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Topical application of liposomal cobalamin hydrogel for atopic dermatitis therapy

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Topical vitamin B_{12} was shown to be effective for atopic dermatitis. However, vitamin B_{12} itself is light sensitive and has low skin permeability, thus reducing its therapeutic effectiveness. In the present study, we prepared a liposomal hydrogel of adenosylcobalamin (AdCbl), a vitamin B_{12} derivative, and investigated possible beneficial effects of AdCbl on atopic dermatitis using an NC/Nga murine atopic dermatitis model. AdCbl was loaded into liposomes prepared by a thin film hydration method using a pH gradient method that employed citric acid buffer solution. This resulted in AdCbl-loaded liposomes that were 106.4 ± 2.2 nm in size. The loading efficiency was 40% (of the initial AdCbl amount). Lipo-AdCbl had enhanced skin permeability, being about 17-fold compared with AdCbl-gel. Topical administration of Lipo-AdCbl-gel to 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis-like skin lesions in NC/Nga mice ameliorated lesion intensity scores, dorsal skin thickness, and total serum IgE in a concentration-dependent manner. Other preparations, including AdCbl solution, AdCbl cream, liposomes alone, and a mixture of AdCbl solution and liposomes had little effect. Taken together, our findings indicate that Lipo-AdCbl-gel has protective effects against atopic dermatitis symptoms, and suggest that it may be of benefit in the treatment of human inflammatory skin diseases.

1. Introduction

Atopic dermatitis (AD) is a common, chronic, inflammatory skin disease that often presents with flares, and can be complicated by recurrent skin infections (Lee et al. 2005; Yatsuzuka et al. 2007). It is classified as an immediate hypersensitivity reaction (type I) similar to allergic rhinitis, bronchial asthma and food allergy. There might be a genetic predisposition to develop atopic conditions (Helm 2004; Abramovits 2005). Although AD is associated with increased serum immunoglobulin E (IgE), the etiology and underlying mechanism of AD remain unclear. Currently, antihistaminic agents and steroids are the main agents used to treat atopic dermatitis, but they produce side effects such as infection, secondary adrenocortical insufficiency, diabetes, and pigmentation. Therefore, there is an unmet medical need for treatment of atopic dermatitis that has improved effectiveness and is more tolerable by patients.

Cobalamin (vitamin B_{12}), which is soluble in aqueous solution, has a complicated structure consisting of a cyclic porphyrin moiety and a nucleotide with alpha-glycoside bonds (Jacobs and Wood 2003). Cobalamin is important for the normal functioning of the brain and nervous system and for the formation of blood. In addition, it is involved in the metabolism of every cell in the body, especially affecting the synthesis and regulation of DNA, fatty acid synthesis, and energy production (Wang et al. 2007). Also, previous reports indicate that cobalamin protects against dermatitis by reducing the production of nitric oxide and proinflammatory cytokines (Yamashiki et al. 1992; Stucker et al. 2004; Januchoiwski 2009). However, cobalamin appears to be unstable in the presence of light, and has low skin permeability due to its high molecular weight and hydrophilic properties, all of which reduce its therapeutic effects (Wang et al. 2007; Naik et al. 2000).

Liposomes are nano-scale vesicles used as chemical or biological drug carriers (Sharma and Sharma 1995; Drummond et al. 1999). In particular, liposomes as a drug delivery system have been designed to mimic the phospholipid bilayers of biological membranes (Drummond et al. 1999; Bajoria and Sooranna 1998). The membrane of the liposome has a high structural similarity with skin lipids, and thus offers many advantages such as strong tissue affinity, biodegradability and low toxicity, which promote their increasingly wider application in transdermal delivery systems (Choi and Maibach 2005; Sinico et al. 2005).

In this study, we investigated the topical effects of liposomal adenosylcobalamin (Lipo-AdCbl) against DNCB-induced atopic dermatitis-like skin lesions in NC/Nga mice. First, we prepared liposomal adenosylcobalamin (Lipo-AdCbl), a vitamin B_{12} derivative, by a thin film hydration method and by a pH gradient method using citric acid buffer solution. Second,

Table: Physical properties of Lipo-AdCbl

Formulations of liposomes (HSPC: CHOL; mass ratio)	Mean particle diameter (nm)	Zeta potential (mV)	AdCbl loading efficiency (%)
[1] 3: 0	–	–	–
3: 1	106.4 ± 2.2	0.3 ± 0.2	38.8 ± 0.4
3: 2	142.1 ± 1.2	1.2 ± 0.4	22.2 ± 0.8
3: 3	227.1 ± 1.2	0.7 ± 0.1	11.5 ± 0.8

[1] Physical properties could not be characterized because of formulation of lipid aggregates. Results represent mean ± S.D. for three experiments.

Lipo-AdCbl was formulated into a hydrogel for topical application and the skin permeation of Lipo-AdCbl-gel was evaluated using a Franz diffusion cell with excised mouse skin. Last, the effects of Lipo-AdCbl-gel on atopic dermatitis were examined after topical application of Lipo-AdCbl-gel to DNCB-induced atopic dermatitis-like skin lesions. This was done by measuring the intensity of skin damage, IgE in blood, skin thickness, and the histology of dorsal skin.

2. Investigations and results

2.1. Physical properties of Lipo-AdCbl

The structures of various Lipo-AdCbls were evaluated by measuring their mean particle diameter, zeta potential, and AdCbl loading efficiency. These results are summarized in the Table. The loading efficiency of Lipo-AdCbls was inversely proportional to the CHOL content; the mean particle diameter was proportional to the CHOL content. The formulation for which L-α-phosphatidylcholine (HSPC):CHOL was 3:1 exhibited the highest loading efficiency for AdCbls and the smallest mean particle diameter; formulations with other mass ratios (3:2, 3:3) had lower AdCbl loading efficiencies and larger mean particle diameters. On the other hand, the formulation for which HSPC:CHOL = 3:0 (lipid composition lacking CHOL) exhibited lipid aggregates. Based on these results, the HSPC:CHOL = 3:1 formulation was chosen for further study. The zeta potentials of the prepared liposomes were 0.3 to 1.2 mV due to the use of neutral phospholipids, HSPC and CHOL.

2.2. Permeability study of AdCbl-loaded liposomal gels

Skin penetration efficiency of AdCbl was modeled by applying Lipo-AdCbl-gel and AdCbl-gel to Franz-type diffusion cells, as shown in Fig. 1. Although AdCbl was skin permeable as previously reported (Howe et al. 1967), Lipo-AdCbl-gel showed

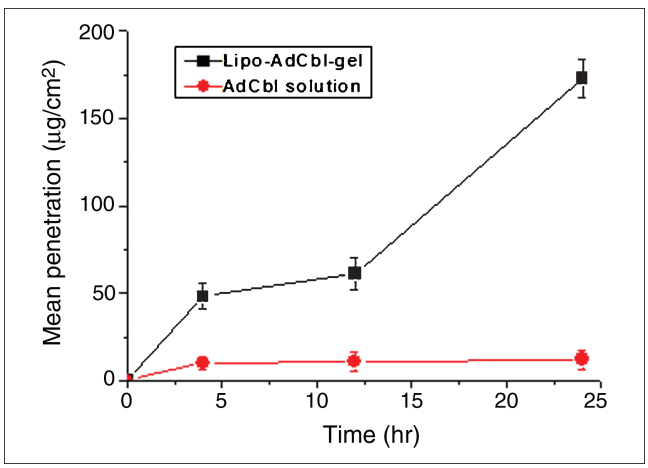
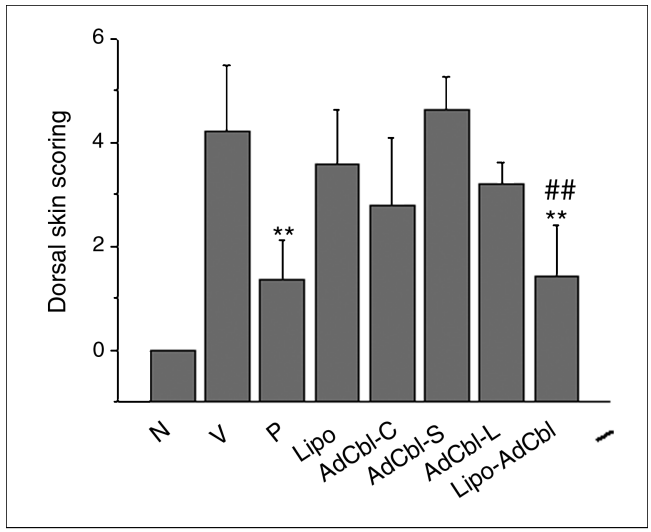
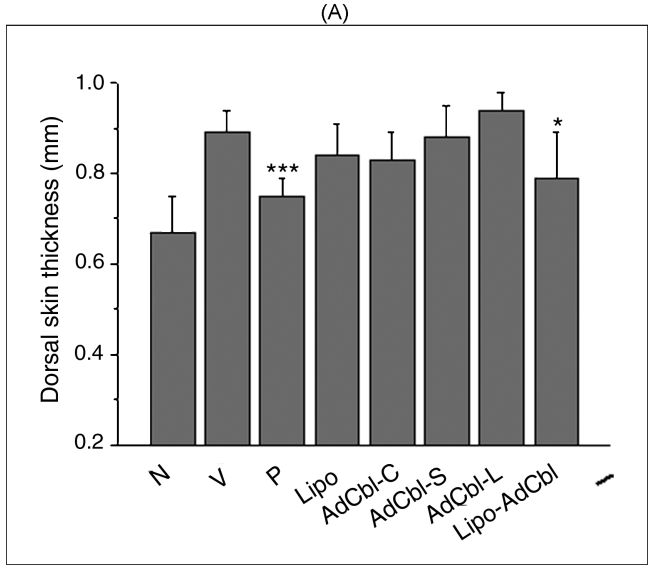


Fig. 1: Cumulative amount of AdCbl permeating mouse skin from different formulations. Results represent mean ± S.D. for three experiments.



(A)



(B)

Fig. 2: Effect of Lipo-AdCbl-gel on atopic dermatitis. A) Effects on dorsal skin scoring, B) Effects on dorsal skin thickness. N: normal control, V: vehicle group, P: Protopic (0.1%), AdCbl-C: 0.7% adenosylcobalamin cream type, AdCbl-S: 0.7% adenosylcobalamin solution, AdCbl+L: mixture of 0.7% adenosylcobalamin and liposome, Lipo-AdCbl: liposomal adenosylcobalamin gel preparation (0.7% AdCbl). $*P < 0.05$, $**P < 0.01$, $***P < 0.005$ vs vehicle. $\#\#P < 0.01$ vs liposome. Results represent mean ± S.E.M. of at least two experiments ($n = 7$).

17 times greater permeability of AdCbl compared to that of AdCbl-gel at 24 h, indicating that the transdermal delivery of AdCbl was enhanced when AdCbl was loaded in liposomes.

2.3. Effects of AdCbl-loaded liposomes on atopic dermatitis

The effects of various AdCbl preparations on lesions in DNCB-induced NC/Nga atopic dermatitis mice were examined. As

shown in Fig. 2A, DNCB (vehicle group) induced substantial skin damage. The Lipo-AdCbl-gel treated group showed marked reductions in DNCB-induced skin damage as determined by the SCORAD severity score. On the other hand, liposomes themselves, a simple mixture of AdCbl and liposomes, and AdCbl cream preparations had little effect on DNCB-induced skin damage. Likewise, skin thickness was reduced only by the Lipo-AdCbl preparation (Fig. 2B). These results indicate that the suppressive effects of AdCbl on DNCB-induced atopic dermatitis-like skin lesions are enhanced by the incorporation of AdCbl into liposomes. Protopic® (0.1%), a commercially available anti-AD agent that is a calcineurin inhibitor showed comparable effects to those for the Lipo-AdCbl-gel preparation.

2.4. Concentration dependence of Lipo-AdCbl-gel effects on atopic dermatitis

Since 0.7% AdCbl in Lipo-AdCbl-gel showed beneficial effects against atopic dermatitis, the concentration dependence of AdCbl effects on atopic dermatitis lesions was examined. As shown in Fig 3A, dorsal skin scoring was reduced by Lipo-AdCbl-gel in a concentration-dependent manner. A significant effect was observed starting at 0.07% Lipo-AdCbl-gel. Similar effects were observed for dorsal skin thickness and serum IgE concentrations (Fig. 3B and3C). These results indicate that Lipo-AdCbl-gel, even at concentrations as low as 0.07 %, can improve the symptoms of AD. In contrast, AdCbl cream had little effect at 0.7 %, suggesting that Lipo-AdCbl-gel may deliver AdCbl to the bloodstream more easily than does AdCbl cream, possibly due to a strong affinity of Lipo-AdCbl-gel with skin.

2.5. Histological study of epidermal tissue

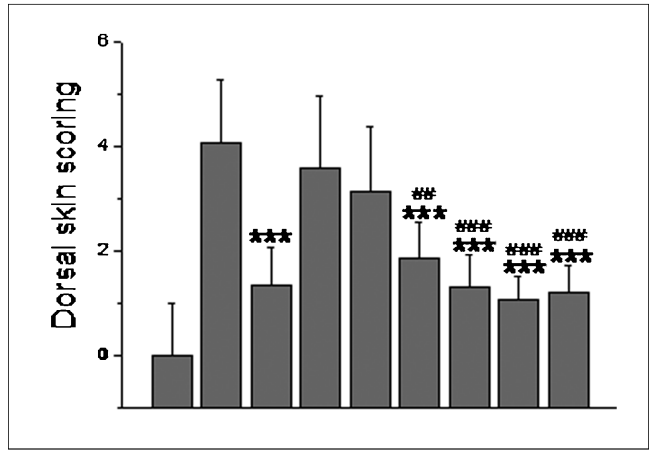
As shown in Fig. 4, histological observation revealed that epidermis and dermis became thicker after DNCB treatment. In particular, the tissue structure of the dermis was loosened, like a sponge. Lipo-AdCbl-gel treatment considerably improved the histology of epidermal tissue such that 74% of the Lipo-AdCbl-gel treated group showed normal histology with decreased hypertrophy and hyperkeratosis in the epidermis.

3. Discussion

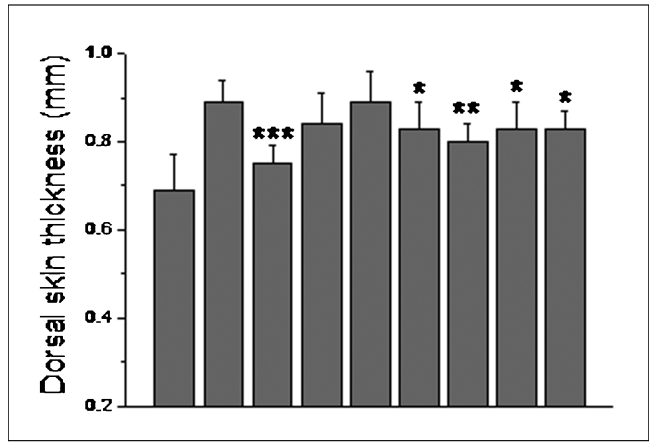
Liposomes enhance drug penetration through the stratum corneum and can be deposited in both the epidermis and dermis, while reducing systemic absorption of the drug. Therefore, liposomes are a suitable drug carrier for the treatment of skin diseases. In this study, we prepared liposomal preparation of AdCbl to compare the beneficial effects of Lipo-AdCbl against atopic dermatitis-like skin lesions with the effects of non-liposomal preparations of AdCbl.

To generate liposomal preparations of AdCbl, CHOL was included in the formulation to increase the physicochemical stability of Lipo-AdCbl. CHOL localizes to the hydrophobic chain of the phospholipids in the liposome bilayer. Therefore, increased CHOL will increase the stability of the liposome bilayer (Kirjavainen et al. 1996), but can also decrease the loading efficacy of AdCbl, especially in the pH gradient method, due to enhanced hydrophobic interactions of AdCbl with the liposome membrane. In addition, increased CHOL can increase the mean particle diameter of the liposome because the mean particle diameter of a liposome with enhanced hydrophobic interactions is difficult to control with an extruder when the liposomes are extruded.

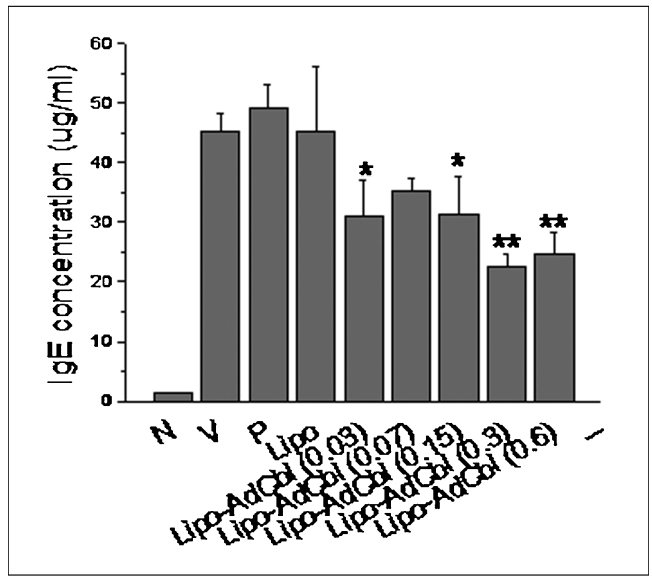
As expected, the skin permeability of Lipo-AdCbl-gel was higher than that of AdCbl-gel in a permeability study using



(A)



(B)



(C)

Fig. 3: Concentration-dependence of Lipo-AdCbl-gel effects on atopic dermatitis. A) Effects on dorsal skin scoring, B) Effects on dorsal skin thickness, C) Effects on serum IgE concentration. N: normal control, V: vehicle group, P: Protopic (0.1%), Lipo-AdCbl: liposomal adenosylcobalamin gel preparation. The number in parenthesis represents the concentration of AdCbl in the liposome preparation. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$ vs vehicle. $\#\#P < 0.01$ vs liposome. Results represent the mean ± S.E.M. of at least two experiments (n = 7).

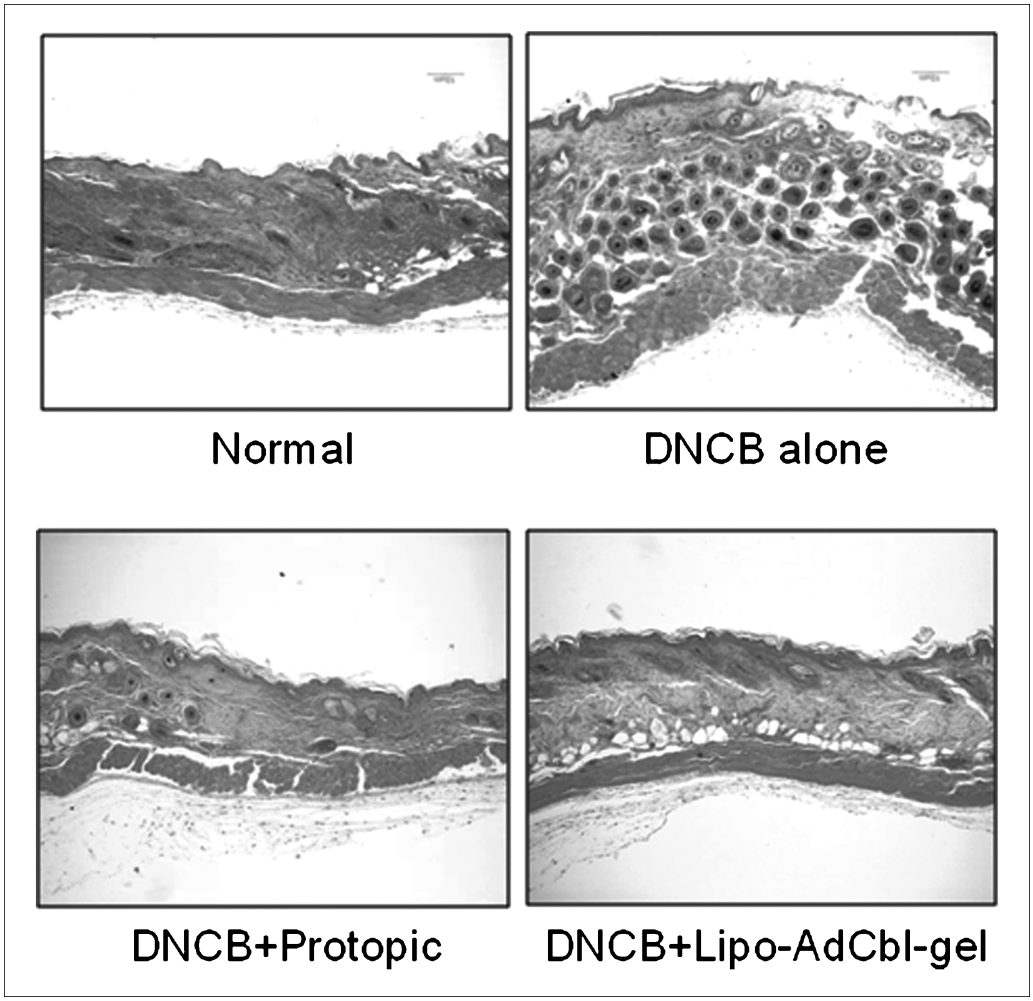


Fig. 4: Histological analysis of dermis and epidermis after Lipo-AdCbl-gel treatment. Results shown are representative pictures from three experiments.

excised mouse skin. Since keratinocytes of the stratum corneum are composed of phosphatidylcholine and sphingomyelin (Cevc et al. 1996; Liu and Hu 2007), and liposome has strong tissue affinity for the stratum corneum due to the phospholipid in the liposome (Benson 2005; Barichello et al. 2006), Lipo-AdCbl appears to induce greater skin permeability of the stratum corneum. The hydrogel of the Lipo-AdCbl-gel contains various chemical enhancers such as propylene glycol, ethanol and glycerin (Spernath et al. 2008), which increases permeability across the stratum corneum (Valenta and Auner 2004). Furthermore, hydrogel containing water and chemical enhancers increase the hydration of the stratum corneum by occluding the skin surface to retard normal moisture loss by transpiration, and are an essential plasticizer for the stratum corneum of the skin (Finnin and Morgan 1999). Thus, Lipo-AdCbl-gel produces greater skin permeability than AdCbl-gel itself due to the synergistic effects of liposomes and enhancers.

Over the last few decades, various animal models of AD have been developed. Among these models, the NC/Nga mouse, an inbred strain with a characteristic phenotype of spontaneous dermatitis, exhibits clinical and histologic characteristics similar to human AD-like lesions when the mice are maintained under conventional conditions but not under SPF conditions (Matsuda et al. 1997; Aioi et al. 2001). Furthermore, an epicutaneous application of chemical antigens such as 2,4-dinitrofluorobenzene (DNFB) evokes contact hypersensitivity reactions in NC/Nga mice, and prolonged dermatitis is observed even after cessation of DNFB application (Tomimori et al. 2005). In the present study, we induced atopic dermatitis-like skin lesions by topical application of DNCB, a chemical hapten similar to DNFB, to the dorsal skin of NC/Nga mice. In this model, the Lipo-AdCbl-gel treated group showed lower dorsal skin scoring and thinner skin thickness compared to that of the other groups, as shown in Fig. 2. It appears that Lipo-AdCbl-gel can easily be delivered into the skin due to the strong tissue affinity of phospholipids of the liposome for the stratum corneum.

As shown in Fig. 3, topical application of Lipo-AdCbl-gel to NC/Nga mice led to reductions in skin damage, skin thickness and serum IgE concentration even at concentration of 0.07%; no effect was observed for either AdCbl solution or liposomes alone. Protopic® (0.1%), a commercially available AD agent and a calcineurin inhibitor, showed effects comparable to those for the Lipo-AdCbl-gel preparation. The concentration used in the present study was similar to that used in a human clinical trial (Stucker et al. 2004). In addition, the suppressive effects of Lipo-AdCbl-gel indicate that AdCbl may inhibit proinflammatory cytokine production, thus reducing the infiltration of inflammatory cells such as mast cells and CD4+ T cells responsible for IgE synthesis. Indeed, Lipo-AdCbl-gel reduced IL-1β production (results not shown) although the effects on NO in tissue/serum and on IFN-γ were undetectable because NO and IFN-γ were not induced by DNCB.

In a separate experiment, we compared the effectiveness of various vitamin B_{12} derivatives including cyanocobalamin, methylcobalamin and adenosylcobalamin. The effects of adenosylcobalamin (AdCbl) were the most potent with regard to reducing skin scoring, skin thickness, and serum IgE under our experimental conditions (results not shown). It was reported

that cyanocobalamin and/or hydroxocobalamin are converted into the active forms of methyl and adenosylcobalamin *in vivo* (Stucker et al. 2004). Liposomal preparations of AdCbl in the present study exhibited enhanced therapeutic effectiveness compared with a methylcobalamin liposomal preparation.

A histology study revealed that Lipo-AdCbl-gel treatment improves skin histology, showing marked decreases in hypertrophy and hyperkeratosis in the epidermis. These results suggest that Lipo-AdCbl is delivered more efficiently to the dermal layer of epidermal tissues by increased skin permeability and then the AdCbl in Lipo-AdCbl is released from liposomes, producing beneficial effects against DNCB-induced atopic dermatitis-like skin lesions. Protopic® (0.1%) also restored the histology of skin tissue to normal conditions. Taken together, our findings suggest that Lipo-AdCbl-gel possesses high therapeutic efficacy against murine atopic dermatitis. As expected from previous reports (Stucker et al. 2004), no macroscopic adverse effects, including body weight changes, were observed in the Lipo-AdCbl-gel treated group.

4. Experimental

4.1. Materials

L-α-Phosphatidylcholine (soy hydrogenated, HSPC) and cholesterol (CHOL) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Adenosylcobalamin (AdCbl) was purchased from Interquim S. A. (Barcelona, Spain). Carbopol® 940, hydroxyethylcellulose (Natrosol), Nikkol® (HCO-60) and methylparaben were purchased from Neveon Inc. (Cleveland, OH, USA). Disodium EDTA, 2,4-dinitrochlorobenzene (DNCB), triethylamine (TEA), propylene glycol and glycerin were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). All other materials were of analytical grade and used without further purification.

4.2. Preparation of Lipo-AdCbl

AdCbl-loaded liposomes (Lipo-AdCbl) were prepared according to a thin film hydration method. AdCbl was loaded into the liposomes via a pH gradient method using citric acid (Li et al. 1998; Hwang et al. 1999). Briefly, HSPC and CHOL (3:1 mass ratio, total lipid content 12.80 mg/ml) were dissolved in 5 ml of chloroform, dried into a thin film on a rotary evaporator (Buchi Rotavapor R-200, Switzerland) and then suspended in 350 mM citric acid. The liposomal solution was extruded through a polycarbonate filter (pore sizes 200 and 100 nm, Whatman, USA) using an extruder (Northern Lipids Inc., USA). Free citric acid was removed by cellulose dialysis tubes (MWCO 12,000∼14,000, Viskase Co, IL, USA) for 24 h at 4 °C. The liposomal solution and a 5 mg/ml AdCbl solution were mixed and then incubated for 1 h at 60 °C. The mixture was dialyzed for 24 h at 4 °C to remove free AdCbl. The Lipo-AdCbls were stored at 4 °C until use. The concentration of AdCbl in the liposomes was measured by UV-Vis spectrophotometry at 525 nm (UV-mini, Shimadzu, Japan). Liposome solution (500 μl) was mixed by vortexing with 1 ml of chloroform. After centrifugation at 13,000 rpm for 5 min, the supernatant was obtained and the loading efficiency was calculated according to the following equation:

$$\text{Loading efficiency}\,(\%) = \text{Fi/Ft} \times 100 \qquad (1)$$

where Fi is the concentration of AdCbl loaded in the liposomes and Ft is the initial concentration of added AdCbl. The particle size and zeta potential of the liposomes were measured by light scattering with a particle size analyzer (ELS-Z, Otuska, Japan).

4.3. Preparation of liposomal AdCbl containing gel or AdCbl containing cream

Lipo-AdCbl containing gel was prepared as described below under conditions in which light was blocked. Twenty-six grams of Lipo-AdCbl solution were added to 10.0 g of deionized distilled water. Then, 0.3 g of carbopol 940 was added in small amounts until completely dissolved at 40 °C. Six grams of TEA, 0.05 g of Natrosol, 0.05 g of disodium EDTA, 0.2 g of methylparaben, 0.3 g of Nikkol®, 10 g of glycerin and 3 g of ethyl alcohol were added to the solution as a base, an emulsifying agent and a solvent, respectively. Deionized distilled water was added to provide 100 g of total solution. The mixture was agitated at room temperature and 3,000 rpm with a homomixer (MS-280D, MTOPS®, South Korea) until the solution became uniform.

AdCbl containing cream was prepared as described below under conditions in which light was blocked. As the aqueous phase, 1.0 g of sodium hydroxide was added to 70 g of deionized distilled water. Then, 70 mg of AdCbl was dissolved at 90 °C with a magnetic stirrer. The oil phase, which consisted of 13 g of stearic acid, 4 g of lanolin, 2 g of sucrose fatty acid ester and 2 g of isopropyl myristate, was completely mixed at 90 °C with a magnetic stirrer. The aqueous and oil phases were thoroughly mixed while the two phases were added slowly at 90 °C using a homomixer. The cream was cooled to 40 °C while being continuously mixed with the homomixer at 3,000 rpm until the cream became uniform; it was then cooled to room temperature. Gas bubbles in the AdCbl-containing cream were removed under vacuum.

4.4. Animals

Female BALB/c mice (7 weeks old, 18–20 g) and female NC/Nga mice (7 weeks old, 18–22 g) were purchased from Harlan Inc. (IN, USA). All animal experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH), and approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Chemical Technology.

4.5. In vitro permeation measurements with mouse skin

Female BALB/c mice were sacrificed by ether inhalation and the skin was cut to a size adequate for permeation studies (Mayorga et al. 1996; Kirjavainen et al. 1999). The permeation study was carried out at 37 °C using a Franz-type diffusion cell (exposed skin diameter = 2 cm) and a Franz-type diffusion cell magnetic stirrer (poly 15, H+P Labortechnik AG, Germany). The donor compartment contained 2 ml of Lipo-AdCbl-gel (0.7% AdCbl, m/v ratio) or AdCbl-gel (0.7% AdCbl, m/v ratio). The receiver compartment contained 12.5 ml of phosphate buffered saline solution (PBS, pH 7.4). At scheduled time intervals up to 24 h, aliquots of 200 μl were removed from the receiver compartment and replaced by an equal volume of fresh PBS solution (pH 7.4). The amount of AdCbl retained in the applied skin and in the receiver compartment was determined by UV–Vis spectrophotometry at 525 nm.

4.6. Preparation of an atopic dermatitis-like animal model

To compare the efficacy of AdCbl in different formulations, NC/Nga mice (n = 7/group) were anesthetized with avertin and dorsal skin hair was shaved using a hair-clipper. Two days later, NC/Nga mice were sensitized by application of 300 μl of 1% DNCB in acetone/olive oil (AOO) (3:1) onto dorsal skin. Four days after sensitization, mice were challenged weekly for 5 weeks by application of 300 μl of 0.4% DNCB in AOO (3:1) at the same site, and were unchallenged for up to 7 weeks thereafter. At 4 weeks after the first treatment of 0.4% DNCB, Lipo-AdCbl-gel (0.03–0.7%) was applied twice a day for 3 weeks, and the intensity of skin damage and the levels of IgE in blood were measured weekly. At the end of drug treatment, mice were sacrificed by CO_2 asphyxiation, and skin thickness and histology were measured in tissue sections.

4.7. Evaluation of the effects of Lipo-AdCbl-gel on atopic dermatitis

Intensity of AD was determined according to the SCORAD scale (Scoring Atopic Dermatitis) based on the following criteria: 0 points for showing no symptoms, 1 point for mild indication of symptoms (mild), 2 points for moderate symptoms (moderate), and 3 points for severe symptoms (severe). The five symptoms considered in scoring were edema, oozing/crusting, excoriation, erythema and lichenification.

Measurement of total IgE in serum is routinely used in the diagnosis of allergic disorders (Olivry et al. 2008; Wang et al. 2008). To measure IgE, blood was withdrawn from the tail vein at the indicated times, centrifuged at 500 × g for 10 min at 4 °C and stored at –20 °C until use. The total IgE levels in serum were measured by IgE ELISA kit (Sphibayagi, Japan).

After mice were sacrificed by CO_2 asphyxiation at the end of the experiment, epidermal tissue from the back of the mice was lightly pinched and the folded part of the skin was measured using a dial gauge (Mitutoyo, Japan). For histological analysis, a piece (about 0.5 × 1.0 cm) of epidermal tissue was excised, fixed in 4% formalin, embedded in paraffin and sliced into 8 μm slices. The tissue slices that adhered to slides were dyed with hematoxylin and eosin and studied under a light microscope (eclipse TE2000U, Nikon, Japan). Images were taken at 40× magnification.

4.8. Statistical analysis

Data are expressed as either means ± S.D. or means ± S.E.M. of three experiments. Statistical significance was evaluated using Student's *t*-test, and $p < 0.05$ was considered as statistically significant.

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