Overexpression of Human Intestinal Oligopeptide Transporter in Mammalian Cells via Adenoviral Transduction

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Purpose. Our goals are to establish an *in vitro* screening system and to evaluate a new approach in improving oral absorption of peptides and peptide-like drugs by overexpression of the human intestinal oligopeptide transporter (hPepT1). This study characterizes the expression of hPepT1 in human intestinal Caco-2 cells, rat intestinal epithelial cells (IEC-18), and human cervix epithelial cells (Hela) after adenoviral transduction.

Methods. A recombinant replication-deficient adenovirus carrying the hPepT1 gene was made and used as a vector for the expression of hPepT1. The increase in the uptake permeability of cephalexin and Gly-Sar was determined. The effects of time, dose, apical pH, and substrate specificity were evaluated.

Results. A significant increase in the uptake permeability of Gly-Sar and cephalexin was found in all three cell lines after viral transduction. The increase of Gly-Sar permeability in Hela, IEC-18, and Caco-2 cells was 85-, 46-, and 15-fold respectively. Immunoblotting using an antibody against hPepT1 detected high levels of a 85-98-kDa protein in all three infected cell lines. Substrate permeability was dependent on time of infection, inward pH gradients, and multiplicity of infection (MOI). Decreased infectivity and lower hPepT1 expression were observed in differentiated Caco-2 cells. The uptake was inhibited by dipeptides and β-lactam antibiotics but not amino acids.

Conclusions. Adenoviral infected Hela cells displayed a pronounced level of hPepT1 expression with a low background and high specificity to dipeptides. These features make this system a useful tool for screening of potential substrates. The success of overexpression of hPepT1 in Caco-2 and IEC-18 cells may lead to a novel approach in improving oral absorption of peptides and peptidomimetic drugs.

KEY WORDS: gene expression; hPepT1; Caco-2 cells; adenovirus; drug screening.

INTRODUCTION

Oral peptide drug delivery has been of interest for decades due to increasing knowledge of transport mechanisms participating in solute uptake from the intestine. It has been shown that di- and tripeptides, β-lactam antibiotics, angiotensin-converting enzyme inhibitors, renin inhibitors, and some peptide prodrugs are actively transported into the intestinal mucosal cell by a specific carrier system that is different from those involved in the transport of free amino acids (1). cDNA clones encoding a proton-coupled oligopeptide transporter have been isolated from the intestine of rabbits, humans, and rats by an expression cloning technique using Xenopus laevis oocytes (2–4). Numerous studies have focused on its functional analysis (5), structural determinants of substrate recognition (6–7), cellular models for binding and transport (8), and tissue distribution (9–10). Comparison of the amino acid sequence of these three transporters revealed a high degree of similarity, with the human oligopeptide transporter (hPepT1) having 81% and 83% identity to rabbit and rat PepT1, respectively (4). The predicted structure of hPepT1 consists of 708 amino acids which give a core molecular size of 78,810 Da with 12 membrane-spanning domains and a long hydrophilic loop facing the extracellular site (3).

Gene delivery, using a variety of viral and synthetic vectors, provides a novel strategy for treating diseases and delivery of therapeutic proteins. In particular, the development of replication-deficient recombinant adenoviral vectors has been very successful in the past decade (11). These vectors have been used for gene transfer to various cell types of animals with high efficiency, including hepatocytes, endothelial cells, skeletal muscle cells, respiratory airway epithelial cells, and cardiac myocytes (12). We have constructed an adenoviral vector, Ad.RSVhPepT1, which contains the hPepT1 gene under the control of the Rous sarcoma virus (RSV) promoter to examine the effect over expression of the hPepT1 gene in a number of tissue culture cell lines, including Caco-2, IEC-18, and Hela cells.

MATERIALS AND METHODS

Materials

[^{3}H]-Glycyl-sarcosine (400 mCi/mmol) was synthesized by Moravek Biochemicals (Brea, CA). All other chemicals were purchased from Sigma (St. Louis, MO) unless specified. Cell culture reagents were obtained from Gibco (Grand Island, NY) and culture supplies were from Corning (Corning, NY) and Falcon (Lincoln Park, NJ).

Cell Culture

Caco-2 cells were obtained from American Type Culture Collection (ATCC HTB37, Passage number 40-55) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% MEM nonessential amino acids, 1 mM Sodium pyruvate, and 1% L-glutamine. IEC-18 cells (ATCC CRL1589, untransformed primary epithelial cells from rat ileum, ref. 13), Hela and 293 cells were also obtained from ATCC. All cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

Construction and Preparation of Ad.RSVhPepT1

Ad.RSVhPepT1 is a replication-defective adenoviral vector based on a genomic backbone of adenovirus type 5 with a deletion of E1A, E1B, and portions of the E3 regions that

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impair replication (14–15). The recombinant adenoviral vectors were generated by homologous recombination (16) between human adenovirus serotype 5 derivative sub360 and a plasmid, pAd.RSVhPepT1, containing the Rous sarcoma virus (RSV) promoter, human PepT1 gene, and SV40 early region polyadenylation signal in 293 cells (constructed by University of Iowa Vector Core, Dr. Beverly L. Davidson, Director). Clones carrying the hPepT1 gene were identified by infection of Hela cells followed by cephalexin uptake measurement 48-hour post-infection. After an additional two rounds of plaque purification, high titer stocks were made in 293 cells following standard methods (17). The resulting viral stocks contained 3–6 $\times$ 10^{12} particles per ml as determined by OD measurement at 260 nm. Viral titers were determined by plaque-forming assay in 293 cells and expressed as multiplicity of infection (MOI, plaque-forming unit per cell).

Adenoviral Infection of the Cells

Caco-2, IEC-18, and Hela cells were seeded onto 12 or 24 well plates at a density of 10^4, 5×10^4, and 1×10^5 cells/cm^2, respectively. The infection conditions were as follows in all studies unless otherwise specified. Hela cells were infected 24 hours after seeding and uptake was measured 24 hours post-infection. IEC-18 and Caco-2 cells were infected 3 days after seeding and uptake was determined 3 days post-infection.

Western Blot Analysis for hPepT1

All the equipment and chemicals used in Western blot analysis were obtained from Bio-Rad (Hercules, CA) unless otherwise specified. Caco-2 and IEC-18 cells were infected with Ad.RSVhPepT1 at a MOI of 75 pfu/cell. For the dose-dependent study, Caco-2 cells were infected at a MOI of 1.5, 15, 35.7, and 75 pfu/cell. Cells were resuspended and solubilized by boiling in sample buffer containing 5% 2-mercaptoethanol and 2% SDS (Sigma) at 100°C for 5 minutes. The samples, which contained 20 μg of cell protein, were separated by SDS-PAGE with 7.5% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The blots were blocked overnight with 5% nonfat dry milk in PBS. After washing with PBS, the blots were incubated for 3 hours at 25°C with a 1:500 dilution of a rabbit anti-hPepT1 serum (provided by Dr. Wolfgang Sadee, University of California, San Francisco, CA). The anti-hPepT1 serum was raised against the C-terminal 15 amino acids of hPepT1. They were further incubated for 30 minutes at 25°C with a donkey anti-rabbit horseradish peroxidase-conjugated antibody diluted 1:1,000 and probed using the enhanced chemiluminescence system (ECL, Amersham).

Uptake Measurement

The culture medium was removed and the cells were washed with PBS pH 7.4 and 0.5 ml of the uptake medium containing 1 mM ^{3}H-labeled Gly-Sar or cephalexin was added. The uptake medium contained 145 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM MES (pH 5–6.5) or 5 mM Hepes (pH 7–7.5). The osmolarity of the uptake medium was 300 $\pm$ 5 mmol/kg as measured by Vapor Pressure Osmometer (Wescor Corp., Logan, Utah). After incubation for a specific time at room temperature, the buffer was removed and the cells were washed three times with ice-cold PBS and dissolved with water containing 1% TritonX-100. The uptake of Gly-Sar was measured by a liquid scintillation spectrometry (Model LS6000, Beckman, Fullerton, CA). Cephalexin uptake was determined by HPLC.

Data Analysis

The amount of protein was measured by Protein Assay DC Kit (Bio-Rad, CA) based on the Lowry method (18). The uptake permeability can be calculated by the following equation (19):

$$P_{uptake} = \frac{dQ}{dt} \times \frac{1}{A \times C_0 \times \mathrm{Pr}}$$

Co is the starting concentration of substrate, A is the total surface area of the plate, and dQ/dt is the initial rate of uptake. The values are normalized by the protein amounts (Pr) and expressed as cm/sec/mg protein. Each experimental point was determined with duplicate or triplicate wells. The results are expressed as means $\pm$ s.d. of these replicates. The statistic significance was performed by Student's t-test.

RESULTS

Expression of hPepT1 in Adenovirus Infected Cells

The expression of hPepT1 in Caco-2, IEC-18, and Hela cells after Ad.RSVhPepT1 infection at 75 pfu/cell is shown in Fig. 1. A significant increase ($p < 0.001$) of Gly-Sar permeability in infected Hela (85-fold), IEC-18 (46-fold), and Caco-2 (15-fold) cells was seen. The uptake of cephalexin, another known substrate for hPepT1, was increased 19 and 28 times in Caco-2 and IEC-18 cells, respectively. For the purpose of negative control, cells were infected with Ad.RSVlacZ at the same MOIs and no significant increase of substrate permeability was observed (data not shown). The uptake in uninfected Caco-2 cells had the highest background, which lead to the lowest increase of uptake permeability.

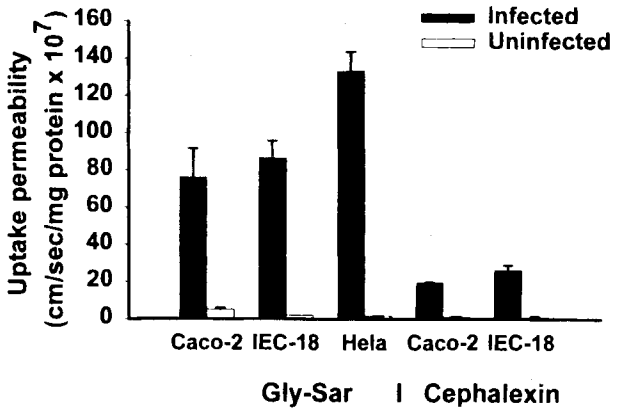

Fig. 1. Comparison of uptake permeability of Gly-Sar and cephalexin in Caco-2, IEC-18, and Hela cells. IEC-18 and Caco-2 cells were infected with Ad.RSVhPepT1 at a MOI of 75 pfu/cell while Hela cells were infected at a MOI of 15 pfu/cell. All the increase of permeability after adenoviral infection showed significant over control ($p < 0.001$).

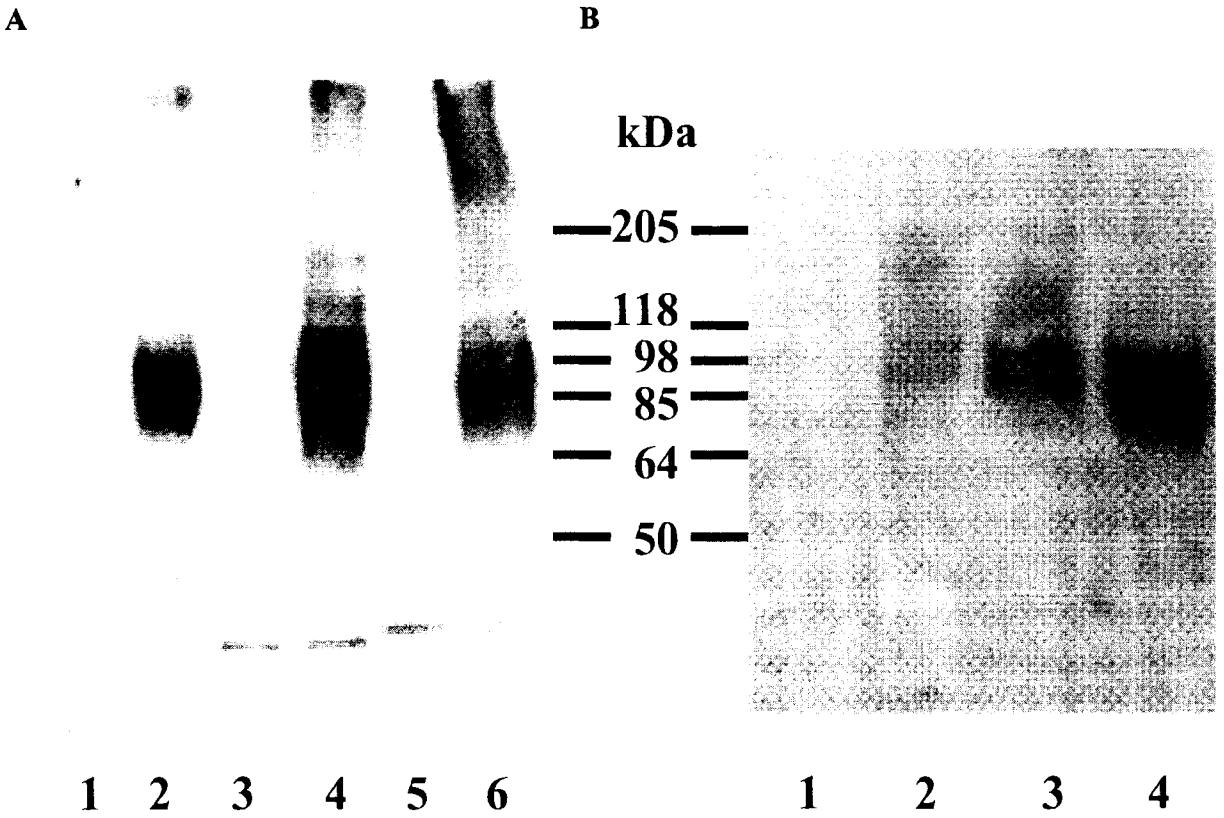

Fig. 2. Expression of hPepT1 in Hela, IEC-18, and Caco-2 cells. Twenty μg of cell lysate were loaded into SDS-polyacrylamide gels and hPepT1 was detected with a rabbit anti-hPepT1 antibody. (A) IEC-18 and Caco-2 cells were infected at a MOI of 75 pfu/cell and collected 3 days after infection. Hela cells were infected at 15 pfu/cell and collected 24 hours post-infection. Lanes 1 and 2, uninfected and infected IEC-18 cells; Lanes 3 and 4, uninfected and infected Caco-2 cells; Lanes 5 and 6, uninfected and infected Hela cells. (B) Caco-2 cells were infected at increasing MOIs. Lanes 1–4, infected cells at 1.5, 15, 37.5 and 75 pfu/cell respectively.

Western Blot Analysis for hPepT1

Immunoblotting with a rabbit anti-hPepT1 antibody was carried out with the cell lysates from Caco-2, IEC-18 and Hela cells. A 85-98-kDa protein was detected in all infected cells while uninfected ones showed no detectable band (Fig. 2A). In dose-dependent study, increasing intensity of the hPepT1 band was seen in cell lysates when Caco-2 cells were infected with Ad.RSVhPepT1 at an increasing MOI from 15 to 75 pfu/cell (Fig. 2B). The expression corresponding to 1.5 pfu/cell was detected only after overexposure of the blots.

pH Dependence of Drug Uptake

To determine whether the adenoviral-mediated expression of hPepT1 is proton dependent, the uptake of Gly-Sar in Caco-2 and Hela cells was measured at extracellular pH of 5.0, 6.0, 6.5, and 7.5 (Fig. 3). The permeability was decreased as pH increased from 5.0 to 7.5, indicating the requirement of a proton gradient for drug uptake. The results are consistent with the reported activity of H^+/peptide cotransporter.

Human PepT1 Expression in Caco-2 and IEC-18 Cells After Infection at Different Times

To determine whether the stage of cell differentiation affects the infectivity of adenoviruses and the expression of hPepT1, Ad.RSVhPepT1 was added to Caco-2 cells at different times (3, 5, 14, 21 days) after seeding at a MOI of 15 and 75 pfu/cell. The uptake of cephalexin and Gly-Sar was measured 3 days after infection. The results are shown in Table I. At day 3, Caco-2 cells have reached 80–90% confluence but have few cell to cell contacts during this exponential growth phase (data not shown). After infection at 75 pfu/cell, the permeability

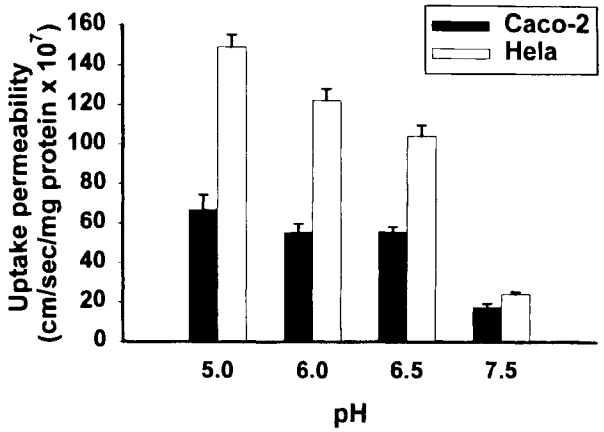

Fig. 3. pH dependence of Gly-Sar uptake in Caco-2 and Hela cells. Caco-2 cells were infected at a MOI of 75 pfu/cell while Hela cells were infected at 15 pfu/cell. Gly-Sar uptake at pH 7.5 was significantly decreased as compared to uptake at pH 5.0, 6.0, and 6.5 ($P < 0.005$).

Table I. The Uptake Permeability of Gly-Sar and Cephalexin in Caco-2 Cells After Infection at Different Times and Doses

Time of infection[a]	Uptake permeability (cm/sec/mg protein) × 10^7					
	Gly-Sar			Cephalexin		
	MOI 75	MOI 15	Uninfected	MOI 75	MOI 15	Uninfected
Day 3	75.9 (15.5)[b]	n.d.	5.0 (1.0)	19.4 (0.7)	8.0 (0.2)	1.0 (0.3)
Day 5	25.0 (1.3)	14.8 (0.2)	7.5 (0.3)	7.1 (3.2)	2.4 (0.4)	1.0 (0.1)
Day 14	36.1 (0.3)	28.5 (1.8)	13.5 (2.6)	5.9 (2.4)	1.9 (0.4)	1.4 (0.3)
Day 21	32.4 (4.9)	31.2 (5.6)	12.5 (0.8)	n.d.[c]	n.d.	n.d.

[a] Cells were infected at determined times at a MOI of 15 and 75 pfu/cell and the uptake study was performed 3 days after infection.
[b] Data are the result of two separate experiments and the standard deviations are in parenthesis.
[c] n.d. = not determined.

of cephalexin and Gly-Sar was increased 15–19 times over uninfected cells. Gene expression decreased when cells reached confluence at day 5 and decreased further when fully differentiated cells were infected at day 14 or 21. These results are consistent with reported low transfection efficiency in differentiated Caco-2 cells due to a reduced expression of integrins, the cell surface receptors associated with adenoviral internalization (20–21). In order to evaluate how long hPepT1 expression lasts after adenoviral transduction, undifferentiated Caco-2 cells (3 days post-seeding) were infected with Ad.RSVhPepT1 at different MOIs and cephalexin uptake was examined on different days after infection. Cephalexin uptake revealed that for uninfected cells the permeability remained constant over a 10 day period, while it increased slightly when cells were infected at a MOI of 1.5 pfu/cell (Fig. 4). After infection at 15 pfu/cell, a 4- to 8-fold increase of cephalexin uptake in Caco-2 cells was observed throughout the studied time, indicating that the expression of hPepT1 was maintained for at least 10 days.

Substrate Specificity of hPepT1

hPepT1 is selective for dipeptides, tripeptides, and amino β-lactam antibiotics (4). Figure 5 shows the results of hPepT1

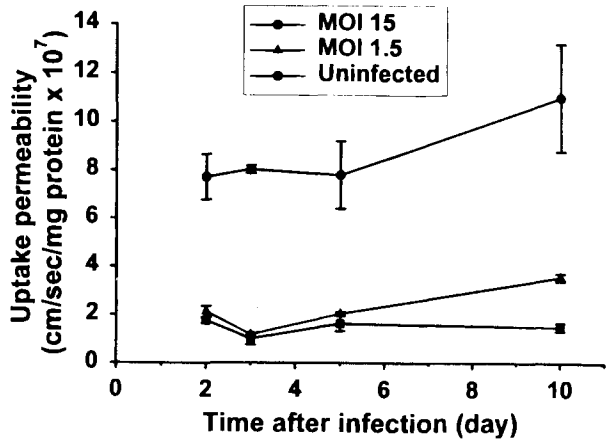

Fig. 4. Time course of hPepT1 expression in Caco-2 cells. Caco-2 cells was infected at a MOI 15 (●) and 1.5 (▲) pfu/cell. The uptake of cephalexin was measured at determined times after infection. The uptake in uninfected Caco-2 cells is also shown (■). After infection at 15 pfu/cell, cephalexin permeability was increased significantly ($p < 0.05$) throughout the studied time (up to 10 days after infection) as compared to that in uninfected Caco-2 cells.

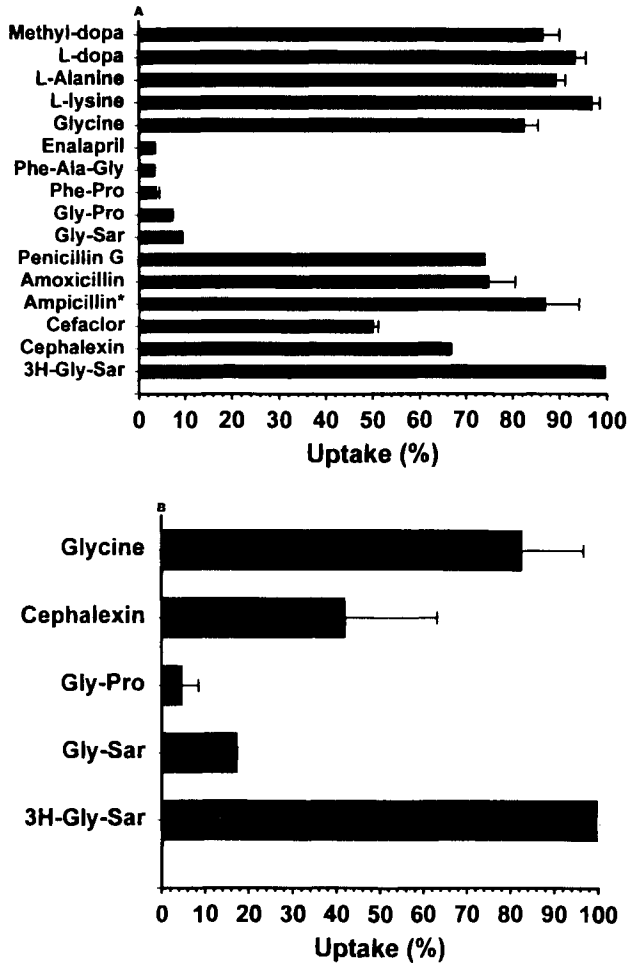

Fig. 5. Competition for ^{3}H-Gly-Sar uptake by dipeptides, amino acids and other β-lactam antibiotics in Hela (A) and Caco-2 (B) cells. (A) After 1-day infection, Hela cells were incubated for 30 minutes at pH 6.0 with 1 mM ^{3}H-Gly-Sar in the presence or absence of 20 mM of the inhibitors. The uptake of ^{3}H-Gly-Sar in the absence of inhibitors was 1.55 nmole/min/mg protein and was defined as 100%. *The concentration of ampicillin was 40 mM. (B) After 3-day infection, the uptake permeability of 1 mM ^{3}H-Gly-Sar in Caco-2 cells with or without various inhibitors (20 mM) was measured at pH 6.0 and expressed as % control (in the absence of inhibitors).

uptake competition studies in Ad.RSVhPepT1 transduced Hela (5A) and Caco-2 (5B) cells. In these studies, the ability of unlabeled amino acids, di-, tripeptides, antibiotics, and some peptide-like compounds to inhibit the uptake of ^{3}H-Gly-Sar (1 mM, specific activity 500 μCi/mmol) was studied. At a concentration of 20 mM, β-lactam antibiotics showed 15–50% inhibition of ^{3}H-Gly-Sar uptake while di- and tripeptides displayed over 90% inhibition. In contrast, free amino acids, which are not a substrate for hPepT1, had no effect on ^{3}H-Gly-Sar uptake. Enalapril, an ACE inhibitor which is known to be transported by hPepT1, showed 59% inhibition while L-dopa and methyl-dopa which are transported by an amino acid transporter (22), had no effect on ^{3}H-Gly-Sar uptake.

DISCUSSION

Since gene expression provides an alternative approach for delivery of peptide and protein drugs, many studies have focused on how to improve gene transduction efficiency and the level of protein expression for therapeutic purposes. In addition, the strategy of developing a cell line which overexpresses a protein for structure/function studies represents a new paradigm in the field of drug absorption and metabolism. In recent years, we have used recombinant adenoviral vectors to study gene transduction in Caco-2 cells (20–21). In the current study, the uptake of Gly-Sar and cephalexin was significantly increased in Ad.RSVhPepT1 infected cells. This high-level expression of hPepT1 was confirmed in Western blot studies. Furthermore, the possible correlation between hPepT1 levels and doses of Ad.RSVhPepT1 (Fig. 2B) has strongly indicated that the elevated Gly-Sar uptake was specific for the overexpression of hPepT1.

There was no significant cytopathic effect caused by adenoviral treatment up to 75 pfu/cell in IEC-18 and Caco-2 cells (data not shown). When Caco-2 cells were infected 3 days postseeding at a MOI of 150 or 300 pfu/cell, cells stopped growing and some dead cells were found localized at the edge of the culture plates. These results agree with the finding that adenoviral vectors slow cell proliferation and increase cell death in cultured human airway epithelial cells (23). However, the cytotoxicity at high doses of adenovirus was not observed in differentiated Caco-2 cells (infected at day 14 or 21 after seeding), indicating greater stability toward viral infection in differentiated cultures.

Caco-2 cells readily form differentiated monolayers which share a number of characteristics with intestinal epithelium and are often used for drug screening in pharmaceutical development. Unfortunately with respect to screening peptide prodrugs and peptidomimitic drugs for intestinal transport, Caco-2 cells are an inadequate model because of low expression of hPepT1, which fails to yield good *in vitro: in vivo* correlations of permeability (24). Our results demonstrate that the hPepT1 mediated uptake of Gly-Sar in infected Caco-2 cells was significantly increased over uninfected cells. This significant enhancement may provide a better screening system compared to traditional Caco-2 culture systems.

In conclusion, we have established a system that uses Ad.RSVhPepT1-infected cells for screening of potential substrates for hPepT1. This system presents many advantages over traditional Caco-2 cells, which are by far the most commonly used cell line for drug screening in the pharmaceutical industry. In addition, the success of overexpression of hPepT1 in Caco-2 and IEC-18 cells suggests a new approach to improving oral absorption of peptides and peptidomimetic drugs.

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