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Research paper

Topical delivery of 5-aminolevulinic acid-encapsulated ethosomes in a hyperproliferative skin animal model using the CLSM technique to evaluate the penetration behavior

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ABSTRACT

Psoriasis, an inflammatory skin disease, exhibits recurring itching, soreness, and cracked and bleeding skin. Currently, the topical delivery of 5-aminolevulinic acid-photodynamic therapy (ALA–PDT) is an optional treatment for psoriasis which provides long-term therapeutic effects, is non-toxic and enjoys better compliance with patients. However, the precursor of ALA is hydrophilic, and thus its ability to penetrate the skin is limited. Also, little research has provided a platform to investigate the penetration behavior in disordered skin. We employed a highly potent ethosomal carrier (phosphatidylethanolamine; PE) to investigate the penetration behavior of ALA and the recovery of skin in a hyperproliferative murine model. We found that the application of ethosomes produced a significant increase in cumulative amounts of 5–26-fold in normal and hyperproliferative murine skin samples when compared to an ALA aqueous solution; and the ALA aqueous solution appeared less precise in terms of the penetration mode in hyperproliferative murine skin. After the ethosomes had been applied, the protoporphyrin IX (PpIX) intensity increased about 3.64-fold compared with that of the ALA aqueous solution, and the penetration depth reached 30–80 μm. The results demonstrated that the ethosomal carrier significantly improved the delivery of ALA and the formation of PpIX in both normal and hyperproliferative murine skin samples, and the expression level of tumor necrosis factor (TNF)-α was reduced after the ALA–ethosomes were applied to treat hyperproliferative murine skin. Furthermore, the results of present study encourage more investigations on the mechanism of the interaction with ethosomes and hyperproliferative murine skin.

1. Introduction

Psoriasis is a chronic skin disorder which can be completely reversed with appropriate therapy [1]. The optimal therapy increases patient compliance and decreases the short- and long-term adverse effects from therapy [2]. Psoriasis, from moderate to severe, is classified into five types, and treatment strategies depend on the condition of the patient. Traditional treatments can cause remission of symptoms; however, it should be used with caution, and the effective outcome is limited [3,4]. Moreover, biologic-immune-response modifiers are under development [5].

Advanced research has pointed out that topical photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA) (a second-generation photosensitizer) is a treatment option for psoriasis covering a large area and is a now safe and effective treatment which may more readily be organized and the long-term implications assessed [6]. The main mechanism of PDT on psoriasis, an inflammatory skin disease, is modulation of cellular functions which lead to induction of transcription factors (especially AP-1 and NF-κB) but not to cellular death [7]. PDT is a composite technique which requires three basic elements: a photosensitizer, light irradiation, and singlet oxygen. Indeed, ALA is a form of a drug precursor, in that the photosensitizer, protoporphyrin IX (PpIX), is formed in vivo after the exogenous application of ALA. The major limitation of PDT is the poor penetration of ALA into the skin or skin lesions. Recently, numerous strategies were proposed to improve these penetration problems. The notable numbers of ALA preparations include clinical usage of Levlan Kerastick® and a clinical trial of PD P 506 A, BF-200 ALA [7]. However, the high concentration of 20% ALA for clinical usage increases skin irritation and costs.

A practical and important step is to use an animal study which mimics a disease model for the initial development of pharmaceutics, and a suitable animal skin disease model can be used as a tool

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for screening a series of drug [8]. Therefore, we need an animal model of psoriasis-like to represent the syndrome and make the formulation screening simply [3].

Accordingly, the problem of the poor penetration of ALA remains unresolved; our previous study reported that ethosomal carriers had great potential for delivering PpIX into the skin [9]. Therefore, our purpose is to establish an animal model of hyperproliferative disease to mimic the clinical situation and to provide an evaluation of the skin penetration/absorption behavior.

2. Materials and methods

2.1. Materials

ALA and phosphatidylethanolamine (PE, commercial grade) were purchased from Sigma Chemical (St. Louis, MO, USA). Other chemicals used in the study were of reagent grade. The fibroblast cell line (Hs68) was purchased from the Culture Collection and Research Center (CCRC) of the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan). Cell culture media and supplements were obtained from GIBCO Invitrogen (GRAND Island, NY, USA).

2.2. Induction of hyperproliferative murine skin

Female nude mice (ICR, Foxnl) at 7–8 weeks old were used in the study. In order to mimic hyperproliferative murine skin, a tape-stripping technique was used [10]. The stripping procedure was repeated at the same site of dorsal skin with cellophane tape (3 M Scotch®, MN, USA) twice daily for 5 days. The treated skin was excised when the transepidermal water loss (TEWL) (TM300, Courage & Khazaka, Köln, Germany) level reached 8–10 $g/m^2/h$. This animal experiment was reviewed and approved by the Institutional Animal Care Committee at Kaohsiung Medical University.

2.3. Evaluation of hyperplasic skin

Skin specimens were fixed in 4% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Each sample was measured at three random sites for three samples by light microscopy (Axiovert200, Zeiss, Oberkochen, Germany). The epidermal thickness was randomly measured from the basal layer to the granulosum/SC junction by Image Pro-plus 5.0 (Media Cybernetics, Silver Spring, MD, USA).

2.4. Preparation of ethosomes

Ethosomes were prepared according to the thin-film hydration method [9]. PE (0.13 mM) was dissolved in chloroform and methanol (2:1, v/v) in a 250-ml round-bottom flask. The mixture was evaporated in a rotary evaporator above the transition temperature of the phospholipid, PE, at 60 °C, and solvent traces were removed under a vacuum overnight. The film was hydrated with 5 ml of 0.1% ALA dissolved in a 15% (v/v) ethanol solution above the lipid transition temperature for 30 min. The vesicle suspension was dispersed by a probe sonicator (UP50H, Dr. Hielscher GmbH, Teltow, Germany) at 25 W for 1 min. The entrapment efficiency was about 10%.

2.5. In vitro topical delivery of the ethosome carriers

We measured the topical delivery of ALA by using a modified Franz vertical diffusion assembly [11]. Normal nude mouse skin and hyperproliferative nude mouse skin were used as the barrier membrane. The donor medium consisted of 0.5 ml of an ethosomal formulation or control solution (0.1% ALA in distilled water), and the receptor medium consisted of 4.2 ml of pH 5 citrate–phosphate buffer. The available diffusion area was 0.785 cm^2. The stirring rate of the receptor was 600 rpm, and the temperature was maintained at 37 °C. At appropriate intervals, 100-μl aliquots of the receptor medium were withdrawn and immediately replaced by an equal volume of fresh buffer. The concentration of ALA was measured by high-performance liquid chromatography (HPLC). The cumulative amount of ALA was examined at the end of the in vitro experiments (12 h) ($n = 3$).

2.6. HPLC analysis of ALA

The fluorescence derivation of ALA samples was based on a modification of the Hantzsch reaction, in which amine compounds react with acetylacetone and formaldehyde [12]. The acetylacetone reagent was prepared by the addition of 15 ml acetylacetone, 10 ml ethanol, and 75 ml deionized water. The acetylacetone reagent (3.5 ml), 0.45 ml of a 10% formaldehyde solution, and 50 μl of the ALA sample were vortex-mixed for about 3 s. The mixture was heated in an aluminum-block heater to 100 °C for 15 min and then cooled in an ice bath.

The ALA contents of the various samples were analyzed with an HPLC system consisting of a Waters 515 HPLC pump, a Waters 715 sample processor, and a Waters 474 fluorescence detector (Waters, Milford, MA, USA). A reverse-phase column [Lichrospher RP-18, 250 × 4 mm, 5 μm, (Merck, Darmstadt, Germany)] was used, with the column oven set to 40 °C. The mobile phase consisting of methanol–water–acetic acid (50:50:1) was used at a flow rate of 0.7 ml/min. The excitation and emission wavelengths of the fluorescence detector were set to 378 and 467 nm, respectively. All samples were allowed to stand in the dark.

2.7. In vivo topical delivery of the ethosome carriers

Both normal and hyperproliferative murine skin samples were employed in the in vivo examination to clarify differences in the penetration behavior between normal and hyperproliferative murine skin. A glass cylinder with an available area of 0.785 cm^2 was placed on the dorsal skin of a mouse with glue. The ethosome formulation (100 μl) was added to each cylinder for 4 h. At the end of the incubation period, the formulation was removed, and the skin was wiped 10 times with a cotton cloth. The amount of ALA-induced PpIX retained in the skin was measured by confocal laser-scanning microscopy (CLSM). All procedures were carried out in the dark to prevent the influence of ambient light.

2.8. CLSM study

For analysis of PpIX expression, CLSM was used to scan the fluorescence signal of PpIX at different skin depths. The excised nude mouse skin was positioned on the microscopic slide with the SC side face to the coverglass. The CLSM (FV 500, Olympus, Tokyo, Japan) was carried out with an argon laser beam with excitation at 488 nm and emission at 590 nm. Each skin sample was sliced in sections of 6–10 μm thicknesses through the *Z*-axis by CLSM. The PpIX intensity and the permeated depth were detected by CLSM with Fluoview software. Since CLSM is not able to be calibrated, we used arbitrary unit to compare the data.

2.9. Cell viability test

Cell viability was determined by a 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum bromide (MTT) assay. Fibroblasts (Hs68) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics at 37 °C in a

humidified atmosphere containing 5% CO_2. Hs68 fibroblasts at 5×10^3 cells/well were seeded in 96-well plates. ALA or ALA-encapsulated in the ethosomal carrier (20 μl) was added and incubated for 24 h. We then added 50 μl of the MTT solution (50 μg/ml) to each well and incubated the wells for 4 h. In order to dissolve the water-insoluble formazan, MTT/medium was removed from each well, and 100 μl of dimethyl sulfoxide (DMSO) was added to each one. The quantity of cell survival was measured by an enzyme-linked immunosorbent assay (ELISA) reader (FLx800, BIOTEK, Burlingame, CA, USA) at 540 nm.

2.10. Determination of tumor necrosis factor (TNF)-α

Excised skin was extracted with 100 μl of lysis buffer (Biovision, Mountain View, CA, USA) and sonicated for 1 min in an ice bath. After centrifugation at 16,000*g* for 20 min, the supernatant was removed, and the protein content was quantified. The protein content was determined by the BSA protein assay reagent (Bio-Rad, Hercules, CA, USA). The TNF-α analysis was performed with an ELISA kit (BMS607/2INST, Bender MedSystems, Vienna, Austria) and detected at 450 nm.

2.11. Statistical analysis

Data are expressed as the mean ± SD. Statistical analysis was performed using unpaired Student's *t*-test with WINKS software. A 0.05 level of probability was used as the level of significance. An ANOVA test was also used if necessary.

3. Results

3.1. Histology of hyperproliferative murine skin

The histological images of normal and hyperproliferative murine skin are shown in Fig. 1. The histological features of normal nude mouse skin were a well-defined SC, epidermis, and appendages (Fig. 1A). The histological features of hyperproliferative murine skin were stacking in the SC and elongation in the epidermis (Fig. 1B). The epidermal thickness was randomly measured from the basal layer to the granulosum/SC junction. The thickness of normal murine skin was 30.54 ± 2.68 μm, while that of hyperproliferative murine skin was 61.06 ± 2.97 μm.

3.2. In vitro evaluation of the ability of the ethosomal carrier to deliver ALA

We studied both normal and hyperproliferative murine skins to evaluate the ability of the ethosomal carrier to deliver ALA and the penetration behavior. The penetration behaviors are shown in Fig. 2 and Table 1. The cumulative amounts (CAs) of the control group (consisting of the ALA aqueous solution) between normal and hyperproliferative murine skins did not significantly differ. Moreover, the original data showed a less-precise trend of the order of the penetration mode of the control group in hyperproliferative murine skin, and the zero- and first-order relation coefficients were 0.9099 and 0.9040, respectively. Generally, the CAs of the ethosomal carrier showed significant improvement in normal and hyperproliferative murine skin, and the ranges of increases were 5- to 26-fold. In addition, the penetration mode in hyperproliferative murine skin was first-order.

3.3. In vivo CLSM-observed PpIX intensity vs. penetrated depth

To clarify the penetration behavior of ALA-induced PpIX synthesis between the normal skin and the hyperproliferative murine skin models, we employed the CLSM technique to observe the PpIX intensity and penetration depth as shown in Fig. 3. The total intensity of PpIX in hyperproliferative murine skin with the ALA aqueous solution and ALA-encapsulated in the ethosome carrier (PE) were 255.25 ± 42.09 and 930.07 ± 96.32, respectively. In addition, the maximum penetrated depth of PpIX in hyperproliferative murine skin was 30 μm and extended to approximately 80 μm.

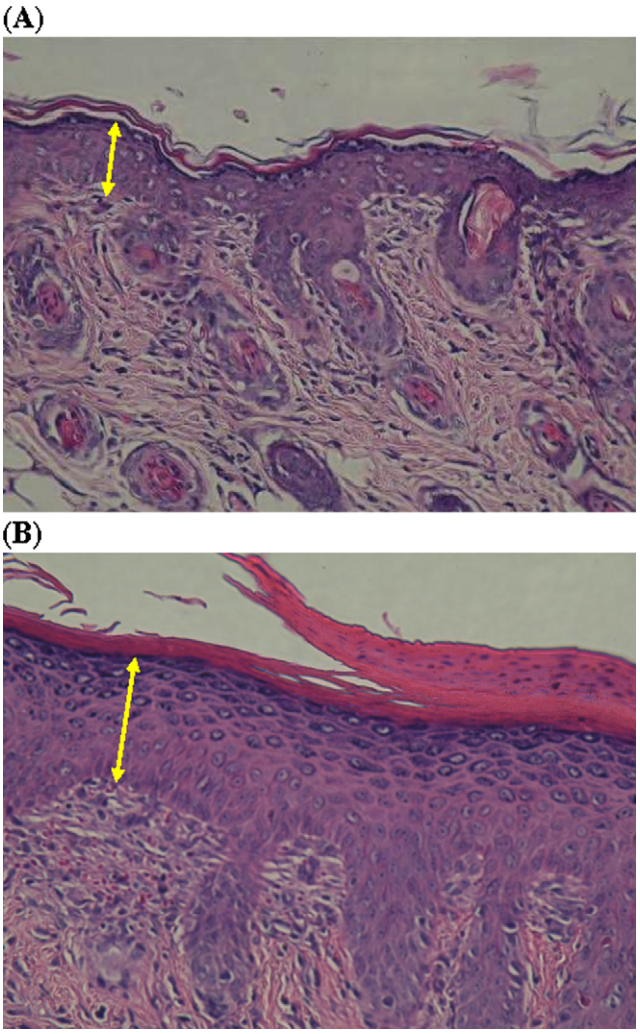


Fig. 1. Histologic examination of nude mouse dorsal skin in (A) non-treated control skin and (B) skin subjected to a tape-stripping technique. Arrows indicate the thickness of the epidermis (magnification 400×). (For interpretation of the references to colours in this figure, the reader is referred to the web version of this paper.)

3.4. In vitro evaluation of cell viability

Safety of the ethosomal carrier was determined by examining skin fibroblasts (Hs68) with the MTT assay as shown in Fig. 4. The effect of the cell viability was tested after a 24-h incubation period. The addition of the ALA aqueous solution resulted in a slight decrease in the cell viability compared with the blank ($p > 0.05$). Moreover, there was no significant difference between the blank and the treatment with ALA-encapsulated in the ethosome carrier (PE).

3.5. Inhibition of TNF-α with application of the ethosomal carrier

To determine the levels of epidermal disruption after applying the ethosome carrier, we used TNF-α as an indicator. As shown in Fig. 5, application of ALA dissolved in 15% EtOH resulted in a

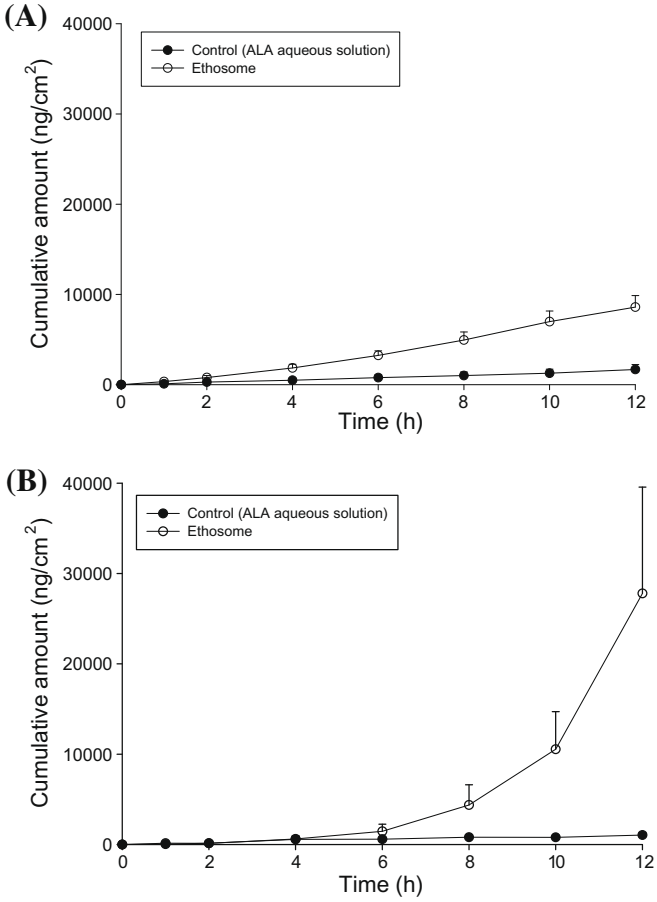


Fig. 2. In vitro cumulative amount-time profiles of the topical penetration of 5-aminolevulinic acid (ALA) in an aqueous solution and in ethosomes for 12 h in (A) normal murine skin and (B) hyperproliferative murine skin. Data represent the mean ± SD (n = 3).

Table 1
In vitro cumulative amount and enhancement ratio of 5-aminolevulinic acid across normal skin and hyperproliferative murine skin with control and phosphatidylethanolamine-treated ethosomes for 12 h. Data represent the mean ± SD (n = 3).

Formulation	CA in normal murine skin $(ng/cm^2)_{0-12\ h}$	CA in hyperproliferative skin $(ng/cm^2)_{0-12\ h}$
Control (ALA aqueous solution)	1670.43 ± 548.89	1047.28 ± 300.99
Ethosome (PE)	8590.36 ± 1281.46	27783.00 ± 11,772
ER[a]	5.14	26.53

[a] ER (the enhancement ratio) = (cumulative amount of the PE ethosome group/cumulative amount of the control group).

slight decrease in skin TNF-α levels, but no significant difference ($p > 0.05$) was detected. In contrast, ethosomes caused about a 20% decrease in TNF-α levels compared to that of the untreated group.

4. Discussion

Recently, ALA–PDT was considered to have greater safety for long-term treatment among the various therapeutic strategies for psoriasis skin disease. However, the major limitation of the therapy is the poor penetration behavior across skin of ALA. Most researchers have utilized healthy skin to investigate the penetration behavior which cannot precisely predict a drug's behavior in disordered skin. Moreover, in our previous experiment, all ethosomal formulations containing ALA were characterized, and topical ALA-encapsulated in PE ethosomes applied to normal mouse skin produced high PpIX intensities in the epidermis [9]. We tried to employ the hyperproliferative murine skin model to investigate the penetration behavior of the ethosomal carrier (PE). In both the normal and the hyperproliferative murine skin samples, results showed that the ethosomal carrier significantly increased the in vitro delivery of ALA and the in vivo intensity of PpIX.

Indeed, there are many factors may cause the differences between human and rodents skin, and even with different application sites of the same species. Therefore, clarifying differences in skin has remained an important issue. The major differences involve the thickness of the skin/SC, the composition and organization of intercellular SC lipids (including the amounts of free fatty acids, cholesterol, long-chain ceramides, and triglycerides), and the distributing density of hair follicles [8]. Previous studies showed that mouse skin generally has higher penetration rates than does human skin, because the SC (17 vs. 9 μm) and epidermis (47 vs. 29 μm) of human skin are thicker than those of mouse skin [1,8].

On the other hand, psoriatic lesions obviously differ from normal skin in abnormal SC stacking, which leads to the formation of scales, reduces the granular layer, elongates the epidermal rete, and enlarges the blood vessels. None of these features, except epidermal rete, if at all, exist in the mouse model. In this study, the stripping technique followed that of previous studies, which is an easy method to handle. [10,13,14]. Moreover, the results were similar to actual conditions of patients with mild to severe psoriasis. The epidermal thickness is the distance from the basal layer to the stratum granulosum/SC junction. Overall, the distance increased when using the stripping technique compared to the distance in the control. In addition, observable changes in the SC existed with abnormal stacking. The animal model could represent the hyperproliferative human skin and make the formulation screening simply.

Nano-sized particles as vehicles offer a potential way for topical application to deliver drugs to local lesions. Indeed, nanoporous pathways with a width of tens of nanometers exist in the SC. Hence, a vehicle with a suitable size ranging 30–40 nm should be able to travel across the SC [15]. Our previous work demonstrated that the properties of PE ethosome represented the particle size, and zeta potential was 163.5 ± 0.9 and −53.5 ± 0.9, respectively. However, traditional liposomes do not efficiently enter the deep skin. They are mostly confined to the upper layer of the epidermis, and partially associate with the particle size of vesicle and intercellular of skin. The composition of lipids, the optimized ratio of the additive mixture, the charge of the vesicles, the entrapment efficiency of the drug, and the type of skin barrier should be considered [16]. In contrast, deformable ethosome carriers can disrupt or weaken the skin structure, while enhancing drug delivery through the skin barrier. Vesicles are composed of phospholipids, ethanol, and water, and these components synergistically influence penetration through the skin. Ethanol, an aliphatic chain, acts as an enhancer which disrupts the skin barrier by lipid extraction [17]. On the other hand, ethanol increases the fluidity and flexibility of ethosomes, which are caused by modulating the mobility of polar lipid heads of lipid molecules.

In the in vitro penetration study, we found that the ALA aqueous solution did not permeate across either normal or hyperproliferative murine skin. This indicates that hydrophilic molecules do not penetrate into hydrophobic skin. Moreover, due to the inhomogeneity of hyperproliferative murine skin, the penetration mode represented a lack of precise order especially for the ALA aqueous solution. Indeed, discrepancies in penetration behaviors in psoriatic or hyperproliferative lesions have been described by several studies. Most clinical investigators employed a keratolytic agent as a pretreatment or surgery to remove the scaling on the skin surface [18,19].

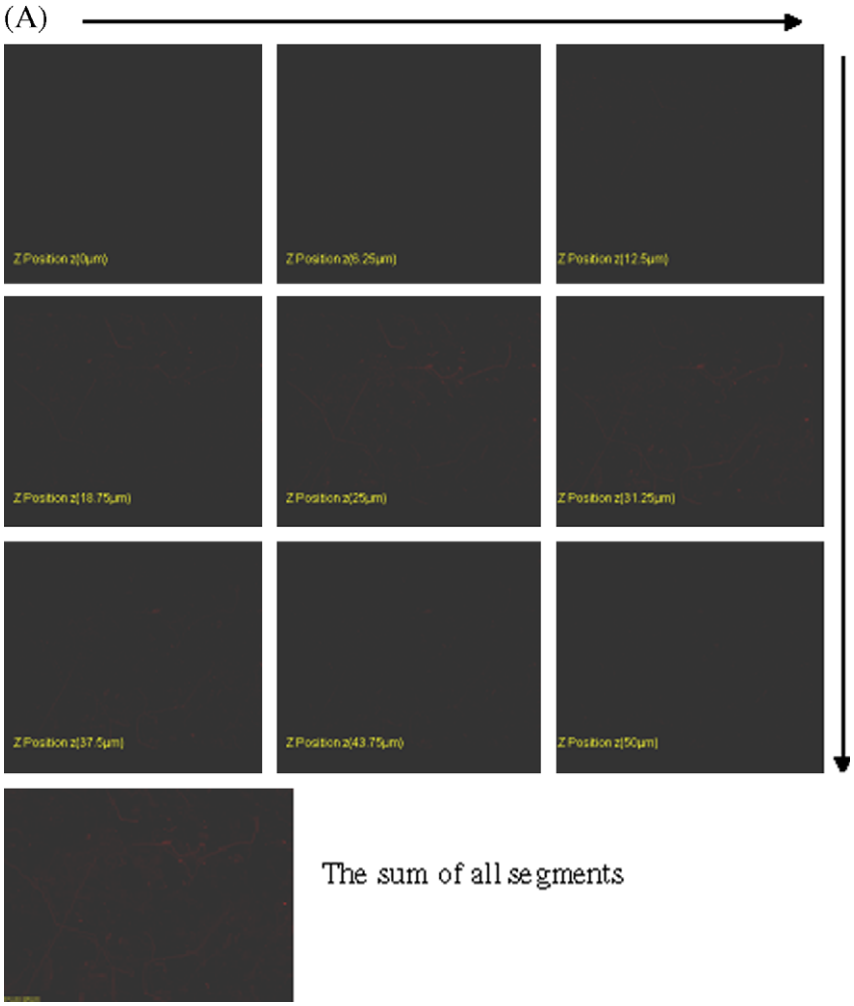
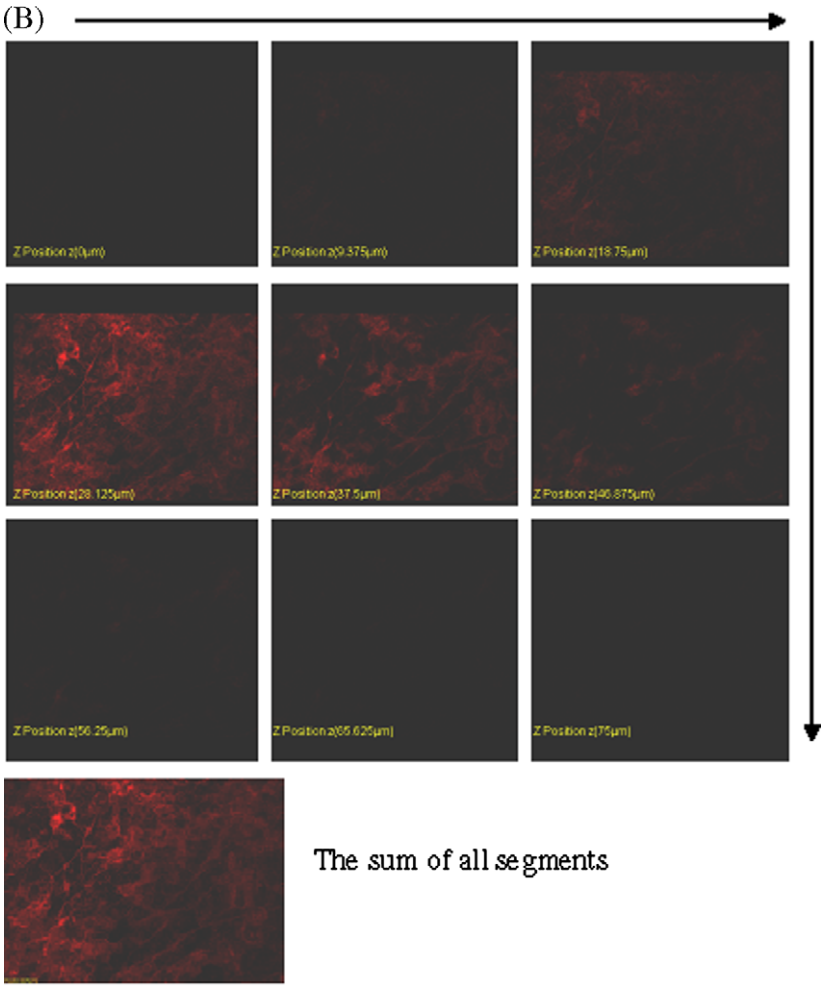


Fig. 3. Confocal laser-scanning microscopic (CLSM) micrographs of the PpIX intensity in hyperproliferative murine skin after in vivo topical administration for 4 h. (A) Control (a 5-aminolevulinic acid aqueous solution), (B) ethosomes, and (C) profile of skin depth versus intensity of PpIX. The full thickness was divided into nine segments from the surface of the skin (left to right, top to bottom). Images below the photographs of the nine segments are the sum of all segments. Data represent the mean ± SD (n = 3). Arbitrary units (A.U.). (For interpretation of the references to colours in this figure, the reader is referred to the web version of this paper.)

In present study, we confirmed the safety of ethosomes by examining the cell viability of a skin fibroblast cell line. The results demonstrated that ALA dissolved in 15% EtOH caused a slight decrease in the viability of fibroblast cells. On the other hand, ethosomes themselves caused no significant difference when compared with the blank. The phenomenon provides some hints which indicate that the composition of PE may play a particular role in protecting skin. As is known, when skin is exposed to ethanol, a chemical reagent, mechanical insults, ultraviolet light, chemicals, and pathogenic microorganisms, the skin barrier or function may be influenced [20]. Moreover, there are various types of skin damage which result in changes in the structure of the skin. One type the damage occurs in intercellular lipid areas, and it may create gaps which increase the amount of drug. Barry and his colleagues reported that disruption of the skin can be repaired by phospholipids [21].

It is important to provide a platform to mimic the clinical situation in an in vivo animal model of hyperproliferative skin disease for pharmaceutical research. As it is known that using vesicles with a proper composition should increase the transport of drug across normal skin. In this study, we employed ethosomal carriers to evaluate the penetration potential into hyperproliferative murine skin in an in vivo situation. Results of CLSM examination confirmed that ALA entrapped in the ethosomal carrier successfully delivered PpIX into hyperproliferative murine skin. Previous studies demonstrated that the thickness of the mouse SC was 9 μm, and the epidermis was 29 μm. Compared to our result, the maximal absorption of PpIX had a broad distribution of 20–50 μm, which indicates that PpIX remained in the lower epidermis and passed through the upper dermis. Numerous skin diseases are located in the lower epidermis such as basal cell carcinoma and squamous cell carcinoma [22], and ethosomal carriers can have an influence at this depth of skin. Actually, the stripping technique caused the epidermis to become about 2-fold thicker than normal; therefore, we assumed that PpIX remained in the epidermis.

In addition, a discrepant phenomenon was found between the in vitro and the in vivo studies. Due to the precursor characteristics of ALA, PpIX formed in the in vivo situation. We used the cumulative amount of ALA as a penetration indicator in the in vitro study, and the results indicated that ALA existed in hyperproliferative murine skin rather than in normal skin after the application of ethosomes. However, we also used PpIX as a fluorescence marker in the in vivo situation, and the results indicated that PpIX levels existed in normal murine skin rather than in hyperproliferative murine skin with the application of ethosomes (compared to our previous data) [9]. In general, there are several major steps which influence the formulation and depth in skin. First, ALA-encapsulated vesicles (ethosomes) were transported to the SC of the skin.



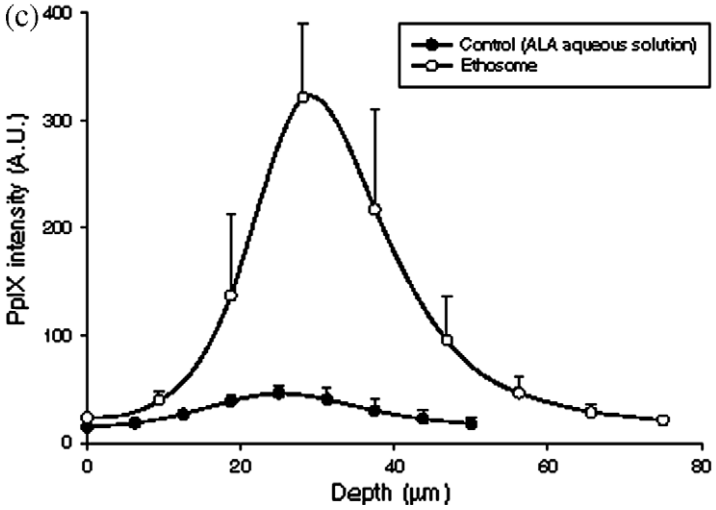


Fig. 3 (*continued*)

Second, ALA was released from the vesicles and located in the skin. Third, most PpIX formed from ALA. In addition, the incubation time of ALA, the combination with additives, and the kind of skin surface also influence the penetration depth and intensity of ALA and ALA-induced PpIX [23].

Our data inferred that there were different mechanisms of PpIX formation between normal and hyperproliferative murine skin. As mentioned earlier, the pathology of hyperproliferative murine skin presented stacking in the SC and thickening of the epidermis which apparently changed the barrier function. Certainly, increased uptake of ALA by pathologic tissue may also have been responsible for the increased formation of PpIX. However, our data demonstrated that ALA could efficiently be transported into hyperproliferative murine skin, but the limitation was the transformation of

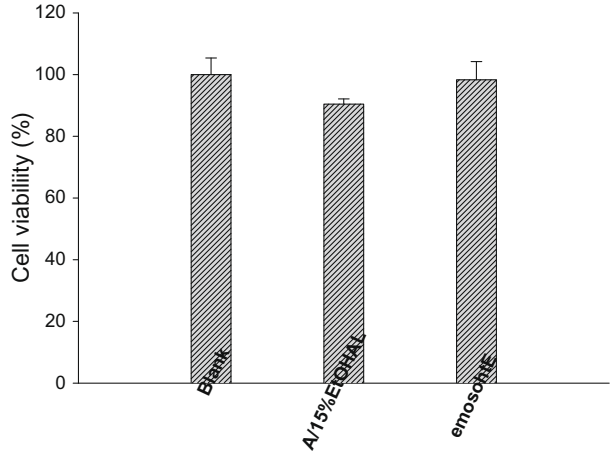


Fig. 4. Cell viability of fibroblasts following treatment with a hydroalcoholic solution or ethosomes with 5-aminolevulinic acid. Data represent the mean ± SD (n = 6).

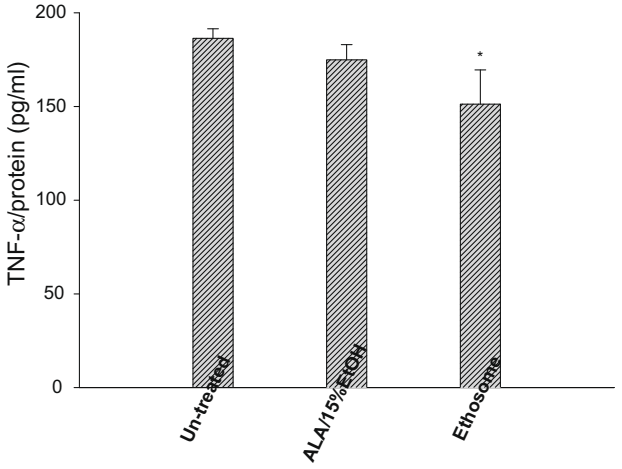


Fig. 5. Determination of the contents of skin tumor necrosis factor (TNF)-α per unit of protein after application of an 5-aminolevulinic acid aqueous solution or ethosomes for 4 h. Data represent the mean ± SD (n = 3). $^{*}p < 0.05$, compared with the untreated group.

PpIX. The conversion of PpIX from ALA remains a key factor. ALA selectively accumulates in abnormal SC or psoriatic plaque; however, porphyrin and heme synthesis rates may differ [24,25]. Currently, the rate-limiting enzyme in the heme synthesis pathway is ALA synthase, which is inhibited by the buildup of heme in the system. After moving through the body, ALA is converted in cells to PpIX via a number of sequential chemical reactions. However, the real rate-limiting enzyme in the ALA–PpIX pathway is still currently an ongoing study. Such enzymes in the ALA–PpIX pathway may play a particular role, but the importance enzymes in various cell types or diseases may differ [26,27]. According to those were mentioned earlier, we assumed that the limitation was PpIX formation from ALA associated with an enzyme in hyperproliferative murine skin.

Tape stripping was used to disrupt the permeability barrier, and a cascade of changes occurred in epidermal metabolism including increased lipid synthesis and DNA synthesis, and an enhanced production of cytokines. In particular, increased synthesis of TNF-α and other cytokines plays a role in psoriatic skin [28]. Generally, cytokines initiate epidermal hyperproliferation and cutaneous inflammation. We determined that TNF-α is an indicator of perturbation of the barrier function. We found that after application of ethosomes, a prominent decrease in TNF-α of about 18.8% was noted, which confirmed that the ALA–ethosome carrier recovered the skin.

5. Conclusions

To sum up, we confirmed that application of ethosomal carriers with ALA in hyperproliferative murine skin can improve the penetration of ALA and the formation of PpIX and significantly reduce TNF-α in this disordered skin compared to that after application of an ALA aqueous solution. The present work contributes to our understanding of the behavior and outcome of penetration of hyperproliferative murine skin. Furthermore, the mechanism of the interaction of ethosomes with hyperproliferative murine skin needs to be elucidated.

References

[1] M.A. Lowes, A.M. Bowcock, J.G. Krueger, Pathogenesis and therapy of psoriasis, Nature 445 (2007) 866–873.
[2] A. Khachemoune, S. Guillen, Psoriasis: disease management with a brief review of new biologics, Dermatol. Nurs. 18 (2006) 40–43.
[3] P.M. Witman, Topical therapies for localized psoriasis, Mayo Clin. Proc. 76 (2001) 943–949.
[4] M.P. Schön, W.H. Boehncke, Psoriasis, N. Engl. J. Med. 18 (2005) 1899–1912.
[5] A.B. Gottlieb, Psoriasis: emerging therapeutic strategies, Nat. Rev. Drug Discov. 4 (2005) 19–34.
[6] P. Fergin, Photodynamic therapy of dermatoses other than non-melanoma skin cancer, Australas. J. Dermatol. 46 (Suppl. 3) (2005) S27.
[7] A. Klein, P. Babilas, S. Karrer, M. Landthaler, R.M. Szeimies, Photodynamic therapy in dermatology – an update 2008, J. Dtsch. Dermatol. Ges. 6 (2008) 839–845.
[8] B. Godin, E. Touitou, Transdermal skin delivery: predictions for humans from in vivo, ex vivo and animal models, Adv. Drug Deliv. Rev. 59 (2007) 1152–1161.
[9] Y.P. Fang, Y.H. Tsai, P.C. Wu, Y.B. Huang, Comparison of 5-aminolevulinic acid-encapsulated liposome versus ethosome for skin delivery for photodynamic therapy, Int. J. Pharm. 356 (2008) 144–152.
[10] M. Demerjian, M.Q. Man, E.H. Choi, B.E. Brown, D. Crumrine, S. Chang, T. Mauro, P.M. Elias, K.R. Feingold, Topical treatment with thiazolidinediones, activators of peroxisome proliferator-activated receptor-gamma, normalizes epidermal homeostasis in a murine hyperproliferative disease model, Exp. Dermatol. 15 (2006) 154–160.
[11] Y.W. Chien, Transdermal drug delivery and delivery systems, in: Drugs and the Pharmaceutical Science, Novel Drug Delivery System, second ed., vol. 50, Marcel Dekker Inc., New York, United States of America, 1992, p. 338 (Chapter 7).
[12] H. Oishi, H. Nomiyama, K. Nomiyama, K. Tomokuni, Fluorometric HPLC determination of delta-aminolevulinic acid (ALA) in the plasma and urine of lead workers: biological indicators of lead exposure, J. Anal. Toxicol. 20 (1996) 106–110.
[13] F.O. Nestle, B.J. Nickoloff, Animal models of psoriasis: a brief update, J. Eur. Acad. Dermatol. Venereol. 20 (2006) 24–27.
[14] M. Denda, L.C. Wood, S. Emami, C. Calhoun, B.E. Brown, P.M. Elias, K.R. Feingold, The epidermal hyperplasia associated with repeated barrier disruption by acetone treatment or tape stripping cannot be attributed to increased water loss, Arch. Dermatol. Res. 288 (1996) 230–238.
[15] G. Cevc, Lipid vesicles and other colloids as drug carriers on the skin, Adv. Drug. Deliv. Rev. 56 (2004) 675–711.
[16] M.M. Elsayed, O.Y. Abdallah, V.F. Naggar, N.M. Khalafallah, Deformable liposomes and ethosomes: mechanism of enhanced skin delivery, Int. J. Pharm. 322 (2006) 60–66.
[17] D. Bommannan, R.O. Potts, R.H. Guy, Examination of the effect of ethanol on human stratum corneum in vivo using infrared spectroscopy, J. Control. Rel. 16 (1991) 299–304.
[18] T. Smits, C.A. Robles, P.E. van Erp, P.C. van de Kerkhof, M.J. Gerritsen, Correlation between macroscopic fluorescence and protoporphyrin IX content in psoriasis and actinic keratosis following application of aminolevulinic acid, J. Invest. Dermatol. 125 (2005) 833–839.
[19] E. Angell-Petersen, R. Sørensen, T. Warloe, A.M. Soler, J. Moan, Q. Peng, K.E. Giercksky, Porphyrin formation in actinic keratosis and basal cell carcinoma after topical application of methyl 5-aminolevulinate, J. Invest. Dermatol. 126 (2006) 265–271.
[20] K.R. Feingold, Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis, J. Lipid Res. 48 (2007) 2531–2546.
[21] E.A. Essa, M.C. Bonner, B.W. Barry, Electroporation and ultradeformable liposomes; human skin barrier repair by phospholipid, J. Control. Rel. 92 (2003) 163–172.

[22] S.T. McGillis, H. Fein, Topical treatment strategies for non-melanoma skin cancer and precursor lesions, Semin. Cutan. Med. Surg. 23 (2004) 174–183.
[23] C. Fritsch, P. Lehmann, W. Stahl, K.W. Schulte, E. Blohm, K. Lang, H. Sies, T. Ruzicka, Optimum porphyrin accumulation in epithelial skin tumours and psoriatic lesions after topical application of delta-aminolaevulinic acid, Br. J. Cancer 79 (1999) 1603–1608.
[24] N.C. Zeitouni, A.R. Oseroff, S. Shieh, Photodynamic therapy for nonmelanoma skin cancers. Current review and update, Mol. Immunol. 39 (2003) 1133–1136.
[25] S.H. Ibbotson, Topical 5-aminolaevulinic acid photodynamic therapy for the treatment of skin conditions other than non-melanoma skin cancer, Br. J. Dermatol. 146 (2002) 178–188.
[26] M.C. Aalders, N. van der Vange, W.M. Star, H.J. Sterenborg, A mathematical evaluation of dose-dependent PpIX fluorescence kinetics in vivo, Photochem. Photobiol. 74 (2001) 311–317.
[27] S.L. Gibbs, B. Chen, J.A. O'Hara, P.J. Hoopes, T. Hasan, B.W. Pogue, Protoporphyrin IX level correlates with number of mitochondria, but increase in production correlates with tumor cell size, Photochem. Photobiol. 82 (2006) 1334–1341.
[28] L.C. Wood, S.M. Jackson, P.M. Elias, C. Grunfeld, K.R. Feingold, Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice, J. Clin. Invest. 90 (1992) 482–487.