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Enhancement of intestinal absorption of macromolecules by spermine in rats

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Summary. The aim of this study was to investigate the enhancing effect of polyamines on intestinal absorption of fluorescein isothiocyanate-labeled dextran (MW 4400, FD-4) in the in situ loop study and in vivo oral absorption study. Absorption of FD-4 from the jejunum was significantly enhanced by 5 mM spermine without serious membrane damage in the jejunum. An in vivo oral absorption study was also performed, and plasma FD-4 levels increased significantly after co-administration of 30 mM spermine. In the in vitro transport studies with Caco-2 cells, prolonged incubation with spermine resulted in a gradual decrease in transepithelial electrical resistance. This finding suggests that the absorption-enhancing mechanism of spermine partly includes opening the tight junctions of the epithelium via the paracellular route. These results indicate that excess oral ingestion of polyamines may have widespread health effects via the modulation of the intestinal epithelial barrier function.

Keywords: Polyamines – Fluorescein isothiocyanate-labeled dextran – Intestinal absorption – in situ loop – Oral absorption – Caco-2 cell monolayers

Abbreviations: C10, sodium caprate; C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin; FD-4, fluorescein isothiocyanate-labeled dextran 4400; HBSS, Hanks balanced salt solution; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); LDH, lactate dehydrogenase; MES, morpholinoethane sulfonic acid; TEER, transepithelial electrical resistance

Introduction

The polyamines spermidine and spermine and their precursor putrescine are ubiquitous polycationic compounds found in all mammalian cells (Tabor and Tabor, 1984; Pegg, 1986). They are essential for the maintenance of cell growth in many tissues, including the gastrointestinal tract, and intracellular levels of polyamines are maintained within narrow limits. At physiological pH levels, the polyamines are positively charged and can interact with many anionic structures including DNA, RNA, and protein (Watanabe et al., 1991; Medina et al., 2003).

The polyamines are present in variable amounts in almost all kinds of food or foodstuffs. In humans, a typical diet might contribute hundreds of micromoles of polyamimes per day to the gut lumen (Bardocz et al., 1995; Nishibori et al., 2007), and ingested food might be the major source of polyamines in the lumen of the upper small bowel. Exogenous polyamines play a role in the maintenance of normal growth and function in the intestinal tract (McCormack and Johnson, 1991; Deloyer et al., 2001). Oral administration of the polyamines spermine or spermidine induces precocious maturation of the intestine (Harada et al., 1994; Kaouass et al., 1994; Capano et al., 1994; Greco et al., 2000; Peulen et al., 2004; Deloyer et al., 2005). The enhancement of maturation is dose-dependent, and spermine is more effective than spermidine (Dufour et al., 1988), and putrescine has relatively little effect (Etienne-Poncin et al., 1989). Oral polyamines have also been shown to have beneficial effects in healing (Wang and Johnson, 1990, 1992) and prevention (Mizui et al., 1987; Buzas et al., 1996; ter Steege et al., 1999) of gastrointestinal damage.

The gastrointestinal tract acts as a barrier against the invasion of exogenous proteins into the body. It is generally recognized that macromolecules such as proteins are not easily absorbed from the gastrointestinal tract in an intact form. In reality, however, the barrier function of the small intestinal epithelium in mammals is incomplete and to a limited extent, macromolecules can pass from the lumen to the circulation (Sanderson and Walker, 1993;

Tsume et al., 1996). The mechanisms of the absorption of macromolecules include paracellular passage and transcellular endocytosis (Heyman et al., 1982; Marcon et al., 1989; Atisook and Madara, 1991; Yan et al., 2004; Takano et al., 2004).

Thus far, several polycationic substrates, such as poly-L-arginine (McEwan et al., 1993; Miyamoto et al., 2001; Ohtake et al., 2003a, b), poly-L-lysine (McEwan et al., 1993), and chitosan derivatives (Illum et al., 1994; Schipper et al., 1997; Kotze et al., 1997; Dodane et al., 1999), also have been shown to affect tight junction permeability in the epithelium. In addition, some experiments have demonstrated that spermine or spermidine can induce an increased transport of poorly absorbable compounds from intestinal mucosa (Mendizabal and Naftalin, 1992; Osman et al., 1998; Miyake et al., 2006). However, few reports are available about the enhancing effect of polyamines on the intestinal absorption of macromolecules in rats.

In the present study, fluorescein isothiocyanate-labeled dextran (MW 4400, FD-4) was chosen as a water-soluble model compound and the effects of polyamines on intestinal absorption in rats were examined. The main aim of this study was to investigate the enhancing effect of polyamines on intestinal absorption of FD-4 in an in situ jejunum loop study and an in vivo oral absorption study. The second purpose of this study was to investigate the mechanisms of the absorption-enhancing effect of polyamines by measuring FD-4 permeation and transepithelial electrical resistance (TEER) in the human intestinal Caco-2 cell line. Because the effects of polyamines on intestinal absorption can be mediated by a positive charge, we chose poly-L-arginine as control to study the absorption-enhancing effects of polyamines.

Materials and methods

Materials

Spermine was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). FD-4 and poly-L-arginine (MW ca. 48 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of research grade.

Animals

SD rats were purchased from Tokyo experimental Animals (Tokyo, Japan) and maintained in an environmentally controlled room (25 °C) with a 12-h light/dark cycle and allowed access to standard laboratory chow (Oriental Yeast Co. Ltd., Tokyo) and water ad libitum. Rats weighting about 280 g (250–300 g) were randomly assigned to each experimental group.

Cell cultures

Caco-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 50 U/ml penicillin G, and 50 μg/ml streptomycin in a humidified 5% CO_2-95% air at 37 °C. Caco-2 cells were seeded onto polycarbonate filters (pore size, 0.4 μm; area, 4.7 cm^2) in Costar Transwell 6-well plates at a seeding density of 5.0×10^4 cells/cm^2 and were grown for 3 weeks.

Ex vivo studies in rats (in situ closed loop technique)

Rats were fasted for 18 h before the experiment and were anesthetized with urethane (1 g/kg, ip). Ten cm of a jejunal or ileal loop were prepared after the lumen was washed out by saline. FD-4 was dissolved at a concentration of 1 mM in phosphate buffered saline (PBS; pH 7.4) containing 1–15 mM polyamines. The solution (1 ml) was introduced into the loop via a microsyringe, and blood was withdrawn from the jugular vein at designated times. Blood samples were centrifuged (18,000 × g, 5 min) and the plasma concentration of FD-4 was determined spectrophotometrically.

Assessment of membrane damage

After 1 h of administration of spermine into the jejunum, PBS buffer was recovered for determination of lactate dehydrogenase (LDH) leakage. The release of LDH was examined using a commercially available kit (Wako Pure Chemicals). Histopathological observation of the intestinal mucosa of the spermine-administrated rat was also done. The rat jejunum was filled with 10% neutral formalin, prepared for histological specimens, stained with hematoxylin-eosin, and examined by light microscopy. Histopathological evaluation was carried out by the senior pathologists at the Hist Science Laboratory Co., Tokyo.

In vivo studies in rats

Rats were fasted for 18 h before the experiment. Spermine was dissolved at a concentration of 30 mM in saline and 1 ml of solution was orally administrated. After 1 h, 1 ml of 4 mM FD-4 solution containing 30 mM spermine was orally administrated. Blood was withdrawn from the jugular vein of rats anesthetized with ether at designated times after administration of FD-4. The plasma concentration of FD-4 was determined using a fluorescence spectrophotometer.

In vitro transport studies

The transport media were in accordance with those described by Ranaldi et al. (2002). Solutions of spermine (5 and 15 mM) and poly-L-arginine (0.05 and 0.25% w/v) were prepared in Hanks balanced salt solution (HBSS) buffered at pH 6.0 with 10 mM morpholinoethane sulfonic acid (MES) containing 100 μM FD-4. Before each experiment, the confluent monolayers were washed twice with PBS and re-equilibrated for 50 min in the absence of the polycationic substance with HBSS containing 10 mM MES at pH 6.0 in the apical compartment and HBSS containing 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) at pH 7.4 in the basolateral compartment. After removal of the medium, Caco-2 cells were treated for 4 h at 37 °C with the polycation solution containing FD-4. After the addition of FD-4 solution to the apical side, a 100 μL aliquot of sample was withdrawn from the basolateral side at 20-min intervals, followed by the addition of 100 μl of HBSS (pH 7.4) to maintain a constant serosal volume. Controls were run in every experiment with solutions containing FD-4 without the dissolved polycationic substance.

Measurement of the TEER

The TEER of the cell monolayers was measured at 37 °C using a Millicell ERS meter (Millipore Corp., Bedford, MA). TEER measures were started during the pre-equilibration time in HBSS and continued during the treatment, recording values every 20 min over the 4-h experiment. Control experiments were done under the same conditions without dissolved polycations. Average TEER values for untreated cell monolayers were in the range of 600–1000 $\Omega \cdot cm^2$.

Determination of FD-4

Sample solutions were diluted with 0.1 M borate buffer solution (pH 8.5) and the fluorescence intensity of FD-4 in the sample solutions was determined at an excitation wavelength of 495 nm and an emission wavelength of 515 nm using a JASCO fluorescence spectrophotometer FP-750.

Results

Evaluation of FD-4 absorption by the in situ intestinal loop technique

Small intestine absorption was evaluated by the in situ closed intestinal loop technique using FD-4 as a model of macromolecule absorption in the rat. The concentration of FD-4 appearing in plasma was determined at designated times over a 3 h period. The time course of FD-4 concentration in plasma after its administration into the jejunal loop in the presence of spermine is shown in Fig. 1A. When 1 ml of spermine solution containing FD-4 was administered into the intestinal loop, the absorption of FD-4 from the jejunum was enhanced by spermine, and 5 mM spermine solution significantly increased the absorption of FD-4 compared with controls (PBS containing FD-4). An increase in FD-4 absorption was remarkable after 1 h of administration. The same tendency was found to occur on administration of 1 ml of 15 mM spermine solution, whereas the lower concentrations of spermine (1 mM solution) slightly but significantly increased the absorption of FD-4. In contrast, in the ileum, no significant increase of FD-4 absorption was observed in the presence of 5 or 15 mM spermine (Fig. 1B). The degree of absorption-enhancing effect of spermine in the jejunum was greater than that in the ileum. The absorption of FD-4 from the jejunum was also enhanced by spermidine in the same manner, whereas putrescine had no effect on the absorption of FD-4 from the small intestine (Fig. 2). On the other hand, treatment with poly-L-arginine, which is known to be the polycationic absorption enhancer, elevated plasma FD-4 levels without delay after administration into the jejunal loop.

To assess the toxicity of spermine in rat jejunum, we performed histopathological investigations and examined LDH leakage into the intestinal lumen (Figs. 3 and 4). The toxicity of spermine was also compared with that of

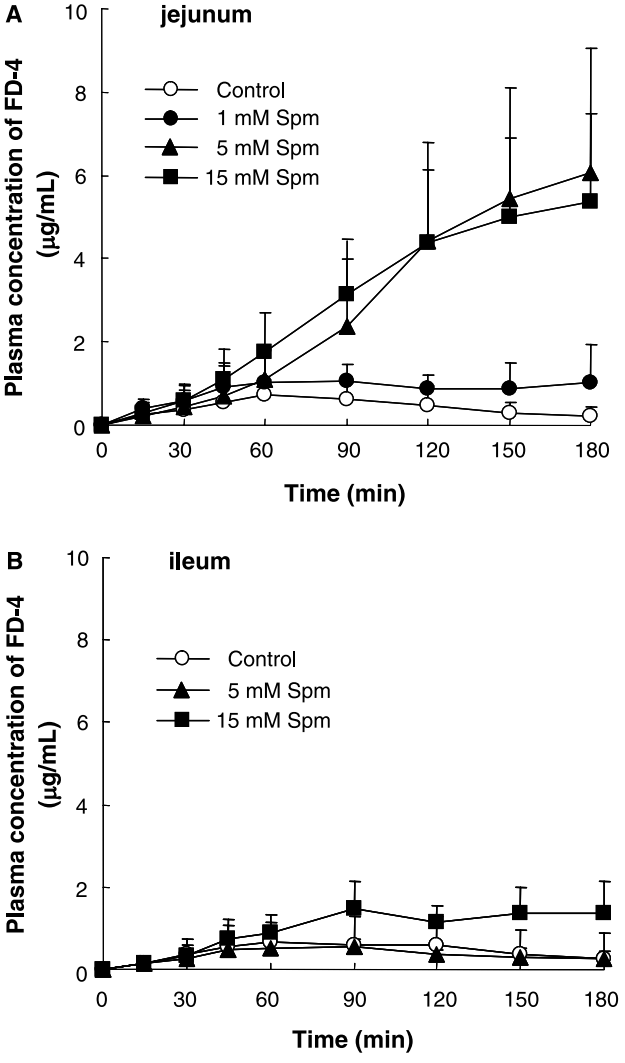

Fig. 1. Effect of spermine (Spm) on FD-4 absorption in the in situ rat jejunum (**A**) or ileum (**B**) loop study. The lumen was washed out by saline and each concentration of 1 ml of drug solution was introduced into the loop of jejunum or ileum. Data are means ± SD ($n = 5$–9)

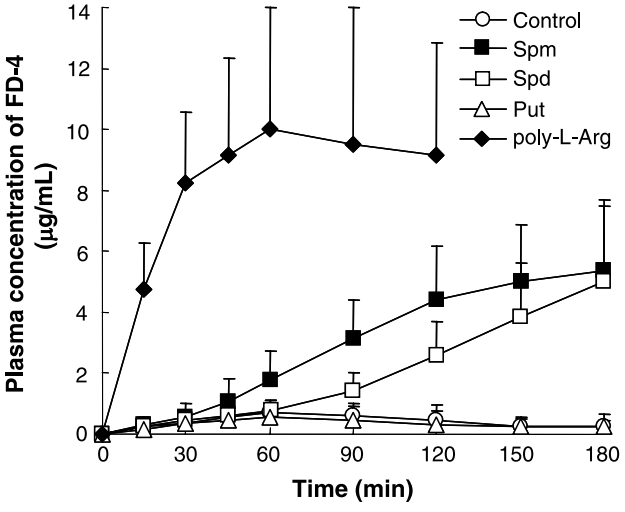

Fig. 2. Effect of polyamines and poly-L-arginine (poly-L-Arg) on FD-4 absorption in the in situ rat jejunum loop study. The rat jejunum was treated with 1 ml of FD-4 solution in the presence of Spm (15 mM, $n = 8$), spermidine (Spd; 15 mM, $n = 8$), putrescine (Put; 15 mM, $n = 5$) or poly-L-Arg (2.5 mg/ml, $n = 3$). Data are means ± SD

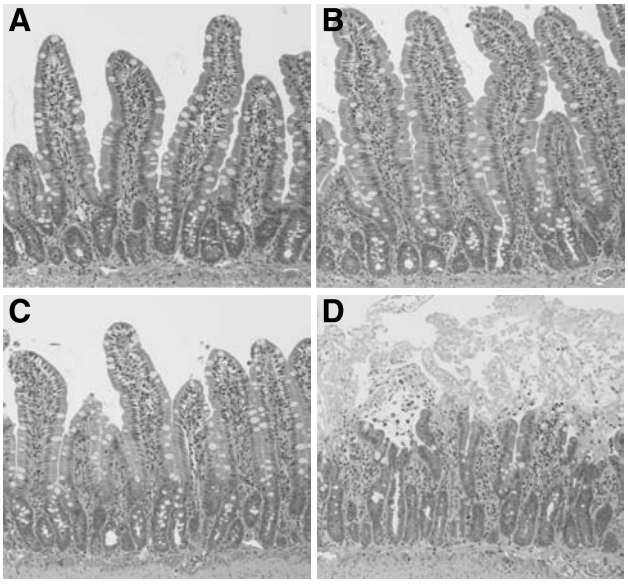

Fig. 3. Histopathological observation of the spermine treated jejunum. Micrographs were taken at 1 h after administration into the jejunal loop. **A** Control, **B** Spm (5 mM), **C** poly-L-Arg (2.5 mg/ml), **D** C10 (40 mg/ml)

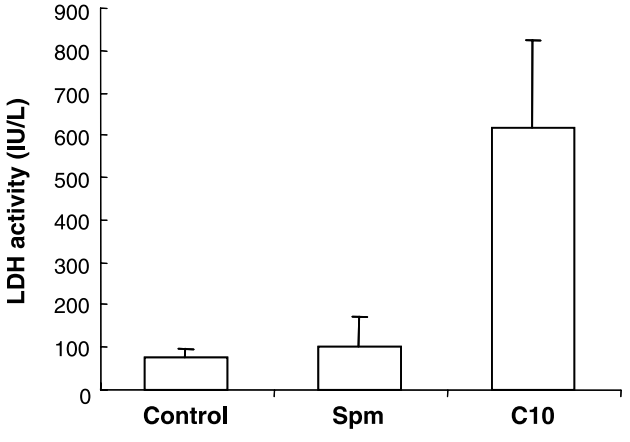

Fig. 4. LDH leakage from the jejunum treated with spermine. After a 1 h treatment with 1 ml solution of Spm (5 mM) or C10 (40 mg/ml), PBS was added to the jejunum, and the solution was recovered for the determination of LDH leakage. Data are means ± SD ($n = 3$–4)

sodium caprate (C10), a clinically used enhancer of absorption. Figure 3 shows the typical photographs of the jejunum 1 h after administration of FD-4 solution control, spermine, poly-L-arginine, and C10. No significant structural alterations in the epithelial membrane were observed after spermine or poly-L-arginine administration compared with controls (Fig. 3B and C), although a slight disarranged layer of the villous mucosal epithelium was seen. For poly-L-arginine, a slight focal accumulation of goblet cells and infiltration of cells, including granulocytes, between epithelial cells were observed. In contrast, a high concentration of C10 produced marked damage, including erosion in the membrane structure (Fig. 3D). In agreement with the histopathological results, a marked increase in the release of LDH was observed in the presence of C10, indicating high toxicity to the intestinal membrane. In contrast, spermine caused an only minor release of LDH compared with control levels, suggesting low toxicity (Fig. 4).

Evaluation of FD-absorption in vivo

The absorption-enhancing effect of spermine was further evaluated by an in vivo study. The time course of FD-4 concentration in plasma after oral co-administration of FD-4 and spermine in rats is shown in Fig. 5. When FD-4 was orally administered alone, limited absorption was observed. Plasma FD-4 levels increased significantly compared with control after the co-administration of spermine.

In vitro transport of FD-4 and TEER across Caco-2 cell monolayers

The effects of polyamines and poly-L-arginine on the TEER of the Caco-2 cell monolayers are shown in Fig. 6A.

Incubation with poly-L-arginine resulted in a pronounced and immediate reduction in TEER values in a concentration-dependent way compared with the control group. In contrast, a high concentration of spermine (15 mM) was able to cause decrease in TEER values compared with the control, although no significant decrease in TEER of the cell monolayers was observed by incubation with 5 mM spermine. Prolonged incubation with 15 mM

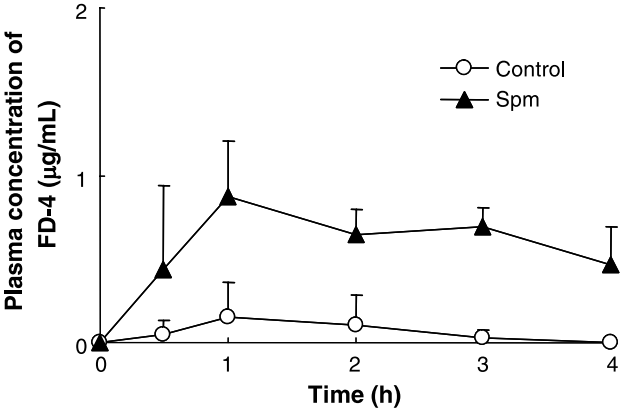

Fig. 5. Effect of spermine on FD-4 absorption after oral administration to rats. FD-4 was co-administered orally with Spm (1 ml of 30 mM solution) after 1 h of pre-administration of Spm (1 ml of 30 mM solution). Data are means ± SD ($n = 3$)

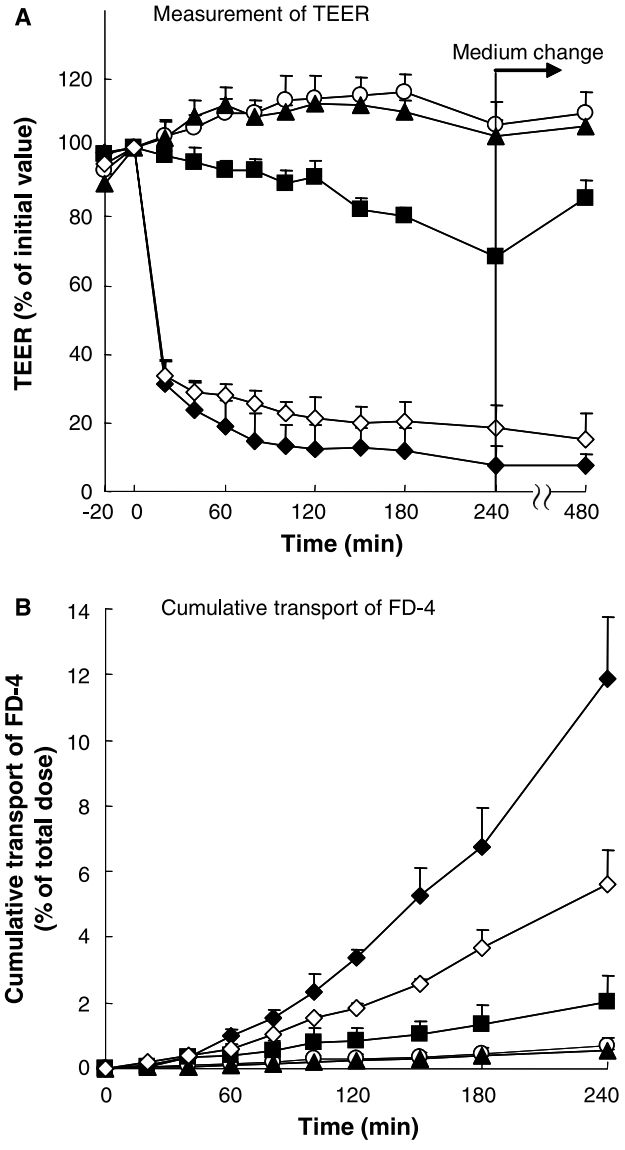

Fig. 6. Effect of spermine on the TEER (**A**) and the transport of FD-4 (**B**) across Caco-2 cells monolayers. Control (○), 5 mM Spm (▲), 15 mM Spm (■), 0.5 mg/ml poly-L-Arg (◇), 2.5 mg/ml poly-L-Arg (◆). Data are means ± SD ($n = 4$)

spermine, up to 4 h, resulted in a gradual decrease in TEER from initial values.

When the monolayers were incubated with fresh culture medium after removal of the spermine solution and repeated washing of filters with PBS, a clearer tendency to recover to initial values was observed (Fig. 6A), suggesting the reversibility of the effect of polyamines on TEER of the cell monolayers.

The permeation profiles of spermine and poly-L-arginine are shown in Fig 6B. In agreement with the TEER results, poly-L-arginine produced a marked concentration-dependent increase the transport of FD-4. In contrast, incubation with 15 mM spermine resulted in a progressive increase in the permeability of the tight junctions in Caco-2 cells as shown by a gradual decrease in TEER, although treatment with 5 mM spermine did not reveal an increased permeation of FD-4.

Discussion

In the present study, we investigated the enhancing effect of polyamines on the intestinal absorption of FD-4 as a water-soluble macromolecule in an in situ jejunum loop study, in vivo oral absorption study, and in vitro transport study using Caco2-cell monolayers. It has been reported that the plasma concentrations of poorly absorbed compounds are markedly increased by the addition of several known absorption enhancers such as C10 (Tomita et al., 1995; Lindmark et al., 1998; Hayashi et al., 1999) and medium-chain alkyl saccharides (Murakami et al., 1992; Uchiyama et al., 1996, 1999), as well as poly-L-arginine, in the in situ closed loop methods. In these all cases, the plasma peaks generally appeared very early, that is, within 1 h after administration. In the case of spermine, an increase in FD-4 absorption was remarkable after 1 h, and the time to reach the peak plasma level of FD-4 was delayed to 3 h after administration. Similar plasma level vs time profiles have been found to occur during the treatment of a C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) as a claudin modulator (Kondoh et al., 2005).

The absorption-enhancing abilities of the absorption enhancers vary considerably according to the site of the gastrointestinal tract. Murakami et al. (1992) reported that the enhancing effects of n-dodecyl β-maltopyranoside, an alkyl saccharide, on the absorption of carboxyfluorescein displayed considerably larger increases in lower regions, and the rectum was determined to be the most effective site. Kondoh et al. (2005) reported that treatment with C-CPE enhanced absorption of FD-4 in the jejunum but not the colon. Our results show that spermime is a more effective absorption enhancer in the jejunum than in the ileum. These results suggest that spermine has tissue-specific effects, although the reason for the regional differences in the transport of FD-4 are not clearly understood. In vivo oral absorption studies also revealed an increased absorption of FD-4 after co-administration of spermine. This result is indicative of the fact that dietary or luminal polyamines may play a role in gastrointestinal absorption of undigested nutritional substances.

Thus far, several reports have shown that polycationic compounds, including poly-L-arginine and chitosan

derivatives, have the potential to promote the transmucosal permeation of macromolecules without causing severe epithelial damage (Ranaldi et al., 2002; Ohtake et al., 2003a, b). One mechanism for the enhancing effect of polycations could be the initiation of an ionic interaction between the positive charge and the negative charges on the surface of the cell membrane (McEwan et al., 1993; Schipper et al., 1997; Miyamoto et al., 2001). Therefore, interaction of the positively charged amino groups of spermine with the negative membrane components, might influence intestinal permeability.

It has been reported that the binding- and absorption-enhancing effects of chitsan on epithelial cells are mediated through their positive charge (Schipper et al., 1997). In addition, the degree of the amino group in the chitosan derivatives may influence the degree of interaction between the positive charge and the cell-surface negative charges, as well as the molecular weight of the chitsan derivatives. The interaction of chitosans with the cell membrane results in a structural reorganization of tight junction-associated proteins, which is followed by enhanced transport through the paracellular pathway (Schipper et al., 1997; Dodane et al., 1999; Ranaldi et al., 2002). It has been also reported that, for poly-L-arginine, the charge density of the polycations is related to the ionic interaction, and that the number of positive charges in one poly-L-arginine molecule could be one of the most important factors for determining the degree of enhancement of the absorption (Miyamoto et al., 2001). In the present study, putrescine did not increase the absorption of FD-4 in the in situ closed loop experiments, even though putrescine was administered at a concentration of 15 mM. The reason may be due to difference of charge density of spermine and putrescine.

In the case of in vitro transport studies with Caco-2 cells, the permeable-enhancing effect of spermine was low compared with the in situ jejunal loop studies. The reason for this finding might be due to tissue-specific effects of spermine, because Caco-2 cells originate from human colon adenocarcinoma cells (Hidalgo et al., 1989). On the other hand, incubation with poly-L-arginine resulted in a marked accumulation of FD-4 in the acceptor compartment compared with controls, in agreement with the TEER results. Measurement of TEER is believed to be a good indication of the tightness of the junction between cells. Decreases in TEER indicate the opening of tight junctions, resulting in the enhancement of absorption via the paracellular pathway. In this study, the prolonged incubation with 15 mM spermine resulted in a gradual decrease in TEER. This finding suggests that the absorption-enhancing mechanism of spermine partly includes opening the tight junctions of the epithelium via the paracellular route. Osman et al. (1998) reported that spermine at high concentrations (10–50 mM) appeared to mainly affect intestinal transcellular permeability, probably due to some effect on cell membrance integrity, while having little or no effect on paracellular permeability in the in vitro studies in rats. FD-4 may be transported across the epithelium by both transcellular and paracellular routes in the presence of various compounds that have an absorption-enhancing effect (Marttin et al., 1997; Schipper et al., 1997; Nakamura et al., 2003), although we did not determine the main transport pathway of FD-4 with co-administrated spermine in the present studies.

Highly effective absorption enhancers, such as C10, often cause damage to the intestinal mucosal membrane. Kondoh et al. (2005) also reported that C10 caused remarkable damage to the small intestinal membrane in the in situ loop assay at a similar concentration as used in this study. In contrast, treatment with spermine did not induce serious intestinal damage in the present study. Miyake et al. (2006) recently reported that spermine can improve intestinal absorption of a poorly absorbable drug after oral administration to rats without causing any significant changes in the gastrointestinal tract. We also examined the reversibility of spermine on TEER of Caco-2 cell monolayers and observed a clear tendency to recover to the initial value. This result also suggests that the plasma membrane was not permanently damaged by treatment with spermine.

Cellular polyamines play a critical role in maintenance of the intestinal epithelial integrity. It has been reported that polyamines are implicated in the regulation of the intestinal epithelial barrier function and that depletion of cellular polyamines increases epithelial paracellular permeability at least partially by inhibiting expression of various tight junction proteins (Guo et al., 2005). Namely, polyamines are also required for normal function of tight junctions. In intestinal epithelial cells, intracellular polyamine concentrations are high and maintained by endogenous polyamine biosynthesis and uptake of polyamines from the gastrointestinal lumen (McCormack and Johnson, 1991).

In adult humans, the daily intake of polyamines from a typical diet is estimated at hundreds of micromoles, and significant differences in polyamine concentrations and distribution patterns are observed between food groups (Bardocz et al., 1995; Nishimura et al., 2006; Nishibori et al., 2007). Thus, beans show high concentrations of spermidine and spermine, vegetables have higher levels of putrescine and spermidine, and fish, shellfish, meat, and

nuts have high levels of spermine (Nishibori et al., 2007). Thus, different dietary balances and dietary habits might lead to variations in polyamine intake. In addition, luminal polyamines rapidly disappear from the duodenal and jejunal lumen into the circulation (Uda et al., 2003). Although the exact mechanisms of the absorption-enhancing effect of polyamines are still unclear, it is possibly related to the specific molecular structure of polyamines.

As factors that induce maturation of the digestive system, that is, precocious intestinal and pancreatic maturation (Romain et al., 1998), dietary polyamines could play a role in the prevention of food allergy. Indeed, oral administration of spermine induces precocious maturation of the intestine resulting in the cessation of macromolecular transport in suckling rats (Harada et al., 1994).

Modulation of the intestinal epithelial barrier function by ingested luminal polyamine may influence the induction of food allergies. An enhanced intestinal permeability by daily ingestion of polyamines may be implicated in regulation of the induction of tolerance because oral tolerance induction is related to a gradual and continuous absorption of the food allergen (Stokes et al., 1983; Akiyama et al., 2001). After oral sensitization, an increase in intestinal permeability by polyamines may facilitate the absorption of allergen into the circulation and lead to the anaphylaxis response, as the immediate hypersensitivity response to antigen.

In conclusion, we found that polyamines, especially spermine, can enhance the intestinal absorption of FD-4, a hydrophilic macromolecule. These results indicate that excess oral ingestion of polyamines may have widespread health effects.

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