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ORIGINAL ARTICLE

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Aerosol delivery of liposomal all-*trans*-retinoic acid to the lungs

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Abstract *Purpose*: To optimize the delivery of all-*trans*-retinoic acid (ATRA) to lung tissue, we determined the potential of vehiculating the drug in liposomes (L-ATRA) and delivering it via aerosol. Liposomes may provide a means to prevent local irritation of lung tissue and reduce pulmonary toxicity, prolong therapeutic levels and generate high drug concentrations at the tumor sites. Cumulatively, this would result in reduced systemic toxicity and enhanced drug efficacy. *Methods:* Previous studies have shown that liposomes can serve as excellent carriers for otherwise poorly soluble ATRA. Delivery of ATRA to the lung tissue of mice was accomplished by nebulization of L-ATRA. The liposomes in the aerosol were relatively uniform (309 ± 138 nm), stable, and retained the drug well. *Results*: The drug was effectively delivered at high concentrations (10 ± 2 μg/g of tissue) to the lungs of mice and was retained for at least up to 96 h after a single exposure to L-ATRA aerosol. No appreciable levels of ATRA were detected in the blood or the liver of treated mice. The aerosol-delivered ATRA was biologically active as demonstrated by its ability to induce the expression of tissue-type transglutaminase. *Conclusion*: Aerosol delivery of L-ATRA offers an effective way to deliver high levels of ATRA to the lung without apparent pulmonary toxic effects.

Key words Liposome · All-*trans*-retinoic acid · Aerosols · Lung

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Introduction

Retinoids are natural and synthetic analogs of vitamin A that normally play a critical role in growth, vision, reproduction, differentiation, and immune functions [1, 2]. A relationship between vitamin A and cancer was first noted when experimentally induced vitamin A deficiency was shown to lead to preneoplastic lesions and neoplasms [3, 4]. Recent observations in patients with acute promyelocytic leukemia, cervical cancer, and squamous cell carcinomas (SCC) of the skin have kindled further interest in using retinoids as chemotherapeutic agents [5–7].

Epithelial tissues undergo biochemical, morphological and functional changes if deprived of or treated with an excess of retinoids. Because SCCs are derived from epithelial cells, they may be ideal targets for treatment with retinoids. Epithelial cancers of the head and neck or lungs are a devastating group of diseases that account for approximately 30% of cancer deaths [8]. Retinoids are known to be potent modulators of epithelial differentiation and carcinogenesis [9]. For example, 13-*cis*-retinoic acid is potent in suppressing oral carcinogenesis as well as preventing second primary tumors [10]. Clinical trials using retinoic acid for the treatment of oral leukoplakia, a premalignant lesion of oral cancer, have shown regression of existing disease and prevention of new disease and progression of the disease [11]. This effect is reversed when the treatment is withdrawn. However, like many other anticancer drugs, chronic administration of retinoids in patients is associated with toxic effects [9].

Liposomal incorporation of retinoids may provide an alternative way of delivering retinoids without the

resulting toxic effects. Incorporation of drugs in liposomes has been shown to sequester the drug at tumor locations, protect the drug from rapid metabolism, and amplify the drug's therapeutic effect [12]. More importantly, incorporation in liposomes may allow the retinoids to be delivered by aerosol directly to target sites in the aerodigestive tract, which is otherwise not possible for lipophilic drugs like all-*trans*-retinoic acid (ATRA). Oral administration of retinoids allows only low levels of drugs to reach the aerodigestive tract especially the lungs. The advantage of the aerosol mode of delivery is that the drug is deposited more uniformly over the respiratory tract, leading to local levels of the drug that may far exceed the levels achieved by systemic administration [13].

Previous studies with liposomal-ATRA (L-ATRA) have shown that L-ATRA is less toxic than the free drug, inhibits the growth of malignant cell lines, and induces biological effects [14]. We report here the feasibility of delivering L-ATRA to mice by aerosolization. The amount of ATRA delivered to the respiratory tract, the characteristics of the aerosolized liposomes, the aerosol particles, and the biological activity of the ATRA thus delivered were studied. Preliminary toxicology of L-ATRA delivered by aerosol was determined.

Materials and methods

Mice

Age-matched ICR male mice (8 weeks old, weighing 23–25 g) were purchased from Harlan-Sprague (Houston, Tx.) and housed in cages. They were fed mouse chow and water *ad libitum*.

Preparation of liposomes

Multilamellar liposomes were prepared from lyophilized powder of the lipids dipalmitoylphosphatidylcholine (DPPC) and stearylamine (SA) (Avanti Polar Lipids, Alabaster, Ala.) in the ratio 9:1 w/w. The lipids and ATRA (Argus Pharmaceuticals, Woodlands, Tx.) in a drug:lipid ratio of 1:10 (w/w) were dissolved in tertiary butanol. The drug-lipid mixture was then freeze-dried in a lyophilizer and stored away from light at −20 °C [14]. Before use, the liposomes were reconstituted in 10 ml water, vortexed, and incubated at a temperature higher than the transition temperature of the lipids (> 42 °C) for about 15–20 min in a waterbath and sonicated for about 20 min. The final drug concentration was 0.5 mg ATRA/ml. The size of liposomes before and after aerosolization was determined using a Nicomp Submicron Particle Sizer-Model 370 (Nicomp, Santa Barbara, Calif.). To some vials, ≃0.5% (w/w) of fluorescent lipid (Rhodamine-PC; Avanti Lipids) was added before lyophilization.

Aerosol delivery

A Raindrop nebulizer (Puritan-Bennett Co., Los Angeles, Calif.) was used to generate aerosol particles of the L-ATRA. The nebulizer had a single air jet and a 10-ml reservoir and generated an air flow of 10 l/min. Each preparation was placed in the reservoir and aerosolized for 25–30 min. Groups of 10–25 mice were placed in special air-tight cages and allowed to inhale the aerosols for 30 min. At each of a series of time-points ranging from 15 min to 96 h, four animals were removed from the cage and killed by cervical dislocation. Untreated mice were used as controls. For multiple-dose treatment, animals were exposed for 30 min to aerosolized L-ATRA every day for 5 consecutive days. After each treatment the mice were sacrificed to collect the lungs for later analysis.

Quantitation of ATRA and lipids by HPLC

ATRA was quantified by reverse-phase high-performance liquid chromatography (HPLC) with monitoring at 350 nm (Millipore Corp., Millford, Mass.). All measurements were made at ambient temperature on a stainless-steel HPLC column (μBondapak C18 3.9 × 300 mm column; Millipore Corp.). The mobile phases were 100% methanol (solvent B) and methanol/water (65:35) containing 10 m*M* ammonium acetate and 0.05% formic acid (solvent A). The solvents were setup on a linear gradient at a flow rate of 2 ml/min. ATRA had a retention time of 25–26 min at 70% of solvent B and 30% of solvent A. This assay system was able to detect ATRA at 1.0 μg/ml and was linear up to 200 ug/ml concentration.

Lipids were also quantitated by HPLC using a previously described protocol [15]. A Waters 717 WISP automatic sampler injector and a Spherisorb S5 amino column (25 cm × 4.66 mm, 5 μm) was used with acetonitrile, methanol, and 10 m*M* ammonium/trifluoroacetic acid, pH 4.8 (64:28:8 v/v/v) as mobile phase. Peaks were detected with a mass evaporative detector (Sedex 55, Sedere, France) and quantitated with an integrator. A 50-μl aliquot of each aerosol sample in methanol was injected and the DPPC in each sample was analyzed.

Aerosol particle characteristics of L-ATRA

Aerosol particle size was determined using an Andersen Cascade Impactor (Andersen Samplers, Atlanta, Ga.). Calculations of aerodynamic mass median diameter and geometric standard deviation were based on gravimetric and chemical determinations of samples collected over a 5-min sampling period [16]. The ATRA and the lipids deposited on the filters were obtained by soaking and shaking the filters in 10 ml absolute methanol for about 1 h. Total concentrations of ATRA and the lipids in the aerosol were determined by collecting 2-min aerosol samples in all-glass impingers containing 20 ml water. The all-glass impingers were calibrated to collect 12.5 l of air per minute. The concentrations of ATRA and lipids in the samples collected were determined by HPLC as described above.

Collection of samples from mice

At predetermined time-points, aerosol-treated and untreated animals were killed by cervical dislocation. Blood was drawn by cardiac puncture and the lungs and livers were removed. The tissues were homogenized in buffer (10 m*M* EDTA, 200 mg ascorbic acid, and 2 ml acetic acid in 200 ml distilled water) and retinoic acid was extracted twice in methanol containing antioxidant (1 mg/ml butahydroxytoluene). The buffer and methanol extracts were then dried together and reconstituted in 300 μl methanol, a 25-μl sample of which was used for HPLC analysis.

Biological activity of L-ATRA

To study the biological activity of the aerosol-delivered L-ATRA, alveolar macrophages were obtained from the lungs of control and treated mice. Briefly, the lung tissue was placed on ice and lavaged two or three times with cold RPMI medium. The cells obtained from five animals with similar treatment were pooled and centrifuged. The cell pellet was then resuspended in RPMI medium containing 10% fetal calf serum and incubated for about 2 h in a six-well plate. After incubation, nonadherent cells were removed by washing the wells twice with medium and the adherent cells were

scraped into phosphate-buffered saline (PBS). The cells were then centrifuged and the pellet resuspended in lysing buffer (PBS containing 0.5% Triton X-100), sonicated, and assayed for transglutaminase (TGase) activity as described previously [17]. The cell lysates were also analyzed for TGase levels by immunoblotting, using a monospecific monoclonal antibody (CUB74, Neomarkers, Fremont, Calif.) as previously described [17]. Alternatively, alveolar macrophages were allowed to adhere to plastic coverslips for 2 h, and after washing were examined under a fluorescent microscope for the uptake of fluorescent lipid-tagged liposomes.

Toxicity studies

Tissue sections were fixed in buffered formalin, embedded in paraffin, and cut into 10-μm thick sections. Lung sections obtained from untreated and treated mice were examined for histological changes after staining with hematoxylin and eosin. Serum was isolated from treated and untreated animals and assayed for liver enzymes (SGOT, SGPT). Frozen sections of mice lungs were also taken before and after aerosolized L-ATRA treatment for visualization of fluorescent lipid-tagged liposomes in the lungs.

Results

Characteristics of aerosol L-ATRA

Liposomes used for aerosolization were made of the neutral lipid DPPC and the positively charged lipid SA in the ratio 9:1 (w/w). The drug to lipid ratio was 1:10, with a final concentration of ATRA of 0.5 mg/ml. On reconstitution, liposomes measured 807 ± 165 nm on average, and after 20–30 min of aerosolization with the Puritan-Bennett Raindrop nebulizer, they measured 309 ± 138 nm in the aerosol. Reservoir samples were taken during aerosolization for particle sizing.

The characteristics of the aerosol particles (such as their size and the amount of drug associated with them) generated by the nebulizer were also studied. The mass median aerodynamic diameter of the aerosol particles was 1.44 μm, with a geometric standard deviation of 2.6. The aerosol so generated contained on average 6.5 μg of ATRA per liter of aerosol (Fig. 1). On the basis of this value, the estimated retained amount of ATRA for a single 30-min treatment per day was calculated as follows [16, 18]: estimated aerosol dosage retained = aerosol concentration (μg/l) × minute volume (l/min) × duration of treatment (min) × retention factor = 6.5 μg/l × 0.025 l/min × 30 min × 0.3 = 1.5 μg per treatment.

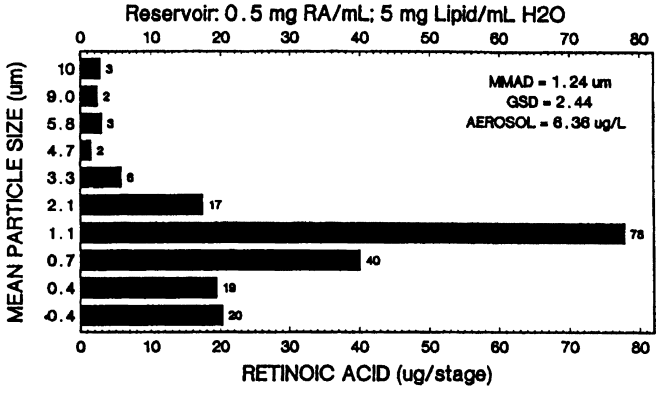


Fig. 1 Aerosol characteristics of L-ATRA. The particle size distribution of the L-ATRA aerosols was measured using an Andersen cascade impinger as described in Materials and methods. ATRA concentrations were measured by HPLC. The characteristics of L-ATRA presented here are from one representative experiment

Analysis of the collected aerosols by HPLC showed that the ratio between ATRA and the lipids was at the initial value of 1:10 (data not shown), suggesting that L-ATRA retained its integrity throughout the process of aerosolization.

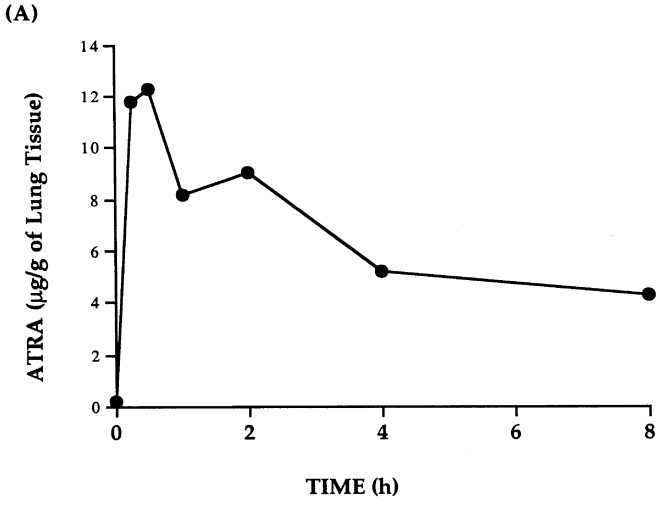
Levels of ATRA in the lungs of mice exposed to L-ATRA aerosols

Following a 30-min exposure to L-ATRA-containing aerosol (0.5 mg of ATRA/ml), mice were sacrificed at 15 min or halfway through exposure, at the end of exposure (30 min), and at different times ranging from 1 h to 96 h after exposure. For each time-point, three or four mice were sacrificed, and the lungs were used individually for drug analysis. The extraction efficiency of ATRA from lung using buffer and methanol was determined to be 80%. Analysis of the lung samples by HPLC indicated that the highest levels of ATRA (10 ± 2 μg/g) occurred at the end of treatment, i.e. 30 min (Fig. 2A). Drug levels had dropped 2 h after treatment to about 5–6 μg/g, after which the levels were maintained until 96 h (Fig. 2B). The first half-life of ATRA in the lungs ($T_{1/2}\alpha$) was 1.25 h, and the second half-life from 1–96 h ($T_{1/2}\beta$) was 6.1 h. Lungs from untreated control mice showed a moderate level of ATRA (0.2 ± 0.002 μg/g). These results suggest that ATRA, when incorporated in liposomes, can be delivered by aerosolization, and the ATRA thus delivered was retained at levels of 5 ± 0.2 μg of ATRA/g of lung tissue at least until 96 h after treatment. Blood and liver samples showed no detectable levels of ATRA after aerosol L-ATRA treatment, suggesting that the drug was retained predominantly in the lung tissue.

A group of mice were given aerosolized L-ATRA for 30 min every day for 5 consecutive days. At the end of each treatment, the animals were sacrificed, and the lungs were extracted for ATRA determination. After the first treatment, the mean level of ATRA in the lungs was 13.2 μg/g of tissue. After administration of the second dose, the levels of ATRA were about 6.8 μg/g tissue and remained at 6 ± 2 μg/g even after five treatments (Fig. 3). Thus, multiple treatments for 5 consecutive days did not cause any greater accumulation of ATRA compared to the single treatment.

Uptake of L-ATRA

In sections of lung from mice exposed to aerosolized L-ATRA for 30 min, fluorescent liposomes were found



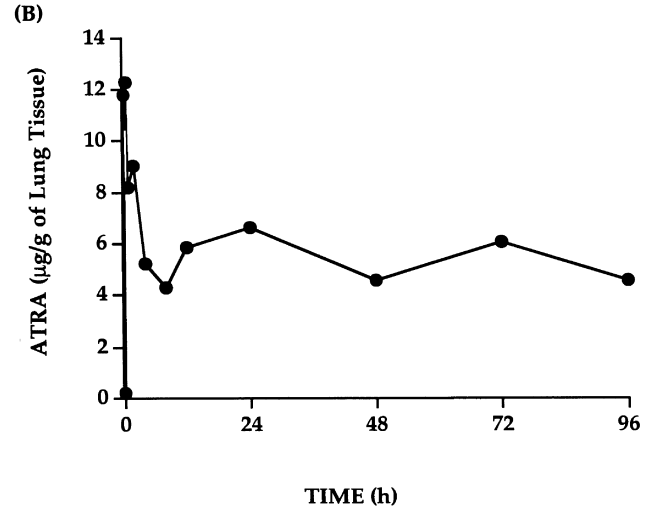


Fig 2A,B ATRA levels (μg/g) in the lungs of mice exposed to a single dose of aerosolized L-ATRA. Mice exposed to aerosols were sacrificed (four mice per time-point) at the indicated times, the lungs were removed, and the drug was extracted and analyzed by HPLC as described in Materials and methods. (**A** accumulation of ATRA over 8 h, **B** accumulation over 96 h following exposure). Values shown are the mean ATRA levels (μg/g of lung tissue) from at least three animals

adhered to the pulmonary epithelial cells (Fig. 4). Also, lungs were removed from animals 1 h after treatment and lavaged with cold medium. Cells were allowed to adhere to coverslips, and fluorescent liposomes were observed within the cells (Fig. 5). Thus, in mice that inhaled aerosolized L-ATRA, deposits of liposomes in the lungs and alveolar macrophages were observed.

Biological activity of aerosolized L-ATRA

Alveolar macrophages express the enzyme tissue-type TGase ($TGase_C$) [19]. This enzyme is induced in response to retinoic acid treatment [20]. Cell lysates of alveolar macrophages lavaged from treated and untreated mice were subjected to the TGase enzyme assay. The results shown in Fig. 6A demonstrate that alveolar macrophages from untreated animals had a low basal TGase activity (20 nmol/h per mg protein). However, although cells from L-ATRA-aerosolized mice

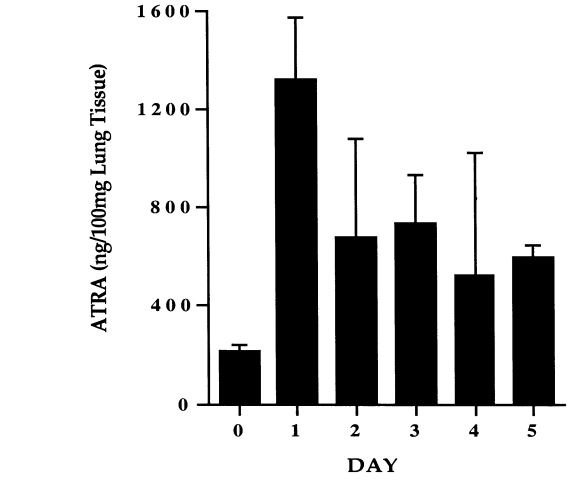


Fig. 3 ATRA in the lungs of mice exposed to multiple doses of aerosolized L-ATRA. Mice were exposed to aerosolized L-ATRA for 30 min daily for 5 consecutive days. After each dose, four mice were sacrificed and the lungs were analyzed for ATRA. Presented are the mean ATRA levels (μg/g tissue)

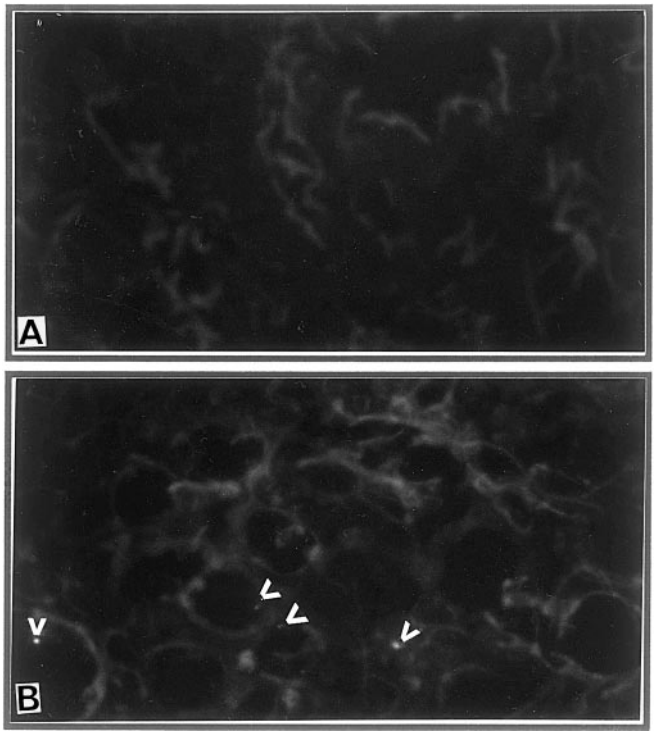


Fig. 4A,B Frozen sections of lung from untreated **(A)** and aerosolized L-ATRA-treated **(B)** mice. The *arrows* indicate the location of fluorescent-tagged liposomes

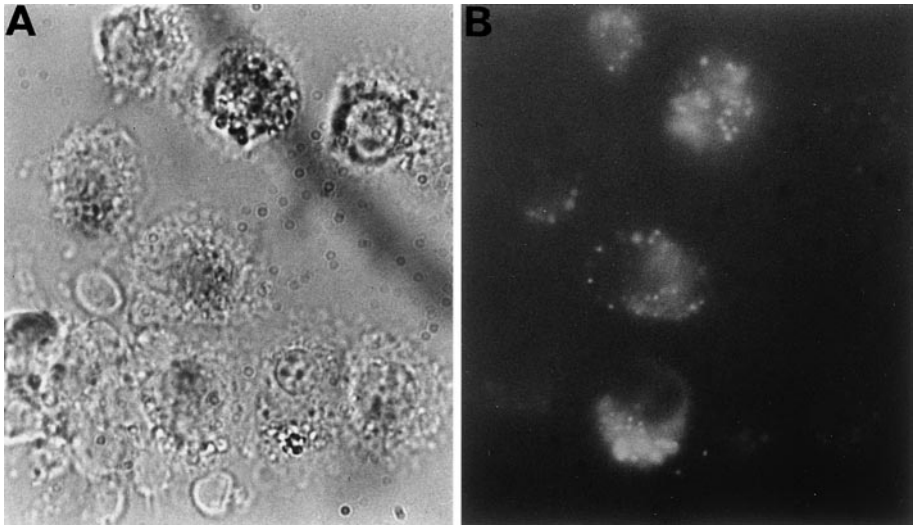


Fig 5A,B Uptake of fluorescent lipid-tagged L-ATRA by alveolar macrophages lavaged from mice exposed to a single treatment of aerosol containing L-ATRA (**A** phase-constrast microscopy, **B** fluorescent microscopy)

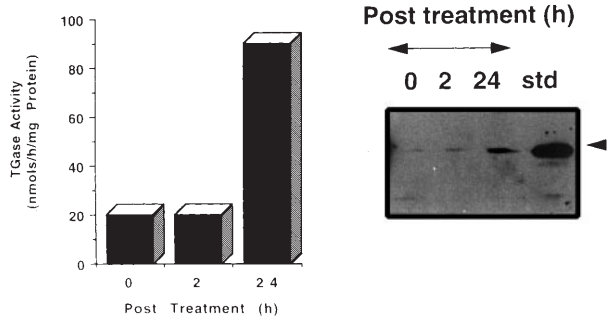


Fig. 6 $TGase_c$ activity and levels in alveolar macrophages of mice exposed to aerosolized L-ATRA. Mice were exposed to single dose of aerosol containing L-ATRA. After 2 h or 24 h, the mice were sacrificed and the macrophages lavaged. Macrophages were lysed, and the lysates were analyzed for enzyme activity and for $TGase_c$ protein levels by western blotting, as described in Materials and methods (*std* purified $TGase_c$ from guinea pig liver)

sacrificed after 2 h showed no appreciable increase in the levels of TGase, 24 h after treatment, the enzyme activity levels had increased by 4.5 times (90 nmol/h per mg protein; Fig. 6A). The increase in enzyme activity was due to ATRA alone, as 'empty liposomes' did not induce any TGase even at high concentrations (data not shown).

Moreover, immunoblot analysis of alveolar macrophage lysates, using anti-TGase antibody, revealed no detectable TGase protein from untreated mice or mice 2 h after treatment. However, by 24 h after treatment the alveolar macrophages showed significant accumulation of TGase protein. L-ATRA-induced TGase was identical to the purified TGase from guinea pig liver, as suggested by the identical size of the immunoreactive band. Thus, the ATRA reaching the lungs was biologically active and could induce both the protein and activity levels of TGase.

Discussion

Retinoids modulate the in vitro growth and differentiation of a variety of transformed cell types, including melanomas, leukemias, and SCCs [1, 2, 5–7, 9, 10]. Based on this property, retinoids have been used effectively in therapeutic regimens for prevention and treatment of various dermatological conditions, acute promyelocytic leukemia, as well SCCs of the aerodigestive tract, skin and cervix [6, 7, 10]. However, like many anticancer drugs, administration of ATRA is associated with undesirable toxic effects both in experimental models and in patients [11, 21]. Liposomes have been evaluated both clinically and experimentally as a delivery system for mitigating the toxic effects associated with administration of drugs such as doxorubicin, vincristine, amphotericin, and retinoids [14, 22–25]. 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, altered rates of metabolism, and decreased toxicity due to altered pharmacokinetics [26–28]. For example, free ATRA when administered in CD-1 mice, exerted toxic effects at 25–30 mg/kg of body weight. However, in liposome-encapsulated form the animals could tolerate much higher doses of ATRA (120 mg/kg) probably due to altered drug distribution in target tissues [24].

Of 14 different formulations tested in our laboratory, liposomes composed of DPPC and SA seemed to be the least toxic [14]. The addition of the positive-charged SA dramatically increased uptake of ATRA, which was also well retained over a 24 h period by squamous carcinoma cells. The L-ATRA was also biologically active and caused the differentiation of cells in culture as well as in a spheroid model [14]. Liposomal incorporation was also seen to reduce the rate of cellular and microsomal metabolism of ATRA. The amount of metabolites

secreted into the medium was decreased by 15%, and the levels of intact ATRA in the cell doubled. Thus, liposomes were able to protect the drug from the metabolic enzymes of the host [28]. This contention is consistent with our previous results demonstrating that microsomes isolated from the livers of free ATRA-treated rats catabolize [^{3}H]ATRA faster than microsomes isolated from L-ATRA-treated rats [26]. Similarly, in leukemia patients the plasma drug levels are maintained over long periods of time following administration of L-ATRA [27].

ATRA is currently being administered to patients as an oral formulation. This would probably allow only very low levels of the drug to reach the tumor sites in the aerodigestive tract and lungs. L-ATRA can be administered intravenously, but owing to its particulate nature, a major fraction of the dose is taken up by the reticuloendothelial system. If the drug could be targeted directly to the aerodigestive tract by way of aerosols, much higher concentrations of the drug could be achieved with minimal toxicity. Free ATRA, owing to its lipophilic properties, cannot be aerosolized, so its incorporation in liposomes provides an efficient way for the drug to be delivered by aerosol to the lung and upper aerodogestive tract. In this study, we demonstrate the possibility of administering L-ATRA by aerosol directly to the tumor-bearing areas of the aerodigestive tract and lungs. The L-ATRA was homogeneous in size after aerosolization and maintained its integrity during the aerosolization process. After a single dose of aerosolized L-ATRA, mice were able to retain about 5 μg of ATRA/g of lung tissue, for up to 96 h. Liposomes incorporating the drug as well as a fluorescent lipid marker were located in lung sections (Fig. 4), as well as within alveolar macrophages lavaged from treated mice (Fig. 5). It has already been shown that liposomes bearing a positive or negative charge accumulate in the lungs to a greater extent than neutral vesicles (29).

Gilbert et al. [16] have demonstrated that antiviral drugs like enviroxime when incorporated in liposomes and delivered by aerosol can accumulate to significant levels in lungs. The drug accumulation is preferentially seen in the epithelial cells lining the bronchi and the bronchioles. The present study demonstrates that L-ATRA delivered by aerosol accumulates in lungs to a great extent and, more importantly, retains its biological activity as revealed by increased expression and activity of TGase in alveolar macrophages of treated mice. It has been demonstrated that aerosol administration of amphotericin B liposomes significantly increases both the mean time of survival and percent survival in mice with systemic infections of *Candida* [30]. Multiple treatments were undertaken to study the possibility of increasing the levels of ATRA delivered to the lungs. In our studies, multiple treatments (single dose every day for 5 days) did not show greater accumulation of ATRA in the lungs when compared with single treatments (Figs. 3 and 4). Daily treatment for 5 days may induce enhanced clearance of L-ATRA from the lungs probably due to the induction of RA-metabolizing enzymes such as hp450RA1. The hp450RA1 enzyme has recently been cloned and shown to be acutely induced in response to ATRA treatment in non-small-cell lung carcinoma [31]. Based on these observations, weekly treatment over an extended period of time may be able to achieve higher levels of ATRA in the target tissues.

No gross or microscopic pathologic changes were observed in the lungs of mice treated with single or multiple doses of aerosolized L-ATRA. Liver function tests were normal, demonstrating that L-ATRA was nontoxic. Gilbert et al. [30], Wyde et al. [32], and Waldrep et al. [33] have also shown that exposure to liposome aerosols causes no adverse effects in mice and humans. Our results suggest that aerosol delivery of L-ATRA could be an efficient and nontoxic way of delivering higher levels of drug to treat cancers of the upper aerodigestive tract and lungs. Further evaluation in animal models should provide support for using this mode of delivery in clinical settings.

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