Interaction of Liposomes with Cultured Cells: Possible Involvement of Lipid Peroxidation in Cell Growth Inhibition

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Systematic analyses of the interaction between liposomes and cells were examined. Liposomes were found to affect the growth of mouse NIH 3T3 cells depending upon their size, net charge, and cholesterol content. Among the charged compounds, stearylamine was the most inhibitory and showed complete inhibition of cell growth at 100 μM. The cholesterol-rich and small unilamellar vesicles were more suppressive compared to the cholesterol-poor and multilamellar ones, respectively. The binding assay of liposomes to the cells showed a positive correlation between liposome binding and the extent of growth inhibition. Suppression of liposome uptake by inhibitors of the cytoskeletal system and energy metabolism were suggestive of an endocytotic mechanism for the cellular uptake of liposomes. The growth inhibitory effect seemed secondary to the intracellular uptake of liposomes, and peroxidation of incorporated lipids would lead to cellular damage. Therefore, it is highly recommended that potential growth inhibitory effects associated with the particular composition and other properties of liposomes should be carefully assessed in any human studies, especially for long-term use.

Keywords liposome; fibroblast NIH 3T3; cell growth inhibition; peroxidation; endocytosis

Introduction

Recent investigations on liposomes have led to the development of their application to medicine, such as *in vivo* drug delivery system,[1] and acting as a "depot" or time release system.[2] It is also possible to convey the drugs to specific organs by modifying the size and surface properties of the liposomes.[3] Recently applications of liposomes for the introduction of foreign deoxyribonucleic acid (DNA) into the target cells were also reported.[4] Several mechanisms of interaction between liposome components and cells *in vitro* and *in vivo* have been proposed: adsorption,[5] phospholipid exchange,[6] endocytosis[7] and fusion.[8] When liposomes are applied for the drug delivery system, it is highly recommended that they are as innocuous as possible in individuals. However, experimental information related to this aspect is rather limited. Some types of liposomes were shown to be cytotoxic to tumor cells.[9] A possible mechanism for cytotoxicity has been proposed. The incorporated lipid components of liposomes, probably phosphatidylcholine, were hydrolyzed by phospholipase activity resulting in fatty acid liberation. The free fatty acids, especially unsaturated ones, may cause cytotoxicity.[10] Liposomes have already been administered on a small scale to humans and little inhibitory effects simply due to liposomal components have been reported.[11] It is probable that liposomes will be administered on a larger scale for clinical purposes of drug carrier in the near future. The present studies were undertaken to characterize the long-term effects of liposomes on cell growth in terms of the vesicle type, lipid composition or charge. Furthermore, in order to delineate the mechanism(s) of growth inhibition by liposomes, we investigated the interaction of liposomes with fibroblasts especially focused on lipid peroxidation.

Materials and Methods

Materials Phosphatidylcholine (PC) was extracted from egg yolk as described by Singleton *et al.*[11] Bovine brain phosphatidylserine (PS) was purchased from Sedary Research Laboratories (London, Ontario). Cardiolipin (CL), stearylamine (SA) and dicetylphosphate (DCP) were obtained from Sigma Chemicals (St. Louis, MO). Cholesterol (Chol) was the product of Nakarai Chemicals (Kyoto, Japan). Glycerol tri[1-^{14}C]palmitate ([^{14}C]TG) was purchased from Amersham (Bucking Hamshire, England). [Methyl-^{3}H]thymidine ([^{3}H]thymidine) and trypsin were from American Radiolabeled Chemicals (St. Louis, MO) and GIBCO (Grand Island, NY), respectively.

Cell Cultures Mouse NIH 3T3 cells supplied by Dr. K. Kohno (Gifu Pharmaceutical College) were maintained in Dulbecco's modified eagle medium (GIBCO) containing 10% fetal bovine serum. The cells were subcultured every 3—4 d.

Preparation of Liposomes Appropriate volumes of the lipid stock solutions in benzene were mixed at the molar ratios as given in Table I. The lipids were dried and hydrated with 1 ml of Ca, Mg-free phosphate-buffered saline (PBS). Two types of liposomes were made. One type was prepared by sonication for 2 h at 45 °C in a Branson B-12 bath type sonifier at 60 W. Small unilamellar vesicles (SUVs) were fractionated ranging 80 to 100 nm in diameter by gel-exclusion chromatography on a Sephacryl S-1000 column (1.6 × 65 cm) eluted with a flow rate of 0.7 ml/min according to the methods of Reynolds *et al.*[13] Calibration was carried out using polystyrene beads of 85 ± 5.5, 109 ± 2.7, 220 ± 5.9 nm (Nissin EM Co., Ltd., Tokyo) in diameters. And then liposomes were sterilized by filtering through an Ekicrodisc 13 (pore size, 0.2 μm) (Gelman Sciences Japan Ltd., Tokyo). The other type of liposome was prepared as follows. The hydrated lipids were vortexed for 15 min at 45 °C and then extruded through an Ekicrodisc 13 (poresize, 0.45 μm) to prepare multilamellar vesicles (MLVs). The sizes of the liposomes were determined by freeze-fracture electron microscopy showing that the liposomes were 80—100 nm in diameter for SUV and 200—400 nm for MLV. Lipid concentrations were determined by phospholipid phosphorus analysis.[14]

Microassay for Cell Viability Effects of liposomes on cell viability were measured by dye uptake methods as described by Ito.[15] Cells in 96-well trays (3 × 10^{3} cells/well) were cultured overnight at 37 °C, and then the desired concentrations of liposome suspensions were added to the wells (final volume 200 μl/well). The cells were further incubated for 5 d prior

TABLE I. Lipid Composition of Liposomes

Lipid composition	Molar ratio	Probable net charge	Liposome type
PC : Chol	7 : 2 , 4 : 5	Neutral	SUV, MLV
PC : Chol : SA	7 : 2 : 1, 4 : 5 : 1	Positive	SUV, MLV
PC : Chol : DCP	7 : 2 : 1, 4 : 5 : 1	Negative	SUV, MLV
PC : Chol : PS	7 : 2 : 1, 4 : 5 : 1	Negative	SUV, MLV
PC : Chol : CL	7 : 2 : 1, 4 : 5 : 1	Negative	SUV, MLV

PC, phosphatidylcholine (egg); Chol, cholesterol; CL, cardiolipin; SA, stearylamine; PS, phosphatidylserine; DCP, dicetylphosphate. SUV, small unilamellar vesicle; MLV, multi lamellar vesicle.

to fixing with 10% formalin and stained with 0.05% crystal violet. After two washings with PBS, the dye was eluted with 0.1% acetic acid in 50% ethanol. The absorbance (A) at 590 nm each sample was measured using an enzyme immunoassay (EIA) reader (Bio-Rad Model 2550). Wells without cells were set for blank, and those with cells, but without liposome, for 100% viability. The percent cell viability was determined according to the following formula:

$$\% \text{ cell growth} = A \text{ (sample)}/A \text{ (without liposome)} \times 100$$

The percent absorbance is directly proportional to the number of alive cells.

[^{3}H]Thymidine Uptake Cells were cultured in 96-well clusters (3×10^3 cells/well). Three days after the addition of liposomes, the cells were pulsed for 4 h with [^{3}H]thymidine (1 μCi/well). After solubilization with 0.1% sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA)-precipitable fractions were harvested on glassfibered filterpaper using a cell harvester (Waken). The radioactivities of TCA-precipitable fractions were counted with a scintisol (ACSII) in a Beckman LS-9000 scintillation counter.

Liposomes Binding Assay The cell suspensions (5×10^4 cells/2 ml) were incubated with [^{14}C]TG-labeled liposomes composed of eggPC : Chol : R : [^{14}C]TG (7 : 2 : 1 : 0.5, molar ratio) for 2 h at 4 °C without fetal calf serum or albumin. After incubation the cells were rapidly washed with PBS at 4 °C for three times. The cells were then removed from the plates using a cell scraper (Costar) and were collected on glass fiber mats. Filters were dried and the cells were solubilized by incubation with *N*-chlorosuccinimide (NCS) tissue solubilizer (Amersham). The radioactivity of the samples was counted in a scintisol.

Liposome Uptake Assay Cells were first preincubated with various metabolic inhibitors for 30 min at 37 °C at the following concentrations: sodium azide (N_3Na), 5 mM; deoxyglucose, 50 mM; cytochalasin B, 20 μg/ml; sodium fluoride (NaF), 10 mM; osmium tetroxide (OsO_4), 2% (w/v); glutaraldehyde, 4% (v/v). After a further 2 h-incubation with liposomes (eggPC : Chol : DCP : [^{14}C]TG 7 : 2 : 1 : 0.3, molar ratio, 300 μM, 3×10^5 dpm), the cells were washed twice with PBS and the cell-associated radioactivities were measured.

Measurement of Lipid Peroxides The levels of lipid peroxides or malondialdehyde (MDA) precursors were measured by thiobarbituric acid (TBA) reaction according to the method of Kogure *et al.*[16] with a slight modification. The cells were incubated for 3 d containing each liposome (300 μM) under the same condition as described in the "microassay for cell viability" section. Scraped cells were divided into two batches for measurements of lipid peroxides and glutathione content. To each sample, 0.5 ml of 7% (w/v) SDS, 2 ml of 0.1 N HCl, 0.3 ml of 10% (w/v) phosphotungstic acid, and 1 ml of 0.5% TBA were added in this order, and the reaction mixtures were immediately vortexed. The samples were heated at 95 °C for 45 min in boiling water and then immediately cooled in ice. Five ml of *n*-butanol was added to the system and mixed for 15 s. The *n*-butanol layer was withdrawn after centrifugation at $1100 \times g$ for 10 min, and the fluorescence intensity was measured at 565 nm (ext. 535 nm) by spectrofluorometry (Hitachi MPE-3). The amounts of TBA reactants were calculated in comparison with a standard MDA solution prepared by hydrolysis of 1,1,3,3-tetraethoxy propane, one mole of which yields one mole MDA under these conditions. The total protein was assayed by the method of Lowry *et al.*[17] using bovine serum albumin as a standard.

Measurement of Glutathione Concentration The other halves of the above cell fractions were used for measurement of glutathione (GSH). GSH was measured according to Boyne *et al.*[18] Cells were homogenized in an all-glass apparatus in 10 volumes of 6% TCA. The filtrate (1 ml) was treated with 5 ml 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) (2 mg/ml) dissolved in 0.2 M phosphate buffer, pH 8.0. The absorbance of thiophenol anion produced was recorded at 412 nm for 2 min after the addition of DTNB. The GSH concentration was determined by a standard curve using authentic GSH.

Results and Discussion

Effects of Liposomes on the Cell Viability The liposome composition similar to those used in other laboratories, where the long-term clinical use of liposomes has been aimed, were investigated in the present studies.[19] We first examined the viability of cells cocultured with various types of liposomes for a long-period of time (5 d). As shown in Table II, these compositions of liposomes dose-dependently reduced the number of viable NIH 3T3 cells. Susceptibilities of the cells to liposomes varied depending upon vesicle type and composition. As to SUVs, the SA-containing liposomes were the most inhibitory among the lipids examined irrespective of the cholesterol content. Nearly complete cell death was observed at a liposome concentration of 100 μM. Other charged lipid-containing liposomes, such as CL and PS, were more suppressive compared with those without charged lipids. Experiments with mice reported that positively-charged liposomes were more toxic *in vivo* than negatively-charged or neutral liposomes.[20] Cholesterol content in liposomes also affected the viabilities of the cells. Charged lipid-containing liposomes with a high cholesterol (eggPC : Chol : R 4 : 5 : 1) are more suppressive than those with a lower cholesterol content (eggPC : Chol : R 7 : 2 : 1). For example, 300 μM CL-containing liposomes with 20% cholesterol showed no significant inhibition, whereas at the same concentration CL-containing liposomes with 50% cholesterol were inhibited by 25%. These results are consistent with those in the previous report on the cytotoxic effect of cholesterol.[21] Effects of the vesicle type were also examined as to SA- and CL-containing liposomes. SUVs (80—100 nm) were more inhibitory than MLVs (<200 nm in diameter) with the same lipid composition, as shown in Table II. No effects on cell viability were observed below 100 μM of liposomes with SUV. Allen *et al.* also demonstrated that it is necessary to keep empty liposome concentrations to levels below 60 μM in order to prevent cultured cells from liposome cytotoxicity.[22]

Thymidine Uptake The effects of liposomes on cell growth were examined by [^{3}H]thymidine incorporation 3 d after the addition of the liposomes, when SA-containing liposomes had not caused complete cell death. Stronger suppression of [^{3}H]thymidine uptake was observed in cells treated with positively-charged liposomes (SA) than in those

Table II. Inhibitory Effects of Liposomes on Viability of NIH 3T3 Cells

Liposome concentration (μM)	SUV EggPC : Chol : R (7 : 2 : 1)					SUV EggPC : Chol : R (4 : 5 : 1)					MLV EggPC : Chol : R (7 : 2 : 1)			
	SA	DCP	CL	PS	EggPC : Chol R is nothing	SA	DCP	CL	PS	EggPC : Chol R is nothing	SA	CL	SA	CL
	(Inhibition % control)													
30	9.1±5.1	0	0	0	0	0	0	0	0	0	24.5±4.1	0	27.2±4.5	0
100	94.1±3.1	0	0	0	0	92.9±1.5	0	0	0	0	25.1±5.8	8.0±4.0	27.3±4.0	1.7±2.1
300	95.2±2.8	11.4±4.5	0	27.2±5.5	0	100	0	25.0±6.5	25.0±5.5	13.6±5.6	58.8±5.9	14.2±5.1	70.5±7.6	24.6±4.5
1000	97.4±1.9	20.7±6.4	35.9±6.4	52.3±5.2	8.0±2.1	100	87.5±2.2	85.7±5.7	51.1±6.6	76.1±5.4	91.7±2.1	14.8±4.1	97.6±2.2	65.3±6.6
3000	100	96.6±2.1	100	100	38.1±5.5	100	100	100	100	88.6±6.0	100	20.5±7.0	100	66.0±5.3

Inhibition was expressed as percent of control treated with medium alone. Values represent the means±S.D. of five different experiments.

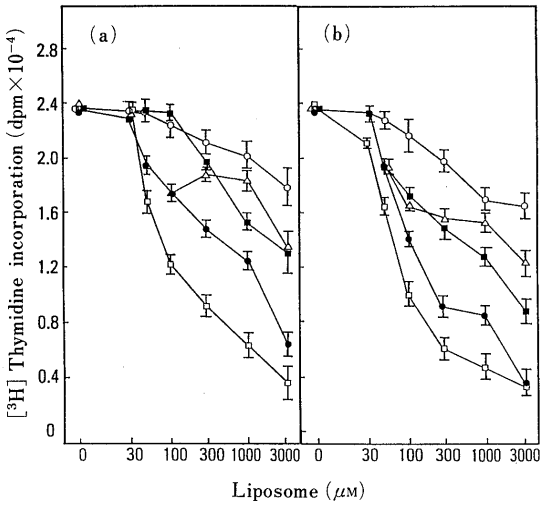

Fig. 1. Effects of Liposomes on [^{3}H]Thymidine Uptake

(a) SUV, eggPC : Chol : R (7 : 2 : 1, molar ratio), (b) SUV, eggPC : Chol : R (4 : 5 : 1, molar ratio). R: △, DCP; □, SA; ■, CL; ●, PS. ○, eggPC : Chol (a, 7 : 2; b, 4 : 5, molar ratio. R is nothing). Values represent the means±S.D. of five different experiments.

with negatively-charged or neutral liposomes (Fig. 1). Moreover, [^{3}H]thymidine uptake was dependent upon the cholesterol content (Fig. 1(a), (b)). Dose–response curves of [^{3}H]thymidine uptake by liposomes with 50% cholesterol shifted to the left compared to those with 20% cholesterol (Fig. 1(b)), indicating that increased liposomal cholesterol reduces the uptake of [^{3}H]thymidine. These results were in good accordance with those observed in Table II.

Binding of Liposomes to the Cells In order to understand the differences in the inhibitory effects among liposome composition, bindings of the liposomes to the cells were examined. The binding of SA-containing liposomes, which showed the most suppressive effect on cell growth, was significantly greater than the other types of liposomes (Table III). Magee *et al.* demonstrated that the cells were protected from virus infection by cationic liposomes containing antiviral antibodies to a degree of up to 10000 times compared with those in the presence of free antibodies.[23] It is conceivable that the enhanced association is due to electrostatic interaction between the positively-charged liposomes and the negatively-charged cells.

Uptake of Liposome by the Cells Since little intracellular uptake of some liposomes occured in the cells which were not strongly endocytotic, it was likely that at least a part of the toxic effects acted at the cell surface.[24] Although the mechanism(s) for the growth inhibitory effects of liposomes was poorly understood, it might involve the effects at the cell surface or during intracellular processing of liposomes. There are four possible modes of liposome uptake: 1) adsorption of liposomes, 2) exchange of phosphatidylcholine molecules between liposomes and cells, 3) fusion of liposomes with the cell membrane, and 4) endocytotic process. Experiments with various metabolic inhibitors were undertaken to characterize the modes of liposome uptake. As shown in Table IV, inhibitors for the cellular energy metabolism or the cytoskeletal system significantly reduced the uptake of vesicles. Prefixation of cells with glutaraldehyde or OsO_4 strongly inhibited the uptake of vesicles. Cytochalasin B reportedly suppresses endocytosis (phagocytosis and macropinocytosis, but not micropinocytosis) of a wide range of materials in diverse cell types.[25] Either deoxyglucose or NaN_3 alone moderately suppressed the liposome uptake, whereas the combination of these inhibitors markedly suppressed the uptake, indicating a synergistic effect of the two inhibitors. The liposome uptake experiments showed that the inhibitors which blocked cytoskeletal systems were more potent than the inhibitors for energy metabolism. These results suggested that liposomes were taken up by endocytosis. If this is the case, the possible sites of liposomes affecting cell growth might be inside the cells but not at the plasma membrane. In erythrocytes, positively charged liposomes have been reported to cause membrane perturbation leading to hemolysis.[26] However, the endocytotic incorporation of positively charged liposomes containing stearylamine was also demonstrated.[27] Although the direct membrane perturbing effects of positively charged liposomes would occur immediately after their addition, SA-containing liposomes did not affect cell growth within 24 h. Significant growth inhibition was observed only more than 24 h after the addition of the liposomes (data not shown). Thus, it seems reasonable to consider that cell growth inhibition may be caused by endocytotic incorporation rather than by the direct effects at the outer membrane surface.

Malondialdehyde and Glutathione Concentration It is well known that various peroxides cause cytotoxic effects. This may be true in our observations of cultured cells. As previously reported, the liposomes taken up by hepatocytes were catalyzed by lysosomal enzymes and free fatty acids were released.[28] The released polyunsaturated fatty acids are converted to their peroxy radicals and then to hydro- and endoperoxides.[29] As shown in Table V, marked increases of lipid peroxides were observed in cells incubated with

TABLE III. Binding of Liposomes to NIH 3T3 Cells

R	Cells (%)	Supernatant (%)
SA	5.05±0.88[a]	94.95±0.48[a]
PS	3.66±0.36	96.34±0.36
CL	3.55±0.28	96.45±0.28
DCP	3.01±0.22	96.99±0.22
EggPC : Chol (7 : 2)	3.21±0.31	96.79±0.31
R is nothing		

Liposomes were composed of eggPC : Chol : R : [^{14}C]tripalmitin (7 : 2 : 1 : 0.5, molar ratio). Values represent the means±S.D. of three different experiments. *a*) Statistically significant compared with PS, CL, DCP and eggPC : Chol (7 : 2) at $p<0.05$.

TABLE IV. Uptake of [^{14}C]Tripalmitin-Labeled Liposomes by NIH 3T3 Cells

Treatment	Uptake (% of control)
Control	100
NaN_3	87.9±5.0
Cytochalasin B	28.1±2.9
OsO_4	7.6±0.6
Glutaraldehyde	27.1±6.4
NaN_3 + deoxyglucose	18.0±4.7
Deoxyglucose	86.8±7.8
NaF	84.7±3.9

Values are expressed % of medium control. Values represent the means±S.D. of four different experiments.

TABLE V. Levels of Glutathione and Malondialdehyde after Treatment with Liposomes

R	Malondialdehyde (nmol/mg protein)	Glutathione (nmol/mg protein)
Control	0.41±0.04	74.2±4.3
SA	0.65±0.06	18.0±1.4
DCP	0.61±0.16	51.0±3.9
PS	0.49±0.08	28.7±3.5
CL	0.56±0.18	47.5±1.8
PC:Chol (7:2)	0.41±0.03	49.2±3.5

The levels of malondialdehyde and glutathione were measured by thiobarbituric acid reaction and SH reaction, respectively. Values represent the means±S.D. of five different experiments.

liposomes. The TBA-reactive substances were produced in larger amounts in cells treated with SA-containing liposomes than in those with neutral and negatively-charged liposomes. Also, the various extents of decreases in glutathione levels were observed in the presence of liposomes. In PS-containing liposomes, the level of decreased GSH did not correlate well with the increased level of MDA. Mechanism(s) for growth inhibition, other than lipid peroxidation, may be operating in a PS-containing system. Christophersen[30] suggested that GSH reacts enzymatically with some organic peroxide intermediate(s) which otherwise forms MDA. Thus, GSH may interrupt the autocatalytic chain reaction of lipid peroxidation. A close correlation between low GSH levels and the accumulation of MDA in intact cells has been documented. It is conceivable that GSH deficiency *per se* leads to lipid peroxidation and that it at least partially causes the observed cell growth inhibition. *In vitro* administration of liposomes for longer periods demonstrated cell growth inhibition to various extents depending on the liposomal size, net charge and cholesterol content. Although these *in vitro* results would not immediately reflect *in vivo* effects of liposomes, it is conceivable that some types of liposomes might be cytotoxic during long-term *in vivo* administration. Therefore, one should remember that some type of liposomal vehicle as a drug carrier could become cytotoxic when used at high doses for longer periods. The obtained results should be carefully assessed in any human studies.

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