[^{3}H]ATRA (1% DMSO) or [^{3}H]ATRA incorporated in DSPC/cholesterol or DOTAP/cholesterol liposomes (1 μM ATRA) at 37 or 4 °C. After incubation, the cells were washed thoroughly with Hank's buffered salt solution (HBSS) 5× and solubilized with 1.0 ml 0.3 M NaOH with 0.1% Triton X-100. Aliquots were taken to determine the radioactivity, using a liquid scintillation counter (LSA-500, Beckman Coulter, Inc., Fullerton, CA, USA), and the protein content. The radioactivity data were normalized with respect to the protein contents of the cells.

2.7. Cytotoxicity study

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT) assay was performed by the method described previously [25,27]. The A549 cells were plated on a 96-well cluster dish at a density of 1×10^4 cells/0.28 cm^2. Twenty-four hours later, the medium containing various concentrations of ATRA, bare liposomes, or ATRA incorporated in liposomes was added to the plates. After 48 h of incubation, the medium was removed and 5 mg/ml MTT solution was added to each well. Cells were incubated for 4 h at 37 °C in 5% CO_2 and then 10% sodium dodecyl sulfate (SDS) solution was added followed by incubation overnight to dissolve formazan crystals. The absorbance was measured at wavelengths of 570 nm in a microplate photometer (Bio-Rad Model 550, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.8. DNA fragmentation study

The A549 cells were plated on a 6-well cluster dish at a density of 1×10^5 cells/10.5 cm^2. Twenty-four hours later, the medium containing 1.0 μM ATRA (1% DMSO), bare liposomes, or ATRA incorporated in liposomes was added to the plates. After 48 h of incubation, the supernatant cells and adherent cells were collected. After centrifugation, the pellets were resuspended in 0.2 ml PBS (−). These cells were incubated with 10 μl proteinase K (10 mg/ml), 66.6 μl RNase A (1.5 mg/ml) and 10 μl 10% SDS solution for 30 min at 37 °C, and then incubated with 300 μl NaI solution (6 M NaI, 13 mM EDTA, 0.5% sodium-*N*-lauroylsarcosinate, and 26 mM Tris–HCl, pH 8.0) for 15 min at 60 °C. This was followed by the addition of 500 μl isopropanol and centrifugation at 20,000×*g* for 30 min to extract the total DNA. After agarose electrophoresis at 100 V for 25 min and labeling with ethidium bromide, total DNA was visualized by UV.

2.9. Measurement of cell apoptosis

The A549 cells were plated on a 6-well cluster dish at a density of 1×10^5 cells/10.5 cm^2. Twenty-four hours later, the medium containing 1.0 μM ATRA (1% DMSO), bare liposomes, or ATRA incorporated in liposomes was added to the plates. After 48 h of incubation, cells were trypsonized, collected and centrifuged. After washing in phosphate-buffered saline, 100 μl HEPES buffer including 10 μl annexin V-fluorecsein and 10 μl propidium iodide (PI) were added and cells were resuspended. Labeled cells were counted by flow cytometry (Becton Dickinson Co., Inc., Franklin Lakes, NJ, USA). Annexin V-FITC labeling was measured at 518 nm on the FL1-channel (FITC-detector) and PI staining at 620 nm on the FL2-channel (phycoerytrin-detector).

2.10. Quantification of TIG3 mRNA

The A549 cells were plated on a 6-well cluster dish at a density of 1×10^5 cells/10.5 cm^2. Twenty-four hours later, medium containing 1.0 μM ATRA (1% DMSO) or ATRA incorporated in liposomes was added to the plates. After 48 h incubation, total RNA was extracted using a MagExtractor-RNA kit and MagExtractor System (Toyobo Co., Ltd., Osaka, Japan). The total RNA was subjected to reverse transcription, which was performed by reverse transcription using an RT–PCR core kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Real-time RT–PCR was performed with a LightCycler® 350S system (Roche Diagnostics GmbH, Mannheim, Germany) in LightCycler capillaries using a commercially available master mix containing SYBR–Green I [28]. After the addition of the primers (final concentration=0.2 μM) and the template DNA to the master mix, 30 cycles of denaturation (94 °C for 1 s), annealing (58 °C for 10 s) and extension (72 °C for 10 s) were performed. After the completion of PCR amplification, a melting curve analysis was performed. Primers were designed by the Nihon Gene Research Laboratories, Inc. (Miyagi, Japan). The sequences for tazarotene-induced gene 3 (TIG3) primers were as follows: 5′–ACCATGAGTAC-CAACCACG–3′ and 5′–CCACACCGACTTCAACCTT–3′. The primers produce a PCR fragment of 177 bp. The quality of RNA and cDNA synthesis was determined by amplification of the glyceraldehyde phosphate dehydrogenase (GAPDH) gene as the internal control. The primer sequences for the GAPDH primers were as follows: 5′–TGAACGGGAAGCTCACTGG–3′ and 5′–TCCACCACCCTGTTGCTGTA–3′. The primers produced a PCR fragment of 307 bp.

2.11. Statistical analysis

Statistical comparisons were performed by Student's *t*-test for two groups, and one-way ANOVA for multiple groups. $P<0.05$ was considered indicative of statistical significance.

3. Results

3.1. Physicochemical properties of liposomes

The zeta potentials and the particle sizes of the liposomes are shown in Table 1. The zeta potentials of ATRA incorporated in DOTAP/cholesterol or DSPC/cholesterol liposomes were about +50 and −3 mV indicating that DOTAP/cholesterol or DSPC/cholesterol liposomes are cationic and neutral liposomes. The particle sizes of ATRA incorporated in DOTAP/cholesterol or DSPC/cholesterol liposomes were about 125 and 110 nm. The zeta potentials and particle sizes of DOTAP/cholesterol or DSPC/cholesterol liposomes without

Table 1
The zeta potential and mean particle size of ATRA incorporated in DSPC/cholesterol or DOTAP/cholesterol liposomes

Formulations	Zeta potential (mV)	Particle size (nm)
ATRA incorporated in DOTAP/cholesterol liposomes	51.2±0.80	132±57.3
ATRA incorporated in DSPC/cholesterol liposomes	−3.11±0.30	115±40.0

Each value represent the mean±S.D. of at least three experiments.

ATRA were the same as those of ATRA incorporated in DOTAP/cholesterol or DSPC/cholesterol liposomes [20,29].

3.2. ATRA incorporation of liposomes

The incorporation ratio of ATRA in neutral liposomes and DOTAP/cholesterol liposomes was 95.6±0.42% (n=3) and 97.5±0.38% (n=3), indicating that most of the ATRA was tightly incorporated in DSPC/cholesterol liposomes and DOTAP/cholesterol liposomes.

3.3. Cytotoxic effects of ATRA or ATRA incorporated in liposomes

In the present study, DOTAP/cholesterol and ATRA were mixed at a molar ratio of 100:1. To assess the anti-tumor activity in terms of A549 cells, the cytotoxic effects were examined by MTT assay. In a preliminary experiment, the cytotoxicity of bare DOTAP/cholesterol liposomes was evaluated by varying the concentration of bare cationic liposomes. Higher cytotoxicity was observed in a concentration-dependent manner for DOTAP/cholesterol liposomes; however, no significant cytotoxicity was observed at a concentration of 100 μM bare DOTAP/cholesterol liposomes (data not shown). In the following experiments, therefore, the concentrations of DOTAP/cholesterol liposomes and ATRA for the application to cells were adjusted to 100 and 1.0 μM, respectively, to minimize the cytotoxic effects of DOTAP/cholesterol liposomes.

After application of free ATRA to A549 cells, cytotoxic effects were observed in a concentration-dependent manner (Fig. 1A). In order to examine whether the cytotoxic effects could be enhanced by incorporation into each of the liposomes, ATRA was incorporated into neutral liposomes or DOTAP/cholesterol liposomes. Although ATRA and ATRA incorporated in neutral liposomes did not induce cytotoxicity, a significantly higher cytotoxicity was induced in ATRA incorporated in DOTAP/cholesterol liposomes at an ATRA concentration of 1.0 μM (Fig. 1B).

3.4. Cellular uptake study of ATRA or ATRA incorporated in liposomes

At 37 °C, ATRA uptake was enhanced by incorporation into liposomes, especially in DOTAP/cholesterol liposomes (Fig. 2A). At 4 °C, on the other hand, little uptake of [^{3}H] ATRA was observed and there was no significantly difference among these formulations until 6 h (Fig. 2B).

3.5. Quantitative PCR analysis of TIG3 mRNA of ATRA or ATRA incorporated in liposomes

Fig. 3 shows the effect of the liposomal formulations on the induction of TIG3 mRNA at an ATRA concentration of 1.0 μM. After application of free ATRA, ATRA incorporated in DSPC/cholesterol liposomes, and bare DOTAP/cholesterol liposomal formulations, TIG3 mRNA did not differ significantly from the control (no treatment). However, a significantly higher expression of TIG3 mRNA was observed when ATRA was incorporated into DOTAP/cholesterol liposomes.

3.6. Apoptosis induction of ATRA or ATRA incorporated in liposomes

To study whether 1.0 μM ATRA incorporated in DOTAP/cholesterol liposomes could induce apoptosis, this was

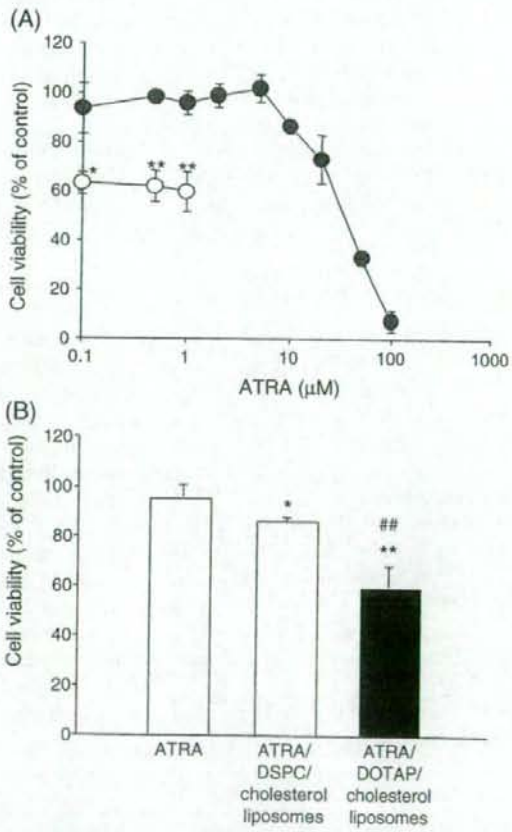

Fig. 1. (A) Cell viability after application of ATRA (●) and ATRA incorporated in DOTAP/cholesterol liposomes (○) at various concentrations for 48 h in A549 cells. Each value represents the mean±S.D. of four experiments. (B) Cell viability after application of 1.0 μM ATRA, ATRA incorporated in DSPC/cholesterol liposomes, or DOTAP/cholesterol liposomes at an ATRA concentration of 1.0 μM for 48 h in A549 cells. Cell viability was determined by MTT assay. Each value represents the mean±S.D. of four experiments. Significant difference $^*P<0.05$ and $^{**}P<0.01$ vs. ATRA, $^{\#\#}P<0.01$ vs. DSPC/cholesterol liposomes.

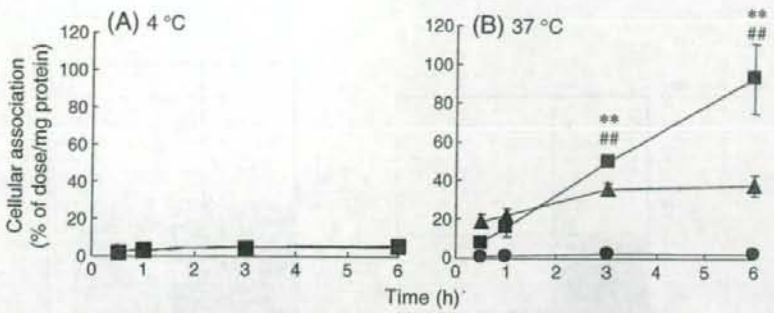

Fig. 2. Cellular association time courses of [^{3}H]ATRA (●), [^{3}H]ATRA incorporated in DSPC/cholesterol liposomes (▲), or DOTAP/cholesterol liposomes (■) at 4 °C (A) and 37 °C (B) in A549 cells. The ATRA concentration was 1.0 μM. Each value represents the mean±S.D. of four experiments. Significant difference $^{\#\#}P<0.01$ vs. ATRA, $^{**}P<0.01$ vs. ATRA incorporated in DSPC/cholesterol liposomes.

examined by flow cytometric analysis and DNA fragment extraction. H_2O_2 was treated as a positive control for apoptosis [30]. Flow cytometric analysis demonstrated that phosphatidylcholine-exposing cells was significantly increased by ATRA incorporated in DOTAP/cholesterol liposomes (27.5±1.80%) compared with free ATRA liposomes (7.66±5.14%), and bare DOTAP/cholesterol liposomal (6.62±5.43%) formulations (Figs. 4 and 5).

Fig. 6 shows the analysis of DNA extracted from A549 cells after application of free ATRA, ATRA incorporated in DSPC/cholesterol liposomes, ATRA incorporated in DOTAP/cholesterol liposomes, and bare liposomal formulations. It was found that giant DNA fragments accumulated in the nucleus of cells treated with ATRA incorporated in cationic liposomes. These results indicate that apoptosis could be efficiently induced by ATRA incorporated in cationic liposome in A549 cells.

4. Discussion

The mean particle sizes of ATRA incorporated DOTAP/cholesterol and DSPC/cholesterol liposomes were about 125 and 110 nm, respectively. On the other hand, the zeta potentials of ATRA-incorporated DOTAP/cholesterol and DSPC/cholesterol liposomes were +50 and −3 mV, respectively. Since the mean particle sizes of these two liposomes were almost identical, the effect of the liposomal charge on ATRA action could be analyzed.

To investigate the cellular uptake mechanisms of ATRA in A549 cells, the cellular association was analyzed at 4 and 37 °C using [^{3}H] ATRA. At 37 °C, ATRA uptake was enhanced by incorporation into liposomes, especially in DOTAP/cholesterol liposomes (Fig. 2A). At 4 °C, ATRA uptake of both liposomes was significantly lower than at 37 °C (Fig. 2B). These results suggested that ATRA is efficiently taken up by

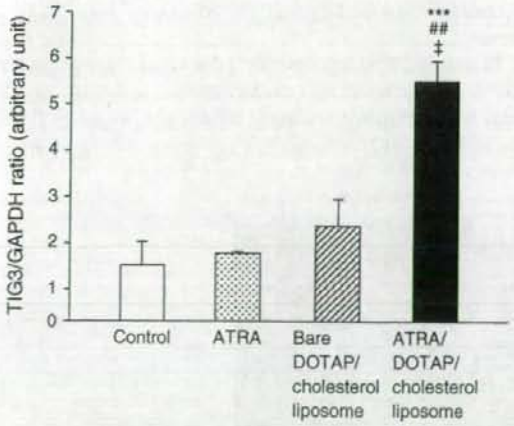

Fig. 3. Quantitative PCR analysis of TIG3 mRNA levels in A549 cells. Cells were incubated with 1.0 μM ATRA, bare DOTAP/cholesterol liposomes, or ATRA incorporated in DOTAP/cholesterol liposomes for 48 h in A549 cells. As a control, the cells were incubated with 1% DMSO. The results are expressed as arbitrary units of the TIG3 level divided by the GAPDH level. Each value represents the mean±S.D. of four experiments. Significant difference $^{***}P<0.001$ vs. (1% DMSO), $^{\#\#}P<0.01$ vs. ATRA, and $^{\ddagger}P<0.05$ vs. bare DOTAP/cholesterol liposomes.

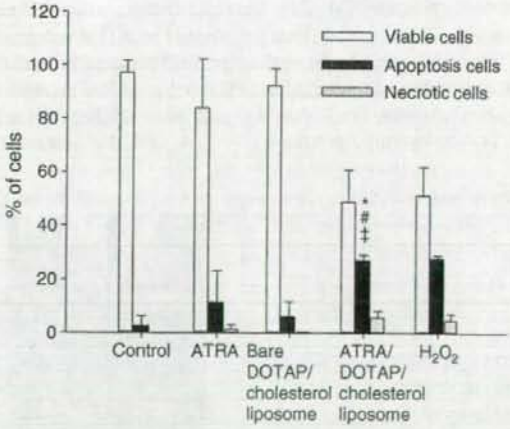

Fig. 4. Flow cytometric analysis of A549 cells treated with 1.0 μM ATRA, bare DOTAP/cholesterol liposomes, or ATRA incorporated in DOTAP/cholesterol liposomes for 48 h in A549 cells. After treatment, the cells were stained with annexin V-fluorescein and PI. As a control, cells were incubated with 1% DMSO and 400 μM H_2O_2. Each value represents the mean±S.D. of four experiments. Significant difference $^{*}P<0.05$ vs. control (1% DMSO), $^{\#}P<0.05$ vs. ATRA, and $^{\ddagger}P<0.05$ vs. bare DOTAP/cholesterol liposomes.

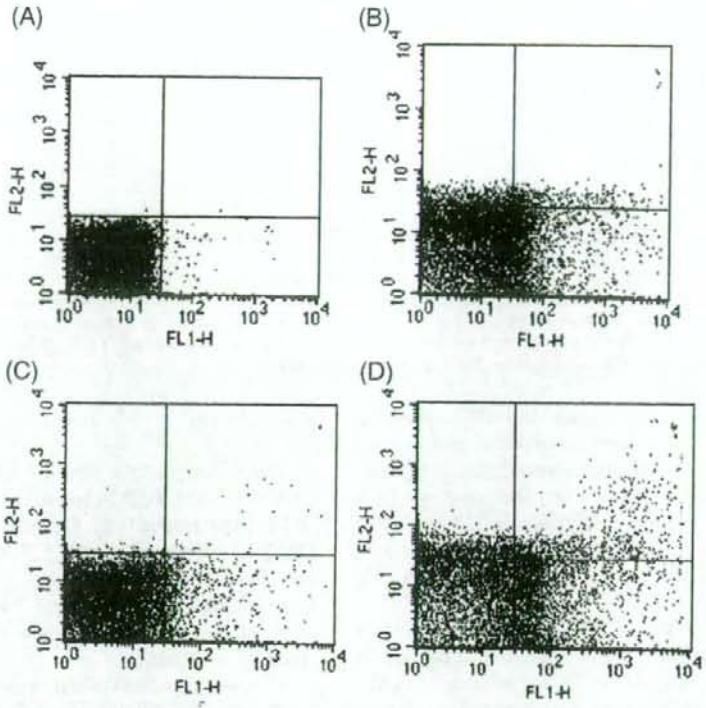

Fig. 5. Typical flow cytometric analysis of A549 cells treated without (A) or with 1.0 μM ATRA (B), bare DOTAP/cholesterol liposomes (C), or ATRA incorporated in DOTAP/cholesterol liposomes (D) for 48 h in A549 cells.

endocytosis when it is incorporated in DOTAP/cholesterol liposomes in A549 cells. This uptake mechanism agrees with the results of the uptake of plasmid DNA complexed with cationic liposomes [31–33]. The cellular association difference between 37 and 4 °C reflects the amount of ATRA internalized in the A549 cells. As far as the types of liposome formulations are concerned, the internalized ATRA by ATRA incorporated in DOTAP/cholesterol liposomes was much higher than that in DSPC/cholesterol liposomes (Fig. 2A and B). Since ATRA incorporated in DOTAP/cholesterol liposomes had positive surface charge (Table 1), they could interact with the cellular membrane, which possesses a negative charge, through electrostatic interaction, and be effectively internalized into the cells.

In order to evaluate whether cytotoxicity was enhanced by ATRA incorporated in DOTAP/cholesterol liposomes, MTT assay was performed. Recently, Manna and Aggarwal [15] and Higuchi et al. [12] reported that no growth inhibitory effect in

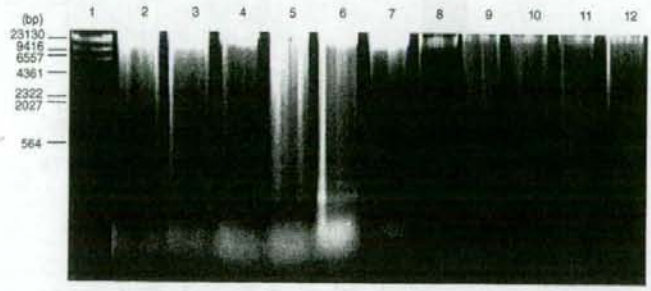

Fig. 6. Agarose gel electrophoresis of DNA extracted from A549 cells incubated with 1.0 μM ATRA, ATRA incorporated in DOTAP/cholesterol or DSPC/cholesterol liposomes and bare DOTAP/cholesterol or bare DSPC/cholesterol liposomes for 48 h in A549 cells. Lane 1: DNA marker. Lanes 2–4: DNA from cells treated with ATRA at ATRA concentrations of 0.5, 1.0, and 50 μM, respectively. Lanes 5–6: DNA from cells treated with ATRA incorporated in DOTAP/cholesterol liposomes at ATRA concentrations of 0.5 and 1.0 μM. Lanes 7–8: DNA from cells treated with DOTAP/cholesterol liposomes at total lipid concentrations of 59 and 117 μM. Lanes 9–10: DNA from cells treated with ATRA incorporated in DSPC/cholesterol liposomes at ATRA concentrations of 0.5 and 1 μM. Lanes 11–12: DNA from cells treated with DSPC/cholesterol liposomes at total lipid concentrations of 27 and 55 μM. DNA was extracted from about 5×10^5 cells and loaded in each well.

A549 cells was observed at ATRA concentrations of 2.0 and 1.0 μM, although the growth of other ATRA sensitive cell lines was inhibited at these concentrations. We also observed a similar result showing that there was no cytotoxic effect below 10 μM free ATRA (Fig. 1A), indicating that A549 cells are ATRA-insensitive (resistant). In the present study, we demonstrated that when ATRA was incorporated in cationic liposomes composed of DOTAP/cholesterol, a potent cytotoxic effect was exhibited even at 0.1, 0.5, and 1.0 μM (Fig. 1B), indicating that the cytotoxicity in A549 cells can be efficiently induced by ATRA incorporated in DOTAP/cholesterol liposomes. These cytotoxicity results support our hypothesis that intracellular delivery of ATRA should be able to overcome ATRA resistance in A549 cells.

To investigate whether ATRA incorporated in DOTAP/cholesterol liposomes could induce apoptosis, flow cytometric analysis and extraction of DNA fragments were performed. The flow cytometric analysis demonstrated that phosphatidylcholine-exposing cells were significantly increased by ATRA incorporated in DOTAP/cholesterol liposomes compared with free ATRA, and ATRA incorporated in DSPC/cholesterol liposomes (Figs. 4 and 5). Furthermore, the giant DNA fragments accumulated in the nucleus of cells treated with ATRA incorporated in cationic liposomes (Fig. 6). These findings strongly suggest that apoptosis can be induced by ATRA incorporated DOTAP/cholesterol liposomes in A549 cells.

The TIG3 gene is a retinoic acid inducible class II tumor suppressor gene down-regulated in several human tumors and malignant cell lines [34]. To explore the mechanism of cytotoxicity and induction of apoptosis by ATRA incorporated in DOTAP/cholesterol liposomes, we examined the expression of mRNA of TIG3. TIG3 mRNA was not induced by free ATRA in A549 cells at an ATRA concentration of 1.0 μM. This observation agrees with the results reported by Higuchi et al. [12]. On the other hand, we showed that the amount of TIG3 mRNA increased in A549 cells treated with ATRA (1.0 μM) incorporated in DOTAP/cholesterol liposomes in A549 cells. In addition, the induction of TIG3 mRNA (Fig. 3) is in agreement with the cytotoxicity (Fig. 1) and apoptosis (Figs. 4–6) results. These observations provide evidence that TIG3 induction by ATRA incorporated in DOTAP/cholesterol liposomes induces cytotoxicity and/or apoptosis in A549 cells.

Higuchi et al. also reported that ATRA-induced TIG3 expression takes place through the nuclear retinoid pathway [12]. As shown in Fig. 2, we demonstrated that ATRA were efficiently internalized in cells by incorporation into DOTAP/cholesterol liposomes and this phenomenon corresponds to the expression of TIG3 mRNA (Fig. 3). Therefore, these observations lead us to believe that ATRA is efficiently delivered to the nucleus by incorporation into DOTAP/cholesterol liposomes.

Recently, much effort has been devoted to the development of cationic liposome-mediated gene delivery systems due to their favorable characteristics. Since pDNA complexed with cationic liposomes (i.e. lipoplexes) accumulate in the lung immediately after intravenous administration [35], they lead to a high gene expression in that tissue [36–39]. Recently, we have reported that intravenously injected interferon encoding plasmid DNA complexed with cationic liposomes prevents lung metastases in a mouse lung CT26 metastasis model [40]. Since ATRA are incorporated in liposomal lipid membranes, ATRA are able to be introduced into the cytokine gene therapy system without the inhibition of the lipoplex formation via electrostatic interaction. Thus, the combination of cytokine therapy and differentiation therapy might be used to improve the treatment of NSCLC in the near future.

In conclusion, we have demonstrated that ATRA incorporated in cationic liposomes composed of DOTAP/cholesterol are efficiently internalized into A549 cells, producing potent cytotoxic and apoptosis-inducing effects on ATRA-insensitive (resistant) A549 cells. The enhanced expression of TIG3 mRNA tumor suppressor gene by ATRA incorporation into DOTAP/cholesterol liposomes might partly explain the mechanism of enhanced cytotoxicity and/or apoptosis in A549 cells. These observations provide valuable information to help in the design of differentiation therapy by ATRA in NSCLC.

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References

[1] A.I. Spira, M.A. Carducci, Differentiation therapy, Curr. Opin. Pharmacol. 3 (2003) 338–343.
[2] M.E. Huang, Y.C. Ye, S.R. Chen, J.R. Chai, J.X. Lu, L. Zhoa, L.J. Gu, Z.Y. Wang, Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia, Blood 72 (1988) 567–572.
[3] S. Castaigne, C. Chomienne, M.T. Daniel, P. Ballerini, R. Berger, P. Fenaux, L. Degos, All-*trans* retinoic acid as a differentiation therapy for acute promyelocytic leukemia: I. Clinical results, Blood 76 (1990) 1704–1709.
[4] D.G. Kim, B.H. Jo, K.R. You, D.S. Ahn, Apoptosis induced by retinoic acid in Hep3B cells in vitro, Cancer Lett. 107 (1996) 149–159.
[5] K. Hayashi, H. Yokozaki, K. Naka, W. Yasui, R. Lotan, E. Tahara, Overexpression of retinoic acid receptor β induces growth arrest and apoptosis in oral cancer cells, Jpn. J. Cancer Res. 92 (2001) 42–50.
[6] Y. Choi, S.Y. Kim, S.H. Kim, J. Yang, K. Park, Y. Byun, Inhibition of tumor growth by biodegradable microspheres containing all-*trans*-retinoic acid in a human head-and-neck cancer xenograft, Int. J. Cancer 107 (2003) 145–148.
[7] Y.F. Lee, B.Y. Bao, C. Chang, Modulation of the retinoic acid-induced cell apoptosis and differentiation by the human TR4 orphan nuclear receptor, Biochem. Biophys. Res. Commun. 323 (2004) 876–883.
[8] C. Di, S. Liao, D.C. Adamson, T.J. Parrett, D.K. Broderick, Q. Shi, C. Lengauer, J.M. Cummins, V.E. Velculescu, D.W. Fults, R.E. McLendon, D.D. Bigner, H. Yan, Identification of OTX2 as a medulloblastoma oncogene whose product can be targeted by all-*trans* retinoic acid, Cancer Res. 65 (2005) 919–924.
[9] S.J. Freemantle, M.J. Spinella, E. Dmitrovsky, Retinoids in cancer therapy and chemoprevention: promise meets resistance, Oncogene 22 (2003) 7305–7315.

[10] D. DiSepio, C. Ghosn, R.L. Eckert, A. Deucher, N. Robinson, M. Duvic, R.A. Chandraratna, S. Nagpal, Identification and characterization of a retinoid-induced class II tumor suppressor/growth regulatory gene, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 14811–14815.
[11] A. Deucher, S. Nagpal, R.A. Chandraratna, D. DiSepio, N.A. Robinson, S.R. Dashti, R.L. Eckert, The carboxy-terminal hydrophobic domain of TIG3, a class II tumor suppressor protein, is required for appropriate cellular localization and optimal biological activity, Int. J. Oncol. 17 (2000) 1195–1203.
[12] E. Higuchi, R.A. Chandraratna, W.K. Hong, R. Lotan, Induction of TIG3, a putative class II tumor suppressor gene, by retinoic acid in head and neck and lung carcinoma cells and its association with suppression of the transformed phenotype, Oncogene 22 (2003) 4627–4635.
[13] J. Geradts, J.Y. Chen, E.K. Russell, J.R. Yankaskas, L. Nieves, J.D. Minna, Human lung cancer cell lines exhibit resistance to retinoic acid treatment, Cell Growth Differ. 4 (1993) 799–809.
[14] B.M. van der Leede, C.E. van den Brink, P.T. van der Saag, Retinoic acid receptor and retinoid X receptor expression in retinoic acid-resistant human tumor cell lines, Mol. Carcinog. 8 (1993) 112–122.
[15] S.K. Manna, B.B. Aggarwal, All-*trans*-retinoic acid upregulates TNF receptors and potentiates TNF-induced activation of nuclear factors-κB, activated protein-1 and apoptosis in human lung cancer cells, Oncogene 19 (2000) 2110–2119.
[16] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Adv. Drug Delivery Rev. 46 (2001) 3–26.
[17] S. Kawakami, P. Opanasopit, M. Yokoyama, N. Chansri, T. Yamamoto, T. Okano, F. Yamashita, M. Hashida, Biodistribution characteristics of all-*trans* retinoic acid incorporated in liposomes and polymeric micelles following intravenous administration, J. Pharm. Sci. 94 (2005) 2606–2615.
[18] G. Thurston, J.W. McLean, M. Rizen, P. Baluk, A. Haskell, T.J. Murphy, D. Hanahan, D.M. McDonald, Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice, J. Clin. Invest. 101 (1998) 1401–1413.
[19] S. Krasnici, A. Werner, M.E. Eichhorn, M. Schmitt-Sody, S.A. Pahernik, B. Sauer, B. Schulze, M. Teifel, U. Michaelis, K. Naujoks, M. Dellian, Effect of the surface charge of liposomes on their uptake by angiogenic tumor vessels, Int. J. Cancer 105 (2003) 561–567.
[20] W. Yeeprae, S. Kawakami, F. Yamashita, M. Hashida, Physicochemical and pharmacokinetic characteristics of cationic liposomes, Pharmazie (in press).
[21] S. Kawakami, J. Wong, A. Sato, Y. Hattori, F. Yamashita, M. Hashida, Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice, Biochim. Biophys. Acta 1524 (2000) 258–265.
[22] S. Kawakami, C. Munakata, S. Fumoto, F. Yamashita, M. Hashida, Novel galactosylated liposomes for hepatocyte-selective targeting of lipophilic drugs, J. Pharm. Sci. 90 (2001) 105–113.
[23] S. Kawakami, K. Yamamura, T. Mukai, K. Nishida, J. Nakamura, T. Sakaeda, M. Nakashima, H. Sasaki, Sustained ocular delivery of tilisolol to rabbits after topical administration or intravitreal injection of lipophilic prodrug incorporated in liposomes, J. Pharm. Pharmacol. 53 (2001) 1157–1161.
[24] M. Teshima, S. Kawakami, K. Nishida, J. Nakamura, T. Sakaeda, H. Terazono, T. Kitahara, M. Nakashima, H. Sasaki, Prednisolone retention in integrated liposomes by chemical approach and pharmaceutical approach, J. Controlled Release 97 (2004) 211–218.
[25] S. Kawakami, F. Yamashita, M. Nishikawa, Y. Takakura, M. Hashida, Asialoglycoprotein receptor-mediated gene transfer using novel galactosylated cationic liposomes, Biochem. Biophys. Res. Commun. 252 (1998) 78–83.
[26] P. Opanasopit, Y. Higuchi, S. Kawakami, F. Yamashita, M. Nishikawa, M. Hashida, Involvement of serum mannan binding proteins and mannose receptors in uptake of mannosylated liposomes by macrophages, Biochim. Biophys. Acta 1511 (2001) 134–145.
[27] S. Kawakami, A. Sato, M. Nishikawa, F. Yamashita, M. Hashida, Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes, Gene Ther. 7 (2000) 292–299.
[28] Y. Hattori, S. Kawakami, S. Suzuki, F. Yamashita, M. Hashida, Enhancement of immune responses by DNA vaccination through targeted gene delivery using mannosylated cationic liposome formulations following intravenous administration in mice, Biochem. Biophys. Res. Commun. 317 (2004) 992–999.
[29] C. Managit, S. Kawakami, F. Yamashita, M. Hashida, Effect of galactose density on asialoglycoprotein receptor-mediated uptake of galactosylated liposomes, J. Pharm. Sci. 94 (2005) 2266–2275.
[30] T. Geiser, M. Ishigaki, C. van Leer, M.A. Matthay, V.C. Broaddus, H_2O_2 inhibits alveolar epithelial wound repair in vitro by induction of apoptosis, Am. J. Physiol.: Lung Cell. Mol. Physiol. 287 (2004) L448–L453.
[31] F. Sakurai, R. Inoue, Y. Nishino, A. Okuda, O. Matsumoto, T. Taga, F. Yamashita, Y. Takakura, M. Hashida, Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression, J. Controlled Release 66 (2000) 255–269.
[32] D. Lleres, J.M. Weibel, D. Heissler, G. Zuber, G. Duportail, Y. Mely, Dependence of the cellular internalization and transfection efficiency on the structure and physicochemical properties of cationic detergent/DNA/liposomes, J. Gene Med. 6 (2004) 415–428.
[33] C.M. Wiethoff, J.G. Koe, G.S. Koe, C.R. Middaugh, Compositional effects of cationic lipid/DNA delivery systems on transgene expression in cell culture, J. Pharm. Sci. 93 (2004) 108–123.
[34] K. Lotz, T. Kellner, M. Heitmann, I. Nazarenko, A. Noske, A. Malek, A. Gontarewicz, R. Schafer, C. Sers, Suppression of the TIG3 tumor suppressor gene in human ovarian carcinomas is mediated via mitogen-activated kinase-dependent and -independent mechanisms, Int. J. Cancer 116 (2005) 894–902.
[35] S. Kawakami, F. Yamashita, K. Nishida, J. Nakamura, M. Hashida, Glycosylated cationic liposomes for cell-selective gene delivery, Crit. Rev. Ther. Drug Carrier Syst. 19 (2002) 171–190.
[36] N. Zhu, D. Liggitt, Y. Liu, R. Debs, Systemic gene expression after intravenous DNA delivery into adult mice, Science 261 (1993) 209–211.
[37] S. Li, L. Huang, In vivo gene transfer via intravenous administration of cationic lipid–protamine–DNA (LPD) complexes, Gene Ther. 4 (1997) 891–900.
[38] S. Kawakami, S. Fumoto, M. Nishikawa, F. Yamashita, M. Hashida, In vivo gene delivery to the liver using novel galactosylated cationic liposomes, Pharm. Res. 17 (2000) 306–313.
[39] S. Kawakami, A. Sato, M. Yamada, F. Yamashita, M. Hashida, The effect of lipid composition on receptor-mediated in vivo gene transfection using mannosylated cationic liposomes in mice, STP Pharm. Sci. 11 (2001) 117–120.
[40] F. Sakurai, T. Terada, M. Maruyama, Y. Watanabe, F. Yamashita, Y. Takakura, M. Hashida, Therapeutic effect of intravenous delivery of lipoplexes containing the interferon-β gene and poly I: poly C in a murine lung metastasis model, Cancer Gene Ther. 10 (2003) 661–668.

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Inhibition of liver metastasis by all-*trans* retinoic acid incorporated into O/W emulsions in mice

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Abstract

All-*trans* retinoic acid (ATRA) was incorporated into lipid emulsions in an attempt to alter its distribution characteristics and improve its inhibition of liver cancer metastasis. Lipid emulsions composed of egg phosphatidylcholine, cholesterol, and soybean oil were the optimized carriers for ATRA delivery, as shown by the submicron particle size and high incorporation efficiency. The particle size and zeta potential of ATRA incorporated into emulsions were about 133 nm and −11 mV, respectively. *In vitro* drug release study demonstrated that the release of ATRA from emulsions was sustained in the absence and present of bovine serum albumin, suggesting that ATRA was stable when incorporated in emulsions. After intravenous administration in mice, [^{3}H]cholesteryl hexadecyl ether incorporated into emulsion, which is the inherent distribution of emulsions, accumulated gradually mainly in the liver. The blood concentration and hepatic accumulation of [^{3}H]ATRA incorporated into emulsion was significantly higher than that of serum dissolving [^{3}H]ATRA, which represent the original distribution characteristic of free ATRA. In a murine liver metastasis model by colon adenocarcinoma, the liver metastasis number and liver weight were significantly reduced and the survival time of mice was prolonged following intravenous injection of ATRA incorporated into emulsions.

Keywords: All-*trans* retinoic acid; Lipid emulsions; Liver metastasis; Biodistribution; Controlled release

1. Introduction

Retinoids, natural or synthetic derivatives of Vitamin A, are normal regulators of embryonic development, vision, reproduction and other physiological processes by affecting differentiation, proliferation and apoptosis (Sun and Lotan, 2002). Low serum retinol levels may be a risk factor for the incidence of second liver tumors, and retinoids have a potential antineoplastic effect on liver cancer (Soprano et al., 2004; Okuno et al., 2004). All-*trans* retinoic acid (ATRA), an active metabolite of retinol, has been shown to exert anti-cancer activities in a number of cancer cells and tissues (Otsuki et al., 2003; Arce et al., 2005). Recently, it has been extensively used for the treatment of acute promyelocytic leukemia (APL) (Lengfelder et al., 2005). However, a gradual decrease in the ATRA concentration in the blood circulation after prolonged treatment (Muindi et al., 1992) and highly variable bioavailability after oral administration was observed (Ozpolat et al., 2003). Therefore, a parenteral formulation may provide a reliable approach for ATRA administration.

It is known that tumor metastasis is an important prognostic factor affecting the survival time of cancer patients and the liver appears to be target organ of tumor metastasis (McCarter and Fong, 2000). Multiple metastases in the liver occur from many primary tumor sites, and these multiple nodules are unresectable (Ravikumar et al., 1990). To overcome liver metastasis, various chemotherapeutic agents and combined therapy with radiotherapy are used (Webber et al., 1978). However, the effect of anti-cancer agents is not enough and is limited by toxicity. An alternative approach using non-cytotoxic anti-cancer agents, such as retinoids, was investigated (Garcia-Alonso et al., 2005).

In recent years, considerable emplasis has been placed on the development of new formulations of ATRA that are suitable for intravenous administration. However, the poor aqueous solubility of ATRA hampers its administration in solution form. Attempts have been made to develop parenteral formulations of ATRA by loading it in lipophilic carriers to overcome its solubility limitation. ATRA dispersal systems such as liposomes (Estey et al., 2005; Kawakami et al., 2006), solid lipid nanoparti-

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cles (Lim et al., 2004), polymeric micelles (Zuccari et al., 2005; Kawakami et al., 2005), and phospholipid-based microemulsions (Hwang et al., 2004) have been developed for parenteral administration purposes. Among these approaches, liposomes have been a promising parenteral delivery system for ATRA because of their superior ability to maintain plasma concentrations of ATRA over oral administration. In particular, clinical trials have demonstrated that parenteral liposomal ATRA offers potential pharmacological advantages over oral administration for APL therapy (Ozpolat and Lopez-Berestein, 2002). However, the limited solubility of ATRA within the liposomal membrane allowed only large vesicle multilamellar-type liposomes to provide efficient ATRA loading. In this regard, other carriers of submicron size and effective ATRA loading will provide an alternative for ATRA parenteral delivery for liver targeting.

Lipid emulsions are considered to be superior to liposomes as they can be produced on an industrial scale, are stable during storage, are highly biocompatible, and have a high solubilizing capacity as far as lipophilic drugs are concerned because they have an oil phase in particulate form, so they can dissolve large amounts of drugs (Kawakami et al., 2000a; Hashida et al., 2005). Moreover, small lipid emulsions have been widely used as long circulating carriers and for the administration of lipid nutrients, and have become useful parenteral drug delivery systems. It has been reported that particular lipid drug carriers mimic the metabolism of plasma lipoprotein or chylomicron and are preferentially taken up by the liver (Tomii, 2002). This phenomenon is also expected in small lipid emulsions with phosphatidylcholine and cholesterol located at the surface since they are the major lipid constituents of plasma lipoprotein. These triglyceride-rich emulsions attract apolipoproteins, are hydrolyzed by lipoprotein lipase, convert into remnants and are subsequently taken up by the liver (Rensen et al., 1997). Therefore, improved solubility, sustained and prolonged blood concentration, and enhanced liver uptake by lipid emulsion could be advantageous to target ATRA to the liver and treatment of liver metastasis.

The purpose of this study was to investigate lipid emulsions as a delivery system for ATRA. We hypothesize that ATRA uptake by the liver can be enhanced by incorporating it into optimized emulsions. In this study, we have shown that ATRA was strongly incorporated into emulsions without significant release. The biodistribution characteristics of ATRA delivered by emulsions after intravenous administration showed increased liver accumulation when compared to the injection of free ATRA. Finally, we evaluated the efficiency of ATRA-incorporated emulsions against hepatic metastasis by measuring the number of metastatic colonies on the liver surface, tissue weight of the liver, and survival of mice.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EggPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-polyethylene glycol 2000 (DSPE-PEG_{2000}) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol (Chol) and Clear-Sol I were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Soluene-350 was purchased from Packard Co., Inc. (Groningen, The Netherlands). ATRA and soybean oil were obtained from Wako Pure Chemicals Industry Ltd. (Osaka, Japan). HCO-60 was obtained from Nikko Chemical Co. Ltd. (Japan). [^{3}H]Cholesteryl hexadecyl ether (CHE) and [^{3}H]ATRA were purchased from NEN Life Science Products Inc. (Boston, MA, USA). RPMI1640 medium was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Biowhittaker (Walkersville, MD, USA). Bovine serum albumin, fraction V (BSA) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All other chemicals were of the highest purity available.

2.2. Preparation of ATRA-incorporated emulsions

Emulsions were prepared by the method described previously with slight modification (Takino et al., 1994; Ishida et al., 2004; Managit et al., 2005). Briefly, a mixture of EggPC/Chol (60:40, molar ratio) or EggPC/DSPE-PEG_{2000}/Chol (50:10:40, molar ratio) and ATRA (1:20, molar ratio of total lipid) was first dissolved in chloroform. After vacuum drying and desiccation, soybean oil (7:3, weight ratio of total lipid) was added to dissolve the dry film and sterile phosphate-buffered saline pH 7.4 (PBS) was added for hydration. The emulsions were then sonicated (200 W) at 4 °C under a current of nitrogen gas using an ultrasonic sonicator (US300, Nissei Inc., Tokyo, Japan) for 30 min. The preparations were passed through a 0.45 μm filter to remove precipitated ATRA and protected from light under nitrogen gas at 4 °C and had been used within 1 week. For radiolabeling preparations, [^{3}H]ATRA or [^{3}H]CHE (50 μCi) was added to the lipid mixture before formation of the thin film layer. Other preparation steps were the same as described above.

2.3. Characterization of the preparations

The lipid content of the emulsions was determined by a cholesterol E-test Wako kit (Wako Pure Chemical Industry Ltd., Osaka, Japan). The amount of ATRA in the formulations was determined by UV absorption at 360 nm (UV–vis Spectrophotometer, Shimazu Co. Ltd., Kyoto, Japan) after dissolving the formulations in a mixture of dimethyl sulfoxide (DMSO) and water (DMSO: water = 9:1 by volume). The particle size and zeta potential of the emulsions were measured by Zetasizer Nano Series (Malvern Instruments Ltd., Worcestershire, UK).

2.4. Animals

Male ddY mice (5 weeks old, 20–25 g) and CDF1 mice (male, 4 weeks old, 18–22 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were carried out in accordance with the guidelines for Animal Experiments of Kyoto University.

2.5. In vitro release study

The dialysis membranes (Spectra/Por® membrane MWCO 3500, Spectrum Laboratories Inc., Roncho Dominguez, CA, USA) were mounted in diffusion chambers. Three ml of PBS containing 0.5% Tween-20 was added to the receiver side and the diffusion chamber was maintained at 37 °C. ATRA dissolved in 1N NaOH (0.25 mg/ml) or ATRA incorporated into emulsions were diluted with PBS to the concentration of ATRA 33.33 μg/ml and 3 ml of diluted ATRA was added to the donor side. The samples were withdrawn from the receiver side at specific times and analyzed for ATRA concentration by UV spectrophotometer at 360 nm. To investigate the effect of BSA on ATRA release, PBS containing 4% BSA was used instead of PBS at each step of the method described above. These studies were performed in triplicate for each sample.

2.6. Biodistribution study

A biodistribution study was performed by the method described previously (Kawakami et al., 2000b; Yeeprae et al., 2005). [^{3}H]ATRA dissolved in serum or radioactivity labeling emulsions were injected into the tail vein of ddY mice at an ATRA dose of 0.6 mg/kg. At each collection time point, blood was collected from the vena cava under anesthesia, and the mice were then killed. The liver, spleen, kidney and lung were excised, washed with saline, blotted dry and weighed. Ten microliters of blood and a small piece of each tissue were precisely weighed and digested in 0.7 ml Soluene-350 by incubating overnight at 45 °C. Following digestion, 0.2 ml isopropanol, 0.2 ml 30% hydrogenperoxide, 0.1 ml 5N HCl, and 5.0 ml Clear-Sol I were added. The samples were stored overnight and the radioactivity was measured using a liquid scintillation counter (LSA-500, Beckman, Tokyo, Japan).

2.7. Experimental liver metastasis and treatment with ATRA

CT26, mouse colon adenocarcinoma cells were routinely growth with RPMI1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (All from Invitrogen Co., Carlsbad, CA, USA) in 5% CO_2, humidified air at 37 °C. The cells were harvested from 2-day-old subconfluent cultures by trypsin and the cell concentration was adjusted to 10^6 cells/ml by Hank's balance salt solution (HBSS). CT26 cells (0.1 ml) were then implanted by intrasplenic injection to CDF1 mice under pentobarbital anesthesia. ATRA dissolved in 5% HCO-60 solution, ATRA incorporated into emulsions, and empty emulsions were repeatedly injected through the tail vein at an ATRA dose of 0.6 mg/kg/day or total lipid dose of 120 mg/kg/day, from days 3 to 7 after tumor inoculation (6–8 mice per group). The survival of mice was recorded up to 28 days after tumor inoculation. In a different set of experiments, mice were sacrificed on day 14 and the number of tumor nodules on the liver surface and the liver weight were measured.

2.8. Statistical analysis

Statistical comparisons were performed by Student's *t*-test for two groups, and one-way ANOVA for multiple groups. Statistical analysis of survival curves was done with the log-rank test. $P < 0.05$ was considered significant.

3. Results

3.1. Physicochemical properties of ATRA incorporated into emulsions

The particle size intensity of the prepared ATRA emulsions is shown in Fig. 1. The particle size of ATRA incorporated into emulsions was 133.2 ± 3.41 nm ($n = 3$), similar to preparations without ATRA. The zeta potential of emulsions was -11 ± 4.5 mV ($n = 3$), shifted from the neutral or slightly negative sign of empty emulsions (data not shown). The particle size and zeta potential of the emulsions remained constant over 1 month at 4 °C but the particle size was slightly increased at room temperature at 30 days as shown in Fig. 2. The percent recovery of ATRA in emulsions when compared with the initial concentration of ATRA was $88.9 \pm 4.3\%$ ($n = 3$) with the final concentration of ATRA up to 0.123 mg/ml of emulsions.

3.2. Release of ATRA from ATRA incorporated into emulsions

In order to investigate ATRA binding with the emulsions, the release of ATRA was performed using a dialysis membrane since it retained the emulsions and allowed the transfer of only free ATRA into the receiver side. Fig. 3 shows the *in vitro* diffusion of ATRA through the dialysis membrane. ATRA in the free form rapidly diffused through the membrane and reach equilibrium within 72 h whereas the diffusion of ATRA incorporated into the emulsions was very slow, less than 10% at 72 h. In the presence of 4% BSA in the donor and receiver sides, the diffusion of ATRA incorporated into the emulsions was almost the same as without

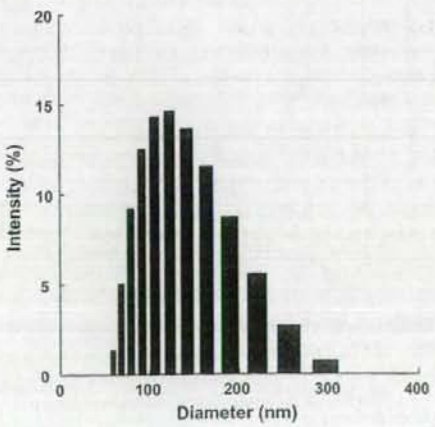

Fig. 1. Size distribution of ATRA-incorporated emulsions.

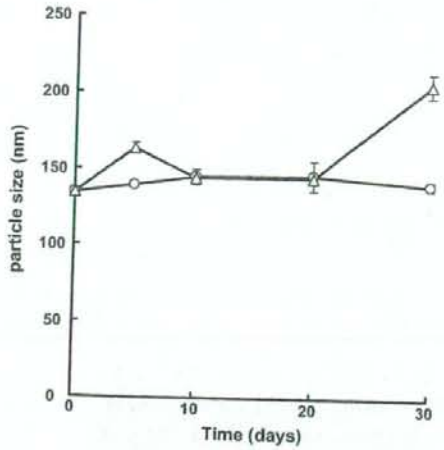

Fig. 2. The mean particle sizes of ATRA in emulsions within 30 days. Emulsions were protected from light under nitrogen gas and stored at 4 °C (○) or room temperature (△) until measurement.

BSA. The results imply that ATRA was stably incorporated into the emulsions even in the presence of BSA.

3.3. Biodistribution of [^{3}H]CHE and [^{3}H]ATRA labeling emulsions

The distribution characteristics of empty emulsions, free ATRA, and ATRA incorporated into emulsions after intravenous administration into mice was investigated. The empty emulsions labeled with [^{3}H]CHE were eliminated from the circulating blood and accumulated in the liver for up to 60 min, and were retained in the liver for over 240 min (Fig. 4).

Serum dissolved in [^{3}H]ATRA was rapidly eliminated from the blood circulation and recovered in the liver within the first

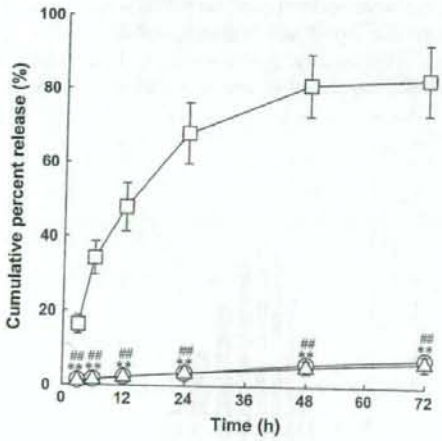

Fig. 3. The diffusion of ATRA through a dialysis membrane within 72 h (□) and *in vitro* release of ATRA from ATRA incorporated into emulsions in the presence (△) or absent (○) of 4% BSA. Each value represents the mean ± S.D. of three experiments. Statistically significant differences from the diffusion of free ATRA in the presence or absent of BSA ($^{**}P < 0.001$ or $^{\#\#}P < 0.001$, respectively).

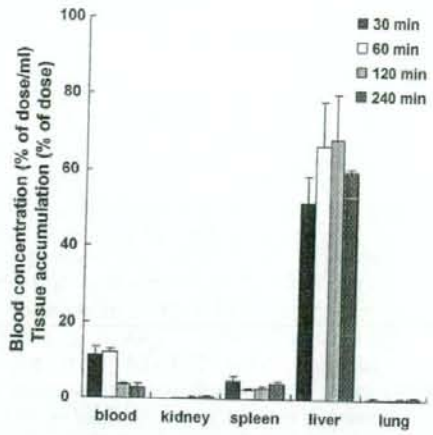

Fig. 4. Distribution profiles of [^{3}H]CHE incorporated into emulsions after intravenous injection in mice. Results are expressed as the means + S.D. of three mice.

30 min, and was rapidly eliminated from the liver (Fig. 5A). When ATRA was incorporated into emulsions, ATRA was retained in the blood circulation longer than free ATRA and increasingly accumulated in the liver and spleen for up to 60 min

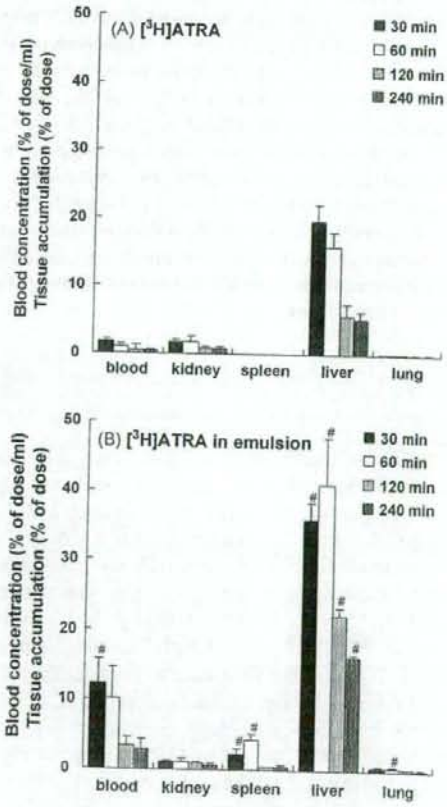

Fig. 5. Distribution profiles of serum dissolved in [^{3}H]ATRA (A) and [^{3}H]ATRA-incorporated emulsions (B) after intravenous injection in mice. Results are expressed as the means + S.D. of three mice. Statistically significant differences from the distribution of [^{3}H]ATRA ($^{\#}P < 0.01$).

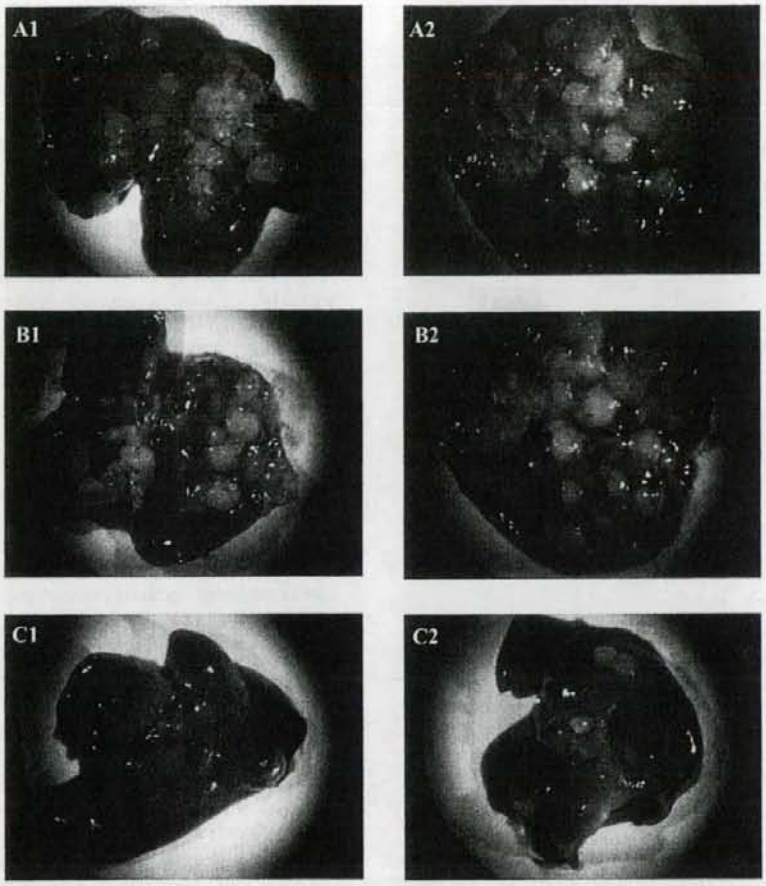

Fig. 6. Metastatic colonies of CT26 tumor cells on the liver surface of mice injected with saline (A), ATRA in 5% HCO-60 micelles (B) or ATRA in emulsion (C) on days 3–7 after tumor inoculation. Mice were sacrificed at day 14 after tumor inoculation. Two different mice in the same treatment group are shown in the figure.

before being gradually eliminated (Fig. 5B). Modification of the emulsion surface by DSPE-PEG_{2000} did not further increase the liver accumulation of ATRA although it was retained in the blood circulation longer than free ATRA (data not shown). For both free ATRA and ATRA incorporated into emulsions, no significant accumulation in the kidney and lung was observed.

3.4. Anti-liver cancer metastasis activity of ATRA emulsions

The antitumor efficiency of several formulations of ATRA in the liver metastasis model was studied. When CT26 tumor cells were injected into the spleen of CDF1 mice, metastatic colonies were visibly observed on the surface of the liver, which corresponds to the progressive stage of liver metastasis lesions. On day 14, the number of metastatic colonies on the liver surface was significantly higher in the control mice injected with saline when compared with mice treated with ATRA in 5% HCO-60 micelles or emulsions (Figs. 6 and 7A). Moreover, the ATRA incorporated into the emulsion-treated group showed the most prominent effect over the HCO-60 micelle group as far as the inhibition of liver metastases was concerned. The liver weights correlated with the number of metastatic colonies in the liver (Fig. 7A and B). As shown in Fig. 8, all mice treated with saline or empty emulsions had died by day 18 after tumor inoculation (mean survival time = 16.0 ± 1.41 and 15.9 ± 1.95 days, respectively) while treatment with ATRA in 5% HCO-60 micelles slightly increased the survival time (mean survival time = 19.6 ± 2.30 days). In contrast, the survival time of mice treated with ATRA incorporated into emulsions was much longer than any other treatments with the mean survival time 21.2 ± 5.12 days.

4. Discussion

Most conventional drugs diffuse freely throughout the body and show relatively even tissue distribution due to their low molecular weight. The methodology for manipulating drug distribution in the body and the use of lipid dispersion carrier systems as carriers of lipophilic drugs has attracted particular interest. However, these dispersal systems pose a number of

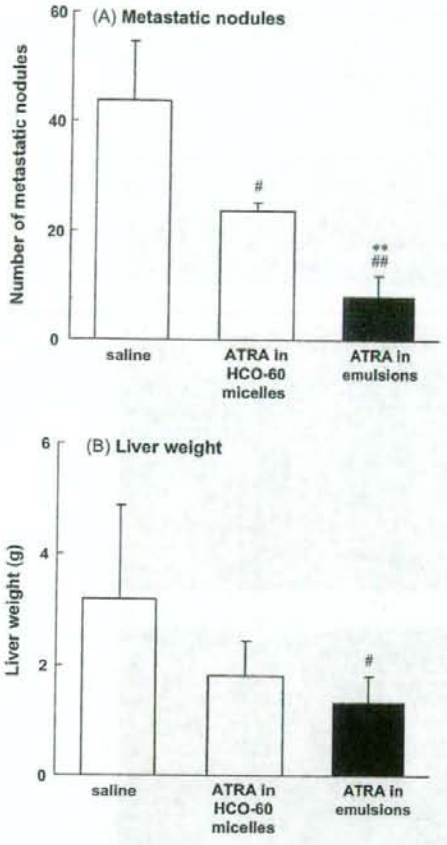

Fig. 7. Number of metastatic nodules of CT26 tumor cells on the liver surface (A) and liver weight (B) of mice intrasplenically injected with cells on day 14. Results are expressed as the mean + S.D. of three mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$, significantly different from mice injected with saline, and $^{**}P<0.01$ significantly different from mice injected with ATRA in HCO-60 micelles.

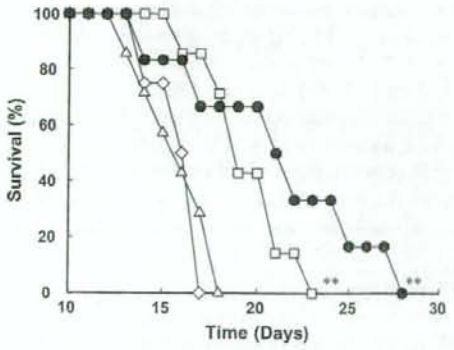

Fig. 8. Survival time of mice inoculated with CT26 tumor cells followed by intravenous injection of saline (△), empty emulsions (◇), ATRA in 5% HCO-60 micelles (□), or ATRA in emulsions (●) on days 3–7 after tumor inoculation. Survival of mice was observed daily for 28 days. $^{**}P<0.05$ significantly different from mice injected with saline.

problems in their application as drug formulations. The main problems are whether it is possible to include sufficient amounts of the drug and the expected effect of the carrier might not be achieved because there is a possibility that the drug might be released immediately after administration. In this regard, lipid emulsions were investigated as alternative carriers to improve solubility and to enhance the liver uptake of ATRA.

In order to prepare small-sized emulsions, which are considered to prolong retention in blood, 30% EggPC and cholesterol in a molar ratio 60:40 and 70% soybean oil were used. This optimized emulsion system improved the solubility of ATRA and exhibit submicron size which were advantage for parenteral administration. We confirmed that the emulsion had a particle size less than 200 nm with narrow size distribution (Fig. 1). The particle size remained constant over 1 month at 4 °C but not at room temperature suggested the appropriated storage condition of the emulsion at 4 °C. The small size and lipid components of the emulsions also benefited the selective accumulation of emulsions in the liver (Fig. 4). This phenomenon corresponds with previous reports that triglyceride-rich emulsions exhibit distribution characteristics similar to plasma lipoproteins of submicron particle size for apolipoprotein binding, lipoprotein lipase-mediated lipolysis and subsequently uptake by the liver (Arimoto et al., 1998; Tomii, 2002).

Since the water solubility of ATRA is lower than 0.1 μg/ml, the poor aqueous solubility of ATRA is the principal obstruction of a parenteral formulation. Therefore, the advantage of lipid emulsions, which posses an oil phase in particulate form and have a high solubilizing capacity for lipophilic drugs like ATRA, might be an interesting alternative. After removing precipitated ATRA by membrane filtration, the recovery of ATRA in the formulations of almost 90% suggested that ATRA was successfully dissolved in the lipophilic phase of the emulsions. For the *in vitro* release study, a lower diffusion of ATRA from the emulsions across the dialysis membrane was observed (Fig. 3), suggesting that ATRA was strongly bound to the emulsions and had sustained and prolonged release characteristics. The results also implied that most of the ATRA was effectively incorporated into the emulsion core because no rapid diffusion of ATRA across the membrane, which was a characteristic of free ATRA, was detected during ATRA release from emulsion. Moreover, the release of ATRA did not alter in the presence of BSA, which is a model hydrophobic component in serum, suggesting that ATRA was effectively retained in the emulsions even after entering the blood circulation.

In order to examine the effect of emulsion formulation on the distribution of ATRA, the biodistribution of serum dissolved in [^{3}H]ATRA and [^{3}H]ATRA incorporated into emulsions was compared. As shown in Fig. 5A, [^{3}H]ATRA was rapidly eliminated from the blood when dissolved in serum, which represents the original distribution of ATRA (Hattori et al., 2000; Kawakami et al., 2002). In contrast, the blood concentration of [^{3}H]ATRA was significantly delayed (Fig. 5B) and this slower elimination of ATRA contributed to longer and higher liver accumulation when ATRA was incorporated into emulsions. This distribution characteristic of [^{3}H]ATRA incorporated into emulsions corresponded to [^{3}H]CHE-incorporated emulsions

(Fig. 4), which is the inherent distribution of emulsions (Takino et al., 1998; Ishida et al., 2004). These results lead us to believe that when ATRA was incorporated in small-sized emulsions, sustained liver targeting could be achieved.

To investigate whether emulsions can be used to target ATRA to the liver for the treatment of hepatogenic diseases, the antitumor activity of ATRA against liver metastasis was studied. The number of metastatic nodules (Fig. 7A), the liver weight (Fig. 7B), and survival of liver metastasis mice (Fig. 8) demonstrated that ATRA incorporated into emulsion exhibited superior activity against liver metastasis when compared with untreated animals, empty emulsions and ATRA dissolved in HCO-60 micelles. The improved efficacy of ATRA in emulsions is attributed to its strong association and preferential accumulation and retention in the liver. The results indicated the potential of emulsions as carriers for ATRA targeting to liver tissue in order to improve the outcome in liver diseases.

In order to enhance the circulation time of ATRA emulsion, DSPE-PEG_{2000} was added to the emulsion system. Although the blood circulation of ATRA delivery by this PEGylated emulsion was longer than free ATRA, the liver accumulation did not further increased. The results are in accordance with the report that modification of emulsion with PEG increase the hydrophilicity of emulsion and sterically prevent the affinity for the reticuloendothelial system and liver uptake (Liu and Liu, 1995). Our results imply that the inclusion of PEG into emulsion may retain the drug in the circulation but not advantageous for the target drug delivery to the liver.

Recently, Hwang et al. (2004) reported that the pharmacokinetic profile of ATRA after the intravenous administration of emulsions was similar to that of sodium ATRA. This discrepancy in the distribution characteristics may be explained by the effect of different emulsion compositions on their stability and the release of ATRA in blood. It has been reported that the cholesterol content in emulsion is important factor to stabilize emulsion against lipolysis in blood (Maranhao et al., 1986; Clark et al., 1991); therefore, our emulsions, which contained more cholesterol, may more effectively deliver ATRA to the target tissue (liver) without degradation of emulsion.

Although the results reveal that bare emulsions could target the drug to the liver, efficiency could be further improved by attaching targeting molecules to their surface that cognate moieties at the site of interest and further enhance their targeting ability. Moreover, the effective delivery of ATRA to the liver by emulsions could be advantageous for various retinoid-associated conditions because the majority (up to 80%) of the total body Vitamin A is stored in the liver, serving as the main source of retinoids that are utilized throughout the body (Kmiec, 2001). In addition, ATRA has been shown to be toxic in long-term use since it has very low tissue specificity. Taking this into consideration, emulsion formulation of ATRA, which control the release of the drug into the blood and accumulate in the liver, is a promising approach to increase efficacy in the liver and decrease side-effects.

In conclusion, the delivery of ATRA by emulsions can reduce the elimination of ATRA from the blood circulation and preferentially accumulate in the liver after intravenous injection. The retention of ATRA in the liver can successfully suppress the progression of liver metastasis in mice injected with colon carcinoma cells. These findings indicate that the effective delivery and retention of ATRA in hepatocytes by emulsion is an efficient approach for the treatment of liver metastasis.

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References

Arce, F., Gatjens-Boniche, O., Vargas, E., Valverde, B., Diaz, 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.

Arimoto, I., Saito, H., Kawashima, Y., Miyajima, K., Handa, T., 1998. Effects of sphingomyelin and cholesterol on lipoprotein lipase-mediated lipolysis in lipid emulsions. J. Lipid Res. 39, 143–151.

Clark, S.B., Derksen, A., Small, D.M., 1991. Plasma clearance of emulsified triolein in conscious rats: effects of phosphatidylcholine species, cholesterol content and emulsion surface physical state. Exp. Physiol. 76, 39–52.

Estey, E., Koller, C., Tsimberidou, A.M., O'Brien, S., Beran, M., Cortes, J., Tirado-Gomez, M., Lopez-Berestein, G., Kantarjian, H., 2005. Potential curability of newly diagnosed acute promyelocytic leukemia without use of chemotherapy: the example of liposomal all-trans retinoic acid. Blood 105, 1366–1367.

Garcia-Alonso, I., Palomares, T., Alonso-Varona, A., Castro, B., Del Olmo, M., Portugal, V., Mendez, J., 2005. Effects of all-trans retinoic acid on tumor recurrence and metastasis. Rev. Esp. Enferm. Dig. 97, 240–248.

Hashida, M., Kawakami, S., Yamashita, F., 2005. Lipid carrier systems for targeted drug and gene delivery. Chem. Pharm. Bull. 53, 871–880.

Hattori, Y., Kawakami, S., Yamashita, F., Hashida, M., 2000. Controlled biodistribution of galactosylated liposomes and incorporated probucol in hepatocyte-selective drug targeting. J. Control. Release 69, 369–377.

Hwang, S.R., Lim, S.J., Park, J.S., Kim, C.K., 2004. Phospholipid-based microemulsion formulation of all-trans-retinoic acid for parenteral administration. Int. J. Pharm. 276, 175–183.

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

Kawakami, S., Ohshima, N., Hirayama, R., Hirai, M., Kitahara, T., Sakaeda, T., Mukai, T., Nishida, K., Nakamura, J., Nakashima, M., Sasaki, H., 2002. Biodistribution and pharmacokinetics of *O*-palmitoyl tilisolol, a lipophilic prodrug of tilisolol, after intravenous administration in rats. Biol. Pharm. Bull. 25, 1072–1076.

Kawakami, S., Opanasopit, P., Yokoyama, M., Chansri, N., Yamamoto, T., Okano, T., Yamashita, F., Hashida, M., 2005. Biodistribution characteristics of all-trans retinoic acid incorporated in liposomes and polymeric micelles following intravenous administration. J. Pharm. Sci. 94, 2606–2615.

Kawakami, S., Suzuki, S., Yamashita, F., Hashida, M., 2006. Induction of apoptosis in A549 human lung cancer cells by all-*trans* retinoic acid incorporated in DOTAP/cholesterol liposomes. J. Control. Release. 110, 514–521.

Kawakami, S., Wong, J., Sato, A., Hattori, Y., Yamashita, F., Hashida, M., 2000b. Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice. Biochim. Biophys. Acta 1524, 258–265.

Kawakami, S., Yamashita, F., Hashida, M., 2000a. Disposition characteristics of emulsions and incorporated drugs after systemic or local injection. Adv. Drug Deliv. Rev. 45, 77–88.

Kmiec, Z., 2001. Cooperation of liver cells in health and disease. Adv. Anat. Embryol. Cell Biol. 161, 1–151.

Lengfelder, E., Saussele, S., Weisser, A., Buchner, T., Hehlmann, R., 2005. Treatment concepts of acute promyelocytic leukemia. Crit. Rev. Oncol. Hematol. 56, 261–274.

Lim, S.J., Lee, M.K., Kim, C.K., 2004. Altered chemical and biological activities of all-trans retinoic acid incorporated in solid lipid nanoparticle powders. J. Control. Release 100, 53–61.

Liu, F., Liu, D., 1995. Long-circulating emulsions (oil-in-water) as carriers for lipophilic drugs. Pharm. Res. 12, 1060–1064.

Managit, C., Kawakami, S., Yamashita, F., Hashida, M., 2005. Uptake characteristics of galactosylated emulsion by HepG2 hepatoma cells. Int. J. Pharm. 301, 255–261.

Maranhao, R.C., Tercyak, A.M., Redgrave, T.G., 1986. Effects of cholesterol content on the metabolism of protein-free emulsion models of lipoproteins. Biochim. Biophys. Acta 875, 247–255.

McCarter, M.D., Fong, Y., 2000. Metastatic liver tumors. Semin. Surg. Oncol. 19, 177–188.

Muindi, J., Frankel, S.R., Miller Jr., W.H., Jakubowski, A., Scheinberg, D.A., Young, C.W., Dmitrovsky, E., Warrell Jr., R.P., 1992. Continuous treatment with all-trans retinoic acid causes a progressive reduction in plasma drug concentrations: implications for relapse and retinoid "resistance" in patients with acute promyelocytic leukemia. Blood 79, 299–303.

Okuno, M., Kojima, S., Matsushima-Nishiwaki, R., Tsurumi, H., Muto, Y., Friedman, S.L., Moriwaki, H., 2004. Retinoids in cancer chemoprevention. Curr. Cancer Drug Target 4, 285–298.

Otsuki, T., Sakaguchi, H., Hatayama, T., Wu, P., Takata, A., Hyodoh, F., 2003. Effects of all-trans retinoic acid (ATRA) on human myeloma cells. Leuk. Lymphoma 44, 1651–1656.

Ozpolat, B., Lopez-Berestein, G., 2002. Liposomal-all-trans-retinoic acid in treatment of acute promyelocytic leukemia. Leuk. Lymphoma 43, 933–941.

Ozpolat, B., Lopez-Berestein, G., Adamson, P., Fu, C.J., Williams, A.H., 2003. Pharmacokinetics of intravenously administered liposomal all-trans-retinoic acid (ATRA) and orally administered ATRA in healthy volunteers. J. Pharm. Pharm. Sci. 6, 292–301.

Ravikumar, T.S., Olsen, C.O., Steele, G., 1990. Resection of pulmonary and hepatic metastasis in the management of cancer. Crit. Rev. Oncol. Hematol. 10, 111–130.

Rensen, P.C., Herijgers, N., Netscher, M.H., Meskers, S.C., van Eck, M., van Berkel, T.J., 1997. Particle size determines the specificity of apolipoprotein E-containing triglyceride-rich emulsions for the LDL receptor versus hepatic remnant receptor in vivo. J. Lipid Res. 38, 1070–1084.

Soprano, D.R., Qin, P., Soprano, K.J., 2004. Retinoic acid receptors and cancers. Annu. Rev. Nutr. 24, 201–221.

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

Takino, T., Konishi, K., Takakura, Y., Hashida, M., 1994. Long circulating emulsion carrier systems for highly lipophilic drugs. Biol. Pharm. Bull. 17, 121–125.

Takino, T., Koreeda, N., Nomura, T., Sakaeda, T., Yamashita, F., Takakura, Y., Hashida, M., 1998. Control of plasma cholesterol-lowering action of probucol with various lipid carrier systems. Biol. Pharm. Bull. 21, 492–497.

Tomii, Y., 2002. Lipid formulation as a drug carrier for drug delivery. Curr. Pharm. Des. 8, 467–474.

Webber, B.M., Soderberg Jr., C.H., Leone, L.A., Rege, V.B., Glicksman, A.S., 1978. A combined treatment approach to management of hepatic metastasis. Cancer 42, 1087–1095.

Yeeprae, W., Kawakami, S., Higuchi, Y., Yamashita, F., Hashida, M., 2005. Biodistribution characteristics of mannosylated and fucosylated O/W emulsions in mice. J. Drug Target. 13, 479–487.

Zuccari, G., Carosio, R., Fini, A., Montaldo, P.G., Orienti, I., 2005. Modified polyvinylalcohol for encapsulation of all-trans-retinoic acid in polymeric micelles. J. Control. Release 103, 369–380.

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Physicochemical and pharmacokinetic characteristics of cationic liposomes

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After intravenous administration of plasmid DNA (pDNA)/cationic liposome complexes, the gene expression is predominantly observed in the lung due to the physicochemical properties of the liposome complexes and the physiology of the lung. To determine the physicochemical properties and distribution behavior of cationic liposomes for lung-selective drug and/or gene delivery systems, *N*-[1-(2,3-dioleyloxy)propyl]-*n,n,n*-trimethylammonium chloride (DOTMA)/cholesterol and 1,2-dioleoyl-3-trimethylammoniopropane (DOTAP)/cholesterol liposomes were studied. The particle sizes of DOTMA/cholesterol and DOTAP/cholesterol liposomes were very similar: 126 and 128 nm, respectively. Furthermore, the zeta potentials of these two liposomes were 51 and 66 mV, respectively. After intravenous injection into mice, both cationic liposomes were rapidly eliminated from the blood circulation and preferentially recovered in the lung. Interestingly, the highest lung accumulation was observed at 1 min, and then, decreased gradually. The distribution characteristics of DOTMA/cholesterol and DOTAP/cholesterol liposomes were almost identical due to the similarities in their physicochemical properties. These results demonstrated that DOTMA/cholesterol and DOTAP/cholesterol liposomes, which possess a positive charge, are promising carriers for lung-selective drug and/or gene delivery systems.

1. Introduction

Recently, much effort has been devoted to the development of cationic liposome-mediated gene delivery systems due to their favorable characteristics. Cationic liposomes condense plasmid DNA (pDNA) into particulates of defined size, protect them from premature degradation in the bloodstream, and interact non-specifically with cell surfaces (Mahato et al. 1997; Huang and Li 1997; Kawakami et al. 2002; Ruozi et al. 2003). Since pDNA complexed with cationic liposomes (i.e. lipoplexes) accumulate in the lung immediately after intravenous administration (Mahato et al. 1995; Sakurai et al. 2001), they lead to a high gene expression in the lung (Zhu et al. 1993; Li and Huang 1997; Song et al. 1997; Kawakami et al. 2000a). Although lung-selective drug and/or gene delivery by cationic liposomes is also expected, there are few reports on the distribution of (bare) cationic liposomes after intravenous administration.

The purpose of this study was to elucidate the physicochemical and distribution characteristics of cationic liposomes for lung-selective delivery systems. *N*-[1-(2,3-dioleyloxy)propyl]-*n,n,n*-trimethylammonium chloride (DOTMA)/cholesterol and 1,2-dioleoyl-3-trimethylammoniopropane (DOTAP)/cholesterol liposomes were selected as cationic liposomes because of the many reports regarding their *in vivo* gene transfection in the lung after intravenous administration (Song et al. 1997; Li and Huang 1997; Templeton et al. 1997; Mahato et al. 1998; Kawakami et al. 2000a; Sakurai et al. 2001; Faneca et al. 2004). [^{3}H]Cholesteryl hexadecyl ether (CHE), a non-degradable maker, was employed as a liposomal marker (Schwiegelshohn et al. 1995; Hattori et al. 2000; Kawakami et al. 2000b; Managit et al. 2003).

2. Investigations, results and discussion

Liposomes are widely used as carriers for a variety of drugs. After intravenous administration, however, they are commonly retained in the blood circulation and removed by the reticuloendothelial system (Papahadjopoulos and Gabizon 1990). Therefore, tissue and/or cell-specific drug delivery is of great importance for a variety of clinical purposes. In order to achieve tissue and/or cell-specific drug delivery, the liposomal surface can be modified with a variety of agents including polyethyleneglycol (Klibanov et al. 1990; Teshima et al. 2004), galactose (Kawakami et al. 2001; Mady et al. 2004), mannose (Kawakami et al. 2000b; Engel et al. 2003), transferrin (Ishida et al. 2001), antibody (Zhang et al. 2003; Yu et al. 2004), RGD-peptide (Dubey et al. 2004), and folate (Pan et al. 2003). In this present study, we analyzed the disposition characteristics of cationic liposomes for tissue and/or cell-specific drug delivery.

Table 1 summarizes the zeta potentials and mean particle sizes of cationic liposomes. The zeta potentials of DOTMA/cholesterol and DOTAP/cholesterol liposomes were

Table 1: Mean particle sizes and zeta potentials of cationic liposomes

Cationic liposomes	Mean particle size (nm)	Zeta potential (mV)
DOTMA/cholesterol liposome	125.5 ± 4.1	51.2 ± 2.3
DOTAP/cholesterol liposome	127.6 ± 7.9	66.5 ± 1.7

Each value represents the mean ± S.D. values (n = 3)

51.2 and 66.5 mV, respectively. As shown by the zeta potential, the surface charge of each particle was positive. The mean particle sizes of DOTMA/cholesterol and DOTAP/cholesterol liposomes were 126 and 128 nm, respectively. Therefore, there was no significant difference in physicochemical properties between DOTMA/cholesterol and DOTAP/cholesterol liposomes.

After intravenous administration of [^{3}H]CHE labeled DOTMA/cholesterol and DOTAP/cholesterol liposomes, they were rapidly eliminated from blood circulation (Fig. 1). The highest amount of lung accumulation of both liposomes reached about 280% of dose/g of tissue at 1 min and then decreased gradually. Their concentrations in the liver and spleen were relatively low; i.e., about 40–50% and 35% of dose/g of tissue, respectively (Fig. 2).

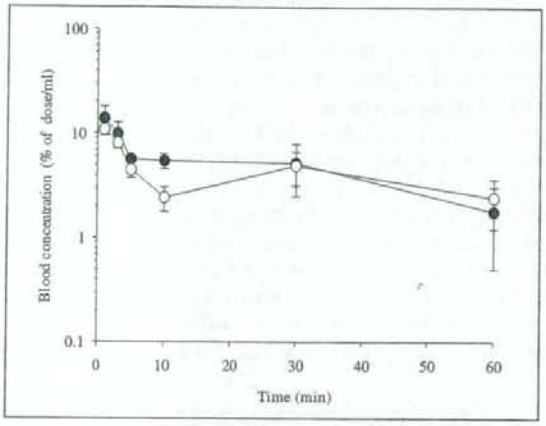

Fig. 1: Blood concentration of [^{3}H]CHE-labeled DOTMA/cholesterol (●) and DOTAP/cholesterol (○) liposomes following intravenous injection in mice. Each value represents the mean ± S.D. (n = 3)

Once the [^{3}H]CHE labeled liposomes were taken up by the organ, they began to redistribute. To avoid this complication, pharmacokinetic analysis was performed during a relatively early period (10 min) when the tissue uptake efflux was negligible. Table 2 summarizes the AUC, and tissue uptake index for representative organs after intravenous administration of cationic liposomes. This pharmacokinetic analysis revealed that the disposition of cationic liposomes was characterized by a small AUC. The tissue uptake index for the lung was the greatest among the tissues examined. The lung uptake index of DOTMA/cholesterol was 130000 µl/h/g, thus, the figure for DOTAP/cholesterol liposomes was around 220000 µl/h/g which was quite closed to the pulmonary plasma flow rate 260000 µl/h/g (Gerlowski and Jain 1983). There was a slight difference in pharmacokinetics between DOTMA/cholesterol and DOTAP/cholesterol liposomes, however, these cationic liposomes were predominantly accumulated in the lung after intravenous administration.

As for the other cationic liposome systems, Litzinger et al. (1996) investigated the biodistribution characteristics of the cationic liposomes 3β-(*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl)cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) liposomes. After intravenous injection into the tail vein of mice (at 5 min) DC-Chol/DOPE liposomes accumulated extensively in the liver (about 75% of the dose), whereas less accumulated in the lung (about 10% of the dose). The distribution differences between DC-Chol/DOPE liposomes and DOTMA/cholesterol liposomes (Fig. 2A) correspond to the observation involving gene delivery systems that there is lower gene expression in the lung after intravenous administration of pDNA complexed with DC-Chol/DOPE liposomes (Kawakami et al. 2000c) compared to pDNA complexed with DOTMA/cholesterol liposomes (Kawakami et al. 2000a).

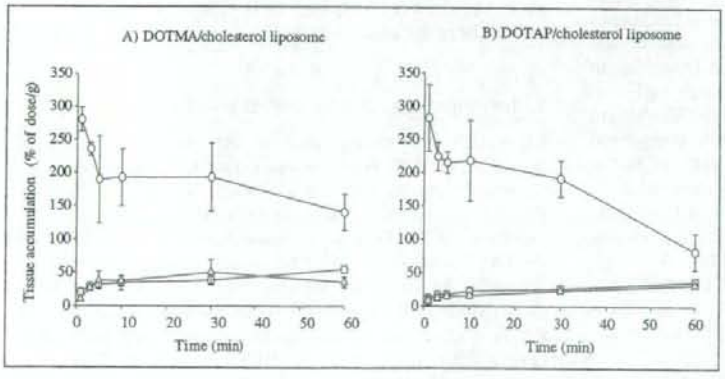

Fig. 2:
Tissue accumulation of [^{3}H]CHE-labeled DOTMA/cholesterol (A) and DOTAP/cholesterol (B) liposomes following intravenous injection in mice. Radioactivity was determined in the lung (○), liver (□) and spleen (△). Each value represents the mean ± SD (n = 3)

Table 2: Area under the blood concentration-time curve (AUC) and tissue uptake index of [^{3}H]CHE-labeled liposomes after intravenous injection into mice[a]

Cationic liposomes	AUC[a]	Tissue uptake index (µl/h/g)[a]		
	(% of dose · h/ml)	CL_{lung}	CL_{liver}	CL_{spleen}
DOTMA/cholesterol liposome	1.40	131218	27387	16947
DOTAP/cholesterol liposome	1.00	216178	22193	15260

[a] The AUC and tissue uptake index were calculated for the periods up to 10 min after injection. An average of three experiments is shown

We previously analyzed the physicochemical and disposition characteristics of pDNA complexed with DOTMA/cholesterol liposomes (Sakurai et al. 2001). When pDNA was complexed with DOTMA/cholesterol liposomes at a charge ratio of 2.24, the zeta potential and particle size were 46 mV and 187 nm, respectively. In this study, the zeta potential of DOTMA/cholesterol liposomes (about 51 mV) was higher than that of pDNA complexed with DOTMA/cholesterol liposomes (about 46 mV), suggesting neutralization by negatively charged pDNA. Although the particle size of DOTMA/cholesterol liposomes was almost the same between these studies, the particle size of DOTMA/cholesterol liposomes (about 126 nm) was smaller than that of pDNA complexed with DOTMA/cholesterol liposomes (about 187 nm), suggesting an interaction with negatively charged pDNA.

In our previous study, about 80% of [^{32}P]pDNA complexed with DOTMA/cholesterol liposomes had accumulated in the lung 1 min after injection (data not shown). A high degree of accumulation of lipoplexes would explain the gene expression level in the lung although it remains unclear why lipoplexes accumulate in the lung. Some studies have suggested that lipoplexes aggregate with blood components via electrostatic interaction and become entrapped in the lung capillaries (McLean et al. 1997; Sakurai et al. 2001). Although the zeta potential of DOTMA/cholesterol liposomes was higher than that of pDNA complexed with DOTMA/cholesterol liposomes, only 50% of the dose (the lung weight was about 0.2 g) of DOTMA/cholesterol liposomes accumulated in the lung 1 min after intravenous injection (Fig. 2). This difference in lung accumulation may be explained by the fact that the particle size of DOTMA/cholesterol liposomes was smaller than that of pDNA complexed with DOTMA/cholesterol liposomes.

Recently, Tan et al. (2001) reported that when pDNA was injected into the tail vein of mice 2–5 min after the injection of cationic liposomes (DOTAP/cholesterol liposomes), 50–80% lower levels of proinflammatory cytokines, including TNF-α, IL-12, and IFN-γ, were observed compared with lipoplex administration. Moreover, the sequential injection technique resulted in a 2- to 5-fold higher level of transgene expression in the lung while transgene expression at 10 or 20 min sequential injection intervals was at a level comparable with that of lipoplex administration. As far as sequential injection-mediated gene transfection is concerned, the distribution characteristics of cationic liposomes need to be investigated to clarify their mechanism. As shown in Fig. 2, the lung accumulation of DOTAP/cholesterol liposomes decreased gradually from 1 to 5 min. Subsequently, the radioactivity in the lung fell gradually from 5 to 30 min, suggesting a lower interaction with negatively charged endogenous components in the blood. Thus, these results may support the fact that an effective interval for sequential injection is within 5 min after administration.

In conclusion, we have demonstrated that DOTMA/cholesterol and DOTAP/cholesterol liposomes, which posses a positive charge, accumulate efficiently in the lung after intravenous administration. This observation provides valuable information to help in the design of cationic liposomes for drug and gene delivery.

3. Experimental

3.1. Materials

DOTMA were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). DOTAP was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol and Clear-Sol I were obtained from Nacalai Tesque, Inc. (Kyoto, Japan), and Soluene-350 was purchased from Packard Instrument Co., Inc. (Groningen, Netherlands). [^{3}H] CHE was purchased from NEN Life Science Products, Inc. (Boston, MA, USA). All other chemicals were of the highest purity available.

3.2. Preparation of liposomes

The preparation of liposomes was reported previously (Kawakami et al. 2000b, 2004). Briefly, a mixture of DOTMA or DOTAP and cholesterol was dissolved in chloroform and evaporated to dryness in a round-bottomed flask. Then, the lipid film formed was resuspended in 5 ml 5% dextrose solution. After hydration, the dispersion was sonicated for 5 min (200 W). Each resulting suspension was passed five times through a 0.45 μm pore size polycarbonate membrane filter (Millipore Co., Bedford, MA, USA). The concentration of liposomes was adjusted to 2.5 mg/ml total lipids based on radioactivity measurement. Radiolabeled liposomes were prepared by addition of [^{3}H]CHE (50 μCi) to the lipid mixture before formation of a thin film layer.

3.3. Measurement of particle sizes and zeta potentials

The particle sizes and zeta potentials of cationic liposomes without radioisotope were measured by laser-Doppler electrophoresis and dynamic light-scattering spectrophotometric methods using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK).

3.4. Distribution study

Five-week-old female ICR mice (20–30 g) were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the US National Institutes of Health and the Guideline for Animal Experiments of Kyoto University. [^{3}H]CHE (1.0 μCi/100 μl)-labeled liposomes were injected into the tail vein of mice at a dose of 25 mg/kg. At predetermined time points, blood was collected from the vena cava under anesthesia and the mice were then sacrificed. The liver, spleen, and lung were collected, washed with saline, blotted dry, and weighed. Ten microliters of blood and a precisely weighed small amount of each tissue (20–30 mg) were digested in 0.7 ml Soluene-350 by incubating overnight at 45 °C. Following digestion, 0.2 ml isopropanol, 0.2 ml 30% hydrogen peroxide, 0.1 ml 5 N HCl, and 5.0 ml Clear-Sol I were added. The samples were stored overnight and the radioactivity was measured in a liquid scintillation counter (LSC 6100, Aloka, Tokyo, Japan).

3.5. Calculation of tissue uptake index

Tissue distribution data were evaluated using the organ clearances as reported previously (Takakura et al. 1990; Kawakami et al. 2000b; Ishida et al. 2004). Briefly, the tissue uptake rate can be described by the following equation:

$$\frac{dX_t}{dt} = CL_{uptake} \cdot C_b \quad (1)$$

where X_t is the amount of [^{3}H]-labeled liposomes in the tissue at time t, CL_{uptake} is the tissue uptake clearance, and C_b is the blood concentration of [^{3}H]CHE-labeled liposomes. Integration of Eq. (1) gives

$$Xt = CL_{uptake} \cdot AUC_{(0-t)} \quad (2)$$

where $AUC_{(0-t)}$ represents the area under the blood concentration-time curve from time 0 to t. Eq. (2) divided by C_b gives

$$\frac{X_t}{C_b} = \frac{CL_{uptake} \cdot AUC_{(0-t)}}{C_b} \quad (3)$$

The CL_{uptake} value can be obtained from the initial slope of a plot of X_t/C_b vs. $AUC_{(0-t)}/C_b$.

The tissue uptake index was described as the uptake clearance per gram of tissue examined (μl/h/g).

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References

Dubey PK, Mishra V, Jain S, Mahor S, Vyas SP (2004) Liposomes modified with cyclic RGD peptide for tumor targeting. J Drug Target 12: 257–264.

Engel A, Chatterjee SK, Al-arifi A, Riemann D, Langner J, Nuhn P (2003) Influence of spacer length on interaction of mannosylated liposomes with human phagocytic cells. Pharm Res 20: 51–57.

Faneca H, Simoes S, Pedroso de Lima MC (2004) Association of albumin or protamine to lipoplexes: enhancement of transfection and resistance to serum. J Gene Med 6: 681–692.

Gerlowski LE and Jain RK (1983) Physiologically based pharmacokinetic modeling: Principles and applications. J Pharm Sci 72: 1103–1127.

Hattori Y, Kawakami S, Yamashita F, Hashida M (2000) Controlled biodistribution of galactosylated liposomes and incorporated probucol in hepatocyte-selective drug targeting. J Control Release 69: 369–377.

Huang L, Li S (1997) Liposomal gene delivery: a complex package. Nat Biotechnol 15: 620–621.

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

Ishida O, Maruyama K, Tanahashi H, Iwatsuru M, Sasaki K, Eriguchi M, Yanagie H (2001) Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors *in vivo*. Pharm Res. 18: 1042–1048.

Kawakami S, Fumoto S, Nishikawa M, Yamashita F, Hashida M (2000a) In vivo gene delivery to the liver using novel galactosylated cationic liposomes. Pharm Res 17: 306–313.

Kawakami S, Wong J, Sato A, Hattori Y, Yamashita F, Hashida M (2000b) Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice. Biochim Biophys Acta 1524: 258–265.

Kawakami S, Sato A, Nishikawa M, Yamashita F, Hashida M (2000c) Mannose receptor-mediated gene transfer into macrophage using novel mannosylated cationic liposomes. Gene Ther 7: 292–299.

Kawakami S, Munakata C, Fumoto S, Yamashita F, Hashida M (2001) Novel galactosylated liposomes for hepatocyte-selective targeting of lipophilic drugs. J Pharm Sci 90: 105–113.

Kawakami S, Yamashita F, Nishida K, Nakamura J, Hashida M (2002) Glycosylated cationic liposomes for cell-selective gene delivery. Crit Rev Ther Drug Carrier Syst 19: 171–190.

Kawakami S, Hattori Y, Lu Y, Higuchi Y, Yamashita F, Hashida M (2004) Effect of cationic charge on receptor-mediated transfection using mannosylated cationic liposome/plasmid DNA complexes following the intravenous administration in mice. Pharmazie 59: 405–408.

Klibanov AL, Maruyama K, Torchilin VP, Huang L (1990) Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett 268: 235–237.

Li S, Huang L (1997) *In vivo* gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. Gene Ther 4: 891–900.

Litzinger DC, Brown JM, Wala I, Kaufman SA, Van GY, Farrell CL, Collins D (1996) Fate of cationic liposomes and their complex with oligonucleotide *in vivo*. Biochim Biophys Acta 1281: 139–149.

Mady MM, Ghannam MM, Khalil WA, Repp R, Markus M, Rascher W, Muller R, Fahr A (2004) Efficient gene delivery with serum into human cancer cells using targeted anionic liposomes. J Drug Target 12: 11–18.

Mahato RI, Kawabata K, Takakura Y, Hashida M (1995) *In vivo* disposition characteristics of plasmid DNA complexed with cationic liposomes. J Drug Target 3: 149–157.

Mahato RI, Takakura Y, Hashida M (1997) Nonviral vectors for *in vivo* gene delivery: physicochemical and pharmacokinetic considerations. Crit Rev Ther Drug Carrier Syst 14: 133–172.

Mahato RI, Anwer K, Tagliaferri F, Meaney C, Leonard P, Wadhwa MS, Logan M, French M, Rolland A (1998) Biodistribution and gene expression of lipid/plasmid complexes after systemic administration. Hum Gene Ther 9: 2083–2099.

Managit C, Kawakami S, Nishikawa M, Yamashita F, Hashida M (2003) Targeted and sustained drug delivery using PEGylated galactosylated liposomes. Int J Pharm 266: 77–84.

McLean JW, Fox EA, Baluk P, Bolton PB, Haskell A, Pearlman R, Thurston G, Umemoto EY, McDonald DM (1997) Organ-specific endothelial cell uptake of cationic liposome-DNA complexes in mice. Am J Physiol 273: 387–404.

Pan XQ, Wang H, Lee RJ (2003) Antitumor activity of folate receptor-targeted liposomal doxorubicin in a KB oral carcinoma murine xenograft model. Pharm Res 20: 417–422.

Papahadjopoulos D, Gabizon A (1990) Liposomes designed to avoid the reticuloendothelial system. Prog Clin Biol Res 343: 85–93.

Ruozi B, Forni F, Battini R, Vandelli MA (2003) Cationic liposomes for gene transfection. J Drug Target 11: 407–414.

Sakurai F, Nishioka T, Yamashita F, Takakura Y, Hashida M (2001) Effects of erythrocytes and serum proteins on lung accumulation of lipoplexes containing cholesterol or DOPE as a helper lipid in the single-pass rat lung perfusion system. Eur J Pharm Biopharm 52: 165–172.

Schwiegelshohn B, Presley JF, Gorecki M, Vogel T, Carpentier YA, Maxfield FR, Deckelbaum RJ (1995) Effects of apoprotein E on intracellular metabolism of model triglyceride-rich particles are distinct from effects on cell particle uptake. J Biol Chem 270: 1761–1769.

Song YK, Liu F, Chu S, Liu D (1997) Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. Hum Gene Ther 8: 1585–1594.

Takakura Y, Fujita T, Hashida M, Sezaki H (1990) Disposition characteristics of macromolecules in tumor-bearing mice. Pharm Res 7: 339–346.

Tan Y, Liu F, Li Z, Li S, Huang L (2001) Sequential injection of cationic liposome and plasmid DNA effectively transfects the lung with minimal inflammatory toxicity. Mol Ther 3: 673–682.

Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, Pavlakis GN (1997) Improved DNA: liposome complexes for increased systemic delivery and gene expression. Nat Biotechnol 15: 647–652.

Teshima M, Kawakami S, Nishida K, Nakamura J, Sakaeda T, Terazono H, Kitahara T, Nakashima M, Sasaki H (2004) Prednisolone retention in integrated liposomes by chemical approach and pharmaceutical approach. J Control Release 97: 211–218.

Yu W, Pirollo KF, Rait A, Yu B, Xiang LM, Huang WQ, Zhou Q, Ertem G, Chang EH. (2004) A sterically stabilized immunolipoplex for systemic administration of a therapeutic gene. Gene Ther 11: 1434–1440.

Zhang YF, Boado RJ, Pardridge WM (2003) Absence of toxicity of chronic weekly intravenous gene therapy with pegylated immunoliposomes. Pharm Res 20: 1779–1185.

Zhu N, Liggitt D, Liu Y, Debs R (1993) Systemic gene expression after intravenous DNA delivery into adult mice. Science 261: 209–211.

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Note

Inhibition of pulmonary metastasis in mice by all-trans retinoic acid incorporated in cationic liposomes

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Abstract

The purpose of this study was to investigate whether all-trans retinoic acid (ATRA), an active metabolite of retinal, incorporated in cationic liposomes composed of 1,2 dioleoyl-3-trimethylammonium propane (DOTAP)/cholesterol could inhibit established metastatic lung tumors by delivery to the pulmonary tumor site after intravenous injection. After intravenous injection in mice, the highest lung accumulation of [^{3}H]ATRA was observed by the DOTAP/cholesterol liposomes formulation, while other formulations including [^{3}H]ATRA dissolved in serum or [^{3}H]ATRA incorporated in distearoyl-L-phosphatidylcholine (DSPC)/cholesterol liposomes produced little accumulation in the lung. In mice used as a model of lung cancer metastasis, ATRA incorporated in DOTAP/cholesterol liposomes, injected intravenously, reduced the number of tumor nodules compared with free ATRA or ATRA incorporated in DSPC/cholesterol liposomes. These results suggest that ATRA incorporated in cationic liposomes would be an effective strategy for differentiation therapy of lung cancer metastasis.

Keywords: Cationic liposomes; All-trans retinoic acid; Pulmonary metastasis; Targeting

1. Introduction

All-trans retinoic acid (ATRA), an active metabolite of retinal, has been shown to exert anti-cancer activities in a number of types of cancer cells and tissues. The activity of ATRA is mediated by regulation of a variety of forms of gene expression through ATRA-dependent activation of retinoic acid receptors (RAR) and retinoid X receptors (RXR) in the nucleus of cancer cells, leading to growth inhibition, differentiation, and apoptosis of cancer cells [1]. Recent advances have led to a better understanding of the mechanism of apoptosis using cultured cell lines [2–5]. To date, few reports have been published about the *in vivo* application of ATRA. Since many reports have established the anti-cancer potential of ATRA *in vitro*, we believe that the use of a cancer tissue-selective drug delivery carrier would achieve cancer differentiation therapy for *in vivo* application. However, there are two factors to be overcome as far as ATRA delivery is concerned: one is low water solubility of ATRA (logPCoct (the logarithmic *n*-octanol/water partition coefficient)=6.6) and the other is a lack of cancer selectively. These two problems must be solved in order to design an effective ATRA carrier system.

Recently, we have demonstrated that most ATRA are incorporated in the liposomal membrane due to their high lipophilicity [6]. Since ATRA contain a carboxyl group, ATRA would be more stably incorporated in cationic liposomes. In addition, cationic liposomes are internalized by endocytosis in these cells; therefore incorporated ATRA in the cationic liposomes will be efficiently delivered to the cells. More recently, we have reported the induction of apoptosis by ATRA incorporated in cationic liposomes composed of 1,2 dioleoyl-3-trimethylammonium propane (DOTAP)/cholesterol in A549 human lung carcinoma cells, which are insensitive (resistant) to the growth inhibitory effects of ATRA *in vitro* [7]. In addition, we have demonstrated that about 50% of cationic liposomes composed of DOTAP/cholesterol accumulate in the lung 1 min after intravenous injection [8]. These results prompted us to investigate whether ATRA incorporated in cationic liposomes composed of DOTAP/cholesterol could inhibit established metastatic lung tumors by delivery to pulmonary tumor site after intravenous administration.

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In the present study, the distribution characteristics of ATRA incorporated in DOTAP/cholesterol liposomes were examined after intravenous injection in mice. Then, the therapeutic potential against pulmonary tumor nodules of intravenously injected CT-26 cells and mouse colon carcinoma cells were also evaluated. Results were compared with free ATRA or ATRA incorporated in distearoyl-L-phosphatidylcholine (DSPC)/cholesterol liposomes, which have an almost neutral charge [9].

2. Materials and methods

2.1. Materials

DOTAP, DSPC, were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and Sigma-Aldrich Co. (St. Louis, MO, USA). Cholesterol and Clear-Sol I were obtained from Nacalai Tesque, Inc. (Kyoto, Japan), and Soluene 350 was purchased from Packard Co., Inc. (Groningen, Netherlands). RPMI1640 medium was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Biowhittaker (Walkersville, MD, USA). ATRA was purchased from Wako Pure Chemicals Industry, Ltd. (Osaka, Japan). [^{3}H]ATRA was purchased from NEN Life Science Products, Inc. (Boston, USA). All other chemicals were of the highest purity available.

2.2. Solubility of ATRA

The solubility of ATRA in water was determined by mixing the excess amount of ATRA in water and stirred overnight at 37 °C. The undissolved ATRA was separated from the solution by filtration through 0.45 μm filter. The amount of ATRA dissolved in water was determined by spectrophotometer at 340 nm (UV–visible Spectrophotometer, Shimazu Co., Ltd., Kyoto, Japan).

2.3. Preparation of liposomes

The liposomes were prepared by the method described previously [7,10]. The liposomes, DOTAP/cholesterol (50:50 M ratios) and DSPC/cholesterol (60:40 M ratios), were prepared with or without ATRA. ATRA was mixed at the molar ratio of 0.2, 1 or 5:100 of total lipid liposomes. In the biodistribution experiments, liposomes were labeled with a tracer amount of [^{3}H]ATRA. The mixture, with or without [^{3}H]ATRA, was first dissolved in chloroform. After vacuum drying and desiccation, pH 7.4 phosphate-buffered saline was added for hydration. The preparation was then sonicated and passed through a 0.45 μm filter for sterilization. The zeta potential and mean particle size of the liposomes was measured using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK).

2.4. Cell lines

CT-26 cells were routinely grown with RPMI1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Invitrogen Co., Carlsbad, CA, USA) in 5% CO_2 humidified air at 37 °C.

2.5. Animals

ddY mice (5-weeks old, male, 20–25 g) and CDF_1 mice (4-weeks old, male, 18–20 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of Kyoto University.

2.6. Biodistribution study

The biodistribution study was performed by the method described previously [9]. [^{3}H]ATRA dissolved in serum or [^{3}H]ATRA incorporated in liposomes were injected into the tail vein of ddY mice. At a given time periods, blood was collected from the vena cava under anesthesia, and mice were killed. Then, the liver, kidney, spleen, and lung were excised, washed with saline, blotted dry, and weighed. Ten microliters blood and a small piece of each tissue were incubated with 0.7 ml Soluene 350 overnight at 45 °C. Following digestion, 0.2 ml isopropanol, 0.2 ml 30% hydrogen peroxide, 0.1 ml 5.0 N HCl, and 5.0 ml Clear-Sol I were added in this order. The samples were stored overnight and the radioactivity was measured using a liquid scintillation counter (LSA-500, Beckman, Tokyo, Japan).

2.7. Experimental pulmonary metastasis and treatment

The pulmonary metastasis model was prepared by the method described previously [10]. The ATRA dose was 0.585 mg/kg according to the report by Shimizu et al. who demonstrated the hepatic antitumor effects produced by ATRA incorporated in sterylglucoside liposomes [11]. CT-26 cells were trypsinized and injected into the tail vein of CDF_1 mice (5×10^4 cells/0.2 ml HBSS). ATRA dissolved in 5% HCO-60 (Nicco Chem. Co., Tokyo, Japan) solution, ATRA incorporated in DSPC/cholesterol or DOTAP/cholesterol liposomes or bare DOTAP/cholesterol liposomes were repeatedly injected via the tail vein at an ATRA dose of 0.585 mg/kg/day or a total lipid dose of 120 mg/kg/day, from day 3 to day 7 after tumor inoculation. On the 12-day after tumor inoculation, mice were killed and their lungs were extracted. The number of the tumor colonies on the surface of the lung was counted.

2.8. Evaluation of liver toxicity

After intravenous injection of ATRA incorporated in DOTAP/cholesterol liposomes, liver toxicity was evaluated. ATRA and lipid were injected at a dose of 0.585 and 120 mg/kg in ddY mice, the serum ALT (alanine aminotransferase) and AST (aspartate aminotransferase) concentrations were measured with kits using the UV-Rate method according to the manufacturer's (Wako Pure Chemical Industries, Ltd., Osaka, Japan) directions.

2.9. Statistical analysis

Multiple comparisons between groups were made by the Tukey–Kramer test. $P<0.05$ was considered to be indicative of statistical significance.