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Immunohistochemical localization of acidic fibroblast growth factor in normal human enterochromaffin cells and related gastrointestinal tumours

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Abstract Acidic fibroblast growth factor (aFGF) is a member of the structurally related heparin-binding growth factor family. The best studied members of this family are aFGF and basic FGF (bFGF), which are potent mitogens and differentiation factors for mesoderm-derived cells, including fibroblasts. This study was designed to verify the immunohistochemical expression of aFGF in normal human endocrine cells of the gut and in related endocrine tumours. We examined normal gastrointestinal mucosa from seven different subjects and 41 gut endocrine tumours from different sites, including stomach, duodenum, and small and large intestine, using an aFGF polyclonal antibody with no cross-reactivity for bFGF. We localized aFGF in a fraction of serotonin-producing enterochromaffin (EC) cells of the normal gut, while it was absent in gastrin (G), CCK, secretin (S), somatostatin (D) and glicentin (L) cells. aFGF immunoreactivity was also expressed in serotonin producing EC cell tumours, but not in other functional types of gut endocrine neoplasms investigated, including gastric ECL cell, duodenal somatostatin and gastrin cell, and rectal L cell tumours. A positive correlation was found between expression of aFGF and the amount of tumour fibrous stroma, suggesting that aFGF may be involved in proliferation and activity of stromal fibroblasts.

Key words Acidic fibroblast growth factor · Gastrointestinal endocrine tumours · Carcinoid tumours · Immunohistochemistry

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Introduction

Acidic fibroblast growth factor (aFGF or FGF-1) belongs to a large family of homologous polypeptide growth factors that have an affinity for heparin and glycosaminoglycans [10, 29]. This family also includes basic fibroblast growth factor (bFGF or FGF-2), *int*-2 (FGF-3), Kaposi sarcoma FGF (FGF-4), FGF-5, FGF-6, and keratinocyte growth factor (FGF-7) [10, 24, 29]. aFGF is a single-chain anionic polypetide with a molecular weight of about 18,000, coded by a gene positioned on human chromosome 5 between bands 5q31.3 and 5q33.2 [38]. It was found to be chemotactic for fibroblasts, and able to promote angiogenesis and proliferation of fibroblasts and other mesoderm-derived cells, including heart myocytes [3, 10, 17, 19, 20, 22, 24, 26, 29]. Its biological properties, including the ability to stimulate angiogenesis and formation of granulation tissue, suggest a possible role in physiological wound healing [19, 29]. In addition, aFGF has been proved to be a neurotrophic factor, which regulates neuronal cell differentiation and survival both in vitro and in vivo [25, 48, 49]. Although aFGF does not seem to have a direct role as an autocrine factor in tumour progression, it could act as a paracrine factor stimulating angiogenesis necessary for neoplastic cell growth [29]. However, the precise role and function of aFGF in normal and neoplastic tissues is not known.

aFGF is widely distributed in human and other mammalian tissues. It was primarly found in neural tissue, including brain [31, 33], hypothalamus [32] and retina [14, 16], and has been extracted or immunohistochemically detected in bone matrix [27], kidney [23], uterus [9], pancreas [21], parathyroid [45], breast [4] and salivary glands [40]. Information on aFGF localization in endocrine cells of the human gut is lacking.

Endocrine tumours of the gastrointestinal tract (carcinoids) are slow-growing neoplasms, which often show striking fibroblastic reaction in the tumour stroma, proliferation of smooth muscle within the tumours, pronounced serosal fibrosis, obliterative elastic sclerosis of mesenteric blood vessels and, sometimes, fibroelastosis

of the inside surfaces of the cardiac chambers and valvular leaflets, mainly of the right side, resulting in so-called carcinoid heart disease. In particular, these features are typical of midgut serotonin-producing enterochromaffin (EC) cell tumours (argentaffin carcinoids). Previous studies have considered the possible role of TGFα, TGFβ, PDGF, insulin-like growth factor-I and bFGF in the stimulation of stromal fibrosis and in neoplastic growth regulation of gastrointestinal endocrine tumours [1, 5, 7, 12, 13, 42].

Studies on localization of aFGF in endocrine neoplasms and related normal endocrine cells of the human gut have not been previously reported. We performed immunohistochemical investigations to characterize aFGF production by normal and neoplastic endocrine cells of the gut using specific antibodies for aFGF. We observed aFGF expression in serotonin-producing EC cells of the normal gastrointestinal mucosa and in intestinal EC cell tumours (argentaffin carcinoids).

Materials and methods

We studied samples of normal human stomach and small and large bowel collected at surgery from seven different subjects (four males, three females). We also examined 41 endocrine tumours of the gastrointestinal tract (7 ECL cell tumours and 2 poorly differentiated neuroendocrine carcinomas of the stomach, 1 gastrin cell tumour and 1 somatostatin cell tumour of duodenum, 1 jejuneal well differentiated endocrine carcinoma, 9 ileal, 8 appendiceal, 4 right colon and 2 rectal serotonin-producing EC cell tumours, 1 appendiceal and 5 rectal glicentin- and PP-producing L cell tu-

Table 1 Summary of clinico-pathological data and immunohistochemical staining of 41 endocrine tumours of the gastrointestinal tract (*ne* not evaluated, *aFGF* acidic fibroblast growth factor, *Glic* glicentin-glucagon, *5HT* serotonin, *PP* pancreatic polypeptide, *SubP* substance P, *Som* somatostatin)

No.	Sex	Age	Site	Type	Size (cm)	Fibrous stroma[b]	Metastases	Markers (% of positive cells)					
								Som	PP	Glic	5HT	SubP	aFGF
1	F	46	Stomach	Type 1 [44]	1.5	+	No	0	0	0	0	0	0
2	F	65	Stomach	Type 1	ne	ne	No	0	0	0	0	ne	0
3	F	90	Stomach	Type 1	ne	ne	No	0	0	0	0	0	0
4	M	76	Stomach	Type 1	2	++	No	0	0	0	0	0	0
5	M	43	Stomach	Type 3 [44]	2	+	Node	0	0	0	0	ne	0
6	M	26	Stomach	Type 3	2	+	Node/liver	0	0	0	0	0	0
7	M	44	Stomach	Type 3	2	++	Node/liver	0	0	0	0	ne	0
8	M	68	Stomach	NEC [44]	3	+	Node/liver	20	0	0	0	ne	0
9	M	52	Stomach	NEC	5	+	Node	0	0	0	0	ne	0
10	F	52	Duodenum	D-cell	1.5	+	Node	100		0	0	ne	0
11[a]	F	42	Duodenum	G-cell	1	++	No	0	0	0	0	0	0
12	F	38	jejunum	Undefined-cell	1.2	+	Node/liver	0	0	0	0	ne	0
13	F	67	Ileum	EC cell	2	++	Node/liver	0	0	0	100	100	5
14	M	52	Ileum	EC cell	2	+++	Node/liver	0	0	0	40	100	80
15	M	ne	Ileum	EC cell	2.5	++	No	0	0	0	90	40	60
16	F	46	Ileum	EC cell	1.5	++	Node	0	0	0	30	70	70
17	M	59	Ileum	EC cell	2.5	++	Ne	0	0	0	100	60	70
18	M	58	Ileum	EC cell	2.5	++	Node/liver	0	0	0	70	40	70
19	M	39	Ileum	EC cell	3	+++	Node	0	0	0	60	90	5
20	F	75	Ileum	EC cell	1.5	+++	Liver	0	0	0	100	60	80
21	F	69	Ileum	EC cell	2	++	Node	0	0	0	10	30	40
22	M	53	Appendix	EC cell	2.5	+++	No	0	0	0	90	100	30
23	M	25	Appendix	EC cell	0.5	++	No	0	0	0	70	90	5
24	F	95	Appendix	EC cell	2	++	No	0	0	0	100	10	40
25	F	14	Appendix	EC cell	0.6	+++	No	0	0	0	10	ne	70
26	F	17	Appendix	EC cell	0.5	++	No	0	0	0	30	0	60
27	F	24	Appendix	EC cell	0.2	+++	No	0	0	0	100	20	60
28	M	27	Appendix	EC cell	0.5	+++	No	0	0	0	100	10	80
29	F	21	Appendix	EC cell	0.3	+	No	0	0	0	100	0	3
30	F	24	Appendix	L cell	1.5	+++	No	60	60	100	0	0	0
31	F	ne	Right colon	EC cell	ne	ne	Liver	0	0	0	100	ne	20
32	ne	ne	Right colon	EC cell	ne	+++	Ne	0	0	0	30	50	5
33	F	50	Right colon	EC cell	ne	++	Omentum	0	0	0	90	0	5
34	M	46	Right colon	EC cell	8	++	Liver	0	0	0	100	80	5
35	F	51	Rectum	L cell	0.7	+	No	0	5	0	0	0	0
36	F	66	Rectum	L cell	ne	ne	No	3	40	15	0	ne	0
37	M	39	Rectum	L cell	0.3	+++	No	0	90	0	0	ne	0
38	M	54	Rectum	EC cell	2	++	No	10	0	0	80	0	30
39	F	66	Rectum	L cell	1.7	+	No	0	0	0	0	0	0
40	M	66	Rectum	L cell	1	+	No	0	20	10	0	0	0
41	M	70	Rectum	EC/L cell	2	+++	No	0	0	40	30	0	10

[a] Immunoreactivities for gastrin 34 and C-terminus gastrin-CCK-cerulein were found only in case 11
[b] See "Materials and methods"

Table 2 Antibodies and antisera used

Antibodies/antisera	Clone/code	Dilution	Source
Neuron specific enolase (NSE)	A589	1:200	Dako, Copenhagen, Denmark
Chromogranin A (CgA)	Phe5	1:50	Enzo Diagnostics, New York, USA
Chromogranin B (CgB)	B11	1:4000	Dr. Siccardi, University of Milan, Italy
Glicentin-glucagon (Glic)	B37-1	1:2500	Milab, Malmo, Sweden
Pancreatic polypeptide (PP)	CA08327	1:4000	Cambridge Research Biochemicals, Cambridge, UK
Somatostatin (Som)	A566	1:500	Dako
Somatostatin (Som)	786 OC	1:10	Labometrics, Milan, Italy
Secretin (Secr)	B33-1	1:500	Milab
Serotonin (5HT)	YC5	1:50	Biogenesis, Bournemouth, UK
Substance P (Sub P)	B45-1	1:640	Milab
C-terminus gastrin-CCK-cerulein (Cer)	B4	1:10000	Farmitalia, Milan, Italy
Gastrin 34 (Gastr)	RA-08–090	1:500	Cambridge
Acidic fibroblast growth factor (aFGF)	06–101	1:100	UBI, Lake Placid, NY, USA
Basic fibroblast growth factor (bFGF)	05–118	1:100	UBI

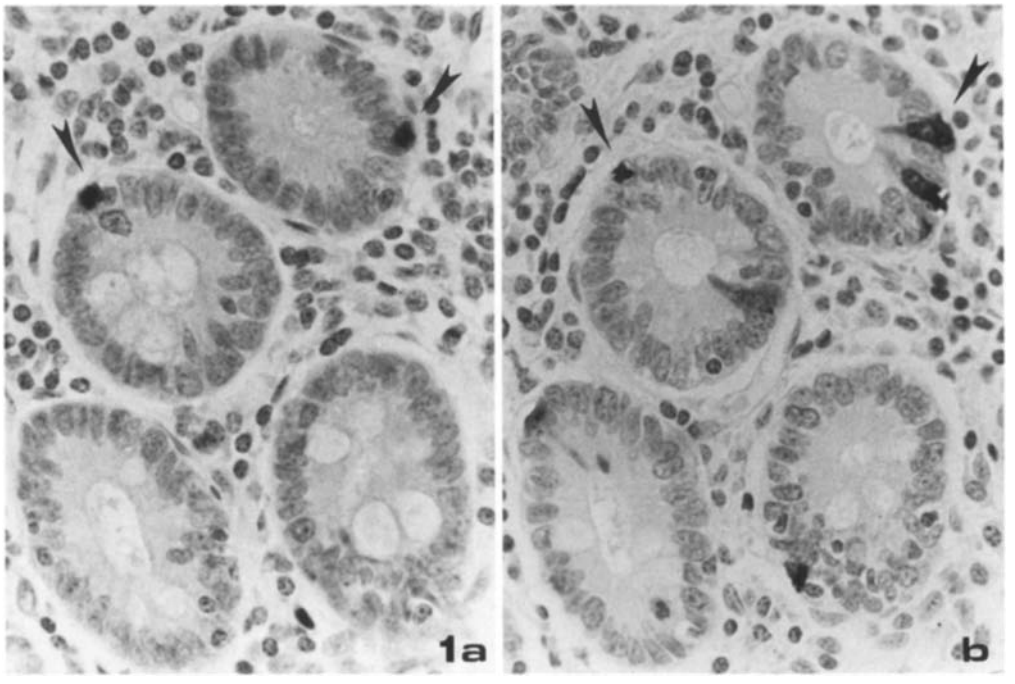

Fig. 1 **a** Acidic fibroblast growth factor (aFGF) and **b** serotonin immunoreactivities in normal human duodenal mucosa. Only a subgroup of serotonin-positive cells (*arrows*) shows aFGF immunoreactivity. ×400

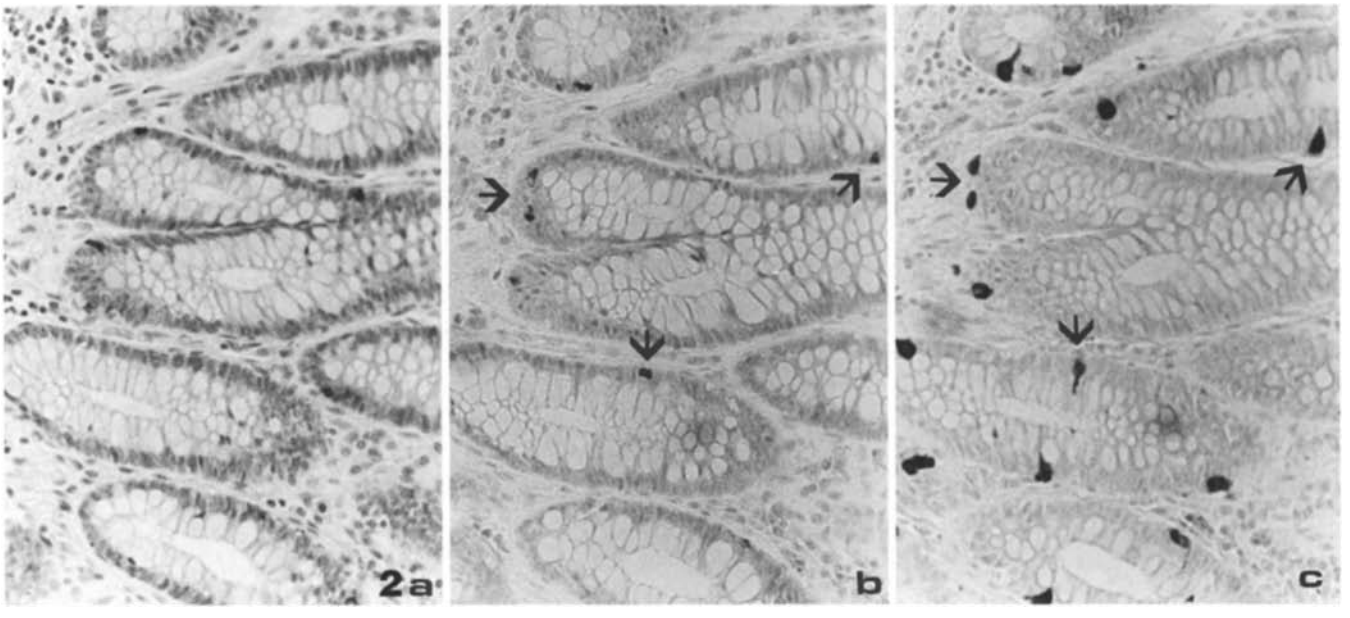

Fig. 2 **a** Glicentin, **b** aFGF and **c** serotonin immunoreactivities in normal human rectal mucosa. Glicentin-positive cells are unreactive with anti-aFGF antibodies which, in contrast, recognize a subpopulation of serotonin-positive cells (*arrows*). ×250

mours). The clinico-pathological data for these tumours are given in Table 1.

For all histological, histochemical and immunohistochemical studies, tissues were fixed in buffered formalin (formaldehyde 4% w/v and acetate buffer 0.05 M) and routinely embedded in paraffin. Serial sections were stained with haematoxylin-eosin (H&E) for histological evaluation, with van Gieson stain for the evaluation of the fibrous stroma, and with Grimelius' silver impregnation for argyrophil cell detection. The amount of fibrous stroma was scored on van Gieson stained sections into three categories: (+) scant, when the fibrous stroma represented less than 10% of the neoplastic tissue; (++) intermediate, when it was from 10% to 50%, and (+++) abundant when it was more than 50%.

A panel of immunohistochemical stains was performed on serial reverse face sections using the antibodies and antisera listed in Table 2. Sections, cut at 3 μm and mounted on slides coated with poly-L-lysine, were deparaffinized and hydrated through graded alcohol to water. The endogenous peroxidase activity was quenched by plunging sections in 3% hydrogen peroxide for 10 min. Primary antibody incubation was done at 4°C for 18–20 h, followed by the avidin–biotin complex (ABC) detection procedure according to Hsu et al. [28]. The sections were then immersed in 0.03% 3,3'diaminobenzidine tetrahydrochloride and counterstained with Harris' haematoxylin. The sections stained for glicentin and somatostatin (Dako, polyclonal) were pretreated for 10 min with 0.003% subtilisin (Sigma, P4789; protease type XXVII or Nagarse protease) in 0.05 M TRIS-buffered saline pH 7.4, while sections stained for aFGF were pretreated with 0.01 M citrate buffer pH 6 (2 × 5 min) in a microwave oven at 650 W. Colocalization studies were performed using both 3 μm serial reverse face sections [18] and double immunostain performed according to Mason et al. [35] or Lan et al. [30]. Specificity controls consisted of absorption of each antiserum with 10–50 nM/ml of the antigen and related or unrelated amines or peptides, including bFGF (PBH, Hannover, Germany) and serotonin conjugated with bovine serum albumin (BSA)-pNH_2-phenylalanine (Prof. R. Buffa, University of Milan), and omission of the first layer, and use of control tissues with or without the pertinent antigen.

Results

aFGF was detected by immunohistochemical analysis in scattered endocrine cells present in the mucosa of the whole gastrointestinal tract. The immunohistochemical staining was clearly granular and diffuse in the cytoplasm. The staining was prevented by absorption with 10 nM aFGF, but not by absorption with 20 nM bFGF or 50 nM serotonin. Gastric mucosa showed rather scanty aFGF-immunoreactive (IR) cells, which were particularly rare in the fundus. In contrast, aFGF-IR cells were numerous in the mucosa of duodenum, jejunum and ileum, as well as in that of the right colon and rectum. In adjacent 3-μm sections aFGF-IR cells stained strongly with serotonin antibodies (Fig. 1), while failing to react with antisera or antibodies directed against C-terminus gastrin-CCK-cerulein, somatostatin, glicentin (Fig. 2) and secretin. Double labelling studies confirmed that all aFGF-IR cells were serotonin positive (Fig. 3) and proved that a fraction (about 1/3) of serotonin-IR cells failed to stain with aFGF antibody. Double immunostains confirmed that aFGF was not contained in somatostatin- (Fig. 4), gastrin-CCK-cerulein-, glicentin- and secretin-immunoreactive cells. No substance P-positive cells were found in the normal gastrointestinal mucosa.

Sections of normal gastrointestinal mucosae from seven subjects expressed few bFGF reactive endocrine cells,

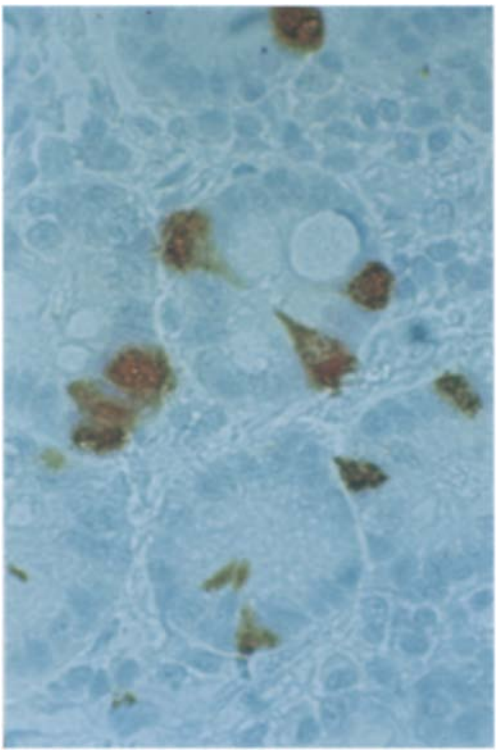

Fig. 3 Normal human duodenal mucosa: colocalization of serotonin (*brown*) and aFGF (*red*) in some enterochromaffin cells. aFGF revealed with anti-rabbit immunoglobulins conjugated to alkaline phosphatase, followed by enzyme development with FastRed as chromogen. ×630

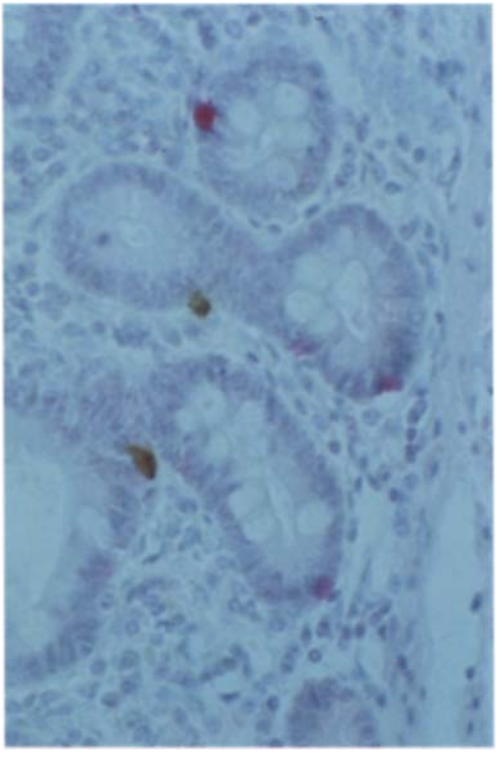

Fig. 4 Normal human duodenal mucosa: different localization of somatostatin (*brown*) and aFGF (*red*) in endocrine cells. aFGF revealed with anti-rabbit immunoglobulins conjugated to alkaline phosphatase, followed by enzyme development with FastRed as chromogen. ×400

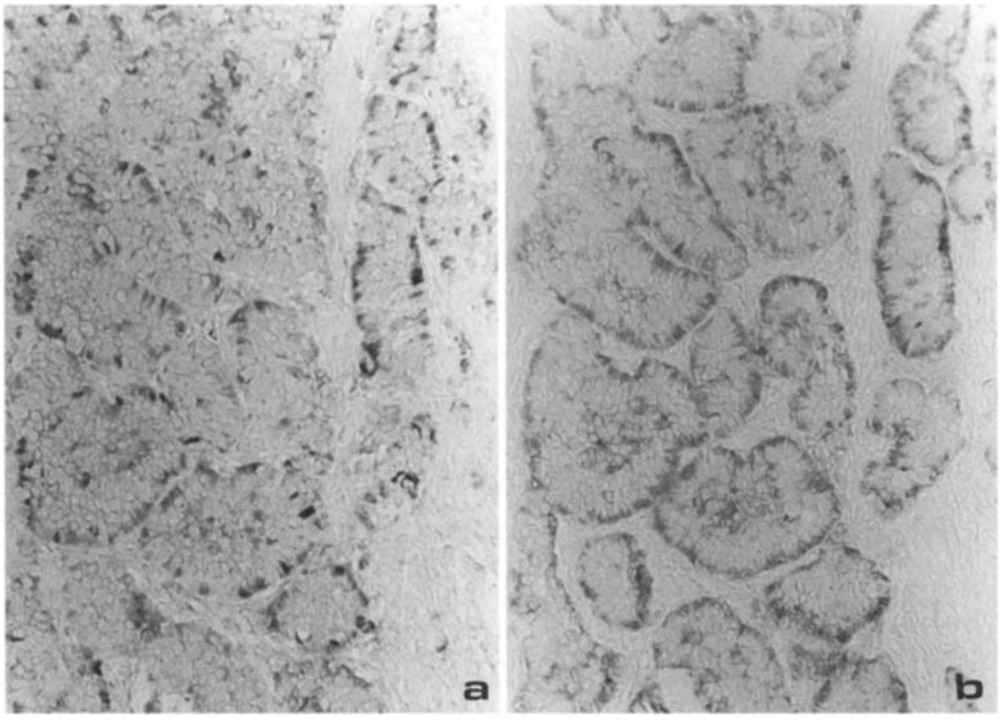

Fig. 5 **a** aFGF and **b** serotonin immunoreactivities in an ileal endocrine tumour. ×200

which were more numerous in the gastric antral mucosa and were very few in the small intestinal mucosa.

In 23 of the 41 gastrointestinal endocrine tumours examined, immunostaining for aFGF showed significant numbers of positively staining cells and was restricted to cases with serotonin-positive cells (Fig. 5). Most (81%) of the aFGF- and serotonin-IR cells were also substance P positive. The percentage of aFGF positive cells, varied from tumour to tumour, ranging from 5% to 100%, with a mean of 40%. The mean percentage of serotonin-IR cells, in the same neoplams, was 75% (ranging from 10% to 100%), indicating that, as in the normal gastrointestinal mucosa, aFGF is expressed only in a subpopulation of serotonin cells. In tumours coexpressing aFGF and substance P the mean percentage of aFGF-IR cells was 42%, while that of substance P-IR cells was 50.8% (ranging from 10% to 100%). None of the tumours examined, including ECL cell gastric carcinoids, showed positive staining for bFGF.

The amount of fibrous stroma was comparatively evaluated in 22 serotonin- and aFGF-positive EC cell tumours and in 15 serotonin- and aFGF-negative tumours (Table 3). The former group was associated with abundant or intermediate fibrous stroma in 21of 22 cases (95.5%), while only 1 (4.5%) case had scanty stroma. In contrast, of the 15 serotonin- and aFGF-negative tumours, only 2 (13.3%) showed abundant fibrous stroma, and only 3 (20%) presented an intermediate fibrous stroma. The majority of these neoplasms (10/15, 66.7%) had scanty fibrous stromal reaction.

No relationships were found between expression of aFGF and malignancy of the tumour, the marker being about equally expressed in benign appendiceal tumours and in malignant ileal and colonic endocrine neoplasms (see Table 1).

Table 3 Relationship between amount of fibrous stroma and serotonin and aFGF expression in gut endocrine tumours. (figures in round brackets are percentages)

		Fibrous stroma		
		Scant	Intermediate	Abundant
Serotonin + aFGF+	tumours	1 (4.5)	12 (54.5)	9 (41)
Serotonin- aFGF-	tumours	10 (66.7)	3 (20)	2 (13.3)

Discussion

We have shown that antibodies against aFGF identify a population of endocrine cells scattered in the normal gastrointestinal mucosa as well as in tumours composed of EC cells, which are known to produce serotonin and tachykinins. The aFGF-IR cells represent a fraction of serotonin-IR cells of the normal gut, while they are not related to other functional types of endocrine cells, such as gastrin (G), CCK, secretin (S), somatostatin (D) and glicentin (L) cells.

aFGF immunoreactivity was also expressed in serotonin- and substance P-producing (EC) cell tumours (argentaffin carcinoids), but not in other functional types of endocrine tumours of the gut investigated, including gastric ECL cell, duodenal somatostatin and gastrin cell tumours, and rectal L cell tumours. These findings indicate that aFGF may be considered to be a specific marker for EC cell tumours and that it should be used in addition to serotonin and tachykinins (substance P, neurokinin A and B and neuropeptide K) to characterize these lesions.

In addition, our results demonstrate that, at least in our experimental conditions, the distribution of aFGF in endocrine cells of the gut and related tumours, appears to be different from that of bFGF. The bFGF-IR cells were most numerous in the normal gastric mucosa where only few aFGF-IR cells were found, and in contrast aFGF-IR cells were numerous in the small intestine, where bFGF-IR cells were absent or very few. The absence of bFGF reactivity that we found in EC-cell tumours should indicate that this growth factor, unlike aFGF, cannot be regarded as a tumour marker. However, our findings contrast with those of Alhman et al. [1], which showed a strong immunopositive reaction with bFGF antiserum in tumour cells of 30 neuroendocrine tumours of the digestive system. These different results may be due to the different conditions of tissue processing and use of different anti-bFGF antibodies. In this context, in the paper of Ahlman et al. [1] there is no mention of the specificity tests proving that the bFGF antibodies employed were not cross-reactive with aFGF. Technical problems may also explain the lack of bFGF-IR that we found in gastric ECL cell tumours, which contrasts with the positivity shown by Bordi et al. [7] in the same type of tumours.

It is well known that intestinal EC cell tumours (argentaffin carcinoids) are associated with significant stromal fibrosis when there is invasion of the muscular layer, which may be combined with peritoneal adhesions and intestinal obstruction. Moreover, a "carcinoid heart disease" characterized by large fibrous plaques on the endocardial surface of cardiac chambers and of valvular cusps, mainly of the right side, may be associated with the carcinoid syndrome. Although serotonin and other vasoactive substances have been implicated as responsible for the fibrosis, the relationship between these substances and desmoplastic reaction has not been well defined. Experimental data have shown that serotonin alone does not have a mitogenic effect on Chinese hamster lung fibroblasts, but it can potentiate the mitogenic effect caused by bFGF [46]. Some authors [1, 5, 12, 13, 41, 42] have shown that midgut endocrine tumours produce several growth factors, including PDGF, TGFα, TGFβ_1, TGFβ_2, TGFβ_3, insulin like growth factor-I and bFGF, that may interfere with mechanisms regulating stromal reaction, angiogenesis and tumour growth.

Our results have shown a correlation between aFGF expression and the amount of fibrous stroma present in the gut endocrine tumours (Table 3), suggesting that aFGF might be involved in proliferation and activity of stromal fibroblasts. Other trophic responses to the production and local release of aFGF by EC cell tumours, (not examined in the present study) may include proliferation of smooth muscle tissue within the tumour stroma or peritumour gut wall [8, 37] and elastic sclerosis of medium size mesenteric arteries and veins [2, 43]. The proliferation of smooth muscle cells within the tumours and in the extratumoural muscularis mucosae of stomachs harbouring gastric ECL cell carcinoids has been well documented by Bordi et al. [8]. In gastric tumours, the proliferative response of smooth muscle cells has been supposed to be induced by bFGF. Since aFGF is known to be a potent mitogen for smooth muscle cells [10], like bFGF, the hypertrophy of the muscularis propria underlying the carcinoid tumours of the small intestine [37] and contributing to the mechanism of small bowel obstruction might be induced by this growth factor. Our preliminary unpublished observations (A.M. Chiaravalli, S. La Rosa, C. Capella, 1996) showing the presence of a significant component of α-smooth muscle actin immunoreactive cells within the stroma of 10 out of 13 aFGF-positive EC cell tumours, as against 3 out of 11 aFGF-negative non EC cell gastrointestinal endocrine tumours, further support the hypothesis of an involvment of aFGF in smooth muscle proliferation.

A possible role of aFGF in the pathogenesis of characteristic obliterative elastic sclerosis of peritumour and mesenteric blood vessels associated with EC cell carcinoids [2, 43] appears questionable, since the effect of aFGF on the production of elastic fibers is not known and experimental studies have shown that aFGF promotes vascular repair and inhibits intimal thickening in response to intravascular damage [6].

The possible functional role of aFGF produced by tumour cells in the proliferation of stromal fibroblasts and smooth muscle cells has to be substantiated by the demonstration of aFGF receptors in the target mesenchymal cells. In this context, it is interesting to recall that aFGF is synthesized as a cytosolic protein without a signal sequence, but it can be exported from cells by an unknown mechanism [15]. The biological activity of aFGF is mediated by high affinity transmembrane receptors consisting of an intracellular tyrosine kinase domain and an extracellular binding domain with immunoglobulin loop-like motifs [24]. The role of aFGF relative to other fibroblast stimulating factors, such as IGF-I, PDGF, TGFα, TGFβ, and their possible interactions remain to be established.

In addition to or as an alternative to paracrine stimulation of tumour stromal cells as an explanation, a possible regulatory effect of aFGF on normal and neoplastic EC cell function might be suggested. Although no data are available on the effect of aFGF on EC cells, other cellular models indicate that aFGF may be an autocrine cell regulator. In this context, it is interesting to recall that aFGF is an intrinsic component of retinal photoreceptor cells, where it has been localized by both immunocytochemical and in situ hybridization techniques [34]. In addition, rod outer segments contain specific receptors for aFGF, as has been demonstrated by Mascarelli et al. [34]. aFGF has also been shown to enhance rhodopsin synthesis in newborn rat photoreceptor cells in vitro. All these findings seem to indicate that aFGF may have an important role in photoreceptor physiology. A similar regulating role seems to be exerted by aFGF on pancreatic acini, where is known to stimulate amylase release [11].

Recent immunohistochemical studies have demonstrated much more intense expression of aFGF in malignant tumours than in normal tissues. Elevated intracellu-

lar aFGF immunoreactivity has been reported in brain tumours [36, 47]. In carcinomas, aFGF is overexpressed in pancreatic carcinoma [50], oral carcinoma [39], and salivary gland carcinoma [40]. In certain types of malignancies the overexpression of aFGF seems to correlate with advanced tumour stages [50]. At variance with these findings, aFGF immunoreactivity of EC cell tumours is not related to malignancy, being present both in benign appendiceal tumours, and in malignant ileal and colonic tumours. It is not overexpressed in neoplastic EC cells relative to normal EC cells.

In conclusion, our results show that aFGF is expressed in a fraction of normal gastrointestinal serotonin producing EC cells; is constantly expressed by gut serotonin-producing endocrine tumours and should therefore be considered a marker for gut EC cell tumours (argentaffin carcinoids), being useful for differentiation of these tumours from other functional types of gastrointestinal endocrine tumours; and may be involved, in conjuction with other growth factor peptides, in the stromal fibroblast reaction frequently found in these endocrine neoplasms.

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