Investigation of Transport Mechanism of Exendin-4 across Madin Darby Canine Kidney Cell Monolayers

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The purpose of this study was to investigate the transport mechanism of exendin-4 using Madin Darby canine kidney (MDCK) cell monolayer as an in vitro model of the human intestinal barrier. The roles of active and passive mechanisms of exendin-4 in the cell models were well studied and the corresponding contributions of the transcellular and paracellular pathway to exendin-4 transport were also evaluated. Moreover, the apparent permeability coefficient (Papp) values of exendin-4 were determined in the presence of chitosan, sodium decanoate and ethylenediaminetetraacetic acid (EDTA) to further confirm the relative transport mechanism and to evaluate their potential utility in future formulation design. The results revealed the low transport capacity of exendin-4 (Papp 0.10±0.06×10⁻⁶ cm/s). And exendin-4 transport across the cell models was time and concentration-dependence, direction and energy-independent, and similar to the passive transport marker. Drug efflux and active transport were not observed. In the presence of absorption enhancers, the Papp value significantly increased up to 2.2–11.9 folds without apparent cytotoxicity, which is comparable to that of the paracellular transport marker. And the order of enhancement was to the effect of chitosan>EDTA>sodium decanoate, and the order of safety was sodium decanoate=chitosan>EDTA. These findings demonstrated that exendin-4 transport across MDCK cell monolayer mainly by passive paracellular pathway, which agrees with the result of confocal laser scanning microscopy. And these absorption enhancers can be used as potential safe ingredients to improve oral efficacy of exendin-4.

Key words: peptide; transepithelial transport mechanism; paracellular transport; absorption; enhancer

Exendin-4 is a glucagon-like peptide-1 (GLP-1) receptor agonist possessing glucoregulatory activities similar with the mammalian GLP-1, consisting of 39 naturally-occurring amino acids, which is clinically used for the treatment of type 2 diabetes by subcutaneous injection. Exendin-4 has been found to show many beneficial antidiabetic bioactivities including induction of glucose-dependent insulin secretion, suppression of high glucagon secretion, slowing of gastric emptying to modulate nutrient absorption, reduction of food intake and body weight, improving pancreatic endocrine function and increasing β-cell mass. Exendin-4 parenteral solution has been approved by U.S. Food and Drug Administration (FDA) for the treatment of type 2 diabetes in 2005. However, its therapeutic application is limited due to the incompliance caused by twice-daily subcutaneous injections, such as pain and expensive costs. To improve patient compliance, many studies focused on the development of its oral administration.

However, the development of oral exendin-4 preparations remains to be a huge challenge. A previous report revealed that the oral bioavailability of exendin-4 solution was too low (0.0053%) to suitable for oral route of administration. One of the most important factors related to the oral absorption of a drug is its permeability across the gastrointestinal lining. Currently, a few approaches have been employed to improve the mucosal permeation of exendin-4, including conjugation or physical complex with cell penetrating peptides, chemical modification with biotin, and exploitation of certain carrier systems such as chitosan nanoparticles. Although the oral bioavailability and bioactivity were improved to some extent compared with exendin-4 solution in above studies, there is no detail information on the mechanism of its absorption across the human epithelial intestinal barrier. However, understanding the permeation behavior of exendin-4 in the intestine will be beneficial to enhance its oral efficacy, which is also closely related to the design of peroral formulations.

The aim of the present study was to investigate the capacity and characteristics of exendin-4 transport across the Madin Darby canine kidney (MDCK) cell monolayer model, and to reveal its absorption mechanism in intestinal tract. In general, drug absorption across the intestinal epithelium may occur through concentration gradient-driven passive diffusion. For hydrophilic and large molecules, such as peptides, which have the poor distribution into the cell membrane, transportance via paracellular pathway is generally involved. However, some peptides can also be absorbed through intestine via active transports. We sought to confirm which premise are true in the case of exendine-4. The objectives of this study were: (1) to determine whether exendin-4 is absorbed by passive or active way; (2) if passive way, to confirm the contributions of transcellular and paracellular pathway mainly involved in exendin-4 transport; (3) the effect of absorption enhancers, chitosan, sodium decanoate and ethylenediaminetetraacetic acid (EDTA) on the transport of exendin-4. Our study may shed light on the successful development of exendin-4 oral formulations in the future.

MATERIALS AND METHODS

Materials: Exendin-4 was purchased from GL Biochem
Co., Ltd. (Shanghai, China). Propranolol and digoxin were purchased from NICPBP (Beijing, China). Fluorescein, chitosan (CS) and sodium decanoate (C10) were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), non-essential amino acids solution (NEAA), penicillin–streptomycin solution, trypsin solution, were obtained from Invitrogen (Carlsbad, CA, U.S.A.). 24-well Millicell (0.6 cm² and 0.1 µm) was purchased from Millipore Corporation. Twenty four-well cluster trays were purchased from the CostarCorning Corporation. Exendin-4 and fluorescein isothiocyanate (FITC)-exendin-4 were purchased from GL Biochem Co., Ltd. (Shanghai, China). 1,1’-Dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) was from Molecular Probes Inc. (Carlsbad, U.S.A.). All other chemical products were commercially available and of analytical grade.

Methods. Analysis Methods The quantification of exendin-4 was performed by Agilent 1200-6410 Triple Quadrupole LC-MS/MS system (Agilent Technologies, U.S.A.). The method of detection was outlined in the paper by Kehler et al.15 The amount of propranolol and digoxin were measured by the HPLC (Agilent 1200, U.S.A.). The detection condition of HPLC was described elsewhere.6,17 Concentration of fluorescein was determined with Fluoroskan Ascent FL (Thorno, U.S.A.).

Cell Culture The MDCK cell lines were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown and maintained in DMEM supplemented with 10% FBS, 1% NEAA, and 1% penicillin-streptomycin solution. The cultures were kept at 37°C, 5% CO₂ humified incubator.

For transport studies, cells were seeded at a density of 5×10⁴ cells/cm² onto 24-wells Millicell. The medium was replaced every day. Following an incubation period in 6–8 d in culture, the cell monolayers were used for drug transport experiments. MDCK and Caco-2 cells with the passage numbers of 70–78 and 52–54 were used in this study, respectively. The formation of confluent monolayers and tight junctions was confirmed by measuring the paracellular transportation of fluorescein and the transepithelial electrical resistance (TEER) using a Millicell ERS® voltmeter (Millipore, U.S.A.). The MDCK cell monolayers that displayed a TEER of 400–600 Ω cm² with the blank TEER values were used in the experiments. The Caco-2 cell monolayers that displayed a TEER of 400–600 Ω cm² were used in the experiments. The apparent permeability coefficient \( P_{app} \) (cm/s) of the compounds was calculated by the following equation:

\[
P_{app} (\text{cm/s}) = \frac{(dQ / dt) \times (1/A \times C_d)}{A}
\]

Where \( dQ/dt \) is the increase in the amount of drug in the receiver chamber per time interval, \( C_d \) is the initial concentration in the donor compartment, \( A \) is the permeation area of the cell culture insert.

To investigate the effect of energy depletion on \( P_{app} \) values, sodium azide (Na₃N, 10 mM), sodium fluoride (NaF, 2 mM) and 2-deoxyglucose (2-DG, 50 mM) were added to both compartments. After 30 min incubation at 37°C, medium was removed, 200 µM exendin-4 and 10 µM digoxin were added to the basolateral compartment, respectively, and the \( P_{app} \) values were determined.

Cell Uptake Study At the end of the transport experiment (2h), the amount of compounds taken up by cell was detected. For this assay, monolayer cells were washed three times with ice-cold buffer, and repeatedly froze and thawed in −80°C and 37°C for 5 times. The cell lysate was then centrifuged at 10000 rpm for 5 min, and the concentration of the compound in the supernatant was assayed.

The Influence of Absorption Enhancer CS, C10 and EDTA on the Transport The MDCK cell monolayers were washed three times with HBSS or HBBS without Ca²⁺/Mg²⁺ (D-HBBS) and equilibrated for 30 min at 37°C prior to performing the experiment. CS solution (0.1%) and C10 solution (1 mM) were prepared using HBSS and EDTA solution (0.1 mM) was prepared with D-HBSS. Exendin-4 solution (200 µM), fluorescein solution (1 mM) and propranolol solution (25 µM) with/without absorption enhancer were added to the apical compartment for 2h at 37°C. Transport studies were performed and the \( P_{app} \) value was measured and calculated under various conditions.

TEER Measurement The TEER was measured before and after the transport experiment. After experiment the monolayers were washed three times with HBBS and filled up with complete medium for 24 h and the TEER values were measured to evaluate the regeneration of cell monolayers. The TEER values measured before the experiment were taken as the initial values expressed as 100%. All subsequent TEER values are expressed as a percent of the initial value. The TEER values (Ω cm²) were calculated according to the following equation:

\[
\text{TEER} = (R - R_{blank}) \times A
\]

Where \( R \) is the measured resistance across a cell monolayer, \( R_{blank} \) is the resistance of a blank well, and \( A \) is the surface area of the Millicell filter (0.6 cm²).

Lactate Dehydrogenase (LDH) Measurement At the end of the experiment, the release of LDH in the apical solutions in which cells treated with or without absorption enhancers was determined using LDH kit (CytoTox96® non-radioactive cytoxicity Promega Corporation, Madison, WI, U.S.A.) to assess the damage of cell monolayers. Cells treated with the cytotoxic reagent was used as positive control in whose value was set as 100%.

Confocal Laser Scanning Microscopy MDCK cells were incubated with HBBS containing 125 mM of FITC-exendin-4 at 37°C for 4h. Cells were then washed 5 times with HBBS and then were fixed with 4% paraformaldehyde for 30 min,
and it was counterstained with 5 µM DiI for 20 min. The fixed cells were observed using an Olympus FV1000 Confocal Laser Scanning Biological Microscope (CLSM). The excitation wavelength of FITC and DiI was 488 nm and 543 nm, respectively.

Statistical Analysis All results are expressed as mean±S.D. (standard deviation) of five replicates. Statistical comparisons were performed by Student’s t-test for two groups with a p value of less than 0.05 being considered statistically significant.

RESULTS

Time and Concentration Effect of Exendin-4 Transport To confirm whether exendin-4 transport has character of saturation, the time course of exendin-4 (200 µM, 4 h) transport across MDCK cell monolayers was studied at 37°C in the apical to basolateral (AP-to-BL) direction (Fig. 1). The amount of exendin-4 transported increased linearly from 0 to 4 h with no latent period (R=0.99), indicating no saturable phenomenon occurred over the time period studied.

The effect of concentration on the exendin-4 transporting across MDCK cell monolayers in the AP-to-BL direction after 2 h incubation at 37°C was investigated (Fig. 2). It can be seen, the cumulative permeation amount of exendin-4 across the cell monolayers was gradually increased with the increasing concentration in AP side. The transport of exendin-4 showed linearity within a concentration range of 50–500 µM (R=0.94), and was not saturated over the entire concentration range examined. The Papp (0.11–0.15×10⁻⁶ cm/s) was found to be almost unchanged throughout the concentration investigated.

These results showed that exendin-4 transport across MDCK cell monolayers was time and concentration-dependent, which were the typical characteristics of passive diffusion.18–20

Energy-Related Effect of Exendin-4 Transport In order to determine the possible involvement of an active transport mechanism in exendin-4 transport across MDCK cell monolayers, the Papp of exendin-4 (200 µM, 2 h) in the AP-to-BL direction was measured at 37°C and 4°C, respectively, meanwhile the Papp of fluorescein and propranolol were measured as controls. Fluorescein and propranolol are generally acknowledged markers of paracellular and transcellular passive permeability, respectively. Changing temperature should have no or slight effect on their transport because passive diffusion does not require energy.18 As shown in Fig. 3, the transport of exendin-4 was decreased but not abolished at 4°C, its Papp was reduced 2.0-fold compared with that at 37°C. The similar effect of temperature was also found in control compounds, the Papp of fluorescein and propranolol were reduced 2.1-fold and 3.2-fold, respectively. The Papp value of exendin-4 through MDCK cell monolayers at 37°C from AP to BL was 0.10±0.06×10⁻⁶ cm/s (shown in Fig. 3), a permeability value even lower than that of fluorescein (1.28±0.09×10⁻⁶ cm/s), but significantly lower than that of propranolol (23.22±0.15×10⁻⁶ cm/s). Further studies were conducted using Caco-2 cell monolayers which are more sufficient than MDCK in the expression of efflux and active transporters.21 The results were shown in Table 1, which indicated that the transport of exendin-4 probably did not involve an energy-dependent active transport. In addition, the efflux ratio (P_{B→A}/P_{A→B}) of

Table 1. Bidirectional Transport and the Effect of ATP-Depletion on Papp of Exenatide and Digoxin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bidirection</th>
<th>ATP-depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P_{app}×10⁻⁶ (cm/s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AP-BL</td>
<td>BL-AP</td>
</tr>
<tr>
<td>Digoxin</td>
<td>0.07±0.01</td>
<td>0.57±0.05***</td>
</tr>
<tr>
<td>Exenatide</td>
<td>0.027±0.006</td>
<td>0.030±0.002</td>
</tr>
</tbody>
</table>

***p<0.001, significant difference from control. Values are expressed as the mean±S.D. for 3–4 different cell monolayers.
exendin-4 suggested no direction-dependent transport.

Taken together, exendin-4 transport across \textit{in vitro} cell monolayers was not energy-dependent, which is similar to that of passive diffusion markers. It also showed that no direction-dependent transport involved, and low $P_{\text{app}}$ value is similar to that of paracellular marker. All of these indicated that the mechanism of exendin-4 absorption across MDCK cell monolayers was passive transport, and likely through paracellular pathway.

**Cellular Uptake of Exendin-4** To investigate to which extent the paracellular and transcellular pathways contributed to the passive transport of exendin-4 (200 $\mu$M, 2 h) across MDCK cell monolayers, the intracellular content of exendin-4 and its total accumulation in the basolateral compartment were measured after 2 h incubation. The results in Table 2 showed that the amount of exendin-4 incorporated within the cells was 0.05% of the dose initially added to the AP compartment, the ratio of cellular uptake and total accumulation in the basolateral was 0.17. Meanwhile the ratio of fluorescein uptake into cells and total accumulation in the basolateral was 0.16, and that of propranolol was 0.37. These results demonstrated that paracellular pathway was mainly involved in the passive transport of exendin-4 rather than transcellular transport.

**Confocal Laser Scanning Microscopy** The transport pathway of exendin-4 across MDCK cell model was also studied using confocal laser scanning microscopy with FITC-modified exendin-4. Figures 4a–c is a series of three images of a MDCK monolayer under a confocal laser scanning microscope. Figure 4b provided the interesting insight to membrane outline due to the localization of DiI (light gray) in the cell membrane of MDCK monolayer. The green fluorescence in Fig. 4a resulted from FITC conjugated to exendin-4. Figure 4c is a composite of Figs. 4a and b. As evidenced in Fig. 4c, the light gray fluorescence resulted from FITC-exendin-4 is located between the intercellular spaces, which clearly shows the paracellular pathway of exendin-4 across MDCK cell monolayer.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Uptake (%)$^a$</th>
<th>Transport (%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exenatide</td>
<td>0.05±0.02</td>
<td>0.30±0.01</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>0.31±0.10</td>
<td>1.98±0.20</td>
</tr>
<tr>
<td>Propranolol</td>
<td>18.52±0.16</td>
<td>50.12±0.01</td>
</tr>
</tbody>
</table>

$^a$ The percentage of the amount taken up into cell monolayers. $^b$ The percentage of the amount transported from apical to basolateral compartment. Values are expressed as the mean±S.D. for five different cell monolayers.

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**Fig. 3. Temperature Effect on the Transport of Exendin-4 across MDCK Cell Monolayer**

The $P_{\text{app}}$ of (A) exendin-4, (B) fluorescein and (C) propranolol transport from AP to BL compartment was measured at 37°C and 4°C and Open and hatched columns represent the different transport temperature. The concentrations of exendin-4, fluorescein and propranolol were 200 $\mu$M, 1 $\mu$M and 25 $\mu$M, respectively. Results are represented as the mean±S.D. of five different cell monolayers.

**Fig. 4. Confocal Micrograph Images of DiI-Stained MDCK Cell Monolayers Incubated with FITC-Exendin-4 for 4 h at 37°C**

FITC-exendin-4 (light gray) for drug, DiI (light gray) for cell membrane.
Table 3. The Effect of Absorption Enhancers on $P_{app}$ of Exenatide, Fluorescein and Propranolol

<table>
<thead>
<tr>
<th>Compound</th>
<th>D-HBSS $P_{app} \times 10^{-6}$ (cm/s)</th>
<th>HBSS $P_{app} \times 10^{-6}$ (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control$^a$</td>
<td>EDTA$^b$</td>
</tr>
<tr>
<td>Exenatide</td>
<td>0.36±0.02</td>
<td>3.74±0.17***</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>3.57±0.03</td>
<td>36.49±1.12***</td>
</tr>
<tr>
<td>Propranolol</td>
<td>14.86±0.83</td>
<td>15.76±0.12</td>
</tr>
</tbody>
</table>

$^a)$ Without any absorption enhancers. $^b)$ The group of EDTA was performed in D-HBSS, and the groups of CS, C10 were performed in HBSS. $**p<0.001$; significant difference from control. Values are expressed as the mean±S.D. for five different cell monolayers.

Table 4. The Effect of Absorption Enhancers on TEER and LDH Release

<table>
<thead>
<tr>
<th></th>
<th>TEER (Ωcm$^2$) 0 h</th>
<th>TEER (Ωcm$^2$) 2 h</th>
<th>TEER (Ωcm$^2$) 5 h</th>
<th>TEER (Ωcm$^2$) 10 h</th>
<th>LDH (%) 2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>292±6.11</td>
<td>292±4.04</td>
<td>306±7.57</td>
<td>302±7.50</td>
<td>7.03±1.15</td>
</tr>
<tr>
<td>Exendin-4</td>
<td>309±9.71</td>
<td>305±4.16</td>
<td>325±5.69</td>
<td>299±9.07</td>
<td>6.95±1.52</td>
</tr>
<tr>
<td>CS</td>
<td>300±17.00</td>
<td>116±3.60***</td>
<td>219±4.16**</td>
<td>310±11.10</td>
<td>7.26±1.55</td>
</tr>
<tr>
<td>C10</td>
<td>273±14.57</td>
<td>120±0.58***</td>
<td>133±5.29**</td>
<td>265±10.07</td>
<td>6.34±1.50</td>
</tr>
<tr>
<td>EDTA</td>
<td>308±2.52</td>
<td>30.3±6.51***</td>
<td>47.3±2.09**</td>
<td>125±3.05***</td>
<td>6.90±1.30</td>
</tr>
</tbody>
</table>

$p$ value for double comparison was compared with the group of HBSS. $**p<0.01$, $***p<0.001$; significant difference from control. Values are expressed as the mean±S.D. for five different cell monolayers.

Transport after Alteration of the Tight Junction Barrier. To further confirm the passive paracellular transport mechanism of exendin-4 across MDCK cell monolayers and to evaluate the potential utility of absorption enhancers in future formulation design, the $P_{app}$ of exendin-4 (200 μM, 2h) in the AP-to-BL direction at 37°C was measured after the tight junction of cells were opened (shown in Table 3). CS, C10 and EDTA were added with different concentration (based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) data) to alter the tight junction. The cytotoxicity of the three kinds of these enhancers to MDCK cell monolayers was evaluated by measuring the recovery of TEER value and release of LDH (results shown in Fig. 5 and Table 4).

By the treatment with 0.1 mM EDTA in the D-HBSS (2h), the TEER of MDCK cell monolayers reduced to about 10% of the original value and gradually recovered after removal of EDTA during 24h, indicated the reversible opening of cell tight junctions. After EDTA treatment, the $P_{app}$ of exendin-4 was considerably increased by approximately 10.4-fold compared with that under the condition of untreated cell monolayers. A similar transport enhancing was found in the study of paracellular marker fluorescein (10.2-fold).

However, EDTA treatment has no significant effect ($p>0.05$) on the $P_{app}$ of transcellular maker propranolol. The $P_{app}$ values of exendin-4 and fluorescein in D-HBSS untreated with enhancers were significantly higher compared with those in HBSS, which also resulted from the effect of Ca$^{2+}$ deficiency in D-HBSS on tight junctions of cell monolayers. These data also suggested that the paracellular route through the tight junctions was the major pathway for the transport of exendin-4 across the MDCK cell monolayers.

In this experiment, exendin-4 transport also was studied in the HBSS containing 0.1% CS and 1 mM C10, respectively. The $P_{app}$ of exendin-4 across MDCK cell monolayer was considerably increased in the presence of CS and C10 with 11.9-fold and 2.2-fold, respectively. The similar remarkable enhancing effect of CS and C10 were found at the study of paracellular marker fluorescein (5.1-fold and 1.5-fold, respectively). However, there was no significant effect observed on the permeation of transcellular maker propranolol ($p>0.05$). These results further confirmed the passive paracellular transport mechanism of exendin-4 across the MDCK cell monolayers.

As shown in Fig. 5, at the end of transport experiment, the TEER value was reduced to about 40% of the original value by inclusion of CS and C10 for 2h, and the decreased TEER value indicated the opening of cell tight junctions. After removal of CS and C10, TEER value was gradually going up, and almost recovered completely at 10h. However, at the same time point (at 10h), the TEER value of EDTA group was still markedly lower than the original value ($p<0.05$) as shown in Table 4. The amounts of LDH released of CS, C10 and EDTA groups had no significant difference compared with those of HBSS control group ($p>0.05$), suggested that above enhancers on the concentrations used in these studies showed no cytotoxicity. These data indicated that CS, C10 and EDTA on these concentrations were proved...
to significantly increase exendin-4 transport, the order of enhancement effect was CS->EDTA>C10, and the order of safety was C10>CS->EDTA.

DISCUSSION

Exendin-4 is the first GLP-1 mimetic drug approved by U.S. FDA for clinical use, with noticeable curative effect on type 2 diabetes. However, to date, exendin-4 is only available in injection forms in clinic, including the novel PLGA microsphere Bydureon™ (a long-acting formulation), though considerable attention has been given to other formulations. Injection route has potential disadvantages in therapeutic utility, such as pain and expensive costs. One of the favored routes of exendin-4 administration is oral delivery due to its patient compliance and acceptance. However, because many peptides are highly ionized and hydrophilic, their capability of permeating intestinal epithelium barriers is poor. So attempts to understand the permeation behavior of peptides in the intestine are very important to improve their oral efficacy. To our knowledge, the present study is the first report which provides the detail information of the trans-membrane capacity and related transport mechanisms of exendin-4 across in vitro cell model.

In this study, MDCK cell monolayer was chosen as in vitro model. Since oral absorption capacity is a crucial selection criterion at the early stage of drug development and formulation screening, there is an urgent need for quick and reliable drug absorption assessment models. During the past few decades, the Caco-2, MDCK, LLC-PK1and HT-29-H cell monolayers have been gradually accepted as in vitro permeability models. Although Caco-2 cell monolayers have been approved by FDA as a standard permeability screening assay to predict the absorption of subtox drugs, it is inaccurate for prediction of drugs absorbed paracellularly or via delivery carrier in many cases because of its “tighter” tight junctions compared with the small intestine. MDCK cell, derived from a normal male cocker spaniel, could differentiate into columnar epithelium and form tight junctions when cultured on semi-permeable membranes. Its main advantages include the shorter cell culture times (7 d) compared to Caco-2 cell (21 d), and the similar tight junctions of cell monolayers to that of physiological small intestine. MDCK cell monolayer have been considered as an alternative for Caco-2 for studying absorption capacity and transport mechanisms of passive transport drugs in small intestine, especially for paracellular passive transport drugs. However, the expression of transporters in MDCK cells was considered different or insufficient compared with those of Caco-2 cells because of the canine kidney origin. So in the present study, Caco-2 cells were used in the bidirectional transport and energy-dependent active transport study.

The transport of drugs across the intestinal epithelium may occur by three main routes: passive transport, active transport and endocytosis transport. Both the carrier-mediated active transport and receptor-mediated endocytosis transport processes generally require energy, with typically saturable phenomenon of transporter or receptor. Drugs may also cross the intestinal epithelium into the blood by passive transport route which is concentration gradient driven, energy-independent and transport unsaturated. The passive diffusion is further divided into transcellular pathway (through the cell membrane) and paracellular pathway (through the tight junctions between the cells). Lipophilic molecules (such as propranolol) could easily cross the cell membrane by transcellular diffusion. By contrast, hydrophilic molecules (such as fluorescein) that are not recognized by a carrier cannot penetrate into the hydrophobic membrane and thus traverse the epithelial barrier via the paracellular pathway. Exendin-4 is extremely watersoluble (>1 mg/mL), with relative large molecular weight (MW 4186.6 Da). As a zwitterion molecule, its iso-electric point is around 4.5 so it is negative charged at the intestinal physiological pH of 6–7. On the basis of above physicochemical features, exendin-4 is supposed to be transported through intestinal epithelium through passive transport route, mainly by paracellular pathway. An additional possible pathway for the transport of exendin-4 was the GLP-1 receptor-mediated endocytosis, because low amounts of GLP-1 receptors were found presenting in human pancreas, intestine, lung, kidney, breast, and brain. In addition, organic anion transporting polypeptide (OATP) family, such as OATP1A2 (also named SLCO1A2) and OATP2B1 (also named SLCO2B1), may also contribute to the cellular uptake of exendin-4, which has been reported to mediate intestinal absorption of several drugs.

The first step of this study was to demonstrate whether active transport and endocytosis transport is involved in the exendin-4 transport across MDCK cell monolayers. Experimental results of time and concentration effect showed that the cumulative permeation amount of exendin-4 was increased linearly with the concentration or time increasing, and no saturation phenomenon was observed over all time period and concentration range, which were the typical characteristics of passive diffusion. Results of temperature effect and bidirectional transport showed that exendin-4 transport across MDCK cell monolayers can not be significantly affected by temperature change and it is not energy-dependent, which is similar to passive diffusion markers. There is no efflux observed either. In addition, the results of ATP depletion experiment clearly demonstrated that no active transport mechanism is involved in the transport of exendin-4. Taken together, we concluded that exendin-4 transport across MDCK cell monolayers was passive transport. The next step of this study was to identify the involved transport route of passive diffusion which maybe occurred via paracellular or transcellular pathway. The amount of drug uptake in the cells was measured. The data showed that there was a small part of exendin-4 taken up into the cells, similar to that of paracellular marker. After treatment by paracellular absorption enhancers (EDTA, CS and C10) to open the tight junction of MDCK cell monolayers, the $P_{app}$ of exendin-4 was considerably increased (up to 2.2–11.9 folds) compared with that in untreated cell monolayers, and the enhancing extent is similar with paracellular marker, but different with transcellular marker. These results confirmed that paracellular passive transport was the primary pathway for the exendin-4 across the MDCK cell monolayers, which also agree very well with the direct evidence provided by the experiments of confocal image of MDCK cells exposed to FITC-exendin-4. Although adequate evidences to support the passive paracellular transport mechanism, we still can not exclude the possible contribution of GLP-1 receptor and OATP family-mediated endocytosis, because these receptors' expression might exist in human intestine. However,
the contribution of the endocytosis pathway to the exendin-4 transport across the intestinal mucosa is minority if it really exists.

According to the properties of solubility and permeability, oral drugs have been classified into biopharmaceutics classification system (BCS) class I to IV. Result abovementioned showed that the transport capacity of exendin-4 was very low ($P_{\text{app}} = 0.1 \times 10^{-5} \text{cm/s}$), so exendin-4 should be categorized into BCS class III-high solubility and low permeability drug. For the drugs of BCS class III, improving permeability is the most essential way to improve the oral bioavailability. As drug absorption is mainly by paracellular passive pathway, the transport of exendin-4 will be severely restricted by the presence of the tight junctions between intestinal epitheliums and will resultantly lead to the low bioavailability. There are four strategies generally employed to increase the permeability of paracellular drugs: (1) modification with a liposoluble substance to increase transcellular flux; (2) modification with a substrate for a carrier to increase carrier-mediated active transport; (3) co-administration with paracellular enhancers to increase paracellular flux; (4) using delivery vectors to increase one or more of above transport routes. In the case of exendin-4, there are also a few approaches which have been employed to improve its oral bioavailability. Khafagy et al. reported that using the complex of exendin-4 with cell penetrating peptides (CPPs) significantly improved the oral bioavailability which was 1.8% relative to the intravenous route.9 The CPPs enhancing absorption seemed to be through an active and transcytosis-like mechanism.33 An oral delivery system of pH-sensitive exendin-4 loaded chitosan nanoparticles was reported, and its bioavailability (dose, 300 µg/kg) was 14.0±1.8% relative to its subcutaneous counterpart (dose, 50 µg/kg). The significant improvement of drug transport by chitosan nanoparticles have been attributed to transient opening of the tight junctions between epithelial cells.13

Co-administration with paracellular enhancers is an attractive strategy to improve oral absorption because of the ease of incorporating into the conventional oral dosage forms without the need for complex or expensive formulation techniques. In addition, it could avoid degradation of the active agent by intracellular enzymes, which is another problem for peptide and protein drugs. However, enhancers may cause toxicity in vivo, probably due to that the improved transport is the result of nonspecific mechanisms. An ideal absorption enhancer would probably improve the intestinal absorption of drugs without severe membrane damage or local irritation in their presence. The development of in vitro models of the intestinal epithelium, such as Caco-2 and MDCK cell monolayers, have provided excellent tools to identify the enhancing effect and toxicity of enhancers.34 In this study, chitosan, sodium caprate and EDTA were used to reversibly open the tight junction of cells, which considerably increased the transport of exendin-4 across MDCK cell monolayers (up to 2.2–11.9 folds). Moreover, the cytotoxicity data indicated that these enhancers may be used as safe and effective excipients alone or combined to improve the oral efficacy of exendin-4, which sheds light on the future formulation design.

CONCLUSION

In this study, the transepithelial transport capacity, properties and mechanism of exendin-4 across MDCK cell monolayers model were well investigated for the first time. The results of our experiments showed that the transport capacity of exendin-4 was very weak, and was categorized into BCS class III. Exendin-4 transport across the MDCK cell models was time- and concentration-dependence, direction and energy-independence, and can be significantly improved when the tight junction of cells opened. These transport characteristics suggested that exendin-4 transport across the intestinal epithelium mainly by passive paracellular pathway, which agrees very well with the result of confocal laser scanning microscopy. And chitosan, sodium decanoate and EDTA may be potential safe excipients to improve oral efficacy of exendin-4. This study is useful in designing oral formulation for exendin-4 with improved bioavailability.

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