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Minimal hydrolysis of epidermal growth factor by gastric fluid of preterm infants

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SUMMARY **Epidermal growth factor (EGF), present in high concentrations in milk, may play a role in growth of the gastrointestinal tract. Resistance to proteolytic degradation in the stomach is necessary if ingested EGF is to function within the gastrointestinal tract. Although EGF stability to low pH and proteases predicts gastric survival, the extent of digestion in the stomach remains to be defined. Consequently, we measured gastric degradation of ^{125}I-human recombinant EGF in preterm infants with an *in vitro* method in which EGF was incubated at 37°C with stomach fluid at pH 1·8, 3·2, and 5·8 followed by analysis of degradation products. As maximal acid proteolytic activity is present one hour after feeding in preterm infants, fluid was obtained at that time from 18 infants with a mean gestational age at birth of 30·4 (3·0) (SD) weeks and a postnatal age of 26·3 (12·7) days at sampling. Incubations for up to 60 minutes revealed minimal loss of trichloroacetic acid precipitable radioactivity, in contrast to the substantial hydrolysis of iodinated casein which occurred under the same conditions. Chromatography of reaction mixtures on Sephadex G-25 showed a single major peak of radioactivity which coeluted with EGF. Epidermal growth factor also retained >75% of its ability to bind to anti-EGF affinity columns and placental membrane receptors after incubation with gastric fluid. These data support the concept of substantial gastric survival of ingested EGF in a potentially biologically active form in preterm infants.**

The milk of several species has growth promoting effects *in vivo* and *in vitro*.[1,2] The gastrointestinal tract of pigs, rabbits, dogs, and rats of milk fed newborns has been shown to have increased weight, protein, and DNA content compared with unfed or formula fed controls.[3–7] In tissue culture, milk is mitogenic for several cell lines, and in some cases it may replace serum in the media.[8–10] Among the several growth promoting activities in milk, epidermal growth factor (EGF) has emerged as one of the foremost.[11,12] A polypeptide with a molecular weight of about 6000 daltons,[13] EGF is present in high concentrations in human milk and colostrum but wide variations in individual levels exist.[14–16] Some[14] but not other[15] studies have found higher EGF concentrations in colostrum than in mature milk. Although EGF is also produced in the submandibular and Brunner's glands,[17–20] endogenous concentrations, studied thus far only in rodents, are low during the neonatal period,[17,19] leading to speculation that milk may be a major source of EGF for the suckling mammal.

An increasing body of evidence suggests that EGF may be trophic for various regions of the gastrointestinal tract, including stomach, small intestine, colon, pancreas, and liver.[21–33]. In addition, studies in rats support the concept of absorption of intact EGF from the gastrointestinal tract with delivery to peripheral tissues during the neonatal period.[34–36] In human infants, the role of EGF in early development remains to be defined, but it is possible that it may act within the gut or be absorbed and act at distant body sites. Promotion of growth might be especially important for premature infants, who often show

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growth delay and to which additional growth promoting activities could be valuable. In addition, EGF has been shown to be a potent inhibitor of gastric acid secretion[37 38] and to have gastrointestinal cytoprotective effects,[39 40] and it is possible that this role could also be ascribed to milk EGF. For such phenomena to occur, resistance to digestive processes within the stomach and intestines is required, at least in part: EGF must survive in either its intact form or as an active proteolytic digestion product. Although EGF resistance to low pH and a number of proteolytic enzymes *in vitro* has been shown,[13] the extent of digestion of dietary EGF in the human infant is unknown. Accordingly, we evaluated the gastric digestion of human EGF by measuring its digestibility by stomach fluid, as this is the first digestive secretion to which the growth factor is exposed after ingestion. Because of the potential importance of EGF for the preterm infant, gastric fluid was obtained from a group of such infants after feeding induced stimulation of gastric secretion.

Methods

COLLECTION OF GASTRIC FLUID

This project was approved by the Human Subjects Committee of the University of Arizona Health Sciences Center. Informed parental consent was obtained before collection of samples. Eighteen preterm infants (nine boys, nine girls) with a gestational age at birth of 30·4 (3·0) mean (SD) weeks were studied at a postnatal age of 26·3 (12·7) days. All infants were receiving intermittent orogastric feedings of either a preterm formula (15 infants) or fortified human milk (three infants). Between 0800 and 1200 hours on the morning of collection, the infants received a test feeding of 17·9 (6·4) ml/kg body weight.

Because previous studies have shown maximum gastric acid proteolytic activity at 60 minutes after a feed in premature infants,[41] gastric fluid was aspirated at this time into plastic containers on ice. After a 1:5 dilution with normal saline and homogenisation with a glass Teflon homogeniser, the fluid was assayed directly for proteolytic activity and EGF degradation as described. In some experiments, fluid was stored under liquid nitrogen for up to five days; such storage did not affect the rate of hydrolysis of casein nor did it alter the digestibility of EGF.

ANALYSIS OF HYDROLYSIS OF EGF

Experiments were designed to investigate the proteolytic activity of gastric fluid toward EGF and the nature of the reaction products. The samples of gastric fluid had measured pH values between 5 and 6, in accordance with previously reported values for gastric pH after milk feedings in the newborn.[42 43] To evaluate EGF degradation under these conditions, hydrolysis was measured in the presence of 0·1 M maleate buffer, pH 5·8; in addition, measurements were also performed in the presence of 0·05 N HCl, pH 1·8 (as used in the classical Anson assay for gastric peptic activity[44]) and with 0·1 M glycine-HCl, pH 3·2, as the latter pH approximates that of the unfed newborn stomach.[45 46] The rate of hydrolysis of EGF was measured at 37° in a 0·15 ml reaction mixture containing 0·05 ml of gastric fluid and 150 ng of human recombinant EGF[47] labelled with ^{125}I.[48] This EGF has been extensively characterised and is identical to gamma urogastrone.[49] After incubation for various time periods, the reaction was terminated by the addition of 1·0 ml 10% trichloroacetic acid (TCA) containing 0·2% bovine serum albumin and chilling on ice. Hydrolysis was determined by measuring the generation of acid soluble radioactivity as previously described.[50 51] In all experiments, the hydrolysis of iodinated bovine alpha casein (Sigma) to TCA-soluble material was measured as a control.[50] Such assays were done under conditions giving linearity with respect to amount of gastric fluid and incubation time, and results were expressed as micrograms casein hydrolysed/hour in the standard reaction mixture. For EGF, hydrolysis was expressed as the per cent of label remaining acid precipitable after a 60 minute incubation.

ANALYSIS OF REACTION PRODUCTS

Reaction mixtures prepared as above but containing 1 ng EGF were incubated for up to one hour at 37°C and terminated by the addition of cold 0·1 M Tris-HCl, pH 8·0. This resulted in pH values above neutrality and consequent obliteration of proteolytic activity. Reaction products were analysed by three methods, each of which was chosen to assess the structural intactness of EGF by different criteria:

Gel filtration

Mixtures were chromatographed on Sephadex (Pharmacia) G-25 columns (24×1 cm) equilibrated and eluted with 0·05 M phosphate buffer, pH 7·4, containing 0·075 M NaCl. Forty 1 ml fractions were collected and counted in a gamma counter. Intact EGF typically elutes in the void volume, giving a single major peak of radioactivity (Figure). In some experiments, this peak was rechromatographed on Sephacryl S-200 (Pharmacia) using the same buffer.

Affinity chromatography

Reaction mixtures were applied to a 2×1 cm Sepharose 4B (Sigma) column to which monospecific polyclonal rabbit anti-human EGF had been complexed.[34] Columns were eluted with Hank's solution,

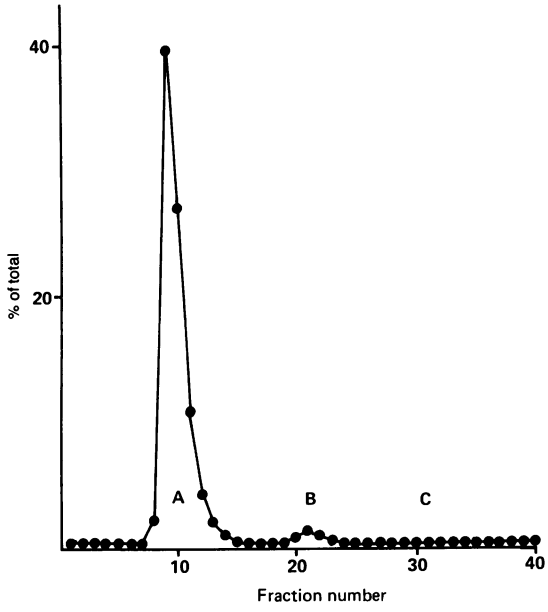

Figure *Gel filtration chromatography of EGF incubated with gastric fluid. Labelled EGF was incubated with gastric fluid pooled from five infants and subjected to chromatography on Sephadex G-25 as described in Methods. (A) denotes position of intact EGF, which elutes in the void volume as determined with blue Dextran. Inorganic iodine elutes in peak (B) with the column volume, and iodotyrosines show retarded elution at (C). Identical profiles were obtained with incubations for 0 and 60 minutes at pH 5·8 and 3·2.*

pH 7·4, and the percentage of retained radioactivity was determined. Typically, greater than 90% of the radioactivity in an EGF preparation was retained by the columns; boiling reaction mixtures containing labelled EGF for seven minutes resulted in reduction of binding to affinity columns to less than 2% of original values.

Receptor binding

Reaction mixtures were assayed for binding to human placental EGF receptors essentially as described by Hock and Hollenberg[52] and Hock *et al.*[53] Under these conditions, 33–35% of added labelled EGF binds at 25°C, and non-specific binding, measured in the presence of a 1000-fold excess of unlabelled EGF, is 5–10%.

For the latter two methods, results were expressed as a percentage of a corresponding zero time sample, prepared by incubation with boiled gastric fluid; this gave values in each assay identical to that of EGF alone.

STATISTICAL ANALYSIS

Data are expressed as mean (standard deviation (SD)) of determinations on each gastric fluid. Comparison between different groups was performed using analysis of variance and Student's unpaired *t* test, with significance accepted at $p<0·05$.

Results

Analysis of hydrolysis of EGF by gastric fluid is shown in the Table. Incubations carried out for up to one hour revealed no increase in acid soluble radioactivity, with over 98% of the added label remaining TCA-precipitable. In contrast, iodinated bovine casein incubated under identical conditions was substantially hydrolysed to soluble material; the rate of casein degradation was significantly greater at the acid pH values than at pH 5·8, in keeping with the known pH optima of gastric proteases.[44 54]

Chromatographic analysis of EGF which had been exposed to human gastric fluid for one hour *in vitro* is shown in the Figure. A single major peak of radioactivity eluted in the void volume of the column (peak A), a position identical to that of intact EGF. Minimal amounts of label were present in a second peak (B) which eluted at the position of inorganic iodine and other undefined peptides[34] and no material was present at the position of iodotyrosines (C); there was no increase in the quantity of the iodine peak when compared with a zero time sample. To assess the possibility of aggregation of breakdown products with EGF, peak A was rechromatographed on Sephacryl S-200; a single peak was obtained which eluted in a position corresponding to intact EGF (data not shown). This material also retained 80·9% of its ability to bind to placental receptors compared to unincubated EGF. Similar results were obtained with incubations at pH 3·2 and 5·8.

The percentage of EGF which retained its ability to bind to anti-EGF affinity columns and placental

Table *EGF degradation by gastric fluid*

	pH		
	5·8	*3·2*	*1·8*
TCA precipitable material	99·5 (0·5) [6]	98·0 (2·4) [16]	99·0 (0·7) [11]
Antibody binding	97·8 (4·2) [7]	97·7 (9·7) [14]	
Receptor binding	74·9 (18·9) [6]	78·9 (18·1) [6]	
Hydrolysis of casein (μg/h)	1·23 (1.09)* [14]	4·77 (6·17) [14]	10·48 (9·47) [14]

Values represent the mean (SD) of determinations on the number of samples shown in square brackets. For each method, the amount of EGF detected after incubation for 60 minutes at 37°C is expressed as a percentage of that in a zero time sample. *Different from values at lower pH, $p<0·05$.

membrane receptors after incubation with gastric fluid is also shown in the Table. Binding to affinity columns was reduced by less than 3%, and greater than 75% of the ability to bind to placental EGF receptors was retained after incubation with gastric fluid.

No difference in gastric fluid EGF degradation was observed between breast fed and formula fed infants.

Discussion

These experiments support the concept of substantial gastric survival of dietary EGF in premature infants. The inability to detect hydrolysis of EGF to acid soluble peptides suggests the absence of proteolytic cleavage of the primary structure of the peptide. Although this type of assay is generally quite sensitive when used with radioactive substrates,[55] it may not, however, detect the generation of peptide fragments which do not contain tyrosine, the amino acid residue which is iodinated.[48] Human EGF, a 53 amino acid peptide with three interconnected loops held by disulphide bonds, contains tyrosine at positions 13, 22, 29, 37, and 44,[56] and it is possible that fragments cleaved from the peptide between these residues or at either the carboxy or amino terminus might escape detection.

As the antisera utilised for our affinity columns was polyclonal, the retention of substantial antibody binding ability of EGF after exposure to gastric fluid similarly does not permit conclusions regarding specific regions of the molecule. The data from the affinity columns, however, do indicate substantial preservation of tertiary structure of the peptide, the loss of which, as with heat treatment, resulted in obliteration of binding to our columns. Moreover, the retention of substantial receptor binding ability also indicates some tertiary structure preservation; indeed, at least partial preservation of carboxy terminal residues is implied by our findings, since Hollenberg and Gregory[57] and Cohen *et al*[58] observed that loss of residues 49–51 results in nearly complete loss of receptor binding ability. In contrast, proteolytic removal of amino terminal residues, which may result in more limited reduction in receptor binding,[59 60] is compatible with our data, especially as such losses might also escape detection in the TCA assay. The reduction of receptor binding by approximately 25% may be caused by a minor alteration of tertiary structure by the gastric fluid.

Petrides *et al*[16] have observed that beta-urogastrone is the predominant form of EGF in human milk. The EGF utilised in our experiments is identical to gamma urogastrone, one of the two major EGF species present in human urine and differing from beta-urogastrone by absence of the carboxy-terminal arginine.[57 61] Both beta- and gamma-urogastrone are resistant to proteases *in vitro* and have identical biological activity.[57] That gastric digestion of these two forms could differ in the infant is unlikely but possible.

The gastric fluid used in these assays was obtained one hour after a milk feeding, a time of maximal acid proteolytic activity.[41] Our control assays with casein as substrate show that the fluid has an active proteolytic capacity which is greatest at low pH. Milk feedings are known to buffer gastric acid in the infant,[42 43] and the resulting rise in pH may be responsible for the lower proteolysis as observed in these control assays. Nevertheless, EGF degradation was undetectable at all of the pH values tested, including 5·8, which approximates the value of the stomach pH one hour postprandially in the infant.[42 43]

Although we have not shown biological activity of EGF after treatment with gastric fluid, receptor binding is known in general to correlate with mitogenicity.[13] The gastric survival of dietary EGF in a potentially biologically active form thus has several possible implications for the preterm infant. For example, gastric acid secretion is relatively low in the premature during the first four weeks postnatally,[62] and we may speculate that EGF might play a role in the inhibition of such secretion.[37 38] Appropriate studies correlating gastric acid secretion and dietary EGF intake in premature infants remain to be performed. Although data regarding the gastric trophic effects of EGF are also lacking in man, such effects have been well documented in rodents.[22 30–33]

Gastric survival of EGF suggests that at least some of the active peptide present in the diet might reach the intestine. Although the extent of intestinal digestion of EGF is unknown in man, experiments in suckling rats suggest substantial survival and absorption in an intact form.[34–36 63] Although the extent of biological action of dietary EGF in the premature infant remains unknown, its survival in a potentially biologically active form suggests the availability for possible action within the stomach and perhaps other regions of the gastrointestinal tract. The susceptibility of dietary EGF to gastric luminal hydrolysis in term infants or in preterm infants with progressive postnatal age remains to be explored.

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