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Polyethylene glycol-coated liposomes for oral delivery of recombinant human epidermal growth factor

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Abstract

The present study was to investigate the feasibility of oral delivery of recombinant human epidermal growth factor (rhEGF). Polyethylene glycol (PEG)-coated liposomes containing rhEGF was prepared and evaluated for their stability and permeability in Caco-2 cells. In the animal study, we also determined plasma concentration and gastric ulcer healing effect after oral administration of rhEGF liposomes or the solution. Encapsulation of rhEGF into liposomes, suppressed the degradation in Caco-2 cell homogenate compared with the solution. The flux of rhEGF from dipalmitoylphosphatidylcholine (DPPC) liposome across Caco-2 cell monolayer from the apical to basolateral side was three times greater than that from phosphatidylcholine (PC) liposome or the solution. After oral administration of rhEGF liposomes or the solution in rats, the area under the concentration–time curve (AUC) of rhEGF increased 1.7- and 2.5-fold for PC and DPPC liposomes, respectively. The gastric ulcer healing effect was significantly increased in DPPC liposome compared with PC liposome and the solution. The enhanced curative ratio of rhEGF encapsulated into DPPC liposome may be due to the resistance to enzyme degradation, higher permeability and increased plasma AUC. Therefore, PEG-coated liposomes containing rhEGF could be used as an oral delivery formulation with enhanced encapsulation efficiency.

Keywords: rhEGF; Liposome; DPPC; PEG-coated; Caco-2; Oral delivery

1. Introduction

Human epidermal growth factor (rhEGF) is a single-chain polypeptide containing 53 amino acid residues (MW = 6045) and three disulfide bridges (Senderoff et al., 1994). rhEGF stimulates the proliferation and differentiation of epithelial tissues, such as the intestinal mucosa, corneal epithelial tissue, lung and trachea epithelial (Carpenter and Cohen, 1979). Moreover, rhEGF also inhibits gastric acid secretion (Bower et al., 1975; Elder et al., 1975; Gregory, 1975; Konturek et al., 1981b) and protect gastroduodenal mucosa against tissue injury induced by ulcergenic agents (Konturek et al., 1981a; Kirkegaard et al., 1983). We have reported that oral bioadhesive gels containing rhEGF was effective against induced acute and chronic gastric ulcers in rat, but the healing efficacy was not complete (Han et al., 1998b). When rhEGF was orally administered, the bioavailability is very low (1–5%) because of its liability to enzyme and poor gastrointestinal membrane transport ability (Han et al., 1998a). Therefore, most of peptides and proteins including rhEGF, can be mostly administered by the injection formulation. In order to enhance their

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oral bioavailability, many strategies have been tested, such as a co-administration of absorption enhancers which increase the permeability of peptides through the intestinal membrane (Morishita et al., 1993; Hovgaard et al., 1995), a synthesis of their stable derivatives (Bruce and Susanna, 1995) and the development of special dosage forms by utilizing the micro- or nano-particles as a drug carriers (Desai et al., 1996; Jenkins et al., 1994; Takeuchi et al., 2001). However, absorption enhancers showed several safety problems in their clinical use. The bioactivity of peptides might be reduced due to the derivatization process. On the contrary, a drug carrier system has many advantages. First of all, it is possible to control the release rate of peptides from particles after oral administration. Liposomes are one of the most potent candidates for such carrier systems. However, the efficacy of liposomes as an oral delivery system for peptide drugs has not yet been established. Many researchers have investigated the stability of liposomes in the GI tract (Iwanaga et al., 1997, 1999; Moribe et al., 1999). Liposomes coated with polyethylene glycol 2000 or the sugar chain of mucin found to be resistant to their digestion by bile salts and were useful for an oral delivery system for peptide drugs (Iwanaga et al., 1997, 1999). Furthermore, liposomes with polyethylene glycol (PEG)-derivatized lipid have been reported to increase encapsulation efficiency of nystatin (Moribe et al., 1999).

In this study, we prepared PEG-coated liposomes containing rhEGF and evaluated for their stability and the permeation of rhEGF across Caco-2 cells. Furthermore, we also investigated the in vivo absorption and gastric ulcer healing effect after oral administration of rhEGF liposomes to examine the feasibility of PEG-coated liposomes as an oral delivery system of peptides.

2. Materials and methods

2.1. Materials

rhEGF was supplied by Daewoong Pharm. Co. (Seoul, South Korea). The human EGF Quantikine® kit was purchased from R&D systems (Minneapolis, MN, USA). A human colonic epithelial cell line, Caco-2 was obtained from the American Type Culture Collection (Rockville, MD). Phosphatidylcholine (PC), dipalmitoylphosphatidylcholine (DPPC), and dioleoylphosphatidylethanolamine–polyethylene glycol 2000 (DOPE–PEG) were purchased from Avanti polar lipid Inc. (Alabaster, AL, USA). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Trypsin–EDTA was purchased from Gibco Laboratories (Gaithersburg, MD). Dulbecco's modified Eagle's medium, non-essential amino acid solution, L-glutamine, penicillin–streptomycin, HBSS, HEPES, MES and cholesterol (Chol) were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

2.2. Preparation of rhEGF liposomes

The liposomes were prepared according to the method of Bangham (Bangham et al., 1974) with some modifications. Briefly, a film was obtained by rotary evaporation of chloroform solution containing PC or DPPC, Chol and DOPE–PEG at a different molar ratio. The lipid film was flushed with nitrogen for at least 30 min and then hydrated with rhEGF/HBSS buffer (pH 6.5). The liposomes were then extruded through the 0.4 and 0.2 μm polycarbonate membrane filter (Nucleopore®; Costar, MA, USA) and stored at 4 °C. The final lipid concentration was adjusted to 25 mM.

2.3. Liposome characterization

The encapsulation efficiency was calculated from untrapped and total rhEGF amount in liposome. The liposome suspension was centrifuged at 100,000 × *g* for 2 h at 4 °C and untrapped rhEGF was separated. The total amount of rhEGF in liposome was completely lyzed with 5% Triton X-100. Untrapped and total rhEGF amount was measured by HPLC and the encapsulation efficiency (EE) was calculated from the following equation:

$$\mathrm{EE}\ (\%) = \frac{\text{Total rhEFG amount} - \text{Untrapped rhEGF amount}}{\text{Total rhEGF amount in liposome}} \times 100\%$$

The size of rhEGF liposome was determined using a NICOMP 370 Submicron Particle Analyzer (Pacific Scientific, CA, USA). The measurement was conducted in duplicate, and the mean values were used.

2.4. Caco-2 cell culture

Caco-2 cells (passage 36–45) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% non-essential amino acid solution, 100 units/ml penicillin and 0.1 mg/ml streptomycin at 37 °C and 90% relative humidity in an atmosphere containing 5% CO_2. For the transport experiments, cells were grown on a permeable polycarbonate insert (1 cm^2, 0.4 μm pore size: Corning Costar Corp., Cambridge, MA) in 12-Transwell plates, and the medium was changed every 2 days. The integrity of cell monolayers was confirmed by measuring transepithelial electrical resistance (TEER) before the transport study. The TEER value of Caco-2 cell monolyers reached 300–600 Ω cm^2 at 18–25 days of culture.

2.5. Bioactivity assay

The growth stimulating bioactivity of rhEGF in liposome was determined by MTT colorimetric assay (Sheng et al., 1996). Caco-2 cells were seeded into 96-well Corning Costar polystyrene tissue culture plates (5000 cells per well) with 100 μl culture medium. After 3 days, the cells were switched to serum-free culture medium with rhEGF solution or liposomes. The cells were incubated for 72 h and performed MTT colorimetric assay. Briefly, the medium with rhEGF was changed to 100 μl fresh culture medium containing 10 μl of MTT solution (5 mg/ml in PBS) and returned to the incubator. After a 4 h incubation, 100 μl of acid–isopropanol (0.04N HCl in isopropanol) was added to all wells. The color changes were recorded on a microplate reader at 540 nm.

2.6. rhEGF degradation in Caco-2 cell homogenate

Degradation of rhEGF in Caco-2 cell homogenate was measured by incubating cell homogenate with the solution or liposomes. Cell homogenate was prepared by the method of Annaert et al. (1997) with some modification. Cells grown 14 days in three T-flask were scraped and collected in 5 ml of ice-cold transport medium (pH 6.5) and then homogenized. The homogenate was centrifuged at 8000 × g for 10 min at 4 °C and the supernatant was used as the cell homogenate (protein concentration = 1 mg/ml). The liposomes or solution (0.2 ml) was added to the same volume of the cell homogenate and incubated at 37 °C. At the predetermined time, 50% of acetic acid was added to each sample in order to terminate the reaction. After the addition of 5% reduced Triton X-100, the sample was centrifuged at 4000 rpm for 15 min. The concentration of rhEGF in each sample was determined by HPLC.

2.7. Measurement of transepithelial transport

Transepithelial transport were performed in HBSS buffer supplemented with 20 mM of glucose, 9 mM of sodium bicarbonate, and 25 mM of HEPES (pH 7.4). The rhEGF solution or liposomes (0.5 ml) was added to the apical side of the monolayers and the incubation medium (1.5 ml) was added to the basolateral side. The monolayers were incubated for a specified period of time at 37 °C, samples were withdrawn from the basolateral side. The concentration of rhEGF was determined by ELISA method using a Quantikine® kit. Apparent permeability coefficients, P_{app} (cm/s) were calculated according to

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{ACo}$$

where dQ/dt is the permeability rate, Co is the initial concentration in the donor chamber and A is the surface area of the monolayer.

2.8. In vivo oral administration experiment

Male Sprague–Dawley rats (270–300 g) were purchased from Samyook Animal Company (Kyonggi-do, Korea) and housed for at least 2 days. Before the experiment, SD rats were fasted for 18–24 h. Under pentobarbital anesthesia, the femoral artery and vein of rats were cannulated with polyethylene tubing (PE-50) for blood sampling and saline supplement, respectively. rhEGF solution or liposomes was administered orally (1.4 mg/kg) to rats and 300 μl of blood was collected at 0, 2, 4, 7, 10, 20, 30, 45, 60, 90 min. Blood samples were immediately centrifuged at 3000 rpm for 15 min and the plasma was collected and stored at −20 °C. The plasma concentration of rhEGF was determined by ELISA method using Quantikine® kit.

2.9. Gastric ulcer healing test

Acute gastric ulcer healing test was investigated in rats (230–250 g) after inducing acute gastric ulcers by ethanol (Han et al., 1998b). After a 24 h fast, ethanol was instilled in a volume of 1 ml into the stomach of rats to induce acute gastric ulcers. After 4, 10, 24 h following the induction of ulcers, the rats as a test group were instilled orally (400 μg/kg) with PC or DPPC liposome, the rats as control group were instilled with rhEGF solution and the rats with no treatment were left as a normal group. At 30 h after the induction of ulcers, the rats were killed, and the stomach was removed and then opened along the greater curvature. In order to evaluate the degree of gastric mucosal injury, the length of ulcerated mucosa was measured (in mm) and the curative ratio was calculated from ulcer length using following equation:

$$\text{Curative ratio} = \frac{\text{Length of ulcer without treatment} - \text{Length of ulcer after treatment}}{\text{Length of ulcer without treatment}} \times 100\%$$

2.10. Determination of rhEGF

The encapsulation efficiency and remaining of rhEGF in Caco-2 homogenate were determined by HPLC. The HPLC system was composed of a model PU-980 pump, a model UV-975 UV-Vis detector, a model LC-Net II control borwin integrator and a model AS-950-10 autoinjector. Samples were injected into a 20 μl sample loop. Separation was achieved using a 10 μm reversed phase C_{18} column (Vydac; 2.5 mm × 250 mm, 10 μm). The mobile phase was composed of acetonitrile, triethylamine and water (pH 6.5) in 250:2.2:850 (v/v/v) ratio. The flow rate was 1 ml/min and detection was monitored at 214 nm.

3. Results and discussion

3.1. Effect of lipid components on the encapsulation efficiency and particle size

The encapsulation of a water-soluble proteins and peptides in liposomes is commonly correlated with the lipid composition and encapsulated solution. Thus, given the high cost of rhEGF, it would be important to elucidate the impact of each lipid component on the encapsulation efficiency. The lipid compositions of various liposomes tested are shown in Table 1. PEG derivatized lipids influenced the encapsulation efficiency. As the lipid molar ratio of DOPE–PEG increased, the encapsulation efficiency of rhEGF increased significantly. It is consistent with the DSPE–PEG increased the encapsulation of amphotericin B into liposome (Moribe et al., 1998). The mechanism has been suggested to involve complex formation between amphotericin B and DSPE–PEG (Moribe et al., 1998). Thus, the fact that DOPE–PEG increased encapsulation efficiency of rhEGF may be explained by the complex formation between rhEGF and DOPE–PEG. Other lipid components of liposomes, Chol also increased the encapsulation efficiency of rhEGF. Chol is known to increase the rigidity of the liposomal membrane. Thus, it suggests that the encapsulation enhancing effect of Chol might have resulted from the reduced release of rhEGF from more rigid liposomes containing Chol (Kim et al., 1999). Taken together, from the investigations on the encapsulation efficiency, PC:Chol:DOPE–PEG (10:5:1) liposome was selected. In the DPPC/Chol/DOPE–PEG (10:5:1) liposome, encapsulation efficiency was similar to the PC/Chol/DOPE–PEG liposome. On the other hand, in the absence of DOPE–PEG, encapsulation efficiency was significantly decreased for both PC and DPPC liposomes.

In addition, we also investigated the effect of lipid composition on the size distribution. The results summarized in Table 1. In all cases, particle size was not changed significantly and it might be due to the presence of DOPE–PEG. Although the mechanism by which DOPE–PEG influenced the size distribution of liposome is not clear, it is possible that DOPE–PEG increased the polarity of the liposomal surface, preventing the aggregation of extruded liposome.

3.2. Effect of rhEGF concentration on the encapsulation efficiency

The concentration of rhEGF also affected encapsulation efficiency. As shown in Fig. 1, as rhEGF concentration increased from 0.3 to 0.5 mg/ml, the encapsulation of rhEGF was significantly increased. However, the encapsulation efficiency slightly

Table 1
Encapsulation efficiency (EE, %) and particle size of rhEGF in liposomes

Liposome composition	rhEGF concentration (mg/ml)	EE[a] (%)	Size[b] (nm)
PC:Chol:DOPE–PEG (10:5:0.5)	0.3	5.1 ± 0.1	251.7
PC:Chol:DOPE–PEG (10:5:0.8)	0.3	6.6 ± 0.9	271.4
PC:Chol:DOPE–PEG (10:5:1.0)	0.3	9.1 ± 0.9	281.2
PC:Chol:DOPE–PEG (10:2.5:1.0)	0.3	7.3 ± 0.7	280.4
PC:Chol:DOPE–PEG (10:5:1.0)	0.5	15.6 ± 1.7	289.9
PC:Chol (10:5)	0.5	8.3 ± 0.3	286.4
DPPC:Chol:DOPE–PEG (10:5:1.0)	0.5	18.5 ± 1.4	306.1
DPPC:Chol (10:5)	0.5	10.9 ± 0.5	301.7

[a] Mean ± S.D. of two preparation.
[b] Mean value of two preparation.

increased as rhEGF concentration increased from 0.5 to 1.5 mg/ml. Thus, considered the high cost of rhEGF, 0.5 mg/ml rhEGF was used to prepare liposomes.

3.3. Bioactivity assay

In order to investigate the bioactivity of encapsulated rhEGF, the growth stimulating activity was measured. In Caco-2 cells, rhEGF solution showed growth stimulating activity and maximal activity was 151.9 ± 27.4% of control (no treatment with rhEGF) at 10 ng/ml. In PC and DPPC liposomes, the growth stimulating activity of rhEGF at 10 ng/ml were 151.8 ± 11.7 and 165.7 ± 18.3% of control, respectively. The growth stimulating activity of rhEGF had no significant difference in the solution, PC and DPPC liposomes, indicating the encapsulated rhEGF into liposome had bioactivity similar to free rhEGF.

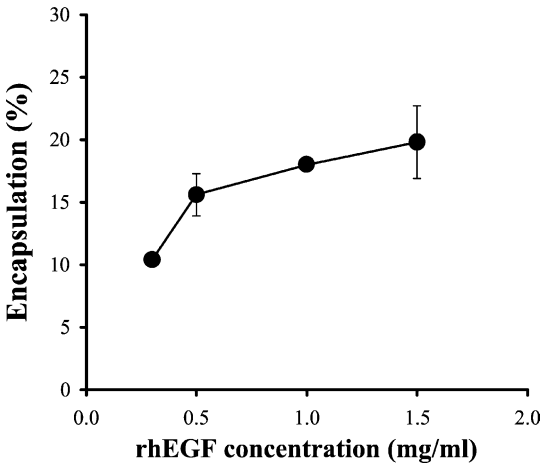

Fig. 1. Effect of rhEGF concentration on the encapsulation efficiency in PC:Chol:DOPE–PEG (10:5:1) liposomes.

3.4. rhEGF degradation in the Caco-2 cell homogenate

The ability of liposomes to prevent the enzymatic degradation of rhEGF in GI tract was investigated in vitro by using Caco-2 cell homogenate. As shown in Fig. 2, rhEGF degraded rapidly according to first-order kinetics in the Caco-2 cell homogenate. The apparent half-life of rhEGF was about 60 min, representing the rapid enzymatic hydrolysis of rhEGF. In rhEGF liposomes, about 25% of rhEGF were degraded after 60 min. In DPPC liposome, rhEGF was

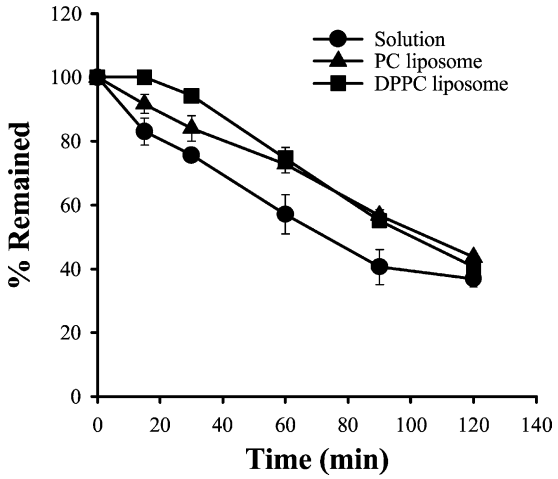

Fig. 2. The degradation of rhEGF in the Caco-2 cell homogenate. (●) Solution; (▲) PC liposome; (■) DPPC liposome. Data are expressed as the mean ± S.D. ($n = 3$).

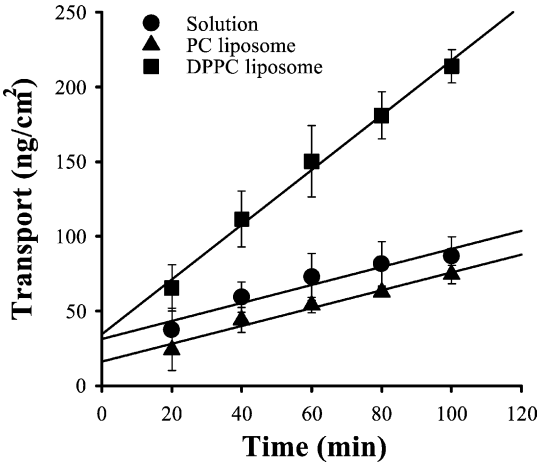

Fig. 3. The apical to basolateral transport of rhEGF across the Caco-2 cell monolayers. Data are expressed as the mean ± S.D. ($n = 3$).

not degraded for 30 min. These results indicated that encapsulation of rhEGF into liposome significantly suppressed the degradation of rhEGF in Caco-2 cell homogenate. However, after 120 min of incubation, rhEGF degraded about 60% in both cases, may be due to low encapsulation efficiency of rhEGF. Therefore, the improvement of encapsulation efficiency of rhEGF could completely suppress the degradation of rhEGF in GI tract.

3.5. rhEGF transport in Caco-2 cell monolayers

The development of liposomes for oral delivery of peptides aims at improving the stability of peptides in the GI tract and the absorption rate of peptides. Thus, the effect of liposomes for rhEGF transport in Caco-2 cell monolayers was investigated. A representative flux of rhEGF across the Caco-2 cell monolayers, when the drug in solution or liposomes was loaded on the apical side of the cell monolayers, is shown in Fig. 3. The flux was essentially linear for a period of up to 60 min. The flux of rhEGF in PC liposome was not significantly different from the flux of rhEGF in solution. Interestingly, the flux of rhEGF in DPPC liposome was three times greater than that the flux of rhEGF in solution. This suggested that DPPC liposome could be used for the improvement of rhEGF transport. However, the particle size and encapsulation efficiency showed no differences in PC and DPPC li-

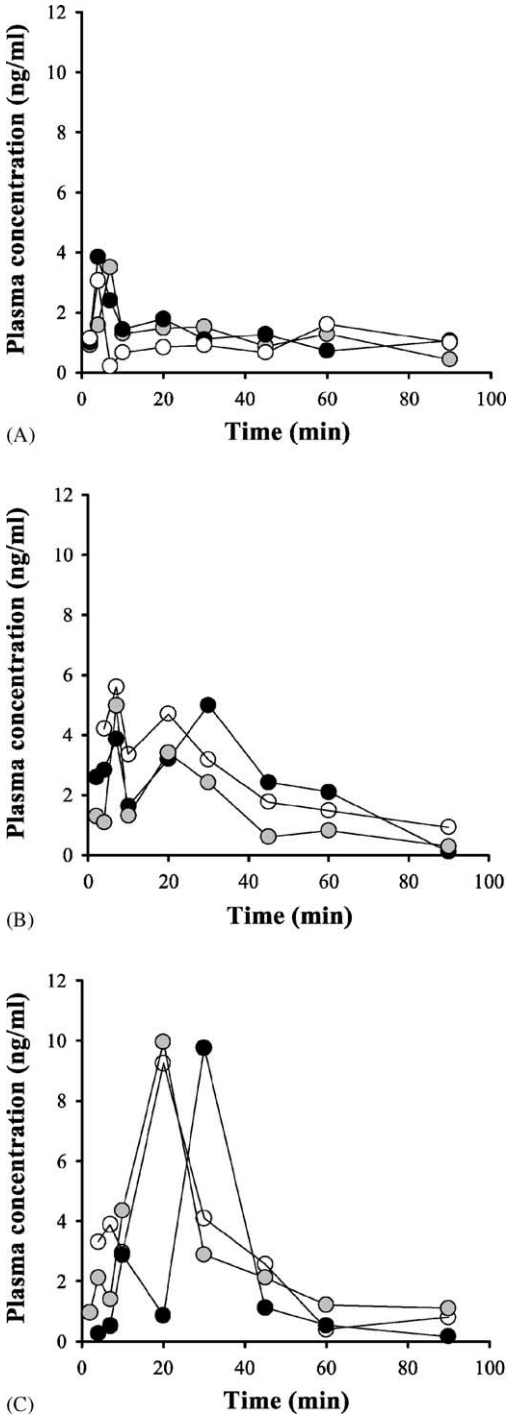

Fig. 4. The plasma concentration time profile of rhEGF after oral administration of solution or liposomes to rats. (A) Solution; (B), PC liposome; (C) DPPC liposome.

Table 2
Pharmacokinetic parameters of rhEGF after oral administration of solution or liposomes to rats (1.4 mg/kg)[a]

Parameters	Solution	PC liposome	DPPC liposome
C_{max} (ng/ml)	3.45 ± 0.37	5.19 ± 0.35	9.65 ± 0.37
T_{max} (min)	5.0 ± 1.7	14.6 ± 13.2	23.3 ± 5.8
AUC (ng/min ml)[b]	495.5 ± 23.0	879.5 ± 259.2	1238.8 ± 132.9

[a] Each data is expressed as mean ± S.E. ($n = 3$–4).
[b] Calculated up to 120 min.

posomes. Therefore, together with suppression of enzyme degradation, the increase of rhEGF permeability in DPPC liposome may be due to DPPC liposome maintained high rhEGF concentration in apical side of Caco-2 cell monolayers at the initial 60 min. However, in PC liposome, the apparent permeability was not increased. Thus, the effect of lipid composition on the rhEGF permeability was not negligible. A report using an isolated, perfused, intestinal-loop model has indicated that liposomes composed of higher phase transition (Tm) lipid are internalized to a greater extent than liposomes consisting of lower Tm lipids, although once intracellular absorption has occurred liposomes of high Tm lipids are more extensively metabolized (Anderson et al., 1999). Therefore, the increase of rhEGF permeability may be explained by the higher Tm composition in DPPC (41 °C) compared with PC (−7 °C). Although, the mechanism is not clear, it is possibly due to the suppression of enzyme degradation or the effect of lipid composition, such as DPPC.

3.6. Plasma concentration of rhEGF after oral administration

The plasma concentration of rhEGF after oral administration of solution or liposomes to rats are shown in Fig. 4 and some relevant parameters are listed in Table 2. After oral administration of rhEGF solution, the plasma concentration reached its peak at 5 min and declined thereafter. However, the plasma concentration of rhEGF exhibited double peak after oral administration of liposomes. Some pharmacokinetic parameters, such as the C_{max} and T_{max} were increased in liposomes compared with rhEGF solution. Area under the plasma concentration time curves ($AUC_{0–120}$) also increased in liposomes and DPPC liposome exhibited highest AUC compared with PC liposome and the solution. These results indicated that lipid composition affected oral bio-availability of rhEGF. Taken together with in vitro degradation and transport, the AUC of rhEGF from DPPC liposome increased 2.5-fold compared with the solution, this may be explained by suppression of enzyme degradation and improvement of permeability. The AUC increases by PC liposome may be due to the suppression of enzyme degradation.

The phenomenon of the multiple peaking profiles exhibited by the liposome is also reported by vancomycin liposome, suggested that preabsorptive phenomenon was possibly related to delayed gastric emptying (Anderson et al., 2001). In another study, Iwanaga et al. (1999) demonstrated that PEG-coated liposome interacted strongly with the intestinal mucous layer, leading to its slow transit in the intestine. Therefore, PC and DPPC liposomes exhibited multiple peaks may be due to the delayed gastric emptying by liposome systems.

3.7. Gastric ulcer healing effect

Gastric ulcer healing effect was determined by the measurement of the length of ulcerated mucous after

Table 3
Curative ratio of ethanol-induced gastric ulcers after oral administration of rhEGF solution and liposomes to rats at a dose of 400 μg/kg[a]

Group	Length of acute ulcer (mm)	Curative ratio (%)
No treatment	81.3 ± 10.8	–
Solution	65.2 ± 7.5	19.8 ± 12.0
PC liposome	60.1 ± 4.7*	26.1 ± 5.8
DPPC liposome	41.0 ± 9.0*	49.6 ± 11.1**

[a] rhEGF solution and rhEGF liposomes were orally administered to the rats after 4, 10, 24 h following the induction of ulcers. Each data is expressed as mean ± S.E. ($n = 4$–5).
* $P < 0.05$, significantly different from the no treatment group by Student's t-test.
** $P < 0.01$, significantly different from the solution group by Student's t-test.

oral administration of rhEGF liposomes or the solution. To test acute gastric ulcer healing, rhEGF solution was administered at a dose of 400 μg/kg to acute gastric ulcer rats as a control group, and PC and DPPC liposomes were administered with same dose as a test group. As shown in Table 3, the decline of ulcer length of the PC and DPPC liposomes were faster than that of the control group about 1.3- and 2.5-fold, respectively, indicated that the PC and DPPC liposomes were effective for acute gastric ulcer healing than rhEGF solution. This result is consistent with in vivo result that PC and DPPC liposomes increase the plasma AUC of rhEGF for 120 min.

4. Conclusion

PEG-coated liposomes have the potential for improving the GI stability and absorption of rhEGF. DPPC liposome seems to have advantages compared with PC liposome, due to its resistance to enzyme degradation, high permeability through biological membrane and acute ulcer healing effect. Moreover, it suggests that the DPPC liposome coated with PEG might have a potential as an oral delivery system for other protein and peptide drugs with improved encapsulation efficiency.

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