = 6/4 0.1%$DiIC_{18}$ (FK506 free)	162.7 ± 6.1	−12.4 ± 0.5	0.2 ± 0.0	—
EPC/DCP = 6/4 0.1%$DiIC_{18}$ (FK506 10%)	352.7 ± 57.5	−13.9 ± 3.1	0.5 ± 0.0	45.2 ± 14.6

tion of isoflurane and intraperitoneal administration of chloral hydrate (400 mg/kg per rat) dissolved in PBS. To measure the effect of ItP alone, two pieces of absorbent cotton (surface area of 1 cm^2) moistened with 100 μL PBS were placed on the dorsal skin and attached to either the cathode or the anode side of Ag–AgCl electrodes (3M Health Care, Minneapolis, MN, U.S.A.), connected to a power supply (TTI Ellebeau, Inc., model TCCR-3005, Tokyo, Japan). ItP was then performed on the dorsal skin of normal rats for 1 h with a constant current of 0.34 or 1.0 mA/cm^2. For delivery of the ItP-blank liposomes, absorbent cotton with 100 μL liposomes was placed on the dorsal skin of normal rats and attached to the cathode (anode: 100 μL PBS), followed by 1 h ItP with a constant current of 0.34 mA/cm^2. After a specified period of time from the end of ItP treatment, rats were euthanized and skin tissue was excised and used in the following experiments.

FK-Lipo ItP Treatment in Psoriasis Model Rats Psoriasis model rats were prepared as previously reported.[20] Briefly, rats were anesthetized as described above. Under isoflurane anesthesia, 60 mg of IMQ cream was applied topically onto the shaved dorsal skin, after which the rats were allowed to recover from anesthesia. IMQ treatment was performed a total of 4 times over 24 h to induce psoriasis, and the resultant rats were used in the following experiments. To evaluate intradermal distribution and anti-psoriatic efficacy, 1 h ItP treatment with FK-Lipo was performed in psoriasis model rats. At 24 h after the 4th IMQ treatment, IMQ-induced psoriasis rats were anesthetized with chloral hydrate as described above. Absorbent cotton (1 cm^2) containing FK-Lipo in 100 μL PBS (25 μg equivalent as FK506) was placed on the dorsal skin, and attached to the cathode (anode: 100 μL PBS), followed by 1 h of ItP (0.34 mA/cm^2). Rats were euthanized after 23 h from the end of ItP treatment and the skin tissues under the cathode were collected and used in the following experiments.

FK-Ointment Treatment in Psoriasis Model Rats A commercial formulation of FK506 (FK-ointment) was used as a reference material in this study. FK-ointment-treated rats were divided into 2 groups: 1-h treatment and 24-h treatment. At 24 h after the 4th IMQ treatment, 25 mg FK-ointment cream (25 μg equivalent as FK506) was applied topically onto the back psoriatic skin of rats in both groups, with a treatment area of 1 cm^2. After 1 h, the cream in the FK-ointment 1-h group was wiped off, while that of the 24-h group was not. At 48 h after the 4th IMQ treatment, animals in both groups were euthanized and the skin tissues under the treatment areas were collected and used in the following experiments.

Hematoxylin–Eosin (H&E) Staining The excised skin was embedded in Optimal Cutting Temperature (OCT) Compound in Tissue Tech Cryomold No. 3 (Sakura Finetek), and subsequently frozen with ethanol and dry ice and cut into 10 μm sections using a cryostat (CM3050S, Leica Biosystems, Nussloch, Germany). The 10-μm thick frozen sections were mounted onto glass slides (Matsunami Glass Ind., Ltd., Osaka, Japan) with Perma Fluor Aqueous Mounting Medium (Thermo Fisher Scientific, Waltham, MA, U.S.A.). $DiIC_{18}$ fluorescence in the skin sections was observed using a confocal laser scanning microscope (LSM700, Carl Zeiss, Jena, Germany). The delivery depths of liposomes from the skin surface were measured using the BZ-9000 image analysis software. The 10-μm thick frozen sections were stained with H&E for histopathological examination. Sections were fixed with 4% paraformaldehyde for 10 min. After washing with PBS, the sections were stained with Mayer's hematoxylin solution for 10 min, washed with distilled water, and subsequently stained with 0.125% eosin Y solution for 1 min at room temperature. Next, the skin sections were dehydrated with 80–100% ethanol, and finally washed with xylene. After cleaning, sections were mounted with a hydrophobic mounting medium. Thereafter, the prepared 10-μm sections of each group were observed using a phase-contrast microscope (BZ-9000, Keyence, and Osaka, Japan). Average epidermis layer thickness was determined from 20 images per rat using the BZ-9000 image analysis software.

Quantitative Analysis of mRNA Expression by Real-Time RT-PCR Skin samples collected from each group were immersed in Allprotect Tissue Reagent (Qiagen) to stabilize RNA before extraction. Next, 200–250 mg of the collected skin was homogenized in 5 mL of QIAzol Lysis reagent (Qiagen) using Tissue Ruptor II (Qiagen). After incubation at room temperature for 5 min, purified total RNA was extracted according to the protocol provided with the RNeasy Plus Midi Kit (Qiagen). The concentration of total RNA was quantified using Nano Drop 8000 (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Total RNA (200 ng) was mixed with 2 μL of 5 × PrimeScript RT Master Mix (Perfect Real Time, TaKaRa Bio); the total volume was adjusted to 10 μL with ribonuclease (RNase)-free water, and then reverse transcription reaction (37 °C for 15 s) and reverse transcriptase inactivation (85 °C for 5 s) were performed using a MJ Mini Personal Thermo Cycler (Bio-Rad, Hercules, CA, U.S.A.) to obtain cDNA. The mRNA levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α, tumor growth factor (TGF)-β and IL-10 were quantitated by real time-PCR. Real time-PCR was performed using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus, TaKaRa Bio) and Thermal Cycler Dice® Real Time System III (TaKaRa Bio). The Real time-PCR conditions were as follows: heat denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The dissociation steps were: 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. The primers were synthesized by Eurofins Genomics, and the sequences of those used for real time-PCR are shown in Table 2. mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method by normalization rela-

Table 2. Primer Sequences Used for Real Time-PCR

Primer	Forward (5′–3′)	Reverse (5′–3′)
GAPDH	CCCCCAATGTATCCGTTGTG	TAGCCCAGGATGCCCTTTAGT
IL-6	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC
IL-10	GCCTGCTCTTACTGGCTGGA	TCTGGCTGACTGGGAAGTGG
TGF-β	AGATTCAAGTCAACTGTGGAG	AAGCCCTGTATTCCGTCTC
TNF-α	CGTAGCAAACCACCAAGCA	CCCTTGAAGAGAACCTGGGAGTA

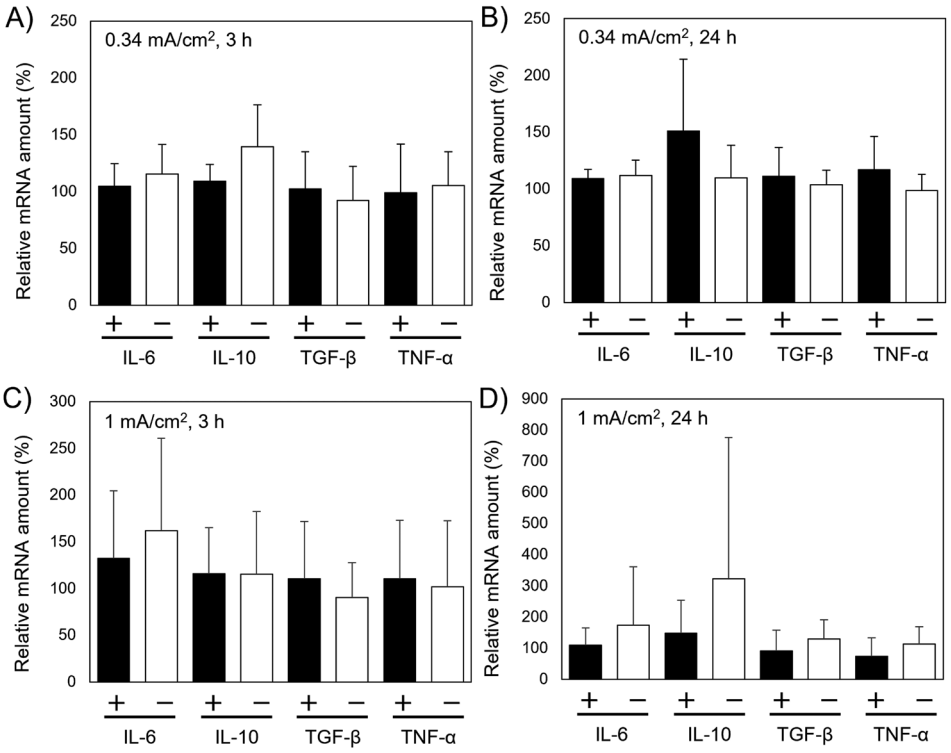

Fig. 2. mRNA Levels of Inflammatory Cytokines in the Skin Following ItP

Relative mRNA expression levels for each cytokine in the skin (anode side:+, cathode side:−) were measured at A) 3 h and B) 24 h after normal ItP (0.34 mA/cm^2), and C) 3 h and D) 24 h after strong ItP (1.0 mA/cm^2). Data are mean ± S.D. ($n = 3$).

tive to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, and relative transcript levels (as percentages) were calculated to evaluate differences between groups.

Statistical Analysis Statistical differences were evaluated by one-way ANOVA followed by least significant difference (LSD) *post-hoc* test. Comparisons between two groups were accomplished by a Student's *t*-test. All data are expressed as means ± standard deviations (S.D.). *p*-Values <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of ItP on Expression Levels of Inflammatory Cytokines in the Skin To evaluate the effect of ItP alone on induction of inflammation in the skin, the mRNA levels of inflammatory cytokines after ItP were evaluated by real time-PCR. In this study, the skin inflammatory conditions were analyzed by altered levels of key cytokines, including IL-6, TNF-α, TGF-β and IL-10. Among these, IL-6, TNF-α, and TGF-β act as key pro-inflammatory cytokines, while IL-10 is mainly responsible for anti-inflammatory effects.[21] Furthermore, TNF-α correlates with psoriasis severity,[22] and is considered a therapeutic target of psoriasis.[23] Results demonstrated that ItP treatment (0.34 mA/cm^2, 1 h) did not show significant effects on mRNA levels. As shown in Figs. 2A, B, ItP induced a slight increase in mRNA expression, while significant increases were not observed. In addition, ItP at a constant current of 1.0 mA/cm^2 for 1 h, which is much higher than the normal current condition (0.34 mA/cm^2), induced an increase in mRNA expression for almost all cytokines, especially IL-6 and IL-10 (Figs. 2C, D), but the difference was not statistically significant. These results confirm that ItP under normal conditions (0.3–0.5 mA/cm^2) does not induce inflammation in the skin, while ItP at a stronger current intensity (1.0 mA/cm^2) may induce an inflammatory response.

Effect of ItP on Skin Morphology Based on our previous study, skin morphology, including thickness of the epidermis, changes under inflammatory conditions.[20] To evaluate the influence of ItP on skin morphology after ItP treatment, skin section was subjected to H&E staining and then observed under a microscope. At both 3 and 24 h after ItP, morphologies of skin tissues collected under the cathode and anode were similar to that of non-treated skin (Figs. 3A, B). Based on the results shown in Figs. 2 and 3, ItP can be considered as a safe technology for treatment of skin diseases, such as psoriasis.

ItP of Liposomes on Dorsal Skin of Rats We performed ItP of DiC_{18}-labeled liposomes applied on the dorsal skin of normal rats. No fluorescence was observed in the skin section treated with liposome-free ItP (Fig. 4A), whereas bright fluorescence was observed over a wide area from the skin surface to the epidermal layer in the skin section treated with liposomal ItP (Lipo ItP) (Fig. 4B). Furthermore, fluorescence was also observed in the hair follicles, as well as diffused widely around them. Liposome fluorescence reached an average depth of approx. 61 μm from the surface of the skin. Taken together, these results confirm that the liposomes used in this study can be delivered into the skin tissue by ItP. The stability of liposomes intradermally delivered *via* ItP has been investigated in our previous work. In our previous publication,[24] we prepared double-labeled liposomes, in which the lipid membrane was labeled fluorescently with nitrobenzoxadiazole (NBD) (green) and inner aqueous phase was labeled with rhodamine (red). After *in vivo* ItP of the double labeled liposomes on rat dorsal skins, we observed the intradermal distribution of NBD-

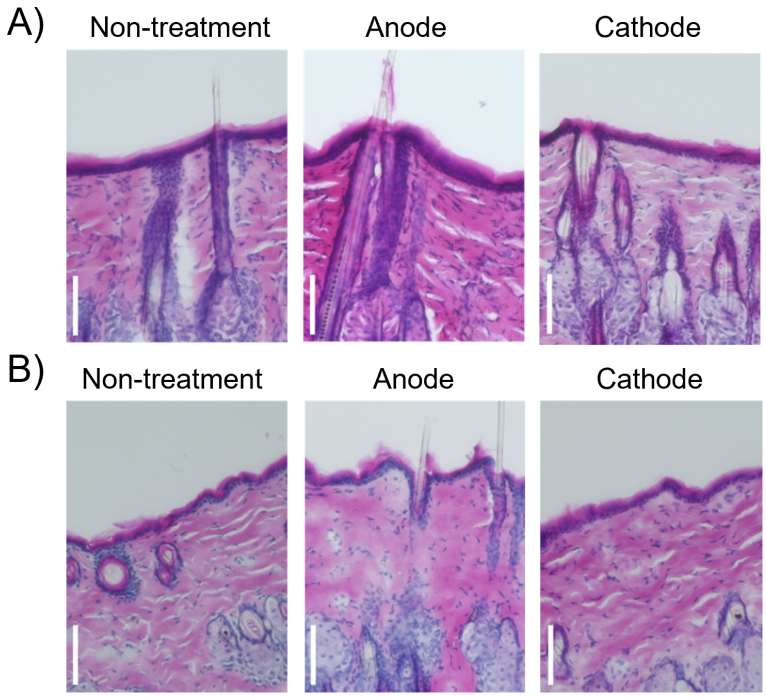

Fig. 3. Morphology of the Skin after ItP

After ItP (0.34 mA/cm^2), skin sections were prepared and subjected to H&E staining. A) 3 h and B) 24 h after ItP. Scale bars = 100 μm.

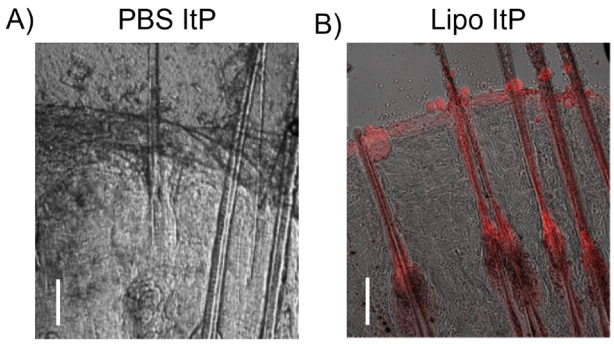

Fig. 4. Intradermal Distribution of Liposomes after ItP

Intradermal distribution of ItP-administered DiIC$_{18}$-labeled liposomes was observed by confocal laser scanning microscope. Skin sections obtained after A) ItP of PBS as a negative control, and B) ItP of DiIC$_{18}$-labeled liposomes. Scale bars = 100 μm.

labeled liposome membranes and encapsulated rhodamine by confocal laser scanning microscopy. The results showed that the green fluorescence of NBD and the red fluorescence of rhodamine were co-localized and could be observed as a yellow color, indicating that rhodamine was encapsulated in the liposomes distributed in the skin. From these results, it was suggested that the liposomes retained their vesicular structure even after ItP. Based on our previous study, we consider that FK-Lipo would also be delivered into skin retaining the structure of liposomes in a stable condition.

Effect of FK-Lipo ItP on Inflammatory Cytokine Expression in Psoriatic Rat Skin In Table 1, the mean diameter of the liposomes increased by encapsulation of FK506. We considered that the increase in mean diameter after FK506 encapsulation was due to the gradual aggregation formation of FK-Lipo as a hydrophobic colloid. FK506 has three OH groups in its structure, all of them are far apart, each locating in the interface of the liposome membranes. In this case, the other hydrophobic part, except for OH groups, may be present as a lying form in the liposome membranes. Hence, the hydrophobic part of FK506 may be exposed at the membrane interface to some extent. On the other hand, the surface of FK-Lipo was negatively charged because of the component of anionic lipid DCP. Thus, the FK-Lipo were hydrophobic colloids. Since hydrophobic colloids aggregate loosely in the presence of a small amount of electrolyte, it is assumed that a loose aggregation of FK-Lipo occurred, resulting in the larger particle size and PDI. As a supporting data to our speculation, the mean diameter of FK506 free liposomes was about 162.7 nm, which increased about 2.2 folds to nearly 352.7 nm in FK-Lipo. A stereoscopic agglomeration of FK-Lipo may contain six free liposomes gathering together to show a twice diameter.

IMQ, a ligand for toll-like receptors, was utilized to induce psoriatic rat models and assess the therapeutic efficacy of different FK506 formulations. After IMQ treatment, the back skin developed visual skin lesions consisting of redness, scaling and skin thickening from the 2nd day, which progressively intensified until the 6th day of the experiment. Time course of drug treatment is shown (Fig. 5A). TNF-α and IL-6 act as major psoriasis mediators and were evaluated as indicators of the initiation of psoriasis. Significantly higher levels of TNF-α (2.97-fold) and IL-6 (7.19-fold) in the IMQ group were observed in comparison with the non-treated group, which confirmed the development of psoriasis model rats (Figs. 5B, C). After ItP of FK-Lipo on the psoriatic skin, the anti-inflammation efficacy in the FK-Lipo ItP group was compared with that of the groups treated with commercially-available FK506 formulations. TNF-α levels were reduced to 70% in the FK-Lipo ItP group and 36% in the FK-ointment 24-h group, relative to the IMQ group, while no reduction in TNF-α levels (related to the IMQ group) was observed in the FK-ointment 1-h group on the 6th day of study. Although no

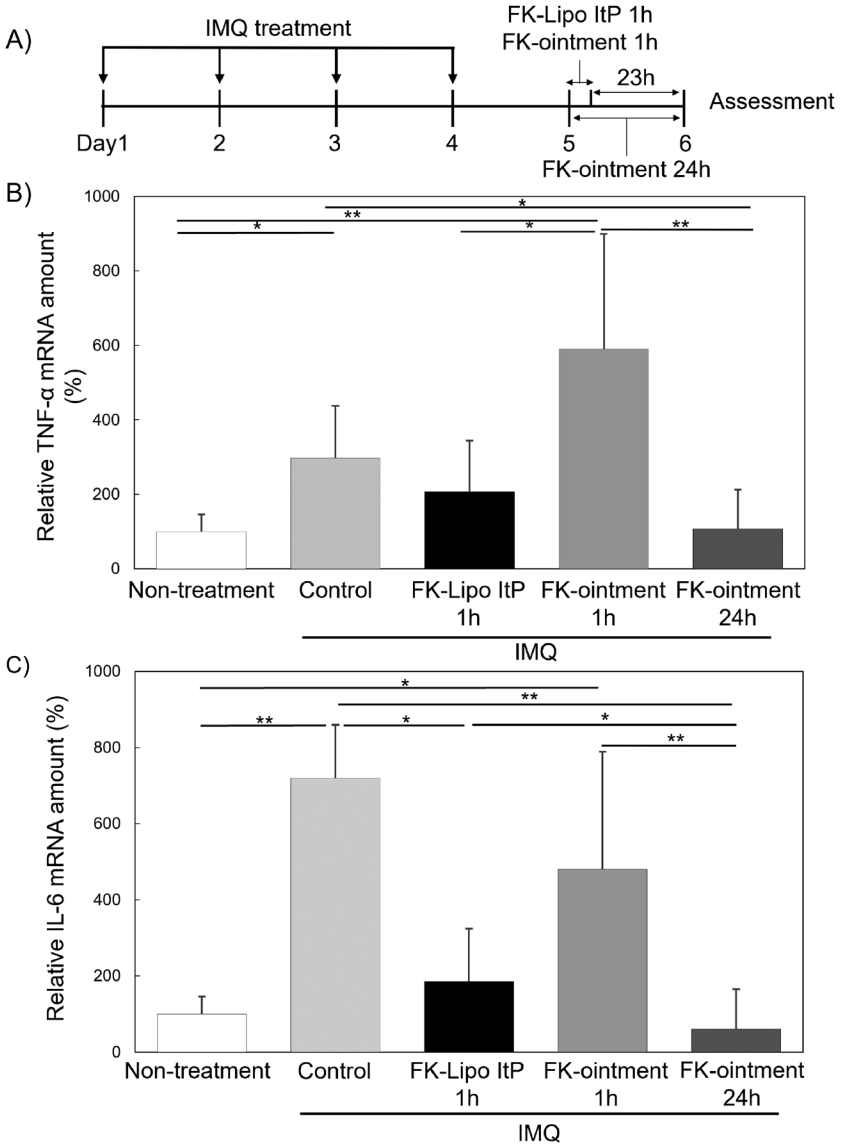

Fig. 5. Effect of FK-Lipo ItP on mRNA Levels of the Inflammatory Cytokines TNF-α and IL-6 in IMQ-Induced Psoriasis Model Skin

A) The time course of drug treatment. Relative inflammatory cytokine mRNA levels of B) TNF-α and C) IL-6 were evaluated by RT-PCR. Data points are mean ± S. D. from at least 3 measurements. $^{**}p < 0.01$; $^{*}p < 0.05$.

significant difference was observed between the FK-Lipo ItP and IMQ groups, TNF-α levels in the FK-Lipo ItP group tended to decrease (Fig. 5B). IMQ-induced IL-6 expression was significantly reduced by FK-Lipo ItP (26%) and FK-ointment for 24-h (8.4%). IL-6 levels in the FK-ointment 1-h group also decreased (67%), but the difference was not statistically significant. Thus, the FK-Lipo ItP group showed a similar significant IL-6 reduction effect to that of the FK-ointment 24-h group, and a >2-fold higher effect than the FK-ointment 1-h group (Fig. 5C).

These results demonstrate that ItP of FK-Lipo effectively suppresses inflammatory cytokines in a relatively short period of time.

Effect of FK-Lipo ItP on Epidermis Thickness of Psoriatic Rat Skin IMQ treatment induced thickening of the epidermis. H&E staining showed a mean epidermal thickness of 22.1 μm in the non-treated group and a 4.26-fold increase to 94.2 μm in the IMQ group, indicating that IMQ application also led to hyperplasia of keratinocytes (Figs. 6A, B). The epidermis thickness decreased to 79.0 μm in the FK-Lipo ItP group (84% of IMQ group) (Figs. 6C, F). However, FK-ointment did not significantly affect epidermal thickness (Figs. 6D–F). Previous studies using cyclosporine-encapsulated liposomes showed a positive correlation between the reduction of inflammatory cytokines and the anti-thickening effect in IMQ model animals.[25] According to this previous report, inflammatory cytokine levels in the FK-ointment 24-h group were the lowest so the strongest anti-thickening effect was also expected to appear in the same group. In our study, however, a significant anti-thickening effect was detected only in the FK-Lipo ItP group. This thickening effect is noteworthy, as current psoriasis treatment is mostly based on symptom control. It is likely that the significant anti-thickening effect is due to sustained release of liposomes delivered into the skin by ItP.[26]

We attempted dialysis experiment of FK-Lipo to evaluate the *in vitro* release profile under the same conditions performed in the previous publication.[27] However, FK506 was

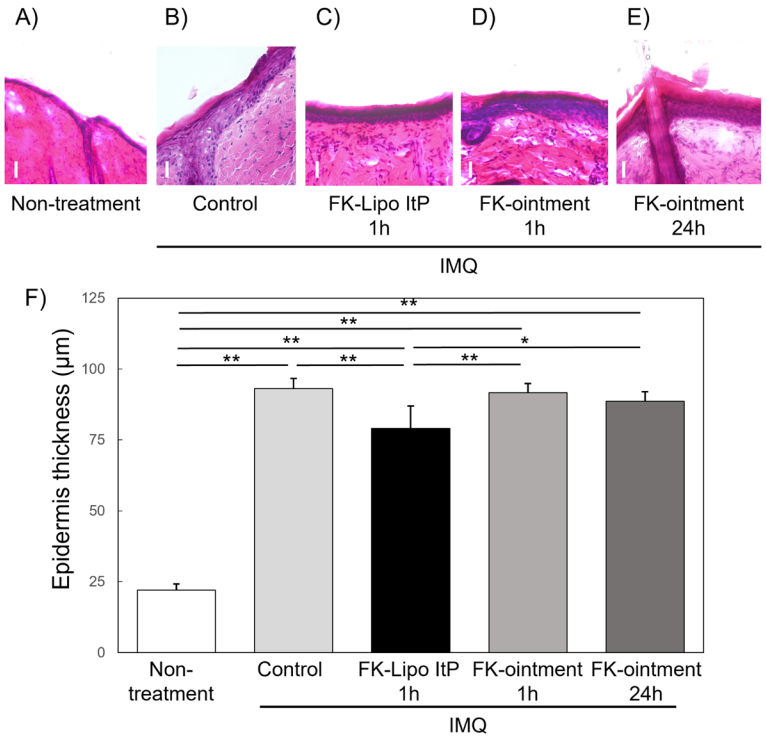

Fig. 6. Effect of FK-Lipo ItP on Epidermis Thickness in Psoriasis Model Skin

Skin sections of A) non-treated group, B) IMQ group, C) FK-Lipo ItP group, D) FK-ointment 1-h group and E) FK-ointment 24-h group were subjected to H&E staining. F) The average thickness of the epidermis layer was measured from 20 images for each rat. Data are mean ± S.D. ($n = 3$). $^{**}p < 0.01$; $^{*}p < 0.05$. Scale bars = 100 μm.

not detected in the dialysis external fluid. The considerable reason is that FK506 was not released into the aqueous system due to its very high hydrophobicity, or even if it was released, it was undetectable because of the extremely small amount. The FK506-loaded liposomes in this reference were different from ours because the component contained surfactant (bile acid). Probably, the micelles consisting of bile acid enabled the elution of hydrophobic FK506. To further investigate the *in vitro* release of FK506 from FK-Lipo, we focused on the method of another reference.[28] In this paper, PBS containing surfactant (Tween 80) was used as the release solution to increase the FK506 solubility released from MPEG-poly lactide polymer nanoparticles containing FK506. The FK506/MPEG-PLA nanoparticles would not be disintegrated even in a buffer containing Tween 80 because those nanoparticles were composed of polymer macromolecules poly lactide. On the other hand, in the present study, liposomes were composed of lipids only. If it were suspended in a buffer containing surfactant, the liposome membranes would collapse and the drug would be released at once, so the FK506 release characteristics cannot be measured accurately. Currently, there is a big dilemma of whether adding a surfactant to dissolve FK506 or not, because the addition will also destroy the liposome formulation. Thus, we concluded that it is difficult to measure the sustained release profile of FK-Lipo *in vitro*.

Therefore, we focused on the *in vivo* sustained release of FK506. However, it was difficult to visualize intradermal distribution of FK506 and measure only FK506 released from liposomes in skin. Furthermore, to study the release and absorption of FK506 in the skin, we additionally attempted to measure the concentration in the blood after ItP of FK-Lipo by HPLC. However, the FK506 concentration in the blood was below the detection limit. Then, we thought that a sustained release of FK506 from liposomes would improve the therapeutic effect in a time-dependent manner. As shown in Supplementary Fig. 1, the suppression rate of skin thickening in FK-ointment group increased from 2.7% (after 23 h) to 4.1% (after 47 h). On the other hand, in the FK-Lipo/ItP group, the suppression rate increased from 16% (after 23 h) to 22% (after 47 h). Therefore, the therapeutic effect of FK-Lipo was approximately 4.3 times greater than that of FK506 already present in the skin by FK-ointment (1.4%). Thus, the therapeutic effect of FK-Lipo ItP was time-dependent, suggesting a sustained release of FK506 from liposomes. This suggests that the sustained release effect of liposomal formulations is effective in the treatment of psoriasis. Therefore, ItP should contribute to aggravating the anti-thickening effect associated with liposomal formulations.

CONCLUSION

Results of this study confirm that ItP is a safe drug delivery technology that does not adversely affect the expression levels of inflammatory cytokines or thickening of the skin. Furthermore, the combination of FK-Lipo and ItP exhibited anti-psoriatic efficacy in an IMQ-induced psoriatic rat model, resulting in both improved histopathological features of psoriatic skin and also reduced levels of TNF-α and IL-6. Therefore, the combination of FK-Lipo and ItP is suggested to be an efficient treatment method for psoriasis.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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