Gastrointestinal Absorption of Recombinant Human Insulin-Like Growth Factor-I in Rats

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ABSTRACT
The GI absorption of recombinant human insulin-like growth factor-I (rhIGF-I) and its improvement were investigated in rats. The $^{125}$I-rhIGF-I rapidly degraded to the trichloroacetic acid-soluble form in the small-intestinal contents, but it was relatively stable in the gastric and large-intestinal contents and in the subcellular fraction of the small-intestinal mucosa. To protect rhIGF-I from degradation in the small-intestinal contents, the effect of some adjuvants was examined and their degradation was markedly inhibited by the presence of aprotinin or casein. After p.o. administration of $^{125}$I-rhIGF-I at the dose of 1.0 mg/kg, trichloroacetic acid-precipitable radioactivity in the plasma was periodically determined. We found that a considerable amount of rhIGF-I was absorbed into the systemic circulation and that the bioavailability was 9.3%, which is much greater than that of insulin. The coadministration of aprotinin and that of casein enhanced the bioavailability further: 46.9% and 67.0%, respectively. Radioimmunoassay using a monoclonal antibody for rhIGF-I confirmed the high bioavailability of immunoreactive rhIGF-I. From gel chromatography of plasma, the radioactivity in the plasma was found to be in the form of high-molecular-weight complexes. The mechanism for the uptake of rhIGF-I by intestinal mucosa may be absorptive-mediated endocytosis.

It has become possible to produce biologically active peptides and proteins that are therapeutically applicable by means of recombinant DNA technology. rhIGF-I, a peptide composed of 70 amino acids residues with a molecular weight of 7649, is one of them. IGF-I is an essential factor that controls the growth-promoting action. It has considerable homology with proinsulin but exerts its biological actions through specific IGF-I receptors (Humbel, 1990).

Recently, rhIGF-I has been used clinically to treat both Laron dwarfism, in which the function of the growth hormone receptor is deficient, and insulin-resistant diabetes. However, because frequent s.c. injections are needed for the therapy, the patients experience great discomfort. To improve the quality of life of such patients, an alternative method of administration is needed. We have already studied the nasal route as one substitute for s.c. injection and showed its utility as a novel route of administration of rhIGF-I in rats (Ukai et al., 1996). However, p.o. administration is the most convenient route, and the development of its formulation for oral dosage would be of great value. Furthermore, IGF-I must be absorbed from the GI tract of the suckling newborn, because IGF-I in the maternal milk may play a role in regulating its postnatal development (Xu, 1996). Recently, Vacher et al. (1995) and Xu and Wang (1996) reported the absorption of IGF-I from the GI tract of neonatal calves and neonatal pigs, respectively. Furthermore, several protease inhibitors, including casein, are reported to be in the milk they ingest (Rao et al., 1993). However, there is no information on the GI absorption of IGF-I in adult animals.

In the present study, to investigate the possibility of p.o. administration of rhIGF-I, we examined the GI absorption of rhIGF-I and its improvement in adult rats using several protease inhibitors.

Materials and Methods

Materials
The following drugs and chemicals were kindly provided by or obtained from the sources indicated: rhIGF-I, a monoclonal antibody (McAb) for rhIGF-I and $^{125}$I-rhIGF-I (Fujisawa Pharmaceutical Co., Osaka, Japan), aprotinin (Teikokuzouki Pharmaceutical Co., Tokyo, Japan), casein (Nacalai Tesque Inc., Kyoto, Japan), DM$\beta$CD (Funa-koshi Co., Tokyo, Japan), SGC, FD4 (average molecular weight 4400), colchicine, poly-L-lysine hydrobromide (molecular weight

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ABBREVIATIONS: IGF-I, insulin-like growth factor-I; rhIGF-I, recombinant human insulin-like growth factor-I; McAb, monoclonal antibody; FD4, fluorescein isothiocyanate-dextran 4000; DM$\beta$CD, dimethyl $\beta$-cyclodextrin; SGC, sodium glycocholate; TCA, trichloroacetic acid; BBM, brush border membrane; BSA, bovine serum albumin; AUC, area under the plasma concentration versus time curve; MRT, mean residence time; C$\text{max}$, maximum plasma concentration; $T_{\text{max}}$, time to reach C$\text{max}$; RIA, radioimmunoassay; CL$\text{abs}$, absorption clearance; IGFBP, IGF binding protein; DNP, 2,4-dinitrophenol.
about 1000), proteamine (Grade IV, from salmon), poly-L-glutamic acid sodium salt (molecular weight about 1000) (Sigma Chemical Co., St. Louis, MO) and other reagents were used without further purification.

**Animals**
Male Sprague-Dawley rats weighing 200 to 300 g (Charles River Japan, Inc., Yokohama, Japan) were used as the animal model.

**Preparation of Luminal Contents and Mucosal Subcellular Fractions**
The luminal contents of the GI tract were prepared according to the method of Asada et al. (1994). Briefly, under urethane anesthesia, the GI tract was excised. The luminal contents of the stomach were collected by flushing 15 ml of artificial gastric juice (pH 1.2), and the contents of the jejunum (the 20 cm below the ligament of Treitz), ileum (the 20 cm above the ileocecal junction) and large intestine (from colon to anus) were collected by flushing 15 ml of isotonic phosphate buffer (pH 7.4).

Mucosal subcellular fractions of the small intestine were prepared according to the method of Bai and Chang (1995), with slight modification. The intestinal mucosa of each segment was scraped off, suspended in 0.3 M sucrose buffer (pH 7.0) and then homogenized using a blender homogenizer (Nihon Seiki Ltd., Tokyo). The homogenate was centrifuged at 100,000 × g at 4°C for 1 h to separate each fraction. The resulting supernatant was used as the cytosol fraction, and the pellet was divided into two fractions. One was resuspended with 0.3 M sucrose buffer (pH 7.0) as a BBM fraction, the other with 1 M acetate/NaOH buffer (pH 4.5) as a lysosomal fraction.

**Degradation Study**
The degradation of 125I-rhIGF-I in the luminal contents and mucosal subcellular fractions was examined according to the method of Bai and Chang (1995). The incubation mixture consisted of 50 mM Tris/HCl buffer (pH 7.5) (or artificial gastric juice (pH 1.2) or 1 M acetate/NaOH buffer (pH 4.5)), 125 mM NaCl, 1% BSA, 8 μg/ml of 125I-rhIGF-I, some adjuvants (0.8 μg/ml of aprotinin, 10 mg/ml of casein, 20 mg/ml DMJ/CD or 2.4 mg/ml of SOG) and a luminal content (0.008–0.12 mg protein) or a subcellular fraction (0.4–2.2 mg protein). The experiment of 125I-rhIGF-I degradation was performed at 37°C, and the incubation periods were 0, 1, 5, 10, 15 and 30 min. To stop proteolysis, 200 μl of 15% TCA was added to 200 μl of the incubation mixture. The resulting mixture was then centrifuged at 1600 × g for 10 min. We assumed that the TCA-precipitable radioactivity indicates the intact rhIGF-I, whereas the degraded rhIGF-I remains soluble in TCA solution, and we calculated the degrading activity (nmol/min/g protein).

**Absorption Study**

**Oral administration experiments.** The p.o. administration of 125I-rhIGF-I alone or with aprotinin or casein was carried out by a gastric sonde after fasting for 16 h. Doses of 125I-rhIGF-I, aprotinin and casein were 1.0, 4.0 and 10 mg/kg as saline solution (5 ml/kg), respectively. The blood samples were periodically taken from the cannulated femoral artery, and TCA-precipitable radioactivity in plasma was determined. In this study, rhIGF-I was determined by RIA using a McAb for rhIGF-I. Similar experiments were also carried out for FD4 as a hydrophilic reference drug.

**In vitro everted sac experiments.** Under urethane anesthesia, the jejunum of jejunum was excised after washing out of the luminal side with 20 ml of isotonic phosphate buffer (pH 7.4). The isolated jejunum was divided and inserted into 5-cm segments. Half a milliliter of the isotonic phosphate buffer was introduced into the serosal side, and both ends of the segment were ligated. The resulting everted sac was placed in the incubation medium (10 ml), pH 7.4 isotonic phosphate buffer containing 80 ng/ml of 125I-rhIGF-I and 10 ng/ml of casein with or without some adjuvants (1 mM poly-L-lysine, 1 mM protamine, 1 mM poly-L-glutamic acid, 1 mM DNP or 0.1 mM colchicine) and was bubbled with 95% O2/5% CO2 throughout the experiments. The incubation was performed for 30 min at 37°C, and then the everted sac was twice immersed for 5 min in 20 ml of the fresh isotonic buffer (pH 7.4, 4°C) to remove the nonspecifically adsorbed 125I-rhIGF-I on the mucosal surface. To remove the acid-sensitively adsorbed 125I-rhIGF-I, we immersed the everted sac in the acidic washing solution (pH 3.0, 0.2 M acetic acid/0.5 M NaCl, 10 ml) for 10 min in an ice-cold water bath. After recovery of the serosal solution, the tissue was homogenized and mixed with 4% acetic acid. The mixture was centrifuged at 3000 rpm for 10 min. TCA-precipitable radioactivity in the resulting supernatant and in the serosal solution was determined.

**Gel Chromatography of Rat Plasma**
The gel chromatography of rat plasma was performed as described previously (Kimura et al., 1994). Briefly, 2 ml of a plasma sample was applied to a Sephacryl S200HR (Pharmacia LKB Biotechnology, Tokyo) column (16 mm I.D. × 600 mm) previously equilibrated with 10 mM phosphate buffer (pH 7.4) containing 0.02% NaN3 and 25 mM EDTA. The elution was carried out at a flow rate of 28.8 ml/h, and the fractions (2.4 ml each) were collected. The plasma samples applied were as follows: a plasma at 1 h after p.o. administration of 125I-rhIGF-I alone, a plasma at 3 h after p.o. administration of 125I-rhIGF-I with casein, a plasma at 5 min after i.v. administration of 125I-rhIGF-I and a 125I-rhIGF-I-spiked plasma.

**Assay Procedures**
The 125I-rhIGF-I in the plasma and GI-luminal contents was determined by TCA precipitation. For plasma, 1 ml of Krebs-Henseleit bicarbonate buffer containing 5% BSA was added to 50 μl of a sample plasma, and then 1 ml of 15% TCA was added to the mixed solution. As for GI-luminal contents, 200 μl of 15% TCA was added to 200 μl of the incubation mixture. Then the mixtures were centrifuged at 3000 rpm for 15 min, and the radioactivities in the precipitate and the supernatant were counted as unchanged and degraded rhIGF-I, respectively, by a γ-counter (Aroka, Tokyo). The radioactivity in the effluent of gel chromatography was determined directly by a γ-counter. The immunoreactive rhIGF-I was determined by RIA using a McAb for rhIGF-I as reported previously (Suzuki et al., 1987;
Kimura et al., 1994). Neither casein nor aprotinin cross-reacts with McAb for rhIGF-I. The plasma glucose level was determined by a glucose-oxidase method using an assay kit (Iatrochrome GLU-LQ, Iatron Laboratories Co., Tokyo). FD4 was determined by means of a spectrofluorophotometer (Shimadzu FR-540, Kyoto) at 495 nm and 512 nm for the excitation and emission wave lengths, respectively. Protein concentrations of each GI content and subcellular fraction were determined by the method of Lowry et al. (1951) with BSA as a standard.

Data Analysis

The plasma concentration-time data were analyzed noncompart-mentally on the basis of the statistical moment theory (Yamaoka et al., 1978). The moments, the AUC and the MRT, were calculated by the trapezoidal method with a monoexponential extrapolation of the terminal phase. The bioavailability of rhIGF-I after intraintestinal or p.o. administration was calculated from the AUC using the value after the i.v. administration. In the in situ single-pass perfusion experiments, the CLabs at steady state was calculated by the following equation:

$$\text{CL}_{\text{abs}} = \frac{Q_{\text{in}} \cdot C_{\text{in}} - Q_{\text{out}} \cdot C_{\text{out}}}{C_{\text{in}}}$$

where $Q_{\text{in}}$ and $Q_{\text{out}}$ are the flow rates of inflow and outflow perfusates, respectively, and $C_{\text{in}}$ and $C_{\text{out}}$ are the concentrations in inflow and outflow perfusates, respectively.

Statistical Analysis

Statistical significance was evaluated by using Student’s $t$ test.

Results

Degradation of rhIGF-I in GI contents and mucosa. Figure 1 shows the degradation of rhIGF-I in the GI contents. As is evident from the figure, $^{125}$I-rhIGF-I rapidly degraded to the TCA-soluble form in the small-intestinal contents, but it was relatively stable in the gastric and large-intestinal contents. The calculated degradation rates in the GI contents are summarized in table 1. The maximal degradation rate was 326 nmol/min/g protein in the ileum. Some adjuvants could inhibit the degradation, especially aprotinin and casein, which inhibited it by 70% to 95%.

Table 1 also shows the degrading activity in the intestinal mucosa. In contrast to the GI contents, rhIGF-I was relatively stable in any subdivision of the intestinal mucosa, and there was no site difference through the intestine.

Intestinal absorption of rhIGF-I and its improvement. Figure 2 shows the plasma concentration-time curves of TCA-precipitable radioactivity, regarded as unchanged, after p.o. administration of $^{125}$I-rhIGF-I (1.0 mg/kg) with or without aprotinin (4.0 mg/kg) or casein (10.0 mg/kg). The bioavailability of rhIGF-I without adjuvants was 9.3 ± 0.7%. The bioavailability was further increased by the coadministration of aprotinin and that of casein to 46.9 ± 1.9% (P < .001) and 67.0 ± 4.8% (P < .001), respectively.

To confirm the absorption of rhIGF-I, immunoreactive rhIGF-I in the plasma was determined after oral administration of unlabeled rhIGF-I. Figure 3 shows the plasma immunoreactive rhIGF-I concentration-time profiles. The bioavailability values of rhIGF-I administered alone, with aprotinin and with casein were 11.7 ± 3.5%, 41.9 ± 12.3% (P < .05) and 87.8 ± 11.0% (P < .001), respectively.

The site difference of rhIGF-I absorption in the intestine was also examined by an in situ loop method. As shown in figure 4, a marked site difference was observed; the rank order of the absorbability was jejunum > ileum > large intestine (table 2).

Figure 5 shows the changes in the plasma glucose level after p.o. administration of rhIGF-I (1.0 mg/kg). The hypoglycemic effect of rhIGF-I was evaluated in terms of the difference between the AUC of glucose (0–12 h) after p.o. administration of rhIGF-I and that after saline administration. The decreased AUC values of glucose were 44.0 ± 21.1, 204.4 ± 32.8 (P < .01, compared with rhIGF-I alone) and 316.4 ± 29.3% · h (P < .001, compared with rhIGF-I alone) for rhIGF-I alone, with aprotinin and with casein, respectively, whereas that for i.v. administration was 321.7 ± 24.3% · h (P < .001, compared with oral dosing of rhIGF-I alone). The marked hypoglycemic effect of rhIGF-I was recognized especially in the case of coadministration with aprotinin or casein, which indicates that the bioactive rhIGF-I was substantially absorbed into the systemic circulation.

Form of rhIGF-I in plasma. Figure 6 shows elution profiles of the plasma radioactivity on a Sephacryl S200HR column. The peaks in the high-molecular-weight region, but no peak of free rhIGF-I, were detected 1 h after p.o. administration of $^{125}$I-rhIGF-I alone and 3 h after its p.o. administration with casein. The peaks agreed well with high-molecular-weight complexes after the i.v. administration.

Saturable absorption of rhIGF-I. To estimate the absorption kinetics of rhIGF-I, we performed in situ single-pass perfusion studies in the presence of casein at 2.0 mg/ml to protect rhIGF-I from the degradation in the perfusate. Table 3 shows the values of CLabs for rhIGF-I and FD4 in rat jejunum. The CLabs values for rhIGF-I were much higher than that for FD4 and were dependent on its initial concentration, which suggests the saturation of rhIGF-I absorption.
Furthermore, we noted acid-washable binding to the mucosal surface and internalization into mucosal tissue. When the initial concentration was 405.2 ng/ml, the absorption rate was 54.2 ± 6.5 ng/min/20 cm, and the acid-washable and internalized rhIGF-I were 39.7 ± 5.4 cm and 76.1 ± 33.9 ng/20 cm, respectively.

**Characterization of rhIGF-I transport.** To clarify the mechanism of the transport of rhIGF-I, we examined the effect of inhibitors on the uptake and transport of 125I-rhIGF-I across the jejunal mucosa. The results are summarized in table 4. The transport of 125I-rhIGF-I to the serosal side was significantly inhibited by low temperature, by DNP as an uncoupler of oxidative phosphorylation, by polycations (poly-L-lysine and protamine) and by colchicine as an inhibitor of microtubular assembly, but not by a polyanion (poly-L-glutamic acid). The amount in the intestinal tissue was also decreased. The total amount of 125I-rhIGF-I taken up and transported tended to be inhibited by these treatments except for the addition of poly-L-glutamic acid. Poly-L-lysine in particular significantly inhibited the accumulation of 125I-rhIGF-I in the intestinal tissue and its transport to the serosal side.

**Discussion**

IGF-I, a basic peptide composed of 70 amino acids, has been shown to mediate the growth-promoting action of growth hormone (Humbel, 1990). Recently, recombinant DNA technology has made it possible to supply rhIGF-I, and it is now being used clinically to treat Laron dwarfism and insulin-resistant diabetes. However, it is administered by frequent s.c. injections. The purpose of our study was to develop the p.o. dosage form of rhIGF-I to improve the quality of life of these patients.

The p.o. delivery of biologically active peptides is a very attractive but difficult project. There are two main barriers to the GI absorption of peptide drugs: degradation by proteases in the GI lumen and mucosa and the poor membrane permeability that results from their macromolecular and hydrophilic properties (Lee, 1990). For example, insulin is exten-
sively degraded in the small-intestinal lumen by pancreatic serine protease (Asada et al., 1994) and in the cytosol fraction of epithelial cells by insulin-degrading enzyme (Yamamoto et al., 1994; Bai and Chang, 1995). In addition, insulin forms the hexamer in aqueous solution (Porter et al., 1983), which results in poor penetration across the mucosal membrane.

The rhIGF-I was also extensively degraded in rat small-

Fig. 2. Plasma concentration profile of TCA-precipitable radioactivity after p.o. administration of 125I-rhIGF-I alone or with aprotinin or casein in rats. Plasma TCA-precipitable radioactivity was expressed as the amount of rhIGF-I equivalent. Doses of 125I-rhIGF-I, aprotinin and casein were 1.0, 4.0 and 10.0 mg/kg, respectively. Results are expressed as the mean ± S.E. of 4 to 6 experiments. ○, 125I-rhIGF-I alone; △, with aprotinin; ●, with casein.

Fig. 3. Plasma concentration profile of immunoreactive rhIGF-I after p.o. administration of rhIGF-I alone or with aprotinin or casein in rats. Plasma rhIGF-I was determined by RIA using McAb for rhIGF-I. Doses of rhIGF-I, aprotinin and casein were 1.0, 4.0 and 10.0 mg/kg, respectively. Results are expressed as the mean ± S.E. of 4 to 6 experiments. ○, rhIGF-I alone; △, with aprotinin; ●, with casein.

Fig. 4. Plasma concentration profiles of TCA-precipitable radioactivity after the administration of 125I-rhIGF-I alone or with adjuvants into the loops of jejunum (panel A), ileum (panel B) and large intestine (panel C). Plasma TCA-precipitable radioactivity was expressed as the amount of rhIGF-I equivalent. Doses of 125I-rhIGF-I, aprotinin and casein were 1.0, 4.0 and 10.0 mg/kg, respectively. Results are expressed as the mean ± S.E. of 3 to 4 experiments. ○, 125I-rhIGF-I alone; △, with aprotinin; ●, with casein.
intestinal contents, but unlike insulin, it was relatively stable in mucosal cells (table 1). As for the degradation of IGF-I in mucosal cells, Bai (1995), using rat colon enterocytes, reported that IGF-I is not degraded by insulin-degrading enzyme and that proteasome-like activities may result in IGF-I degradation, but not to a substantial extent. We have shown that rhIGF-I is extensively metabolized in the lysosomal fraction of the kidney (Kimura et al., 1994). In the small intestine, however, the metabolism of rhIGF-I in the lysosomal fraction was not so rapid. As for the degradation in GI contents, rhIGF-I was degraded very rapidly in the contents of the jejunum and ileum but was more stable in the stomach and large intestine. Similar results have been reported by Xian et al. (1995). They also showed that casein and (to a lesser extent) BSA and lactoferrin can protect IGF-I from degradation in the stomach or duodenal flushings, but the mechanisms for this protection are not clear. We confirmed the strong inhibitory effect of casein on rhIGF-I degradation in the intestinal contents (fig. 1).

**TABLE 2**

<table>
<thead>
<tr>
<th>Site</th>
<th>Adjuvant (n)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>MRT (h)</th>
<th>Bioavailability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunum</td>
<td>None (5)</td>
<td>0.7 ± 0.1</td>
<td>676.1 ± 51.3</td>
<td>2.8 ± 0.1</td>
<td>35.6 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Aprotinin (5)</td>
<td>1.5 ± 0.6</td>
<td>725.5 ± 74.7</td>
<td>2.9 ± 0.2</td>
<td>38.6 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>Casein (3)</td>
<td>1.3 ± 0.3</td>
<td>797.4 ± 52.0</td>
<td>3.0 ± 0.1</td>
<td>45.1 ± 3.4***</td>
</tr>
<tr>
<td>Ileum</td>
<td>None (5)</td>
<td>4.2 ± 1.1</td>
<td>369.1 ± 21.9</td>
<td>3.2 ± 0.1</td>
<td>17.9 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Aprotinin (5)</td>
<td>4.0 ± 0.6</td>
<td>492.2 ± 53.3</td>
<td>3.5 ± 0.1</td>
<td>22.3 ± 2.8*</td>
</tr>
<tr>
<td></td>
<td>Casein (3)</td>
<td>3.3 ± 1.3</td>
<td>446.6 ± 50.2</td>
<td>3.5 ± 0.1</td>
<td>26.7 ± 2.9**</td>
</tr>
<tr>
<td>Large intestine</td>
<td>None (5)</td>
<td>5.2 ± 0.5</td>
<td>146.8 ± 37.9</td>
<td>3.8 ± 0.1</td>
<td>5.6 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Aprotinin (5)</td>
<td>3.2 ± 0.8</td>
<td>270.3 ± 35.6</td>
<td>3.4 ± 0.1</td>
<td>15.7 ± 1.7*</td>
</tr>
<tr>
<td></td>
<td>Casein (3)</td>
<td>5.3 ± 0.7</td>
<td>66.0 ± 10.2</td>
<td>3.8 ± 0.2</td>
<td>3.6 ± 0.4</td>
</tr>
</tbody>
</table>

Doses of 125<sup>I</sup>-rhIGF-I, aprotinin and casein were 1.0, 4.0 and 10 mg/kg, respectively. Results are expressed as the mean ± S.E. * P < .05, ** P < .01, *** P < .001, compared with no adjuvant.

**TABLE 3**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Initial Concentration (ng/ml)</th>
<th>n</th>
<th>C&lt;sub&gt;lab&lt;/sub&gt; (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>125&lt;sup&gt;I&lt;/sup&gt;-rhIGF-I</td>
<td>88.1</td>
<td>5</td>
<td>0.212 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>405.2</td>
<td>5</td>
<td>0.122 ± 0.017*</td>
</tr>
<tr>
<td></td>
<td>1372.0</td>
<td>5</td>
<td>0.105 ± 0.016**</td>
</tr>
<tr>
<td>FD4</td>
<td>1457.6</td>
<td>3</td>
<td>0.011 ± 0.000</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± S.E. * P < .05, ** P < .01, compared with rhIGF-I (88.1 ng/ml).

IGF-I and IGF-II in the milk may be important in the differentiation of the GI tract and other organs of the newborn. Therefore, the growth factors must reach the intestine...
and pass through the intestinal wall intact. Indeed, Philipp et al. (1995) showed that milk-borne IGFs are stable in the GI tract of neonatal rats and remain biologically active for as long as 30 min after ingestion. Vacher et al. (1995) and Xu and Wang (1996) showed the absorption of p.o. administered IGF-I in neonatal calves and neonatal pigs, respectively. Rao et al. (1990) reported that the peptidase inhibitors are present in both the acid-soluble fraction and the acid-precipitable fraction (casein) of rat milk and that the inhibitory activity of casein is heat-stable whereas that of the acid-soluble fraction is heat-labile. They further investigated the peptidase inhibitors in the acid-soluble fraction of rat milk and reported the presence of at least three peptidase inhibitors (Rao et al., 1993), but the precise characteristics of the inhibitory activity of casein remain unknown. One of the reasons why suckling neonates can absorb IGF-I may be the stabilization of the peptide by protease inhibitors in milk they ingest. Therefore, we selected casein as one of the possible candidates for the peptidase inhibitor.

Aprotinin, a proteasome inhibitor, also markedly protected rhIGF-I from degradation in the intestinal contents (table 1). On the other hand, DMβCD, which showed protective activity against rhIGF-I degradation in nasal mucosa (Ukai et al., 1996), and SGC, which inhibited the degradation of insulin in nasal mucosa (Hirai et al., 1981), did not sufficiently inhibit rhIGF-I degradation in the intestinal contents (table 1). Consequently, only casein and aprotinin were found to be effective inhibitors.

The bioavailability of rhIGF-I after p.o. administration was 9.3% (fig. 2), which is much greater than that of insulin (negligible value) (Yamamoto et al., 1994). This significant absorption of rhIGF-I may be due to the fact that rhIGF-I, unlike insulin, does not form the hexamer (DeMeyts, 1994) and to its relative stability in the intestinal mucosa, especially in intracellular fractions (table 1). Another possibility is its uptake by a specialized mechanism, which will be discussed later. The bioavailability of rhIGF-I was markedly increased by the coadministration of aprotinin or casein (fig. 2). This result was confirmed by the determination of plasma immunoreactive rhIGF-I (fig. 3) and by the hypoglycemic activity (fig. 5). Each method of determining the blood level of peptide has advantages and drawbacks, so it is difficult to estimate the bioavailability of the entire peptide. However, all three analytical methods indicated the quantitatively equivalent effect of casein and aprotinin on the absorption of rhIGF-I, which suggests that the results in the present study could be more credible.

In the in situ loop experiment (fig. 4), the degradation of rhIGF-I in the intestinal lumen was minimal, because the lumen was washed well before administration of the drug. This could explain the high bioavailability after dosing of rhIGF-I alone and the smaller increment in bioavailability that resulted from the coadministration of casein or aprotinin. Furthermore, these results indicate that the marked enhancement of p.o. bioavailability was due to protection of rhIGF-I from degradation in the intestinal contents. This study also showed that the absorbability of rhIGF-I was differed in the different intestinal sites (fig. 4). The peptide was absorbed rapidly in the jejunum and more slowly in the ileum. The rapid appearance of rhIGF-I in the portal vein after administration in the jejunal loop was confirmed (data not shown). The absorption was much slower in the large intestine than in the small intestine. However, the absorption from the large intestine was markedly enhanced by aprotinin. This may be due to the permeability-enhancing property of aprotinin (Gotoh et al., 1995). We also found that the AUC of FD4 after administration into the jejunal loop at a dose of 1.0 mg/kg was increased from 4.90 ± 0.04 μg · h/ml to 29.6 ± 2.6 μg · h/ml by aprotinin, whereas casein almost never changed the AUC value of FD4 (5.67 ± 0.03 μg · h/ml). This suggests that casein is a safer adjuvant to enhance the absorption of rhIGF-I.

As for the absorption mechanism, the CLinfra values for rhIGF-I determined in three concentrations were much larger than that for FD4 and showed the saturation to be dependent on concentration (table 3). Because rhIGF-I is a basic peptides, absorptive-mediated endocytosis is suggested as the possible uptake mechanism (Terasaki et al., 1989). The significant acid-washable adsorption on the mucosal surface supports this hypothesis. Furthermore, as shown in the experiments using the everted sac of the jejunum (table 4), the uptake of rhIGF-I was inhibited by polycations (poly-L-lysine and protamine), but not by a polyanion (poly-L-glutamic acid). In addition, the passage may involve endocytosis, because the transport was inhibited by DNP and colchicine. These data suggest that rhIGF-I is absorbed by absorptive-mediated endocytosis rather than receptor-mediated endocytosis.

It is known that IGF-I in the plasma exists mostly as two complexes, 50-kDa and 150-kDa, with two specific binding proteins, IGFBP-2 and IGFBP-3, respectively. These complex formations are very important to maintaining a high plasma level of IGF-I (Humbel, 1990). Our previous paper showed that rhIGF-I forms the 50-kDa complex first and that the 150-kDa complex is formed slowly in the plasma (Kimura et al., 1994). After p.o. administration of 125I-rhIGF-I alone or with casein, the plasma radioactivity (fig. 6A and B) was not found in the fraction of the unbound form (fig. 6D) but was found in higher-molecular-weight regions that agree with those where it was found after i.v. administration (fig. 6C). This result suggests that p.o. administered rhIGF-I is present as the 50-kDa and the 150-kDa complexes, but not as the unbound form. Xu and Wang (1996) reported a similar result in neonatal pigs.

In conclusion, we have shown that the immunoreactive and bioactive rhIGF-I could appear in the systemic circulation.

### Table 4

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Amount Transported in 30 Min (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>None (4°C)</td>
<td>—</td>
<td>1.2 ± 0.4**</td>
</tr>
<tr>
<td>DNP</td>
<td>1.0</td>
<td>1.3 ± 0.1*</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.1</td>
<td>0.3 ± 0.1***</td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>1.0</td>
<td>0.8 ± 0.2**</td>
</tr>
<tr>
<td>Protamine</td>
<td>1.0</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>Poly-L-glutamic acid</td>
<td>1.0</td>
<td>1.2 ± 0.7</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± S.E. of 3 to 4 experiments. * P < .05, ** P < .01, *** P < .001, compared with untreated control.
after p.o. administration and that coadministration with either of the protease inhibitors aprotinin and casein enhanced its bioavailability significantly. Furthermore, we suggest that rhIGF-I is absorbed via a specialized transport mechanism, absorptive-mediated endocytosis. These results strongly support the feasibility of the p.o. administration of rhIGF-I.

Acknowledgments

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References


