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Interaction of liposome-associated all-trans-retinoic acid with squamous carcinoma cells

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Summary. Because of their antiproliferative and differentiation-inducing properties, retinoids have been used clinically as therapeutic and chemopreventive agents against squamous-cell carcinomas (SCC). As is the case for many therapeutic agents, however, the administration of retinoids is associated with toxic effects. Because encapsulation of certain drugs in lipid vesicles (liposomes) has been shown to result in reduced toxic effects, we studied the in vitro interaction of liposome-encapsulated all-*trans*-retinoic acid (L-ATRA) with a SCC line (MDA 886Ln) and its multicellular tumor spheroid (MTS) model. Various L-ATRA formulations were tested for incorporation of retinoic acid, toxic effects against human red blood cells, uptake and retention by tumor cells, and antiproliferative effects against SCC. Of the different formulations tested, L-ATRA containing diphosphatidyl palmitoylcholine (DPPC) and stearylamine (SA; 9:1, w/w) showed optimal drug incorporation, high stability, and minimal toxicity toward red blood cells and was highly efficacious in delivering ATRA and, thus, in inhibiting the growth of MDA 886Ln and its MTS model. DPPC: SA L-ATRA inhibited the expression of the enzyme keratinocyte transglutaminase in epidermal cells as effectively as did the free drug. These results suggest that liposomes can serve as an effective carrier system for the delivery of retinoids to SCC.

Key words: Liposomes – Retinoids – Squamous-cell carcinoma – Transglutaminase

Introduction

Numerous reports have described the effects of retinoids on the growth and differentiation of normal, premalignant, and malignant cells in tissue explants, cell cultures, and established cell lines [6]. Recent observations in patients with acute promyelocytic leukemia [12], cervical cancer [9], and metastatic squamous-cell cancers (SCC) of the skin [8] have kindled further interest in exploring retinoids as therapeutic agents for cancer treatment. All-*trans*-Retinoic acid (ATRA) induces cell differentiation in some human acute myeloid leukemia patients. In vitro observations and several case reports have suggested that leukemic cells of acute promyelocytic leukemia are sensitive to the differentiative effects of retinoids [1, 3].

Retinoids have a vital role in the normal differentiation and growth of epithelial tissues. Epithelial cells manifest biochemical, morphological, and functional changes if deprived of or given an excess of retinoids. In cases of vitamin A deficiency, the columnar and transitional epithelium in several organs is replaced by stratified squamous keratinized cells [13]. These changes are reversible, however, and repletion of vitamin A causes the ciliated and mucus-secreting columnar cells to reappear. Because they are derived from epithelial tissues, SCC may be ideal targets for retinoid therapies. Retinoids have been shown to induce antiproliferative and differentiative effects in various SCC cell lines as well as in multicellular tumor spheroids (MTS) [7, 19, 21]. They have also been shown to inhibit the expression of various biochemical markers of squamous differentiation, including the enzyme keratinocyte transglutaminase ($TGase_k$) and its substrates involucrin and loricrin as well as other cross-linked envelope proteins [7]. There is evidence that retinoids can suppress the development of oral SCC in experimental animals exposed to various carcinogens [2, 23]. In leukoplakia, a premalignant lesion of oral cancer, retinoids cause the existing disease to regress and prevent the formation of new lesions and the progression of existing disease [5].

Administration of retinoids at therapeutic doses leads to acute or chronic hypervitaminosis syndrome, resulting in

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symptoms such as hepatosplenomegaly, dry skin, bone pain, and headaches as well as long-lasting teratogenicity [24]. Encapsulating retinoids in liposomes may provide an alternative way of delivering the drug without the resultant toxicity. Liposomal encapsulation is known to decrease drug toxicity, sequester the drug as particles at tumor locations, protect the drug from rapid metabolism, amplify its therapeutic effect, and improve the solubility of lipophilic drugs such as retinoids [25]. Because of their potential for reducing drug toxicity, liposomes are currently being evaluated both clinically and experimentally as a drug delivery system. In this study we examined the ability of liposomes to deliver ATRA to and induce the differentiation of SCC cell lines. Since the charge and the fluidity of liposomal membranes play important roles in the delivery of entrapped drugs to the target cells, various lipid compositions were tested to identify the formulation that would most effectively deliver ATRA to the SCC cell line MDA 886Ln and its MTS model.

Materials and methods

Cell culture. Cell line MDA 886Ln was derived from a lymph-node metastasis of an SCC of the larynx in a 64-year-old patient at the M.D. Anderson Cancer Center [18]. The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (ICN Flow, Irvine, Calif.) supplemented with 10% fetal calf serum and antibiotics (100 μg streptomycin/ml and 100 U pencillin/ml). Spheroids were initiated by plating logarithmic-phase cells onto petri dishes precoated with 1.25% agarose in normal medium as described earlier [18, 19]. Normal human epidermal keratinocytes (NHEK; Clonetics Corporation, San Diego, Calif.) were also used and were in their second or third passages at the time of assay. These cells were cultured in KGM medium, which is a modified MCDB 153 formulation (also purchased from Clonetics Corporation).

Preparation of liposomes. Various lipids in chloroform (Avanti Polar Lipids, Alabaster, Ala.) were dried on a rotary evaporator (Brinkmann Instruments Inc., Westbury, N.Y.). The dried lipids were dissolved in tertiary butanol containing 0.2 mg ATRA/ml (provided by Argus Pharmaceuticals Inc., The Woodlands, Tex.). Aliquots of the drug-lipid mixture were placed in small vials, each containing 200 μg of ATRA and 10 mg of lipids. The vials were freeze-dried and the lyophilized mixture was then stored away from light at –80° C. Before their use, the contents of each vial were reconstituted in 1 ml of saline, vortexed, and incubated for about 10 min at the transition temperature of the lipids (>42° C). The size of liposomes obtained using this procedure ranged from 2 to 4 μ*M* with the average size being 2.5 μ*M*, as determined by a Nicomp Submicron Particle Sizer-Model 370 (Nicomp, Santa Barbara, Calif.). To some vials, 5 μCi of [^{3}H]-ATRA (specific activity, 49.3 Ci/mmol; New England Nuclear, Boston, Mass.) was added before lyophilization so as to study the stability, uptake, and retention of the encapsulated drug.

Incorporation efficiency and stability. The reconstituted liposomes containing [^{3}H]-ATRA were spun down in a microfuge (10,000 rpm; 15 min) and duplicate samples of the supernatants were counted for radioactivity. The pellet was resuspended to the original volume with saline, and two 5-μl samples each were counted for radioactivity. The encapsulation efficiency was calculated as follows:

$$\text{Encapsulation efficiency} = \frac{\text{CPM in pellet}}{\text{CPM in pellet} + \text{CPM in supernatant}} \times 100,$$

where CPM represents the counts per minute.

The stability of the liposomes was studied by diluting 1 ml of the liposomal suspension containing [^{3}H]-ATRA (1 μCi) in 5 ml of serum-containing medium. The diluted suspension was placed in a membrane dialysis bag with a 6,000 to 8,000-Da cutoff (Sigma Chemical Co., St. Louis, Mo.) and dialyzed against medium at 37° C. A sample containing tritiated free ATRA was similarly analyzed for comparison. At the indicated time points, 50-μl aliquots of the outside medium were counted (in duplicate). To determine the leakage of ATRA from the dialysis bag, the ratio of the released [^{3}H]-ATRA to the total amount of [^{3}H]-ATRA added was calculated.

Toxicity to red blood cells. Venous blood was drawn from healthy individuals and mixed with an equal volume of Alsever's solution. The cells were washed twice and a 1% suspenion (v/v) of the packed red cells was prepared in phosphate-buffered saline (PBS; pH 7.2). Of the cell suspension, 1 ml was incubated for 4 h at 37° C with 25 μg of free ATRA or liposomal ATRA (L-ATRA). At the end of the incubation period the tubes were centrifuged for 15 min at 10,000 g and red blood cell (RBC) lysis was determined by measuring the release of hemoglobin into the supernatant at an absorbance of 550 nm. The maximal RBC lysis was measured by the release of hemoglobin by hypotonic shock and was used to calculate the percentage of lysis induced by free ATRA or L-ATRA [11].

Uptake and retention. From 50,000 to 100,000 cells (MDA 886Ln) were seeded in each well in 24-well plates and allowed to grow for 3–4 days. When the cells were 50%–75% confluent, they were treated with 1 μ*M* of either free ATRA or L-ATRA containing [^{3}H]-ATRA (1 μCi) as a tracer, and the cultures were continued at 37° C for another 24 h. At the end of incubation, the cells were washed three times and lysed by repeated cycles of freezing and thawing. The cell lysates were subsequently solubilized in 1 *N* NaOH and counted for radioactivity. The protein content was determined by the Bradford method using bovine albumin as the standard. To determine the retention of the drug following 24 h of incubation with free ATRA or L-ATRA, the medium was aspirated, cells were washed, and fresh medium was added. At different time points the cells were washed and lysed and the protein content and radioactivity retained were determined.

To study the uptake of the drug by spheroids, the MTS were individually cultured in 48-well cluster dishes and exposed to 1 μ*M* of free ATRA or L-ATRA for the appropriate periods. Eight MTS were exposed for each condition and then divided into two groups of four each for counting. Following incubation, MTS were rinsed three times in PBS and solubilized in 100 μl of 1 *N* NaOH-10% sodium dodecyl sulfate solution; 50-μl aliquots were counted for radioactivity.

Inhibition of proliferation. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Sigma) was used to determine the cell growth-inhibiting ability of free ATRA and L-ATRA [4]. In brief, 1×10^4 cells (MDA 886 Ln) were plated in 200 μl of medium per well in 96-well plates. After 24 h of incubation, the cells were treated with 1 μ*M* of free ATRA or L-ATRA. The cultures were incubated for 4 days. At the end of incubation, 25 μl of MTT (5 mg/ml) was added to each well. After 4 h of incubation, 100 μl of lysing buffer was added and the cultures were incubated overnight. The following day, the amount of formazan formed was measured by reading the plates at 570 nm absorbance.

The MTS were individually cultured in 24-well culture dishes precoated with 1.25% agarose [19]. The MTS were measured on day zero using a reticle. Free ATRA or L-ATRA was then added at concentrations ranging from 10^{-6} to 10^{-10} *M*. MTS were remeasured on day 6. Relative growth was defined as the ratio of the diameter measured on day 6 to that measured on day zero.

Uptake of liposomes. In the liposomes, 0.1% of fluorescent lipid [l-phosphatidyl ethanolamine dipalmitoyl *N*-(5-fluoresceinthiocarbamoyl); Sigma] was incorporated along with ATRA. MDA 886Ln cells were grown on coverslips and incubated with these fluorescent liposomes for 24 h. The coverslips were then washed, mounted on a slide, and observed under a fluorescent microscope for the uptake of liposomes. MTS were exposed to fluorescent liposomes for similar

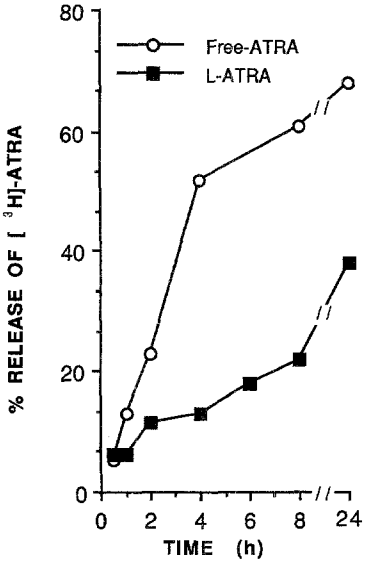

Fig. 1. Stability of DPPC: SA liposomes. The liposomal suspension (–■–) containing [^{3}H]-ATRA was diluted in serum-containing medium, placed in a dialysis bag, and dialyzed against medium at 37° C. Free ATRA (–○–) was similarly prepared; at the indicated time points, aliquots from outside medium were counted to quantitate the released radioactivity

periods. After incubation, they were rinsed and embedded in O.C.T. medium (Miles, Elkart, Ind.). The MTS were cut into sections ranging from 4 to 20 μm in thickness. The coverslips and the sections were examined under a Nikon inverted fluorescent microscope [22].

Transglutaminase activity. Transglutaminase (TGase) activity was determined by studying the Ca^{2+}-dependent incorporation of [^{3}H]-putrescine (Amersham Corp., Arlington Heights, Ill.) into *N,N*-dimethyl casein (Sigma). NHEK were grown in serum-free KGM medium for 8 days, after which the cells were cultured with or without 10^{-8} *M* of free ATRA or L-ATRA. At 2 days after the incubation, cells were washed twice with PBS and the cell monolayers were subjected to three freeze-thaw cycles. The cells were then scraped into 1 ml of PBS and centrifuged for 10 min at 2,000 *g*. Buffer containing 0.5% Triton X-100 was added to the pellet to solubilize the membrane-associated proteins, and the mixture was centrifuged again. The supernatant was removed and used for determining the protein content and TGase activity as described elsewhere [15]. TGase expression in NHEK cells was also studied by immunohistochemical staining with an anti-human keratinocyte TGase monoclonal antibody (anti-$TGase_K$; Biomedical Technologies Inc., Stoughton, Mass.).

Histological analysis. MTS from growth assays were fixed in 10% buffered formalin, embedded on paraffin, and histologically examined as described earlier [22].

Table 1. Characteristics of various liposomal-ATRA formulations. Data are given as the average values ± SD from at least three independent experiments

Composition	Encapsulation of ATRA (%)[a]	Uptake (pmols of ATRA per mg protein)[b]	Growth inhibition (%)[c]	RBC toxicity (%)[d]
Neutral:				
PC	84.0±11	610±60	69.5±3	3.19
DMPC	95.2±1	730±150	65.4±5	4.07
PC + CHOL	91.1±6	800±400	59.8±1	19.95
DPPC	91.8±2	2900±1900	71.6±9	4.38
Positively charged (cationic):				
PC + CHOL + SA (9:3:1)	88.1±12	1200±400	66.8±2	68.53
DMPC + SA (9:1)	75.5±11	2600±200	55.7±1	9.77
DPPC + SA (9:1)	88.8±5	5600±300	60.0±5	2.75
Negatively charged (anionic):				
PC + PS (9:1)	74.3±11	400±30	55.6±3	22.2
PC + CHOL + PS (9:3:1)	76.5±7	330±120	60.7±2	22.2
PC + CHOL + PS (7:3:1)	66.9±6	500±80	32.1±7	22.2
DMPC + DMPG (9:1)	28.9±7	1200±180	59.3±1	28.8
DMPC + DMPG (7:3)	85.7±2	1300±10	66.8±2	38.8
DMPC + PS (9:1)	88.8±2	220±20	64.7±5	39.5
DPPC + PS (9:1)	89.0±5	2100±160	65.0±5	4.18

[a] Reconstituted liposomes were spun down and the supernatant and resuspended pellets were counted for radioactivity as described in Materials and methods. The ratio was calculated to obtain the encapsulation efficiency (%)

[b] From 50,000 to 100,000 cells/well were plated and allowed to grow to confluency. Free ATRA and L-ATRA (1 μ*M*) labeled with [^{3}H]-ATRA was added and the incubation was continued for an additional 24 h. The cells were then washed and lysed and the radioactivity was counted. The protein content in cell lysates was determined using a Bio-Rad protein-assay kit

[c] MDA 886Ln cells were grown in 96-well plates for 1–2 days, and then 1 μ*M* of free ATRA and L-ATRA was added and incubation was continued for an additional 4 days. At the end of the incubation period, cell viability was determined by the MTT reduction test as described in Materials and methods

[d] In all, 25 μg of free ATRA and L-ATRA was incubated with 1 ml of RBC suspension (1%) and incubated for 4 h. The degree of ATRA-induced membrane damage was determined by measuring hemoglobin release at 550 nm

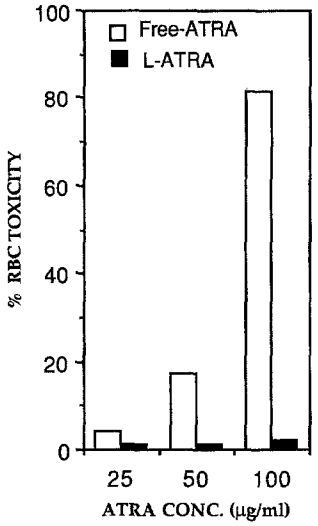

Fig. 2. Toxicity of free ATRA and L-ATRA against human RBCs. A 1% suspension of RBCs was incubated with different doses of free ATRA (*empty bars*) and L-ATRA (*solid bars*) for 4 h. RBC lysis was determined at the end of the incubation period by measuring the release of hemoglobin into the supernatant at 550 nm absorption. The results are shown as averages of triplicate values from a representative experiment

Results

Incorporation efficiency and stability of L-ATRA

As shown in Table 1, the incorporation or encapsulation efficiency of ATRA by most of the liposomal formulations was above 75%, but we considered those with an efficiency of over 85% to be ideal. Furthermore, the toxicity of various liposome formulations against RBCs was in general proportional to their drug-encapsulation efficiency. For example, liposomes composed of the anionic lipid phosphatidylserine (PS), when present along with cholesterol (CHOL) and phosphatidylcholine (PC), incorporated less ATRA than most of the other liposome formulations and were relatively more toxic to RBCs. Of the various liposomal formulations studied, those composed of dipalmitoylphosphatidylcholine and stearylamine (DPPC: SA) showed the optimal combination of encapsulation efficiency, low toxicity to RBC, and effective delivery of ATRA to the target cells. We therefore elected to characterize this particular formulation further.

The stability of the DPPC: SA liposomes containing ATRA was also studied by dialyzing free ATRA and L-ATRA labeled with [^{3}H]-ATRA against serum-containing medium. Figure 1 shows that [^{3}H]-ATRA in both free and liposomal forms could leak out of the dialysis bag, but in the case of L-ATRA the leakage was gradual; by 24 h, only 38% of the liposome-incorporated ATRA could be detected in the outside medium. In contrast, free ATRA leaked out much more rapidly; by 8 h, most of the drug (60%) could be detected in the outside medium.

Toxicity to RBCs

Since ATRA is a highly lipophilic agent, it can partition into lipid bilayers, thus resulting in destabilization of the cell membranes. We therefore used ATRA-induced damage to RBCs as an in vitro model to study the toxicity of free ATRA and L-ATRA. In an initial experiment, we tested the various L-ATRA formulations for their toxicity toward RBCs. A 25-μg/ml concentration of L-ATRA was incubated with the RBCs for 4 h. ATRA-induced damage to RBCs was quantified by determining the hemoglobin release at 550 nm absorbance (A_{550}). As shown in Table 1, L-ATRA

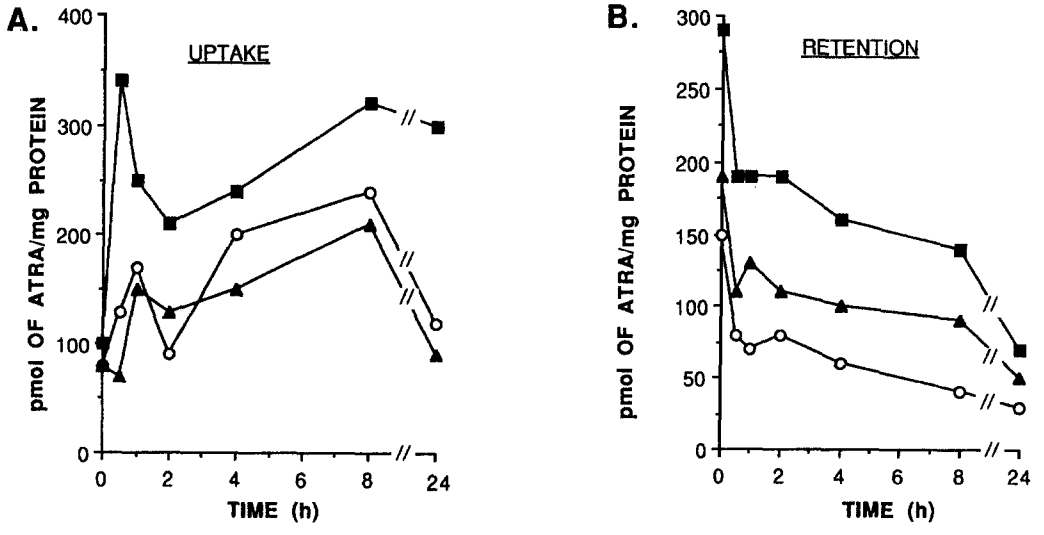

Fig. 3A, B. Uptake and retention of free ATRA and L-ATRA by MDA 886Ln cells. Cells were plated in 24-well plates and allowed to grow up to 50%–75% confluency. A 1-μ*M* concentration of tritiated free ATRA (–○–), DPPC L-ATRA (–▲–), or DPPC: SA L-ATRA (–■–) was then added and incubated. At different time points the cells were lysed and analyzed for the uptake of ATRA (**A**). For retention studies, after a 24-h incubation, the cells were washed and fresh medium was added. Then, at different time points the cells were lysed and analyzed for ATRA retention (**B**). The results are shown as averages of quadruplicate values from a representative experiment.

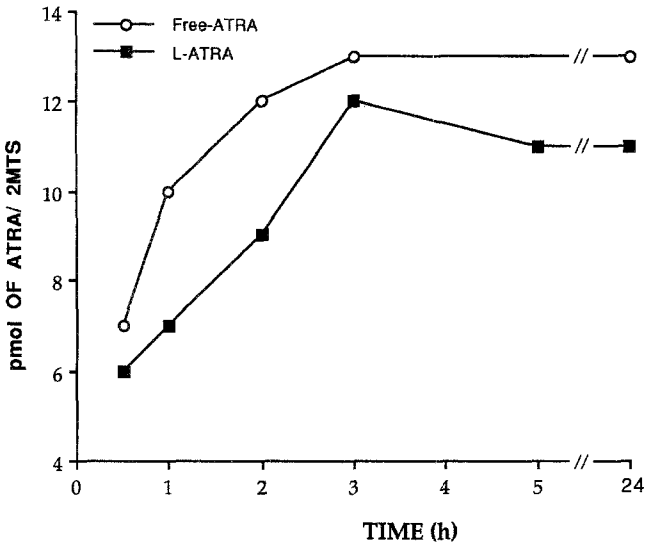

Fig. 4. Uptake of free ATRA and L-ATRA by MDA 886Ln MTS. MTS were cultured individually in 48-well cluster dishes and treated with 1 μ*M* of [^{3}H]-free ATRA (–○–) or DPPC: SA L-ATRA (–■–). At the indicated time points, 8 MTS each were rinsed in PBS, solubilized, and counted to determine the uptake of labeled ATRA. The results are shown as mean values from four observations

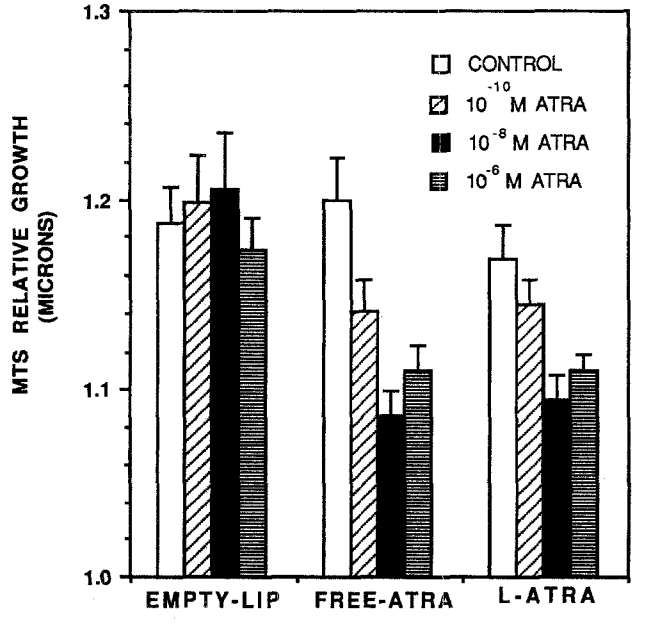

Fig. 5. Dose-dependent inhibition of MDA 886Ln MTS growth by free ATRA and DPPC: SA L-ATRA. MTS were measured for their size before incubation and after 6 days of treatment with free-ATRA, L-ATRA, or "empty" liposomes containing lipids without ATRA (*EMPTY-LIP*) at doses equivalent to that used for delivering L-ATRA. The results are shown as the mean diameter (in μm) of 6 MTS treated under each condition

composed of dimyristoylphosphatidylcholine (DMPC), DPPC, and DPPC: SA exhibited the least toxicity toward RBCs. These data also suggest that liposomes composed of cholesterol (CHOL) or phosphatidyl serine (PS) tend to be more toxic toward RBCs.

The DPPC: SA liposomal formulation containing varying doses of ATRA was further tested for toxicity to RBCs. Figure 2 shows that even at doses of ATRA as high as 100 μg/ml, the DPPC: SA liposomes protected the RBCs against ATRA-induced damage. DPPC: SA liposomes without ATRA ("empty" liposomes) were used as controls. The empty liposomes were nontoxic at all the doses tested (data not shown).

Uptake and retention

The uptake of L-ATRA was studied by culturing the MDA 886Ln cells for 24 h in the presence of 1 μ*M* of ATRA encapsulated in various liposomal formulations labeled with 1 μCi of [^{3}H]-ATRA. Of the different formulations tested, DPPC: SA liposomes were taken up most efficiently by MDA 886Ln cells (Table 1). Moreover, it is evident from the uptake of the various L-ATRA formulations that liposomes composed of neutral lipids such as DPPC and DMPC interact well with the MDA 886Ln cells. The addition of a cationic lipid such as SA further augmented the degree of L-ATRA interaction with the target cells, leading to an increased uptake of the drug; the uptake of DPPC: SA liposomes by MDA 886Ln cells was 5,600 pmol ATRA/mg protein, that of DMPC: SA liposomes was 2,600 pmol ATRA/mg protein, and that of PC: CHOL: SA (9: 3: 1) liposomes was 1,200 pmol ATRA/mg protein.

To confirm that the DPPC: SA liposomes could deliver ATRA more efficiently than the other liposome formulations, we compared the uptake of free ATRA and L-ATRA composed of DPPC or DPPC: SA over a 24-h period (Fig. 3A). During the first 8 h of incubation, the uptake of L-ATRA in the DPPC: SA liposomal form was rapid and time-dependent (300 pmol ATRA/mg protein); during the same period, the uptake of free ATRA and that of DPPC L-ATRA was equivalent (200 pmol ATRA/mg protein). At the end of the 24-h incubation, the uptake of ATRA presented in the DPPC: SA liposomal form was much higher than that of free ATRA or DPPC L-ATRA (Fig. 3A). Furthermore, the retention profile of ATRA by the MDA 886Ln cells (Fig. 3B) shows that by 8 h after removal of the drug from the medium, DPPC: SA L-ATRA was retained to a greater extent by the cells than was the free drug or DPPC L-ATRA. By 24 h, free ATRA and DPPC: SA L-ATRA were retained to an equal extent.

MTS were individually cultured and exposed to 1 μ*M* of free ATRA or DPPC: SA L-ATRA for different periods. After 2 h of incubation, the amount of free drug taken up by MTS was greater than that of the liposomal drug. However, at 24 h after the incubation, there was no significant difference in the uptake of free ATRA versus L-ATRA (Fig. 4).

Inhibition of cell growth

The ability of cells to transform soluble MTT into formazan was used to determine the number of living cells. Incubation of MDA 886Ln cells in the presence of various L-ATRA formulations (equivalent to a 1-μ*M* ATRA concentration) resulted in 30%–70% inhibition of cell growth

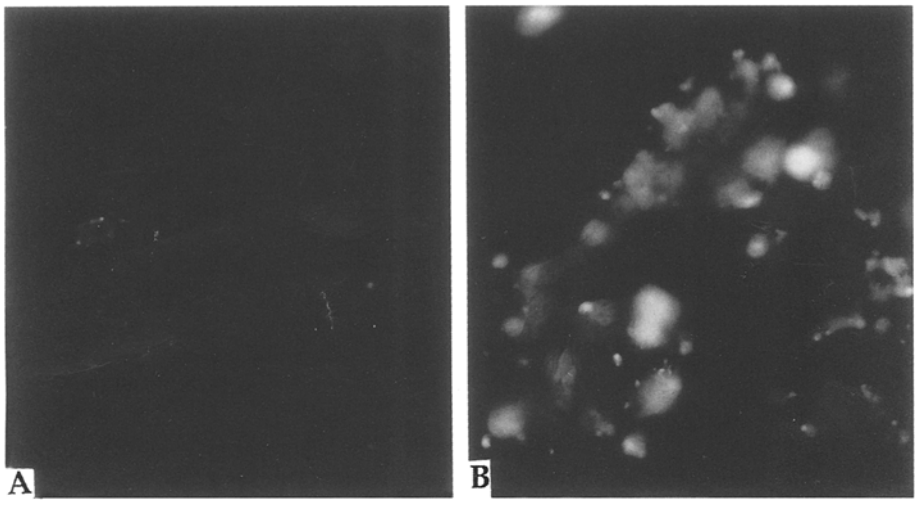

Fig. 6A, B. Uptake of fluorescent liposomes by MDA 886Ln cells. The cells were grown on coverslips until they were confluent and were then incubated with 1 μ*M* of fluorescent lipid-tagged DPPC L-ATRA (**A**) or DPPC: SA L-ATRA (**B**) for 24 h. The coverslips were removed, washed, and observed under a fluorescent microscope

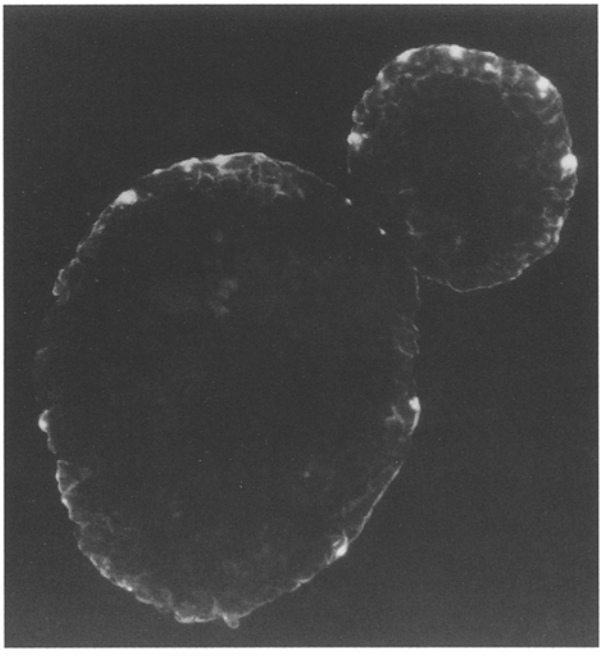

Fig. 7. Uptake of fluorescent liposomes by MDA 886Ln MTS. The MTS were incubated with fluorescent lipid-tagged DPPC: SA L-ATRA. After 24 h of incubation they were rinsed and embedded in O.C.T. medium. The MTS were then sectioned, mounted, and observed under a fluorescent microscope

(Table 1). DPPC: SA L-ATRA applied under these conditions caused 60% inhibition of the growth of MDA 886Ln cells.

Inhibition of spheroid growth was studied by culturing the MTS with 10^{-6}–10^{-10} *M* of free ATRA and DPPC: SA L-ATRA for 6 days. The diameters of the MTS were measured on day zero and then again on day 6 of ATRA treatment. Whereas the untreated control MTS grew without restraint, the growth of those treated with free ATRA was increasingly inhibited with increasing doses of the drug. In the case of L-ATRA, 10^{-8} *M* of the drug had the maximal effect on the growth of the spheroids (Fig. 5). Treatment of MTS with free or L-ATRA induced morphological changes (data not shown) similar to those observed earlier with free ATRA [20].

Uptake of fluorescent liposomes

For further study of the interaction of the DPPC: SA liposome formulation (in terms of its uptake) with SCC, fluorescent liposomes were used to visualize the uptake of L-ATRA by the cells and MTS. L-ATRA composed of DPPC or DPPC: SA phospholipids was used to confirm further that the positive charge provided by the inclusion of SA would make a difference in the liposomal uptake by the MDA 886Ln cells. As described in Materials and methods, a fluorescent lipid was included as a tracer. The uptake of the DPPC: SA liposomes by MDA 886Ln cells (Fig. 6B) was considerably higher than that of DPPC liposomes (Fig. 6A). Thus, a positive charge on the liposomal surface resulted in a significant increase in the uptake of L-ATRA. Liposomes were seen adhering to the cells as well as within the cytoplasm of the individual cells.

A similarly strong interaction of DPPC: SA liposomes was observed with MTS as well (Fig. 7). After 24 h of incubation, most of the liposomes were seen adhering to the periphery of the MTS. Prolonged incubation would probably reveal liposomes penetrating the core of the MTS, as L-ATRA has been shown to inhibit the differentiation of the cells in the core of MTS [22].

Inhibition of TGase activity by free ATRA and L-ATRA

The biological activity of DPPC: SA L-ATRA was determined by studying the activity of the enzyme $TGase_K$ in normal epidermal cells. On incubation of a confluent cul-

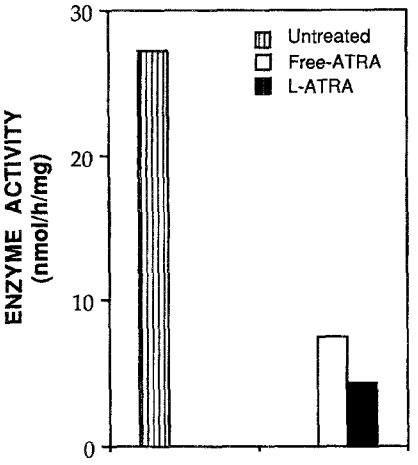

Fig. 8. Inhibition of $TGase_K$ activity expression by free ATRA and DPPC: SA L-ATRA in normal human epidermal keratinocytes (NHEK). The NHEK cells were grown to confluency (8 days) and incubated in the presence of medium alone (*striped bar*) or medium containing 10 n*M* of free ATRA (*empty bar*) or L-ATRA (*solid bar*). At the end of the incubation period (2 days), cells were washed, lysed, and assayed for enzymatic activity as described in Materials and methods. The results are shown as averages of three observations with less than 10% standard deviation from the mean

ture of NHEK with different doses of free ATRA or DPPC: SA L-ATRA, the expression of the enzyme was inhibited even at relatively low doses (10 n*M*) of ATRA (Fig. 8). Treatment of these cells with empty liposomes showed no significant effect on the modulation of differentiation markers. In another experiment, after the cells had grown to confluency, 1 μ*M* of free ATRA or L-ATRA was added and the cells were cultured for 2 additional days. Cytochemical staining using the anti-$TGase_K$ antibody revealed that L-ATRA was as effective as the free drug in inhibiting the expression of $TGase_K$ expression as compared with untreated controls (data not shown).

Discussion

Retinoids modulate the in vitro growth and differentiation of a variety of cell types, including squamous-cell carcinomas [8, 10, 19, 20]. On the basis of this property, retinoids have been used effectively in therapeutic regimens for the prevention of SCC [5]. Because administration of ATRA, like many other anticancer drugs, at the therapeutic dose level is associated with undesirable toxic effects both in experimental models and in patients, liposomes are being evaluated clinically and experimentally as an alternative delivery system for retinoids. The potential advantages of liposome delivery include increased activity due to specific targeting, amplified therapeutic effect due to packaging of numerous drug molecules in each liposome, and decreased toxicity due to altered pharmacokinetics.

It was shown in CD-1 mice that the toxic dose of free ATRA was 25–30 mg/kg. However, no apparent toxic effect was observed when the animals were injected with L-ATRA at a dose of up to 120 mg/kg [11]. Thus, liposome encapsulation dramatically protected the animals from the toxicity of ATRA. The results reported herein for the in vitro RBC-lysis model confirm these observations. Among the 14 L-ATRA formulations tested, DPPC: SA seemed to be the least toxic and retained the drug well over a considerable period, even in the presence of serum proteins.

A number of investigators have obtained advantageous therapeutic effects by using liposomes that include the positively charged lipid SA [16, 17]. On the other hand, positively charged lipids can have toxic effects on cells in culture and on erythrocytes [26]. However, toxicity tests done with erythrocytes from different animal species have suggested that whereas those from the horse, guinea pig, and rabbit are highly susceptible to liposomes containing SA, erythrocytes from humans, sheep, chickens, and cattle are less susceptible [26]. The liposome formulation used in the present study contained DPPC: SA at 9:1 (w/w) and proved to be much less toxic than the 13 other L-ATRA formulations studied.

The uptake of L-ATRA by MDA 886Ln cells has previously been shown to inhibit the growth of cells and to induce biological effects [22]. Initial experiments showed that of the 14 different lipid combinations tested, DPPC: SA liposomes were taken up to the maximal extent by the cells (Table 1). Incubation of SCC monolayers (MDA 886Ln) with fluorescent-labeled L-ATRA also suggested that the addition of a positively charged lipid to liposomes caused a dramatic increase in their interaction and uptake (Table 1; Figs. 3, 6). The lipids could be seen not only in association with the membranes but also within the cells.

It was recently reported that the growth and differentiation of a spheroid model of SCC is modulated by ATRA [19–21]. Furthermore, liposomes were shown to be effective in delivering retinoic acid to MTS and in producing biological effects within its three-dimensional structure [22]. Our experiments on the uptake of [^{3}H]-ATRA by MTS revealed that free and liposomal drug were taken up to an equal extent by the spheroids. On incubation of fluorescent liposomes with the MTS, the liposomes were seen to adhere strongly to the spheroids and to penetrate them (Fig. 7). In a similar study, fluorescent-labeled DMPC: dimyristoyl phosphatidylglycerol (DMPG; 7: 3) liposomes were used [22]. The uptake by both monolayers and MTS of MDA 886Ln cells was shown to be very minimal, suggesting that lipid composition is a major factor in the effective delivery of ATRA to the cells. As suggested by the results presented herein, the positive charge on the liposomes conferred by SA causes a stronger interaction of the liposomes with the SCC cell membranes, thereby delivering ATRA very efficiently (Fig. 6).

Several studies have shown that ATRA can suppress cell growth in cultured cell lines [7, 10, 15]. Assays for inhibition of the growth of the MDA 886Ln cell line and its spheroid model indicated that the antiproliferative effects of free ATRA and L-ATRA were equivalent. Other researchers have reported that L-ATRA has a greater in vitro antiproliferative effect than does free ATRA [14]. This effect could be due to increased uptake and/or to increased solubility of the retinoids.

ATRA has been documented to inhibit the squamous differentiation of both normal and malignant epidermal cells [10]. During squamous differentiation, epidermal cells

express phenotype markers such as $TGase_K$ as well as keratins and TGase substrates such as loricrin and involucrin, and retinoids modulate these markers [6]. Cytochemical staining of untreated normal human epidermal cells also showed a large accumulation of $TGase_K$ following their squamous differentiation. Treatment with 1 μM of free ATRA or L-ATRA inhibited differentiation as revealed by suppression of $TGase_K$ activity and expression in these cells (Fig. 8). Free ATRA and DPPC: SA L-ATRA inhibited the differentiation of epidermal cells to the same extent, suggesting that the encapsulated ATRA retains its efficacy.

In summary, the data presented herein suggest that liposomal retinoids have growth-inhibitory effects comparable with those of the free drug. On in vitro treatment of the SCC cell line MDA 886Ln with various liposomal formulations, we observed that the positively charged liposomes composed of DPPC: SA showed a high affinity toward malignant squamous cells and induced minimal toxic effects over a wide range of ATRA concentrations. The biological activity of L-ATRA was confirmed by its ability to suppress the proliferation of malignant cells and inhibit the expression of the differentiation marker $TGase_K$ in normal cells. Further experiments to investigate the in vivo toxicity and efficacy of this L-ATRA formulation may offer a promising lead to an effective and less toxic delivery of retinoic acid to tumors of the upper aerodigestive tract.

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