Enteroglucagon and experimental intestinal carcinogenesis in the rat

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SUMMARY To assess the association between the putative intestinal trophic hormone enteroglucagon and the development of intestinal tumours, four groups of 20 rats underwent either jejunal transection or 20%, 50%, or 80% proximal small bowel resection. Tumours were induced with azoxymethane 10 mg/kg weekly for 12 weeks. At 26 weeks there was a promotion of colonic neoplasia from a median of 0.5 (range 0–3) per rat in the transection group to 1.0 (0–3) in the 50% resected group (p<0.01) but no significant promotion in the 80% resection group. In the small bowel, increasing resection resulted in a progressive promotion of tumours from a median of 1.0 (range 0–3) per rat in the transection group to 2.0 (0–5) in the 50% resection group (p<0.001) and 3.0 (0–11) in the 80% group (p<0.01). Plasma enteroglucagon was measured at 2, 16, and 26 weeks and was raised seven-fold in the 80% resected group (p<0.001). There was a significant correlation between enteroglucagon concentrations and number of duodenal tumours but not colonic tumours. Crypt cell production rate in the duodenum increased from 11.5±1.9 to 29.2±1.4 cells/crypt/h in the 80% resected group (p<0.001) and showed a close correlation with both enteroglucagon levels and tumour promotion in the small bowel. There were no changes in crypt cell production rate in the colon with resection. This study shows a close association between enteroglucagon concentrations, promotion of tumours and crypt cell production rate in the duodenum but not in the colon.

Small bowel resection promotes the development of colonic tumours in carcinogen treated animals and causes a moderate hyperplastic response in the colon. As hyperplasia is a feature of some premalignant lesions of the colon, promotion of tumours by intestinal resection has been attributed to this response. Hyperplasia, at least in the small intestine, is in part under hormonal control and enteroglucagon is the favoured candidate hormone mediating this response. Changes in trophic hormones may play a role in the promotion of colonic cancer. The aim of this study is to assess any association between enteroglucagon, intestinal cell proliferation, and the promotion of tumours induced by small bowel resection in an experimental rat model.

Methods

ANIMALS Eighty male Wistar rats weighing between 200 and 240 g were housed in groups of four in animal quarters with a 12 hour day/night cycle and were fed a standard pelleted rat food (Labsure, Poole, Dorset) with water ad libitum. Animals were randomly allocated to four groups. Group 1 underwent jejunal transection and reanastomosis alone and group 2 to 4 underwent 20%, 50%, and 80% proximal small bowel resection as measured from ligament of Treitz to ileocaecal junction. All operations were done under inhalational anaesthesia with 50% O₂/50% N₂O plus 3% halothane. Black silk interrupted sutures (5/0) were used for all anastomoses. One week after recovery from surgery all animals received the first of 12 weekly subcutaneous injections of azoxymethane 10 mg/kg (Ash Stevens, Detroit, USA). Rats were weighed weekly and weight gain expressed by subtracting the starting...
weight of each animal from all subsequent weights to accentuate any differences between the groups. Animals were examined daily and were killed when moribund according to previously defined criteria\textsuperscript{14} or at the end of the study at 26 weeks. A full post mortem examination was carried out. The stomach, small bowel, caecum, and colon were dissected free, opened longitudinally, washed in saline and blotted dry. Site, size, and luminal appearance of all tumours were recorded and tumours with their adjacent normal bowel were fixed in 10% formalin in phosphate buffered saline before preparation for histological examination. Samples of duodenum, terminal ileum, caecum, and descending colon were taken for estimation of crypt cell production rate.

**CRYPT CELL PRODUCTION RATE (CCPR)**

A thymomokinetic technique was used to assess the rate of cell proliferation in the crypts of Lieberkuhn.\textsuperscript{15,16} At 0800 on the morning of being killed each animal received an intraperitoneal injection of vincristine 1 mg/kg (Oncovin, Eli Lilly, Basingstoke). Rats were killed at intervals from 30 to 180 mins after injection. Samples of bowel were fixed in Carnoy’s fixative for four hours and stored in 70% ethanol. Tissues were bulk stained by the Feulgen reaction and individual crypts microdissected. The number of metaphase arrested cells in 10 intact crypts for each site in each animal was counted and CCPR was derived as the slope of the line produced by least squares linear regression analysis of the mean number of metaphase arrested cells plotted against time.

**HORMONE ASSAYS**

Samples of blood were taken by tail vein puncture under light ether anaesthesia at two and 16 weeks and by cardiac puncture at 26 weeks. Samples were placed in heparinised tubes to which had been added 0·02 ml (400 KIU) apronin (Trasylol) per ml blood. Plasma was separated immediately by centrifugation and samples frozen on dry ice and stored at −20°C pending assay.

Enteroglucagon was measured by a standard radioimmunoassay technique described in full elsewhere.\textsuperscript{17} Two assays were carried out, one with an antibody (R50) which fully crossreacts with glucagon and one with an antibody (RCSS) directed to pancreatic glucagon which shows no crossreaction with glucagon. Enteroglucagon is derived by subtracting the small proportion of pancreatic glucagon from total glucagon immunoreactivity. This combined assay procedure will detect changes of 12 pmol/1 between adjacent plasma samples with 95% confidence.

**HISTOLOGY**

Formalin fixed tissues were embedded in paraffin blocks and multiple 5 μm longitudinal sections were cut through bowel and tumours. Sections were stained with haematoxylin and eosin, and tumours were classified as benign or malignant according to whether there was invasion through the muscularis mucosa.\textsuperscript{18}

**STATISTICAL ANALYSIS**

The Mann-Whitney U test was used to assess differences in tumour number between the four groups except for ear canal tumour data which were analysed by Fisher’s exact test. Enteroglucagon results are expressed as mean ± standard error of the mean (SEM) and significance levels calculated by Student’s unpaired t test. The Spearman rank correlation coefficient was used to assess correlation between entero glucagon levels, CCPR, and tumour numbers.

**Results**

**MORTALITY AND WEIGHT GAIN**

There was no significant difference in operative mortality between the four groups and overall mortality was 10%. Early deaths, related to the development of intestinal tumours, were significantly more common in the 80% resected group (p<0·001, Fisher’s exact test) (Table 1). In groups 1 and 2 there were no early deaths, in group 3 there was one early death caused by intraperitoneal haemorrhage from a duodenal tumour. In group 4, two died from intraperitoneal haemorrhage from duodenal tumours and the remaining eight fulfilled the criteria for early death and were humanely killed. In these animals, the cause was related to large obstructing upper small intestinal or colonic tumours. There were no differences in the rate of weight gain between four and twenty weeks (Fig. 1). Only the 80% resected group lost weight as a result of surgery and this was regained by two weeks. After 20 weeks the 80% resected group lost weight.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Resection 20%</th>
<th>Resection 50%</th>
<th>Resection 80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operative mortality</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Disease related deaths</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>10*</td>
</tr>
<tr>
<td>Number remaining</td>
<td>18</td>
<td>17</td>
<td>17</td>
<td>7</td>
</tr>
</tbody>
</table>

*p<0·001, Fisher’s exact test.
Enteroglucagon and rat gastrointestinal tumours

The animals also developed external ear canal tumours. Histologically, these were papillomas or papillary carcinomas. Only one of 18 rats in the transected group developed these tumours compared with eight out of 17 rats in the 50% group and four of seven surviving animals in the 80% group (p<0.025) (Table 3).

**Enteroglucagon**

There was a seven-fold increase in enteroglucagon concentrations from 53±4.4 pmol/l in the transection group to 358±60 pmol/l in the 80% resection group (p<0.001). This response was present at two weeks and was maintained to the end of the study at

<table>
<thead>
<tr>
<th>Colon</th>
<th>Adenocarcinoma</th>
<th>Adenoma</th>
<th>N/C</th>
<th>Small bowel</th>
<th>Adenocarcinoma</th>
<th>Adenoma</th>
<th>N/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans</td>
<td>8 (67)</td>
<td>4 (33)</td>
<td>0 (0)</td>
<td>15 (88)</td>
<td>2 (12)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>20%</td>
<td>9 (60)</td>
<td>5 (33)</td>
<td>1 (7)</td>
<td>19 (68)</td>
<td>6 (21)</td>
<td>3 (11)</td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>18 (62)</td>
<td>11 (38)</td>
<td>0 (0)</td>
<td>30 (68)</td>
<td>12 (27)</td>
<td>2 (5)</td>
<td></td>
</tr>
<tr>
<td>80%</td>
<td>5 (38.5)</td>
<td>5 (38.5)</td>
<td>3 (23)</td>
<td>43 (70)</td>
<td>9 (15)</td>
<td>9 (15)</td>
<td></td>
</tr>
</tbody>
</table>

N/C=Tumours not accurately classifiable into benign or malignant.
Table 3  Tumours in each site

<table>
<thead>
<tr>
<th></th>
<th>Colon</th>
<th>Duodenum</th>
<th>Ear canal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>Transected</td>
<td>0.5</td>
<td>0-3</td>
<td>1.0</td>
</tr>
<tr>
<td>20% Resected</td>
<td>1.0</td>
<td>0-3</td>
<td>2.0</td>
</tr>
<tr>
<td>50% Resected</td>
<td>1.61</td>
<td>0-3</td>
<td>2.44</td>
</tr>
<tr>
<td>50% Resected Survivors</td>
<td>1.65</td>
<td>0-3</td>
<td>2.53</td>
</tr>
<tr>
<td>80% Resected</td>
<td>0-76</td>
<td>0-2</td>
<td>3.59</td>
</tr>
<tr>
<td>80% Resected Survivors</td>
<td>1-0</td>
<td>0-2</td>
<td>6.00</td>
</tr>
</tbody>
</table>

*p<0.05, †p<0.01, ‡p<0.001 vs transected group.

26 weeks. In the 50% resection group there was a small increase in enteroglucagon from 53.5±4.4 pmol/l in the transection group to 90.5±15.9 pmol/l at 26 weeks (p<0.05). Twenty per cent resection produced no significant changes in enteroglucagon concentrations (Fig. 3). There was a strong positive correlation between enteroglucagon concentrations and the number of duodenal tumours (r_s=0.479, p<0.001) (Fig. 4) but no significant correlation was seen between colonic tumour number and enteroglucagon concentrations (Fig. 5).

**Crypt cell production rate (CCPR)**
Small bowel resection resulted in a progressive rise in CCPR in the duodenum from 11.5±1.9 cells/
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Enteroglucagon and crypt/h in the 80% resected group only the 80% resected group ileum, CCPR 31-7±9-9 tumours denal Fig. 6 colonic tumours. This study treated carcinogen 50% small bowel. In the 80% resected group, a raised CCPR compared with transection from 13-6±2-6 to 31-7±9-9 cells/crypt/h but this was not statistically significant. No changes occurred in CCPR in either the caecum or descending colon after resection (Fig. 6). There was a positive correlation between the increase in duodenal CCPR and number of duodenal tumours (r=0-99, p<0-01) but no correlation was found between caecal or colonic CCPR and colonic tumours.

Discussion

This study confirms previous reports that small bowel resection promotes intestinal neoplasia in carcinogen treated animals.1-4 19 20 In the colon, maximal promotion of neoplasia was achieved with 50% small bowel resection. Eighty per cent resection resulted in fewer tumours than in the 50% group. This may be because there were only seven animals in this group surviving to 26 weeks and the incidence may have been higher if a larger proportion of animals had survived. Further, subtotal resection may result in a degree of malnutrition which may protect against the development of colonic tumours.19 Although rate of weight gain was the same between the four groups from four to 20 weeks, there was a fall in the average weight of the 80% resected group after this time.

In the duodenum, proximal small bowel resection resulted in a progressive promotion of neoplasia with increasing resection (Table 3). This change closely parallels CCPR in this site further confirming the association between hyperplasia and neoplasia previously reported.20 21 Hormonal factors have been implicated in the trophic response of small bowel after resection. For example, luminal factors alone cannot explain the hyperplasia in isolated Thiry-Vella loops after resection.7 In rats connected by vascular parabiosis, small bowel resection of one parabiont is reported as producing hyperplasia of the other.8 The suggestion that enteroglucagon may be important in the control of intestinal cell proliferation comes from several observations. In a patient with an enteroglucagon secreting renal tumour, gross villous hyperplasia was noted, but on resection of this tumour, intestinal morphology returned to normal.22-23 In several studies in the rat, a close correlation has been found between plasma enteroglucagon and the proliferative status of the bowel in isolated Thiry-Vella loops of small bowel.10-13 Finally, partly purified enteroglucagon has been shown to increase the incorporation of 3H-thymidine into DNA of isolated cultured guinea pig ileum.24 While a positive correlation does not prove that there is a causal relationship, it does indicate that such a relationship may exist. We speculate that enteroglucagon may mediate, in part, the hyperplastic response in the duodenum and that, either directly or through this hyperplastic response, may promote duodenal neoplasia. The close association between duodenal tumour number and enteroglucagon does not negate this hypothesis (Fig. 4).

In the colon, small bowel resection has been shown to result in a mild hyperplastic response.5 25-28 This adaptive response, however, is limited and occurs with resections greater than 50% of proximal small bowel or distal resections. We can find only one report of a physiological systemic factor mediating colonic hyperplasia29 and enteroglucagon's role in the regulation of colonic mucosal growth is not known. In pharmacological doses, both gastrin30 and epidermal growth factor31 have been reported to exert a trophic effect on the colon but it is not known whether these agents act physiologically. In this study, a seven-fold rise in enteroglucagon in the 80% resected group did not result in increased crypt cell turnover either in the descending colon or the caecum, which suggests that enteroglucagon probably neither mediates colonic hyperplasia nor is associated with the promotion of colonic tumours by small bowel resection. We could show no changes in rate of cell proliferation in the colon with proximal small bowel resection and therefore no association between proliferative status of the colon and neoplasia. Measurement of CCPR is a less sensitive indicator of cell turnover in the colon than in the small bowel, however, and a small hyperplastic response might therefore not be observed. In addition, any small colonic hyperplastic response may have been masked in this study by the relatively large standard errors at this site, which may be because of individual differences in animals due to

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**Fig. 6** Crypt cell production rate (CCPR) in the four sites. (***p<0.001 vs transection group).
the presence of colonic and duodenal tumours. This study therefore does not refute previous reports associating hyperplasia of the colon with neoplasia in carcinogen treated rats. 4, 20

Tumours of the external ear canal develop from Zymbal’s glands in response to azoxymethane administration. 32 A promotion of ear canal tumours by small bowel resection was observed in the 50 and 80% intestinal resected groups in this study. The promotion of ear canal tumours by resection suggests a systemic factor as a mediator though other effects such as changes in host defence due to the resection may be responsible. While there is a similar pattern between enteroglucagon release and the promotion of ear canal tumours, further studies are required to clarify any possible role.

In conclusion, 80% small bowel resection resulted in a seven-fold rise in enteroglucagon concentrations and this rise closely correlates with both hyperplasia and promotion of tumours in the duodenum. This indicates that hormonal factors may play a role in the development or progression of intestinal tumours. In the colon, on the other hand, there is no evidence from this study that enteroglucagon either mediates hyperplasia or the promotion of tumours.

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