Degradation of IGF-I in the adult rat gastrointestinal tract is limited by a specific antiserum or the dietary protein casein

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Abstract

To investigate the potential of IGF-I peptides as therapeutics in the gut, the survival profiles of a bolus of \( ^{125}\)I-labelled IGF-I (8.6 ng) in vivo in various ligated gut segments of fasted adult rats have been examined. The intactness of IGF-I tracer in the flushed luminal contents was estimated by trichloroacetic acid precipitation, antibody and receptor binding assays. It was found that IGF-I was degraded very rapidly in duodenum and ileum segments with a half-life \( (t_{1/2}) \) of 2 min by all three methods. IGF-I was slightly more stable in the stomach \( (t_{1/2}=8, 5 \text{ and } 2.5 \text{ min by the above three methods}) \), and considerably more stable in the colon \( (t_{1/2}=38, 33 \text{ and } 16 \text{ min as judged by the three methods}) \). Rates of degradation in gut flushings in vitro were similar to the in vivo rates except for the colon, where IGF-I was proteolysed more rapidly in vivo.

As a means of developing gut-stable and active forms of IGF-I, several approaches were examined for their effectiveness in prolonging IGF-I survival in the upper gut. It was found that the extension peptide on the analogue, \( LR^3 \)IGF-I did not protect IGF-I, nor did association with IGF-binding protein-3. However, an IGF-I antiserum was effective in prolonging IGF-I half-life in duodenum fluid by 28-fold. Charge interaction between IGF-I and heparin could also protect IGF-I in the stomach but not in duodenum flushings. Furthermore, casein (a non-specific dietary protein) and to a lesser extent, BSA and lactoferrin, were effective in preserving IGF-I structural integrity and receptor binding activity in both stomach and duodenum fluids. It can be concluded that IGF-I cannot be expected to retain bioactivity if delivered orally because of rapid proteolysis in the upper gut, but the use of IGF antibodies and casein could represent useful approaches for IGF-I protection in oral formulae.


Introduction

The gastrointestinal tract is highly responsive to subcutaneously infused insulin-like growth factor-I (IGF-I) in normal rats (Steeb et al. 1994) and under a variety of conditions including the partially resected small bowel (Lemmey et al. 1991). These responses suggest that IGF-I peptides have potential therapeutic applications to stimulate gut growth and repair. Therapeutically, oral administration would have the advantage of convenience, direct delivery to the site of action and reduction of potential side-effects by limiting exposure of other tissues. However, little evidence is available on the bioactivity of orally delivered IGF-I peptides. The presence of IGF-I and -II receptors in the intestinal epithelium of rats (Laburthe et al. 1988) and pigs (Schober et al. 1990) suggests that orally administered IGFs may exert mitogenic responses in the gut, especially in newborn animals (Koldovsky et al. 1992, Zumkeller 1992). Young et al. (1990) observed that in suckling rats, oral gastric administration of a low dose of IGF-I increased the activities of lactase and sucrase of jejunal brush border, but did not stimulate intestinal growth. In adult animals, there is one report that chronic, intraluminal infusion of 10 nm IGF-I stimulated growth of the rat ileal mucosa (Olanrewaju et al. 1992).

A major factor limiting the bioactivity of orally delivered IGFs is likely to be their susceptibility to a range of proteases including gastric pepsin, small bowel proteases, notably trypsin, chymotrypsin and elastase, as well as colonic enzymes which are largely bacterial in origin (Lehninger 1982, Britton & Koldovsky 1989). The stability of IGF-I in the gut has only been examined previously in gut flushings in vitro. These studies reported that IGF-I, as a non-glycosylated protein, appears to be degraded rapidly in vitro in jejunal and ileal luminal flushings, but slower in duodenal and stomach flushings of adult rats (Koldovsky et al. 1992). In contrast, IGF-I is stable in flushings from newborn rats (Koldovsky et al. 1992), probably reflecting the poorly developed luminal digestion in the neonatal period (Britton & Koldovsky 1989) and the presence of protease inhibitors in the milk (Rao et al. 1993). However, it is not clear if in vitro studies reflect the
luminal stability of IGF-I in vivo where brush border proteolysis could contribute to IGF-I degradation. Moreover, there is as yet no information on the stability of IGF-I in the colon. The present studies were therefore designed to examine systematically the IGF-I survival in various regions of the gastrointestinal tract of adult rats in vivo as well as in the gut flushings in vitro.

We have also evaluated several approaches to prolong IGF-I survival in the gut luminal fluids. IGF-I was either covalently extended at the N-terminus, or non-covalently complexed with a specific binding protein or an antibody, to assess whether the extension and complex formation would mask the cleavage sites. Alternatively, IGF-I was bound to heparin via charge interaction, or mixed with non-specific proteins, to determine if charge interaction or substrate substitution could preserve IGF-I from proteolysis.

Materials and Methods

Materials

Recombinant human IGF-I and LR3IGF-I (receptor grade) were provided by GroPep Pty Ltd (Adelaide, Australia). LR3IGF-I has an Arg replacing Glu at position 3 and a N-terminal 13-residue extension peptide (MF PAMPPLLFLVFN). The IGF-I and LR3IGF-I peptides were labelled with 125I (Amersham Australia) by the chloramine T method (Van Obberghen-Schilling & Pouyssegur 1983), with their respective final specific activities of 63-0 and 61-2 μCi/μg.

Aprotinin (a serine protease inhibitor) was obtained from Boehringer Mannheim Australia (Sydney). A rabbit antiserum against IGF-I (used at a final dilution of 1:80 000 in RIAs) was from GroPep Pty Ltd. A normal rabbit serum was obtained from Dako Corporation (Carpinteria, CA, USA), and an anti-(rabbit IgG) sheep IgG was from Silenus Laboratories (Melbourne, Australia). Ovine IGF-binding protein-3 (IGFBP-3), purified from plasma (Carr et al. 1994), was a gift from Ms P Grant and Dr P Walton (Cooperative Research Centre for Tissue Growth and Repair, Adelaide, Australia). Bovine casein (sodium salt), bovine lactoferrin, BSA (RIA grade) and heparin were obtained from Sigma (St Louis, MO, USA). Sephadex G-50 and the Superose 12 FPLC column were obtained from Pharmacia (Uppsala, Sweden).

In vivo degradation of IGF-I

The rate of degradation of 125I-labelled IGF-I in the gut was measured in male Sprague-Dawley rats (180–210 g) after 24 h fasting. Rats were anaesthetized with brietal/nembutal (9:1), and the gut was exposed after mid-line incision. Two gut segments were ligated on each rat: either duodenum (5 cm distal duodenum plus 1 cm jejunum) and ileum (6 cm distal ileum ending at 2 cm from caecum), or whole stomach and colon (6 cm proximal colon, beginning at 2 cm from caecum). Into each segment, a bolus of 125I-labelled IGF-I (8-6 ng) in 0-5 ml 0-2% BSA (w/v) saline was injected, and incubated for 2-5, 5, 7-5, 10 or 20 min in the duodenal and ileal segments, and for 5, 10, 20, 30 or 60 min in the stomach and colonic segments. Six animals were used for each time-point.

At the appropriate time-point, the gut segments were removed from adjoining mesentery, excised and immediately flushed with 3-5 ml chilled buffer. Tris (75 mmol/l) in 0-9% saline (pH 7-5) plus 0-1% BSA, and a 25 mmol glycine/l in saline (pH 2) containing 0-1% BSA and 11-4 μg aprotinin/ml were used respectively to flush the stomach and intestinal segments (small bowel and colon). These conditions were shown in initial experiments to neutralize effectively the gastric acidity and completely inhibit peptic activity of the stomach contents, or to inhibit the intestinal proteolysis. The gut segment tissue from each region was collected after flushing for measurement of residual radioactivity. For time 0 control, gut segments were flushed with the appropriate buffers before addition of 125I-labelled IGF-I to the luminal flushings in vitro.

Gut flushings were centrifuged at 4 °C at 4000 g for 30 min. The supernatants were collected and stored on ice prior to counting of triplicate 100 μl aliquots using a γ-counter. The pellet fractions of the centrifuged flushings as well as the excised gut tissues were also counted. The absorbed radioactivity was then estimated by the difference between the amount infused and the sum of the amounts recovered from the gut lumen (supernatant plus pellet fractions) and gut tissue.

In vitro degradation in luminal flushings

To compare with in vivo survival in the rat gut, IGF-I stability was also examined in vitro in gut flushings. In each of nine rats, the same four gut segments were ligated as in the in vivo study, and their luminal contents were flushed with 5 ml saline containing 10 mmol Tris/l (pH 7-4) for the intestinal segments (duodenum, ileum and colon), or with 5 ml saline containing 50 mmol glycine/l (pH 3-2) for the stomach. To obtain sufficient volume for the in vitro studies, flushings from the equivalent segments of three rats were pooled, providing a total of three pools of flushings from the nine rats. After centrifugation, flushing supernatants were stored on ice for in vitro degradation experiments within 2 h. For this purpose, 100 μl aliquots were added to 150 μl saline containing 0-1% BSA and 170 pg 125I-labelled IGF-I or LR3IGF-I, and incubated at 37 °C for similar periods of time as in the in vivo study. In attempts to prolong IGF-I survival, stomach or duodenal flushings were also incubated with the radiolabelled IGF-I in the presence of an excess amount of (a) IGFBP-3, (b) an
IGF-I antiserum, (c) heparin or (d) non-specific carrier proteins, casein, BSA or lactoferrin.

To complex IGF-I with IGFBP-3 or the antiserum, 125\(^{I}\)-labelled IGF-I (1-02 ng, 120 000 c.p.m.) was incubated overnight at 4 °C with an excess amount of either ovine IGFBP-3 (1-2 µg), or rabbit antiserum (1:1000) in 900 µl 50 mmol NaH\(_2\)PO\(_4\)/l buffered saline (pH 7-2) containing 0-1% BSA. Aliquots of 150 µl were then incubated with duodenal flushings as described above. These conditions were shown in preliminary chromatography studies using G-50 Sephadex or Superose 12 FPLC column to result in more than 80 or 92% association of the 125\(^{I}\)-labelled IGF-I with the binding protein or antiserum respectively.

The in vitro degradation was stopped by adding 500 µl chilled 0-25% BSA and 200 µl chilled 50% trichloroacetic acid (TCA) or, in experiments where receptor binding was measured, by adding 250 µl 0-1 mol Tris/l (pH 8) for stomach flushing or 250 µl 0-1 mol Na acetate/l (pH 4) containing 0-05% aprotinin for intestinal flushing. The % intact IGF-I peptide was measured with TCA precipitation as well as receptor binding as described below. The in vitro degradation studies were carried out three times using independent pools of gut flushings.

**TCA, antibody and receptor precipitation assays**

The intactness of radiolabelled IGF-I in the degradation studies was estimated within 2 h by one or more of the following precipitation methods.

**Percentage of radiolabel precipitated by TCA** The radioactivity of triplicate aliquots (100 µl) of samples was counted in a γ-counter prior to the addition of 700 µl chilled 0-25% BSA and 200 µl 50% TCA. After vortexing and incubation on ice for 1 h, samples were centrifuged at 4000 g for 20 min at 4 °C. The supernatants were removed, and radioactivity in the TCA-precipitated pellets was measured and expressed as a % of the total radioactivity in the aliquots. The maximum TCA precipitability of undegraded 125\(^{I}\)-labelled IGF-I or LR\(^{3}\)IGF-I was 94%.

**Percentage of immunoreactive radiolabelled peptide** Immunoprecipitation of 125\(^{I}\)-labelled IGF-I or its degraded fragments in the samples was carried out as modified from Owens et al. (1990). Aliquots of samples (50 µl) were mixed with an excess amount of rabbit anti-IGF-I antiserum (1:6000) in 250 µl 30 mmol NaH\(_2\)PO\(_4\)/l (pH 7-5) containing 10 mmol EDTA/l, 0-02% proteamine sulphate, 0-05% (v/v) Tween-20 and 0-025% NaN\(_3\). After 16 h at 4 °C, 10 µl 1:100 diluted normal rabbit serum and 50 µl 1:40 diluted sheep antirabbit IgG were added and mixed, and the tubes were incubated at 4 °C for 30 min. Then 1 ml of 5-5% polyethylene glycol (molecular weight 6000) in 0-9% NaCl was added, before the bound radioactivity was pelleted by centrifugation (as for TCA precipitation) and the supernatant aspirated. The c.p.m. in the blank controls (without the antiserum added) was subtracted from the total bound c.p.m. to give the antibody-bound radioactivity. Under these conditions, the maximum immunoprecipitation of undegraded radiolabelled IGF-I was 87%.

**Percentage of radiolabel that bound receptors** Receptor precipitation of 125\(^{I}\)-labelled IGF-I or its fragments in the samples was performed as modified from Owens et al. (1985) and Read et al. (1986), using human placenta membrane proteins as a source of IGF-I receptor (prepared as described by Cuatrecasas 1972). Briefly, a 50 µl sample was incubated with excess amount of membrane proteins (320 µg) in 300 µl 0-1 mol Tris/l (pH 7-4) containing 0-5% BSA. After 16 h at 4 °C, 1 ml chilled 0-01 mol Tris/l (pH 7-4) containing 0-1 mol CaCl\(_2\)/l and 0-1% BSA was added, mixed and incubated for 10 min, followed by centrifugation for 30 min at 4000 g at 4 °C and supernatant aspiration. Residual c.p.m. in control blanks (without membrane proteins added) was subtracted from the total bound c.p.m. to obtain membrane-bound radioactivity. The maximum receptor binding of the 125\(^{I}\)-labelled IGF-I was 30%.

**Statistical analysis**

All data are expressed as mean ± s.e.m. unless otherwise specified. Rates of degradation were analysed by two-way ANOVA with SuperANOVA software (Abacus Concepts, Inc., Berkeley, CA, USA).

**Results**

**IGF-I survival in different gut segments in vivo**

To compare the rates of IGF-I degradation in various parts of the gut, 125\(^{I}\)-labelled IGF-I was infused as a bolus into the four ligated gut segments for varying lengths of time. The amount of radioactivity recovered from the gut lumen or the gut tissue was calculated as a percentage of the amount infused (Fig. 1). In stomach, 80% of the infused radioactivity was left in the lumen following a 5-min incubation, with only 10% bound to the stomach wall. The unaccounted 10% of infused radioactivity was presumed to have been absorbed into the circulation. These proportions remained constant for incubation of up to 60-min duration. In the duodenum and ileum, luminal radioactivity declined progressively over a 20-min period while the tissue-bound portion remained unchanged, resulting in a significant increase in the counts presumably absorbed into blood. In the colon, luminal radioactivity also decreased with time, but more slowly than in the small bowel. There was a reciprocal increase in tissue-bound counts and a small rise in absorbed radioactivity after 30-min incubation.
For each time-point, the amount of TCA, antibody or receptor-precipitable radioactivity in luminal flushings was determined and expressed as a percentage of the time 0 control. The proportion of infused radiolabelled peptide recovered intact in the segment lumen was then calculated as (c.p.m. recovered in lumen/c.p.m. infused) × % luminal radioactivity precipitated by TCA, antibody or receptor. In stomach (Fig. 2), intact IGF-I disappeared with a half-life (t1/2) of 8 min as judged by the relatively crude measure of TCA precipitation, which separates peptides on the basis of size only. Loss of immunoreactivity was more rapid, with a t1/2 of 5 min, while receptor-binding activity was lost very rapidly with a t1/2 of only 2-5 min. Degradation was much more rapid in the duodenum and ileum, with a t1/2 of 2 min as quantitated by the above three assays. In colon, however, IGF-I appeared much more stable, as shown by all three precipitation methods with the respective t1/2 values of 38, 33 and 16 min.

**IGF-I survival in gut flushings in vitro**

Apart from the *in vivo* stability study, IGF-I survival was also examined *in vitro* in the various luminal flushings. 125I-Labelled IGF-I was incubated with luminal flushings for a similar time-course as in the *in vivo* study, and its stability was quantitated by TCA precipitation as well as receptor binding.

IGF-I degradation in the stomach flushing was slower than in the *in vivo* study as judged by TCA precipitation, but occurred at a similar rate if quantitated by receptor binding (Fig. 3 and Table 1). In the duodenum and ileum flushings, IGF-I was hydrolysed rapidly as in the *in vivo* study, with a t1/2 of 2 min as examined by both methods. In the colon flushing, IGF-I seemed more stable than in colonic lumen *in vivo*. In the colon flushing, only 20% was degraded to TCA-soluble peptide after 60 min, compared with 65% degradation over the same time-period in the colon *in vivo*. On the other hand, the rates of degradation *in vivo* and *in vitro* were very similar in the small bowel and also in the stomach, suggesting that the luminal proteases account for virtually all digestion of IGF-I in the upper gut. In view of this similarity, the *in vitro* incubations in gut flushings were used in subsequent studies for identifying strategies to increase IGF-I stability in the upper gut lumen.

**FIGURE 1.** Time-course of radioactivity recovery following 125I-labelled IGF-I infusion into the different ligated gut segments: (a) stomach, (b) duodenum, (c) ileum and (d) colon. Radioactivity recovered at each time-point from the luminal flushings (○) or gut segment tissue (△) is shown as a % of the amount of radioactivity infused at time 0. Radioactivity not recovered from the flushings or the gut tissues was presumed to have been absorbed from the gut (■). Data are expressed as mean ± S.E.M. of six independent experiments.
Prolongation of IGF-I survival in gut luminal flushings

A number of strategies were evaluated for their effectiveness in increasing IGF-I survival in upper gut flushings, the regions with the highest proteolytic activity.

Extension peptide To investigate whether an extension peptide to the N-terminus of IGF-I can protect the growth factor from luminal degradation, gut stability of IGF-I was compared with that of LR³IGF-I, an analogue with Glu³ substituted by Arg and a 13-residue extension peptide at the N-terminus. ¹²⁵I-Labelled LR³IGF-I was degraded in rat luminal flushings with a t₁/₂ by TCA precipitation of 50, 2, and over 60 min in the stomach, duodenum, ileum and colon, respectively. The degradation time-courses (not shown) were virtually identical to those of IGF-I in all four gut regions (Fig. 3a), demonstrating that this extension peptide could not protect native IGF-I from being degraded.

Complexed with IGFBP-3 In order to assess whether complex formation between IGFBP-3 and IGF-I could protect IGF-I in the gut, ¹²⁵I-labelled IGF-I was incubated with an excess amount of an ovine IGFBP-3 overnight at 4°C. The quantitative binding between IGF-I tracer and IGFBP-3 was confirmed in Sephadex G-50 gel filtration (Fig. 4). Most of the intact IGF-I (about 80% as calculated from the area under the curve) bound to the IGFBP-3, as shown by the forward-shifted elution peak. However, this association between IGFBP-3 and IGF-I did not prolong IGF-I survival in the duodenum flushings, as indicated by the identical time-courses of degradation of free and bound IGF-I (not shown), both with a t₁/₂ of 2 min.

Complexed with antibodies Previous workers have shown that IGF-I antibodies are capable of prolonging IGF-I survival in the plasma through mechanisms which may include protection from enzymatic degradation (Aston et al. 1989, Kerr et al. 1990, Stewart et al. 1993). To investigate whether an antiserum could similarly preserve IGF-I in the small intestine, ¹²⁵I-labelled IGF-I was incubated overnight with a 1:1000 diluted rabbit

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**FIGURE 2.** Survival of ¹²⁵I-labelled IGF-I in *vivo* in the lumen of ligated rat gut segments: (a) stomach, (b) duodenum, (c) ileum and (d) colon. ¹²⁵I-Labelled IGF-I (8-6 ng) was infused into gut segments and flushed out after incubating for the time-periods shown. Intact IGF-I in the flushings was estimated by TCA precipitation (△), antibody (■) and receptor binding (○), and expressed as % of time 0 control. The proportion of infused radiolabelled peptide recovered intact in the segment lumen was then calculated as (c.p.m. recovered in lumen/c.p.m. infused) × % luminal radioactivity precipitated by TCA, antibody or receptor. Data are expressed as mean ± s.e.m. of six independent experiments.
antiserum, and its association with immunoglobulins was confirmed by FPLC gel filtration prior to incubation in rat duodenal flushings. Approximately 92% of radiolabelled IGF-I was bound to immunoglobulins under these conditions, as shown by a forward-shifted eluted peak on the FPLC profile (Fig. 5a). The 1:1000 dilution of this antiserum provided an excess amount of antibodies to IGF-I, since at a final dilution of 1:85 000, this antiserum bound 35–45% of iodinated human IGF-I. When a normal rabbit serum with the same dilution was used, no forward-shifted eluted peak was observed (data not shown).

In the duodenal flushing, the antibody-complexed IGF-I was considerably more stable than the free IGF-I (P<0.001) (Fig. 5b), with their respective t½ values of 56 and 2 min as judged by TCA precipitation. This antibody-mediated protection of IGF-I is specific, since a normal rabbit serum of the same dilution could not preserve IGF-I (P>0.05).

**Complexed with heparin** It has been previously shown that IGF-I can interact with heparin via charge interaction (Li et al. 1990). To investigate whether such an interaction could be utilized to protect IGF-I against gut proteolysis, 125I-labelled IGF-I was incubated for 60 min at 37°C with stomach or duodenum flushings in the presence of increasing concentrations of heparin. In stomach flushings,
incubation, and heparin conferred no stability advantage to IGF-I in the duodenal flushings, possibly reflecting the different cleavage specificities of duodenal enzymes (data not shown).

**Alternative substrates** In addition to the above studies, in which we exploited the specific interactions of $^{125}$I-labelled IGF-I with IGFBP-3 (1:2 µg) or a 1:1000 diluted specific antiserum (with a total protein concentration of 70 µg/ml), we also determined if the provision of alternative substrates of much higher protein concentrations would preserve IGF-I in the gut. The high concentrations of non-specific proteins used in these studies (up to 40 mg/ml) would presumably protect IGF-I by distinct mechanisms to the specific interactions between IGF-I and IGFBP-3 or anti-IGF-I antibodies. Radiolabelled IGF-I was co-incubated with three different proteins, including two acidic proteins, casein and BSA, and one basic protein, lactoferrin. Radiolabelled IGF-I was incubated in stomach and duodenal flushings for 60 min with increasing concentrations of each of the three proteins, and the stability of IGF-I was measured by TCA precipitation as well as receptor binding. In stomach, casein was the most effective protein, with near complete inhibition of IGF-I degradation at casein concentrations of 10 mg/ml or higher, as determined by either TCA precipitation or receptor binding (Fig. 6). BSA and lactoferrin were nearly as effective as casein by TCA precipitation, but considerably less effective when stability was measured by receptor binding. Thus, BSA and lactoferrin at the highest concentration tested (40 mg/ml) conferred only 40% and 60% protection of IGF-I receptor-binding activity respectively.

All three proteins were less protective in the duodenal flushings than in the stomach flushings. Nevertheless, casein remained the most effective protein, with 40 mg/ml conferring maximum protection, at which IGF-I remained 80% intact by TCA precipitation or 36% intact by receptor binding after 60 min incubation (Fig. 6). In the presence of 40 mg/ml BSA or lactoferrin, only 64% or 46% IGF-I remained intact respectively by TCA precipitation, with very little receptor binding activity retained (Fig. 6).

Time-courses of degradation were then carried out to determine the half-life of IGF-I in the presence of the optimal concentrations of casein: 10 mg/ml in stomach flushings and 40 mg/ml in duodenal flushings. BSA was also tested at the same concentrations. Consistent with the 60-min incubation experiment, addition of 10 mg/ml casein to stomach flushings completely protected IGF-I from degradation, as assessed by either TCA precipitation or receptor binding (results not shown). Addition of BSA to stomach flushings increased the half-life of IGF-I from 3 min to 4 min by receptor binding, and from 50 min to greater than 60 min by TCA precipitation. In the duodenal flushings, 40 mg/ml casein or BSA prolonged the half-life of IGF-I by receptor binding from 2 min to 35 or

![Figure 5](https://via.placeholder.com/150)

**FIGURE 5.** Association between $^{125}$I-labelled IGF-I and an IGF-I antiserum and the stability of antibody-complexed IGF-I in rat duodenum luminal flushings. (a) $^{125}$I-Labelled IGF-I alone (□), or after being complexed with IGF-I antibodies (▲) after overnight incubation at 4 °C with a 1:1000 diluted rabbit anti-IGF-I antiserum in 50 mmol NaH$_2$PO$_4$-buffered saline (PBS, pH 7-2) with 0-1% BSA, was chromatographed on a Superose 12 FPLC column at 0-5 ml/min with the above PBS. The radioactivity (c.p.m.) of each fraction (0-5 ml) was determined. (b) $^{125}$I-Labelled IGF-I alone or after being complexed with excess IGF-I antibodies was incubated with duodenal flushing for the time-periods shown. The intact IGF-I was estimated by TCA precipitation, and expressed as a % of the time 0 control. The normal serum with the same dilution (▲) was also used as a negative control. Data show mean ± S.E.M. of three independent trials.

48% IGF-I remained intact after 60-min incubation in the absence of heparin, and heparin conferred partial protection of IGF-I in a dose-dependent manner with maximum protection (75% intact) observed at a heparin concentration of 2 mg/ml (not shown). This protection was completely inhibited by 1-5 mol NaCl/ml, suggesting that charge interaction between heparin and IGF-I partially masks pepsin cleavage sites on IGF-I. In the duodenal flushings, only 18% IGF-I remained intact after 60-min incubation.
6 min respectively. The corresponding values by TCA precipitation were from 2 min for free peptide, to over 60 min in the presence of BSA, and complete stability with casein.

To determine the importance of charge interaction between the negatively charged proteins (casein and BSA) and the positively charged peptide (IGF-I) in the protective action of the two acidic proteins, the dose–responses to casein, BSA and lactoferrin shown in Fig. 6 were repeated at 1·5 mol NaCl/l instead of the physiological concentration of 0·15 mol/l. High salt concentration had no significant effect on the dose–response curves of casein, BSA or lactoferrin (results not shown), arguing against a charge interaction as the mechanism of protection by the negatively charged proteins, casein and BSA.

**Discussion**

Using *in vivo* and *in vitro* degradation techniques, we have determined the survival patterns of IGF-I in four segments of fasted adult rat gut or gut luminal flushings. Duodenum and ileum were found to be the most active sites of IGF-I degradation, followed by stomach, while IGF-I was most stable in the colon. IGF-I was hydrolysed at similar rates in the duodenum and ileum segments, confirming the finding that the proteolytic activity is similar throughout the small intestine (Lepkovsky et al. 1966). We have also shown that TCA precipitation generally gave the highest estimates of intact peptide, with antibody binding intermediate between TCA precipitation and receptor binding. Receptor binding can be considered to be the most definitive test of biologically active peptide, as it directly tests the ability to interact with cell surface receptor. Furthermore, we showed that the survival time–courses of IGF-I in aliquots of luminal flushings *in vitro* can approximately represent those in the stomach and small bowel *in vivo*. This indicates that in the upper gut, luminal proteases account for the majority of the degradation activity.

In the colon segment, there was a significant amount (up to 40%) of radioactive material bound to colonic tissue by 60 min, in contrast to the stomach and small intestine where the proportion of radioactivity bound to tissue was minimum and did not increase with time. The radioactive material bound by colonic tissue was likely to represent either intact IGF-I or large fragments, otherwise they

**Figure 6.** Prolongation of IGF-I survival in rat stomach or duodenum luminal flushing by casein (■), BSA (●) or lactoferrin (△). 125I-Labelled IGF-I was incubated for 60 min at 37 °C with (a, b) stomach or (c, d) duodenum flushing in the presence of increasing concentrations of proteins. The % of intact IGF-I was estimated by both TCA precipitation (a, c) and receptor binding (b, d), and expressed as a % of time 0 control. Data show means of two independent measurements.
would have been absorbed into blood. Ribbons et al. (1994) have similarly conducted an in vivo luminal stability study with radiolabelled epidermal growth factor at the same proximal colon region. They observed that, after incubation for 4 h, most of the radioactivity had transferred to the colonic tissue, and that more than 80% of the radioactivity in the colonic tissue was TCA-precipitable. Whether this tracer binding was non-specific (to mucin) or specific (to receptor) is unknown.

The IGF-I survival results of the present studies have some apparent discrepancies with the findings of Koldovsky et al. (1992) who performed the IGF-I degradation in vitro in gut luminal flushings from non-fasted rats. They found that in the luminal contents of stomach and duodenum of non-fasted rats, the degradation activity of IGF-I was minimal, while the proteolysis was rapid in the flushings of jejunum and ileum. In contrast, our studies with fasted rats demonstrated that both duodenum and ileum degraded IGF-I at a rapid rate, and that stomach also hydrolysed IGF-I at a fast rate although slower than the small bowel. The reason for this discrepancy is not clear, but it may reflect an effect of fasting on enzyme/substrate ratio and enzyme secretion into the gut. Britton & Koldovsky (1987a) found that in the weanling rats, food deprivation increased gastric proteolytic activity two- to threefold, but decreased trypsin activity and thus proteolysis in mid-small bowel but not ileum.

The rapid degradation of IGF-I in the stomach and small bowel indicates that IGF-I peptides cannot be expected to retain bioactivity when delivered orally. Therefore gut stable or proteolysis-attenuated forms of IGF-I would be required before oral IGF-I could be considered therapeutically. The site-directed mutagenesis approach to alter the protease-sensitive sites has been shown to be effective in improving enzymatic stability of some polypeptides such as exotoxin (Brinkmann et al. 1992). An alternative approach to this involves the linkage of IGF-I, either covalently or non-covalently, to other moieties that can be predicted to confer stability in the gastrointestinal environment. In the present studies we have examined the effectiveness of the presence of an extension peptide, IGFBP-3, an antiserum, heparin, or some non-specific proteins (e.g. casein) in improving IGF-I gut stability.

The 13 residue extension peptide at the N-terminus of LR³IGF-I has the potential to protect the growth factor by steric hindrance of cleavage sites. However, we have demonstrated that the extension peptide on LR¹IGF-I was not effective in prolonging IGF-I survival, possibly because the extension peptide is too short and/or contains protease-sensitive sites. It is known that both pepsin and chymotrypsin can attack the peptide bonds involving the carbonyl group of Phe (F) (Lehninger 1982). Since the extension peptide of LR³IGF-I contains two Phe residues, it is likely to be cleaved rapidly in both stomach or duodenum flushings.

Approximately 75% of IGF peptides in plasma are bound with circulating IGFBP-3 molecules, which couple with the 84–86 kDa glycoprotein known as the acid-labile subunit (ALS) to form a ternary complex (Baxter et al. 1989). It has been shown that IGF-I which is complexed with IGFBP-3 has a greatly prolonged half-life compared with the free peptide in the circulation (Ballard et al. 1993, Lewitt et al. 1993). The presence of IGFBP-3 and other IGFBPs (particularly IGFBP-2) in milk have been reported (see review by Zumkeller 1992, Ho & Baxter 1994), but it is not clear whether they confer any additional stability to IGFs against enzymatic degradation in the neonatal gastrointestinal tract. In the adult rat small intestine flushing, we have demonstrated that the half-life of IGF-I was not extended by complexing with IGFBP-3. This may indicate that the protease-sensitive regions of IGF-I remain exposed in the IGFBP-3 complex. If so, infusion of IGF-I in the ternary complex form with ALS and IGFBP-3 may confer some additional stability. Alternatively, IGFBP-3 itself may be rapidly proteolysed in the duodenum. Certainly IGFBP-3 is known to be susceptible to serine proteases (see review by Baxter 1993), and the small bowel is clearly rich in this group of proteases.

The enhancement of activities of hormones or growth factors by antibodies has been known for many years (Aston et al. 1989). For example, Kerr et al. (1990) showed that serum concentrations of IGF-I were greater in guinea-pigs infused with an IGF-I monoclonal antibody than in control animals, and this difference probably resulted from an extension of the serum half-life of IGF-I. More recently, it has been shown that the infusion of an IGF-I antiserum together with IGF-I could enhance the growth stimulation of the infused IGF-I in mice (Stewart et al. 1993). The mechanisms for these effects are not clear, but may be related in part to the protection of IGF-I against enzymatic degradation, as well as a reduction in clearance due to the increased size. In the present studies, we have shown that, in contrast to the results obtained with IGFBP-3, the half-life of IGF-I complexed with antibodies can be extended in the small bowel. This most likely reflects binding of the polyclonal antibodies at regions of the IGF-I molecule which block the protease-sensitive sites. Therefore the IGF-I-antibody complex may act as a 'slow release' system for IGF-I in the small bowel.

The biological activity of heparin-binding acidic or basic fibroblast growth factors (FGFs) can be strongly potentiated by heparin which stabilizes their molecular conformation by preventing physicochemical or enzymatic degradation (see review by Tardieu et al. 1992). Through ionic interaction, heparin or its mimic, dextran sulphate, has been shown to stabilize basic FGF against digestion by pepsin, trypsin and chymotrypsin (Kajio et al. 1992). In the present studies, we observed that heparin can partially protect IGF-I in the stomach flushings, but not in the duodenum flushings where the proteolytic activity was much higher. The relatively weak protection activity of
heparin on IGF-I survival in the upper gut may reflect the low binding affinity of IGF-I to heparin, compared with the FGFs (Li et al. 1990).

The present studies show that the acidic proteins, casein and BSA, can both protect IGF-I from degradation in stomach or duodenal flushings in a dose-dependent manner, with casein the more effective protein. The results are consistent with the findings of other studies that casein was considerably more effective than BSA in inhibiting somatostatin degradation in rat intestinal flushing (Rao & Koldovsky 1990), and that casein had much higher potency than lactalbumin in preserving epidermal growth factor bioactivity in human jejunal juice (Playford et al. 1993). The mechanisms for this protection are not clear, but could involve binding of IGF-I, a basic protein, to the acidic carrier proteins via a charge interaction. On the other hand, lactoferrin, a basic protein, also protected IGF-I. Furthermore, a high salt concentration (1.5 mol NaCl/l), which would interfere with charge interactions, had no effects on the prolongation of IGF-I survival in the gut by any of the three proteins. Alternatively, casein, BSA and lactoferrin may have provided non-specific protection of IGF-I by acting as an alternative substrate. The greater protection afforded by casein compared with BSA or lactoferrin may indicate that it is a preferred substrate for proteases. Casein is flexible in structure and thus is very susceptible to proteases (Lehninger 1982, Swaisgood 1993). Certainly casein is known to be readily degraded in the stomach and small bowel (Britton & Koldovsky 1987a,b).

In summary, IGF-I is degraded rapidly in the stomach and small intestine, and thus would not provide an oral therapeutic. On the other hand, IGF-I antibodies and the dietary protein, casein, may provide opportunities to preserve IGF-I against luminal digestion.

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