Enhanced Delivery of Retinoic Acid to Skin by Cationic Liposomes

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We studied the delivery of retinoic acid to skin by using cationic liposomes consisting of double-chained cationic surfactant, phosphatidylcholine (PC) and retinoic acid in excised guinea pig dorsal skin. Egg yolk PC liposomes contaning retinoic acid at a molar ratio of 4 : 1 increased the delivery of retinoic acid about two-fold, compared with its addition as an isopropyl myristate solution. Cationic liposomes containing 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) further enhanced the incorporation dependent on the DOTAP content. Liposomes consisting of DOTAP, egg yolk PC, and retinoic acid at a molar ratio of 2 : 2 : 1 induced a 3.7-fold increase in the skin incorporation compared with the egg yolk PC liposomes without DOTAP. Significant difference was not observed when either dimyristoylphosphatidylcholine (DMPC) or dipalmitoylphosphatidylcholine (DPPC) was used instead of egg yolk PC as well as when dimethyldipalmitylammonium was used instead of DOTAP. These results suggest the potential of the use of the cationic liposomes for the intradermal delivery of lipophilic drugs like retinoic acid.

Key words retinoic acid; cationic liposome; skin; drug delivery; double-chained cationic surfactant

Various kinds of double-chained cationic surfactants have been shown to form bilayer vesicles (cationic liposomes) and have been applied to gene delivery.[1,2] Cationic vesicles designed for that purpose are generally intrinsically unstable.[3,4] Although application of cationic liposomes has also been anticipated for the delivery of drugs, peptides, vitamins and lipids and other biologically active compounds to biological cells and tissues, liposomes used for that purpose need to be intrinsically stable in order to retain their contents.[5] In a previous study we revealed that phosphatidylcholines enriched with unsaturated acyl chains stabilized the liposomes of dimethyldialkylammoniums such as dimethyldipalmitylammonium at physiological temperature.[6] In contrast, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) formed stable vesicles at physiological temperature with phosphatidylcholines containing either saturated acyl chains such as dimyristoylphosphatidylcholine (DMPC) or unsaturated acyl chains such as dilinoleoylphosphatidylcholine (DLPC).[7] We also revealed that these cationic liposomes interacted with erythrocytes as cationic vesicles and induced the formation of tightly aggregated structures of several erythrocytes which might also induce fusion of the erythrocytes.[6,7]

The lipid lamella of the stratum corneum of the skin contains a high ratio of negatively charged lipids,[8] which are expected to interact with cationic liposomes. Transfer of some of the bilayer components of the liposomes to the skin is then possibly induced. Therefore, in this work we prepared cationic liposomes containing retinoic acid (*all-trans*-retinoic acid) as a bilayer component, and examined the incorporation of retinoic acid into skin by the cationic liposomes. Although topical retinoic acid has been clinically used with success for the treatment of several cutaneous diseases such as psoriasis or ichthyosis and mild acne,[9] its efficient delivery into skin has not been established yet. We compared the incorporation efficiency by cationic liposomes with those by electrically neutral liposomes and elastic liposomes as well as that by an organic solvent solution.

Experimental

Materials Chloride salt of DOTAP was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Bromide salt of dimethyldipalmitylammonium was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dimyristoryphosphatidylcholine (DMPC, L-α-phosphatidylcholine dimyristoyl) and Dipalmitoylphosphatidylcholine (DPPC, L-α-phosphatidylcholine dipalmitoyl) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *All-trans*-retinoic acid, egg yolk phosphatidylcholine (egg yolk PC), heptaethylene glycol mono-*n*-dodecyl ether and all other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of Sonicated Liposomes Consisting of Phosphatidylcholines, Double-Chained Cationic Surfactants and Retinoic Acid Liposomes consisting of phosphatidylcholines, DOTAP (or dimethyldipalmitylammonium) and retinoic acid were prepared as described previously.[6,7] The liposome constituents were dissolved in chloroform, and the solvent was evaporated under a nitrogen stream. The dried films were prepared by removing the solvent under vacuum evaporation, then hydrated and suspended by vortex mixing in phosphate-buffered saline (PBS) (150 mM NaCl, 10 mM phosphate buffer, pH 7.4) containing 10 mM sodium ascorbate. The final concentration of retinoic acid was 2.5 mM, and the total concentrations of double-chained cationic surfactants and phosphatidylcholines were 10 mM. The suspension was then sonicated with a probe-type sonicator for 10 min at an output power of 80 W at 30 °C under a stream of nitrogen. The vesicular sizes were determined with quasielastic light scattering using an ELS-800 laser particle analyzer (Otsuka electronics, Japan) at a scattering angle of 90°.

Measurement of the Intradermal Concentration of Retinoic Acid Full thickness dorsal skin was excised from male guinea pigs and subcutaneous fat and other extraneous tissues were trimmed. *In vitro* study on the skin incorporation of retinoic acid was examined as described previously.[10] The skin was mounted in a Franz cell with water jackets (37 °C). The available diffusion area was about 0.64 cm^2, and the lower cell volume was about 4.5 ml. The upper cells were filled with 1 ml PBS and the receiver cells were also filled with PBS. The lower cells were stirred at 450 rpm by a magnetic stirrer during 12 h pretreatment of the skin.

After washing both cells, 1 ml liposome suspension containing 2.5 mM retinoic acid was added to the upper compartments, and the incubation experiment was started. A control experiment was also carried out by the addition of 2.5 mM retinoic acid dissolved in isopropyl myristate. After 18 h treatment, the skins were removed from the cells and washed three times with ice-cold methanol. Following room temperature drying, each skin was weighed, minced and placed in 10 ml of methanol, then homogenized using a tissue homogenizer Polytoron (Kinematica AG, Switzerland). The samples were then centrifuged and the supernatant layer was used to determine the concentration of retinoic acid by HPLC. The concentration of retinoic acid was determined by HPLC (L-6000; Hitachi, Tokyo, Japan) with an L-4000 UV detector (Hitachi) at 356 nm. Separation was achieved on a reversed-

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phase column (Mightysil RP-18, 4.6 mm i.d., 250 mm) using a mobile phase consisting of methanol, water and phosphoric acid (900 : 100 : 1, v/v) at a flow rate of 0.70 ml/min. Ferulic acid was used as an internal standard.

Statistical Analysis One way analysis of variance and Bonferroni's *post-hoc* test were used to analyze differences between the sets of data. A p-value less than 0.05 was considered significant.

Results and Discussion

We examined the efficiency of the use of neutral liposomes and cationic liposomes for the *in vitro* delivery of retinoic acid into skin. As shown in Fig. 1, egg yolk PC liposomes contaning retinoic acid at a molar ratio of 4 : 1 increased the delivery of retinoic acid about two fold, compared with the accumulation amount by its addition as an isopropyl myristate solution. The enhancement effect of liposomes on the skin delivery of retinoic acid observed in this study is consistent with the previous findings on facilitated deposition of drugs by liposomes.[11)]

We next examined the delivery of retinoic acid by cationic liposomes using DOTAP, a cationic surfactant with double acyl chains, as bilayer forming double-chained cationic surfactants. Cationic liposomes containing DOTAP enhanced the incorporation of retinoic acid into skin dependent on the content of the double-chained cationic surfactants. Liposomes consisting of DOTAP, egg yolk phosphatidylcholine (PC), and retinoic acid at a molar ratio of 2 : 2 : 1, whose mean diameter was about 108 nm, induced a 3.7-fold increase in the skin incorporation compared with the liposomes without DOTAP, as shown in Fig. 1. It corresponded to a 7.3-fold increase compared with the skin accumulation when retinoic acid was added as isopropyl miristate solution. Liposomes consisting of DOTAP, egg yolk PC, and retinoic acid at a molar ratio of 1 : 3 : 1 had less effect.

We furthermore examined the effects of the acyl chains of the phosphatidylcholines on the incorporation efficiency of retinoic acid. As shown in Fig. 2, similar enhancement effects were observed when either dimyristoylphosphatidylcholine (DMPC) or dipalmitoylphosphatidylcholine (DPPC) with saturated acyl chains was used instead of egg yolk PC which is enriched in unsaturated oleic acid and linoleic acid in its acyl chains.[12)] Similar enhancement effects were also observed when dimethyldipalmitylammonium was used instead of DOTAP as a double-chained cationic surfactant.

Elastic liposomes have been revealed to be useful to deliver drugs, especially hydrophilic drugs into the skin.[13)] Therefore, we also examined the delivery of retinoic acid by using the liposomes consisting of egg yolk PC and heptaethylene glycol mono-*n*-dodecyl ether, whose composition has been revealed to form elastic liposomes,[13)] at a molar ratio of 4 : 1. However, as shown in Fig. 2, the elastic liposomes did not have any stimulatory effect on the skin delivery of retinoic acid compared with the liposomes consisting of egg yolk PC.

The results obtained here suggest that cationic liposomes deliver retinoic acid to the skin as a liposomal constituent dependent on the zeta potential. The composition of the liposomes does not seem to have important effects when phosphatidylcholines are used as major bilayer constituents.

Recently the usefulness of topical delivery of retinoic acid by microemulsions and nanoparticles was reported.[14,15)] The present findings suggest that cationic liposomes also have the potential for efficient delivery of retinoic acid and other lipophilic drugs into the skin, although the depth of delivery of the cationic liposomes in the skin remains to be clarified. Since it has been suggested that the size of the liposomes is also important for the interaction of liposomes with the skin,[16)] the effects of the particle size of the liposomes on the delivery should also be considered.

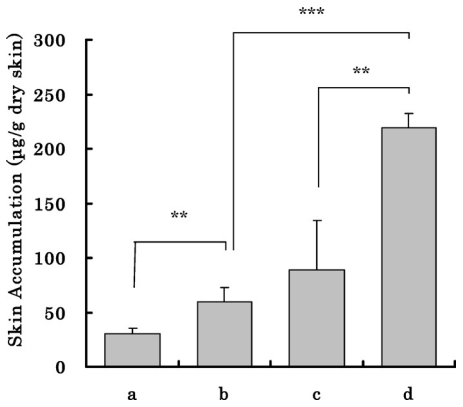

Fig. 1. Effects of Liposomes on the Delivery of Retinoic Acid into Skin after 18 h Treatment

a, isopropyl myristate solution; b, egg yolk PC without DOTAP; c, egg yolk PC and DOTAP (3 : 1); d, egg yolk PC and DOTAP (1 : 1). Final concentration of retinoic acid was 2.5 mM in solution or liposome suspension and total concentration of egg yolk PC and DOTAP was 10 mM. Data are means ± S.D. of four experiments. ** $p<0.01$ (differences between a and b and between c and d), *** $p<0.001$ (difference between b and d).

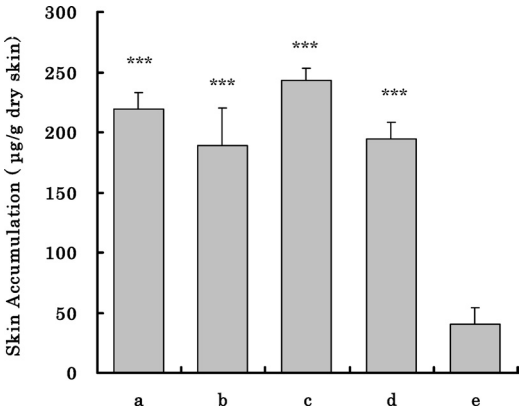

Fig. 2. Effects of Different Phosphatidylcholines and Surfactants in Liposome Compositions on the Delivery of Retinoic Acid into Skin after 18 h Treatment

a, egg yolk PC and DOTAP (1 : 1); b, DMPC and DOTAP (1 : 1); c, DPPC and DOTAP (1 : 1); d, egg yolk PC and dimethyldipalmitylammonium (1 : 1); e, egg yolk PC and heptaethyleneglycol mono-*n*-dodecyl ether (4 : 1). Final concentration of retinoic acid was 2.5 mM in liposome suspension and the total concentration of PC and surfactant in each liposome suspension was 10 mM. *** $p<0.001$, compared with liposomes consisting of egg yolk PC without cationic surfactants.

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