ELSEVIER

International Journal of Pharmaceutics 162 (1998) 29–38

international journal of pharmaceutics

Targeted follicular delivery of macromolecules via liposomes

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Accepted 7 November 1997

Abstract

The hair follicle, hair shaft and sebaceous gland collectively form what is recognized as the pilosebaceous unit. This complex, 3-D structure within the skin, possesses a unique biochemistry, metabolism and immunology. Recent studies have focused on the hair follicle as a potential pathway for both localized and systemic drug delivery. Targeted drug delivery may enhance current therapeutic approaches to treating diseases of follicular origin. In a growing number of topical studies, liposomes have been shown to target drug delivery to the pilosebaceous unit. Presented here is a summary of some of our recent work with a particular focus on studies which have demonstrated targeted follicular delivery via liposomes.

Keywords: Pilosebaceous unit; Hair follicle; Liposomal formulations; Follicular drug delivery

1. Introduction

Increasingly, the hair follicle is gaining acknowledgment as a complex, dynamic structure that may contribute significantly to passive transport of compounds into the skin. Studies within the past half century (MacKee et al., 1945; Montagna, 1954; Feldmann and Mailbach, 1967; Scheuplein, 1967; Rutherford and Black, 1969; Scheuplein et al., 1969; Nicolau et al., 1987; Bidmon et al., 1990; Fabin and Touitou, 1991) have yielded strong qualitative and quantitative evidence of a follicular pathway for a wide variety of compounds, suggesting that this pathway may be more significant than previously assumed. Early reports of a transient follicular pathway were based primarily on qualitative, histological studies of dye and stain localization in the hair follicle (MacKee et al., 1945; Montagna, 1954; Scheuplein, 1967; Rutherford and Black, 1969; Scheuplein et al., 1969). In additional studies, it was found that greatest absorption of some compounds occurred at sites with greatest follicular density (Feldmann and Mailbach, 1967). More recent studies have yielded increasingly quantitative data which characterize follicular transport as a complex phenomenon dependent upon compound and/or vehicle composition, and may occur over several hours (Feldmann and Mailbach, 1967; Nicolau et al., 1987; Bidmon et al., 1990; Fabin and Touitou, 1991).

0378-5173/98/$19.00
PII S0378-5173(97)00409-2

The specific role of the hair follicle in percutaneous transport remains difficult to elucidate due to the lack of an adequate animal model to distinguish follicular from non-follicular transport. Nevertheless, the concept of targeted drug delivery to the hair follicle remains a worthwhile consideration with regard to the potential applications for treating multiple hair follicle-associated dermatological conditions and hair growth abnormalities.

Topically applied liposomes have been used frequently in attempts to enhance percutaneous absorption of several compounds (Mezei, 1988; Schreier and Bouwstra, 1994). In a growing number of studies, topically-applied liposomes have been demonstrated to be efficient in specifically targeting the delivery of a wide variety of compounds into the hair follicle. In this report, recent studies carried out in the laboratories will be discussed with regard to the potential use of liposomes as vectors for targeted transport of macromolecules to the hair follicle and sebaceous gland.

2. Animal models for assessment of follicular delivery

A major limitation in elucidating the follicular pathway is the current lack of an adequate pharmacokinetic model that can clearly distinguish transfollicular from transepidermal percutaneous absorption. Hairless rodents have been used commonly in percutaneous absorption studies, but cannot exclusively represent the transepidermal pathway since they are not follicle-free. Follicles are present in these rodents that are underdeveloped and very different from those of hairy animals (Osborne and Hatzenbuhler, 1990). Follicle-free skin, created by scarred formation after burning (Schaefer et al., 1990), must still be further characterized to ensure that the resultant skin is not different in other ways from skin with hair follicles.

In addition to the lack of a well-characterized model, problems associated with assessment techniques have also made elucidation of the follicular route difficult. In tape stripping studies, incomplete stripping may result in artificially high detection of compounds in the residual skin. Conversely, tape stripping may also result in underestimation of follicular deposition if follicular contents are stripped away. In microscopic visualization studies, skin must be carefully sectioned and minimally fixed to avoid cross-contamination of sections. Autofluorescence within both the skin and the hair follicle necessitates careful interpretation of fluorescence microscopy studies.

The Syrian hamster ear has been proposed as an excellent animal model for the human sebaceous gland (Plewig and Luderschmidt, 1977; Matias and Orentreich, 1983). The ventral side of the Syrian hamster ear is rich in sebaceous glands and resemble human sebaceous glands in that they are large and androgen-sensitive. Typically, the ventral side of the ear is treated with a given volume of a test formulation, usually 50 μl. At either 4, 8 or 12 h, the animals are sacrificed by a lethal dose injection of pentobarbital and the ear is excised and stripped repeatedly with tape to remove the stratum corneum. The ventral ear is then separated from the dorsal ear. The sebaceous glands in the ventral dermis and the cartilage are scraped free with a dull scalpel to avoid any damage to the surrounding dermis. The pilosebaceous material is removed by first placing the ventral dermis on a glass slide, dermis side up, and scraping more intensely than for cartilage removal. A milky white suspension is subsequently collected. The dermis is then examined using a Nikon Diaphot microscope under both light and fluorescence conditions to confirm removal of the sebaceous glands. Dual labeling (active and liposomal lipid) allows us to determine if the active is diffusing independently into the target site or if the active remains associated with constituents of the liposome. Fluorescent probes are employed to gain further insight into the kinetics of deposition of the macromolecules and liposomal bilayers into the substructures of the pilosebaceous units. We have successfully employed both quantitative fluorescence and confocal microscopy to semi-quantitatively determine the extent and time dependencies of deposition of hydrophilic markers, lipid bilayer markers and antibodies within the follicle following the topical application of liposomal formulations.

Table 1
Summary of liposomal formulations used in the studies

CsA formulations

Liposomal formulation	Lipid composition	Mole or weight ratio	Saturation level of entrapped CsA (mg/ml)
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-Interferon formulations

Liposomal formulation	Liposomal composition	Mole or weight ratio	α-IFN concentration (IU/ml)
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

By comparing deposition of the actives in the hairy rat and hamster, we can answer questions concerning how differently actives are deposited into follicles that are fully formed and those that are incompletely formed. The pilosebaceous glands of the hairy rat are much smaller than those of the hamster ear and thus represent a relatively much lower fraction of total follicular pathway into the dermis. The hairs of the hairy rats are clipped carefully with an electric clipper 24 h prior to formulation application. This model promises to be particularly useful for studying the kinetics of deposition in different regions of the fully developed hair follicle. For these studies, excised skin are frozen in OCT solution with liquid nitrogen. The frozen skin is then cryosectioned into 20 μm vertical sections and viewed by confocal microscopy. Since many of the test compounds of interest are not dyes, alternative analytical methodologies are now being employed in the laboratories. In many cases, radiolabels are available and autoradiographic techniques have proven to be quite useful.

3. Topical delivery of peptide drugs into pilosebaceous units

The purpose of this study was to test the hypothesis that nonionic liposomes facilitate the topical delivery of peptide drugs into pilosebaceous units (Niemiec et al., 1995). The hamster ear was used as a model for human pilosebaceous units. The deposition of a hydrophilic protein, α-interferon (α-IFN), into pilosebaceous units and other strata of the hamster ear 12 h 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. The deposition of a hydrophobic peptide, cyclosporin-A (CsA), into pilosebaceous units and other strata of the hamster ear after topical in

Table 2
Distribution of α-IFN in various strata of Syrian hamster ear (expressed as IU $\pm$ S.D.) 12 h after topical in vivo application of various formulations

Strata	Formulation				
	AQ	PC	Non-3	Non-2	Non-1
Pilosebaceous units	2400 $\pm$ 2200	11 000 $\pm$ 3600	2300 $\pm$ 900	6100 $\pm$ 2500	49 500 $\pm$ 13000
Dermis	400 $\pm$ 400	700 $\pm$ 400	200 $\pm$ 100	400 $\pm$ 100	2000 $\pm$ 1500
Cartilage	300 $\pm$ 200	1200 $\pm$ 1600	1200 $\pm$ 1300	2300 $\pm$ 1300	50 500 $\pm$ 34000
Dorsal	300 $\pm$ 200	1100 $\pm$ 1500	900 $\pm$ 1000	1600 $\pm$ 500	37 500 $\pm$ 32500

$n = 4$–7. Applied amount $= 5 \times 10^6$ IU.

vivo application of these liposomal formulations and a hydroalcoholic control solution (HA) were also determined.

The liposomal formulations used are summarized in Table 1. 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 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.

Male Golden Syrian hamsters were anesthetized with sodium pentobarbital and 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 h, the hamsters were sacrificed and the ears removed by cutting across the base and processed to separate the ventral ear strata (dermis, pilosebaceous unit and cartilage) from the dorsal ear.

Table 2 shows the distribution of radiolabeled α-IFN marker in the various compartments of golden Syrian hamster ear 12 h 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 $\gg$ 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 3 shows the distribution of radiolabeled CsA in the various compartments of golden Syrian hamster ear 12 h 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 $\gg$ 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 ($p < 0.01$).

The low levels of both α-IFN and CsA found in the ventral dermis following topical application to

Table 3
Distribution of CsA in various strata of Syrian hamster ear (expressed as $\mu g \pm$ S.D.) 12 h after topical in vivo application of various formulations

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

$n = 3$–6. Applied amount = 125 μg.

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.

An examination of the data in Tables 2 and 3 reveals that the amounts of drug label found in the cartilage and dorsal ear are generally 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.

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.

Thus, 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 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.

4. Topical delivery of liposomal monoclonal antibodies into the hair follicle

Chemically-induced alopecia is a side effect produced by anti-cancer treatment with doxorubicin. Balsari et al. (1994) examined the effect of liposomally-entrapped monoclonal antibodies on alopecia induced rats and demonstrated that topical treatment with liposome-incorporated monoclonal antibodies prevented alopecia. The recent collaborative studies with this group suggest strongly that the follicular route is the primary pathway for penetration.

For these in vivo studies, liposome formulations containing MAD-11 were evaluated with the intent of optimizing their lipid composition and concentration, liposome particle size and charge and extent of drug entrapment using quantitative deposition of liposomal MAD-11 into hairless rat skin. This was determined by radiolabel assay of an ^{125}I-F(ab′)$_2$ IgG antibody. Formulations were applied to the dorsal skin surface for up to 12 h.

Table 4
Distribution of MAD-11 (expressed as a percentage of applied dose ± S.D.) in various strata of rat skin 12 h after in vivo topical application of various formulations containing MAD-11, 0.5 mg/ml

Formulation	Strips 4–9	Strips 10–25	Viable skin	% Recovery
Aqueous	7.31 ± 6.80	0.73 ± 0.42	0.03 ± 0.00	95.5 ± 2.42
PC/CH/PG (sonicated w/MAD-11)	16.7 ± 2.73	3.45 ± 1.37	0.17 ± 0.03	95.3 ± 1.69
PC/CH/PG (with entrapped MAD-11)	6.31 ± 3.75	1.00 ± 0.64	0.04 ± 0.03	101 ± 0.44
Neutral novasome GDL/CH/POE	18.0 ± 5.12	2.80 ± 1.23	0.04 ± 0.00	95.4 ± 4.15
Negative novasome GDL/CH/POE/PS	43.9 ± 4.09	9.48 ± 0.37	0.20 ± 0.08	96.0 ± 1.74

$n = 3$.

The rat was euthanized and the skin was excised, stripped and analyzed for radiolabel. The hamster ear was also used to assess deposition of MAD-11 directly into the sebaceous glands from liposomal formulations.

In another in vivo approach, the effect of formulation on deposition of antibody in fully developed follicles was studied in the hairy rat by assessing the localization of a fluorescent antibody, FITC-MAD-11 by confocal laser microscopy. Rats were euthanized and their excised skins were frozen in OCT solution with liquid nitrogen following application of the delivery systems. The frozen skin was then cryosectioned into 20 μm vertical sections and examined under the confocal microscope to access the depth of penetration of the antibody into the follicle.

Based on the promising studies involving the delivery of other substances into follicles, the Novasome I liposome was the first system tested. A preliminary clinical study carried out in Milano was disappointing in that nonionic liposomes containing MAD-11 offered no protection against doxorubicin-induced alopecia. A concurrent rat study in the laboratories indicated that these liposomes failed to deposit MAD-11 into the deeper skin strata. Based on the intriguing results that a crude phospholipid liposomal preparation similar to the one used in the rat studies had been somewhat active clinically, we began work on optimizing the in-house formulation as a function of lipid concentration, sonication effects and drug entrapment. The basic phospholipid liposomal formulation contained phosphatidylcholine (PC), cholesterol (CH) and phosphatidylserine (PS) at a mole ratio of 1.0:0.5:0.1, respectively. Table 4 summarizes how formulation parameters affect antibody deposition into the skin. The greatest deposition of MAD-11 from phospholipid liposomes into the deeper skin strata was attained by using 75 mg/ml lipid and by sonicating the MAD-11 with the liposomes. Addition of free MAD-11 to empty phospholipid liposomes without sonication or sonication of liposomes before adding MAD-11 both resulted in significantly less deposition into the viable skin of hairless rats. An aqueous MAD-11 formulation, used as a control, was ineffective in transporting MAD-11 into the deeper skin strata of hairless rats. Introduction of a negative charge to the Novasome liposomes by the addition of phosphatidylglycerol (PG) (GDL/CH/POE:PG 52/15/28/5, weight ratio) resulted in increased deposition into the deeper skin strata of hairless rats, with a remarkably elevated amount in the last stratum corneum strips. Sonication of this formulation with MAD-11 did not significantly alter deposition. The results from these liposome studies collectively suggest that a charge–charge interaction may occur between the negatively charged liposome and the positively charged antibody, and this interaction may be needed to transport MAD-11 into the skin.

In the hamster ear studies, the results were similar in that only charged liposomes led to deposition of MAD-11 into the sebaceous glands (0.25% of applied dose from all negatively charged liposomes tested and no deposition from neutral liposomes or aqueous solution), thereby suggesting a follicular route of delivery. Confocal microscopy was also used to view hair follicles to which FITC-MAD-11 in liposomal formulations was applied. In all sections, fluorescent label was

localized in the stratum corneum, hair follicle openings and within the hair follicle. Most importantly, there appears to be a high level of antibody deep within the follicle at the level of the matrix cells.

These studies provide evidence that the composition of liposomes must be custom-tailored to a drug to be transported effectively to the active site. Whereas, the nonionic liposomes described in previous studies have little difficulty in penetrating deep into the hair follicle, they failed to facilitate deposition of the high molecular weight, positively charged antibody. The addition of a negatively charged lipid in the bilayer greatly facilitates the deposition of the antibody to the target sites deep within the hair follicle. These findings suggest that the observed prevention of doxorubicin-induced alopecia may have been mediated through direct penetration of the hair follicle.

5. Topical application of a novel liposome-plasmid DNA formulations in vivo

Topical delivery of gene vectors to cells within the skin is an attractive strategy for gene therapy of many human diseases, including a number of dermatological conditions thought to be mediated by abnormal regulation of soluble cytokines. While it is highly unlikely that macromolecules can permeate the stratum corneum, the presence of follicles and associated structures may not only allow localized delivery to viable skin cells, but may also promote diffusion of transgenic soluble proteins into the surrounding tissue and/or the systemic circulation. The development of pharmaceutical reagents that can mediate transfection of epidermal cells would have far reaching experimental and therapeutic applications. For topical gene therapy to be successful, it will be necessary to optimize delivery of recombinant DNA to accessible target cells within living skin strata using vehicles that can overcome the formidable permeability barriers of the skin and its appendages.

We hypothesized that expression plasmid DNA could be substituted as the charged macromolecule in nonionic liposomal formulations. The goal of this substitution was the development of a topical formulation with two essential physicochemical properties required for transfection of perifollicular skin cells in vivo: (1) transdermal delivery of large amounts of plasmid DNA proximal to perifollicular cells, and (2) intracellular delivery of the DNA into the target cells. Due to the fact that successful gene delivery in vivo is best assessed by the use of theoretically relevant and biologically active transgenes (as opposed to marker transgenes), the cDNA for human interleukin-1 receptor antagonist protein was used as a transgene in the studies. In this report we show that an expression plasmid encoding the cDNA for human IL-1ra protein formulated with nonionic and cationic lipid components can be used as a topical pharmaceutical reagent for the transient transfection of skin cells in vivo.

Expression plasmid DNA for the human interleukin-1 receptor antagonist (IL-1ra) protein was formulated with nonionic:cationic (NC) liposomes or phosphatidylcholine:cationic (PC) liposomes and applied to the auricular skin of hamsters in single and multiple dose protocols. Confocal microscopy identified delivery of plasmid DNA proximal to perifollicular cells, and successful transfection of perifollicular cells was identified by immunohistochemistry and ELISA. Skin treated for 3 days with the NC liposomes had statistically significant levels of transgenic IL-1ra present for 5 days post-treatment. Expression of transgenic IL-1ra was specific to areas of skin treated with NC liposomes but not PC liposomes. The results indicate that the NC liposomes can deliver expression plasmid DNA to perifollicular cells and mediate transient transfection in vivo.

The nonionic/cationic (NC) liposomal formulations used in the experiments contained glyceryl dilaurate (GDL), cholesterol (CH), polyoxyethylene-10-stearyl ether (POE-10) and 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP) at a weight percent ratio of 50:15:23:12. The lipid mixture also contained α-tocopherol (1% by weight of total lipids). Appropriate amounts of the lipids were mixed and melted at 70°C in a sterile polystyrene centrifuge tube. The lipid melt was then filtered through a 0.22 μm filter (Nucleopore®) and the filtrate was reheated

in a water-bath at 70°C prior to being drawn into a sterile syringe. A second syringe containing sterile, autoclaved, double-distilled water was preheated to 65°C and connected via a 3-way sterile stopcock to the lipid phase syringe. The aqueous phase was then slowly injected into the lipid phase syringe. The mixture was rapidly passed back and forth between the two syringes while being cooled under cold tap-water until the mixture was at room temperature and stored at 4°C until use. The total lipid concentration in the suspension was 100 mg/ml.

The ventral side of the male hamster ears were carefully shaved 1 day prior to the experiments. The hamsters were anesthetized and 50 μl of the test formulation containing the pSG5IL-1ra plasmid DNA were applied to the ventral surface of one ear, twice daily for 3 days. The contralateral ear was treated with an equivalent amount of liposomes without plasmid DNA (control). Additionally, a set of control animals was treated as described above with NC liposomes containing pSG5lacZ plasmid DNA. The total amount of lipid applied per ear of NC based liposomes was 15 mg (2.5 mg/dose) and the total lipid applied per ear with the PC-based liposomes was 11.25 mg (1.875 mg/dose). For both NC and PC based formulations the total amount of DNA applied was 1.05 mg (0.175 mg/dose). At 1 day later (15 h after the last application of the test formulations), the hamsters were sacrificed and the ears excised by dissection across the base. Kinetics of transgene expression following topical application of NC liposomal pSG5IL-1ra plasmid DNA and blank NC liposomes was studied by sacrificing treated animals at 1, 3, 5 and 8 days after the last application. Ears of untreated animals were also used as negative controls. All experiments were carried out under non-occluded conditions. At the time of sacrifice, the ears were isolated by sharp dissection, weighed and measured along each border (in order to calculate the surface area exposed to treatment), then processed for either confocal laser scanning microscopy using fluorescently labeled plasmid DNA, Southern analysis, detection IL-1ra by immunohistochemistry or assay of soluble expressed protein from various strata of the hamster ear (Niemiec et al., 1997).

The fluorescent studies showed that the delivery of the labeled DNA into the hair follicles and perifollicular glands appeared to be complete by 24 h post administration. Control animals treated with an aqueous formulation containing an equivalent dose of the fluorescently labeled plasmid failed to show evidence of DNA beyond the superficial epidermis 24 h after topical application. This indicates that perifollicular delivery is a physicochemical property specific to NC liposomal formulations.

Southern analysis data revealed that most of the plasmid DNA present within the skin was in the form of closed circular or linearized plasmid. Analysis of skin samples obtained at various times after the topical application of a single dose showed similar amounts of expression plasmid DNA present within the skin from 12–24 h after treatment. These results indicate that for the first 24 h post-administration, plasmid DNA was not subjected to progressive degradation and suggests that some of the plasmid was delivered intracellularly and may have been protected from digestion by extracellular nucleases.

We next tested the ability of the NC liposomal formulation to mediate transfection of the perifollicular cells proximal to the in vivo location of the delivered expression plasmid DNA. The in vivo expression of transgenic human IL-1ra was initially detected by in situ immunohistochemical staining using a monoclonal antibody specific for the human IL-1ra protein. The NC liposomal formulation also functioned as a transfecting reagent. Transfected human Il-1ra expressing cells were identified within the follicles in the proximal third of the hair shaft and occasionally at the base of the hair shaft. Negative controls treated with aqueous formulations of expression plasmid DNA, or with liposomes alone, failed to show evidence for IL-1ra expressing perifollicular cells.

We then examined the kinetics of hIL-1ra expression within treated skin over an 8 day period following a multiple dose (twice daily for 3 days) topical application protocol. Transgenic expression of human IL-1ra in the skin of the ventral ear was detected at its highest levels on day 1 after application of the final topical dose. The levels of transgene expression remained significantly above

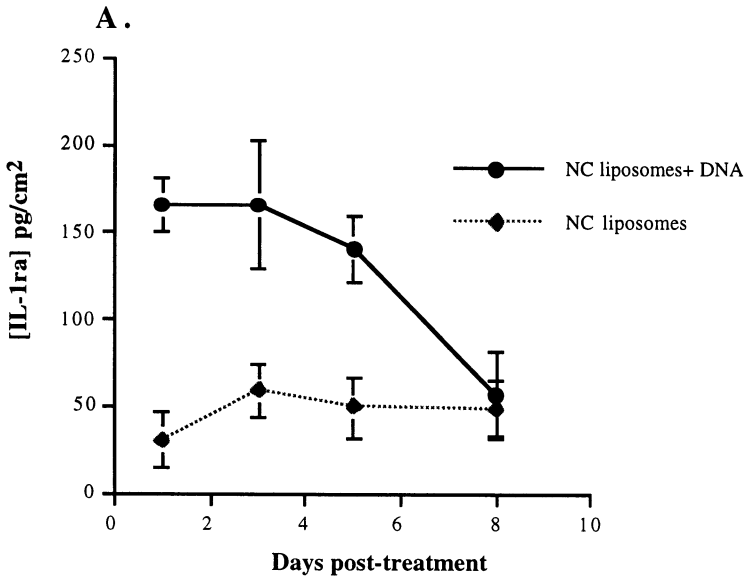

Fig. 1. Expression of human IL-1ra in the ventral skin of the hamster ear following topical in vivo application of nonionic/cationic (NC) liposomes with and without plasmid DNA.

control values ($p < 0.005$) on days 1–5, and had returned progressively to baseline levels by day 8 (Fig. 1). Ear cartilage and dorsal skin were also assayed for transgenic human IL-1ra, however, all of these values were at or below the detection limits of the ELISA (29 pg/ml) and no significant differences were observed between animals treated with NC liposomal DNA and those treated with NC liposomes alone. It was also found that samples of ventral ear, glands, cartilage and dorsal skin obtained from control animals treated with NC liposomes + pSG5lacZ plasmid DNA exhibited transgenic human IL-1ra levels that were below the detection limits of the assay. In addition, no transgenic human IL-1ra was detected in the serum of the treated or control animals. These results suggest that expression of transgenic protein is confined to tissues locally targeted by the NC liposomal pSG5IL-1ra plasmid DNA formulation, and that the diffusion of transgenic IL-1ra protein is largely confined to the microenvironment proximal to the point of topical application. These results corroborate immunohistochemical analysis of treated skin showing expression of transgenic hIL-1ra in vivo.

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