Identification of progastrin derived peptides in colorectal carcinoma extracts

J Nemeth,* B Taylor, S Pauwels, A Varro, G J Dockray

Abstract
The possible production of gastrin by colorectal carcinomas has been studied. Extracts of 44 tumours and adjacent macroscopically normal tissue were examined in radioimmunoassay using the following antibodies: (i) L289 raised to a C-terminal fragment of progastrin; (ii) L6W60 raised to a C-terminal fragment of progastrin; (iii) 109–21 raised to, and reacts with, Gly-extended forms of heptadecapeptide gastrin, that is, biosynthetic intermediates on the pathway producing active gastrin; and (iv) L2 which reacts with amidated, biologically active gastrins. All samples contained detectable material in assays using L6W60; in general, concentrations measured with this antibody were higher than with the other antibodies, and in particular there were higher concentrations in tumour compared with normal tissue extracts. Tumour extracts also contained higher concentrations of immunoreactivity compared with normal tissue, in assays using antibodies L289 and 109–21. In contrast, amidated gastrins were found in similar concentrations in tumour and normal tissue, and concentrations were the lowest of those recorded in the four assays. Separation on Sephadex G50 revealed peaks compatible with progastrin and its C-terminal flanking peptide, and two other peaks that are so far unidentified. In conclusion most colorectal carcinomas contain peptides derived from the gastrin precursor, progastrin, but for the most part these tumours do not convert progastrin into biologically active products.

Methods

PEPTIDES
The C-terminal pentadecapeptide of human progastrin ie preprogastrin 87–101 was obtained from UCB Products (YGWMDFGRRSAEDEN, in the single letter notation); the C-terminal decapeptide of gastrin with Tyr in the first position — that is, (Tyr26) preprogastrin 92–101 (YGRSSAEDEN), was obtained from Multiple Peptide Systems (San Diego, CA, USA); Gly-extended C-terminal octapeptide fragment of G17 ie preprogastrin 86–93 (AYGWMDFG), was a gift from Dr J Walsh. Other human progastrin fragments were obtained from Peninsula Ltd. Human G17 was a gift from the late Professor R A Gregory, and natural human progastrin was isolated from a gastrinoma as previously described.

PATIENTS AND TUMOURS
An unselected, but approximately consecutive, series of 44 patients was studied presenting with colorectal cancers between June 1989 and November 1990 to the University Department of Surgery in Liverpool. All patients had sporadic tumours which were managed electively. Patients who presented as emergencies were excluded, as were patients treated during the same period of time with cancers arising in a...
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**Figure 1:** Schematic representation of human preprogastrin and its major products showing the specificity of antibodies used in the present study. The C-terminal region is shown in an expanded form. Cleavage at the Arg residues by endopeptidase and carboxypeptidase generates Gly-extended gastrin intermediates and the C-terminal tryptic peptide fragment. The Gly-extended intermediate is converted to amidated active products. Antibody L289 reacts with C-terminal fragments of progastrin longer than eight residues, these include intact progastrin, but exclude the C-terminal tryptic peptide. The latter reacts with LW60 which also reveals intact progastrin, MAb 109–21, and L2 react with Gly extended and amidated peptides respectively.

**TABLE I**

<table>
<thead>
<tr>
<th>Operation</th>
<th>%</th>
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</thead>
<tbody>
<tr>
<td>Abdominoperineal excision of rectum</td>
<td>12.5</td>
</tr>
<tr>
<td>Anterior resection of rectum</td>
<td>22.5</td>
</tr>
<tr>
<td>Hartman’s resection of rectum</td>
<td>7.5</td>
</tr>
<tr>
<td>Sigmoid colectomy</td>
<td>22.5</td>
</tr>
<tr>
<td>Left hemicolectomy</td>
<td>5.0</td>
</tr>
<tr>
<td>Transverse colectomy</td>
<td>5.0</td>
</tr>
<tr>
<td>Right, or extended right colectomy</td>
<td>25.0</td>
</tr>
</tbody>
</table>

**TABLE II** Characteristics of antibodies used in radioimmunoassay in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Specificity</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>L289</td>
<td>h prog 87–101*</td>
<td>C-terminus gastrin &gt;8 residues</td>
<td>0.4 pmol/g</td>
</tr>
<tr>
<td>LW60</td>
<td>h prog 87–101*</td>
<td>C-terminus gastrin &gt;5 residues</td>
<td>1.0 pmol/g</td>
</tr>
<tr>
<td>109–21</td>
<td>Gly-extended G6</td>
<td>Gly-Gly amidated gastrins</td>
<td>0.1 pmol/g</td>
</tr>
<tr>
<td>L2</td>
<td>G17</td>
<td>C-terminal amidated gastrins</td>
<td>0.1 pmol/g</td>
</tr>
</tbody>
</table>

*Human progastrin C-terminal pentadecapeptide.

**TABLE III** Specificity of antibody LW60*

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Relative immunochemical potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preprogastrin 22–101</td>
<td>1.0</td>
</tr>
<tr>
<td>YGWMDGIRRSAEDEN</td>
<td>0.8</td>
</tr>
<tr>
<td>YGRRSAEDEN</td>
<td>0.77</td>
</tr>
<tr>
<td>RSAEDEN</td>
<td>0.46</td>
</tr>
<tr>
<td>SAEDEN</td>
<td>0.53</td>
</tr>
</tbody>
</table>

*The relative potency of various peptides in inhibiting binding of label to antibody LW60 is shown. The concentration of peptide required for inhibiting binding of label by 50% was divided by that of progastrin. Peptide sequences are given in the single letter notation.
coupled to bovine thyroglobulin using glutaraldehyde as previously described.10

In radioimmunoassay, antibody LW60 was used at a dilution of 1:65 000 with 2000 cpm of

¹I labelled human progastrin (Tyr⁴ 92–101). Assays were incubated in 1·0 ml 0·02 M sodium
barbitone buffer pH 8·4 containing 0·05% w/v sodium azide and 0·75% v/v Bovumin (Ortho-
diagnostics), for 48 hours at 4°C. Antibody bound and free label were separated by addition
of 100 μl of a suspension of charcoal:dextran:fat free milk powder (10:1·0:5 g in 100 ml distilled
water) and centrifugation at 4°C for 10 minutes.

The specificity of assays with LW60 was established from comparison of the potency of a range of progastrin derived peptides and analogues in inhibiting binding of label (Table III). Peptides longer than the C-terminal hexapeptide were virtually equipotent in inhibiting binding. The standard used routinely was progastrin 93–101. In 10 consecutive assays the mean (SE) of the concentration of standard required for 50% inhibition of binding was 28-0 (1·6) pmol/l. All assays routinely included tubes to assess non-specific binding which was typically less than 5% of total counts. Non-specific binding of all tissue extracts was also determined by including tubes containing extract but no antibody – again non-specific binding was typically less than 5% total counts.

GEL FILTRATION
Samples of selected tumours and control tissue were fractionated on Sephadex G50 columns
(1×95 cm) eluted with 0·05 M ammonium bicarbonate.

STATISTICAL ANALYSIS
Where appropriate the results are presented as means (SE). Comparisons between groups were
made by Student’s t test, or Wilcoxon’s matched pairs test.

Results
PLASMA GASTRIN
In the group as a whole the concentrations of amidated gastrin in fasting plasma were 10·8
(2·9) pmol/l which was well within the normal range for this assay (<30 pM). Five of the 44
patients, however, had raised plasma gastrin concentrations (34, 36, 47, 83, and 84 pM).

TUMOUR GASTRINS
Tissue extracts from all 44 patients contained immunoreactive material detected in assays
using LW60 (Fig 2). The concentrations of immunoreactivity in the tumour extracts (7·53
(0·69) pmol/g) were significantly higher than in adjoining apparently normal colon (3·99 (0·42); t
test, p<0·001). In assays using antibody L289, specific for human progastrin derived peptides
extending beyond the C-terminal region, there were significantly higher concentrations
(p<0·001, Wilcoxon) of immunoreactive material in tumour extracts (1·54 (0·2) pmol/g, in

40 of 44 patients) compared with control samples (of which only nine of 44 contained measurable
activity, Figure 3). The mean concentrations of L289 immunoreactivity in the tumour extracts
were about 20% those detected with LW60. The antibody for the Gly-extended intermediate
showed detectable material in 20 of 44 tumours compared with seven normal samples and again
concentrations were higher in the former than the latter (p<0·001, Wilcoxon) (Fig 4). Finally,
amidated gastrins occurred in detectable amounts in a similar proportion of the tumour
(19 of 44) and control (25 of 44) extracts and mean concentrations in these extracts were also
similar (about 1 pmol/g) (Fig 5).

Figure 2: Scattergram of the distribution of immunoreactive material measured by antibody LW60 in 44 extracts of tumour and the corresponding normal colon.

Figure 3: As Figure 2, showing data obtained using antibody L289. Note that only nine of 44 samples of normal colon mucosa contained detectable immunoreactivity compared with 40 of 44 tumour extracts.
There were no differences in the concentrations of progastrin derived peptides measured by the various antibodies in tumours taken from rectum compared with sigmoid colon (which were the two largest subgroups). In addition, there was no correlation between concentrations measured in any of the four assays and the Dukes' stage of the tumour.

GEL FILTRATION
The samples with the highest concentrations of immunoreactivity in assays with LW60 and L289 were fractionation on Sephadex G50 (Fig 6). The pattern of immunoreactivity with LW60 was variable. A peak corresponding to the C-terminal tryptic fragment of progastrin was consistently identified. In addition, however, there were also peaks of variable proportion that eluted earlier. We have previously seen these peaks in material derived from human antrum where they are generally less than 5% of the total. The amounts of material so far available have been insufficient for full chemical characterisation but we have tentatively identified them as corresponding to G34 and G17 extended through the C-terminus to include the C-terminal fragment of progastrin. A minor peak corresponding to intact progastrin was also identified in assays using LW60. In addition L289 reacted with material that emerged in the characteristic position of progastrin. Unexpectedly, however, L289 also reacted with a further peak of material that has not so far been identified in antral extracts and that emerged relatively late and did not correspond to one of the LW60 peaks. Assays with L2 and 109–21 showed small peaks that had the chromatographic properties of G17 and its Gly-extended variant, respectively (not shown).

Discussion
The main finding of this study is that virtually all
the colorectal cancers studied contained low, but detectable, concentrations of progastrin derived peptides. The same spectrum of peptides was also found in extracts of normal colon mucosa but in still lower concentrations. For the most part, the biologically active forms of gastrin – that is, C-terminally amidated peptides, were found in much lower concentrations than other progastrin derived peptides. Taking these observations together with other data, we conclude that the gastrin gene is likely to be expressed in many colorectal cancers, although the capacity to process progastrin to a biologically active product seems to be poorly developed.

The present study has made use of antibodies that react with progastrin itself, its major active products, Gly-extended biosynthetic intermediates, and its C-terminal tryptic fragment. There are three major steps in the production of amidated gastrins from progastrin: endopeptidase cleavage, carboxypeptidase trimming of Arg residues and conversion of Gly-extended to amidated products. In normal G-cells of the antrum there are approximately similar concentrations of amidated gastrins and the C-terminal progastrin fragment; concentrations of progastrin and of Gly-extended gastrins are about 10% those of the cleaved C-terminal fragment. Assays using antibodies L289 and LW60 provide the capacity to detect intact progastrin (without any processing) or the fragment produced after the first endopeptidase cleavage. This distinction is crucial in the present context, because it appears that material resulting from endopeptidase cleavage is rather commonly encountered whereas material generated at the later stages (Gly-extended and amidated products) is less common. In assays using L289 and LW60, immunoreactive material was more commonly found in tumours than control tissue. We conclude therefore that progastrin is frequently produced by colorectal cancers but the phenotype for processing to amidated products is usually not well developed. Although progastrin derived peptides were more abundant in tumour than control extracts, there were nevertheless detectable amounts of activity in all normal samples using assays with LW60, and in a high proportion of assays of normal samples using antibody L2. It seems possible that this material originates from rare mucosal endocrine cells expressing the gastrin gene. We are presently undertaking further studies using the same panel of antibodies in an attempt to localise each of the precursor antibodies in sections from both normal mucosa and tumours.

Patients with colorectal cancer are reported to have postprandial hypergastrinaemia which returns to normal after tumour resection. Wong et al. consider that the raised gastrin originates in the antrum rather than the tumour. In the present study, basal plasma gastrin concentrations in the group as a whole were within the normal range, although there was a subset in which basal gastrin was raised. This accords with work of others, although the hypergastrinaemic subgroup in our study was a smaller proportion of the total than previously reported (11%, compared with 36–40%). Nevertheless, it seems that colorectal tumours are unlikely to make a consistently important contribution to circulating plasma gastrin, although raised postprandial gastrin of antral origin may be significant as a mitogenic factor in promoting tumour growth.

In peptide producing endocrine cells the main secretory products pass from the Golgi to the cell exterior through the so called regulated route – that is, in secretory granules that are released by exocytosis on stimulation. The post translational processing mechanisms that convert progastrin to amidated peptides are known to operate at the post Golgi level on this pathway. An alternative secretory pathway, the constitutive route, takes material direct from the Golgi to the cell surface, is present in all cells, and is unregulated in the sense that exocytosis is not controlled by external factors. The cellular machinery of the regulated pathway and associated processing enzymes may be poorly represented in colorectal cancer cells, and consequently the apparent failure to process progastrin derived peptides in these tumours is not surprising. The signal sequence of progastrin contains an Arg-Gly-Asp (RGD) sequence which, in many cases, is sufficient to ensure sequestration into the cisternal space of the endoplasmic reticulum so that the peptides we have measured are likely to be localised to the secretory pathway, most probably taking the constitutive route to the exterior. The generally rather low concentrations of material measured in our extracts (compared with antral extracts) are consistent with material passing direct to the cell surface without residence in storage granules.

The potential autocrine growth effects of gastrin in colorectal cancer have previously been studied using cell lines. Our data suggest that the biologically active amidated peptides that are potential mediators of these actions cannot be more than a small proportion of the total progastrin produced. It would appear, however, that some tumours contain more active product than others; thus autocrine growth promoting effects that might be attributable to gastrin are in any case only likely to be a property of a subset of tumours.

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