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Multivesicular Liposomes for Oral Delivery of Recombinant Human Epidermal Growth Factor

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The purpose of the present study was to prepare multivesicular liposomes with a high drug loading capacity and to investigate its potential applicability in the oral delivery of a peptide, human epidermal growth factor (rhEGF). The multivesicular liposomes containing rhEGF was prepared by a two-step water-in-oil-in-water double emulsification process. The loading efficiency was increased as rhEGF concentration increased from 1 to 5 mg/mL, reaching approximately 60 % at 5 mg/mL. Approximately 47% and 35% of rhEGF was released from the multivesicular liposomes within 6 h in simulated intra-gastric fluid (pH 1.2) and intra-intestinal fluid (pH 7.4), respectively. rhEGF-loaded multivesicular liposomes markedly suppressed the enzymatic degradation of the peptide in an incubation with the Caco-2 cell homogenate. However, the transport of rhEGF from the multivesicular liposomes to the basolateral side of Caco-2 cells was two times lower than that of the rhEGF in aqueous solution. The gastric ulcer healing effect of rhEGF-loaded multivesicular liposomes was significantly enhanced compared with that of rhEGF in aqueous solution; the healing effect of the liposomes was comparable to that of the cimetidine in rats. Collectively, these results indicate that rhEGF-loaded multivesicular liposomes may be used as a new strategy for the development of an oral delivery system in the treatment of peptic ulcer diseases.

Key words: rhEGF, Multivesicular liposomes, Loading efficiency, Oral delivery, Peptide

INTRODUCTION

With advance of biotechnology, an increasing number of proteins and peptides have been developed as therapeutic drugs. Although they are highly potent and specific in their physiological functions, most peptide drugs are difficult to be administered orally because of the low bioavailability. In recent years, however, new understandings regarding gastrointestinal physiology and uptake mechanisms have led to a number of novel concepts for oral protein/peptide delivery (Florence, 1997; Russell-Jones, 1996; Aramaki *et al.*, 1993; Chen *et al.*, 1996). In general, a successful delivery of oral protein and peptide drugs depends on the formulation strategies designed to achieve better stability in the intestinal tract, greater GI wall affinity, and higher concentration at the site of delivery.

The liposomes is one of the most popular vehicles for the development of oral drug delivery system of peptide drugs. Indeed, the liposomal encapulation is likely to provide several benefits such as protection of the drug from degradation and enzymatic metabolism by the acidic pH of the stomach and intestinal enzymes (Iwanaga *et al.*, 1997) and improvement of permeability across the gastrointestinal epithelium (Anderson *et al.*, 1999). Previously, we reported that human epidermal growth factor (rhEGF) in the polyethylene glycol-coated liposomes showed improved profiles of stability and permeability (Li *et al.*, 2003).

Recently, it was reported that the multivesicular liposomes can be used to develop sustained release formulations of therapeutic proteins and peptides with a high drug loading capacity (Katre *et al.*, 1998; Ye *et al.*, 2000; Mantripragada, 2002). The multivesicular DepoFoam particles contain a neutral lipid, which is an integral structural component, allowing an unique multivesicular liposomal structure. Multivesicular liposomes consists of multiple nonconcentric aqueous chambers bounded by network of lipid membranes. The particle is reported to contain 95% water in the vesicle, thereby rendering the technology ideal for encapsulation of water-soluble drugs such as peptide

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drugs. The multivesicular DepoFoam technology has been successfully used to deliver several small molecules in a sustained manner. A DepoCytarabine injectable system is currently being evaluated in clinical trials (Murry and Blaney, 2000). However, the potential applicability of the multivesicular liposomes has not been systematically studied for the oral delivery.

rhEGF is a single-chain polypeptide containing 53 amino acid residues and three disulfide bridges (Senderoff *et al.*, 1994). The peptide is reported to stimulate the proliferation and differentiation of epithelial tissues such as intestinal mucosa, corneal epithelial tissues, lung tissues, and trachea epithelia (Carpenter and Cohen, 1979). rhEGF also inhibits gastric acid secretion and protects gastroduodenal mucosa against tissue injury induced by ulcergenic agents (Elder *et al.*, 1975; Gregory, 1975; Konturek *et al.*, 1981; Kirkegaard *et al.*, 1983).

The goal of the present study was to prepare multivesicular liposomes with a high rhEGF-loaded capacity and to examine the feasibility of the system for an oral delivery of peptides. Therefore, we prepared the multivesicular liposomes with several variations in the preparation variables and evaluated the liposomes in terms of the loading efficiency, stability and permeability of the peptide in the GI tract, and gastric ulcer healing effect in rats.

MATERIALS AND METHODS

Materials

rhEGF was supplied by Daewoong Pharm. Co. (Seoul, South Korea). The human EGF Quantikine® kit was purchased from R&D Systems (Minneapolis, MN). A human colonic epithelial cell line, Caco-2, was obtained from the American Type Culture Collection (Rockville, MD). Dimyristoylphophatidylglycerol (DMPG) was from Avanti Polar Lipid Inc (Alabaster, AL), and fetal bovine serum was from Hyclone Laboratories (Logan, UT). Dioleoylphosphatidylcholine (DOPC), triolein, 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 Chemical Co. (St. Louis, MO), and trypsin-EDTA was from Gibco Laboratories (Gaithersburg, MD). All other reagents were of analytical grade and used without further purification.

Preparation of rhEGF-loaded multivesicular liposomes

The rhEGF-loaded multivesicular liposomes was prepared by the method of Kim *et al.* (1983), a two-step water-in-oil-in-water (w-o-w) double emulsification process, with a slight modification. Briefly, 1 mL of an aqueous buffered solution of the rhEGF (0.3-5.0 mg/mL containing various concentration of sucrose) was emulsified with 1.5 mL of a chloroform solution containing phospholipids (13.20 mM DOPC, 2.79 mM DMPG), cholesterol (19.88 mM) and triglyceride (2.44 mM triolein), at ambient temperature for 10 min at 8000 rpm, to obtain a water-in-oil emulsion (the first emulsion). After mixing, 28 mL of a second aqueous solution containing 4% glucose and 20 mM lysine was added to the first emulsion, and the contents were mixed for 1 min at 8000 rpm to form the water-oil-water double emulsion (the second emulsion). The second emulsion was diluted with 42 mL of the second aqueous solution containing 4% glucose and 20 mM lysine. Chloroform was removed by flushing the surface of the mixture at 37°C with nitrogen. The resulting multivesicular liposomes was harvested by centrifugation for 20 min at 12,000 rpm and washed twice with 20 mL of HBSS buffer (pH 6.5). After washing, the pellet fraction (i.e., particles) was resuspended in 3 mL of HBSS buffer solution (pH 6.5).

Characterization of multivesicular liposomes

The loading efficiency was determined by the ratio of the amount of drug in the final liposome suspension to the total amount of drug used in the first aqueous solution. To determine the drug concentration in the final multivesicular liposomal particles, 100 μL of multivesicular liposomes was completely dissolved by the addition of 20 μL of 5% Triton X-100. The concentration of the drug in the mixture was determined by a reverse phase HPLC on Vydac C_{18} column (2.5×250 mm, 10 μm). The HPLC system consisted of a Jasco model PU-980 as a pump, UV-975 UV-Vis detector and LC-Net II control borwin integrator. The mobile phase (pH 6.5) consisted of acetonitrile, triethylamine and water, 200:2.2:800(V/V/V), and was delivered at the flow rate of 1mL/min. The eluent was monitored at 214 nm.

The particle size distribution and median diameter was determined using a NICOMP 370 Submicron Particle Analyzer (Pacific Scientific, CA). The measurement was conducted in duplicate and the mean values were used.

Release of rhEGF from multivesicular liposomes

Rate of release for rhEGF from multivesicular liposomes was measured in the acidic solution (pH 1.2, a simulated intra-gastric condition) and in 10 mM sodium taurocholate in HBSS (pH 7.4, a simulated intra-intestinal condition). The multivesicular liposomes was centrifuged at 10,000 rpm for 10 min and the pellet was resuspended with test solution having a final rhEGF concentration of 200 μg/mL. An aliquot (0.2 mL) of the sample was added in screw-cap Eppendorf tube and then diluted in the release medium. Samples were then incubated under gentle rotating condition (50 cycles/min) at 37°C. Duplicate samples were taken at an appropriate time intervals for 6 h. The sample was centrifuged at 10,000 rpm for 10 min

and the pellet resuspended with fresh 0.2 mL HBSS buffer. The suspension was lysed with 10 μL of 5% Triton X-100 and the concentration determined by HPLC as described previously.

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 under 5% CO_2 and 95% air. The culture medium was replaced every two days. For the determination of the protein by enzymatic degradation, cells were grown for 14 days in three T-flasks. For the transport study, 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. When necessary, the integrity of cell monolayers was confirmed by measuring transepithelial electrical resistance (TEER) before the transport study. The TEER value of Caco-2 cell monolayers reached 300-600 $\Omega \cdot cm^2$ in 18-25 days of culture.

rhEGF degradation in Caco-2 cell homogenate

The stability of rhEGF in Caco-2 cell homogenate was assessed by the incubation of the cell homogenate with the aqueous solution or multivesicular liposomes containing rhEGF. The cell homogenate was obtained essentially by the method of Annaert *et al.* (1997). Briefly, Caco-2 cells grown in three T-flasks were scraped and collected in 5 mL of ice-cold transport medium (pH 6.5); the mixture was then homogenized. The homogenate was centrifuged at 8000×g for 10 min at 4°C and the supernatant collected as the cell homogenate (protein concentration = 1 mg/mL). The multivesicular liposomes or aqueous solution (0.2 mL) containing rhEGF was added to the same volume of the cell homogenate and the mixture incubated at 37°C. At the predetermined time points, an aliquot of the mixture was sampled and transferred to a fresh tube. The reaction was terminated by the addition of 50% of acetic acid to the sample. 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 as described previously.

Measurement of transepithelial transport of rhEGF

The transepithelial transport of rhEGF was studied in HBSS buffer supplemented with 20 mM of glucose, 9 mM of sodium bicarbonate, and 25 mM of HEPES (pH 7.4). An aliquot (0.5 mL) of the rhEGF in an aqueous solution or the multivesicular liposome preparation was added to the apical side of the cell monolayers and 1.5 mL of incubation medium was added to the basolateral side. The monolayers were incubated for a specified period of time at 37°C and samples were withdrawn from the basolateral side. The appearance of rhEGF in basolateral side was measured by ELISA method using a Quantikine® kit. At the end of the experiments, the concentration of free rhEGF concentration in the apical side was also determined by HPLC. The sample was centrifuged at 10,000 rpm for 20 min and the free rhEGF concentration determined in the supernatant. The transport clearance was calculated as described previously (Li *et al.*, 2001).

Gastric ulcer healing test

Acute gastric ulcer healing effect was investigated by the previously described method (Han *et al.*, 1998). Briefly, 1 mL of absolute ethanol was instilled into the stomach of rats (230-250 g) to induce acute gastric ulcers in rats after 24 h of fasting. Various times (i.e., 4, 10, and 24 h) after the induction of ulcers, the rats were orally (400 μg/kg) administered with either rhEGF in aqueous solution or in multivesicular liposomes. Oral administration of cimetidine (120 μg/kg) was used as a positive control. The rats were sacrificed 30 h after the induction of ulcers and the stomach was removed and opened along the greater curvature for the evaluation. The degree of gastric mucosal injury and the length of ulcerated mucosa in millimeter were measured and the curative ratio was calculated using following equation:

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

RESULTS AND DISCUSSION

Characterization of rhEGF-loaded multivesicular liposomes

In general, the loading efficiency of the drug for the multivesicular liposomes may be governed by various factors (aqueous solubility, membrane permeability of the drug and the osmolarity of the drug containing first aqueous solution). In order to prepare multivesicular liposomes with a high loading efficiency for rhEGF, the effect of osmolarity on the encapsulation efficiency was investigated. The osmolarity of the first aqueous solution was adjusted with sucrose and NaH_2PO4. As shown in Fig. 1, the rhEGF loading increased from 28.7 ± 0.9 to $36.28 \pm 1.5\%$ with an increasing sucrose concentration from 3 to 5% at 0.3 mg/mL concentration of rhEGF. However, the loading efficiency decreased by half with an increasing sucrose concentration from 5 to 7% at the same level of rhEGF. Interestingly, with rhEGF at 0.3 mg/mL in 5% sucrose solution, the drug

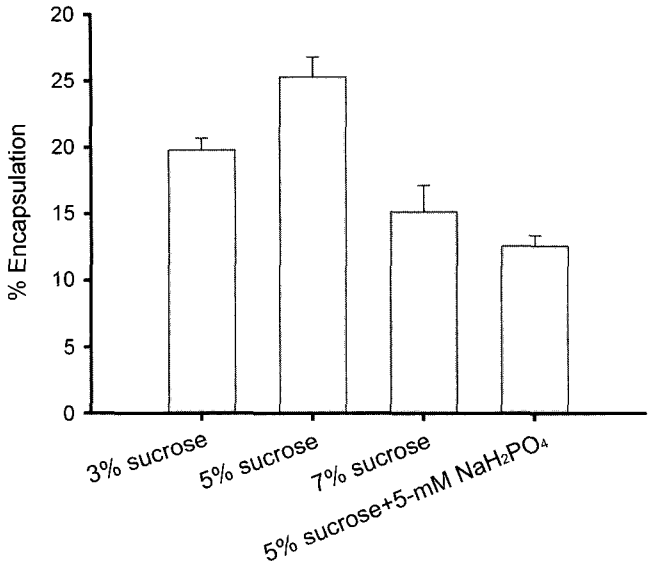

Fig. 1. The effect of sucrose concentration and 50 mM NaH_2PO_4 on the rhEGF encapsulation in multivesicular liposomes. 0.3 mg/mL of rhEGF solution containing sucrose or NaH_2PO_4 was emulsified with chloroform solution containing 13.20 mM DOPC, 2.79 mM DMPG, 19.88 mM cholesterol and 2.44 mM triolein.

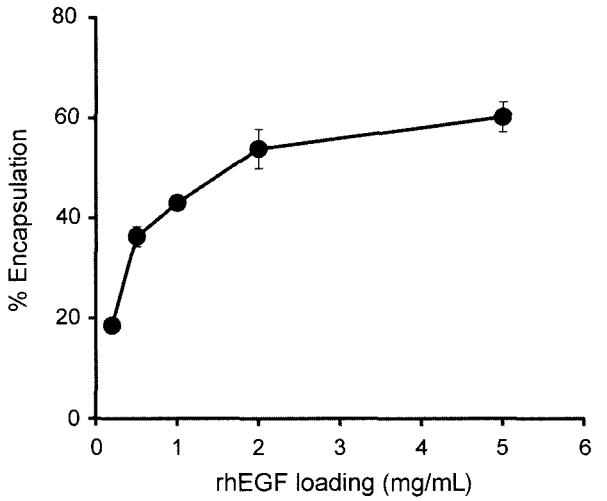

Fig. 2. The effect of rhEGF concentrations on the encapsulation in multivesicular liposomes. 0.3-5.0 mg/mL rhEGF solutions were emulsified with chloroform solution containing 13.20 mM DOPC, 2.79 mM DMPG, 19.88 mM cholesterol, and 2.44 mM triolein.

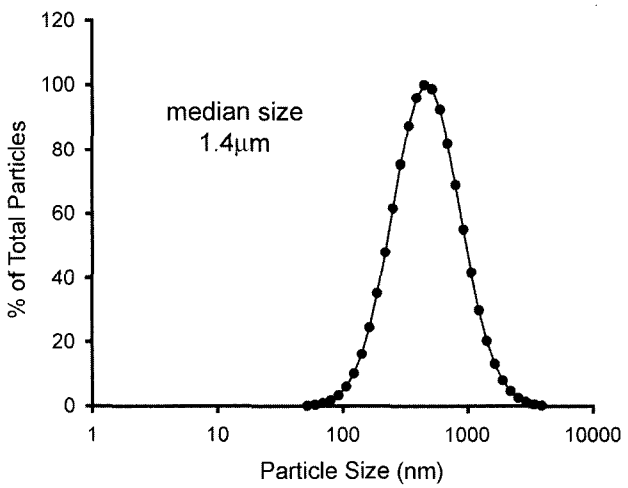

Fig. 3. Representative volume-weighted particle size distribution for multivesicuolar liposomes. The median size of the multivesicular liposomes was 1.4 μm.

loading was significantly decreased by addition of 50 mM NaH_2PO_4. These results collectively indicated that not sucrose concentration, but osmolartity was affected encapsulation efficiency of rhEGF in the multivesicular liposomes and that the maximum encapsulation efficiency was obtained in 5% sucrose solution at 0.3 mg/mL rhEGF in this study.

The effect of the concentration of rhEGF on its loading efficiency was also investigated. In 5% sucrose solution, the rhEGF loading efficiency markedly increased with the rhEGF concentration in the first aqueous solution (Fig. 2). The efficiency was raised to approximately 55% at 2 mg/mL of rhEGF and reached a plateau thereafter to approximately 60% at 5 mg/mL of the rhEGF. Thus, given the high cast of rhEGF, we selected 2 mg/mL of rhEGF in 5% sucrose solution for the preparation of multivesicular liposomes in subsequent studies.

The particle size distribution of the multivesicular liposomes is shown in Fig. 3. The volume-weighted size determination shows a narrow and monomodal distribution with a median diameter of 1.4 μm and over 95% of particles sizing between 0.19 and 5.2 μm.

Release of rhEGF from multivesicular liposomes

The release profiles of rhEGF from multivesicular liposomes are shown in Fig. 4. The release of rhEGF in *in vitro* conditions that mimic the gastrointestinal tracts apparently followed the first-order kinetics. In the acidic condition (pH 1.2), 53% of rhEGF remained stable in the multivesicular liposomes and 47% of rhEGF released from multivesicular liposomes for 6 h. In the simulated intraintestinal condition (pH 7.4), rhEGF release from multivesicular liposomes reached 35% for 6 h. These results indicate that rhEGF is released from the liposomes in a sustained manner for over 6 h periods in the current study condition.

rhEGF degradation in the homogenate of the Caco-2 cell

The ability of the liposomes was investigated for the prevention of enzymatic degradation in the GI tract by using Caco-2 cell homogenate. rhEGF in aqueous solution was

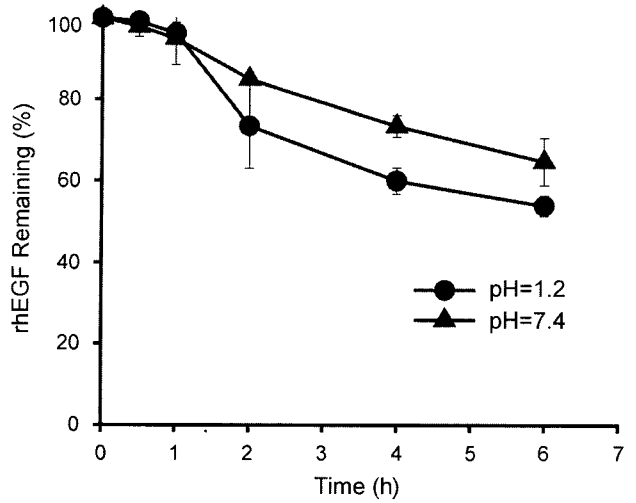

Fig. 4. *In vitro* release characteristics of multivesicular liposomes in the gastrointestinal conditions. (●), in the acidic solution (pH 1.2); (▲), in the presence of sodium taurocholate (10 mM) in HPSS (pH 7.4). Data are expressed as the mean±S.D. (n=2).

disappeared rapidly following the first-order kinetics in the Caco-2 cell homogenate (Fig. 5). As a result, approximately 50% of rhEGF was degraded within 60 min, suggesting a rapid enzymatic hydrolysis occurs for rhEGF in aqueous media. In contrast, degradation of rhEGF in multivesicular liposomes was significantly delayed as evidenced by the fact that about 10% disappeared within the identical time period. This result indicates that the rhEGF-loaded multivesicular liposomes may be an effective vehicle for the enhancement of the stability for the rhEGF in gastrointestinal mucosa.

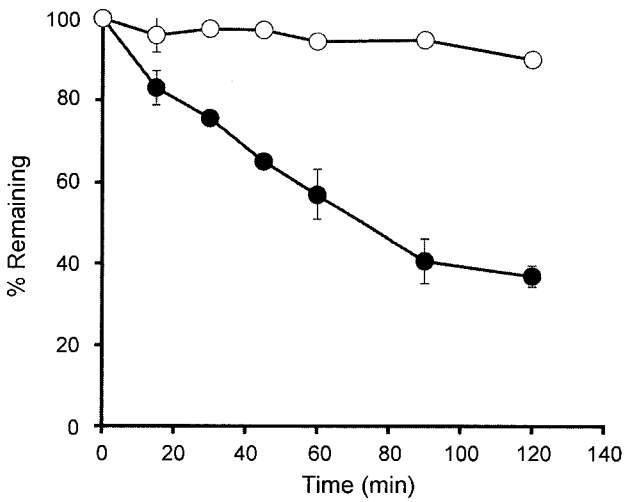

Fig. 5. The degradation of rhEGF in the Caco-2 cell homogenate. Caco-2 cell homogenate and rhEGF-loaded multivesicular liposomes or rhEGF solution was incubated for 120 min at 37°C. (●), rhEGF solution; (○), multivesicualr liposomes. Data are expressed as the mean±S.D. (n=3).

rhEGF transport in Caco-2 cell monolyaers

One of the goals for the development of multivesicular liposomes is the improvement of oral bioavailability of poorly absorbed agents, such as proteins and peptides. Thus, the effect of multivesicular liposomes for rhEGF transport was investigated. Fig. 6 shows the transepithelial transport of rhEGF across Caco-2 cells when the drug contained in an aqueous solution or multivesicular liposomes was applied on the apical side of the cell monolayers. The apical to basolateral flux of rhEGF in aqueous solution proportionally increased with the rhEGF concentration in the range of 0.1 to 0.2 mg/mL. The flux of rhEGF from the multivesicular liposomes to basolateral side of Caco-2 cells was approximately half of that found in the aqueous solution containing rhEGF. In general, free rhEGF is likely to be transported across Caco-2 cell monolayers and, thus, the free protein level in the apical side may be the primary determinant in the rate of transport. In order to investigate this issue, we determined free rhEGF concentration in apical side of Caco-2 cell monolayers immediately after the transport. The transport clearance of rhEGF was calculated by dividing the transport rate by the free rhEGF concentration in the donor chamber. As shown in Table I, the transport clearance for rhEGF was 3.440 ± 0.120 nL/cm^2/min and 1.777 ± 0.298 nL/cm^2/min for rhEGF-loaded multivesicular liposomes and rhEGF in aqueous solution, respectively. This observation indicated that rhEGF in the multivesicular liposomes had a higher permeability compared with of rhEGF in aqueous

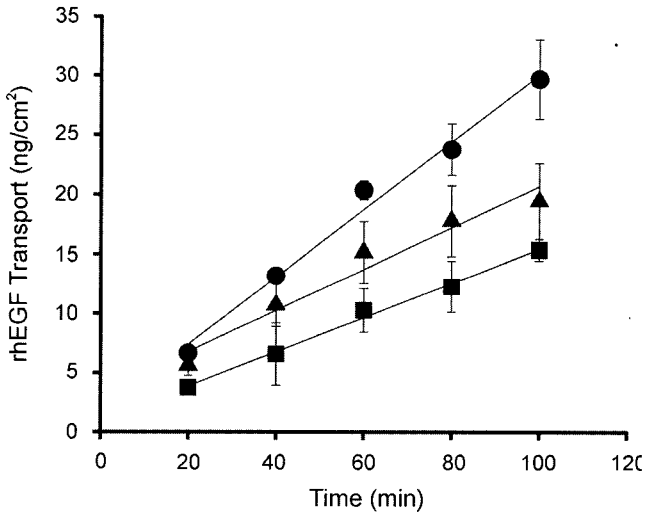

Fig. 6. The apical to basolateral transport of rhEGF across the Caco-2 cell monolayers. 0.5 mL of multivesicular liposomes (pH 6.5) or rhEGF solution (pH 6.5) was added to the apical side and 1.5 mL of the incubation medium (pH 7.4) without the drug was added to the basolateral side. (●) Soution (0.2 mg/mL), (▲) Solution (0.1 mg/mL), (■) Multivesicular liposomes (0.2 mg/mL). Data are expressed as the mean±S.D. (n=3).

Table I. The transepithelial transport of rhEGF in solution or multivesicular liposomes across Caco-2 cell monolayers

Formulation	Apical concentration (mg/mL)		Flux ($ng/cm^2/min$)	Flux/free rhEGF ($nL/cm^2/min$)
	Total rhEGF	Free rhEGF[a]		
rhEGF solution	0.2	0.161±0.007	0.286±0.048*	1.777±0.298
Multivesicular liposomes	0.2	0.042±0.002	0.145±0.005	3.440±0.120*

[a] Free rhEGF concentation in apical side was determined at the end of the experiments.

* P<0.05, significantly different from the other group by student's *t*-test.

Each data is expressed as mean±S.E. (n=4-5).

Table II. Curative ratio of ethanol-induced gastric ulcers after oral administration of rhEGF solution, multivesicular liposomes and cimetidine to the rats.

Group	Dose (μg/kg)	Length of acute ulcer (mm)	Curative ratio (%)
No treatment	-	70.4±10.8	-
Solution[a]	400	56.5± 7.5	19.8±10.0
Multivesicular liposomes[a]	400	41.6± 5.4*	40.9± 7.7*
Cimetidine	120	43.6± 6.8*	38.0± 9.5*

[a] Solution and multivesicular liposomes were orally administered at a dose of 400 μg/kg to the rats after 4, 10, 24 h following the induction of ulcers.

* P<0.05, significantly different from the no treatment group by student's *t*-test.

Each data is expressed as mean±S.E. (n=4-5).

solution, probably due to the fact that DepoForm particles may be taken up Caco-2 cells.

Gastric ulcer healing effect of rhEGF after oral administration

Gastric ulcer healing effect was determined by the measurement of the length of ulcerated mucosa after oral administration of rhEGF-loaded multivesicular liposomes, rhEGF in aqueous solution, or cimetidine (Table II). To test the effect of acute gastric ulcer healing, rhEGF solution or the rhEGF-loaded multivesicular liposomes was given at a dose of 400 μg/kg in rats with acute gastric ulcer. Oral administration of cimetidine (120 μg/kg) was used as a positive control. After oral administration of the rhEGF-loaded multivesicular liposomes, the curative ratio reached 40.9 ± 7.7%. The gastric ulcer healing effect was higher than that of the control group (19.8 ± 10.0%) by about two-fold and was similar with the positive control group (cimetidine, 38.0 ± 9.5%). In this study, we did not directly investigate the underlying mechanism for the enhanced healing effect for rhEGF-loaded multivesicular liposomes. However, since we showed the encapsulated rhEGF formulation was able to suppress the enzymatic degradation and release the protein in a sustained manner in the gastrointestinal fluid, the collective effect of the rhEGF encapsulation may be related to the enhancement in the pharmacological effect.

CONCLUSION

The multivesicular liposomal formulation of rhEGF showed a high encapsulation efficiency, which was able to deliver the peptide in the GI fluid in a sustained manner. The preparation showed a good acute gastric ulcer healing effect probably due to an improvement in the stability and absorption of the drug in GI tract. Collectively, these results indicate that rhEGF-loaded multivesicular liposomes may be used as a new strategy for the development of an oral delivery system in the treatment of peptic ulcer diseases.

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