Effects of longterm epidermal growth factor treatment on the normal rat colon

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Abstract
Background—Epidermal growth factor (EGF) exerts trophic effects on the mucosa of damaged and defunctioned colon, but the effects on the normal large bowel wall are not known.

Aims—To investigate the effect of systemic EGF treatment on growth and morphology of normal rat colon.

Methods—Rats were treated with subcutaneous biosynthetic EGF injections of 150 µg/kg/day for 28 days. The weight of the physiological colonic wall layers and the luminal surface area were measured using quantitative morphometric analysis (stereology). The colon was subdivided into proximal and distal parts.

Results—EGF treatment increased the total colon wet weight by 23% compared with controls (p<0.005). The weight increase occurred in the mucosal (33%) and the submucosal layers of the bowel wall (36%) and there was a 69% increase of the total luminal surface area (p=0.001). In the proximal part of colon of EGF rats there was a 68% increase in mucosal weight (p<0.005) accompanied by a 79% increase in the mucosal surface area compared with controls (p<0.005), whereas submucosal and muscularis propria weights were identical. In distal colon, the mucosal weight increased 28% in the EGF group (p<0.005), the mucosal surface area increased by 72% after treatment (p<0.01). Furthermore there was a 34% increase in the weight of submucosa (p<0.001) in the distal colon among EGF rats.

Conclusions—Treatment of rats with EGF has a stimulating role on the mucosa and luminal surface area of the entire functioning colon and a trophic effect on the submucosa of the distal colon.

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Epidermal growth factor (EGF) is a polypeptide chain consisting of 53 amino acids that belongs to an expanding group of growth factor ligands.1 The EGF family and their related receptors are widely distributed in mammalian species and they play an important part in the growth and differentiation of normal, regenerative, and neoplastic tissues.2 EGF is a powerful mitogen and trophic agent in many tissues including the gastrointestinal tract. The EGF effects are believed to be mediated through stimulation of the polyamine synthesis, as EGF upregulates ornithine decarboxylase activity in the epithelial cells.2 3

Human recombinant EGF is now available and treatment potentials have been investigated in various organ systems.2 In experimental studies, systemic treatment with EGF has been shown to attenuate ulceration or other types of experimental damage to the gastrointestinal tract and to accelerate the healing of such lesions.3-5 In the colon, EGF has a protective effect on the mucosa after trinitrobenzenesulphonic acid (TNB) induced colitis in rats6 7 and EGF increases intestinal anastomotic tensile strength in pigs.8 Reports also exist of EGF use in serious clinical conditions; a case of necrotising enteritis and several cases of congenital microvillus atrophy.9-11

Several in vivo growth promoting actions of EGF on the gastrointestinal tract remain unclear. EGF treatment reduces gut atrophy in the defunctioned rat colorectum and during total parenteral nutrition,12-14 but the effects of systemic EGF on normal colon have not been investigated. The aim of this study was to evaluate the influence of four weeks of systemic EGF treatment on the undamaged rat colon. The colonic wall composition and luminal surface area were investigated using modern stereological techniques.15

Methods

Study animals
The study protocol was conducted on 16 male Wistar rats from our own breed approximately eight weeks old and weighing 155–255 grams. The animals were housed as 21°C and fed a standard laboratory diet. All procedures were carried out in accordance with the Danish law on care and use of laboratory animals.

Study design
The animals were randomly allocated into a treatment group receiving human recombinant EGF (Upstate Biotechnology, New York, USA) and a placebo group receiving isotonic saline. Injections were given subcutaneously twice daily and a total dose of 150 µg/kg/day EGF was given in the treatment group. All the procedures were conducted in a blinded fashion.

Tissue sampling
After 28 days of treatment the animals were transcardially perfused under Mebunal anaesthesia at a pressure of 120 mm Hg with
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The author describes the effects of longterm epidermal growth factor (EGF) treatment on rats colon, focusing on the total colon weight and the weight of different layers. The study used a test system involving the administration of EGF or placebo for 28 days, followed by histological analysis to determine the weight of the colon and its layers. The authors used stereological methods to measure the volume fractions (VF) of the colon layers, and the statistical analysis showed no significant differences between the experimental groups.

Results:
The study involved two treatment groups: EGF and placebo. The results showed no significant weight differences between the groups during the experiment. Blood samples were taken from the periorbital plexus in ether anaesthesia after two weeks and in the Mebunal anaesthesia after four weeks. The results of these tests are described elsewhere. A single animal in the placebo group died during the anaesthetic procedure after two weeks.

The total colon weight was calculated using the following formula:

\[ \text{Total colon weight} = \text{WW} = \sum P_{\text{mucosa}} + \text{WW} \]

Where \( P_{\text{mucosa}} \) is the weight of the mucosa and WW is the weight of the wall. The authors used a non-parametric Mann-Whitney two sample test for comparison between groups, and the probability values less than 5% were considered significant. Results are given as median values.

The authors also measured the weight of the colon and its layers using a test line system and projected (Olympus BH-2, Tokyo, Japan) at random onto a grid with regularly arranged points. The number of points hitting the mucosa (\( P_{\text{mucosa}} \)), the submucosa (\( P_{\text{submucosa}} \)), and the muscularis propria (\( P_{\text{muscularis}} \)) were counted in 24 fields of vision selected evenly among the sections from each colon segment. Points hitting the small amounts of serosa, mesenterical fat, or lymphoid tissue were counted separately (\( P_{\text{extra}} \)). The separate counts were totalised to obtain the total number of points (\( P_{\text{total}} \)) for every field, which was used to calculate the VF of, for example, the mucosa:

\[ \text{VF (mucosa)} = \frac{\sum P_{\text{mucosa}}}{\sum P_{\text{total}}} \]

The VF of each layer was multiplied with the wet weight (WW) of corresponding segments to determine the weight of, for example, the mucosal layer:

\[ \text{weight of mucosa:} \text{VF (mucosa)} \times \text{WW} \]

A specific density of all layers of 1.0 g/cm³ was assumed. The WW constitutes the reference volume (weight). The sum of VF in each animal from proximal and distal segments were multiplied by the total colon weight to determine the weight of the wall layers in the entire colon. This procedure was repeated at a higher magnification (×620) on the mucosal layer to differentiate it into weight of epithelium, lamina propria, and muscularis mucosae.

The mucosal surface area was estimated using a frame with a test line system of cycloids and test points (line length per point \( L_p = 14.5 \) cm). Intersections between cycloids and the borderline between the colonocytes of the epithelium and the lamina propria (I) were counted in 24 fields of vision chosen evenly between the four sections from each segment (Fig 1). Test points hitting the mucosal layer were also counted (P) and included in the mucosal surface area calculation:

\[ \text{surface area:} \sum I \times L_p \times V_{\text{ref}} \]

The mucosal weight was calculated previously and constituted the reference volume (weight) \( V_{\text{ref}} \).

Statistics:
The non-parametric Mann-Whitney two sample test was used for comparison between groups, and probability values less than 5% were considered significant. Results are given as median values.

Results:
Animals in both treatment groups thrived throughout the study. There were no weight differences between the groups during the experiment. Blood samples were taken from the periorbital plexus in ether anaesthesia after two weeks and in the Mebunal anaesthesia after four weeks. The results of these tests are described elsewhere. A single animal in the placebo group died during the anaesthetic procedure after two weeks.

Total colon
As the Table shows, EGF treatment increased the median total colon wet weight by 23% compared with the median weight of colon in

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**Table:**

<table>
<thead>
<tr>
<th>Group</th>
<th>EGF (mg)</th>
<th>Placebo (mg)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total colon (mg)</td>
<td>2683 (3162-2415)</td>
<td>2177 (2432-1879)</td>
<td>†</td>
</tr>
<tr>
<td>Distal colon (mg)</td>
<td>1462 (1059-1793)</td>
<td>1570 (1085-1993)</td>
<td>*</td>
</tr>
<tr>
<td>Mucosa (mg)</td>
<td>1361 (1029-1543)</td>
<td>1344 (1060-1160)</td>
<td>*</td>
</tr>
<tr>
<td>Submucosa (mg)</td>
<td>1712 (1572-1898)</td>
<td>1283 (1280-1326)</td>
<td>†</td>
</tr>
<tr>
<td>Muscularis propria (mg)</td>
<td>480 (374-386)</td>
<td>394 (381-407)</td>
<td>0.73</td>
</tr>
<tr>
<td>Luminal surface area (cm²)</td>
<td>557 (435-765)</td>
<td>556 (494-667)</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Median (range). *p<0.05, **p<0.01, and †p<0.005, EGF v placebo.
control rats (p<0.005). The stereological analysis showed that the colonic growth after EGF treatment was caused by a 33% increase in mucosal weight (p<0.005) and a 36% increase in the submucosal weight (p=0.01). The luminal surface area was increased by 69% (p=0.001) in colon from EGF rats compared with controls (Table).

Proximal colon
Colon segments proximal to the major flexure were increased 35% in wet weight compared with controls (p<0.01) (Table). Analysis showed that the weight increase was caused by growth exclusively in the mucosal layer, where the median weight was 68% higher among EGF treated rats (p<0.005) than in controls (Fig 2). This was accompanied by a 79% increase (p<0.005) in luminal surface area (Fig 3). The weight of submucosa and muscularis propria were similar in the two treatment groups in proximal colon (Fig 2).

Quantification of the mucosa (Fig 4) showed a 64% increase in the epithelium layer (p<0.005) and a 64% increase in the weight of lamina propria (p<0.005). The muscularis mucosae layer is very thin in the proximal part of the rat colon, and we found no difference in the weight of this delicate layer between groups.

Distal colon
The median wet weight of distal colon segments was increased by 28% in EGF rats compared with controls (p<0.05) (Table). In these segments the EGF induced mucosal growth was 28% compared with mucosa in controls (p<0.005) (Fig 2), and seemed less prominent than in proximal colon. The mucosal surface area was increased by 72% (p<0.01) compared with controls (Fig 3). In the distal colon we found a substantial 34% increase in the weight of submucosa among EGF treated rats (p<0.001), while the weight of muscularis propria, as in proximal segments, were equal in the two groups. In the mucosa we found a significant 50% increase in the weight of epithelium among EGF rats (p<0.005) and a 51% increase of muscularis mucosae, where the difference just reached the level of significance (p=0.049). There was a non-significant 20% increase in the lamina propria weight (p=0.064) compared with controls (Fig 4).

For each animal, the weight of proximal mucosa was compared with the weight of distal mucosa to obtain a proximal/distal mucosal ratio in the two treatment groups. This ratio was calculated to further examine the EGF effect on the colonic mucosa and to verify statistically, if there is a diverse response in mucosa of proximal and distal colon to EGF treatment. Figure 5 shows the results and the proximal/distal mucosal ratio tended to be increased in the EGF group (p=0.07).

Discussion
Previous investigations of systemic EGF actions on the colon have focused on the defunctioned or damaged intestine. Foster et al showed that intravenous EGF for 10 days maintained colonocyte proliferation at a normal rate in surgically defunctioned colo-rectum in rats.12 Also in rats, EGF was shown to preserve epithelial proliferation in the colon during total parenteral feeding for eight days,13 and in recent studies to protect against mucosal lesions caused by experimental colitis.6,7 These and comparable studies have unambiguously proposed EGF as a trophic factor in the maintenance of epithelial proliferation in colon. This study is the first to describe the trophic effects of systemic EGF treatment on all the large bowel wall layers and on the luminal surface area of normal functioning colon. It shows that EGF stimulates growth of the mucosal layer and the luminal surface area in both proximal and distal colon and that EGF also has a trophic effect on the submucosa in the distal part of the colon.
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EGF is a peptide hormone which, in addition to its potent mitogenic effects in several epithelial and mesenchymal cell lines, is known to mediate growth, development, and maturation of the gastrointestinal mucosa. Our findings substantiate a proliferative role of EGF on large bowel mucosa and show a significant and longterm (28 days) trophic effect of continuous EGF treatment on normal and well nourished colonic mucosa. The overall weight increase of the mucosal layer affected growth of the epithelial lining as well as the underlying lamina propria, and also in the distal colon a weight increase of the muscularis mucosae. The proportional EGF induced increase in mucosal mass compared with the placebo treated controls was higher in proximal (68%) than in distal (28%) colon. A comparison of the proximal/distal mucosal ratios of the two treatment groups shows a quantitative difference in the growth stimulating response to EGF treatment between mucosa of proximal and distal colon. This is interesting in view of recent studies on samples from human large bowel showing a quantitative variation in the distribution of EGF receptors along the length of the colon.

A previous study on total parenteral fed rats have suggested a stimulatory effect of EGF on the colonic surface area, although these data were obtained only from selected and well orientated colon crypts. In our study we quantified the colonic luminal surface area in an unbiased manner and our data showed a profound surface area increase among EGF treated rats. The surface area increase seemed of equal proportions in proximal and distal colon despite a stronger effect on total mucosal weight in proximal colon segments. This might be explained by the significant effect on epithelial growth in both parts of the colon. Theoretically, the surface area growth can be caused by changes in crypt number or crypt height and diameter, but our data do not confirm this.

In addition to the effects on the mucosa, EGF treatment significantly increased the weight of the colonic submucosal layer. This is in agreement with studies that have shown biological effects of EGF on non-epithelial cells. Specific EGF receptors are found on human fibroblasts, and EGF treatment increases DNA synthesis and cell division in granulation tissue fibroblasts in rats. Kingsnorth et al have shown an increase in tensile strength of intestinal anastomoses in pigs under systemic EGF treatment, and anastomotic strength is derived mainly from collagen fibres of the submucosa. Accordingly this finding supports the idea of a growth stimulating role of EGF in the submucosal layer of intact colon. Furthermore, EGF receptors have been shown on smooth gastrointestinal muscle cells and animal studies have proposed a role for EGF in gastrointestinal muscle contractility. In our study, nevertheless, there were no increases in the external circular or longitudinal muscular layers of the colon.

The data regarding submucosal growth strongly show that there is a differential response in proximal and distal colon to EGF. While there was a pronounced difference and a low p value in distal colon with a small probability of a type I error, the EGF and placebo groups were identical in proximal colon. EGF induced stimulation of submucosal weight could result from a direct mitogen action on the fibroblasts and an increased synthesis of collagen fibres. The connective tissue of the submucosal layer also contains most of the colonic vessels, however, and submucosal growth might reflect an increase in oxygen and nutritional demands of the hypertrophic mucosa. Furthermore, EGF has been shown to possess a definite angiogenic potency and to increase granulation tissue blood flow.

We used stereological methods to obtain precise, quantitative information about the transmural trophic effects of EGF. Although the stereological analyses were based on find-
ings on a limited number of colon sections, these methods do not require any assumptions regarding tissue shape or size. At the practical level during sampling and processing, however, certain simple rules were followed to assure that the information was unbiased. Sampling was done at random along the entire colon length with an equal chance for every portion of the intestine to be sampled. Equally important was the random rotation of specimens in the horizontal plane before sectioning. To account for possible variation in composition or surface area along the length of proximal and distal segments we took four or five samples from each. The variability in volume fraction and surface area estimations between these samples turned out to be small (data not shown) and we could have obtained reliable results using fewer samples. A high counting efficacy in stereological methodology gives a low variability after spending a moderate amount of time and is important in reducing the labour in studies larger than ours.

In conclusion, the reported data show that longterm EGF treatment has a trophic effect on the normal rat colon and stimulates growth in both the mucosal and the submucosal layers. Increase in mucosal weight and luminal surface area occurs along the entire large bowel. Mucosal growth seems most eminent in proximal colon, whereas the submucosal growth is restricted to the distal colon.

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