Influence of Nonionic Liposomal Composition on Topical Delivery of Peptide Drugs into Pilosebaceous Units: An in Vivo Study Using the Hamster Ear Model

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Purpose. The purpose of this study was to test the hypothesis that nonionic liposomes facilitate the topical delivery of peptide drugs into pilosebaceous units. ***Methods.*** The hamster ear was used as a model for human pilosebaceous units. The deposition of a hydrophilic protein, alpha-interferon (α-IFN), into pilosebaceous units and other strata of the hamster ear 12 hours after topical *in vivo* application of three nonionic liposomal formulations, one composed of glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether (Non-1), the second composed of glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether (Non-2) and the third composed of polyoxyethylene-10-stearyl ether/cholesterol (Non-3), a phospholipid-based liposomal formulation (PC) and an aqueous control solution (AQ) was determined. We also determined the deposition of a hydrophobic peptide, cyclosporin-A (CsA), into pilosebaceous units and other strata of the hamster ear after topical *in vivo* application of these liposomal formulations and a hydroalcoholic control solution (HA). ***Results.*** The deposition of α-IFN into the pilosebaceous units was in the order: Non-1 ⩾ PC > Non-2 > Non-3 = AQ. The deposition of CsA into the pilosebaceous units was in the order: Non-1 ⩾ HA > PC > Non-2 = Non-3. ***Conclusions.*** Despite differences in the hydrophobicities and size of the drug molecules, deposition into the various ear strata was significantly enhanced by the Non-1 liposomal system.

KEY WORDS: liposomes; follicular delivery; pilosebaceous unit; cyclosporin A; alpha-interferon.

INTRODUCTION

Previous work in our laboratories showed clearly the utility of the hamster ear model for estimation of deposition of various drugs into sebaceous glands (1). The anatomical and physiologic similarity of the hamster ear pilosebaceous units to that in humans has also been documented (2–4). Recent studies have also indicated that with liposomal formulations, the follicular route may be involved to a greater extent and could be potentially more useful than previously assumed (5–7). Targeted delivery to the follicles and/or sebaceous glands would be beneficial in the alleviation of disease states associated with or originating within these units (7,8). A better understanding of the physicochemical and thermodynamic aspects of drug/liposome transport into and across the pilosebaceous units (hair follicles and sebaceous glands) is therefore necessary. In this study, we describe the extent of deposition of two model peptide drugs, alpha-interferon (α-IFN) and cyclosporin-A (CsA), varying in size and hydrophobicity, into the sebaceous glands of golden Syrian hamster ears following topical *in-vivo* treatment with a variety of liposomal formulations.

MATERIALS AND METHODS

Materials

The synthetic nonionic lipids, glyceryl distearate (GDS), glyceryl dilaurate (GDL) and polyoxyethylene-10-stearyl ether (POE-10), as well as cholesterol (CH) were supplied by IGI Inc., Little Falls, NJ. HEPES free acid was obtained from Sigma Chemical Co., St. Louis, MO. Egg phosphatidylcholine (PC) and egg phosphatidylserine (PS) were obtained from Lipoid KG, Ludwigstrasse, Germany. α-Tocopherol was obtained from Eastman Kodak, Rochester, NY. Alpha-Interferon (α-IFN) and human serum albumin (HSA) were kindly supplied by Hoffman-La Roche, Nutley, NJ. ^{125}I-α-IFN was obtained from NEN Research Products, Boston, MA. Cyclosporin-A (CsA) was provided by the Department of Dermatology, University of Michigan, Ann Arbor, MI. ^{3}H-CsA was obtained from Amersham, UK. Sephadex G-75 was obtained from Pharmacia Inc., Piscataway, NJ. All other chemicals were of analytical grade or better. Water used was double distilled and deionized using a Millipore Milli-Q system.

Preparation of Liposomal Formulations

The liposomal formulations used are summarized in Table I. The total lipid concentration in all preparations was 50 mg/ml. The total α-IFN concentration in all interferon formulations was 1×10^8 IU/ml and the formulations also contained 0.1% HSA. The liposomal suspensions were examined using a Nikon Diaphot light microscope to assure integrity and quality of the liposomal preparations. If lipid particulates were present or if the liposomes were not uniform and spherical the preparation was discarded and a fresh batch was prepared. The CsA liposomal systems were prepared as described earlier (9) so that the bilayers of each of the formulations were saturated with respect to CsA. This procedure was used so that comparisons of drug deposition could be made using formulations of equal thermodynamic activity and equal total lipid concentration (50 mg/ml). The entrapment percent of CsA in the liposomal systems was determined using size exclusion chromatography with Sephadex G-75 columns. Unseparated CsA liposomal formulations containing both entrapped and non-entrapped drug were used in all experiments. All formulations were stored at 4°C overnight before use in *in vivo* experiments.

Nonionic α-IFN Liposomal Formulations

Three nonionic liposomal formulations, one containing GDL:CH:POE-10, the second containing GDS:CH:POE-10, both at a weight percent ratio of 57:15:28, and the third with

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Table I. Summary of Liposomal Formulations Used in the Studies

Liposomal formulation	Lipid composition	Mole or weight ratio	Saturation level of entrapped CsA (mg/ml)
CsA formulations			
Non-1	GDL:CH:POE	57:15:28 (wt)	2.2
Non-2	GDS:CH:POE	57:15:28 (wt)	1.4
Non-3	POE:CH	60:40 (wt)	1.4
PC	PC:CH:PS	1:0.5:0.1 (mole)	1.1
			α-IFN Concentration (IU/ml)
α-Interferon formulations			
Non-1	GDL:CH:POE	57:15:28 (wt)	1×10^8
Non-2	GDS:CH:POE	57:15:28 (wt)	1×10^8
Non-3	POE:CH	60:40 (wt)	1×10^8
PC	PC:CH:PS	1:0.5:0.1 (mole)	1×10^8

POE-10:CH at a weight percent ratio of 60:40 were prepared as follows: Appropriate amounts of the lipids were mixed in a beaker and melted at 75°C. The melt was then drawn into a syringe preheated in a water-bath at 75°C. A second syringe containing 0.05 M isotonic HEPES buffer, pH 7.4, was preheated to 70°C. The two syringes were then connected *via* a 3-way teflon or metal stopcock. The aqueous buffer was then injected into the lipid phase syringe. The mixture was mixed back and forth between the two syringes rapidly several times while being cooled under cold tap water. This process was continued until the mixture was at room temperature. Equal volumes of the empty liposomes were then mixed with α-IFN solution in isotonic 0.05 M HEPES buffer, pH 7.4, containing trace amount of the radioactive marker (^{125}I-α-IFN), 2×10^8 IU/ml cold α-IFN and 0.2% HSA. The total lipid concentration in the final suspension was 50 mg/ml for all formulations. All formulations were stored at 4°C overnight before use in *in vivo* experiments.

Phospholipid-Based α-IFN Liposomal Formulations

PC/CH/PS dehydration-rehydration (DRV) liposomes were prepared by a modification of the method reported by Kirby and Gregoriadis (10). Briefly, appropriate amounts of the various lipids and α-tocopherol (1% by weight of the total lipids) were dissolved in chloroform in a round-bottomed flask. The solvent was then removed using a rotoevaporator under vacuum and the flask containing the film was dried overnight in a desiccator to remove any residual solvent. The film was hydrated with an appropriate amount of isotonic 0.05 M HEPES buffer, pH 7.4, at 40°C for 30 minutes with intermittent vortexing. The resultant suspension was then dehydrated at 50°C under vacuum using the rotoevaporator. When the suspension became very viscous, an amount of distilled water equivalent to that removed (determined by weighing the flask and its contents before and after dehydration) was added back to the suspension and rehydrated at 50°C for 45 minutes. The suspension was then annealed at 40°C for 15 minutes. The total lipid concentration in this preparation was 100 mg/ml. Equal volumes of 2×10^8 IU/ml α-IFN solution in 0.05 M isotonic HEPES, pH 7.4, containing 0.2% HSA and trace amount of ^{125}I-α-IFN were then mixed with empty liposomes to yield suspensions containing 1×10^8 IU/ml α-IFN, 0.1% HSA with a total lipid concentration of 50 mg/ml. The formulation was stored at 4°C overnight before use in *in vivo* experiments.

Aqueous α-IFN Solution

An aqueous α-IFN solution containing 1×10^8 IU/ml cold α-IFN, 0.1% HSA and trace amount of the radioactive marker (^{125}I-α-IFN) in isotonic 0.05 M HEPES buffer, pH 7.4, was used as a control solution.

Nonionic CsA Liposomal Formulations

Three nonionic liposomal formulations containing GDL:CH:POE and GDS:CH:POE at a weight percent ratio of 57:15:28 and POE:CH at a weight percent ratio of 60:40 were prepared as follows: Appropriate amounts of the lipids and CsA along with trace amount of ^{3}H-CsA were accurately weighed in a scintillation vial. The vial was then capped and heated with stirring at 75°C in a water bath to melt the lipids and to dissolve the CsA in the lipid melt. Isotonic 0.05 M HEPES buffer, pH 7.4, preheated in a syringe at 70°C was then added to the clear lipid melt and the mixture was vigorously stirred with cooling under cold tap water. The total lipid concentration in all formulations was 50 mg/ml. All formulations were stored at 4°C overnight before use in *in vivo* experiments.

Phospholipid-Based CsA Liposomal Formulation

PC/CH/PS large unilamellar liposomes (LUV) were prepared by a modification of the reverse-phase evaporation method reported by Szoka and Papahadjopoulos (11). Appropriate amounts of the lipid mixture, CsA and α-tocopherol, along with a trace amount of radiolabeled ^{3}H-CsA were dissolved in a chloroform-methanol mixture (2:1 [v/v]). Isotonic 0.05 M HEPES buffer, pH 7.4, was added to the mixture. The solvent-to-buffer ratio was 6:1 (v/v). If the system was not clear, a small amount of methanol, typically less than 10% of the total solvent volume used, was added to obtain a clear solution. The organic solvents and a small amount of water was then removed using a rotoevaporator at

a temperature above the phase-transition temperature of the lipids. Solvent removal was continued until all foaming ceased. The total lipid concentration in the suspension was 50 mg/ml. The formulation was stored at 4°C overnight before use in *in vivo* experiments.

Hydroalcoholic CsA Solution

A hydroalcoholic solution was prepared by dissolving CsA and trace amount of ^{3}H-CsA in ethanol and mixing the solution with an appropriate volume of 0.05 M HEPES isotonic buffer (pH 7.4) to obtain a final solution containing 2.5 mg/ml CsA in 40% ethanolic buffer.

In Vivo Experiments

Male Golden Syrian hamsters, 10–12 weeks old, were purchased from Charles River Breeding Laboratories (Wilmington, MA) and were maintained for two weeks at a photoperiod of 14 hr of light and 10 hr of darkness to maximize androgen dependent sebaceous gland activity (12) and thus control their size. The hamsters were anesthetized with sodium pentobarbital (10 mg/kg i.p. for the first injection and then 5 mg/kg at 1.5 hour intervals thereafter over the duration of the experiment up to 12 hours). The average surface temperature of the ventral hamster ear monitored every 30 minutes over a 12 hour period using a digital pyrometer was 32.2 ± 1.2°C. A minimum of three ears from three different animals were used for each formulation tested. Following anesthetization, 50 μl of the test formulation were applied to the ventral surface of each ear. All experiments were carried out under non-occluded conditions. At 12 hours, the hamsters were sacrificed and the ears removed by cutting across the base and processed as follows.

The ear was mounted ventral side up on a board using thumb-tacks and stripped about 15–20 times with Scotch tape (#810, 3M Company, St. Paul, MN) to remove the stratum corneum. A shiny and glossy appearance indicated that removal of the stratum corneum was complete. The strips were collected for assay of radiolabeled drug. The stripped ear was then cut across the base to facilitate peeling of the ear. The ears were peeled gently using a pair of forceps such that the ventral ear and the dorsal ear could be processed separately. The peeling procedure was carried out carefully so as to assure that after peeling, the cartilage between the ventral and the dorsal portions of the ear was associated with the dorsal ear. The cartilage was then scraped gently and collected for assay of drug content. The dorsal ear was also assayed for drug. The ventral ear was then placed on a glass slide with the epidermal side down. The sebaceous glands were then scraped using a scalpel. The scraping procedure was carried out three times to ensure complete removal of all the glands. The scraping procedure itself has been validated earlier in our laboratories by examining ears under a Nikon Diaphot inverted light microscope before and after the scrapings (1). The absence of the glands was confirmed by the presence of bright areas under the microscope, after the scraping process. The gland scrapings and the dermis (remainder of the ventral ear) were collected in scintillation vials for assay of peptide content. The strips, gland scrapings, dermis, cartilage and dorsal ear were then assayed using a scintillation counter for CsA (Beckman LS 6000) and a gamma counter for α-IFN (Packard Instruments, Miniaxi Auto-Gamma 5000 Series). To ensure that the radioactivity measured represented intact α-IFN rather than free iodine label, a 10% solution of trichloroacetic acid in buffer was added to the test samples and equilibrated for 24 hours. The mixture was then centrifuged using a Beckman Microfuge and the supernatant representing free iodine label was assayed.

RESULTS AND DISCUSSION

Table II shows the distribution of radiolabeled α-IFN marker in the various compartments of golden Syrian hamster ear 12 hr after topical *in vivo* application of various liposomal formulations and an aqueous control solution. The recovery of total radioactivity was greater than 90% in all cases. The amount of α-IFN found in the pilosebaceous units was in the order: Non-1 ≫ PC > Non-2 > Non-3 = AQ. The amounts of α-IFN found in the cartilage and in the dorsal ear were negligibly low for all formulations except Non-1. Overall, the Non-1 liposomal formulation is far more efficient than the other four formulations tested in facilitating deposition of α-IFN into all of the strata of the hamster ear ($p < 0.01$, two-tailed t-test).

Table III shows the distribution of radiolabeled CsA in the various compartments of golden Syrian hamster ear 12 hr after topical *in vivo* application of various formulations. The recovery of total radioactivity was greater than 95% in all cases. The amount of CsA found in the pilosebaceous units was in the order: Non-1 ≫ HA > PC > Non-2 = Non-3. The amounts of CsA found in the cartilage and in the dorsal ear were negligibly low for all formulations except for the Non-1 and Non-3 liposomes. Overall, the Non-1 liposomal formulation is again more efficient than all the other formulations tested in delivering CsA into all of the strata of the hamster ear ($p < 0.01$).

Table II. Distribution of α-IFN in Various Strata of Syrian Hamster Ear (Expressed as IU ± sd) 12 h After Topical in Vivo Application of Various Formulations[a]

	Formulation				
Strata	AQ	PC	Non-3	Non-2	Non-1
Pilosebaceous units	2400 ± 2200	11000 ± 3600	2300 ± 900	6100 ± 2500	49500 ± 13000
Dermis	400 ± 400	700 ± 400	200 ± 100	400 ± 100	2000 ± 1500
Cartilage	300 ± 200	1200 ± 1600	1200 ± 1300	2300 ± 1300	50500 ± 34000
Dorsal	300 ± 200	1100 ± 1500	900 ± 1000	1600 ± 500	37500 ± 32500

[a] n = 4–7. Applied amount = 5×10^6 IU.

Table III. Distribution of CsA in Various Strata of Syrian Hamster Ear (Expressed as μg ± sd) 12 h After Topical in Vivo Application of Various Formulations[a]

Strata	Formulation				
	HA	PC	Non-2	Non-3	Non-1
Pilosebaceous units	0.77 ± 0.12	0.51 ± 0.06	0.41 ± 0.11	0.38 ± 0.12	2.16 ± 0.52
Dermis	0.18 ± 0.11	0.19 ± 0.11	0.04 ± 0.01	0.04 ± 0.01	0.33 ± 0.09
Cartilage	0.16 ± 0.19	0.00 ± 0.00	0.04 ± 0.03	0.58 ± 0.64	6.09 ± 2.54
Dorsal	0.14 ± 0.18	0.03 ± 0.01	0.01 ± 0.01	1.03 ± 1.19	3.46 ± 1.74

[a] n = 3–6. Applied amount = 125 μg.

The low levels of both α-IFN and CsA found in the ventral dermis following topical application to hamster ventral ear appears to be incongruent with the rather significant and large amounts of the drugs found in the cartilage and dorsal ear especially from the Non-1 liposomal formulation. It is well known that the pilosebaceous unit has a rich and elaborate plexus of capillaries that deliver blood to this highly metabolically active area (13). At the level of the sebaceous glands, offshoots from the major plexus parallel to the follicle engulf the glands and the pilary canal. The vascular system of each follicle is a continuous unit from the dermal papilla to the area around the infundibulum and the sebaceous glands. The supply of blood vessels is proportional to the size of the glands so as to provide the raw materials for the synthesis of sebum. The ventral and dorsal sides of the hamster ear contain several large sebaceous glands that resemble the human sebaceous follicles in shape and organization. Thus, they have 2–3 large glands with a common duct and follicular infundibulum and a small vellus hair. These glands are highly vascularized, and since there is no fatty tissue underlying the hamster ear dermis, clearance of drugs from the gland and the pilary canal areas would be directly channeled into the hypodermal plexus adjacent to or embedded in the cartilage of the ear. Further clearance into the dorsal ear *via* the same plexus network of blood vessels would account for the substantial amounts of drug found in the dorsal ear. Such clearance of drug from the dermis into cartilage and dorsal ear would occur regardless of whether the drug was transported *via* a transepidermal or transfollicular pathway. Recently, Schaefer and co-workers (14,15) demonstrated that despite similar blood flow characteristics in normal and regenerated appendage-free rat skin, increased absorption of steroid drugs was consistently observed with normal skin. They further found that the differences in drug amounts between normal and appendage-free skin was greatest in skin strata at the level of the sebaceous glands (40 to 400 μ depth). Thus, it is evident that depending upon the vehicle used, transport of drugs via follicles and sebaceous glands could be a major pathway for drug absorption.

An examination of the data in Tables II and III reveals in general that the amounts of drug label found in the cartilage and dorsal ear are proportional to the level of drug found in the sebaceous glands. It appears, therefore, that increased deposition into the cartilage and dorsal ear may have resulted from the clearance of the drug by the vast vasculature network from the vicinity of the glands. The presence of substantial amounts of the drug found in the glands themselves coupled with the curiously low amounts in the dermis further suggests a predominant and preferred follicular route of drug deposition from the Non-1 liposomal formulation.

We have previously shown (9,16) that the kinetics of dehydration plays an important role in deposition of CsA into hairless mouse skin from nonionic liposomal formulations. There is reason to believe that this may also be the case with respect to follicular drug delivery. From differential scanning calorimetry, the melting points of GDL, GDS and POE are 30°, 54° and 36°C, respectively. Following topical application, the formulation undergoes dehydration at the temperature of the skin (~32°C) and with the Non-1 liposomal formulation, GDL begins to melt at 30°C resulting in fluidization of the liposomal bilayers and partial release of the lipid components from the bilayer configuration. POE-10 alkyl ethers have been shown to be skin permeation enhancers (17) and the enhanced deposition of CsA from Non-1 formulations indicates the importance of the action of "free" POE-10 alkyl ether as an enhancer. It is also evident from Tables II and III that the Non-3 liposomal formulation is not as effective as the Non-1 liposomal formulation in enhancing drug delivery into the pilosebaceous units. By removing GDL from the liposomal formulation, the melting kinetics change substantially and thus may impede the release of POE-10 stearyl ether from the liposomal bilayers. This appears to be reasonable since POE melts above the temperature of the skin and only a small percentage of POE may be released from the bilayers to provide enhancement of drug deposition. Further, any contributions to enhancer action from GDL itself would be absent in the Non-3 liposomal formulation. It is interesting to note that Non-3 behavior is almost similar to that of Non-2 liposomes. The lack of enhancement by the Non-2 liposomal formulation may be the result of GDS and POE remaining associated within the liposomal bilayers over the duration of the experiment.

It is interesting to note the parallel behavior of the liposomal formulations with respect to the amounts of α-IFN or CsA found in the pilosebaceous units. The excellent correlation (r^2 = 0.996) between the two, despite major differences in hydrophobicity/hydrophilicity, suggests that the relative ability of the liposomal formulations in facilitating deposition of a given drug is independent of the drug. The greater extent of CsA deposition compared to that for α-IFN (based upon percent of applied formulation) for a given formulation may indicate the greater ease of partitioning of the highly hydrophobic CsA into a sebum-rich environment.

In conclusion, Non-1 liposomal formulations facilitate the deposition of both hydrophilic and hydrophobic drugs into pilosebaceous units *via* the follicular route. This study also demonstrates the enormous potential for the use of Non-1 liposomal formulations in targeted drug delivery into the follicles. Although a simple explanation for their action is proposed, the driving force for deposition into the follicles and beyond (cartilage and dorsal ear) is a complex phenomenon greatly dependent on formulation factors.

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