Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 325 (2006) 108–115
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Cytotoxic effect induced by retinoic acid loaded into galactosyl-sphingosine containing liposomes on human hepatoma cell lines

Cecilia Díaz [a,b,*], Ernesto Vargas [a], Omar Gätjens-Boniche [c,d]

[a] *Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica*
[b] *Departamento de Bioquímica, Escuela de Medicina, Universidad de Costa Rica, San José, Costa Rica*
[c] *Escuela de Ciencias y Letras, Instituto Tecnológico de Costa Rica, San Carlos, Costa Rica*
[d] *Unidad de Biotecnología y Biología Molecular, CIDASTH, Instituto Tecnológico de Costa Rica, San Carlos, Costa Rica*

Received 2 February 2006; received in revised form 21 June 2006; accepted 22 June 2006
Available online 30 June 2006

Abstract

Two retinoids, ATRA and 13*cis*RA, were incorporated into liposomes of different composition and charge and added to two hepatoma cell lines with different degree of transformation to measure cytotoxicity by MTT assay.

Retinoid-free cationic liposomes were more toxic than the other kinds (anionic and made only of PC) but were also the best delivery system for retinoic acid to induce specific cytotoxic effects on these tumor hepatoma cell lines.

Galactosyl-sphingosine containing cationic liposomes increased the cytotoxic effect induced by ATRA on Hep3B cells when compared to glucosyl-sphingosine cationic liposomes, but did not improve the effect induced by free retinoid or ATRA loaded into liposomes without glycolipids. This suggests that in this cell line, ATRA is being incorporated by a mechanism mediated by the asialoglycoprotein receptor, but at the same time, non-specific sugar-independent capture is also taking place as well as free diffusion of ATRA directly through the membrane. Galactose-specific effect was not observed in HepG2 cells treated with ATRA or both cell lines treated with 13*cis*RA. In fact, treatment of HepG2 cells with retinoids entrapped into liposomes likely induces proliferation instead of cytotoxicity, a result that interferes with the measurement of cell death by MTT. Compared to the specific effect of ATRA entrapped into cationic liposomes, vesicles made only by PC, did not mediate a specific mechanism, since differences between ATRA in galactosyl- and glucosyl-shpingosine PC-liposomes were not statistically significant.

The specific mechanism was not present in the myoblastic cell line C2C12, where ATRA incorporated into galactosyl- and glucosyl-sphingosine containing cationic and PC-liposomes, was able to induce cytotoxicity at the same extent.

Micelles containing ATRA and galactosyl-sphingosine had a significantly more toxic effect than the retinoid administered together with glucosyl-sphingosine, in Hep3B cells. Also, micelles containing ATRA were more toxic than glycolipid-containing liposomes with ATRA, for both kinds of sphingosines. The same effect was not observed in C2C12 cells, where glycolipid-containing liposomes worked better than micelles, and a sugar-specific mechanism was not seen.

This suggests that, even though galactose-containing cationic liposomes could be a promising approach, a galactose-specific emulsion system could be the best strategy to specifically deliver retinoic acid to liver tumor cells, since it shows tissue specificity (perhaps induced by ASGPR-mediated internalization) and a stronger cytotoxic effect than the retinoid incorporated into liposomes.

Keywords: Retinoic acid; Liposome; Galactose; Hepatocellular carcinoma; Cytotoxicity

1. Introduction

Human hepatocellular carcinoma (HCC) is one of the most deadly and most rapidly increasing type of tumors in the world, representing the fifth worldwide most common malignancy in men and the eight in women (Bosch et al., 2005; Fisher, 2005).

Several chemotherapeutic agents have been used for the treatment of HCC, being the pyrimidine antimetabolite 5-fluorouracil (5-FU), the first drug reported. Some other anti-neoplasic drugs

Abbreviations: HCC, hepatocellular carcinoma; ASGPR, asialoglycoprotein receptor; MTT, 3-(4,5-dimethyl-2-thiazolyl(-2,5-diphenyl-2*H*-tetrazolium bromide)); DMSO, dimethylsulfoxide; PC, phosphatidylcholine; ATRA, all-*trans* retinoic acid; 13*cis*RA, 13*cis* retinoic acid; gal, galactosyl-sphingosine; glu, glucosyl-sphingosine

* Corresponding author. Tel.: +506 2293135; fax: +506 2920485.
E-mail address: cdiaz@cariari.ucr.ac.cr (C. Díaz).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.06.034

that have been used, either alone or in combination, are doxorubicine, epirubicine, cisplatin, interferon-β and γ and others (Leung and Johnson, 2001).

In recent years, it has been suggested that natural and synthetic retinoids could be potentially successful in the treatment of liver cancer (Arce et al., 2005; Muto et al., 1999; Okuno et al., 2001). Retinoids are derivatives of vitamin A with several physiological functions in embryonic development, reproduction, hematopoiesis and other biological processes, due to their ability to regulate differentiation, proliferation and apoptosis (Petterson et al., 2002; Sun and Lotan, 2002).

At pharmacological doses, retinoids induce a variety of effects associated with cancer prevention such as suppression of in vitro cell transformation, inhibition of carcinogenesis in various organs in animal models and a decrease in the occurrence of second primary tumors for epithelial malignancies including HCC (Okuno et al., 2001; Sun and Lotan, 2002).

Since retinoids have been shown to be toxic for long-term use and since they have very low tissue specificity (Lee et al., 1993) some new strategies have to be addressed before using these agents widely.

Liposomes are relatively non-toxic, biodegradable lipid vesicles that can be used for pharmacological delivery. They can alter the distribution and bioavailability of different drugs, representing a good approach for the treatment of several diseases (Felnerova et al., 2004; Kim and Lim, 2002).

Liposomes are lipid membranes mainly composed of a combination of natural or synthetic phospholipids and cholesterol and additional ligands can be also added to increase the specificity for tissue recognition (Felnerova et al., 2004).

One approach to direct liposomes specifically to the liver has been to introduce sugar moieties to the basic phospholipid composition. The idea behind this strategy is to take advantage of the presence of the asialoglycoprotein receptor (ASGPR), present mainly in hepatocytes (Dragsten et al., 1987; Kawakami et al., 1998; Treré et al., 1999; Wu et al., 2002). Since the addition of asialoglycoproteins to liposomes is rather complicated, a more approachable strategy is the incorporation of low molecular weight glycolipids, which can also be recognized by ASGPR (Hashida et al., 2001). For instance, an interesting study that used this approach for the delivery of 5-FU was recently published (Jin et al., 2005). This study showed that 5-FU could more efficiently inhibit tumor cell growth and induce apoptosis when is delivered encapsulated into galactosylceramide liposomes. Even the in vivo anti-neoplasic activity was better obtained when liposome-encapsulated 5-FU was used, and the authors suggest that the mechanism could be partially related to increased drug concentration in liver tumor tissues (Jin et al., 2005).

To reach liver targeting for gene transferring, several kinds of liposomes have been designed too. An interesting approach has been to include some novel galactosylated cholesterol derivatives into the liposomes (Kawakami et al., 1998). This approach could also be applied for pharmacological uses in the delivery of different drugs and medicaments such as chemotherapeutic agents.

In the case of retinoids, some studies have shown that synthetic retinoid *N*-(4-hydroxypheny) retinamide (fenretinide) (Takahashi et al., 2003) and natural retinoid ATRA (Shimizu et al., 2003) entrapped into liposomes containing soybean-derived sterylglucoside mixture have great potential for the treatment of cancer. Both studies showed that these pharmacological preparations are effective by increasing the survival of mice bearing tumor metastasis in the liver.

Some of the most undesirable results of chemotherapy are side effects due to toxicity to normal cells. However, neovasculature present in cancer tissues tends to be poorly formed with gaps between endothelial cells, which could be an advantage in treatments that use relatively big carriers such as liposomes (Kim and Lim, 2002). So, vascular permeability of cancer tissues is a significant physiological factor that should be considered in designing drug delivery systems. Then, encapsulation of therapeutic agents inside carriers is an attractive strategy in cancer treatment since it can minimize drug uptake by sensitive normal tissues.

Since it has been established that retinoids have high potential for the treatment of hepatocellular carcinoma but at the same time their effects are somehow poorly specific in terms of the tissues they affect, we decided to test the effect of these agents delivered into liposomes with some level of specificity for tumor liver cells. The specific aim of this study was to test the cytotoxic potential of two naturally occurring retinoids entrapped into glycolipid-containing liposomes (with galactosyl-sphingosine) in human hepatoma cell lines Hep3B and HepG2, in search of characterizing new potential delivery strategies for the treatment of HCC.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco essential medium, fetal bovine serum, 3-(4,5-dimethyl-2-thiazolyl(-2,5-diphenyl-2H-tetrazolium bromide)) (MTT), cationic and anionic liposome preparations and retinoids were obtained from Sigma Chemical Co. (St. Louis, MO). Egg phosphatidylcholine, D-galactosyl-1β1-1′-D-erythro-sphingosine and glucosyl- β1-1′-D-erythro-sphingosine were purchased from Avanti Polar Lipids Inc. (Alabaster, AL).

2.2. Cell culture and treatment

Two different human hepatoma cell lines, HepG2 and Hep3B and the myoblast cell line C2C12 (American type culture collection, ATCC) were maintained in Dulbecco essential medium in a 37 °C humidified incubator under an atmosphere of 7% CO_2 in air. For the experiments, cells were plated on 96-well plates to confluence and were allowed to adhere overnight. Various concentrations of retinoids (free or loaded into liposome preparations) were added to the plates in 100 μl of fresh medium and cells were incubated for 48 or 72 h periods as indicated in the figures.

2.3. Liposome preparation and drug loading

Retinoids were dissolved in dimethylsulfoxide (DMSO) and added to the chloroform lipid mixture. The samples were dried

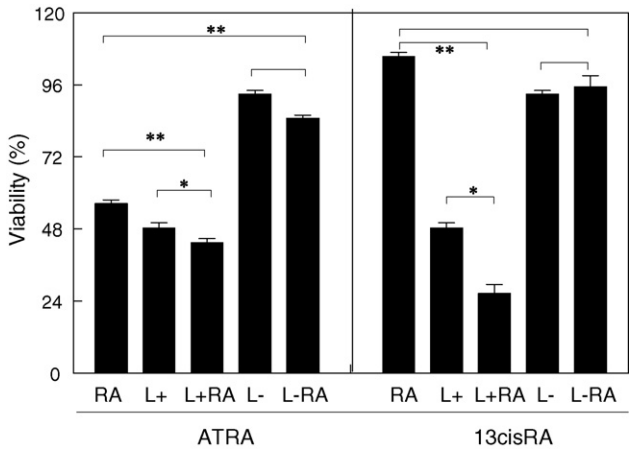

Fig. 1. Cytotoxic effect induced by cationic and anionic liposomes loaded with retinoids ATRA and 13*cis*RA on Hep3B cell line. Confluent cell populations were incubated with liposomes loaded with retinoids at a concentration of 50 μg/ml for 48 h. After treatment, MTT reagent was added to the culture medium and after 2 h of incubation, medium was removed and 95% ethanol was added to the wells to dissolve formazan crystals. Experiments were performed three times, absorbances were recorded at 570 nm and the results are expressed as mean ± S.E. of triplicates from one representative experiment. (L+) cationic liposomes; (L−) anionic liposomes; (RA) retinoic acid. Labels (*) and (**) represent statistically significant differences with $p < 0.05$ and $p < 0.01$, respectively.

under nitrogen and resuspended in cell culture medium (Dulbecco essential medium supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 100 IU/ml penicillin and amphotericin B). Samples were sonicated for 30 min at room temperature in a bath sonicator. The amount of lipid added to each well in the experiments to test liposome cytotoxicity (Figs. 1 and 2) was: 25.2 nmol phosphatidylcholine, 7.2 nmol stearylamine or phosphatidic acid and 3.6 nmol cholesterol. Liposomes containing stearylamine present positive charges (cationic liposomes) and the ones containing phosphatidic acid had negative charges (anionic liposomes). In the following experiments lipid amounts for cationic liposome preparation were reduced five times and

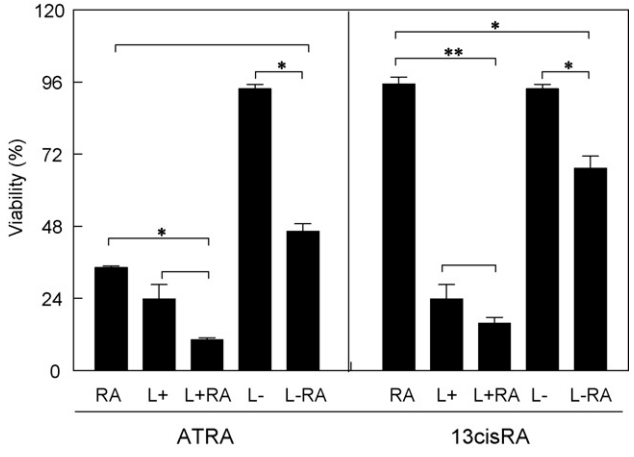

Fig. 2. Cytotoxic effect induced by cationic and anionic liposomes loaded with retinoids ATRA and 13*cis*RA on HepG2 cell line (see details in Fig. 1 legend). Experiments were performed three times and the results are expressed as mean ± S.E. of triplicates of one representative experiment.

the galactosyl- and glucosyl-sphingosine amounts added to the lipid preparation were of 0.5 nmol/well. PC-only liposomes were prepared with 5.2 nmol of egg PC per well and the same amount of sphingosine (0.5 nmol/well). Retinoic acid entrapment has been shown to be very high for multilamellar liposomes, with values ranging between 91 and 95% (Manconi et al., 2002; Nastruzzi et al., 1990). Retinoid incorporation was determined by HPLC using a C18 column (detection at 350 nm) using a mobile phase of acetonitrile, water and acetic acid (84.5:15:0.5) after previous separation of free retinoid by gel filtration using a sepharose 4B column. The result was about 90% of association of the retinoid with liposomes.

2.4. Assessment of cytotoxicity by MTT assay

Cells were treated with retinoids (free or loaded into liposome preparations) for 48 or 72 h and MTT reagent (final concentration of 0.5 mg/ml) was added to the culture medium. After 2 h of incubation at 37 °C, medium was carefully removed and 100 μl of 95% ethanol was added to the wells to dissolve formazan crystals. Absorbances were measured at 570 nm and readings were expressed as viability percentages using the drug solvent (DMSO) or medium as negative controls (100% of viability). Three independent experiments were performed in triplicate and the results shown in the figures represent the means of one of these reproducible experiments.

2.5. Statistical analysis

Paired Student's *t*-test was calculated between the chosen treatments using infostat statistics program.

3. Results

3.1. Cytotoxicity induced by retinoids loaded into cationic and anionic liposomes on hepatoma cells

Liposomes were prepared in the presence of 50 μg/ml ATRA or 13*cis*RA (previously dissolved in DMSO) and then added to both cell lines for 48 h (Figs. 1 and 2). Free ATRA was more efficient than 13*cis*RA inducing cell death in both cell lines, being free 13*cis*RA almost non-toxic at the concentration tested. We observed that cationic liposomes were very toxic for the cells (approximately 50% cytotoxicity for Hep3B and 80% for HepG2), whereas anionic liposomes were basically not toxic (Figs. 1 and 2).

When cytotoxicity of retinoids entrapped into liposomes was tested, we observed that whereas anionic liposomes seem to decrease drug toxicity or had no effect (except for the case of HepG2 cells treated with 13*cis*RA), retinoids loaded into cationic liposomes were more effective than free drugs, especially in the case of 13*cis*RA, which induced 76% of cytotoxicity in Hep3B cells (Fig. 1) and 84% in HepG2 cells (Fig. 2), but was practically non-toxic when was administered free. ATRA entrapped into cationic liposomes was also very toxic, inducing 56% of cytotoxicity in Hep3B cells (Fig. 1) and 90% in HepG2 (Fig. 2) but the difference compared to free drug was

lower due to the high toxicity obtained with cationic liposomes alone.

We decreased liposome concentrations five times for the following experiments (see Section 2) and since anionic liposomes basically did not improve the effect of free retinoids, we did the next set of experiments using only cationic liposomes.

3.2. Cytotoxicity induced by retinoids loaded into cationic liposomes containing galactosyl- and glucosyl-sphingosine on hepatoma cells

When we decreased the concentration of cationic liposomes, we observed a decrease in the toxicity that allowed better conditions for the experiment. Even though in this case, retinoids entrapped into liposomes did not induce more toxicity that free drugs, in the case of Hep3B cells, galactosyl-sphingosine containing liposomes with ATRA were more effective than the ones containing glucosyl-sphingosine (Fig. 3). In the case of treatment with 13*cis*RA, the results were not significantly different in any of the cell lines (Figs. 3 and 4) neither in the case of HepG2 cells treated with ATRA (Fig. 4).

We observed that in some cases the values were over 100% of viability suggesting that proliferation was being induced in some of the treatments with retinoic acid (Ledda-Columbano et al., 2004). This effect could be covering up the cytotoxicity results in the cases where retinoids were better introduced into the cells due to the liposome carriers and proliferation could occur in the first hours of treatment, increasing the number of cells present in the plates.

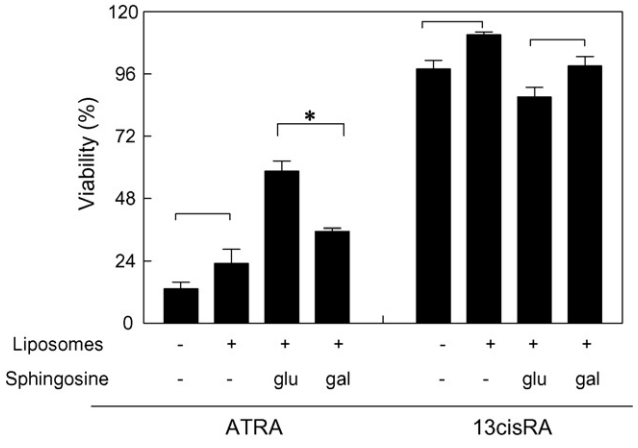

Fig. 3. Cytotoxic effect induced by retinoids loaded into cationic liposomes containing galactosyl- and glucosyl–sphingosine on Hep3B cells. Liposome lipid amount was five times less than the one used in previous experiments (Figs. 1 and 2). In the case of liposomes containing (gal) galactosyl- and (glu) glucosyl-sphingosine, the amount of lipid added was: 0.5 nmol/well. Cells were incubated with liposomes loaded with retinoids for 72 h at a concentration of 50 μg/ml (see details in Fig. 1 legend). (+) Presence; (−) absence of different lipids. Label (*) represents statistically significant differences with $p < 0.05$. All treatments shown contain retinoid. Controls not shown (cationic liposomes, glycolipids, and glycolipids with cationic liposomes, all without retinoid) gave values higher than 90% of viability. Experiments were performed three times and the results are expressed as mean ± S.E. of triplicates of one representative experiment.

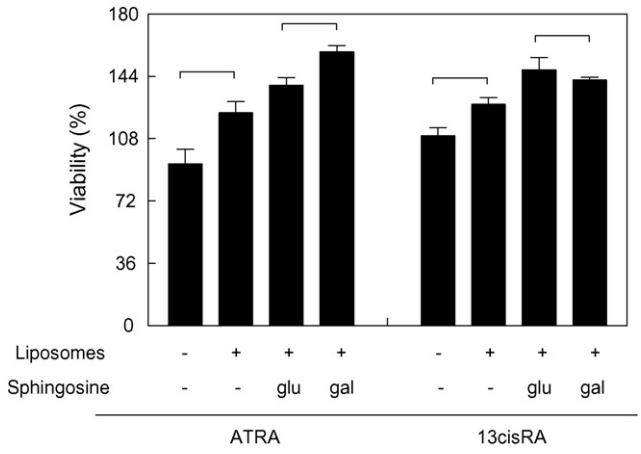

Fig. 4. Cytotoxic effect induced by retinoids loaded into cationic liposomes containing galactosyl- and glucosyl-sphingosine on HepG2 cells. Cells were incubated with liposomes loaded with retinoids for 72 h at a concentration of 50 μg/ml (see details in Fig. 3 legend). (+) Presence; (−) absence of different lipids; (gal) galactosyl-sphingosine; (glu) glucosyl-sphingosine. All treatments shown in the figure contain retinoid. Controls not shown gave values higher than 95% of viability. Experiments were performed three times and the results are expressed as mean ± S.E. of triplicates of one representative experiment.

Interestingly, ATRA loaded into cationic liposomes (devoid of shingosine) was also very toxic on Hep3B cells, showing probably good internalization, since in this experiment, cationic liposomes alone did not show significant toxicity (Fig. 3). We did not observe the same effect in HepG2 cells, where ATRA loaded into cationic liposomes was not toxic (Fig. 4).

HepG2 cells were the ones showing viability percentages closer to 150% in the case of treatment with both retinoids loaded into sphingosine-containing liposomes (either galactosyl- or glucosyl-sphingosine), suggesting an increase in proliferation during the treatments that led to results more difficult to explain. This could also explain why same concentration of free ATRA was not toxic at 72 h (Fig. 4) whereas it was very toxic at 48 h (Fig. 2).

3.3. Cytotoxicity induced by ATRA loaded into neutral liposomes (PC-only) containing galactosyl- and glucosyl-sphingosine on Hep3B cell line

Since cationic liposomes containing ATRA, but devoid of glycolipids, were toxic for Hep3B cells, we decided to change the basic composition of the liposomes, utilizing only egg phosphatidylcholine (PC), trying to understand the effect induced by the retinoids contained in liposomes, on this cell line. We still observed a slightly more toxic effect with ATRA loaded into galactosyl-sphingosine-containing PC-liposomes than the ones containing glucosyl-sphingosine, but the results were not statistically significant (Fig. 5). In this experiment ATRA was not toxic when administered into PC-liposomes, contrary to what was observed with cationic liposomes (Fig. 3). We used, however, a lower concentration of ATRA to test the effect of PC-liposomes (25 μg/ml).

Something interesting we observed in Fig. 5 was that the effect of ATRA together with galactosyl-sphingosine was higher

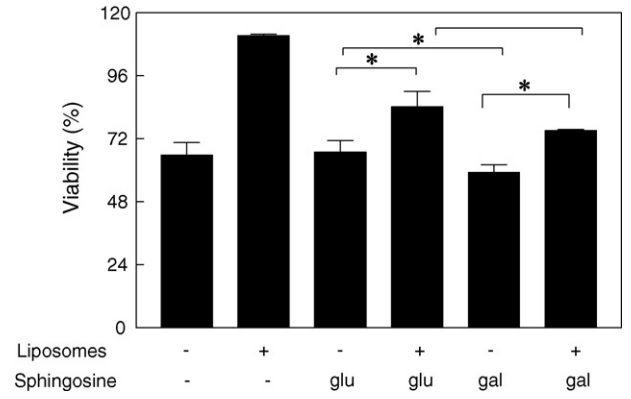

Fig. 5. Cytotoxic effect induced by ATRA-loaded into neutral liposomes (made only with egg PC) containing galactosyl- and glucosyl-sphingosine on Hep3B cells. Cells were incubated with liposomes loaded with ATRA for 48 h at a concentration of 25 μg/ml (see details in Fig. 3 legend). All treatments shown in the figure contain retinoid. All the controls not shown (treatments without retinoid) gave values higher than 95% of viability. Experiments were performed three times and the results are expressed as mean ± S.E. of triplicates of one representative experiment.

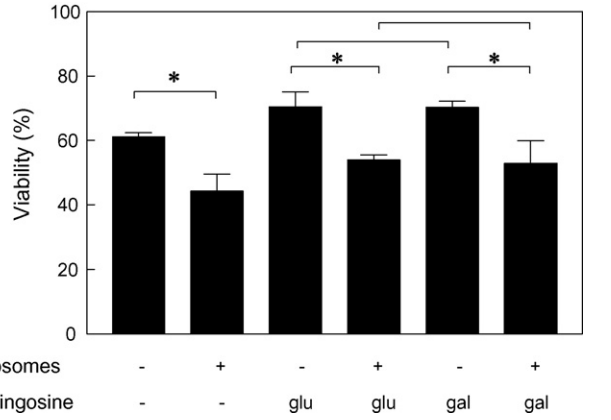

Fig. 6. Cytotoxic effect induced by ATRA-loaded into neutral liposomes containing galactosyl- and glucosyl-sphingosine on C2C12 myoblasts. Cells were incubated with liposomes loaded with ATRA for 48 h at a concentration of 25 μg/ml (see details in Fig. 3 legend). All treatments shown contain retinoid. Controls not shown (treatments without retinoid) gave values higher than 90% of viability. Experiments were performed three times and the results are expressed as mean ± S.E. of triplicates of one representative experiment.

than the results presented for all the other treatments, an effect comparable to cytotoxicity induced by free ATRA. The drug together with glucosyl-sphingosine was toxic too, but the effect was significantly lower than ATRA mixed with galactosyl-sphingosine (Fig. 5). These last results were not observed previously because we did not test sphingosines free of other lipids in the previous experiments.

We have to point out that the cytotoxic effect induced by ATRA mixed with sphingosine was significantly higher than the effect induced by ATRA loaded into sphingosine-containing PC-only liposomes (Fig. 5).

3.4. Cytotoxicity induced by retinoids loaded into neutral (PC-only) and cationic liposomes containing galactosyl- and glucosyl-sphingosine on myoblasts C2C12

To test the effect of retinoids in non-neoplasic cells, we chose a myoblastic cell line, C2C12, and we incubated this cell line with both retinoids loaded into cationic and PC-liposomes containing galactosyl- and glucosyl-sphingosine (Figs. 6 and 7). The drug concentration used was 25 μg/ml (PC-liposomes) and 50 μg/ml (cationic liposomes). As with the hepatoma cell lines, we observed that ATRA was more toxic than 13*cis*RA. In the case of treatment with 13*cis*RA, we did not observe any toxicity (results not shown). In the case of treatment with ATRA, we observed that the drug was more effective when administered into the sphingosine-containing liposomes than when it was mixed only with galactosyl- and glucosyl-sphingosine (Figs. 6 and 7). So, in the case of the myoblasts, sugar-containing sphingosines delivered together with the drug were not as effective as the drug loaded into the sphingosine-containing liposomes, an effect that was observed in Hep3B cells (Fig. 5). Also, not statistically significant differences were observed between the effects obtained with the drug contained into liposomes with galactosyl- and glucosyl-sphingosine, suggesting that the effect was not galactose-specific (Figs. 6 and 7).

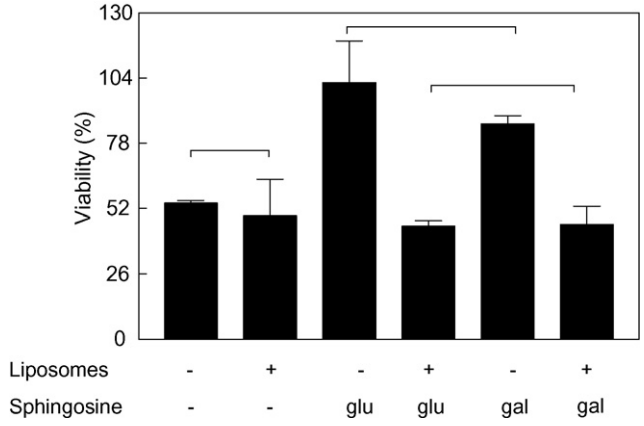

Fig. 7. Cytotoxic effect induced by ATRA-loaded into cationic liposomes containing galacosyl- and glucosyl-sphingosine on C2C12 myoblasts. Cells were incubated with liposomes loaded with ATRA for 48 h at a concentration of 50 μg/ml (see details in Fig. 3 legend). All treatments shown contain retinoid. Controls not shown (treatments without retinoid) gave values higher than 95% of viability. Experiments were performed three times and the results are expressed as mean ± S.E. of triplicates of one representative experiment.

One of the most evident differences between the effect of ATRA in C2C12 and Hep3B cells was that liposomes with the drug were toxic for the myoblasts (approximately 50% of cytotoxicity) whereas same retinoid incorporated into PC-only liposomes did not induce cytotoxicity in the hepatoma cell line Hep3B (Figs. 5–7).

4. Discussion

An ideal drug delivery system should be able to direct drugs exclusively to their desired sites of action, showing minimal toxic exposure to sensitive non-target systems. Also the delivery system itself should be pharmacologically inactive, biodegradable and practically non-toxic (Kim and Lim, 2002). Encapsulation or membrane incorporation of therapeutic agents inside

colloidal carriers has been one of the most interesting strategies in the treatment of diseases, including cancer.

According to the criteria mention above, liposomes fit several of the parameters to be good delivery systems, however they are sometimes cleared very fast from circulation and some lipids could be toxic at certain concentrations (Filion and Phillips, 1997; Chien et al., 2005).

In the case of targeting the liver, an approach that has been tried is the introduction of sugar moieties to the basic phospholipid composition of the liposomes (Dragsten et al., 1987; Kawakami et al., 1998; Treré et al., 1999; Wu et al., 2002). Liver tissue and other few organs in a lesser extent, have the asialoglycoprotein receptor in their membranes, which mediates part of the internalization of glycosylated molecules, including glycolipids (Kawakami et al., 1998; Hashida et al., 2001).

In an study performed by Mehta (1989) it was reported that free ATRA administered to CD1 mice exerted toxic effects at 25–30 mg/kg of body weight while ATRA-loaded into liposomes (made of dipalmitoylphosphatidylcholine, cholesterol and sterylglucoside mixture) could be tolerated to much higher doses (120 mg/kg) possibly due to alterations in drug distribution in target tissues.

We observed that some lipids used for the preparation of the liposomes could be very toxic for the cells, mainly cationic lipids. This is a well known fact since cationic liposomes and nanoparticles have been widely used for a long time for DNA delivery to the cells (Goodman et al., 2004). However, it is also well known that cationic liposomes are one of the best systems for the incorporation of different kinds of substances to the cells (Dass and Su, 2001). Then, we decided to test commercial liposome preparations, either cationic or anionic, to incorporate two different naturally occurring retinoids, ATRA and 13*cis*RA to two hepatoma cell lines with different degrees of transformation. We also made liposomes containing only phosphatidylcholine, to test also potential toxicity of the retinoid ATRA to the hepatoma cell line Hep3B.

We confirmed the toxicity of cationic phospholipids and we observed that retinoids incorporated into positively charged liposomes were significantly more toxic to the cells than the free drugs. The effect was more pronounced in the case of 13*cis*RA, since at the concentration tested here, free retinoid was unable to induce cytotoxicity on any of the cell lines. The effect of ATRA, the other retinoid tested, was very strong in both cell lines, when administered loaded into cationic liposomes.

Negatively charged liposomes, on the other hand, had no effect or even protected the cells from the retinoids, therefore, they do not seem to be very good delivery systems, according to these results. Cationic liposomes, on the other hand, seem to show promising results. However, since cationic liposomes were still very toxic by themselves, we decided to decrease lipid concentration and try the experiment again including sugar-containing sphingosines to increase targeting specificity due to possible recognition by the asialoglycoprotein receptor, highly expressed in liver cells. Since the receptor recognizes galactose and *N*-acetylgalactosamine (Treré et al., 1999), we included galactosyl-sphingosine in the lipid mixture, whereas as a control we used glucosyl-sphingosine, a similar lipid that contains a carbohydrate not specifically recognized by ASGPR.

We determined that retinoids entrapped into galactosyl-sphingosine containing cationic liposomes were not better incorporated into the cells than free retinoids, at least they did not induce higher cytotoxicity at 72 h of treatment. It is evident, however that free hydrophobic drugs permeate plasma membranes very rapidly and liposome endocytosis cannot compete in terms of time of internalization. For instance, it has been shown that retinoid transfer across and between phospholipid bilayers occurs in less than 30 s (Rando and Bangerter, 1982; Nastruzzi et al., 1990), so it would be very difficult to induce an effect faster than that.

Similar to our results, an article using nanoparticles coated with galactose-carrying polymer showed that retinoic acid was always more toxic when added free than when incorporated into nanoparticles, which are internalized through receptor-mediated endocytosis (Cho et al., 2001).

Since we were looking for a delivery system that would allow us the specific incorporation of retinoids into liver cells and at the same time no incorporation or less capture by other tissues, we focused on the results derived from the experiments with sugar-containing sphingosines. So, when we did the comparison between liposomes containing the two different glycolipids, we observed that galactosyl-sphingosine significantly increased the toxicity of the ATRA treatment in Hep3B cells. Therefore, ATRA entrapped into galactosyl-containing liposomes could be incorporated more specifically in these cells through ASGPR, an event that triggers the mechanisms of apoptosis induced by the retinoid inside the cells (Arce et al., 2005).

No significant differences were observed in the treatments with 13*cis*RA in both cell lines and ATRA in HepG2 cells, when we compared the effects of the drug in liposomes containing the different glycolipids. We cannot determine whether 13*cis*RA was not toxic due to lack of cell penetration or the absence of a receptive system (appropriate retinoic acid receptors, for instance) to this retinoid in the cells. We had previously shown that free 13*cis*RA induces cytotoxicity in Hep3B cells at the concentration used here (Arce et al., 2005), but for some reason we did not observed the same effect in this set of experiments. HepG2 cells, on the other hand are not as sensitive as Hep3B to these retinoids (Arce et al., 2005) and the effect was not improved by the incorporation of the drug into liposomes. It would be possible that 13*cis*RA is being better incorporated into the cells, in the same way ATRA is, but the response is not triggered due to lack of further activity.

Some of the results with HepG2 cells treated with free ATRA are difficult to explain, such as the higher than 100% viability results and the fact that free ATRA was very toxic for HepG2 cells in a 48 h treatment (Fig. 2) but did not induce the same toxicity when the treatment was prolonged 24 h more (Fig. 4). It seems that another effect that increases MTT absorbances is covering up the cytotoxicity results, indicating that other kind of methods should be used to measure cytotoxicity (or apoptosis), to better understand these results. A possibility we mentioned in Section 3 is the fact that it has been shown that ATRA can induce proliferation on hepatocytes (Ledda-Columbano et al.,

2004) suggesting that this event could be interfering with the cytotoxicity measurement.

Studies show that hepatocytes and HCC cells have different ASGPR expression (Hyodo et al., 1993; Treré et al., 1999) For example, an study using immunostaining techniques showed that 85% of well differentiated but only 20% of poorly differentiated HCCs were positive for the presence of the receptor (Treré et al., 1999). This could partially explain the differences obtained between the two cell lines utilized in this study. HepG2 cells are somehow less transformed and could express a reduced amount of ASGPR, showing lower retinoic acid internalization by receptor-mediated endocytosis. In any case, the different distribution of ASGPR and the higher expression in more differentiated HCC cells is a finding that can be exploited to improve a more specific chemotherapy approach (Treré et al., 1999).

Due to the apparent non-specific effect of ATRA-loaded into cationic liposomes (without glycolipids), we decided to test another type of liposome, made only with PC, to try to eliminate the intrinsic toxicity due probably to the positive charges. These liposomes did not contain cholesterol as the other preparations, so we expect also some variations in the fluidity of their membranes.

A previous study that measured anti-proliferative effects induced by retinoic acid on leukemic cell lines had shown a better effect when the agents were incorporated into PC-only containing liposomes, compared to free drugs (Nastruzzi et al., 1990). In this study, the authors did not find any toxicity with the empty liposomes as we did, and suggested that the improvement in the effect induced by PC-encapsulated retinoic acid could be due to an increased uptake of the drugs (Nastruzzi et al., 1990).

When we used liposomes made only with PC, we observed no significant differences in toxicity between ATRA-loaded into liposomes containing galactosyl- and glucosyl-sphingosine. We also observed here that ATRA mixed with sugar-containing sphingosine induced a better effect than liposomes containing the retinoid, on Hep3B cells. We hypothesized that micelles formed between sphingosine and hydrophobic retinoid were better incorporated into the cells, perhaps due to their smaller size, since the effect seemed to be still specific and mediated by ASGPR. Emulsions, which have an oil core, in contrast to the aqueous core of liposomes, can provide better systems for hydrophobic molecules, increasing therapeutic efficacy (Kim and Lim, 2002). Emulsion systems have been used for the incorporation of non-polar substances such as protoporphyrin IX (De Rosa et al., 2000) and 1,25-dihydroxyvitamin D3 (Finlay et al., 2002), among others, and clinical trials have been done with emulsion formulations of Paclitaxel (Sonus Pharmaceutical). Galactose-containing emulsion systems have also been tested for liver delivery and internalization of lipophilic drugs through ASGPR-mediated endocytosis (Ishida et al., 2004).

For the last set of the experiments we decided to test the effect of retinoids loaded into PC-only containing liposomes as well as cationic liposomes, in a myoblast cell line (C2C12), as a model for a non-neoplasic cell type and a cell line that has not been reported to contain ASGPR at its surface. We observed that, free ATRA induced the same extent of toxicity than on Hep3B cells, and 13*cis*RA was also not toxic, similar to what was observed in hepatoma cells. Retinoids have been shown to induce several effects on myotubes (long cells formed by the fusion of several myoblasts), including myogenic differentiation and also inhibit cell proliferation in myoblastic cell line C2C12 (Stio et al., 2002).

Interestingly, we did not observed a significant difference between the effect of ATRA incorporated into liposomes containing galactosyl- and glucosyl-sphingosine, so the hypothesis should be that the effect does not seem to be mediated by ASGPR in this cell line. Another intriguing result was that the mixture of ATRA and sphingosine was less toxic than liposomes loaded with the drug, the opposite of what was observed in Hep3B cells. On the other hand, ATRA-loaded into liposomes was also more toxic than free ATRA, an effect that was also not seen on Hep3B.

In conclusion, all these results show that even though liposomes could be a good delivery system for these drugs in the treatment of diseases such as liver cancer, the study of the interactions between lipids and the drugs and lipids and the membranes, as well as the specific mechanisms of internalization (simple endocytosis or receptor-mediated endocytosis), could predict the effect that these agents would have in tumor cells. We hypothesize that cationic liposomes are a good approach to deliver retinoic acid to hepatoma cells, but the concentration of lipids have to be carefully regulated and sugar-containing lipids should be added to the mixture to decrease non-specific effects (decreasing toxicity on normal cells) and increase the specificity by triggering ASGPR-mediated endocytosis. Negatively charged liposomes and PC-only containing liposomes do not seem to be good delivery systems for these agents. Perhaps the best delivery system would be an emulsion prepared with galactose-containing lipids, that would increase the specificity of the mechanism and at the same time improve the efficacy of the internalization due to the smaller size of the micelles compared to liposomes. In vivo studies remain to be carried out to test the effect of these specific micelles and liposomes in liver tumors present in animal models.

Acknowledgements

This work was funded by Consejo Nacional para Investigaciones Científicas y Tecnológicas (CONICIT) and Vicerrectoría de Investigación, Universidad de Costa Rica.

References

Arce, F., Gätjens-Boniche, O., Vargas, E., Valverde, B., Díaz, C., 2005. Apoptotic events induced by naturally occurring retinoids ATRA and 13-*cis* retinoic acid on human hepatoma cell lines Hep3B and HepG2. Cancer Lett. 229, 271–281.

Bosch, F.X., Ribes, J., Cleries, R., Díaz, M., 2005. Epidemiology of hepatocellular carcinoma. Clin. Liver Dis. 9, 191–211.

Chien, P.Y., Wang, J., Carbonaro, D., Lei, S., Miller, B., Sheikh, S., Ali, S.M., Ahmad, M.U., Ahmad, I., 2005. Novel cationic cardiolipin analogue-based liposome for efficient DNA and small interfering RNA delivery in vitro and in vivo. Cancer Gene Ther. 12, 321–328.

Cho, C.S., Cho, K.Y., Park, I.K., Kim, S.H., Sasagawa, T., Uchiyama, M., Akaike, T., 2001. Receptor-mediated delivery of all *trans*-retinoic acid to hepatocyte using poly(L-lactic acid) nanoparticles coated with galactose-carrying polystyrene. J. Controlled Release 77, 7–15.

Dass, C.R., Su, T., 2001. Particle-mediated intravascular delivery of oligonucleotides to tumors: associated biology and lessons from genotherapy. Drug Deliv. 8, 191–213.

De Rosa, F.S., Marchetti, J.M., Thomazini, J.A., Tedesco, A.C., Bentley, M.V., 2000. A vehicle for photodynamic therapy of skin cancer: influence of dimethylsulfphoxide on 5-amino-levulinic acid in vitro cutaneous permeation and in vivo photoporphyrin IX accumulation determined by confocal microscopy. J. Controlled Release 65, 359–366.

Dragsten, P.R., Mitchell, D.B., Covert, G., Baker, T., 1987. Drug delivery using vesicles targeted to the hepatic asialoglycoprotein receptor. Biochim. Biophys. Acta 926, 270–279.

Felnerova, D., Viret, J.F., Gluck, R., Moser, C., 2004. Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs. Curr. Opin. Biotecnol. 15, 518–529.

Filion, M.C., Phillips, N.C., 1997. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. Biochim. Biophys. Acta 1329, 345–356.

Finlay, I.G., Stewart, G.J., Pourgholami, M.H., Akhter, J., Morris, D.L., 2002. The use of lipiodol and medium chain triglyceride as delivery agents for hepatic arterial administration of 1,25-dihydroxyvitamin D3—A potential new treatment for hepatocellular carcinoma. Anticancer Res. 20, 2705–2709.

Fisher, J., 2005. Liver cancer on the rise. Ann. Int. Medicine 142, 1029–1032.

Goodman, C.M., McCusker, C.D., Yilmaz, T., Rotello, V.M., 2004. Toxicity of gold nanoparticles functionalized with cationic and anionic side chains. Bioconjug. Chem. 15, 897–900.

Hashida, M., Nishikawa, M., Yamashita, F., Takakura, Y., 2001. Cell-specific delivery of genes with glycosilated carriers. Adv. Drug Deliv. Rev. 52, 187–196.

Hyodo, I., Mizuno, M., Yamada, G., Tsuji, T., 1993. Distribution of asialoglycoprotein receptor in human hepatocellular carcinoma. Liver 13, 80–85.

Ishida, E., Managit, C., Kawakami, S., Nishikawa, M., Yamashita, F., Hashida, M., 2004. Biodistribution characteristicas of galactosylated emulsions and incorporated probucol for hepatocyte-selective targeting of lipophilic drugs in mice. Pharm. Res. 21, 932–939.

Jin, Y., Li, J., Rong, L.F., Li, Y.H., Guo, L., Xu, S.Y., 2005. Anti-hepatocarcinoma effects of 5-fluorouracil encapsulated by galactosylceramide liposomes in vivo and in vitro. World J. Gastroenterol. 11, 2643–2646.

Kawakami, S., Yamashita, F., Nishikawa, M., Takakura, Y., Hashida, M., 1998. Asialoglycoprotein receptor-mediated gene transfer using novel galactosylated cationic liposomes. Biochem. Biophys. Res. Commun. 252, 78–83.

Kim, C.K., Lim, S.J., 2002. Recent progress in drug delivery systems for anticancer agents. Arch. Pharm. Res. 25, 229–239.

Ledda-Columbano, G.M., Pibiri, M., Molotzu, F., Cossu, C., Senna, L., Simbula, G., Perra, A., Columbano, A., 2004. Induction of hepatocyte proliferation by retinoic acid. Carcinogenesis 25, 2061–2066.

Lee, J.S., Newman, R.A., Lippmann, S.M., Huber, M.H., Minor, T., Raber, M.N., Krakoff, I.H., Hong, W.K., 1993. Phase I evaluation of all-*trans*-retinoic acid in adults with solid tumors. J. Clin. Oncol. 11, 959–966.

Leung, T.W.T., Johnson, P.J., 2001. Systemic therapy for hepatocellular carcinoma. Sem. Oncol. 28, 514–520.

Manconi, M., Sinico, C., Valenti, D., Loy, G., Fadda, A.M., 2002. Niosomes as carriers for tretinoin. I. Preparation and properties. Int. J. Pharm. 234, 237–248.

Mehta, K., 1989. Interaction of liposome-encapsulated retinoids with normal and leukemic cells. Pharm. Skin 3, 74–80.

Muto, Y., Moriwaki, H., Saito, A., 1999. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. New Engl. J. Med. 340, 1046–1047.

Nastruzzi, C., Walde, P., Menegatti, E., Gambari, R., 1990. Liposome-associated retinoic acid. Increased in vitro anti-proliferative effects on neoplastic cells. FEBS Lett. 259, 293–296.

Okuno, M., Sano, T., Matsushima-Nishiwaki, R., Adachi, S., Akita, K., Okano, Y., Kojima, S., Moriwaki, H., 2001. Apoptosis induction by acyclic retinoid: a molecular basis of clonal deletion therapy for hepatocellular carcinoma. Jpn. J. Clin. Oncol. 3, 359–362.

Petterson, F., Dalgleish, A.G., Bissonnette, R.P., Colston, K.W., 2002. Retinoids cause apoptosis in pancreatic cancer cells via activation of RAR-γ and altered expression of Bcl-2/Bax. Brit. J. Cancer 87, 555–561.

Rando, R.R., Bangerter, F.W., 1982. The rapid intermembraneous transfer of retinoids. Biochem. Biophys. Res. Commun. 104, 430–436.

Shimizu, K., Tamagawa, K., Takahashi, N., Takayama, K., Maitani, Y., 2003. Stability and antitumor effects of all-*trans* retinoic acid-loaded liposomes contained sterylglucoside mixture. Int. J. Pharm. 258, 45–53.

Stio, M., Celli, A., Treves, C., 2002. Synergistic effect of vitamin D derivatives and retinoids on C2C12 skeletal muscle cells. IUBMB Life 53, 175–181.

Sun, S.Y., Lotan, R., 2002. Retinoids and their receptors in cancer development and chemoprevention. Crit. Rev. Oncol. Hematol. 4, 41–55.

Takahashi, N., Tamagawa, K., Shimizu, K., Fukui, T., Maitani, Y., 2003. Effects on M5076-hepatic metastasis of retinoic acid and *N*-(4-Hydroxyphenyl) retinamide, fenretinide entrapped in SG-liposomes. Bio. Pharm. Bull. 26, 1060–1063.

Treré, D., Fiume, L., DeGiorgi, B., DiStefano, G., Migaldi, M., Derenzini, M., 1999. The asialoglycoprotein receptor in human hepatocellular carcinomas: its expression on proliferating cells. Brit. J. Cancer 81, 404–408.

Wu, J., Nantz, M.H., Zern, M.A., 2002. Targeting hepatocytes for drug and gene delivery: emerging novel approaches and applications. Front. Biosci. 7, d717–d725.