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EGF- and TGF-α-Like Peptides in Human Fetal Gut

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ABSTRACT. The presence of epidermal growth factor (EGF) and transforming growth factor-α (TGFα) immunoreactivities in fetal human tissues was studied immunohistochemically at different gestational ages. EGF and TGFα immunoreactivities were detected from the 20th gestational wk. EGF immunoreactivity was limited to the small intestine, but TGFα immunoreactive cells were present in the colon also. According to radioreceptor assay, the intestine of a 19-wk-old human fetus contained 10 times more EGF receptor-binding substance than EGF, as measured by immunofluorometric assay. Chromatographic analysis suggests that TGFα-like peptides account for at least part of this activity, as so argues in favor of the presence of TGFα- and EGF-like peptides in the human fetal gut. Whether they are synthesized in the fetus is yet unknown. (*Pediatr Res* 26:25–30, 1989)

Abbreviations

EGF, epidermal growth factor
IFMA, time-resolved immunofluorometric assay
PAP, peroxidase anti-peroxidase
RP-HPLC, reverse-phase HPLC
RRA, radioreceptor assay
TGFα, transforming growth factor-α

EGF and TGFα are structurally related mitogenic polypeptides (1, 2). They share the same receptor, EGF-R. EGF is a potent stimulator of cell multiplication and a modulator of the differentiation and function of cells of various types (1, 3, 4). It inhibits gastric acid secretion and protects the gastric mucosa from damage by the acid (5–7). irhEGF has been detected by immunoassay methods in colostrum and milk, in several other body fluids and in tissue extracts (8, 9). In adults it has been localized immunohistochemically to the submandibular salivary gland, the serous glands of the nasal cavity, the stomach, the Brunner's glands of the duodenum, the Paneth cells of the small intestine, the tubular cells of the kidney, the anterior pituitary, the bone marrow, and the sweat glands (10–13). In a 20-wk-old human fetus it was present in the kidney and the duodenal Paneth cells (12, 13). However, EGF appears in the amniotic fluid only from the 32nd gestational wk (X. Mattila, personal communication). Even at term its concentration is low, only about 0.3–0.96 ng/ml (14, 15), which suggests that it is not synthesized in large quantities during fetal life.

TGFα was discovered in feline sarcoma virus-infected murine cell cultures (16). It induces malignant transformation and anchorage-independent growth. TGFα has been found in fetal mouse tissues (17). TGFα-mRNA has been demonstrated in various human tumors (18), normal human neonatal and adult keratinocytes (19), and rat decidua (20). No TGFα-mRNA is detected in the normal human adult stomach or jejunum (18).

TGFα has been proposed to be the fetal EGF (21). In this study we tested this hypothesis mainly with immunohistochemical methods, using antisera to hEGF and hTGFα to examine human fetuses of different ages.

MATERIALS AND METHODS

Rat tissues. Male Sprague-Dawley rats weighing approximately 400 g were anesthetized with sodium pentobarbital and perfused with physiologic saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The submandibular glands were removed, immersed in 4% paraformaldehyde for 2 h and then transferred to 20% sucrose-0.1 M sodium phosphate buffer for at least 24 h. Cryostat sections were cut at 20 μm for immunohistochemistry.

Human tissues. Fetal tissues (from a total of eight fetuses) were collected at legal abortions: at 14.5 wk the jejunum, liver, adrenals, kidney, lung, and gonads from one fetus, at 19 wk the duodenum, jejunum, ileum, lung, and parotid and submandibular glands from three fetuses, at 20 wk the jejunum, ileum, colon, liver, spleen, adrenals, lung, skin, and urinary bladder from two fetuses, and at 22 wk the stomach, duodenum, jejunum, kidney, parotid glands, liver, adrenals, hypophysis, and brain from two fetuses.

The duodenum was defined as the 10-cm portion distal and adjacent to the stomach, the jejunum as the next 20-cm portion, the ileum as the portion proximal to the ileocecal valve, and the colon as that distal to the ileocecal valve. The tissues were fixed in 4% paraformaldehyde, and those from the 19-, 20- and 22-wk-old fetuses also in acetone-ethanol (30:70), and embedded in paraffin. Biopsy specimens of normal adult duodenum were obtained at routine diagnostic gastroscopy and fixed in 4% paraformaldehyde like the rat tissues.

Primary antisera and peptides. Mouse monoclonal (ascites fluid, code numbers 2 and 4) and rabbit polyclonal (bleed number 2) anti-hEGF antibodies and pure biosynthetic hEGF were gifts from AMGen (Thousand Oaks, CA). rhTGFα and monoclonal hTGFα antibody were from Genentech (South San Francisco, CA).

Dot blot assay. EGF and TGFα were spotted as 2-μl droplets (0.02 to 2 pmol) onto nitrocellulose membrane filters (pore size 0.45 μm, BA 35, Schleicher & Schuell Inc., Keene, NH), and fixed by exposure to paraformaldehyde vapor (30 min at 80°C). For comparison, similar unfixed filters were incubated. Antigen and antisera dilutions (1:1000 or 1:3000) were made in 0.1% BSA-PBS, pH 7.4. The filters were incubated at room tempera-

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ture in a shaker. Nonspecific binding was blocked with 2% BSA-0.1 M PBS, pH 7.4, for 2 h. Incubation with the primary antiserum, preimmune serum, or preabsorbed antiserum (with 0.1–1.0 μM hEGF or hTGFα overnight at +4°C) was for 1 h in 300 μl Eppendorf tubes pressed upward onto the antigen spots. The filters were washed twice for 10 min in 0.1 M PBS, pH 7.4, before addition of the second antiserum (swine antirabbit Ig 1:100, rabbit anti-mouse Ig 1:80, DAKO, Copenhagen, Denmark). Incubation with the peroxidase anti-peroxidase reagent (rabbit-PAP 1:150, mouse-PAP 1:400, DAKO, Copenhagen, Denmark) was carried out for 45 min. The filters were rinsed in 0.05 M Tris-HCL, pH 7.6, for 2 × 10 min. The antigen-antiserum reaction was visualized with 3'3-diaminobenzidine tetrahydrochloride 50 mg/100 ml (Sigma Chemical Co., St. Louis, MO) and 0.05% hydrogen peroxide in 0.05 M Tris-HCl, pH 7.6. The Tris-HCl wash was again repeated and the filters were dried for inspection, densitometric scanning, and photography.

Immunohistochemistry. The acetone fixed tissues were embedded in paraffin and 10-μm sections were cut on coated slides. The paraffin sections were rehydrated in a graded series of alcohols. Tissues fixed in paraformaldehyde were frozen and 20-μm cryostat sections were cut. The immunoreactions were visualized with the FITC technique (22); 0.1 M PBS-0.25% Triton, pH 7.4, was used in all dilutions and washes. Nonspecific binding was blocked with normal swine or rabbit serum (dilutions 1:5, DAKO, Copenhagen, Denmark). Incubations with the primary antiserum, preimmune serum or preabsorbed antiserum (with 1–10 μM hEGF or hTGFα overnight at +4°C) were carried out for 24 h at +4°C. Generally, antisera were diluted 1:1000. After two PBS-T washes the slides were incubated with the second antiserum (FITC-conjugated rabbit anti-mouse and swine anti-rabbit Ig 1:40, DAKO, Copenhagen, Denmark) for 1 h at room temperature. The slides were then washed in PBS and coated with PBS-glycerol (1:1) for examination by fluorescence microscopy.

Tissue extraction. About 10 cm of small intestine from a 19-wk-old human fetus was homogenized in 1 M acetic acid and centrifuged at 35000 rpm for 30 min. The supernatant was collected and divided into 1-ml aliquots, which were lyophilized and dissolved in appropriate buffers for protein determination (23), EGF-IFMA (24), EGF-RIA (25), RRA, and RP-HPLC. EGF-IFMA or EGF-RIA did not cross-react with TGFα.

Radioreceptor assay. Samples, receptor preparation (mouse liver homogenate) and 125I-hEGF (15000 cpm) in Tris-HCl 10 mM-NACl 50 mM-0.25% BSA buffer, pH 7.4, were incubated at room temperature for 90 min. The unbound and receptor-bound label were separated by centrifugation at 3200 rpm for 25 min at +4°C. The radioactivity of the pellet was measured. Unlabeled hEGF was used as a ligand for the standard curve.

RP-HPLC. The lyophilized extract was dissolved in 0.1 M acetic acid and ultrafiltered through a membrane with a 10-KDa cut-off (Ultrafree Pf, PLGC membrane, Millipore Continental Water Systems, Bedford, MA). The filtrate was collected, lyophilized, and dissolved in eluent A. Through a Rheodyne injector valve, 50 μl were injected onto an LKB-Ultropac TSK-ODS 120 T column (4.6 mm × 250 mm, 0.5 μm particle size). The column was eluted with a gradient from eluent A (10% acetonitrile-90% water) to eluent B (65% acetonitrile-35% water). Both eluents contained 0.5% trifluoroacetate and morpholine as additives. The flow rate was 0.8 ml/min. The gradient was started after a 4-min plateau of 90% eluent A. From 5–10 min the percentage of B was raised linearly to 30% and from 11–55 min linearly to 60%. The 0.8 ml fractions were collected. For protein protection, 100 μl 0.1% BSA were added to the aliquots. All fractions were lyophilized and analyzed for both irhEGF and irhTGFα with the dot blot assay using hEGF and hTGFα antibodies, and with EGF-IFMA and RRA. hEGF and hTGFα standards were run similarly. Before running the tissue sample a blank run was made to check for possible carry-over from standard runs.

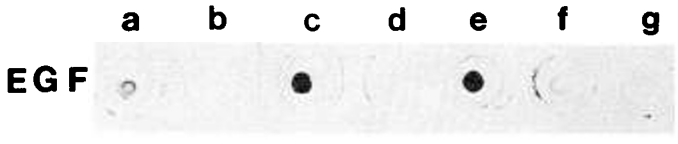

Fig. 1. A total of 0.2 pmol hEGF was spotted on a nitrocellulose filter. Polyclonal hEGF antiserum diluted 1:2000 specifically recognizes hEGF (*a*) as shown by preabsorption with 0.1 μM hEGF (*b*). MAb 2 (*c*) and 4 (*e*) also bind EGF and these reactions are almost abolished by 1 μM EGF (*d* and *f*). 0.1% BSA-PBS was used instead of anti-hEGF as a negative control (*g*).

RESULTS

Specificity of antisera. To verify the affinity of hEGF antibodies to hEGF, we performed model experiments on nitrocellulose filters (Fig. 1). Every hEGF antibody tested recognized hEGF, and preabsorption with 0.1–1 μM hEGF abolished the reaction. The antibodies did not cross-react with hTGFα (data not shown).

In the dot blot experiments anti-hTGFα cross-reacted with hEGF (Fig. 2). Preabsorption with 0.1 μM hTGFα abolished the hEGF reactivity but retained the hTGFα reaction. A 10-fold concentration of hTGFα (1 μM) abolished the hTGFα reactivity. Preabsorption with 1.0 μM hEGF had little or no effect (densitometrically scanned intensities of the dots were almost equal),

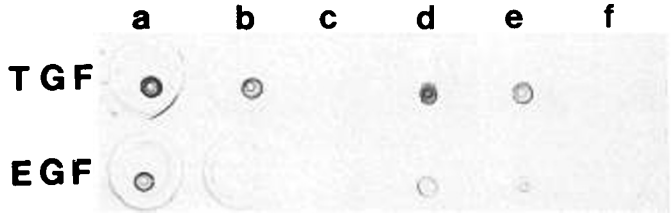

Fig. 2. A total of 0.4 pmol of hEGF and hTGFα were spotted on a nitrocellulose filter. The spots were incubated with anti-hTGFα (*a*), anti-hTGFα preabsorbed with (*b*) 0.1 μM hTGFα, (*c*) 1 μM hTGFα, (*d*) 0.1 μM hEGF, and (*e*) 1 μM hEGF. Then 0.1% BSA-PBS was used instead of anti-hTGFα as a negative control (*f*). The intensities of the dots were scanned densitometrically: the reading was for TGFα plus spotted anti-TGFα 21; for anti-TGFα preabsorbed with 1 μM EGF plus spotted TGFα 20.3; for anti-TGFα plus spotted EGF 17.4; and for anti-TGFα preabsorbed with 1 μM EGF plus spotted EGF 20.9.

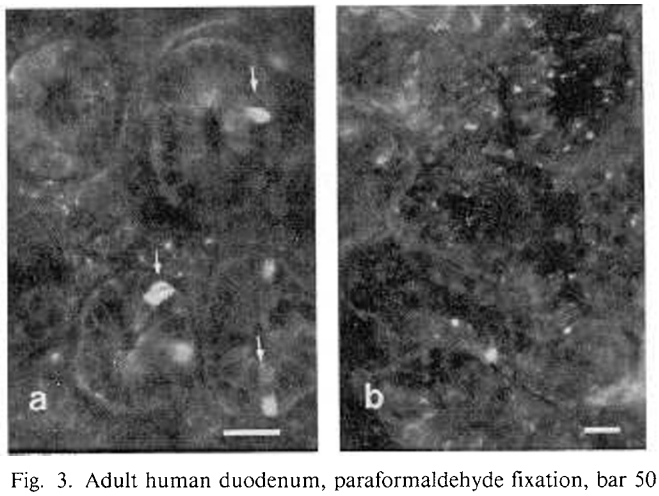

Fig. 3. Adult human duodenum, paraformaldehyde fixation, bar 50 μm. A positive immunoreaction is seen in the Paneth cells, mainly in the basal part of the cell, with polyclonal anti-hEGF 1:1000 (*a*). This reaction is almost abolished after preabsorption of the antiserum by 1 μM hEGF (*b*).

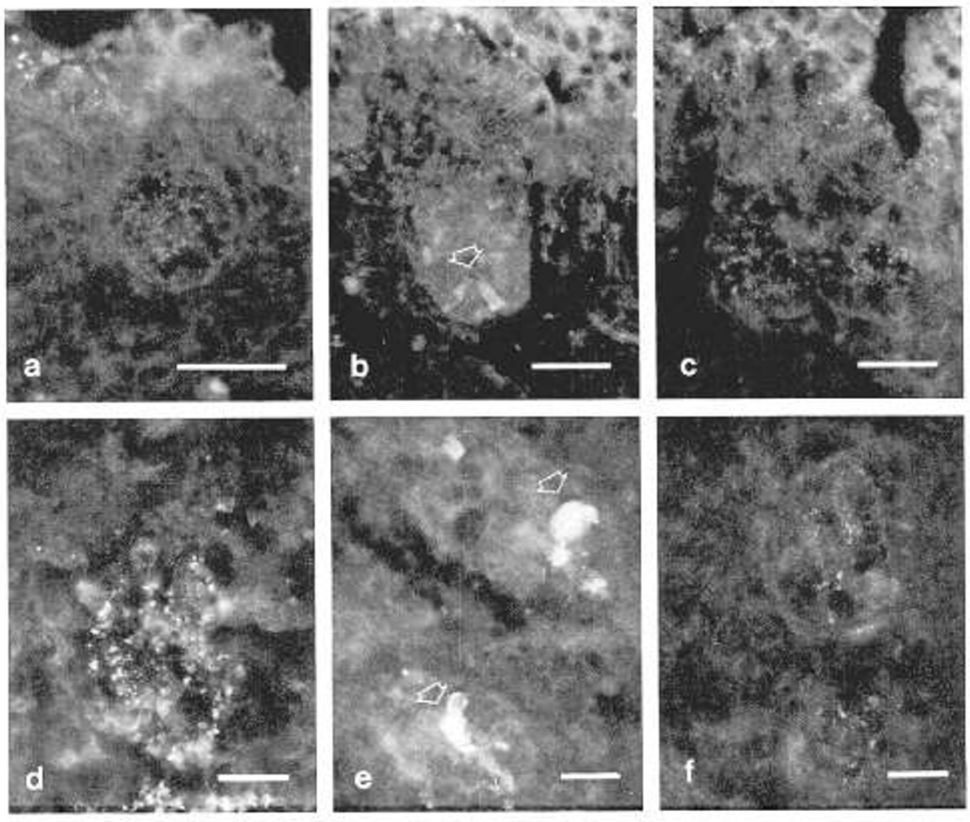

Fig. 4. A 19-wk-old human fetal jejunum (*a* to *c*), bar 50 μm and ileum (*d* to *f*), bar 25 μm, paraformaldehyde fixation. Strong granular flourescence in the epithelium and crypts of Lieberkühn is seen with monoclonal anti-hTGFα (*a* and *d*). Monoclonal anti-hEGF2 gives a similar fluorescence pattern as anti-hTGFα (*c* and *f*). A few cells (*arrows*) staining positively with the polyclonal anti-hEGF diluted 1:250 are seen in the crypts of Lieberkühn (*b* and *e*).

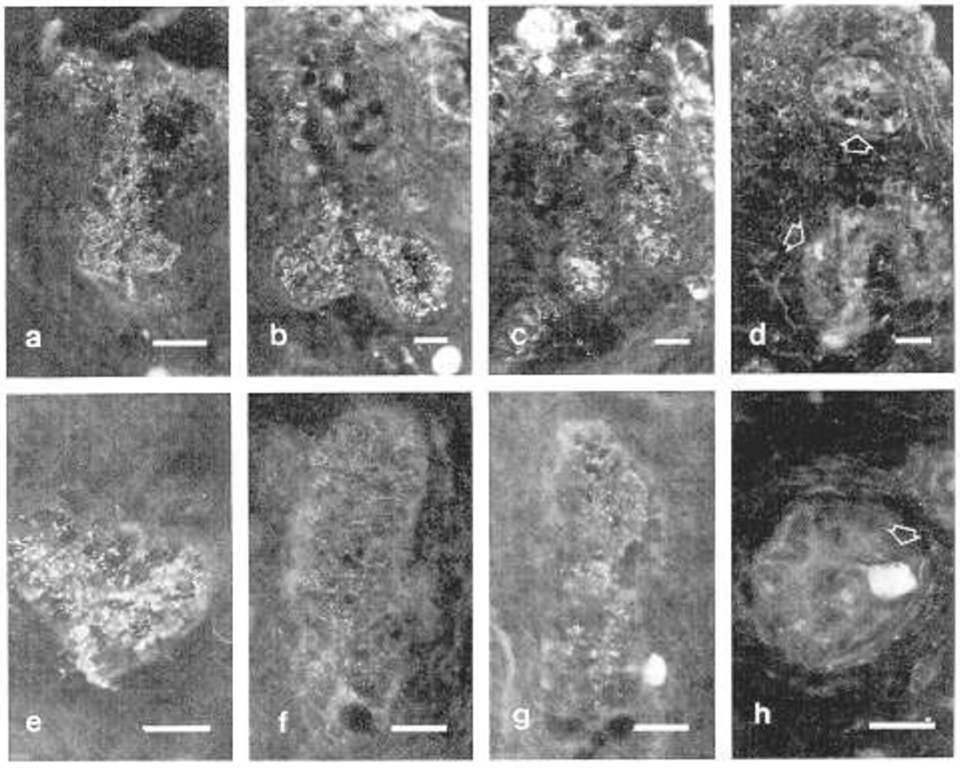

Fig. 5. A 20-wk-old human fetal jejunum, paraformaldehyde fixation, bar 25 μm. Very strong granular fluorescence along the ducts and crypts of Lieberkühn appears with anti-hTGFα (*a* and *e*) and monoclonal anti-hEGF2 (*b* and *f*) and anti-hEGF4 (*c* and *g*) whereas the polyclonal anti-hEGF stains distinct Paneth cells (*arrows*, *d* and *h*).

suggesting that the antibody had a much higher affinity for hTGFα than for hEGF or that the unwanted cross-reaction was due to nonimmunologic factors.

Hence we conclude that the hTGFα immunoreaction is specific in those cellular sites in which it was abolished by preabsorption with hTGFα but not with excess hEGF.

EGF immunohistochemistry. The positive tissue controls were human adult duodenum, in which the fluorescence was seen in the Paneth cells and Brunner's glands (Fig. 3*a*) and human and rat submandibular glands. Preabsorption of the antiserum with 0.001–1.0 μM hEGF diminished the intensity of the fluorescence, but did not abolish it completely (Fig. 3*b*). Similar sections stained with the preimmune serum were generally negative, but occasional fluorescent cells were seen.

The polyclonal anti-hEGF detected hEGF immunoreactive cells in 19- (Fig. 4) and 20-wk-old (Fig. 5) human fetal jejunal crypts of Lieberkühn and Brunner's glands. Fluorescence was also seen in the ileum of a 19-wk-old fetus. To our surprise the monoclonal anti-hEGF recognized much more irhEGF in the jejunum and in the 20-wk-old ileum (Figs. 4–6). The fluorescence pattern was granular and thus different from that obtained with the polyclonal antiserum. The reactions were abolished with 1μM hEGF. Normal rabbit or mouse serum did not stain any structures. Other tissues examined with anti-hEGF were negative. The results are summarized in Table 1.

TGFα immunohistochemistry. The anti-hTGFα caused strong staining of the villi and crypts of Lieberkühn in paraformaldehyde-fixed 19-wk-old human fetal duodenum, jejunum and ileum (Figs. 4 and 7) and in the 20-wk-old fetus it also stained the epithelial and cryptal cells of the colon (Figs. 5, 6, and 8). The fluorescence pattern was linear or formed a granular ring to the peripheral cytoplasm. Replacing the antibody with normal mouse serum gave no staining. Preabsorption of anti-hTGFα with 1 μM hTGFα diminished and with 10 μM hTGFα nearly abolished the fluorescence. One μM hEGF had generally no effect on it, although in some consecutive sections the fluorescence was slightly diminished. The results were similar in acetone-fixed tissues and with the peroxidase anti-peroxidase technique.

We consider that the fluorescence pattern obtained with anti-TGFα represents irTGFα. This is supported by the data from HPLC experiment.

The hTGFα fluorescence was much weaker in a 22-wk-old fetal jejunum. Also, in 22-wk-old fetuses irTGFα was observed

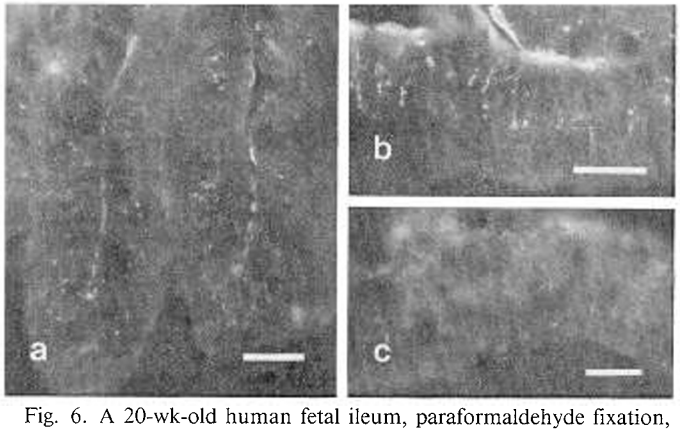

Fig. 6. A 20-wk-old human fetal ileum, paraformaldehyde fixation, bar 25 μm. Moderate staining in the epithelium with anti-hTGFα (*a* and *b*). Both monoclonal hEGF antisera stain rather weakly; the figure shows the fluorescence obtained with anti-hEGF2 (*c*). The polyclonal anti-hEGF did not stain any structures.

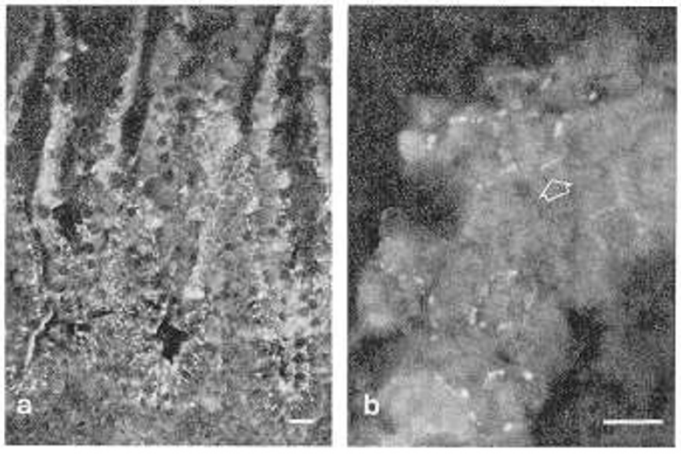

Fig. 7. A 19-wk-old human fetal jejunum stained with hTGFα antibody shows a strong granular fluorescence in the crypts of Lieberkühn (*a*) and the villi (*b*). Bar 25 μm.

Table 1. *Intensity of fluorescence in sections of gastrointestinal tract incubated with hEGF antiserum and preimmune serum after preabsorptions*[a]

Antisera	Source of tissue																
	Rat	Human fetus, age (wk)										Human				
	Adult											Adult				
	PFA	14.5	19			20				22		PFA			AS	
	s	PFA	PFA		AC	PFA			AC	PFA	AC					
		d	j	i	j	j	i	c	j	j	d	d	p	s	p	s
pAE 1:1000	+++	0	+	+	0	+	0	0	+	0	+	++	0	0	++	++
+ 1 μM E	0		0	0		0			0		0	(+)			−	−
+ 0.1	(+)											(+)				
+ 0.01	+											+				
+ 0.001	++											++				
Preimmune (+)(+)	0	0	0	0	0	0	0	0	0		(+)	(+)	0	0		
mAE 1:500	+++	0	++	++	0	++	+	0	−	0	−	−	−	−	++	++
+ 1 μM E	0		0	0		0	0								(+)	(+)
NMS	0		0	0		0	0								0	0

[a] pAE, polyclonal anti-hEGF; mAE, monoclonal anti-hEGF; preimmune, preimmune serum; NMS, normal mouse serum; E, hEGF; +++, very strong fluorescence; ++, strong; +, moderate; (+), weak; 0, no staining; −, not examined; AC, acetone fixation; PFA, paraformaldehyde fixation; c, colon; d, duodenum; j, jejunum; k, kidney; p, parotid gland; s, submandibular gland.

only in a few cells and in the villous epithelium instead of a strong cryptal reaction. All other tissues examined were negative for hTGFα. The results are summarized in Table 2.

IFMA, RIA, and RRA results. According to RRA, an extract of the intestine of a 19-wk-old fetus contained 2.9 ng of an EGF-R-binding and EGF-competing substance per mg protein. According to the EGF-IFMA, it contained only 0.16 ng EGF per mg protein and according to EGF-RIA 0.067 ng EGF per mg protein. Neither EGF-IFMA nor EGF-RIA cross-reacted with TGFα. Thus the intestinal tissue contained approximately 20 times more EGF-R-binding substance than EGF.

RP-HPLC results. A single hTGFα-immunoreactive peak corresponding to the elution time of authentic hTGFα was detected in RP-HPLC analysis of the intestinal extract. No immunoreactivity was found in the irTGFα-peak or in any other fraction with the hEGF antibody (Fig. 9).

In accordance with this RRA detected some receptor binding substance in the irTGFα-peak while EGF-IFMA could not detect any EGF.

DISCUSSION

The results suggest that hTGFα-immunoreactive peptide, which in RP-HPLC shows characteristics of hTGFα and has an affinity for the EGF receptor, is present in developing human intestine. Moreover, an EGF-like peptide was also found in the same tissue.

Although our monoclonal anti-hTGFα reacted in the nitrocellulosa model also with hEGF, the reactivity to hTGFα was much greater. In the RP-HPLC analysis of fragments of 19- and 20-wk-old small intestine, only a hTGFα-immunoreactive fraction was found and it had the same elution time as synthetic hTGFα. Anti-hEGF did not recognize hEGF in any of the fractions. the irhTGFα-peak was also positive in the RRA. However, probably due to insufficient amounts of hEGF in the tissue extract EGF-IFMA did not detect any hEGF. In a 19-wk-old human fetal intestine RRA measured 20 times more receptor binding substance than could be accounted for EGF as measured by EGF-IFMA.

To conclude, despite the anti-hTGFα cross-reactivity with EGF, results of RP-HPLC, RRA, IFMA, and RIA, preabsorption controls, and the weaker fluorescence with the mono- and polyclonal anti-hEGF than with the anti-hTGFα, all suggest the presence of both TGFα- and EGF-like peptides in the fetal gut.

A large number of irhEGF- and irTGFα-containing cells were detected throughout the intestine of 19- to 22-wk-old human fetuses. In the colon a positive fluorescence reaction was obtained only with the anti-hTGFα and in the jejunum the TGFα reaction was much stronger than the hEGF reaction.

Our immunohistochemical results show that the proximal intestine may contain more irEGF and irTGFα than the distal part. This could be associated with the faster rate of the cell cycle in the small intestine (26). The strongest fluorescence is seen in all the segments in the proliferative zone, i.e., the crypts in the small intestine and the lower two-thirds of the colonic crypts. This also suggests a mitogenic role for EGF- and TGFα-like peptides in the intestinal epithelium.

In fetal mice Nexo *et al.* (27) measured a 10-fold higher EGF content by RRA than by RIA, suggesting that the fetal EGF differs from the adult form. Popliker et al. (28) did not find any EGF-mRNA from mouse fetuses, fetal membranes or placentae. The first detectable transcription was in the kidneys at 2 wk postpartum. TGFα-mRNA has been localized predominantly to rat maternal decidua, and not to the embryo itself (20). Most of

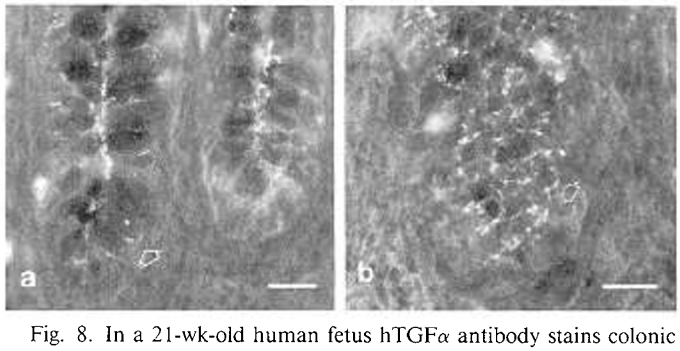

Fig. 8. In a 21-wk-old human fetus hTGFα antibody stains colonic epithelial cells (*arrow, a*) and the ductal crypts (*b*). All EGF antisera failed to stain any structures. Bar 25 μm.

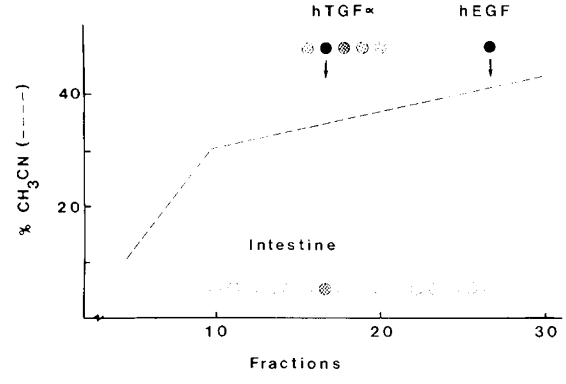

Fig. 9. Reverse-phase HPLC of the 19-wk-old human fetal intestinal extract. LKB-Ultropac TSK-ODS 120 T column with a linear 0–60% gradient of acetonitrile in 0.05% TFA at a flow rate of 0.8 ml/min was used. Then 0.8-ml fractions were collected. All fractions were analyzed for reactivities to both hEGF and hTGFα antibodies. The *dots* represent the intensity of the immunoreaction obtained in the dot blot assay. *Arrows* mark hEGF and hTGFα standards.

Table 2. *Intensity of fluorescence in sections of gastrointestinal tract incubated with hTGFα antiserum after preabsorptions**

	Source of tissue									
	Human fetus, age (wk)									Human
	f19			f20				f22		Adult
	PFA		AC	PFA			AC	PFA	AC	PFA
Antisera	j	i	j	j	i	c	j	j	d	d
mAT 1:1000	+++	+++	++	+++	+++	+	++	+	++	0
1:6000	++	++	–	++	+	+		–	–	
+ 10 μM T	0	0	–	(+)	(+)	(+)		–	–	
+ 1 μM T	(+)	(+)								
+ 1 μM E	++/+	++/+								
NMS 1:1000	0	0	0	0	0	0		0		0

* mAT, monoclonal anti-hTGFα; E, hEGF; T, hTGFα; NMS, normal mouse serum; +++, very strong; ++, strong; +, moderate; (+), weak; 0, no staining; –, not examined; AC, acetone fixation; PFA, paraformaldehyde fixation; c, colon; d, duodenum; j, jejunum; i, ileum.

the luminal EGF in adults originates from the salivary glands and Brunner's glands; sialectomy significantly reduces the gastric content of EGF (29). In our study the source of both irEGF and irTGFα in the intestine during fetal life remains unclear. They could be carried to the fetus by the blood from the placenta or the decidua. Or the fetal kidneys might synthesize and excrete them into the amniotic fluid and the intestinal villi may absorb the peptides from the amniotic fluid swallowed—although with the antibodies used, we could not detect any irEGF/TGFα in the kidney. And finally, the intestine might represent the site of synthesis, as it does in adults. *In situ* hybridization studies will show whether any synthesis does occur in the human fetus.

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