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Effect of liposome encapsulation of tea catechins on their accumulation in basal cell carcinomas

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Summary

Background: (−)-Epigallocatechin gallate (EGCG), the main active polyphenol in green tea, is associated with antioxidant and anticancer activities.
Objective: The aim of this study was to evaluate the feasibility of using liposomes for intratumor distribution of EGCG and its derivative, (+)-catechin.
Method: Liposomes containing egg phosphatidylcholine, cholesterol, or anionic surfactant in the presence of 15% ethanol were prepared. The physicochemical characteristics including vesicle size, zeta potential, drug entrapment, and drug release of liposomal formulations were determined. The liposomes containing EGCG were injected into basal cell carcinomas (BCCs), melanomas, and colon tumors to examine the tumor uptake of the drug. Liposomes were also incubated with a given number of BCC cells, and the cell viability was estimated.
Result: Almost no drug molecules were observed when free EGCG was administered to BCCs. EGCG encapsulated in liposomes with deoxycholic acid (DA) and ethanol increased drug deposition by 20-fold as compared to the free form. The larger vesicle size of this formulation was suggested to be the predominant factor governing this enhancement. The liposomes without ethanol showed low or negligible enhancement on EGCG uptake in BCCs. Liposomes protected EGCG from degradation, resulting in the induction of greater BCC death compared to that by free EGCG at lower concentrations.

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Conclusion: These results suggest that the intratumor injection of liposomes containing EGCG with moderate modification is an effective approach for increasing EGCG deposition in BCCs.

1. Introduction

Green tea polyphenols are antioxidants and have been shown to exhibit chemopreventive and antitumor activities [1,2]. The major and most-active constituent in green tea responsible for these biological effects is (−)-epigallocatechin gallate (EGCG). With respect to the skin, green tea extracts and EGCG itself have been reported to be beneficial in treating UV-induced photodamage, basal cell carcinomas (BCCs), melanomas, and skin papillomas [3—6]. Hence, green tea catechins may be suitable to be the therapeutic agents or adjuvants for treating skin disorders [2,7].

The oral bioavailability of tea catechins is known to be low with a bioavailability of less than 2—5% [8,9]. The systemic clearance of these catechins is also high [8,10]. Hence the direct delivery such as by an intratumor route to localized sites is highly desirable since it would allow local pathologies to be treated without significant systemic side effects. Of the various options for administration, an intratumor injection of drugs is one of the most-promising approaches for solid tumors to minimize side effects and maximize cytotoxicity at the tumor site [11]. However, in most cases, local retention of drugs injected intratumorally is very low because of the large diffusion capability due to their small molecular size. To overcome this problem, drug—carrier systems such as liposomes are suitable because of their favorable characteristics as a biodegradable drug reservoir.

Liposomes are microscopic vesicles consisting of membrane-like phospholipid bilayers surrounding an aqueous medium. The aim of this study was to investigate the effect of liposomal systems on the intratumor accumulation of EGCG. (+)-Catechin and its isomer, (−)-epicatechin, were also used as model drugs since it has shown moderate anticarcinogenic activity toward skin cancer [12]. BCC was used as the skin tumor model since it is the most-common type of skin cancer in the world [13]. Both in vivo drug deposition within the tumor and in vitro cell viability were performed to evaluate the efficacy of liposomes for intratumoral EGCG administration.

2. Materials and methods

2.1. Materials

EGCG, (+)-catechin, (−)-epicatechin, cholesterol (CH), and deoxycholic acid (DA) were purchased from Sigma Chemical (St. Louis, MO, USA). Tween 80 was obtained from Kanto Chemical (Tokyo, Japan). Egg phosphatidylcholine (EPC, 99%) was supplied by Nippon Oil (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and fetal calf serum (FCS) were purchased from Biowest (Nuaillé, France). The BCC cell line (BCC-1/KMC) was kindly provided by Prof. Hsin-Su Yu (Department of Dermatology, National Taiwan University, Taipei, Taiwan). BCC-1/KMC is a long-term culture of human BCC derived from the undifferentiated type of facial BCC tumor on the thermal traumatic scar, which was aneuploidy and subculture for more than 100 passages [14,15]. This immortalized and tumorigenic cell line expresses epithelial markers of keratin filaments and desmosomes. BCC-1/KMC has successfully adapted to grow in RPMI 1640 and at a moderate concentration of calcium (0.4 mM). The melanoma (B16-F0) and colon cancer (HT-29) cell lines were supplied by American Type Culture Collection (Rockville, MD, USA). All other chemicals and solvents were of analytical grade.

2.2. Preparation of liposomes

EPC (4%, w/v), CH (1%, w/v), and other additives were dissolved in a 10 ml volume of a chloroform:-methanol (2:1) solution. The organic solvent was evaporated in a rotary evaporator at 40 °C, and solvent traces were removed by maintaining the lipid film under a vacuum overnight. The films were hydrated with double-distilled water or 15% ethanol in water containing 17.2 mM of the drug using a probe-type sonicator (VCX 600, Sonics and Materials, USA) at 25 W for 30 min. In some experiments, the liposomes were extruded through a LipoFast extruder (Avestin, Canada) using a polycarbonate membrane with a pore size of 200 nm. The liposomal systems used in this study are listed in Table 1.

Table 1 The composition and characterization of EGCG, (+)-catechin, and (−)-epicatechin liposomes by vesicle size, zeta potential, and drug encapsulation

Code	Composition[a]	Size (nm)	Zeta potential (mV)	Encapsulation (%)		
				EGCG	(+)-Catechin	(−)-Epicatechin
F1	EPC + CH = 4:1[b]	131.1 ± 0.3	−0.9 ± 0.4	99.6 ± 0.1	39.5 ± 4.8	31.9 ± 2.7
F2	EPC + CH + DA = 4:1:0.25	378.2 ± 10.9	−26.2 ± 0.9	99.0 ± 0.1	53.4 ± 0.6	62.7 ± 7.8
F3	EPC + CH + Tween 80 = 4:1:1.64	104.6 ± 2.3	−12.1 ± 0.3	84.6 ± 3.8	46.9 ± 4.5	57.8 ± 9.1
F4	F2 extrude	215.8 ± 21.3	−36.1 ± 1.7	98.1 ± 2.5	57.0 ± 6.6	64.7 ± 7.9

EPC, egg phosphatidylcholine; CH, cholesterol; DA, deoxycholic acid. Each value represents the mean ± S.D. (n = 3).
[a] The ratio of liposome composition is weight ratio (%).
[b] All formulations contained a 15% ethanol in the systems except F3.

2.3. Determination of vesicle size and zeta potential

The mean vesicle size and zeta potential of the liposomes were measured by a laser scattering method (Nano ZS® 90, Malvern, UK). Liposomal suspensions were diluted 100-fold with double-distilled water before the measurement. The determination was repeated three times per sample for three samples.

2.4. Drug encapsulation percentage in liposomes

The liposomal suspension with drugs was centrifuged at 48,000 × g and 4 °C for 30 min in a Beckman Optima MAX® ultracentrifuge (Beckman Coulter, USA) in order to separate the encapsulated drug from the free form. The supernatant and precipitate were analyzed by HPLC to determine the encapsulation percentage [16]. A 25 cm long, 4 mm inner diameter stainless RP-18 column (Merck, Darmstadt, Germany) was used. The mobile phase for catechins was 10:90 acetonitrile:2.7% acetic acid/water at a flow rate of 1.2 ml/min. The fluorescence detector was set at 280 nm for excitation and 320 nm for emission. The detection limit of (+)-catechin, (−)-epicatechin and EGCG was 10, 10, and 40 μg/ml, respectively.

2.5. In vitro release

Drug release from liposomes was measured using a Franz diffusion cell. A cellulose membrane (CelluSep® T2, with a molecular weight cutoff of 6000–8000, Membrane Filtration Products, USA) was mounted between the donor and receptor compartments. The donor medium consisted of 1 ml of a liposomal formulation. The receptor medium consisted of 10 ml of pH 7.4 citrate–phosphate buffer. The available diffusion area between cells was 1.539 cm^2. The stirring rate and temperature were kept at 600 rpm and 37 °C, respectively. At appropriate intervals, 300 μl aliquots of the receptor medium were withdrawn and immediately replaced with an equal volume of fresh buffer. The amount of drugs was determined by HPLC.

2.6. In vivo intratumor administration

The BCC and HT-29 cell lines were maintained at 37 °C in RPMI 1640 medium containing 10% FCS and were subcultured twice a week. The B16-F0 cell line was cultured in DMEM and 10% FCS. The solid tumors were obtained by a subcutaneous injection of 10^7 BCC and HT-29 cells or 10^6 B16-F0 cells into the back of female nude mice (Balb/c-nu strain, 6–8 weeks old). The nude mice were purchased from National Laboratory Animal Center, Taipei, Taiwan and were housed under pathogen-free conditions according to Chang Gung University animal care guidelines. All animal experiments were reviewed and approved by the Institutional Animal Care Committee at Chang Gung University. On day 7 as the size of BCCs, melanomas, and colon carcinoma grows to approximately 150, 250, and 300 mm^3, respectively, 50 μl of liposomes was intratumorally injected with a 29-gauge needle. Tumor volume was determined by direct measurement with calipers. The tumor volume was calculated using the formula $w^2 \times l \times \pi/6$, where the length ($l$) is the longest dimension and the width (w) is the dimension perpendicular to the length. At 24 h later, the tumor sites were cut away and weighed.

2.7. Extraction of drug from the tumors

After excising a tumor, it was weighed and minced with scissors, positioned in a glass homogenizer containing 1 ml of 0.1 N HCl, and ground for 5 min

with an electric stirrer. The resulting solution was centrifuged for 10 min at 10,000 rpm and then filtered through a PVDF membrane (with a pore size of 0.45 μm, Millipore, USA). The drug amount in the supernatant was determined by HPLC.

2.8. Cytotoxicity assay

BCC cells were seeded at an initial concentration of 3×10^4 cells/well in 24-well culture plates, and incubated in medium (RPMI 1640 supplemented with 10% FCS). Twenty microliters of liposomes with or without drug diluted with medium was added at 24 h post-inoculation, and plates were incubated in a 5% CO_2 atmosphere at 37 °C for 24 h. After PBS wash, cells were incubated with 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) in RPMI 1640 for 2 h at 37 °C. Formozan crystals resulting from MTT reduction were dissolved by adding 200 μl DMSO and gently agitated for 30 min. The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 550 nm. Cell viability was calculated as the percentage of the control.

2.9. Statistical analysis

Statistical analysis of differences between different treatments was performed using unpaired Student's *t*-test. A 0.05 level of probability was taken as the minimal level of significance.

3. Results

3.1. Size, zeta potential, and drug entrapment of liposomes

The sizes and zeta potentials of the prepared liposomes are shown in Table 1. The liposomes made from EPC and CH in the presence of ethanol (F1) had a relatively small size of 133.1 nm. The further addition of DA (F2) significantly increased ($p < 0.05$) the size of the vesicles. The conventional liposomes without ethanol also produced relatively small vesicles. There were almost no surface charges on F1 because of the negligible charges of phosphatidylcholine, the major component of EPC [17]. The addition of DA (F2), an anionic surfactant, resulted in an increase in the negative charges on the vesicle surface. The absolute zeta potential of F3 vesicles showed a value of −12.1 mV.

EGCG showed a high rate of encapsulation of nearly 100% in liposomes incorporated with ethanol (F1 and F2, Table 1). However, this high trapping efficiency was not observed for conventional liposomes (F3). EGCG always exhibited higher encapsulation than did (+)-catechin or (−)-epicatechin. The encapsulation of both isomers with the same molecular weight in liposomes was similar for all formulations tested ($p > 0.05$).

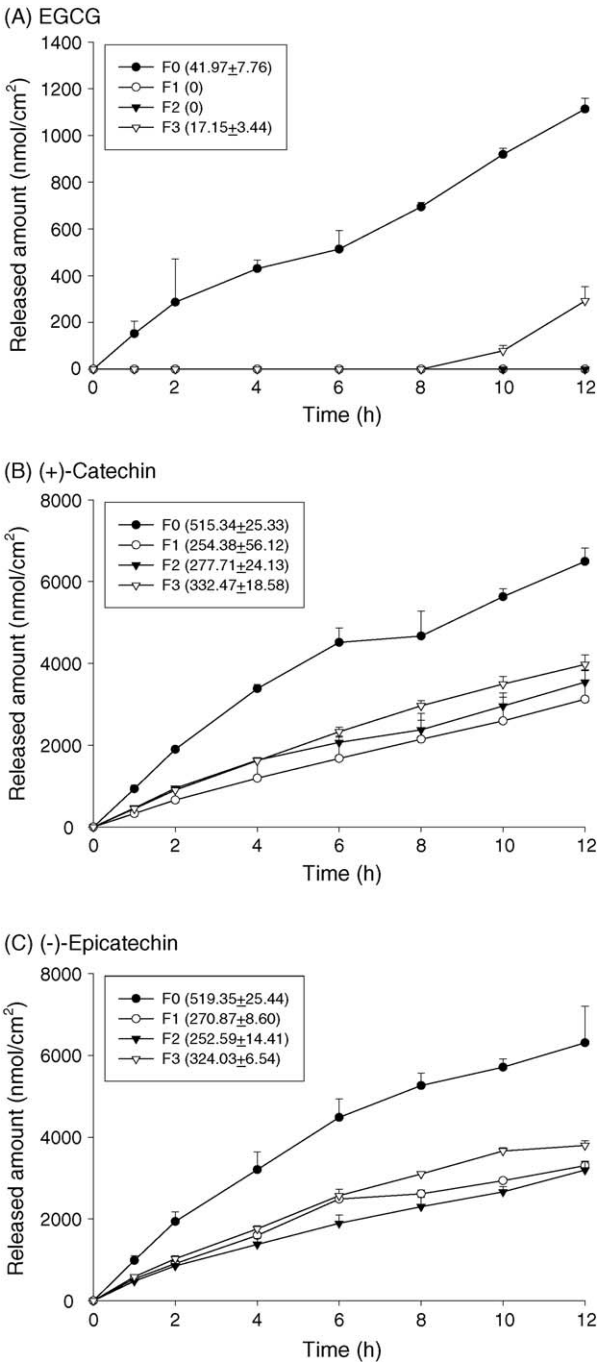

Fig. 1 Release of EGCG (A), (+)-catechin (B), and (−)-epicatechin (C) across a cellulose membrane from a hydroalcoholic solution and liposomes. Each value represents the mean and S.D. ($n = 4$).

Table 2 Tumor uptake of EGCG and (+)-catechin at 24 h from a 15% ethanol solution and various liposome systems after intratumor injection into a basal cell carcinoma

Code	Tumor uptake (nmol of drug/g of tumor)	
	EGCG	(+)-Catechin
F0[a]	2.80 ± 2.02	0.05 ± 0.02
F1	39.26 ± 12.80	27.33 ± 6.75
F2	56.34 ± 9.48	62.71 ± 20.86
F3	6.31 ± 3.45	0.13 ± 0.05
F4	23.57 ± 10.70	1.49 ± 0.45

Each value represents the mean ± S.D. ($n = 6$).
[a] The formulation of code 0 means EGCG or (+)-catechin in a 15% ethanol solution (control group).

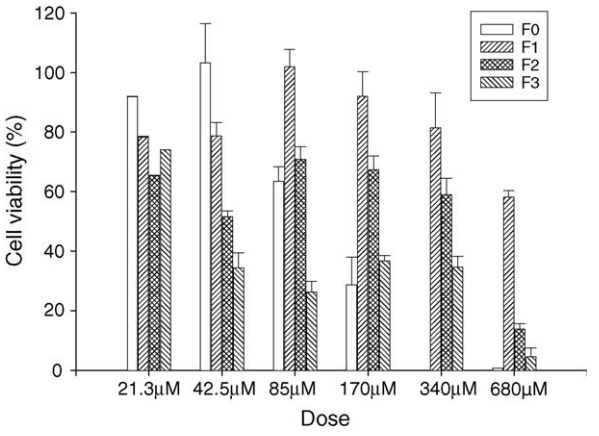

Fig. 2 Viability of basal cell carcinomas following treatment with EGCG in a hydroalcoholic solution or liposomes at various concentrations. Each value represents the mean and S.D. ($n = 6$).

3.2. In vitro release

The released amount of drugs from each liposomal system was plotted as a function of time. The slope of the resulting linear plots was calculated, and the release rate (nmol/cm^2/h) was calculated from the slopes as shown in Fig. 1. The release of all drugs from the hydroalcoholic solution (15% ethanol in water, F0) was significantly higher ($p < 0.05$) than those determined from all of the liposome formulations. The liposomes without ethanol (F3) showed faster diffusion of drugs from the vesicles. There is no or negligible EGCG amounts detected in the receptor phase following the application of liposomes with ethanol (F1 and F2) during a 12 h administration duration.

3.3. In vivo intratumor administration

EGCG and (+)-catechin incorporated with or without liposomes were intratumorally injected into BCCs on the back of nude mice to examine drug deposition. (−)-Epicatechin was excluded in this study because of the similarity of the physicochemical characteristics and release rates between the two isomers (Table 1 and Fig. 1). Table 2 shows the retention of EGCG and (+)-catechin in the BCCs at 24 h after injection. The accumulation of free EGCG and (+)-catechin (F0) was low. Drugs incorporated in liposomes exhibited greater deposition in BCCs, with F2 showing the highest value ($p < 0.05$, Table 2). Liposomes without ethanol (F3) showed the lowest drug deposition among all liposomes examined. Table 2 also demonstrates that the tumor deposition of drugs was significantly reduced ($p < 0.05$) after extruding F2 liposomes (F4).

In addition to BCCs, F2 was also selected for injection into melanomas and colon tumors to examine the tumor deposition of drugs. As shown in Table 3, liposomes with large size can greatly enhanced EGCG deposition in both melanomas and colon tumors. The same effect was observed with liposome-encapsulated (+)-catechin. This indicates the same trend of the effect of liposomes for various solid tumors.

3.4. Cytotoxicity assay

The effects of different concentrations of liposomes (expressed as the EGCG concentration in all cases) on the viability of BCCs studied after a 24 h incubation period, are presented in Fig. 2. The cytotoxicity generally showed a concentration-dependent biphasic effect. At concentrations lower than or higher than 42.5 μM, an inhibitory activity on cell proliferation

Table 3 Tumor uptake (nmol of drug/g of tumor) of EGCG and (+)-catechin at 24 h from a 15% ethanol solution and F2 liposomes after intratumor injection into melanoma or colon tumor

Tumor	Formulation	EGCG	(+)-Catechin
Melanoma (B16-F0)	F0[a]	0.04 ± 0.12	0.49 ± 0.18
	F2	162.56 ± 66.93	13.94 ± 4.26
Colon (HT-29)	F0[a]	7.84 ± 4.35	0.48 ± 0.24
	F2	545.78 ± 174.08	123.88 ± 39.24

Each value represents the mean ± S.D. ($n = 6$).
[a] The formulation of code 0 means EGCG or (+)-catechin in a 15% ethanol solution (control group).

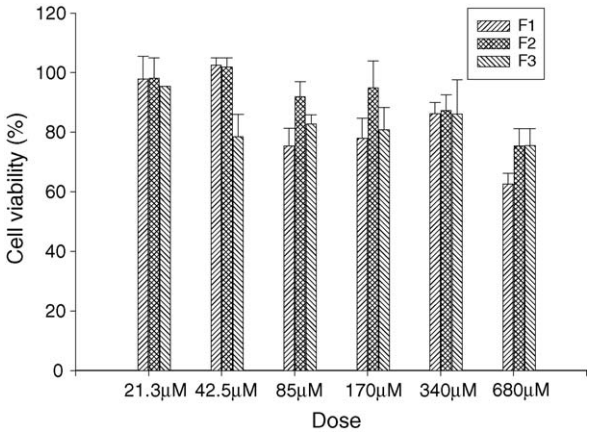

Fig. 3 Viability of basal cell carcinomas following treatment with a hydroalcoholic solution or liposomes without EGCG at various concentrations. Each value represents the mean and S.D. ($n = 6$).

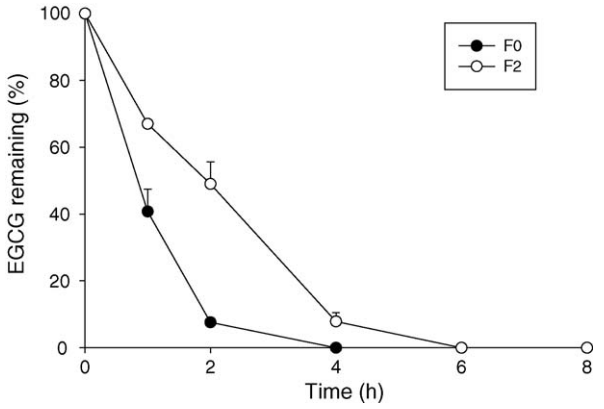

Fig. 4 EGCG remaining in a hydroalcoholic solution or liposomes following incubation for various times in the same medium used for the MTT assay at 37 °C. Each value represents the mean and S.D. ($n = 3$).

appeared for the free EGCG formulation (F0). The antiproliferative activity was negligible for an EGCG dose of 42.5 μM. A similar trend was observed for F1 and F2 liposomes, which showed significant cytotoxicity at concentrations lower than 85 μM and higher than 170 μM. Blank liposomes without EGCG were also used to perform the MTT assay. When the effects of different phospholipid concentrations tested on viability of BCCs were compared, it is clear from Fig. 3 that in most cases except at the highest concentration, the vesicles themselves had no effect on the cytotoxicity. The MTT assay of melanoma and colon tumor was also performed by treating F0 and F2 with EGCG concentration at 42.5 μM as shown in Table 4. Similar to the results of BCCs, the liposomes contributed to higher suppression of cell growth as compared to free EGCG formulation.

EGCG in hydroalcoholic solution and in F2 liposomes was incubated in the medium the same as with the MTT assay, and the residual amounts of EGCG were determined by HPLC as a function of time at 37 °C (Fig. 4). The EGCG remaining percentage is calculated as the residual EGCG amount in medium after incubation divides by EGCG amount in medium at time zero. F2 liposomes loaded with EGCG were quite stable, showing that ~50% of the EGCG still remained at 2 h, while EGCG in the hydroalcoholic solution was unstable, with only ~8% of the EGCG remaining. Free EGCG had been completely degraded by 4 h after incubation.

Table 4 Viability (%) of melanoma or colon tumor following treatment with hydroalcoholic solution (F0) or F2 liposomes with EGCG at 42.5 μM

Tumor	F0	F2
Melanoma (B16-F0)	103.61 ± 7.17	89.02 ± 5.38
Colon (HT-29)	92.36 ± 4.41	76.23 ± 6.83

Each value represents the mean ± S.D. ($n = 6$).

4. Discussion

In contrast to conventional liposomes, vesicles prepared from an ethanolic solution of phospholipids were shown to exhibit high encapsulation efficiency for both hydrophilic and lipophilic drugs [18,19]. The so-called ethosomes contain vesicles with interdigitated fluid bilayers. A 15% ethanol was incorporated in the liposomal systems in this study. The results suggest that ethanol in these liposomal systems can increase the entrapment of EGCG. The encapsulation of (+)-catechin and (−)-epicatechin by liposomes was always lower than that of EGCG. EGCG contains a galloyl group which is absent from the other two catechins. The *n*-octanol/water partitioning coefficient indicates greater lipophilicity of EGCG (16.0) compared to (−)-epicatechin (1.4) [20]. EGCG may strongly locate to the surface of the phospholipids bilayers, resulting in high entrapment within liposomes. The interdigitated fluid membrane of ethanol-containing liposomes may be beneficial to this interaction between EGCG and the bilayers.

(+)-Catechin and (−)-epicatechin showed different manners of liposome entrapment as compared to EGCG. The trapping efficiency of a water-soluble molecule may be reduced by the lack of interactions with the bilayers. The probability of two isomers being incorporated into the aqueous cores inside the bilayers is similar. The incorporation of DA-containing and Tween 80-containing liposomes resulted in a dramatic increase in (+)-catechin and (−)-epicatechin encapsulation (F1 versus F2, $p < 0.05$). Drug

entrapment in conventional liposomes (F3) was comparable to that of DA-containing liposomes (F2 versus F3, $p > 0.05$). Drug leakage from liposomes is often involved in the aggregation or fusion of liposomal membranes [21]. The high surface potential of liposomes (F2 and F3) tends to increase the interbilayer distance owing to electrostatic repulsive forces. This may reduce the leakage of (+)-catechin and (−)-epicatechin from the aqueous cores of liposomes.

For development of liposomes encapsulated with antitumor agents in an in vivo status, it is important to optimize the ability of drug released from vesicles. The pore size of the permeated membrane used was below 10 nm, and free molecules were able to permeate across the membrane. Drug release rates from liposomes were much lower than those determined from the hydroalcoholic solution (control). This may suggest that drugs do not diffuse freely when entrapped in the vesicles. No or negligible EGCG release from liposomes was detected. This may be due to the high entrapment and strong interaction of EGCG within the liposomal bilayers.

Relatively low accumulation in BCCs was achieved with EGCG and (+)-catechin in a hydroalcoholic solution. It is difficult to obtain the desired therapeutic results without side effects by maintaining an effective drug concentration in tumor cells, because the free drug with a small molecular mass rapidly diffuses away from the site of injection. Liposomes with DA and ethanol (F2) showed the greatest ability to enhance local retention of EGCG and (+)-catechin in BCCs. In tumors, the permeability of the capillary endothelium is enhanced compared to that of normal tissues [22]. F2 had the largest structure with an average diameter of 378.2 nm compared to the other formulations. Liposomes with a greater size may be more-readily trapped by the fiber network, and the leakiness of capillary walls may be insufficient to allow their efflux from the interstitial into the vascular space. This result indicates that vesicle size is an important determinant of the retention of these liposomes in the tumor.

The smallest size of F3 may have allowed those liposomal vesicles quickly escape from the tumor interstitium into the vascular space. Another possibility is that free EGCG and (+)-catechin are easily released from liposomes without ethanol (F3, Fig. 1), leading to the invalidity of liposomes for drug delivery in this case. In order to further explore the influence of vesicle size on the tumor uptake of drugs, F2 was extruded from a polycarbonate membrane with a pore size of 200 nm (F4). As shown in Table 1, no difference ($p > 0.05$) was found in encapsulation before or after extrusion. The result of tumor uptake by F4 confirms the importance of vesicle size on drug deposition. The intratumor distribution of F4 was also lower than that of F1, although the vesicle size of F4 (215.8 nm) was larger than that of F1 (131.1 nm). This finding indicates that liposome accumulation by tumors is not always correlated with liposome size. In addition to size, zeta potential and liposomal composition may also play crucial roles in determining tumor uptake. Since the vesicle surface of F4 was highly ionized to a negative charge, the negatively charged proteoglycans in the tumor interstitium may repel F4 liposomes and increase the outflow of F4 from the interstitium to the capillaries [11]. Another explanation is the effect of DA on the phospholipid bilayers. A previous report has suggested that liposomes with rigid bilayers show superior tumor accumulation [23]. The inclusion of DA in the liposomes with ethanol further reduced the rigidity of the bilayers [24], resulting in lower tumor uptake compared to liposomes without DA.

Although F3 showed a rigid structure in the bilayers because of the lack of ethanol and DA, its ability to retain drugs within BCCs was low. This suggests that the size of and drug release from liposomes, rather than bilayer rigidity, still predominate tumor accumulation. The presence of ethanol may also play an important role. The incorporation of ethanol allows the formation of interdigitated and malleable vesicles.

This characteristic of liposomes would accelerate the fusion of vesicles with membranes of fibroblasts (3T3) and delivery of vesicular components together with encapsulation of molecules inside the cell [18].

In addition to BCCs, EGCG has been reported to be beneficial in treating melanomas and colon cancer [25,26]. F2 liposomes were selected to examine the intratumor EGCG amounts in melanomas and colon tumors. The results indicated that liposomes with ethanol and DA have a widespread utility in various solid tumors. The EGCG uptake in melanomas was especially low with injection of the free form (F0, Table 3). This may be due to that melanoma has a vasculature that is characterized by the presence of blood channels and which lacks a well-defined intratumor matrix [27]. The use of liposomes may significantly improve this easy escape of free drug from melanomas. The EGCG deposition in melanoma and colon adenocarcinoma was higher than that in BCCs in the liposomal form (Tables 2 and 3). Melanoma and colon tumor showed an abundant vasculature around the tumor [28,29], contributing to a higher percentage of capillary volume to the whole tumor. The nodular BCCs always lack vasculature. Liposomes-encapsulated EGCG may locate in the interstitial part of the tumor, resulting in the more

concentrated EGCG in the interstitial space of melanoma and colon tumor. Another possible reason may be that BCCs provided higher amounts of protein or enzyme to attack the lipid bilayers of liposomes. The real mechanisms of this effect were not exactly known in the present study and need to be further explored.

BCCs showed similar uptake to liposomes-encapsulated EGCG and (+)-catechin (Table 2). However, the deposition of liposomes-encapsulated EGCG in melanoma and colon tumor was much higher than that of (+)-catechin (Table 3). It can be explained by the higher vasculature system in both tumors. As observed in Table 1, the entrapment percentage of EGCG in F2 liposomes (nearly 100%) is higher than that of (+)-catechin by a two-fold. The drugs in free form may easily escape from tumors by passing through the capillary endothelium, resulting in the greater loss of (+)-catechin as compared to EGCG from tumors with an abundant vasculature.

The intermediate EGCG doses tested in this study did not exhibit a crucial cytotoxic effect towards BCCs. This phenomenon was shown by both the free form and liposomal formulations. The presence of ethanol may have produced this biphasic effect. A previous study reported that ethanol exhibits an antioxidative capacity at low concentrations, while at higher concentrations this effect is reversed [30]. As a result, the cytotoxicity due to EGCG at some concentrations may be partly offset by this particular characteristic of ethanol. The vesicles themselves generally had no effect on the cytotoxicity based on the data in Fig. 3. This indicates that the cytotoxicity toward BCCs was mainly a consequence of the EGCG molecules. It is noticeable that the liposomal systems showed higher antiproliferative activities than the free form ($p < 0.05$) at lower doses (21.3 and 42.5 μM). Not only the BCCs, melanoma and colon cancer cell growth was significantly inhibited by F2 liposomes encapsulated with EGCG at 42.5 μM. It is noteworthy that free EGCG (F0) at 42.5 μM could not induce cell viability of the three cell lines examined here. The stability of EGCG is susceptible to body fluids such as serum [10]. The encapsulation of EGCG by liposomes may protect the drug from oxidation and degradation. As shown in Fig. 4, free EGCG was quickly degraded in a 10% FCS medium. Liposomes provided stable retention of the drug within the vesicles, contributing to the successful anticancer activity of EGCG at lower concentrations. EGCG in liposomes exhibited its cytotoxicity by both direct fusion of vesicles with cells and release from vesicles after a stable retention.

Free EGCG (F0) showed stronger cytotoxicity toward BCCs compared to liposomes at higher concentrations (>85 μM). Although EGCG was not stable in the medium, a large amount of EGCG molecules in the formulations with higher doses still existed after degradation. These intact molecules may have rapidly entered tumor cells to generate the subsequent cell death. The slower leakage of EGCG from liposomes limited the availability of free EGCG. This result may explain the relatively lower viability of BCCs treated by F3 than by the other liposomes, since liposomes without ethanol quickly released the encapsulated EGCG (Fig. 1).

5. Conclusions

The purpose of this study was attempted to assess the feasibility of using liposomes to promote the tumor accumulation of EGCG and its derivatives. The incorporation of DA in the liposomes in the presence of 15% ethanol greatly increased EGCG uptake by the tumor. The key to this enhancement may be due to the vesicle size. Larger vesicles are more-readily trapped in tumors by the fiber network, and the leakiness of capillary walls is insufficient to allow vesicle efflux from the interstitial space. The intratumor distribution of liposomes may also be mediated on the basis of surface charges and the flexibility of the bilayers. Liposomes induced cell death of BCCs more effectively than did the hydroalcoholic solution at lower EGCG doses. This was due to the ability of liposomes to maintain EGCG stability. Therefore the incorporation of liposomes may reduce the drug dose required to avoid side effects evoked by the drugs. From the data presented in this study, liposome delivery systems showed that they can serve as effective tea catechins carriers after moderate formulation design. It was also shown that liposomes were able to provide higher EGCG accumulation within BCCs, and to increase the stability of EGCG inside the vesicles.

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