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Uptake of a Dipeptide by the Dipeptide Transporter in the HT-29 Intestinal Cells

Doo-Man Oh

College of Pharmacy, Taegu Hyosung Catholic University, Kyungsan, Kyungbuk, 713-702, Korea

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HT-29 장관세포에 있는 디펩티드수송체에 의한 디펩티드의 흡수

오두만

대구효성가톨릭대학교 약학대학

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The peptide transporter can be utilized for improving the bioavailability of compounds that are poorly absorbed. Characterization of the dipeptide uptake into the human intestinal epithelial cells, HT-29 was investigated. The uptake of tritiated glycylsarcosine ([^{3}H]-Gly-Sar, 0.1 μCi/m*l*) was measured in confluent or subconfluent HT-29, Caco-2, and Cos-7 cells. Uptake medium was the Dulbecco's Modified Eagle's Media (DMEM) adjusted to pH 6.0. Both HT-29 and Caco-2 cells expressed the dipeptide transporter significantly ($p<0.005$) but Cos-7 did not. Certain portions of passive uptake were observed in all three cell lines. Uptake of Gly-Sar was largest at 7 days after plating HT-29 cells with significant inhibition with 25 mM cold Gly-Sar ($p<0.05$), but expression ratio of the dipeptide transporter was 0.7, suggesting lower expression. The effect of pH on Gly-Sar uptake was not significant in the range of pH 6 to 8. Gly-Sar uptake was also inhibited with 50 mM carnosine, 25 mM Gly-Sar, and 35 mM cephalexin significantly ($p<0.05$). From above results the dipeptide transporter was expressed well in HT-29 cells and was similar to that in the small intestine, suggesting that large amounts of mRNA of the transporter from the cells can be obtained.

Keywords—Dipeptide, Glycylsarcosine, HT-29, Transport, Uptake

Many biologically active peptides or peptide analogs are structurally suitable for the carrier-mediated uptake by mechanisms responsible for transport of small dietary peptides[1]: carnosine, thyroliberin, amino-β-lactam antibiotics such as aminopenicillins and aminocephalosporins,[2-6] angiotensin converting enzyme (ACE) inhibitors,[7-9] and renin inhibitors.[10, 11] Making poorly absorbed but therapeutically active molecules readily absorbable by incorporating molecular features required for the peptide transporter is an attractive approach.[11] It has been suggested that intestinal and renal transports of peptides involve co-transport with proton. Ganapathy and Leibach[12] suggested that in the intact absorptive cell a Na^+ gradient might stimulate peptide transport indirectly by producing a proton gradient via the Na^+/H^+ exchanger.

Molecular cloning and controlled expression of the transporter genes are essential to gain further insights into the biology of peptide transporter. The peptide transporter from the rabbit intestine was successfully expressed in the *Xenopus* oocytes.[13, 14] Recently genes for an intestinal peptide transporter were cloned by an expression cloning in the oocytes[15] or by the monoclonal antibody that blocked cephalex-

in uptake into Caco-2 cells.[16] However amino-acid sequences of two peptide transporters were different, and there is still confusing to clarify their molecular structures. The cloned gene will allow us to fully characterize the molecular features of the transporter for therapeutic applications and to search for homologous genes with similar function.

A human adenocarcinoma cell line, Caco-2, differentiates into polarized, columnar cells spon- taneously that is similar to the small intestine. Caco-2 cells have well-developed microvilli and a polarized distribution of brush-border enzymes. When grown on plastic, monolayers transport ions, sugars, amino acids,[17] bile acids,[18] cephalexin,[19] β-lactam antibiotics,[20,21] thyrotropin-releasing hormone,[22] vitamin B_{12}, and some passively diffused compounds.[23] Recently direct evidences on the dipeptide transport in Caco-2 cells were reported using a dipeptide, glycylsarcosine as a substrate.[24,25] However Caco-2 cells do not produce a mucus layer that is normal in the physiologic condition of the humans. The human intestinal epithelial cell line, HT-29 can be differentiated well in the medium to have microvilli and enterocytic functions.[26,27] Confluent monolayers of HT-29 cells produce tight junctions between cells and contain many brush-border marker enzymes. A HT-29 cell clone has been established as the goblet cells that produce mucin molecules in the cell culture.[28,29] Co-cultures of Caco-2 and HT-29H cell lines were developed.[30] HT-29 cells transport cephalexin,[31] sugar,[32] and taurine.[33] Therefore both Caco-2 and HT-29 cell lines have been using as models for drug absorption.

In order to clone the gene encoding the dipep- tide transporter, it is required to get a large amount and good quality of its messenger RNA (mRNA). Expression of the transporter in *Xeno- pus* oocytes after microinjection of the mRNA should be essential according to the expression cloning method.[34] In this experiment toward cloning the transporter gene, the characteristics of a dipeptide transporter using glycylsarcosine will be studied and possibility of getting its mRNA from the HT-29 cells will be discussed.

MATERIALS AND METHODS

Materials

The radiolabelled glycylsarcosine (Gly-Sar) was obtained from Amersham (Arlington Heights, IL). The specific radioactivity of [^{3}H]-Gly-Sar is 39 Ci/mmole. Cold Gly-Sar, MES (2-[N-morpholino]ethanesulfonic acid), phosphate buffered saline (PBS) and EDTA were purchased from Sigma (St. Louis, MO) and DMEM (Dulbecco's modified Eagle's medium) was obtained from Life Technologie Inc. (N.Y., NY). Other compounds were from Sigma (St. Louis, MO) and all were analytical grades.

Cell Culture

The human colon adenocarcinoma cell lines, HT-29 and Caco-2, and SV 40 transformed African Green Monkey kidney cell line, Cos-7 were obtained from ATCC (Rockville, MD). The cells were grown in T-150 flasks at 37℃ in an atmosphere of 10% CO_2 using Dulbecco's Modified Eagle's medium (DMEM) containing 10% inactivated fetal calf serum. For uptake experiments cells were splitted to 5×10^5 cells per well in 12-well dishes and the medium was replaced every two to three days. The Caco-2 cells were mycoplasma-free and were used between passage number 31 and 48.

Uptake Measurements

The Gly-Sar uptake was measured at 37℃ in a multi-well dish. The uptake medium was a DM- EM containing 0.1 μCi/m*l* of [^{3}H]-Gly-Sar (2.56×10^{-9}M) with or without inhibitors. The pH of uptake medium was adjusted to 6.0 with 1M MES (2-[N-morpholino]ethanesulfonic acid). Before placing the uptake medium,

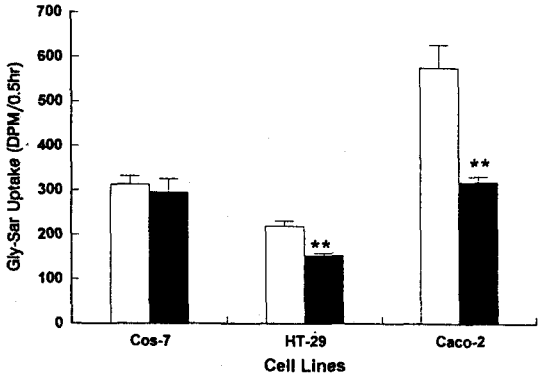

Figure 1—Gly-Sar uptake into Cos-7, HT-29, and Caco-2 cells during 30-min incubation at pH 6.0 with (hatched bar) or without (open bar) 25 mM cold Gly-Sar as an inhibitor. There were significant inhibitions of Gly-Sar uptake with an inhibitor in HT-29 and Caco-2 cells (**$p<0.005$). Each value represents the mean±SE of 4 determinations.

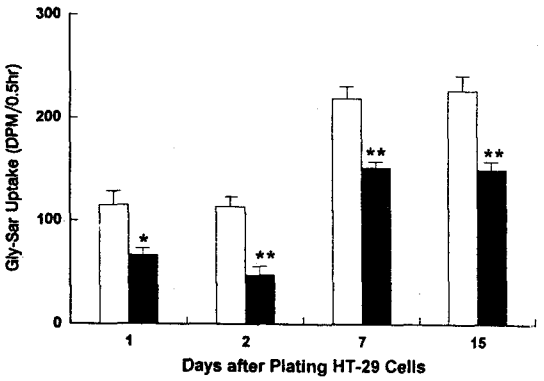

Figure 2—The Gly-Sar uptake into HT-29 cells during 30-min incubation in DMEM (pH 6.0) at 1, 2, 7, and 15 days after plating cells with (hatched bar) or without (open bar) 25 mM cold Gly-Sar as an inhibitor. There were significant inhibitions of Gly-Sar uptake with an inhibitor (*$p<0.05$; **$p<0.005$). Each value represents the mean±SE of 4 determinations.

cells were washed with fresh media several times. Cells were incubated at 37℃ for 30 min. Uptake medium was removed and cells were washed 3 times with cold Phosphate Buffered Saline (PBS) solutions and harvested with 1 m*l* of PBS containing 1 mM EDTA. Cells were transferred to scintillation vials, added 10 m*l* of scintillation cocktails, vortexed, and measured the radioactivity associated with HT-29 cells using a liquid scintillation counter (Beckman LS 6000).

MOPS (3-[N-morpholino]propanesulfonic acid) was used to adjust pH to 7, 7.5 or 8, if needed. The total protein concentration was measured by the Bio-Rad DC protein assay (Bio-Rad, Rich- mond, CA). Briefly 10 μ*l* of cells were taken in 1.5 μ*l* Effendorf tubes and added 15 μ*l* of PBS. 125 μ*l* of Reagent A and 1 μ*l* of Reagent B were added with vortexing, and incubated for 15 min at room temperature before measuring the optical density at 750 nm with a spectrophotometer (Beckman).

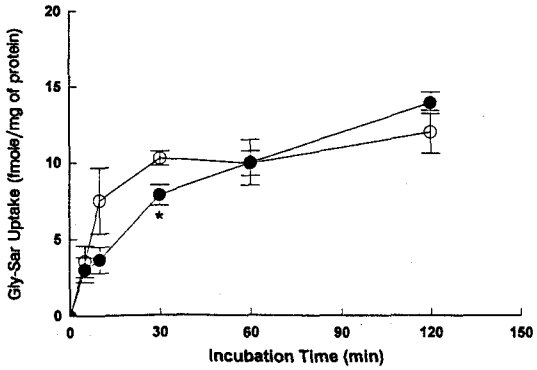

Figure 3—Time course of Gly-Sar uptake into HT-29 cells in the DMEM at pH 6.0. Uptake was measured with (●) or without (○) 25 mM cold Gly-Sar as an inhibitor. Each value represents the mean±SE of 4 determinations (*$p<0.05$).

Table I—*Expression and Inhibition of the Dipeptide Transporter at 1, 2, and 7 Days after Plating HT-29 Cells*

Days after plating cells	Expression Ratio[a]	
	Without inhibitor	With 25 mM Gly-Sar
1	0.94	0.53*
2	0.54	0.23*
7	0.70	0.51

[a]Expression ratio was defined to the ratio of the Gly-Sar uptake in the HT-29 cells to that in the Cos-7 cells. Each value represents the mean value of 4 determinations (*$p<0.05$).

RESULTS AND DISCUSSION

In order to characterize the Gly-Sar uptake into HT-29 cells, a SV40 transformed African Green Monkey kidney cell line, Cos-7 was used as a negative control. Caco-2 cells were used as a positive control.[24,25] Fig. 1 shows the Gly-Sar uptakes into Cos-7, HT-29, and Caco-2

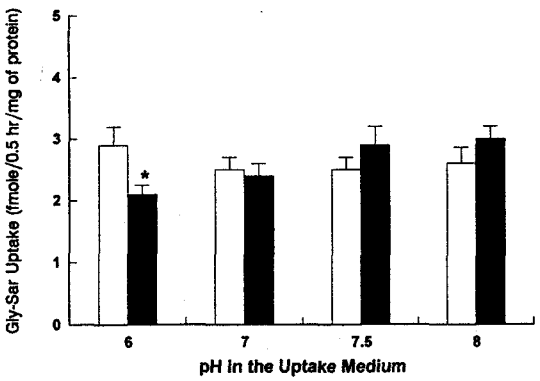

Figure 4—Effect of pH on the Gly-Sar uptake into HT-29 cells. There was a significant difference in Gly-Sar uptake at pH 6.0 compared to others (*$p<0.05$). Each value represents the mean±SE of 4 determinations.

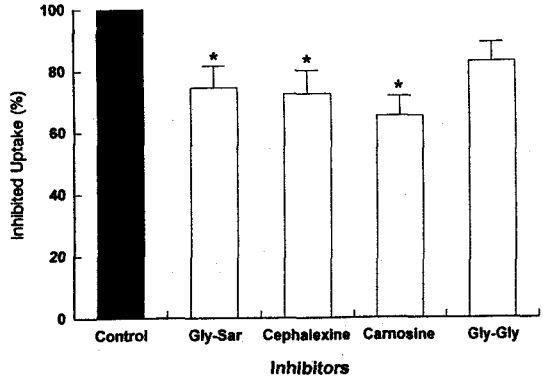

Figure 5—Inhibition of Gly-Sar uptake into HT-29 cells in the presence of 50 mM Gly-Sar, 35 mM cephalexine, 50 mM carnosine, and 50 mM Gly-Gly. There were significant inhibitions except for Gly-Gly (*$p<0.05$). Each value represents the mean±SE of 4 determinations.

cells during 30-minute incubation at pH 6.0 with or without 25 mM cold Gly-Sar. The concentration of [^{3}H]-Gly-Sar was 0.1 μCi/ml (2.56×10^{-9} M). Uptake measurements were performed at 7 days after plating cells. There was no significant inhibition of the uptake into Cos-7 cells in the presence of 25 mM Gly-Sar, indicating that Cos-7 cells did not have any dipeptide transporter but passive absorption only. Both HT-29 and Caco-2 cells showed significant inhibitions of Gly-Sar uptake via the dipeptide transporter in the presence of high concentration of cold Gly-Sar as an inhibitor. Particularly expression in Caco-2 cells was completely inhibited to that in Cos-7 cells. However HT-29 cells expressed the dipeptide transporter less compared to Caco-2 cells. It suggested that Caco-2 cells were differentiated higher than type 2 cells such as HT-29 cells.[35] For example, the activity of the peptide metabolizing enzyme in Caco-2 cells was approximately 7 times higher than that of HT-29 cells.[35]

Fig. 2 shows the Gly-Sar uptake into HT-29 cells during 30-minute incubation in DMEM (pH 6.0) at 1, 2, 7 and 15 days after plating cells. There was significant inhibition of the Gly-Sar uptake with 25 mM cold Gly-Sar at day 1 ($p<0.05$), and further increased the inhibition at 2 to 15 days after seeding ($p<0.005$). There was no more increase in the dipeptide expression after 7 days. Because expression in Cos-7 comes from the passive Gly-Sar uptake only, expression ratio is defined to the ratio of the Gly-Sar uptake in the HT-29 cells to that in the Cos-7 cells.[25] Expression ratio means the carrier-mediated uptake of Gly-Sar or the total expression of dipeptide transporter in HT-29 cells. Expression and inhibition of the dipeptide transporter at 1, 2, and 7 days after plating HT-29 cells are summarized in Table I. It was shown that expression ratio was 0.7 without 25 mM Gly-Sar and 0.51 with the inhibitor at day 7. It suggested that the expression of dipeptide transporter in HT-29 cells was only 50% compared to Cos-7 cells. Seven-day incubation was chosen for further experiments in order to study Gly-Sar uptake.

The time courses of Gly-Sar uptake into HT-29 cells in DMEM (pH 6.0) with or without cold 25 mM Gly-Sar for 120 minutes were shown Fig. 3. The time course of Gly-Sar uptake without the inhibitor showed curvilinear uptake with a plateau after 30 minutes. Gly-Sar uptake was slowed down in the presence of the inhibitor. The initial uptake rate was calculated from the initial slope using a linear

regression. Initial uptake rates with or without high concentration of Gly-Sar were 0.339 ± 0.016 or 0.753 ±0.024 fmole/min/mg of protein, respectively. Again there was a significant inhibition of uptake rate in the presence of 25 mM Gly-Sar ($p<0.05$). It might be compared that in Caco-2 cells the time course of Gly-Sar uptake was linear and that the initial uptake rate was much slower.[25]

The effect of pH on the Gly-Sar uptake into HT-29 cells is shown in Fig. 4. There were no significant differences in uptakes between pH 6 and pH 8. It is generally known that the dipeptide transporter needs a proton gradient for the uptake.[12] Cephalexin was shown to have maximal flux into HT-29 cells at pH 6.2.[31] An inhibition study with 25 mM Gly-Sar was done at all pHs to explain this discrepancy. Only at pH 6, there was a significant difference in Gly-Sar uptake ($p<0.05$). It was suspected that the experimental condition used here did not have a high sensitivity to detect pH effects on the uptake.

The inhibitory effect of structurally similar compounds is one of the most distinctive aspects of active transporter. Cephalexin, carnosine, and other dipeptides such as Gly-Sar and Gly-Gly were transported by the dipeptide transporter.[1,31] Fig. 5 shows that there were significant inhibitions of Gly-Sar uptake in the presence of 35 mM cephalexin, 50 mM Gly-Sar, and 50 mM carnosine except 50 mM Gly-Gly ($p<0.05$). The reason that Gly-Gly did not inhibit the Gly-Sar uptake was not clear. It could be a reason why two different dipeptide transporters were cloned,[15,16] suggesting more than one type of transporters in HT-29 cells.

In summary the characteristics of the dipeptide transporter expressed in HT-29 cells show a carrier-mediated transport, similar to human or animal dipeptide transporters. HT-29 cells could be utilized to get a large amount of mRNA in order to express in *Xenopus* oocytes and to clone the genes encoding the dipeptide transporter. HT-29 cells might be still useful for a model of mucin producing cells, even though Caco-2 cells expressed the dipeptide transporter higher. Expression of the dipeptide transporter using mRNA from HT-29 and Caco-2 cells in *Xenopus* oocytes will be pursued in our laboratory and reported in the future.

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