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Topical liposome delivery of molecules to hair follicles in mice

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Abstract

The hair cycle consisting of growing and resting phases, is subject to widespread disease such as androgenic alopecia or loss of pigment which are in need of effective, targeted therapeutics. In order to develop a hair-follicle delivery system we demonstrate here that phosphatidylcholine liposomes entrapping either the fluorescent dye calcein or the pigment melanin can deliver these molecules into the hair follicle and hair shafts of mice when applied topically. Liposomal delivery of these molecules is time dependent. Negligible amounts of delivered molecules enter the dermis, epidermis or blood stream thereby demonstrating the enrichment of follicle delivery. Naked calcein and melanin are trapped in the stratum corneum and are unable to enter the follicle. The potential of the hair-follicle liposome delivery system for therapeutic use for hair disease is discussed. Copyright © 1997 Elsevier Science Ireland Ltd.

Keywords: Hair disease; Hair follicle; Liposome delivery

1. Introduction

The hair cycle consists of the growing anagen phase with concomitant melanin production, the regression phase of catagen and the resting phase of telogen. There are many diseases of the hair cycle including androgenic alopecia, hair color loss and alopecia areata. Therapeutics for these diseases that would be most effective if they could be specifically targeted to the hair follicle.

To develop a model system for the treatment of hair disease we have utilized the sponge-gel-matrix three-dimensional histoculture system developed by Leighton (1951) [1] to histoculture skin of mice and human scalp on collagen-containing sponges [2–4]. Newly formed hair shafts are produced in vitro from the histocultured skin as demonstrated by increasing hair shaft length with time [2] as well as by translocation of in vitro [^{3}H]thymidine- or [^{35}S]methionine-labeled hair matrix cells to the hair shaft itself.

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In the hair-producing histocultured skin we have observed that liposomes can directly target the hair follicle [3–6]. Phosphatidylcholine-containing liposomes can deliver fluorescent dyes such as calcein [5], pigments such as melanin [3] and high molecular weight DNA [4] including functional genes [6] directly into the hair follicles of histocultured skin. The delivery seems specific in that the liposomes deliver these low and high molecular weight substances to the follicle without apparent delivery to the dermis or epidermis of the histocultured skin [3–6].

Thus, the liposome-follicle drug targeting system seems promising as a new type of drug delivery system (DDS) for hair diseases. However, in order to be of therapeutic use the liposome-follicle DDS must also be effective and selective in vivo. The subject of this report is the high in vivo selectivity of the liposome-follicle-targeting DDS in mice for delivery of fluorescent dyes and melanin, suggesting the development of a novel, effective and safe approach to the study of the hair cycle and therapy of its disorders.

2. Materials and methods

2.1. Preparation of liposomes

Twenty milligrams of egg phosphatidylcholine (PC) were rotary evaporated from a chloroform solution to form a thin film on the walls of a 5 ml round-bottomed flask for 1 h. The dried thin film lipid was suspended in 0.5 ml phosphate buffered saline (PBS, pH 7.4) on a vortex mixer and then sonicated with a Branson probe-type sonicator fitted with a microtip at power level-3, for 8 min to produce multilamellar liposomes. One-half milliliter calcein solution (10 mg/ml) or melanin solution (10 mg/ml), respectively, were added to the sonicated PC liposome solution described above. The calcein or melanin was then entrapped into the liposomes by sonication for an additional 6 min, which was followed by 3 × freeze-thawing. The liposomes were then extruded through a 0.6–1.0 μm size filter and separated from the non-entrapped calcein or melanin by gel-filtration on a Sepharose 4B column eluted with phosphate-buffered saline (PBS).

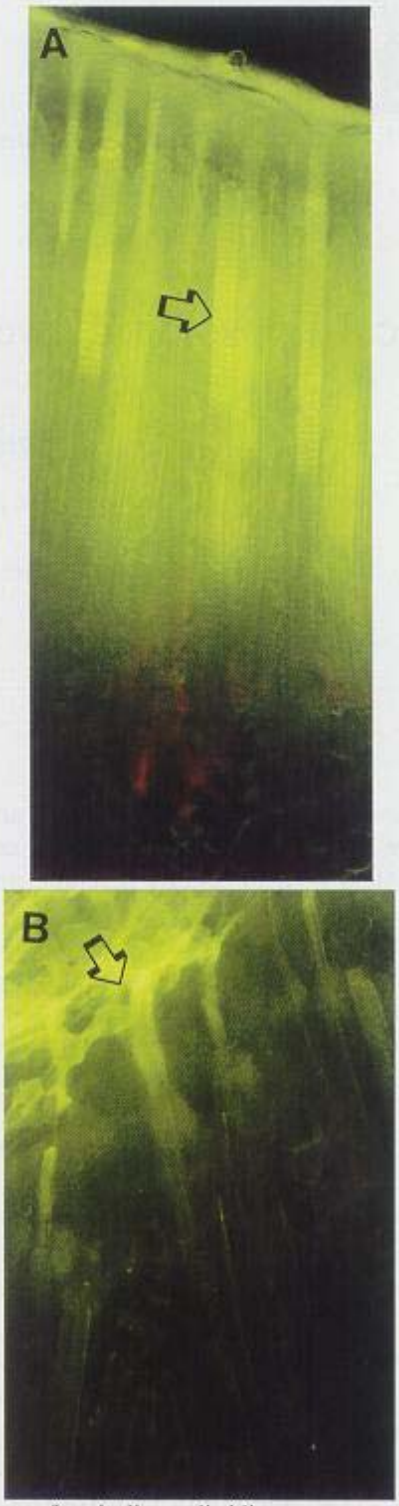

Fig. 1. Delivery of topically applied liposome-entrapped calcein into mouse hair follicles. (A) Shaved white-haired mouse dorsal skin treated with calcein-liposomes with a patch for 20 h in vivo. Note that the liposome-delivered calcein has penetrated into the deep hair shafts and follicles (arrow). (B) Control treated with calcein only. Note that no free calcein could enter the hair shafts and follicles. Free calcein was trapped in the stratum corneum (arrow). Propidium iodide counter-staining. Fluorescent microscopy. Magnification: 100 × .

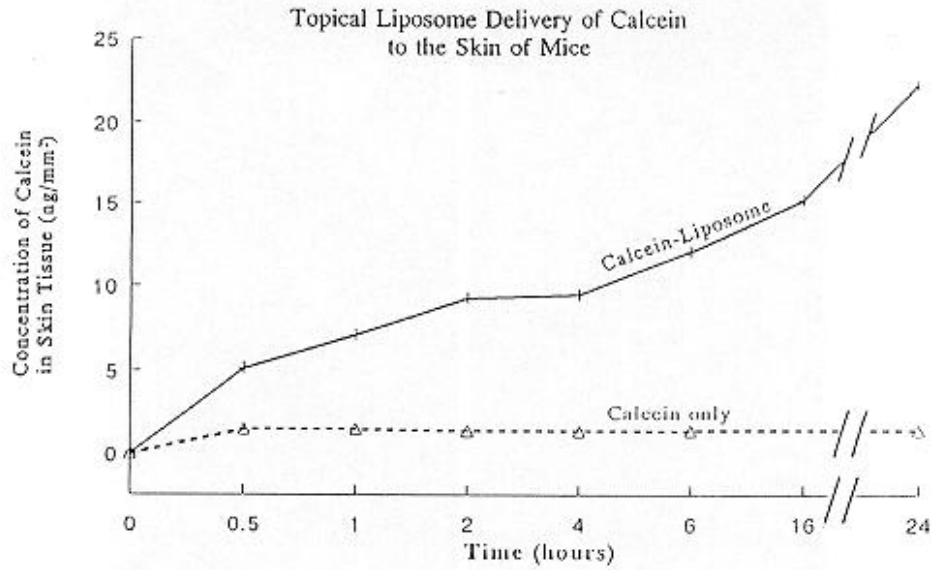

Fig. 2. Time course of delivery of topically applied liposome-entrapped calcein into the skin of the mice compared with naked calcein. Note that the amount of calcein entry into the skin tissue targeted by liposomes increases with time. In striking contrast, almost no naked calcein was delivered to the skin and this did not increase with time.

2.2. Topical in vivo application of liposomes

Two- to four-week-old pre-shaved outbred white-haired mice were used for in vivo topical liposome delivery molecules to hair follicles. Briefly, the liposomes entrapped with calcein or melanin were directly applied on the dorsal skin (approximate area 1.5×1.5 cm^2) of the mice with a sutured 'bandaid' patch to immobilize the liposome preparation and to prevent evaporation. The liposomes were initially re-applied every hour for 6 h with the last preparation remaining overnight for a total of approximately 24 h at which time the skin samples were taken by punch biopsy for analysis. For the time-course experiments one mouse was used for each time point with six punch biopsies taken from each mouse. The punch biopsies were taken at 0.5, 1, 2, 4, 6, 16 and 24 h with the surface of skin cleaned by an alcohol swab in order to eliminate any material remaining on the surface. In the time-course experiments the liposomes were not reapplied. Controls used were the same concentrations of naked calcein or melanin that were entrapped in the liposomes.

2.3. Microscopy to measure efficacy of liposome targeting of calcein and melanin to hair follicles in vivo

After treatment of liposome-entrapped calcein and melanin for 24 h, skin samples were immediately harvested after sacrificing the mice. The skin samples treated with liposome-calcein were cut into very thin pieces of tissue along the vertical direction of the hair follicles and observed by fluorescent microscopy and photographed. For the liposome-melanin treated skin samples, the tissues were first counter-stained by propidium iodide (PI) and then observed by fluorescent microscopy.

2.4. Spectrofluorimetry to measure efficacy of liposome targeting of calcein to hair follicles in vivo

Three samples each containing two pieces of a 2-mm punch biopsy of skin for each time point were put in 2 ml PBS and were sonicated in a water sonication bath for 2 min. The samples were then centrifuged for 10 min in a microcentrifuge at $14\,000 \times g$. The supernatant was then

Fig. 3. Delivery of topically applied liposome-entrapped melanin into mouse hair follicles. (A) Shaved white-haired mouse dorsal skin treated with melanin-liposomes with a patch for 20 h in vivo. Note that the liposome-delivered melanin penetrated into the deep hair shafts and follicles (arrows). (B) Control treated with melanin only. Note that no free melanin could be observed in the hair shafts and follicles and seemed to be trapped in the stratum corneum (arrow). BCECF-AM and propidium iodide counter-staining. Fluorescent microscopy. Magnification: (A) 200 ×, (B) 100 ×.

measured by spectrofluorimetry at an excitation wavelength of 496 nm and an emission wavelength of 517 nm. The concentration of calcein delivered into the skin tissue was determined by comparison with a standard curve.

2.5. Spectrophotometry to measure efficacy of liposome targeting of melanin to hair follicles in vivo

Three samples each containing two pieces of a 2-mm punch biopsy of skin for each time point were put in 2 ml PBS and were sonicated in a water bath for 5 min. The samples were then centrifuged for 10 min in a microcentrifuge at 14 000 × *g*. The supernatant was then measured by spectrophotometry at an absorption wave length of 300 nm. The concentration of melanin delivered into the skin tissue was determined by comparison with a standard curve.

2.6. Determination of calcein in blood after topical application of calcein-liposome

Plasma calcein concentration measurements were made at 0.5, 1, 2, 4, 6 and 24 h after topical application of calcein-liposomes on the back skin of the mice. Blood samples were taken from the lateral tail vein, transferred into a serum separator tube (Vacutainer, Becton Dickinson), and spun at 2000 × *g* for 10 min to isolate the plasma. Calcein was measured by spectrofluorimetry at an excitation wavelength of 496 nm and an emission wavelength of 517 nm. The concentration of calcein in the plasma was determined by comparison with a standard curve.

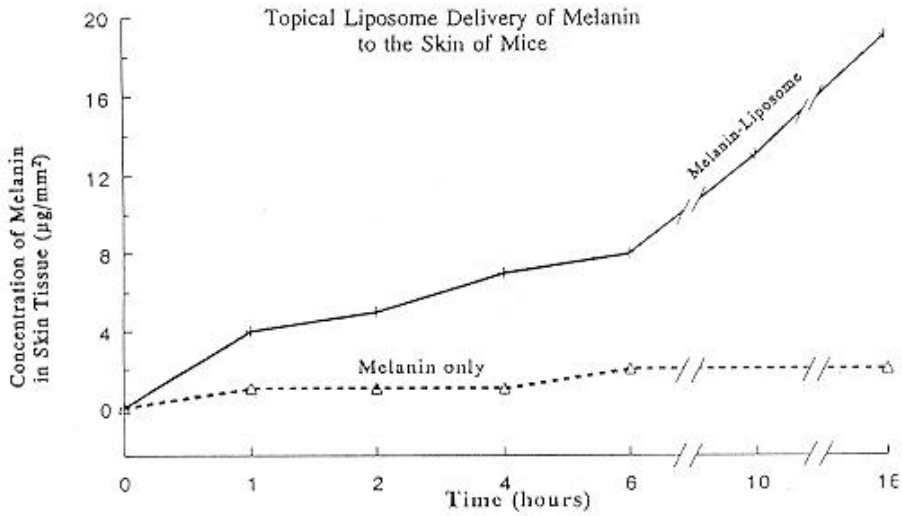

Fig. 4. Time course of delivery of topically applied liposome-entrapped melanin into the skin of mice compared with naked melanin. Note that the amount of melanin entering into the skin tissue delivered by liposomes increased with time. In striking contrast, almost no naked melanin was delivered to the follicles and this did not increase with time.

3. Results and discussion

The liposome delivery of calcein to hair follicles in vivo can be seen in Fig. 1. When calcein entrapped in phosphatidylcholine (PC) liposomes is topically applied to mice, the calcein entered deeply into the hair follicle cells as well as the hair shafts within the follicle (Fig. 1A). In striking contrast, topically applied naked calcein, which is a polar, negatively charged compound, could not enter below the stratum corneum. Indeed, the naked dye was seemingly trapped in the stratum corneum (Fig. 1B).

Fig. 2 shows the time course of entry of liposome-entrapped calcein into the skin of the mice. It can be seen that by 24 h 22 ng/mm^2 of calcein were delivered when the calcein was entrapped in the liposomes. The amount of liposome-delivered calcein increased with time. In striking contrast less than 1.4 ng/mm^2 of naked calcein were delivered and this amount did not increase with time.

Liposome delivery of melanin to hair follicles in vivo can be seen in Fig. 3. Liposome-entrapped melanin, topically applied to the mouse skin entered the hair follicle and hair shaft. Melanin could enter the follicle deeply and also the hair shaft but seemingly not as uniformly as calcein (Fig. 3A). Naked melanin topically applied to the mouse skin could not enter below the stratum corneum and indeed appeared to be trapped in the stratum corneum (Fig. 3B).

Fig. 4 shows the time course of liposome-entrapped melanin into the skin. It can be seen that by 16 h 19 μg/mm^2 of melanin were delivered to the mouse hair follicles, when the melanin was entrapped in the liposomes. In striking contrast less than 2 μg/mm^2 of naked melanin were delivered and this amount did not increase with time.

Thus, the experiments described in this report indeed demonstrate that liposomes can deliver molecules to the hair follicle in vivo. Histological preparations (Fig. 5) also demonstrate that the liposome-targeted melanin was delivered to the hair follicle cells and hair shafts within the follicle. Fig. 5A and Fig. 5B show melanin delivered to hair shafts within the follicle of the white-haired mouse. Note that the liposome-delivered melanin acquires the exact pattern that occurs in naturally melanized mice.

These results are consistent with our previous results demonstrating that liposomes deliver calcein [5], melanin [3] and DNA [4] to the hair follicles of histocultured skin and a functional gene to hair follicles of mice [7]. Initially, in skin

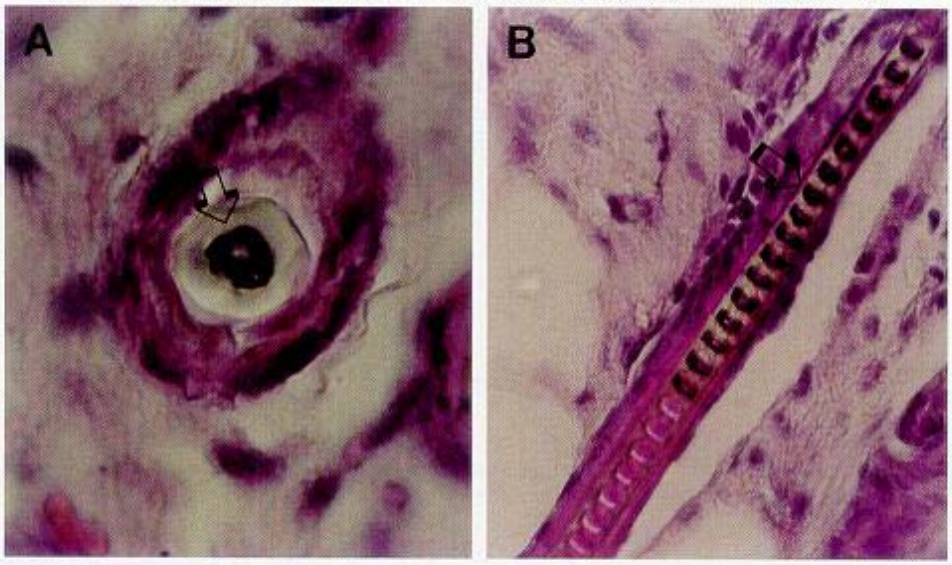

Fig. 5. Hematoxylin- and eosin-stained paraffin-section of white-haired mouse skin topically treated with melanin-entrapped liposomes in vivo for 24 h. (A) and (B) show melanin delivered into the hair shafts within the hair follicles (arrows). Note that the delivered melanin acquires the exact pattern of naturally melanized hair shafts. Magnification: (A) 1000 ×, (B) 200 ×.

histoculture, we observed that liposomes can deliver the dye calcein, melanin, high molecular weight DNA and the active Lac-Z gene into hair follicles of histocultured mouse skin. The results showed that the liposome-entrapped molecules were only delivered into the hair follicles without entry to the epidermis or dermis [3–6]. These results have been confirmed by liposome delivery of the active Lac-Z gene into hair follicles of mice subsequent to topical application [7]. After topical application of liposome-lac-Z gene on mice skin, the expression of the Lac-Z gene was found only in the hair follicles. The tissue outside of hair follicle was completely negative [7]. Here we demonstrate the important result that liposomes can deliver calcein and melanin to the hair follicle of a living animal with the melanin acquiring the normal pattern in the hair shafts of endogenous melanin.

Fig. 6 demonstrates that when calcein-liposomes are topically applied to the mouse skin, calcein is delivered in a time-dependent manner to the skin. In contrast, over the 24 h period of the experiment, no detectable calcein enters the blood circulation. This is a very important result in that it shows that a molecule, in this case calcein, can be delivered to the skin without entry to the systemic circulation, thus indicating that safe follicle delivery of a product is possible. As shown in Figs. 1, 3 and 5, the morphological observations indicate that the vast majority of the molecules delivered by liposomes were located in the hair follicles or hair shafts. Thus, as shown in Figs. 2, 4 and 6, we assume that the measurement of calcein or melanin in the total skin is mainly incorporated into the hair follicles.

Recently, Yarosh et al. [8,9] demonstrated that liposomes can deliver the DNA repair molecule T4 endonuclease V to the skin where it stimulated the removal of UV-light induced cyclobutane pyrimidine dimers with concomitant reduction of squamous-cell carcinomas in mice. Yarosh et al. [9] demonstrated that hair follicles were targeted by the endonuclease-containing liposomes. Similarly Lieb et al. [10] showed delivery of calcein by topically applied multilamellar liposomes to pilosebaceous units in the hamster ear isolated in vitro. Recently, Balsari et al. [11] also reported that liposome can deliver monoclonal antibodies into hair follicles of rats for protection against doxorubicin-induced alopecia. Yarosh [8] also reported very little entry into the blood after topical liposome delivery of the T4 endonuclease. Prausnitz et al. [12] have recently reported that electroporation can cause fluxes in excess of 10 $\mu g/cm^2$ per h of calcein to occur on rodent skin with significant delivery to the blood. This is in contrast to liposome targeting as can be seen in Fig.

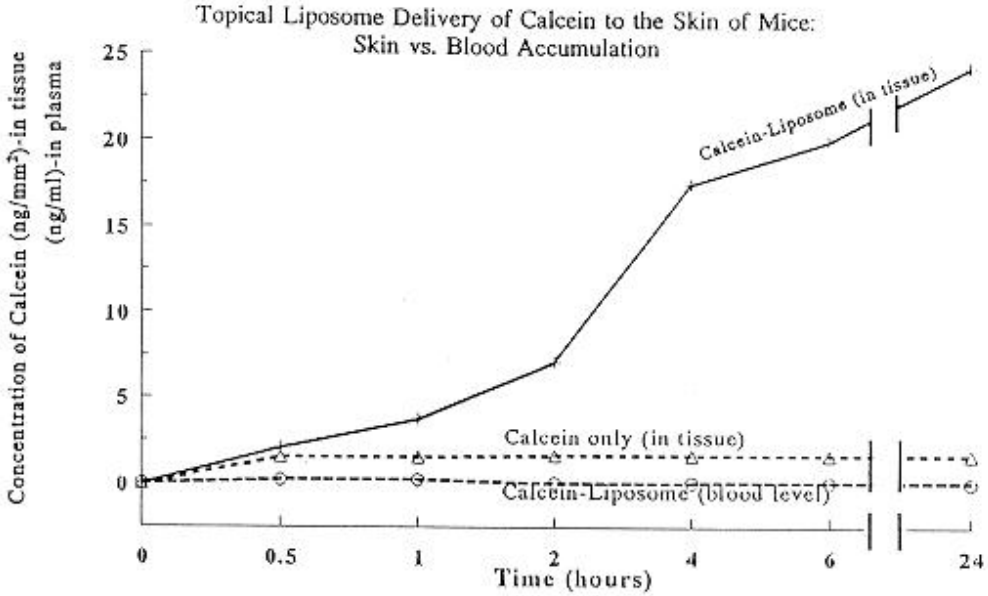

Fig. 6. Calcein liposomes topically applied to mouse skin. Note the increasing delivery over time without entering the blood circulation.

6 which demonstrates that the liposomes indeed deliver calcein to the follicle with little transfer to the blood. The follicle delivery by liposomes with little blood entry is of crucial importance for safety reasons as mentioned above for transferring molecules such as antiandrogens or genes that one wants to deliver only to the follicle.

A major question is how have the liposome-entrapped molecules reached the hair shafts? One hypothesis is that when liposomes are topically applied on the skin, the liposomes penetrate via a 'lipid-rich channel' coating the hair follicles with subsequent entry into the hair follicle keratinocytes. We believe there are two ways of liposome- entrapped molecules reaching the hair shafts. One is that the liposomes first deliver the molecules into the hair matrix cells, then the molecules move to the hair shafts by differentiation and hair growth. Second is that the liposomes may directly penetrate into the hair shafts from the hair tip and the possibly the side of the hair shafts.

In our most recent experiments (unpublished data), we have shown that liposomes can also deliver calcein into hair follicles in histocultured human scalp tissue without detectable calcein being found in non-follicles sites. These data demonstrate that the liposome delivery to hair follicles is effective in human skin.

Thus, the liposome hair-follicle delivery system has great potential to test the effect of drugs and genes on the hair follicle and on the hair process for cure of hair diseases without significant systemic side-effects.

Acknowledgements

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