International Journal of Pharmaceutics 402 (2010) 198–204

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Pep-1 peptide-conjugated elastic liposomal formulation of taxifolin glycoside for the treatment of atopic dermatitis in NC/Nga mice

Myung Joo Kang[a], Jae Yoon Eum[a], Sang Han Park[a], Mean Hyung Kang[a], Kwan Hee Park[a], Sun Eun Choi[a], Min Won Lee[a], Kyung Ho Kang[b], Chil Hwan Oh[b,*], Young Wook Choi[a,**]

[a] College of Pharmacy, Chung-Ang University, 221 Heuksuk-dong, Dongjak-gu, Seoul 156 756, Republic of Korea
[b] College of Medicine, Korea University, 97 Guro-dong, Guro-gu, Seoul 152 703, Republic of Korea

ARTICLE INFO

Article history:
Received 29 July 2010
Received in revised form
15 September 2010
Accepted 24 September 2010
Available online 1 October 2010

Keywords:
Taxifolin glycoside
Elastic liposomes
Pep-1 peptide
Atopic dermatitis
NC/Nga mice

ABSTRACT

In order to develop topical preparations of taxifolin glycoside (TXG) for the treatment of atopic dermatitis (AD), formulations of Pep-1 peptide-conjugated elastic liposomes (Pep1-EL) were examined for their *in vitro* skin permeation profile and *in vivo* therapeutic efficacy. TXG-loaded Pep1-EL – a nanovesicle consisting of phosphatidylcholine, Tween 80, *N*-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine (MPB-PE), and Pep-1 peptide – is 130 nm in size, and has a zeta potential of 25 mV and a deformability index value of 60. Here, we examined the skin permeability of several topical preparations using a Franz diffusion cell mounted with depilated mouse skin and found that formulations of Pep1-EL exhibited superior absorption when compared to aqueous solution, EL or Pep-1 peptide-admixed EL formulations. Both transepidermal water loss and skin surface hydration were also measured using AD-induced NC/Nga mice, and the TXG-loaded Pep1-EL treatment group displayed a significantly expedited recovery in skin barrier function when compared to the controls treated with a TXG aqueous solution ($p < 0.05$). AD-associated immune responses – including serum interleukine-4, immunoglobulin E, and interferon-gamma – were also regulated by topical application of TXG-loaded Pep1-EL. In conclusion, the novel Pep1-EL formulation of TXG shows substantial promise in the treatment of AD as a result of its desirable skin delivery-promoting capability.

1. Introduction

Atopic dermatitis (AD) is a chronically relapsing skin disorder characterized by allergic symptoms such as redness, flaking, and itching (Leung and Bieber, 2003). Pathophysiologically, AD is defined by dysfunction of the stratum corneum that results in excessive transepidermal water losses (TEWL) and the infiltration of allergens into the skin (Watanabe et al., 1991). While the exact causes of AD are not entirely understood, most evidence suggests that complex immune dysregulation and environmental allergenic susceptibility are both involved in the underlying etiology. Several key cytokines have also been implicated in the etiology of AD, most notably interleukins (IL)-4 and -5, both of which activate mast cells and eosinophils and upregulate immunoglobulin E (IgE) production. In contrast to the increased levels of T-helper (Th) 2 cells and Th2-associated cytokines present in AD, serum levels of Th1 cells and Th1-associated cytokines – notably interferon gamma (IFN-γ) and IL-12 – are classically low in this disorder (Byron et al., 1992; Leung, 1995; Akdis et al., 2002). Currently, non-steroidal immunosuppressive agents like cyclosporin and thymopentin are commonly used as first line agents in the treatment of AD and skin inflammatory reactions (Lee et al., 2010). However, as both of these agents have a relatively short duration of action and can cause significant side effects with long-term use, alternative medicines that can be applied safely for longer periods of time are desperately needed.

Taxifolin 3-*O*-β-D-glucopyranoside (TXG), 3′,4′,5′,7′-tetrahydroxyflavonol glucoside, is a major flavonoid isolated from *Rhododendron mucronulatum* and *Silybum marianum* (Takahashi et al., 2001; Kim et al., 2003). A recent study indicates that TXG effectively inhibits microbial antigen-stimulated, dendritic-cell-mediated allergic responses in a dose-dependent manner (Kim et al., 2008). As TXG also reduces serum levels of eosinophils, IgE, IL-4, and IL-5 in NC/Nga mice, it seems to be an ideal drug candidate for a topical AD therapy (Ahn et al., 2010). However, the topical delivery of TXG to the relevant skin layer is markedly limited because of the compound's relative hydrophilicity. Thus,

* Corresponding author at: Department of Dermatology, College of Medicine, Korea University, Seoul, Republic of Korea. Tel.: +82 2 2626 1894; fax: +82 2 2626 1900.
** Corresponding author at: Division of Pharmaceutical Sciences, College of Pharmacy, Chung-Ang University, Seoul, Republic of Korea. Tel.: +82 2 820 5609; fax: +82 2 826 3781.
E-mail addresses: choh@korea.ac.kr (C.H. Oh), ywchoi@cau.ac.kr (Y.W. Choi).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.09.030

the lack of an efficient delivery system remains the primary hurdle in the development of topical TXG preparations (Anigbogu et al., 1996).

To date, several approaches have been explored to facilitate epidermal and/or dermal delivery of therapeutic agents, most notably, chemical enhancers, vesicular carriers, ultrasounds, and microneedles (Trotta et al., 2002; Prausnitz, 2004; Paliwal and Mitragotri, 2006; Rachakonda et al., 2008). Elastic liposomes (ELs) are nanosized vesicles that consist of phospholipids and edge activators and have been widely investigated as adjunctives to increase the topical and/or transdermal delivery of drugs (Cevc and Blume, 2001; Dubey et al., 2006). In comparison to conventional liposomes, ELs are able to respond to external stresses by rapidly changing shape, and penetrate the skin efficiently in terms of quantity and depth (Dubey et al., 2006). A novel approach for improving the skin delivery of therapeutic molecules employs cell-penetrating peptides (CPPs) including Tat (Trans-activating transcriptional activator), YARA, WLR, and R9 peptides (Rothbard et al., 2000; Hou et al., 2007; Lopes et al., 2008). These peptides interact with lipids in the stratum corneum, which subsequently allow entry via macropinocytosis (Lopes et al., 2005; Hou et al., 2007). We previously reported that a Tat peptide-admixed EL system effectively facilitates the delivery of natural immunomodulating compound to the dermal layer (Kang et al., 2010a). In an *in vitro* model, we also demonstrated that the conjugation of Pep-1 peptides – a type of penetrating peptide – to EL remarkably increased the delivery of liposomal drugs into human keratinocytes (HaCaT) when compared to EL alone (Kang et al., 2010b). Accordingly, we hypothesized that the advanced carrier system of Pep-1 peptide-conjugated EL (Pep1-EL) would be an effective vehicle for the topical delivery of TXG as well as serve to amplify TXG's immunomodulatory activity *in vivo*.

Here, we use *in vitro* skin permeation profiles to compare the topical TXG delivery capacity of the Pep1-EL formulation with others of drug solution, EL, and Pep-1 peptide-admixed EL. To assess the efficacy of TXG-loaded Pep1-EL preparations in restoring epidermal barrier function among AD-induced NC/Nga mice, TEWL and skin surface hydration were measured after treatment. Therapeutic improvements of AD were further estimated by tracking serum levels of IL-4, IgE and IFN-γ.

2. Materials and methods

2.1. Animals

Male five-week-old ICR mice and male six-week-old NC/Nga mice were purchased from Orient Bio (Kyungki-Do, Korea) and Central Laboratories, Animal Inc. (Seoul, Korea), respectively. All the mice were housed in plastic cages at a temperature of $22 \pm 3\,^{\circ}C$, with a relative humidity of $50 \pm 20\%$ under 12 h light/12 h dark cycle. All the animal experiments were performed in accordance with NIH guidelines (NIH publication No.85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Chung-Ang University in Seoul, Korea.

2.2. Chemicals and reagents

TXG (purity > 95% assessed by HPLC assay) was provided by the Chung-Ang University Pharmacognosy Laboratory (Seoul, Korea). Soybean phosphatidylcholine (PC), Tween 80, cysteine, phosphate buffered saline (PBS) tablets and trifluoroacetic acid were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Pep-1 peptides (KETWWETWWTEWSQPKKKRKVC, 22mer) were synthesized by Peptron Co. (Taejun, Korea). N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine (MPB-PE) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Sodium carboxymethylcellulose (Na-CMC) and 2,4,6-trinitro-1-chrolobenzene (TNCB) were purchased from Duksan Pure Chemical Co. (Kyungkido, Korea) and Tokyo Chemical Co. (Tokyo, Japan). All the other chemicals and reagents were purchased from commercial sources and were of analytical grade. Twice distilled water was used for all the experiments.

2.3. Preparation of topical formulations

2.3.1. Preparation of solution formulation

TXG aqueous solution (Soln) was prepared by dissolving TXG in distilled water at a concentration of 1.0 (w/v %). Na-CMC (a thickening agent) was then added at a concentration of 0.5 (w/v %) in order to improve application to mouse dorsal skin.

2.3.2. Preparation of EL formulations

The TXG-loaded Pep1-EL formulation was prepared by conjugating Pep-1 peptides to drug-containing EL via the thiol-maleimide reaction, as previously reported (Kang et al., 2010b). TXG-loaded vesicles were initially prepared using a reverse phase evaporation (REV) method (Szoka and Papahadjopoulos, 1978; Wu et al., 2004). Briefly, PC, Tween 80 and MPB-PE were taken in a molar ratio of 89:10:0.5 and dissolved in a mixture of diethyl ether and chloroform (2:1) in a round bottom flask. The aqueous phase containing TXG (50 mg/ml) was then mixed with the organic phase and sonicated in a bath sonicator (Model 2210, Branson Ultrasonics Co., Danbury, CT, USA) for 5 min. After this period of time, the solution was attached to a rotary evaporator (Rotary Evaporator, Super Fit, Ambala, India) to dry the contents under vacuum until a gel formed. The resulting gel was then hydrated with distilled water and extruded 10 times through a 200 nm polycarbonate membrane filter (Millipore, Billerica, MA, USA) to promote the formation of small, unilamellar vesicles. The Pep-1 peptide solution (1 mg/ml) was then added to TXG-loaded vesicles and allowed to react for 12 h at room temperature. An excess amount of cysteine was also added to block any unreacted maleimide groups that remained on the vesicles. Peptidyl liposomes were then isolated from the free Pep-1 peptides, cysteine and unencapsulated TXG through the use of an ultrafiltration-stirred cell (Millipore, Billerica, MA, USA) as described in Hirai et al. (2007). TXG-loaded conventional EL, consisting of PC and Tween 80 (90:10), was prepared using the same procedure. The Pep1-admixed EL (Pep1+EL) was formulated by adding Pep-1 peptides to EL at a concentration of 0.125 mg/ml (w/w %), an equimolar amount to that of the Pep1-EL formulation. All the formulations had a final lipid concentration of 15 mg/ml and drug concentration of 1.0 (w/v %). Na-CMC was also added to all the formulations at a concentration of 0.5 (w/v %) for ease of application.

2.4. Physical characterization of EL formulations

2.4.1. Size and zeta potential

Liposomal vesicles were diluted with an appropriate volume of water and subsequently examined for size distribution and zeta potential using a dynamic light scattering particle size analyzer (Zetasizer Nano-ZS, Malvern Instrument, Worcestershire, UK) with a 50 mV laser at a scattering angle of 90°. All the measurements were carried out under ambient conditions and in triplicate.

2.4.2. Loading amount and loading efficiency

Unencapsulated drug was separated from liposomes using an ultrafiltration-stirred cell as previously described. The liposomal suspensions were held over the filtration membrane (pore size 30 kDa) in pressurized cell (20 psi). The filtrate and supernatant were analyzed for TXG by HPLC. The loading amount per total lipid

was calculated using the equation: $[(T-C)/W] \times 100$. The entrapment efficiency of the liposomes was calculated using the equation: $[(T-C)/T] \times 100$, where T represents the total amount of drug initially added, C represents the amount of drug detected in the filtrate, and W represents the amount of lipid used in the formulations (Touitou et al., 2000).

2.4.3. Elasticity of the vesicle membrane

The vesicle suspension was propelled through a microporous filter using an external pressure of 2.5 bars (Cevc et al., 1998). Vesicle size was monitored through dynamic light scattering measurement and was assessed both before and after filtration. Vesicle membrane elasticity was calculated using the formula (Van den Bergh et al., 1999): $D = J(r_v/r_p)^2$, where D represents the deformability index of the vesicle membrane, J represents the amount of vesicle suspension extruded in 5 min, r_v represents the vesicle size after extrusion, and r_p represents the barrier pore size.

2.5. In vitro skin permeation study

In vitro permeation studies were conducted using vertical Franz diffusion cells, as described previously in Huailiang et al. (2001) and Kang et al. (2010a). Skin specimens were obtained from hairy albino mice. In all the cases, dorsal hair was carefully removed using electric clippers before the tissue was rinsed with a phosphate buffer solution. A circular piece of dorsal skin was then carefully mounted onto the receiver compartment of the diffusion cells so that the stratum corneum directly abutted the donor compartment. The receptor chamber was filled with 10 mM PBS (pH 7.4) solution and maintained at 32 °C throughout the entire process. Each formulation containing the equivalent amount of TXG (15.0 mg) was applied to the skin surface, which had an available diffusion area of 1.76 cm^2. Aliquots of 0.5 ml were withdrawn from the receptor at predetermined time intervals and analyzed by HPLC. The cumulative amount of drug permeation per unit area of skin was plotted as a function of time, and the steady-state permeation rate (J_{ss}) was subsequently calculated from the resulting slope.

2.6. TXG assay by HPLC

The amount of TXG in each solution was quantitatively assessed by HPLC, using a mobile phase of acetonitrile–0.3% acetic acid in water (20:80) at a flow rate of 1 ml/min. The HPLC system consisted of a pump (L-2130), UV detector (L-2400), data station (LaChrom Elite, Hitachi, Japan), and 15 cm C_{18} column (Shiseido, Tokyo, Japan). The column eluant was monitored at 291 nm, and the TXG peak was separated with a retention time of 6.0 min.

2.7. In vivo efficacy and immune-related responses

2.7.1. Induction of AD-like lesions in NC/Nga mice and topical application of TXG

AD-like lesions were artificially induced through the repeat application of TNCB, a well-known Th2 cell activator, to NC/Nga mice as previously described in Matsumoto et al. (2004) and Lee et al. (2010). Briefly, the backs of the mice were injured using tape stripping. One week later, 1% TNCB in a mixture of acetone and ethanol (1:4) was applied daily for two weeks to induce AD. The AD-like lesions presented initially with itching, erythema and hemorrhage, while edematous superficial erosions, deep excoriations, scaling and xerosis developed later (Vestergaard et al., 1999). To facilitate the subsequent topical application of the different formulations, all hair on the backs of the mice was removed one day before the experiment. The mice were then treated daily for three weeks with either drug-free gel base alone (base), or drug-containing topical preparations of Soln and Pep1-EL. A negative control group of five mice was neither sensitized nor received topical treatments.

Table 1
Physical characteristics of TXG-loaded EL preparations.

	EL	Pep1+EL	Pep1-EL
Size distribution (nm)	130.6 ± 3.1	132.2 ± 4.2	131.2 ± 1.9
Polydispersity index	0.11 ± 0.04	0.19 ± 0.03	0.12 ± 0.04
Zeta potential (mV)	−12.4 ± 2.4	7.4 ± 4.3	26.7 ± 3.6
Loading amount (mg/lipid)[a]	0.47 ± 0.03	0.48 ± 0.05	0.44 ± 0.03
Loading efficiency (%)	31.4 ± 2.1	29.8 ± 3.1	29.5 ± 1.8
Deformability index	60.7 ± 6.8	62.5 ± 5.5	58.3 ± 4.4

[a] mg/lipid indicates the amount of drug loaded per total lipid used. Values represent mean ± S.D. ($n = 3$).

2.7.2. TEWL and skin surface hydration assessments

All the measurements were performed in triplicate at each location of skin at a temperature of 22 ± 3 °C and a humidity level of 45 ± 5%. TEWL was measured using an Evaporimeter® (Dermalab, Cortex Technology, Hadsund, Denmark), in accordance with the previously described ventilated chamber method (Yoshizawa et al., 2003; Bornkessel et al., 2005). Skin surface hydration values were measured by using a Corneometer® ASA-M2 (Asahi Biomed, Yokohama, Japan).

2.7.3. Measurement of IL-4, IgE and IFN-γ levels in blood

Serum levels of IL-4, IgE and IFN-γ in treated mice were quantified using a previously described method (Kim et al., 2007). All the blood specimens were collected from the retro-orbital plexus while the mice were sedated under ether anesthesia. The serum was then separated via centrifugation of the whole blood at 12,000 rpm and was subsequently stored at −20 °C until use. IL-4, IgE and IFN-γ levels were measured using the mouse IL-4 ELISA kit, mouse IgE ELISA kit, and IFN-γ ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.8. Statistical analysis

All the data points were expressed as the mean ± S.D. Statistical significance was confirmed by Student's t-test. A value of $p < 0.05$ was considered significant, unless indicated otherwise.

3. Results and discussion

3.1. Physical characteristics of EL formulations

The different EL preparations were characterized by vesicular size, polydispersity index, zeta potential, loading efficiency, and deformability index (Table 1). With the exception of zeta potential, other measured parameters were in a similar range between the different liposomal systems. The mean vesicular size was 130 nm, a value which is widely considered to be an ideal size for skin delivery (Verma et al., 2003). Low polydispersity indices were found to be less than 0.3, indicating a narrow and homogeneous size distribution. Surface charges in the EL were initially measured to be −12 mV, however increased to +27 mV after conjugation of the cationic peptide to the vesicular surface. In a previous study, we found that approximately 370 Pep-1 peptides are conjugated to each vesicle (Kang et al., 2010b). Such Pep-1 peptide modifications of the liposomal surface did not noticeably influence vesicular system loading efficiency. The surface charges of the Pep1-admixed vesicles (Pep1+EL) were reduced to 7 mV via the electrostatic adhesion of the cationic peptide to the liposomal surface. The deformability index of EL preparations – a crucial feature for skin penetration enhancement – was observed to be greater than 50, a value three to four times higher than most conventional liposomes (Gupta et al., 2004; Kang et al., 2010a). The

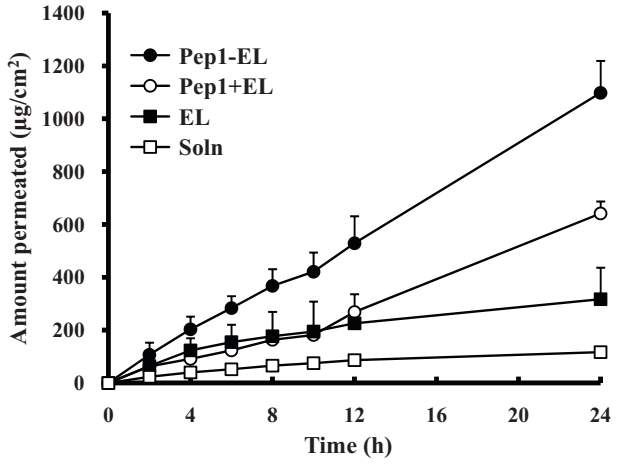

Fig. 1. Permeation profiles of TXG through depilated mouse skin for 24 h in various formulations. Values represent mean ± S.D. ($n = 3$).

incorporation of Tween 80 (an edge activator) fluidized the lipid bilayers, thus increasing vesicular elasticity (El Maghraby et al., 2000). The stress-dependent adaptability of Pep1-EL also imparts the ability to pass through the skin barrier with relative ease, thereby also providing an adjuvant effect for TXG delivery.

3.2. Skin permeation profiles of TXG

The *in vitro* TXG permeation profiles of the topical Soln, EL, Pep1+EL and Pep1-EL formulations are shown in Fig. 1. No distinct lag time was observed in skin permeation study in any of the preparations. The cumulative degree of permeation increased linearly throughout the study, indicating that permeation steady states were reached rapidly, with the slope of the Pep1-EL formulation much steeper than those of the other preparations. When ranked by TXG permeation at 24 h, the order of the formulations was Pep1-EL > Pep1+EL > EL > Soln. The skin permeation parameters of TXG in the various formulations are summarized in Table 2. Compared to Soln preparations, all the EL preparations showed greater values for flux and cumulative permeation amount at 24 h. The addition and/or conjugation of Pep1 peptides to EL further increased the skin delivery of TXG. In particular, Pep1-EL remarkably increased the amount of TXG permeated at 24 h: 1.7-fold versus Pep1+EL, 3.5-fold versus EL, and 9.4-fold versus Soln. For further comparison, the enhancement ratio (ER) was expressed as a relative ratio of the flux values, with the novel Pep1-EL system again showing the greatest incremental increase: about 1.6-fold versus Pep1+EL, 5.1-fold versus EL, and 13.4-fold versus Soln.

This *in vitro* skin permeation study clearly demonstrates that the novel Pep1-EL system combines the advantages of both EL and Pep-1 peptide vehicles, ultimately resulting in superior delivery of TXG into the skin. The high stress-dependent transformability of this system employs the naturally occurring transcutaneous hydration gradient to enable the carrier movement between cells in the stratum corneum, thus allowing the absorption of this formulation into intact skin. Previous studies have demonstrated that EL is able to rapidly enter the deeper layers of the stratum corneum, reaching cells almost as deep as the stratum corneum-viable epidermal junction (Honeywell-Nguyen et al., 2004; Dubey et al., 2006). Moreover, the covalent attachment of Pep-1 peptides to the vesicles further facilitates the movement of nanocarriers across the skin. It has also been reported that several penetrating peptides are able to interact with lipids in the stratum corneum, thus destabilizing the outermost layer of the skin and enhancing permeability (Thoren et al., 2004). A similar study focusing on R9, one type of penetrating peptide, demonstrated that enhanced hydrophilic molecule penetration may occur through the opening of tight junctions (Ohtake et al., 2003). The disassembly of liposomal surface barrier structures by penetrating peptides might also be important for the penetration of TXG-containing vesicles into the viable skin layers. Along these lines, CPPs have also been shown to enter into the epidermis and/or dermis via a macropinocytosis mechanism. Because this process also allows the transfer of any associated cargo molecules, the resulting concentration gradient may also facilitate the delivery of therapeutic compounds to the relevant skin layers.

Table 2
Permeation parameters of TXG in various formulations through depilated mouse skin.

	Soln	EL	Pep1+EL	Pep1-EL
J_{SS} (μg/h/cm²)[a]	3.40 ± 0.14	8.98 ± 2.95	29.3 ± 3.08	45.6 ± 3.50
Permeated (%)[b]	1.37 ± 0.16	3.72 ± 1.79	7.53 ± 0.90	12.9 ± 1.49
ER[c]	1.00	2.64	11.6	13.4

[a] Flux obtained from the slope of the permeation profile in Fig. 1.
[b] Calculated from the cumulative amount permeated at 24 h divided by the amount of drug loaded in the samples.
[c] Enhancement ratio, relative to Soln. Values represent mean ± S.D. ($n = 3$).

Interestingly, Pep1-conjugated EL was able to deliver TXG in large quantities into the skin when compared to Pep1-admixed EL, even if an equimolar amount of Pep-1 peptide was used. These data mirror an earlier report indicating that molecules conjugated with WLR peptide, one type of CPP, were two times more effective in promoting the penetration of proteinous macromolecules into the epidermis than molecules without WLR conjugation (Lopes et al., 2008). Similarly, the topical delivery of cyclosporine A to immune cells (including dermal T cells) was dramatically increased through conjugation with arginine oligomers, another type of CPP, and ultimately resulted in noticeable reductions of inflammation in dermatitis-induced mice (Rothbard et al., 2000). Such findings suggest that, while Pep-1 peptide can be used as a penetration enhancer alone, greater skin penetration efficiency can be achieved through the stoichiometrical conjugation of Pep-1 peptides to therapeutic agents or liposomal carriers.

3.3. In vivo therapeutic efficacy

3.3.1. Recovery of skin barrier function

To evaluate the therapeutic efficiency of the TXG-loaded Pep1-EL system in restoring skin barrier function among AD-induced NC/Nga mice, we measured the TEWL after topical application of various formulations. The content of sphingolipids in the lamellar structure of the stratum corneum is markedly reduced in AD, thus resulting in defective barrier functions and excessive cutaneous water evaporation (Goldstein and Abramovits, 2003). In the control group that was neither sensitized nor received topical treatment, the TEWL was measured at approximately 7−9 g/m²/h throughout the entire experimental period (Fig. 2(a)). In the NC/Nga mice sensitized by TNCB, the TEWL values were initially recorded at about 50 g/m²/h and only decreased minimally after three weeks of treatment with the gel base. In contrast, the mice treated with both the TXG-containing Soln and the Pep1-EL preparations showed remarkable improvements within a week, yielding results that continued through the entire three-week duration. The Pep1-EL-treated group showed the greatest reduction in TEWL values, with significant differences from the Soln-treated group occurring after both the first and second weeks of treatment ($p < 0.05$).

The skin surface hydration values were also significantly reduced in the TNCB-sensitized NC/Nga mice as a result of spontaneous water evaporation from the skin. Whereas the unsensitized controls had a mean skin surface hydrations value of 60 A.U., the sensitized mice showed a value of 20 A.U. (Fig. 2(b)). Notably, a gradual recovery was seen after two weeks of TXG treatment.

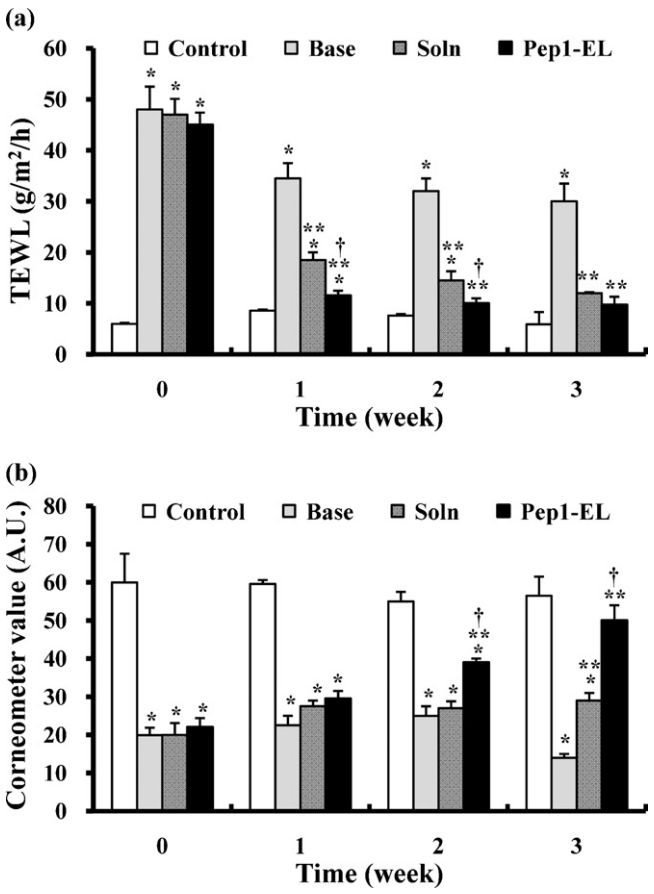

Fig. 2. Changes in TEWL (a) and skin hydration values (b) in NC/Nga mice after topical administration of various formulations: drug-free cream (Base), TXG solution (Soln) and TXG-loaded Pep1-EL (Pep1-EL). Bar represents S.D. ($n = 5$), and statistical analysis was performed using Student's t-test (*$p < 0.05$ versus negative control group; **$p < 0.05$ versus base-treated group; †$p < 0.05$ versus Soln-treated group).

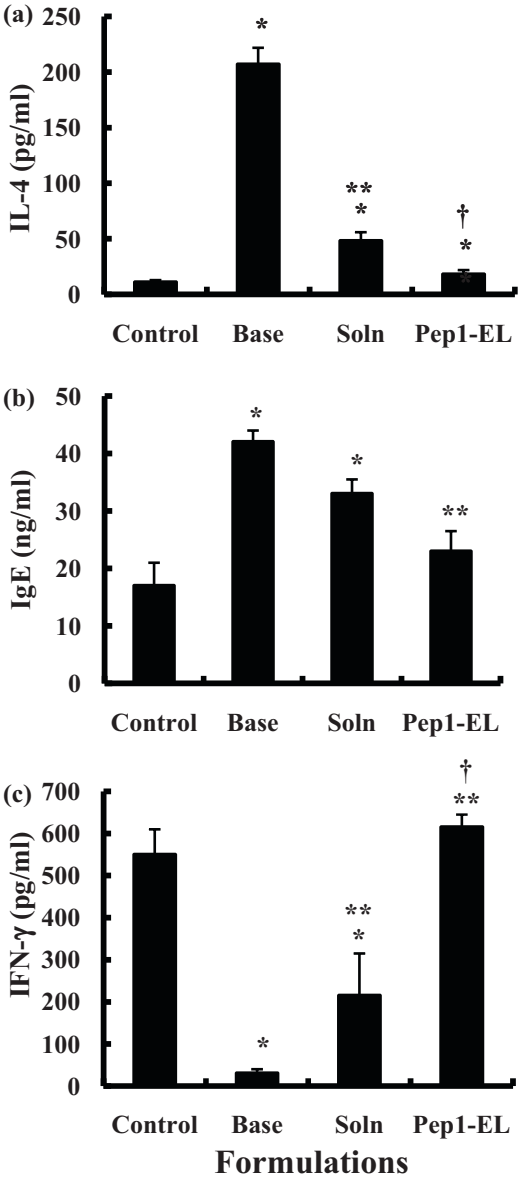

Fig. 3. Alterations of IL-4, IgE and IFN-γ levels in blood after topical application of various formulations: Base, Soln and Pep1-EL. Bar represents S.D. ($n = 5$), and statistical analysis was performed using Student's t-test (*$p < 0.05$ versus negative control group; **$p < 0.05$ versus base-treated group; †$p < 0.05$ versus Soln-treated group).

In particular, Pep1-EL-treated group had roughly equivalent values of skin surface hydration after three weeks treatment as the control group, with significant differences ($p < 0.05$) from the Soln-treated group occurring at two and three weeks. These results indicate that the TXG-loaded Pep1-EL effectively facilitated a rapid and complete recovery in skin barrier function, and correlating well with the data from the *in vitro* skin permeation study. On the other hand, AD-like lesions are typically characterized by clinical symptoms of severe pruritis, dryness and eczema (Gutermuth et al., 2004; Kang et al., 2010a; Lee et al., 2010). The dorsal skin of the base-treated group presented a reddish-colored wound and dryness even after three weeks of treatment. Compared to the base-treated group, the TXG-treated groups manifested considerable reductions in these symptoms, revealing complete recovery within three weeks after treatment (data not shown). In particular, Pep1-EL formulation started to show significant improvement within a week and subsequently brought the best result with complete recovery within two weeks after topical application.

3.3.2. Regulation of IL-4, IgE and IFN-γ levels in blood

To further assess the effect of topically administered TXG-loaded Pep1-EL preparations, we performed immunological analyses of serum IL-4, IgE and IFN-γ levels after three weeks of treatment. Several studies have previously demonstrated that NC/Nga mice with AD-like lesions display a predominance of type-2 immune responses (Byron et al., 1992; Leung, 1995; Matsuda et al., 1997). Upregulation of plasma IgE, an immunologic hallmark of AD, is often evident early and may result from either an increased expression of IL-4 and/or the decreased production of IFN-γ (Jujo et al., 1992). Here, the repeated TNCB stimulation of NC/Nga mice triggered a hyperproduction of IgE, increases in serum IL-4 and the down-regulation of IFN-γ. Interestingly, the topical application of both TXG-containing preparations remarkably altered the serum levels of IgE, IL-4 and IFN-γ, resulting in significant differences with the gel base-treated group ($p < 0.05$). Specifically, the treatment with TXG-containing preparations led to profound reductions in serum IL-4 and IgE levels (Fig. 3(a) and (b)). The serum level of IFN-γ, an important factor in the regulation of Th1 cell development, was also increased 10 or more times by TXG treatment (Fig. 3(c)). In particular, the Pep1-EL-treated group exhibited the best results, showing significant differences from the Soln-treated group in the reduction of IL-4 and IgE and upregulation of IFN-γ expression. These results suggest that TXG-loaded Pep1-EL

formulation effectively regulated Th1/Th2 balance and decreased the production of inflammatory cytokines, ultimately leading to the improvement of AD-like lesions. Our previous study revealed that TXG regulates the production of inflammatory cytokines including IL-4, 5 and 13, and subsequently reduces the inflammation in AD lesional skin (Ahn et al., 2010). It was also found that TXG inhibits production of TNF-α, which activates T cells and produces cutaneous T-cell-attracting chemokines in keratinocytes (Kim et al., 2008). In this way, we believe that the sequestration of the drug in the Pep1-EL system profoundly facilitates the penetration of TXG to the relevant skin layers and amplifies the immune-modulating and anti-inflammatory activities of the compound.

4. Conclusion

This study demonstrates that a novel, advanced EL system, Pep1-EL, combines the substantial advantages of both EL and the Pep-1 peptide, consequently providing remarkable permeation-promoting effects in the topical delivery of a therapeutic agent. In our therapeutic efficacy study in NC/Nga mice with AD-like lesions, a rapid and profound restoration in skin barrier function was observed in the group treated with the TXG-loaded Pep1-EL formulation. Moreover, the formulation normalized multiple immunological parameters including IL-4, IgE, and IFN-γ in NC/Nga mice, with serum levels approaching those of healthy mice. Our results suggest that TXG-loaded Pep1-EL preparations may provide a potential therapeutic tool for the treatment of AD, and should be further investigated.

Acknowledgement

This research was supported by the Seoul Research and Business Development Program (10574).

References

Ahn, J.Y., Choi, S.E., Jeong, M.S., Park, K.H., Moon, N.J., Joo, S.S., Lee, C.S., Choi, Y.W., Li, K., Lee, M.K., Lee, M.W., Seo, S.J., 2010. Effect of taxifolin glycoside on atopic dermatitis-like skin lesions in NC/Nga mice. Phytother. Res. 24, 1071–1077.

Akdis, A.C., Akdis, M., Trautmann, A., Blaser, K., 2002. Immune regulation in atopic dermatitis. Curr. Opin. Immunol. 12, 641–646.

Anigbogu, A.N., Williams, A.C., Barry, B.W., 1996. Permeation characteristics of 8-methoxypsoralen through human skin: relevance to clinical treatment. J. Pharm. Pharmacol. 48, 357–366.

Bornkessel, A., Flach, M., Arens-Corell, M., Elsner, P., Fluhr, J.W., 2005. Functional assessment of a washing emulsion for sensitive skin: mild impairment of stratum corneum hydration, pH, barrier function, lipid content, integrity and cohesion in a controlled washing test. Skin Res. Technol. 11, 53–60.

Byron, K.A., Liberatos, S., Varigos, G.A., Wootton, A.M., 1992. Interferon-gamma production in atopic dermatitis: a role for prostaglandins? Int. Arch. Allergy Immunol. 99, 50–55.

Cevc, G., Blume, G., 2001. New, highly efficient formulation of diclofenac for the topical, transdermal administration in ultradeformable drug carriers, Transfersomes. Biochim. Biophys. Acta 1514, 191–205.

Cevc, G., Gebauer, D., Stieber, J., Schatzlein, A., Blume, G., 1998. Ultraflexible vesicles, transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. Biochim. Biophys. Acta 1368, 201–215.

Dubey, V., Mishra, D., Asthana, A., Jain, N.K., 2006. Transdermal delivery of a pineal hormone: melatonin via elastic liposomes. Biomaterials 27, 3491–3496.

El Maghraby, G.M., Williams, A.C., Barry, B.W., 2000. Oestradiol skin delivery from ultradeformable liposomes: renement of surfactant concentration. Int. J. Pharm. 196, 63–74.

Goldstein, A.M., Abramovits, W., 2003. Ceramides and the stratum corneum: structure, function, and new methods to promote repair. Int. J. Dermatol. 42, 256–259.

Gupta, P.N., Vyas, S.P., Vishwidyalaya, H.S.G., 2004. Transfersomes for vaccine delivery: a potential approach for topical immunization. Med. Chem. Res. 13, 414–426.

Gutermuth, J., Ollert, M., Ring, J., Behrendt, H., Jakob, T., 2004. Mouse models of atopic eczema critically evaluated. Int. Arch. Allergy Immunol. 135, 262–276.

Hirai, M., Minematsu, H., Kondo, N., Oie, K., Igarashi, K., Yamazaki, N., 2007. Accumulation of liposome with Sialyl Lewis X to inflammation and tumor region: application to in vivo bio-imaging. Biochem. Biophys. Res. Commun. 353, 553–558.

Honeywell-Nguyen, P.L., Gooris, G.S., Bouwstra, J.A., 2004. Quantitative assessment of the transport of elastic and rigid vesicle components and a model drug from these vesicle formulations into human skin in vivo. J. Invest. Dermatol. 123, 902–910.

Hou, Y.W., Chan, M.H., Hsu, H.R., Liu, B.R., Chen, C.P., Chen, H.H., Lee, H.J., 2007. Transdermal delivery of proteins mediated by non-covalently associated arginine-rich intracellular delivery peptides. Exp. Dermatol. 16, 999–1006.

Huailiang, W., Chandrasekharan, R., Norman, D.W., Blake, J.R., 2001. Topical transport of hydrophilic compounds using water-in-oil nanoemulsions. Int. J. Pharm. 220, 63–75.

Jujo, K., Renz, H., Abe, J., Gelfand, E.W., Leung, D.Y., 1992. Decreased interferon gamma and increased interleukin-4 production in atopic dermatitis promotes IgE synthesis. J. Allergy Clin. Immunol. 90, 323–331.

Kang, M.J., Eum, J.Y., Jeong, M.S., Choi, S.E., Park, S.H., Cho, H.I., Cho, C.S., Seo, S.J., Lee, M.W., Choi, Y.W., 2010a. Facilitated skin permeation of oregonin by elastic liposomal formulations and suppression of atopic dermatitis in NC/Nga mice. Biol. Pharm. Bull. 33, 100–106.

Kang, M.J., Kim, B.G., Eum, J.Y., Park, S.H., Choi, S.E., An, J.J., Jang, S.H., Eum, W.S., Lee, J., Lee, M.W., Kang, K., Oh, C.H., Choi, S.Y., Choi, Y.W., 2010b. Design of a Pep-1 peptide-modified liposomal nanocarrier system for intracellular drug delivery: conformational characterization and cellular uptake evaluation. J. Drug Target., doi:10.3109/1061186X.2010.511226.

Kim, N.C., Graf, T.N., Sparacino, C.M., Wani, M.C., Wall, M.E., 2003. Complete isolation and characterization of silybins and isosilybins from milk thistle (Silybum marianum). Org. Biomol. Chem. 1, 1684–1689.

Kim, Y.S., Kim, Y., Lee, K.J., Kwon, H.J., Kim, D.S., Kim, T.Y., 2007. Improvement of atopic dermatitis in NC/Nga mice by topical application of CpG hosphodiester-ODN. Int. Arch. Allergy Imm. 144, 315–324.

Kim, Y.J., Choi, S.E., Lee, M.W., Lee, C.S., 2008. Taxifolin glycoside inhibits dendritic cell responses stimulated by lipopolysaccharide and lipoteichoic acid. J. Pharm. Pharmacol. 60, 1465–1472.

Lee, O., Choi, M., Ha, S., Lee, G., Kim, Park, G., Lee, M., Choi, Y., Kim, M., Oh, C.H., 2010. Effect of pedunculagin investigated by non-invasive evaluation on atopic-like dermatitis in NC/Nga mice. Skin Res. Technol. 16, 371–377.

Leung, D.Y., 1995. Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic disease. J. Allergy Clin. Immun. 96, 302–318.

Leung, D.Y., Bieber, T., 2003. Atopic dermatitis. Lancet 361, 151–160.

Lopes, L.B., Brophy, C.M., Furnish, E., Flynn, C.R., Sparks, O., Komalavilas, P., Joshi, L., Panitch, A., Bentley, V.L.B., 2005. Comparative study of the skin penetration of protein transduction domains and a conjugated peptide. Pharm. Res. 22, 750–757.

Lopes, L.B., Furnish, E., Komalavilas, P., Seal, B.L., Panitch, A., Bentley, M.V., Brophy, C.M., 2008. Enhanced skin penetration of P20 phosphopeptide using protein transduction domains. Eur. J. Pharm. Biopharm. 68, 441–445.

Matsuda, H., Watanabe, N., Geba, G.P., Sperl, J., Tsudzuki, M., Hiroi, J., Matsumoto, M., Ushio, H., Saito, S., Askenase, P.W., Ra, C., 1997. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. Int. Immunol. 9, 461–466.

Matsumoto, K., Mizukoshi, K., Oyobikawa, M., Ohshima, H., Tagami, H., 2004. Establishment of an atopic dermatitis-like skin model in a hairless mouse by repeated elicitation of contact hypersensitivity that enables to conduct functional analyses of the stratum corneum with various non-invasive biophysical instruments. Skin Res. Technol. 10, 122–129.

Ohtake, K., Maeno, T., Ueda, H., Natsume, H., Morimoto, Y., 2003. Poly-L-arginine predominantly increases the paracelullar permeability of hydrophilic macromolecules across rabbit nasal epithelium in vitro. Pharm. Res. 20, 153–160.

Paliwal, S., Mitragotri, S., 2006. Ultrasound-induced cavitation: applications in drug and gene delivery. Exp. Opin. Drug Deliv. 3, 713–726.

Prausnitz, M.R., 2004. Microneedles for transdermal drug delivery. Adv. Drug Deliv. Rev. 56, 581–587.

Rachakonda, V.K., Yerramsetty, K.M., Madihally, S.V., Robinson Jr., R.L., Gasem, K.A., 2008. Screening of chemical penetration enhancers for transdermal drug delivery using electrical resistance of skin. Pharm. Res. 25, 2697–2704.

Rothbard, J.B., Garlington, S., Lin, Q., Kirschberg, T., Kreider, E., Mcgrane, P.L., Wender, P.A., Khavari, P.A., 2000. Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. Nat. Med. 6, 1253–1257.

Szoka, F., Papahadjopoulos, D., 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198.

Takahashi, H., Hirata, S., Minami, H., Fukuyama, Y., 2001. Triterpene and flavanone glycoside from Rhododendron simsii. Phytochemistry 56, 875–879.

Thoren, P.E., Persson, D., Esbjorner, E.K., Goksor, M., Lincoln, P., Norden, B., 2004. Membrane binding and translocation of cell penetrating peptides. Biochemistry-US 43, 3471–3489.

Touitou, E., Dayan, N., Bergelson, L., Godin, B., Eliaz, M.J., 2000. Ethosomes novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. J. Control. Rel. 65, 403–418.

Trotta, M., Peira, E., Debernardi, F., Gallarate, M., 2002. Elastic liposomes for skin delivery of dipotassium glycyrrhizinate. Int. J. Pharm. 241, 319–327.

Van den Bergh, B.A., Bouwstra, J.A., Junginger, H.E., Wertz, P.W., 1999. Elasticity of vesicles affects hairless mouse skin structure and permeability. J. Control. Rel. 62, 367–379.

Verma, D.D., Verma, S., Blume, G., Fahr, A., 2003. Particle size of liposomes influences dermal delivery of substances into skin. Int. J. Pharm. 258, 141–151.

Vestergaard, C., Yoneyama, H., Murai, M., Nakamura, K., Tamaki, K., Terashima, Y., Imai, T., Yoshie, O., Irimura, T., Mizutani, H., Matsushima, K., 1999. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. J. Clin. Invest. 104, 1097–1105.

Watanabe, M., Tagami, H., Horii, I., Takahashi, M., Kligman, A.M., 1991. Functional analyses of the superficial stratum corneum in atopic xerosis. Arch. Dermatol. 127, 1689–1692.

Wu, P.C., Tsai, Y.H., Liao, C.C., Chang, J.S., Huang, Y.B., 2004. The characterization and biodistribution of cefoxitin-loaded liposomes. Int. J. Pharm. 271, 31–39.

Yoshizawa, Y., Kitamura, K., Kawana, S., Maibach, H., 2003. Water, salts and skin barrier of normal skin. Skin Res. Technol. 9, 31–33.