Arch Pharm Res Vol 26, No 4, 330-337, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Effect of Excipients on the Stability and Transport of Recombinant Human Epidermal Growth Factor (rhEGF) across Caco-2 Cell Monolayers

In-Wha Kim, Ho-Jung Yoo[1], Im-Sook Song[1], Youn-Bok Chung, Dong-Cheul Moon, Suk-Jae Chung[1], and Chang-Koo Shim[1]

Research Institute Development of Pharmaceutical Resources, Chungbuk National University, San 48, Gaeshin-dong, Hungduk-gu, Cheongju, Chungbuk 361-763, Korea and [1]Department of Pharmaceutics, College of Pharmacy, Seoul National University, San 56-1, Shinlim-Dong, Kwanak-Gu, Seoul 151-742, Korea

(Received October 20, 2002)

The effect of sixteen excipients on the transport of recombinant human epidermal growth factor (rhEGF) across Caco-2 cell monolayers was examined at 37°C. The apparent apical to basolateral (A-B) permeability (P_{app}) of 30 μM rhEGF was 8.15×10^{-7} cm/sec, indicative of a poor level of absorption in the GI tract. The P_{app} was 1.7- and 6.3-fold greater than the P_{app} in the basolateral to apical (B-A) direction and the A-B permeability of mannitol, respectively, and decreased dramatically to a negligible level at 4°C, consistent with a receptor mediated transcytosis of rhEGF. The stability of rhEGF was very poor, undergoing more than 85% degradation in 2 h in the transport medium at 37°C. A significant increase in the P_{app} could be achieved by the addition of certain excipients, as exemplified by 23, 21, 20 and 16-fold increases, in the presence of sodium taurochenodeoxycholate (NaTCDC), sodium taurodeoxycholate (NaTDC), sodium glycodeoxycholate (NaGDC) and sodium laurylsulfate (SLS) (all at a concentration of 1% w/v), respectively. A significant increase in stability could also be achieved by the addition of some of the excipients, as represented by 1% SLS, which nearly completely stabilized the rhEGF. Unfortunately, however, an increase in the P_{app} of rhEGF could not be achieved without a simultaneous and extensive decrease in the integrity of the cell membranes. Thus, more efficient excipients, that specifically enhance the permeation of rhEGF and do not alter the membrane integrity, should be pursued in order to safely enhance the permeation of rhEGF.

Key words: rhEGF, Caco-2 cell monolayer, Absorption enhancers, Stabilizing agents

INTRODUCTION

Epidermal growth factor (EGF), a polypeptide containing 53 amino acid residues, stimulates the proliferation and differentiation of epithelial tissues such as the intestinal mucosa, corneal epithelial tissue, lung, and trachea epithelia (Carpenter and Cohen, 1979). Moreover, recombinant human EGF (rhEGF) acts as a stimulator of the restitution and proliferation of mucosal cells at the margins of ulcer, which supplies cells for the reepithelialization of the mucosal scar surface and the reconstruction of glandular structure, thus accelerating the healing of acute and chronic lesions (Tarnawski *et al.*, 1991; Konturek *et al.*, 1995). Stimulated by these facts, numerous attempts have been made to develop rhEGF for use as an antiulcer agent, especially for oral delivery, for use in the treatment of gastroduodenal ulcers (Palomino *et al.*, 2000).

However, the gastrointestinal (GI) absorption (both rate and extent) of rhEGF is known to be very poor and variable (Shen and Xu, 1998), similar to the cases of other peptide and protein drugs. The low permeability of rhEGF across the GI wall, as well as its extensive degradation or metabolism in the GI tract (Han *et al.*, 1998), may be major

Correspondence to: Chang-Koo Shim and Youn Bok Chung
Address for C.-K. Shim: Department of Pharmaceutics, College of Pharmacy, Seoul National University, San 56-1, Shinlim-dong, Kwanak-gu, Seoul 151-742, Korea
Tel: 82-2-880-7873 , Fax: 82-2-885-8429
E-mail: shimck@plaza.snu.ac.kr
Address for Y.B. Chung: Department of Pharmaceutics, College of Pharmacy, Chungbuk National University, San 48, Gaeshin-dong, Hungduk-gu, Cheongju, Chungbuk 361-763, Korea
Tel: 82-43-261-2824, Fax: 82-43-274-0752
E-mail: chungyb@cbucc.chungbuk.ac.kr

factors that explain its poor GI absorption. In general, various absorption enhancers and/or degradation inhibitors including surfactants (Anderberg *et al.*, 1992), bile salts (Anderberg *et al.*, 1992), fatty acids (Lindmark *et al.*, 1995) and cyclodextrins (Merkus *et al.*, 1991) have been examined for their effect on the absorption of peptide drugs on through nasal (Scipper *et al.*, 1993), rectal (Okada *et al.*, 1982) and subcutaneous routes (Murakami *et al.*, 1993), as well as their transcellular absorption across Caco-2 cell monolayers (Lindmark *et al.*, 1995; Michael *et al.*, 2000). However, only limited information is available on rhEGF, regarding the effect of these absorption enhancers and/or degradation inhibitors except for Murakami *et al.* who reported an increased rectal and subcutaneous absorption in rats in the presence of sodium carboxymethyl cellulose (NaCMC, Murakami *et al.*, 1988) and sodium caprate (NaC10, Murakami *et al.*, 1991).

Thus, the purpose of the present study was to examine the effect of sixteen well-known absorption enhancers and/or degradation inhibitors on the transport of rhEGF across Caco-2 cell monolayers and on the stability of rhEGF during transport experiments. In addition, rhEGF is a drug with a high molecular weight (*i.e.*, Mw of 6 KDa), and is likely to be transported across Caco-2 cell monolayers via an energy-dependent (Sai *et al.*, 1998) and receptor mediated transcytosis (RMT) process (Hidalgo *et al.*, 1989a; Tong *et al.*, 1998). Thus, special emphasis was placed on the effect of these excipients on the RMT of rhEGF. The Caco-2 cell line was selected because it is known to mimic human intestinal epithelial cells in terms of enzymatic metabolism (Hidalgo *et al.*, 1989b) and absorption, specifically, in terms of the expression of rhEGF receptors on the cell surface (Hidalgo *et al.*, 1989a; Tong *et al.* 1998).

MATERIALS AND METHODS

Materials

Highly purified rhEGF (more than 99% purity) was generously provided by Daewoong Pharm. Co. (Seoul, Korea). [^{14}C]Mannitol (50 mCi/mmoL, New England Nuclear, Boston, MA), [^{14}C]inulin (2.4 mCi/g, New England Nuclear, Boston, MA), fetal bovine serum (Hycone Laboratories, Logan, UT), dipotassium glycyrrhizinate (KGrZ, TCI, Tokyo, Japan), cimetidine (TCI, Tokyo, Japan), trypsin-EDTA (Life Technologies Inc., Gaitherburg, MD), Dulbeccos modified Eagles medium, nonessential amino acid solution, penicillin-streptomycin, Hanks balanced salt solution (HBSS), *N*-2-[hydroxylethyl]piperazine-*N'*-ethanesulfonique (HEPES), sodium caprate (NaC10), sodium laurylsulfate (SLS), sodium taurocholate (NaTC), sodium taurodeoxycholate (NaTDC), sodium glycocholate (NaGC), sodium glycodeoxycholate (NaGDC), sodium taurochenodeoxycholate (NaTCDC), sodium tauroursodeoxycholate (NaTUDC), sodium taurolithocholate (NaTLC), dimethyl β-cyclodextrin (DM β-CD), hydroxypropyl β-cyclodextrin (HP β-CD), sorbitol, sodium carboxylmethylcellulose (NaCMC), hydroxylpropylmethylcellulose (HPMC), bestatin (all from Sigma Chemical Co., St. Louis, MO) were used as purchased. Acetonitrile was purchased from Fisher Scientific Co. (Fair Lawn, NJ). All other reagents were of analytical grade.

Caco-2 cell culture

The human colon adenocarcinoma cell line, Caco-2 (American Type Culture Collection, Rockville, MD), was gown as monolayers, in Dulbeccos modified Eagles medium, 10% fetal bovine serum, 1% nonessential amino acid solution, 100 units/mL penicillin and 0.1 mg/mL streptomycin at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. Stock cultures were grown in 75 cm^2 tissue culture flasks and were split 1:3 at 80 to 90% confluency using 0.02% EDTA and 0.05% trypsin. The Caco-2 cells from passage numbers 42 to 55 were seeded on permeable polycarbonate inserts (1 cm^2, 0.4 μm pore size; Corning Costar Co., Cambridge, MA) in 12 Transwell plates at a density of 1 to 1.5×10^5 cells/cm^2. The inserts were fed with complete media at 2 day intervals for the first week and then at daily intervals until they were used for the transport experiments 18 to 25 days after the seeding (Augustijns *et al.*, 1993). The integrity of the cell monolayers was evaluated by measuring transepithelial electrical resistance (TEER) values with an EVOM epithelial volt/ohmmeter (World Precision Instruments, Sarasota, FL). The cell inserts were used for experiments when the resistance reached 300 to 600 Ω·cm^2. In each experiment, the transport of [^{14}C]mannitol was measured in three inserts. The cell monolayers were considered tight when the mannitol transport was less than 0.35% of the dose/hr, corresponding to a P_{app} value of 1.3×10^{-7} cm/sec.

Transepithelial permeability of rhEGF, cimetidine, mannitol, and inulin

Prior to the transport experiments, the cell monolayers were washed twice with incubation medium (pH 7.4, HBSS containing 10 mM HEPES and 25 mM glucose). After each wash, the plates were incubated for 30 min at 37°C, and the TEER value was then determined. The incubation medium on both sides of the cell monolayer was then removed by aspiration (Augustijns *et al.*, 1993).

For measurement of the apical to basolateral (A-B) transport of rhEGF, 0.5 mL of the incubation medium containing rhEGF (30 μM) was added to the apical side, and 1.5 mL of the incubation medium without the drug was added to the basolateral side. The effect of temperature on the transport of rhEGF was examined, when necessary, by comparing the permeabilities measured at 37°C and

4°C. The polycarbonate inserts in the Transwells were moved to wells containing fresh incubation medium every 30 min for 2 h. At 30, 60, 90 and 120 min after the start of the incubation, 150 μL of 0.11% Polysorbate 80 was added to each of the basolateral side (Han *et al.*, 1998) and incubated for 1 h at 37°C to minimize the adsorption of rhEGF. The incubation medium in the basolateral side was then removed and stored at -20°C, for use in a high performance liquid chromatography (HPLC) analysis of rhEGF. Three inserts were used in each transport experiment.

For measurement of basolateral to apical (B-A) transport of rhEGF, 1.5 mL of the incubation medium, containing rhEGF, was added to the basolateral side, and 0.5 mL of incubation medium without the drug was added to the apical side. The inserts were then incubated at 37°C, and a 300 μL aliquot was removed from the apical side at 30 min intervals for 2 h followed by replacement with 300 μL of fresh incubation medium. Three inserts were used in each experiment.

To investigate the effect of excipients on the apical to basolateral transport of rhEGF, each excipient, at a given concentration, was added to the apical side which contained 30 μM rhEGF (Table I). The apical to basolateral transport of rhEGF was then measured as described above, as well as the effect of temperature (*i.e.*, 37°C or 4°C) of the incubation medium on transport.

The effect of excipients on membrane diffusivity and paracellular leakage across the Caco-2 cell monolayer was also examined by measuring the permeability of

Table I. Effects of various excipients on the apical to basolateral permeability of rhEGF (30 μM), mannitol (10 μM), inulin (40 nM) and cimetidine (1 mM) on Caco-2 cell monolayer, and on the degradation of rhEGF in the incubation medium on the cell monolayers, and on TEER values of Caco-2 cell monolayer[a]

	Concentration	P_{app} ($\times 10^{-6}$ cm/sec)				Stability (% remained in 2 h)	TEER (%)
		rhEGF	Mannitol	Inulin	Cimetidine		
Control		0.815±0.310	0.130±0.0277	0.0036±0.00202	1.53±0.235	13.3±4.78	95.4±12.7
NaGC	1 (%)	0.863±0.0295	0.178±0.0704	0.00818±0.00159	6.62±0.542	26.6±5.82	84.2±12.3
NaTC	1 (%)	1.12±0.184	0.223±0.0849	0.00914±0.00165	2.56±0.426	13.1±7.80	73.2±19.7
NaTDC	0.1 (%)	1.83±0.241	0.341±0.0794	0.0100±0.00198	4.29±0.227	52.2±0.565	109±6.25
	0.5 (%)	10.7±2.41	4.82±0.536	0.125±0.00774	50.6±2.35	55.6±1.21	22.2±2.07
	1 (%)	17.2±0.542	11.0±1.16	0.309±0.0212	64.0±5.38	56.4±6.99	1.50±1.16
NaGDC	0.1 (%)	6.34±1.53	4.47±2.35	0.0573±0.0129	4.21±0.400	25.7±3.19	65.5±11.3
	0.5 (%)	12.5±1.91	9.07±2.14	0.212±0.00520	48.1±0.774	33.6±10.4	35.9±6.64
	1 (%)	16.5±0.23	12.3±1.09	0.345±0.00791	55.9±0.660	38.1±5.07	16.9±5.37
NaTCDC	0.1 (%)	2.38±1.06	1.16±0.110	0.00424±0.00161	2.41±0.517	23.4±11.3	89.2±13.4
	0.5 (%)	14.6±1.63	4.74±0.157	0.0508±0.00572	53.9±6.10	41.0±5.11	15.3±6.71
	1 (%)	18.±3.25	12.0±0.387	0.400±0.161	66.6±5.14	46.2±7.42	3.01±2.06
NaTUDC	1 (%)	1.64±0.247	0.421±0.0585	0.0127±0.00309	2.60±0.403	39.1±5.11	68.7±1.07
NaTLC	1 (%)	0.892±0.271	0.168±0.0765	0.00407±0.000657	N.D.[b]	23.7±3.40	77.8±0.92
SLS	0.1 (%)	6.89±1.46	3.76±0.164	0.0827±0.00147	32.5±4.28	54.6±9.87	45.7±10.8
	0.5 (%)	8.76±4.28	6.31±1.47	0.173±0.0226	26.9±2.43	73.4±11.0	20.4±6.12
	1 (%)	13.4±2.79	9.61±3.00	0.329±0.0110	25.6±0.370	103±9.24	2.20±1.66
NaC10	0.1 (%)	0.788±0.0648	0.210±0.0448	0.00424±0.00161	5.41±0.418	12.2±2.94	87.8±13.8
	0.5 (%)	2.52±0.878	0.87±0.178	0.0508±0.00572	10.4±0.449	40.2±8.42	55.7±9.07
	1 (%)	8.61±0.966	8.27±0.752	0.250±0.0184	29.4±6.76	85.2±3.14	40.5±0.567
DM β-CD	0.5 (%)	1.28±0.702	1.27±0.500	0.00942±0.000574	3.24±0.282	57.0±16.6	75.9±11.4
HP β-CD	1 (%)	1.27±0.607	0.186±0.0295	0.00713±0.00130	2.63±0.979	47.3±6.57	81.6±3.18
NaCMC	1 (%)	1.60±0.417	0.0894±0.00536	0.00715±0.00119	1.59±0.132	17.5±3.03	68.6±11.1
Sorbitol	1 (%)	2.54±1.03	0.207±0.0438	0.00715±0.00164	N.D.	12.8±7.60	71.3±2.45
KGrZ	2 (%)	2.04±0.789	0.481±0.0533	0.00131±0.000164	1.07±0.294	22.8±3.07	98.0±6.10
Bestatine	500 (μM)	1.82±0.604	0.507±0.0312	0.00532±0.00201	N.D.	31.0±4.62	70.2±7.74
HPMC	0.5 (%)	1.93±0.304	0.139±0.0302	0.00211±0.000311	2.05±0.669	28.7±4.97	74.4±2.95

[a]The values represent the mean±S.E. of three different experiments.
[b]Not determined.

cimetidine (a representative substance that is known to permeate via passive diffusion, Pade and Stavchansky, 1997), [^{14}C]mannitol and [^{14}C]inulin (markers of paracellular leakage) across the monolayer using the same method. To briefly summarize, HBSS containing cimetidine (1 mM), [^{14}C]mannitol (10 μM) or [^{14}C]inulin (40 nM) was added to the apical side, and the incubation medium in the basolateral side was then removed at 30 (for the case of [^{14}C]mannitol, [^{14}C]inulin) or 15 (for the case of cimetidine) min intervals over a 2 h period.

For each transport experiment, the total amount of rhEGF, cimetidine, mannitol or inulin in the receiver compartment was plotted as a function of time, and the slope of the linear portion of the plot was estimated by linear least regression. The slope was assumed to be the transport rate of the compound, since the surface area of the monolayer membrane used was 1 cm^2. The apparent permeability value (P_{app}) of each compound, expressed in centimeters per seconds, was calculated by dividing the transport rate by the initial concentration of each compound in the donor chamber.

Stability of rhEGF

The effect of excipients on the stability of rhEGF in the incubation medium on the Caco-2 cell monolayers was also examined. The lower side of the Transwell insert was sealed with a Teflon®-P.T.F.E. thread seal tape (Han Yang Chemical Co., Pusan, Korea) in order to block the transport of rhEGF from the apical to basolateral side. After preincubation of the insert for 30 min with incubation medium, the medium was removed, and an incubation medium (0.5 mL) containing rhEGF (30 μM) and excipients at given concentrations (Table I) was then added to the apical side, followed by incubation for 2 h. A 10 μL aliquot of the medium was removed at 30 min intervals for 2 h, collected in ice-cold tubes and stored at -20°C until used in an HPLC analysis of rhEGF.

Analytical methods

The concentration of rhEGF and cimetidine in each sample was analyzed after centrifugation at 10,000 rpm for 15 min using a Hitatchi high HPLC system (L-7110, Tokyo, Japan) with a Shimazu Spectrophotometer UV detector (SPD-6AV, Kyoto, Japan). Vydac Protein and Peptide Analytical C18 column (pore size of 300 Å, Alltech Associates Inc., Deerfield, IL) and μBondapak C18 column (Waters, Milford, MA) were used for the analysis of rhEGF and cimetidine, respectively. In the analysis of rhEGF, a mobile phase consisting of 0.05 M sodium phosphate dibasic (pH 6.5) and acetonitrile (795:205, v/v) was eluted at a flow rate of 1 mL/min, and a wavelength of 214 nm was used for the detection of the compound. In the analysis of cimetidine, a mobile phase consisting of 0.01 M sodium phosphate dibasic (pH 6.9) and acetonitrile (8:2, v/v) was eluted at a flow rate of 1.2 mL/min, and the detection was performed at 230 nm. The height of the peak corresponding to rhEGF or cimetidine was used to calculate the concentration of each drug from their respective standard calibration curves.

For the assay of mannitol and inulin in the receiver compartment, a 1.4 mL aliquot of transport medium in the receiver compartment was transferred to a scintillation vial containing 3 mL of scintillation cocktail (Ultima Gold, Packard, Meriden, CT), and the radioactivity in the vial was measured using a Wallac 1409 liquid scintillation counter (Wallac, Gaithersburg, MD).

RESULTS

Transport of rhEGF across Caco-2 cell monolayers

The apparent permeability (P_{app}) of rhEGF in the A-B direction at a given concentration of 30 μM was calculated to be 8.15×10^{-7} cm/sec (Table I) from Fig. 1 (●). This value was 1.7-fold greater (P<0.05) than its permeability in the B-A direction (4.82±2.3×10^{-7} cm/sec, n=3), indicating possible involvement of carrier system(s) in the A-B transport of rhEGF. Despite the passive diffusion of rhEGF was not likely to exist considering the high Mw (6 kDa) and hydrophilicity of rhEGF, the A-B permeability of rhEGF was 6.3 and 226-fold greater (P<0.05) than the A-B permeability of [^{14}C]mannitol and [^{14}C]inulin (0.130±0.0277 ×10^{-6} and 0.0036±0.00202×10^{-6} cm/sec, Table I), respectively, also indicating the presence of carrier system(s). However, the P_{app} is below the value that is generally required for efficient GI absorption (*i.e.*, 1×10^{-6} cm/sec,

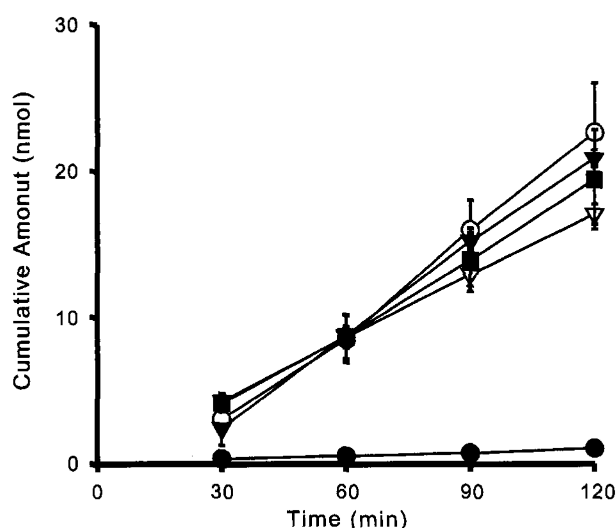

Fig. 1. Representative examples of effects of excipients (1%, w/v) on the time course of apical to basolateral (A-B) transport of rhEGF (30 μM) across Caco-2 cell monolayers at 37°C. ●: Control (*i.e.*, without excipients), ○: NaTCDC, ▼ : NaGDC, ■ : SLS, ▲ : NaTDC.

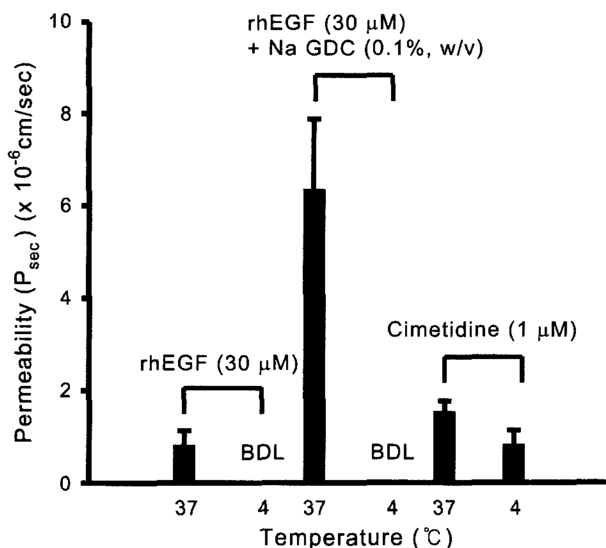

Fig. 2. Effect of temperature on the A-B permeability of rhEGF (30 µM) and cimetidine (1 mM) across Caco-2 cell monolayers. BDL: below the detection limit.

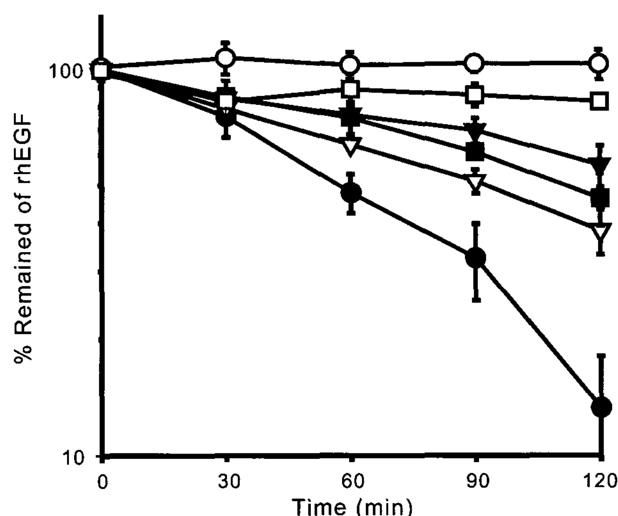

Fig. 3. Representative examples of the effect of excipients (1%, w/v) on the stability (% remained) of rhEGF (30 µM) in the transport medium (pH 7.4) on Caco-2 cell monolayers. ●: Control (*i.e.*, without excipients), ▽: NaGDC ■ : NaTCDC, ▼ : NaTDC, □: NaC10, ○: SLS.

Artursson and Karlsson, 1991), consistent with poor GI absorption of rhEGF observed in rats. Thus, an accelerated permeability would be highly desirable for an acceptable level of GI absorption. The P_{app} of rhEGF decreased dramatically to a negligible level at 4°C (Fig. 2, left two columns), compared to the case of cimetidine, a representative compound transported by passive diffusion (Pade and Stavchansky, 1997) (Fig. 2, right two columns). The above results are consistent with the involvement of receptor mediated transcytosis (RMT) (Hidalgo *et al.*, 1989; Tong *et al.*, 1998) in the transport of rhEGF in Caco-2 cells. This aspect should be taken into account in any attempts to accelerate the permeability of rhEGF.

Effect of excipients on the A-B transport of rhEGF across Caco-2 cell monolayers

Regardless of the mechanism involved, the temporal A-B transport of rhEGF across Caco-2 cell monolayers was increased in the presence of some excipients, as shown for representative examples in Fig. 1, and the P_{app} of which are summarized in Table I. For example, 23, 21, 20 and 16-fold increases could be achieved in the presence of NaTCDC, NaTDC, NaGDC and SLS (1% w/v each), respectively (Fig. 1, Table I). The presence of excipients simultaneously increased the paracellular leakage (or P_{app}) of mannitol (up to more than 90-fold for 1% NaGDC and 1% NaTCDC, for example) and inulin (up to 131-fold for 1% NaTDC, for example) and a corresponding decrease in TEER values (Table I) occurred, consistent with previous reports (Lillienau *et al.* (1992) on bile salts, Anderberg and Artursson (1993) on SLS and NaC10). This indicates that the reduced integrity of intercellular tight junctions of Caco-2 cells might be involved in the increased P_{app} of rhEGF.

Effect of excipients on the stability of rhEGF in the transport medium on the Caco-2 cell monolayer

Peptides and proteins are generally unstable in gastrointestinal fluid, leading to poor bioavailability of the compound *in vivo*. Thus, the stability as well as permeability needs to be considered in any attempts to increase the bioavailability of such compounds. As expected, the degradation of rhEGF in the transport medium was rapid in the absence of excipients: more than 85% of the initial concentration underwent degradation in 2 h of the incubation (Fig. 3). This degradation can be largely attributed to the cell mediated metabolism of rhEGF because no such significant degradation was observed in the transport medium in the absence of cell monolayers (*i.e.*, on the polycarbonate insert). The degradation was accelerated as the concentration of rhEGF was decreased consistent with typical enzyme kinetics, also consistent with cell mediated metabolism. The degradation was substantially retarded by the presence of various excipients. Among the excipients examined, 1% SLS was the most effective (*i.e.*, no degradation in 2 h of the incubation), followed by 1% NaC10, 1% NaTDC and 0.5% DM β-CD (Table I, partly shown in Fig. 3).

DISCUSSION

In the presence of some excipients, especially deoxycholate bile salts, NaGDC, NaTDC and NaTCDC, more

than 20-fold increase in the permeability of rhEGF could be achieved. However, a simultaneous increase in the permeability of mannitol and inulin, as well as a decrease in the TEER value, also occurred, suggesting that the increase in the permeability of rhEGF, in the presence of some excipients, is related to a break down in the integrity of the cell membrane. In this regard, the application of these excipients as practical absorption enhancers would be strongly limited. Fig. 4 shows a relationship between the mannitol leakage and the permeability of rhEGF in the presence of various excipients. A linear relationship was obtained suggesting the involvement of paracellular transport as a factor in increasing in the permeability of rhEGF. However, the slope of the relationship was larger than unity (*i.e.*, 1.3), and was much greater than the slope (*i.e.*, 0.03) of the relationship between mannitol leakage and the permeability of inulin. This indicates that rhEGF, a compound with a Mw of 6 KDa, is more efficiently transported than a similar Mw compound such as inulin (Mw of 5000), which is transported via the paracellular route. Thus, a transport mechanism other than paracellular leakage is needed to explain the transport of rhEGF, consistent with the RMT hypothesis. The inset of Fig. 4 shows the relationship between the rates of increase in the permeation of rhEGF and inulin as a function of the increased rate of mannitol leakage. Again, a linear relationship was observed between rhEGF and inulin, but the slope of the relationship was far less than unity (*i.e.*, 0.2), indicating a less significant increase in the permeability of rhEGF compared to the leakage of mannitol. On the other hand the ratio of the increase in the permeability of inulin was comparable to that of mannitol (inset of Fig. 4, Table I). Thus the excipients appear to open the paracellular route of the cell monolayer, which is 5-6 Å (Knipp *et al.*, 1997) in molecular radius in the absence of excipients, to an extent that allows the permeation of mannitol (3.6 Å) and inulin (11 Å), but not wide enough for the permeation of rhEGF, a large compound with a Mw of 6 KDa. This conclusion is consistent with the report that only substances with molecular weights below 3.5 KDa (15 Å) freely penetrate via the paracellular route (Cromwell *et al.*, 1996).

The P_{app} of rhEGF, when measured at 37°C, was increased 8-fold in the presence of 0.1% NaGDC (Fig. 2), but was decreased dramatically to a negligible level at 4°C (Fig. 2). Such a dramatic decrease in permeability was not observed for cimetidine, a drug that is transported via passive diffusion (Fig. 2, right columns). These results (Fig. 2) support the involvement of RMT in the permeation of rhEGF even in the presence of excipients. In other words, the excipients appear to accelerate the permeation of rhEGF possibly via RMT, but much less compared to the permeation of mannitol and inulin. The increase in the flipping of responsible receptors in the membrane, as the

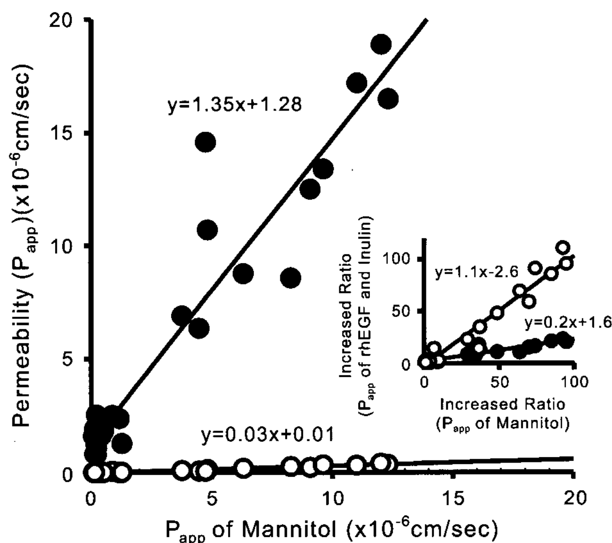

Fig. 4. Relationship between mannitol leakage and A-B permeability of rhEGF (●, 30 μM) and inulin (○, 40 nM) across Caco-2 cell monolayers in the presence of various excipients. The inset indicates the relationship between the increased ratios of corresponding compounds.

result of increased membrane fluidity by the excipients, might represent possible explanations for these findings. The increase in membrane fluidity by the excipients is consistent with the most profound increase (more than 40-fold for 1% NaTDC and 1% NaTCDC, for example) in the permeability of cimetidine, compared to mannitol, inulin and rhEGF (Table I). In summary, paracellular diffusion and/or RMT of rhEGF appear to be increased by excipients, but the increase was minimal compared to mannitol (Fig. 4), inulin and cimetidine (Table I).

The stability of rhEGF could be increased to a satisfactory level by the addition of appropriate excipients, exhibiting the most increase by 1% SLS (*i.e.*, no degradation in 2 h of the incubation), followed by 1% NaC10, 1% NaTDC and 0.5% DM β-CD (Table I, partly shown in Fig. 3). The effect of SLS, NaC10 and bestatin appear to be associated with the inhibition of deamination of the asparagine residue of rhEGF (Son and Kwon, 1995), the inhibition of leucine aminopeptidase activity (Sakai *et al.*, 1997), and the inhibition of selective aminopeptidase B in Caco-2 cells (Han *et al.*, 1998), respectively. The effect of cyclodextrins might be due either to a direct interaction with rhEGF or to complexation with other components that may cause peptide degradation (Haeberlin *et al.*, 1996). KGrZ, HPMC, NaGC, NaTC, sorbitol (an excipient often added to formulations in order to improve the stability of human growth hormone, Costantino *et al.*, 1998) and NaCMC had no significant effect on the stability of rhEGF (Table I).

In the present study, we examined the feasibility of sixteen

excipients as absorption enhancers. The results demonstrate that some excipients increase the stability and permeability of rhEGF increasing the P_{app} up to a level of 1.9×10^{-5} cm/sec, implying a favorable GI absorption of rhEGF in humans. Unfortunately, however, this could not be achieved without affecting the integrity of the cell membranes, as evidenced by a considerable decrease in TEER values and a simultaneous increase in mannitol leakage and the permeability of inluin. Excipients that are specific to the permeation of rhEGF or RMT could not be identified in the present study. Such excipients should be pursued in order to enhance the GI absorption of rhEGF without disturbing the integrity of the intestinal epithelial cells or *in vivo* safety.

ACKNOWLEDGMENT

This work was supported by the Korean Research Foundation (99-005-F00033).

REFERENCES

Anderberg, E. K. and Artursson, P., Epithelial transport of drugs in cell culture. VIII: Effects of sodium dodecyl sulfate on cell membrane and tight junction permeability in human intestinal epithelial (Caco-2) cells. *J. Pharm. Sci.,* 82, 392-398 (1993).

Anderberg, E. K., Nyström, C., and Artursson, P., Epithelial transport of drugs in cell culture. VII: Effect of pharmaceutical surfactant excipients and bile acids on transepithelial permeability in monolayers of human intestinal epithelial (Caco-2) cells. *J. Pharm. Sci.,* 81, 879-887 (1992).

Artursson, P. and Karlsson, J., Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem. Biophys. Res. Commun.,* 175, 880-885 (1991).

Augustijns, P. F., Bradshaw, T. P., Gan, L. S. L., Hendren, R. W., and Tharkler, D. R., Evidence for a polarized efflux system in Caco-2 cells capable of modulating cyclosporin A transport. *Biochem. Biophys. Res. Commun.,* 197, 360-365 (1993).

Carpenter, G. and Cohen, S., Epidermal growth factor (EGF). *Ann. Rev. Biochem.,* 48, 193-216 (1979).

Costantino, H., Carrasquillo, K. G., Cordero, R. C., Mumenthaler, M., Hsu, C. C., and Griebenow, K., Effect of excipients on the stability and structure of lyophilized recombinant human growth hormone. *J. Pharm. Sci.,* 87, 1412-1419 (1998).

Cromwell, R. M. E. M., Shahrokh, Z., Villagran, J., Nguyen, N. Y., Wellton, M., Nguyen, T. H., and Mrsny, J., Flux measurements across Caco-2 monolayers may predict transport in human large intestinal tissue. *J. Pharm. Sci.,* 85, 165-169 (1996).

Haeberlin, B., Gengenbacher, T., Meinzer, A., and Fricker, G., Cyclodextrins-Useful excipients for oral peptide administration. *Int. J. Pharm.,* 137, 103-110 (1996).

Han, K., Choi, M. S., and Chung, Y. B., Site-specific degradation and transport of recombinant human epidermal growth factor (rhEGF) in the rat gastrointestinal mucosa. *Int. J. Pharm.,* 168, 189-197 (1998).

Hidalgo, I. J., Kato, A., and Borchardt, R. T., Binding of epidermal growth factor by human colon carcinoma cell (Caco-2) monolayers. *Biochem. Biophys. Res. Commun.,* 160, 317-324 (1989a).

Hidalgo, I. J., Raub, T. J., and Borchardt, R. T., Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterol.,* 96, 736-749 (1989b).

Knipp, G. T., Ho, N. F. H., Barsuhn, C. L., and Borchardt, R. T., Paracellular diffusion in Caco-2 cell monolayers: Effect of perturbation on the transport of hydrophilic compounds that vary in charge and size. *J. Pharm. Sci.,* 86, 1105-1110 (1997).

Konturek, P. C., Konturek, S. J., Brzozowski, T., and Ernst, H., Epidermal growth factor and transforming growth factor-alpha: Role in the protection and healing of gastric mucosal lesions. *Eur. J. Gastroenterol. Hepatol.,* 7, 933-937 (1995).

Lillienau, J., Schteingart, C. D., and Hofmann, A. F., Physico-chemical and physiological of cholylsarcosine, a potential replacement detergent for bile acid deficiency states in the small intestine. *J. Clin. Invest.,* 89, 420-431 (1992).

Lindmark, T., Nikkilä, T., and Artursson, P., Mechanism of absorption enhancement by medium chain fatty acids in intestinal epithelium Caco-2 cell monolayers. *J. Pharm. Exp. Ther.,* 275, 958-964 (1995).

Merkus, W. H. M. M., Verhoef, J. C., Romeijin, S. G., and Schipper, N. G. M., Absorption enhancing effect of cyclodextrins on intranasally administered insulin in rats. *Pharm. Res.,* 8, 588-592 (1991).

Michael, S., Thöle, M., Dillmann, R., Fahr, A., Drewe, J., and Fricker, G., Improvement of intestinal peptide absorption by a synthetic bile acid derivative, cholylsarcosine. *Eur. J. Pharm. Sci.,* 10, 133-140 (2000).

Murakami, T, Kishimoto, M., Kawakita, M., and Kisaki, M., Enhanced rectal and nasal absorption of human epidermal growth factor by combined use of the absorption promotor and the synthetic polymer in rats. *Eur. J. Drug. Metab. Pharmacokinet.,* 3, 125-131 (1991).

Murakami, T., Kawakita, H., Kishimoto, M., Higashi, Y., Amagase, H., Hayashi, T., Nojima, N., Fuwa, T., and Yata, N., Intravenous and subcutaneous pharmacokinetics and rectal bioavailability of human epidermal growth factor in the presence of absorption promotor in rats. *Int. J. Pharm.,* 46, 9-17 (1988).

Murakami, T., Misaki, M., Kojima, Y., Yamada, M., Yuki, M., Higashi, Y., Amagase, H., Fuwa, T., and Yata, N., Effect of absorption promoters on subcutaneous absorption of human epidermal growth factor in rats. *J. Pharm. Sci.,* 82, 236-239 (1993).

Okada, H., Yamazaki, I., Ogawa, Y., Hirai, S., Yashiki, T., and Mima, H., Vaginal absorption of a potent luteinizing hormone-

releasing hormone analog (leuprolide) in rats I: absorption by various routes and absorption enhancement. *J. Pharm. Sci.,* 71, 1367-1371 (1982).

Pade, V. and Stavchansky, S., Estimation of the relative contribution of transcellular and paracellular pathway to the transport of passively absorbed drugs in the Caco-2 cell culture model. *Pharm. Res.,* 14, 1210-1215 (1997).

Palomino, A., Hernandez-Bernal, F., Haedo, W., Franco, S., Mas, J. A., Fernandez, J. A., Soto, G., Alonso, A., Gonzalez, T., and Lopez-Saura, P., A multicenter, randomized, double-blind clinical trial examining the effect of oral human recombinant epidermal growth factor on the healing of duodenal ulcers. *Scand. J. Gastroenterol.,* 35, 1016-1022 (2000).

Sai, Y., Kajita, M., Tamai, I., Wakama, J., Wakamiya, T., and Tsuji, A., Adsorptive-mediated transcytosis of a basic peptide, 001-C8 in Caco-2 cells. *Pharm. Res.,* 15, 1305-1309 (1998).

Sakai M., Imai, T., Ohtake, H., Azuma, H., and Otagiri, M., Effects of absorption enhancers on the transport on model compounds in Caco-2 cell monolayers: Assessment by confocal laser scanning microscopy. *J. Pharm. Sci.*, 7, 779-785 (1997).

Scipper, N. G. M., Romeijn, S. G., Verhoef, J. C., and Merkus, F. W. H. M., Nasal insulin delivery with dimethyl-β-cyclodextrin as an absorption enhancer in rabbits: Powder more effective than liquid formulations. *Pharm. Res.,* 10, 682-686 (1993).

Shen, W. H. and Xu, R. J., Stability and distribution of orally administered epidermal growth factor in neonatal pigs. *Life Sci.,* 63, 809-820 (1998).

Son, K. and Kwon, C., Stabilization of human epidermanl growth factor (rEGF) in aqueous formulation. *Pharm. Res.,* 12, 451-454 (1995).

Tarnawski, A., Stachura, J., Krause, W. J., Douglass, T. G., and Gergely, H., Quality of gastric ulcer healing: A new, emerging concept. *J. Clin. Gastrienterol.,* 13 (Suppl 1), S42-S47 (1991).

Tong, W. M., Ellinger, A., Shieinin, Y., and Cross, H. S., Epidermal growth factor receptor expression in primary cultured human colorectal carcinoma cells. *Br. J. Cancer,* 77, 1792-1798 (1998).