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POST-TRANSLATIONAL PROCESSING OF GASTRIN IN NEOPLASTIC HUMAN COLONIC TISSUES

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Gastrin has been postulated to stimulate proliferation in colorectal neoplasms. Although gastrin mRNA has been demonstrated to be present in colon cancer cell lines, the intact peptide had not been recovered from human colorectal neoplasms.

We demonstrate that gastrin and its precursors are present in both colorectal neoplasia and adjacent normal-appearing colonic mucosa. In colonic tissue, the glycine-extended precursor form of the peptide is over 10-fold more abundant than the amidated gastrin, and progastrin is more than 700-fold more abundant. In contrast, amidated gastrin in the human antrum is the predominant form of gastrin by a factor of 10. Furthermore, the ratio of gastrin precursors to gastrin is significantly increased in neoplastic colonic mucosa when compared with normal colonic tissue. These data suggest that the processing of gastrin is unique in the human colon and that further differences in processing occur in neoplastic colonic tissue.

Colorectal carcinoma is the second most common cancer in the US, accounting for 156,000 new patients and 58,000 deaths per year.(1) Colorectal carcinoma has an overall survival rate of 50 to 60% at 5 years with current treatment modalities.(2) A recurrent theme found in the study of proliferation of cancers, including breast, thyroid, and prostate carcinomas, is the role of peptides in the promotion of tumor growth.(3) Hormonal manipulation of these tumors has led to significant advances in diagnosis, treatment, and patient outcome. Colon cancer may well be added to this list, as gastrin has been postulated to play a role in the promotion of the growth of colorectal carcinoma *in vivo* via an autocrine mechanism.(4) In fact, serum gastrin levels have been shown to be elevated in some patients with colonic adenomas and carcinomas.(5,6) The return of gastrin levels to the normal range has been reported following resection of these neoplasms.(7,8) Despite this observation, others have examined neoplastic tissue resected at surgery and have been unable to detect gastrin.(9)

Gastrin, a peptide found predominantly in gastric antrum, has multiple effects on the gastrointestinal (GI) tract. These effects include stimulation of parietal cell growth, acid secretion, and trophism for the GI tract during various stages of development.(10) Gastrin is synthesized as a pre-prohormone which requires post-translational modification for its biological activation. The pre-prohormone undergoes several processing steps to yield a carboxyl-terminal glycine-extended precursor, as outlined in Figure 1.(11) The glycine-extended intermediate then undergoes carboxyamidation to yield the amidated, biologically active, hormone. Amidated gastrin itself may exist in various molecular forms in tissue and in serum. G-14, G-17, G-34 and other forms sharing a common amidated carboxyl-terminus have been identified. All circulating forms are roughly equipotent, on a molar basis, for stimulation of gastric acid secretion.

Gastrin has been demonstrated to stimulate the cellular proliferation of several colonic tumor models in a specific manner. Mouse and human colon cancer cell lines and rat colorectal cancer models demonstrate increased proliferation in response to exogenous gastrin, to have

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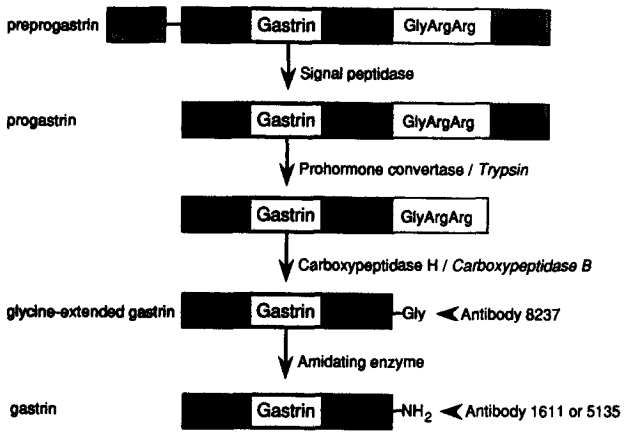

Figure 1. Processing schema for progastrin and antibody specificity for antibodies 1611, 5135, and 8237, as described in the text. Gastrin refers to all molecular forms of gastrin, including sulfated forms, such as G-14, G-17, G-17S, G-34, and G-34S. *In vivo* progastrin undergoes proteolytic cleavage by prohormone convertase and carboxypeptidase H to yield glycine-extended gastrin (G-gly). *In vitro* progastrin is cleaved by trypsin and carboxypeptidase B to yield G-gly. The amidating enzyme cleaves the carboxyl-terminal glycine and replaces it with an amide group to yield the biologically active form of gastrin.

gastrin receptors or binding sites, and the proliferative effects of gastrin are reversed when gastrin-receptor antagonists are applied (12,13,14,15,16).

The source and the role of gastrin in colonic neoplasia is controversial. It has been postulated that gastrin has an autocrine role in colorectal neoplasia, although identification of the intact peptide or its precursors has not been reported in human tissue. Identification of gastrin may be difficult due to multiple factors, including low quantity of processed peptide and assay insensitivity. We hypothesize that gastrin processing may be altered in neoplastic tissue. Our purpose in undertaking this series of experiments was to identify gastrin and its precursors in human neoplastic and non-neoplastic colonic tissue.

Methods

Tissue Collection:

Matched paired samples of tumor and normal-appearing adjacent colonic mucosa were obtained from 15 patients undergoing resection for colorectal carcinoma. Tissue was collected, colonic mucosa was bluntly dissected away from submucosa, and quickly frozen and stored at -70° C.

Peptide Extraction:

Tissue samples were weighed and placed while still frozen into boiling double-distilled water (10 ml/g). The tissues were boiled for ten minutes, homogenized using a high-speed mechanical homogenizer (Polytron, Brinkman, Switzerland), and boiled a second time to insure inactivation of proteolytic enzymes. The extracts were then centrifuged at 30,000G at 4°C for 30 minutes. The supernatant was saved and stored at -70°C until needed.

Radioimmunoassay:

Radioimmunoassays specific for gastrin, glycine-extended gastrin (G-gly), and progastrin were performed as previously described utilizing antibodies 1611, 5135, and 8237, as illustrated in Figure 1 (17,18,19).

For gastrin assay we utilized antibodies 1611 and 5135, generous gifts from Dr. John Walsh (Los Angeles, California). Antibody 5135 is specific for the carboxyl-terminus of the amidated forms of gastrin, cross-reacts 100% with amidated CCK, and <1% with glycine-extended forms of peptides. For confirmation of gastrin immunoreactivity, we utilized antibody 1611, which is specific for the carboxyl-terminus of the amidated forms of gastrin and cross-reacts <1% with CCK and glycine-extended forms of gastrin.

Glycine-extended gastrin was assayed utilizing antibody 8237 which is specific for gastrin and cholecystokinin peptides with carboxyl-terminal glycine extensions but cross-reacts <1% with amidated peptides or peptides with extensions beyond the glycine residue.

Progastrin content was determined using a modified trypsin/carboxypeptidase B reaction coupled with the measurement of G-gly immunoreactivity as described by Rehfeld.(20,21) Briefly, G-gly immunoreactivity was first determined in an aliquot of the sample. 200 µl of the sample were added to 300 µl of phosphate buffer at pH 7.5 and then digested in 500 µl of a trypsin (Sigma, St. Louis, Missouri) solution (100µg/ml) for 30 minutes at room temperature. The sample was boiled for 10 minutes to inactivate the trypsin, and centrifuged for 5 minutes at 1500 RPM at 4° C. 250 µl of the supernatant were placed in 650 µl of the phosphate buffer and 100 µl of carboxypeptidase B (Sigma, St. Louis, Missouri) (750 units/ml) were added for a 30 minute incubation. The reaction was stopped by boiling for 10 minutes, and the samples were frozen at -70°C until assayed with antibody 8237 for G-gly immunoreactivity.

Statistical Analysis:

Determination of means, standard errors, and t-test were performed utilizing Statworks software on a Macintosh SE.

Results

Identification of Gastrin and Gastrin Precursors

We first characterized gastrin content and its processing intermediates in fifteen matched pairs of colonic tissues obtained from patients who had undergone surgical resection for colorectal neoplasia. Samples were obtained from the tumors and from normal-appearing colonic mucosa at least 5 centimeters from the tumor. Individual samples ranged in size from 400 to 2000 mg. Six cancers were right-sided, 6 were left-sided and 3 were rectal adenocarcinomas. No tumors were Dukes A, 1 was Dukes B1 (extending into the muscularis propria), 5 were Dukes B2 (extending through the serosa), 2 were Dukes B3 (involving contiguous structures), 4 were Dukes C1 (≤ 4 regional lymph nodes involved), 2 were Dukes C2 (> 4 regional lymph nodes involved) and 1 was Dukes D (distant metastasis).

As depicted in Table 1, significantly higher concentrations of amidated gastrin were found in the normal appearing mucosa (212±41.8 fmol/g) when compared to the neoplastic mucosa (151±23.5 fmol/g) (p=0.035). Antibody 1611, specific for amidated gastrin, confirmed the presence of amidated gastrin. Neoplastic mucosa contained significantly greater amounts of glycine-extended gastrin (2,710±251 fmol/g) when compared to the normal appearing mucosa (1,820±219 fmol/g) (p<0.001). Differences between progastrin content in the normal colonic tissue (103,000±28,900 fmol/g) and in the neoplastic tissue (124,000±25,000 fmol/g) did not reach statistical significance (p=0.12).

Of particular interest is the relative ratio of glycine-extended gastrin to amidated gastrin, shown in Table 2. The ratio is significantly greater in the neoplastic tissue (27.1±7.78) than in the normal-appearing mucosa (11.7±1.84) (p=0.007). The progastrin to gastrin ratio approached significance (p=0.067) when the normal mucosa was compared to the neoplastic mucosa. The ratios of progastrin to G-gly were not significantly different (p=0.17) when the normal appearing mucosa was compared to the neoplastic tissue.

No relationship between either gastrin or precursor content and location of tumor, Dukes stage, or size of primary tumor were detected during analysis.

Table 1. Gastrin, G-gly, and progastrin content in colonic tissues

	normal colon	carcinoma	p-value (normal vs. cancer)
Gastrin	213±41.8	151±23.5	p=0.035
G-gly	1,820±219	2,700±251	p<0.001
Progastrin	103,000±28,900	124,000±25,000	p=0.12

Note: Results expressed as mean ± S.E.M., fmol/g wet tissue, n=15 matched pairs.

Table 2. Gastrin, G-gly, and progastrin ratios in colonic tissues

	normal colon	carcinoma	p-value (normal vs. cancer)
G-gly/gastrin	11.7±1.84	27.1±7.78	p=0.007
Progastrin/gastrin	706±211	1,390±570	p=0.067
Progastrin/G-gly	73.3±28.4	48.4±9.21	p=0.17

Note: Results expressed as mean ± S.E.M., fmol/g wet tissue, n=15 matched pairs.

Discussion

Although gastrin had been hypothesized to have a proliferative autocrine role in colorectal neoplasia, gastrin has not been previously detected in neoplastic tissue. Our studies are the first to quantitate gastrin and its precursors in human colonic neoplastic and non-neoplastic colonic mucosa.

The presence of gastrin messenger RNA has been reported in colonic neoplastic tissue and colon cancer cell lines without reported recovery of the gastrin peptide.(22,23) The antibodies utilized in our assay allow for the accurate and specific identification and separation of the amidated forms of gastrin and the glycine-extended precursors. The difficulties in detecting the peptide in other studies are attributable to assay sensitivity and specificity; antibody 1611 is specific for amidated gastrin, though specific antibodies for glycine-extended gastrin are not available, allowing for the possibility of cross-reactivity with precursor forms of CCK when antibody 8237 is utilized. Additionally, in a colonic tissue specimen, increased quantities of gastrin precursors were found in relation to processed peptide, possibly indicating altered peptide processing in the colon.(24) Prior studies did not include methods to detect precursor forms of gastrin, which we have shown to be the predominant forms found in human colonic tissue.

A comparison of the gastrin and gastrin precursor content between colonic and antral tissues revealed important differences. Antral tissues contained larger amounts of amidated gastrin, in the range of 860 pmol/g tissue, while the normal-appearing colonic tissue contained lesser amounts of gastrin.(25) In contrast to the G-gly/gastrin ratio in these colonic tissues, normal antral mucosa and cultured endocrine cells contained significantly larger amounts of amidated forms of gastrin when compared to the glycine-extended precursors.(25) Colonic tissues contain gastrin precursors in a greatly increased amount, indicating that the colon processes gastrin through an altered mechanism. The G-cell is the source of gastrin in the human antrum, though the cellular source of the gastrin peptides in the human colon is unknown.

Differences also exist between neoplastic and non-neoplastic colonic tissues. Gastrin content as measured by RIA is slightly, but significantly, decreased in neoplastic tissues compared to paired non-neoplastic tissues. Glycine-extended forms of gastrin are significantly higher in cancer compared with the normal-appearing adjacent colon, and the neoplastic tissues have an increased G-gly/gastrin ratio. Cancer-related changes in the expression or activities of the peptide processing enzymes are likely to account for these differences. Tumor heterogeneity exists in the colonic tissues that we examined, in a manner analogous to gastrinomas examined by our group.(25) This biological variation may help explain the wide range of serum levels of gastrin found in patients with colorectal neoplasia.

The sequence of the gastrin gene, as found in several tumor cell lines, does not differ from the normal human gastrin gene sequence.(23,24) It is possible that regulation of transcription or differential post-translational processing enzyme activity may account for variations in the expression of amidated gastrin. Although these mechanisms were not directly addressed in this study, they may also explain the differences in quantities of gastrin and its precursors present in the colon when compared with the antrum, as regional variability in the activity of enzymes involved in the processing of peptides may occur. Additionally, the difference in the tissue ratios of gastrin/gastrin precursors in colorectal neoplasms is most likely representative of altered post-translational modification of gastrin. The elevated level of gastrin precursors found in the presence of the amidated peptide suggests that multiple enzymes, including the carboxypeptidase and amidating enzymes, as illustrated in Figure 1, are not fully functional, that quantities of the enzymes are limited, or that precursors are abnormally trafficked

within the cells and bypass the processing enzymes. In preliminary studies we have used fast protein liquid chromatography to attempt to characterize the molecular forms of gastrin present and have found forms that co-elute with known forms of gastrin and G-gly.

In nude mice with tumor explants from human colon cancer cell lines and in murine colon cancer cell lines, gastrin receptors have been demonstrated and gastrin antagonists have been shown to inhibit the proliferative response to gastrin and confer a survival advantage for the mice.(25,26) In an analogous manner, we speculate that gastrin and/or its processing intermediates may be involved in the proliferation of some colonic neoplasms via an autocrine mechanism. Pharmacological manipulation of these tumors via receptor antagonists or peptide binding antibodies may lead to advances in therapy for colorectal neoplasia. Further studies are underway to elucidate the mechanism by which gastrin and/or its precursors are involved in colorectal neoplastic proliferation. Additional studies are needed to determine if a carboxyamidation enzyme defect is present and to localize the site of peptide production and receptors at a cellular level within the colon.

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