Effect of epidermal growth factor administration on intestinal cell proliferation, crypt fission and polyp formation in multiple intestinal neoplasia (Min) mice

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ABSTRACT

Recombinant epidermal growth factor (EGF) may be useful to treat severe ulcerative gastrointestinal injury. There is concern, however, that systemic use of this potent mitogen might increase tumour development and/or progression in susceptible subjects. We therefore examined the effect of chronic administration of systemic EGF to multiple intestinal neoplasia (Min) mice, who have a genetic defect in the adenomatous polyposis coli (APC) gene, leading to increased polyp development. Min mice (n = 26) and wild-type littermates (n = 26) received saline or EGF (223 µg of EGF/kg per day) for 4 weeks using subcutaneous osmotic mini-pumps. Cell proliferation and crypt fission were analysed using microdissection techniques and the number and size of polyps in the small and large intestines were determined. EGF increased wet weight and crypt cell proliferation rate by approx. 20 % (all P < 0.01 compared with the relevant control) in the small intestine and colon of both control and Min mice. In both groups, EGF reduced the colonic fission index by approx. 40 % (P < 0.01), but did not affect crypt fission in the small intestine. In Min mice, administration of EGF did not increase numbers of polyps or degree of dysplasia, but resulted in a 40 % increase in the polyp size in the proximal intestine (P < 0.02), but not in the remainder of the small intestine or colon. No polyps were found in control mice given EGF. EGF did not initiate polyp formation in control or Min mice. However, as polyp size is an important determinant for subsequent risk of malignant change in human colon cancer, further studies appear justified.

INTRODUCTION

Recombinant peptides are playing an increasingly important role in the treatment of multiple medical conditions, such as human insulin for diabetes, interferon-γ for hepatitis and colony-derived growth factor for bone marrow rescue. The use of recombinant peptides for luminal gastroenterology is at a much earlier stage, although several peptides (e.g. glucagon-like peptide 2 for short bowel syndrome and keratinocyte-growth factor for mucusitis) have shown promise in animal models and human trials are underway.

Epidermal growth factor (EGF) is a 53-amino-acid peptide that shows particular promise for the treatment of
gastrointestinal disease; its receptor (c-erb1) is expressed throughout the human gastrointestinal tract and several in vitro and animal models of disease have confirmed its potent mitogenic, motogenic and cytoprotective activity against a variety of noxious agents [1,2]. As a result of these studies, several case reports and early clinical trials have been published or are underway for conditions such as necrotizing enterocolitis [3], congenital microvillus atrophy [4], peptic ulceration [5,6] and ulcerative colitis [7].

The normal gastrointestinal tract remains intact due to a finely controlled equilibrium between cell production and loss. Under normal circumstances, the gut undergoes a high rate of cell turnover, secondary only to the haemopoietic system. In addition, intestinal cell mass can also be altered by the relatively poorly understood process of crypt fission, in which small bifurcations in the crypts can ‘unzip’ to create new crypts [8]. Crypt fission plays a major role in intestinal development, but also occurs in adult animals [9,10], and humans [11], especially following damage [12–14].

Although cell proliferation and crypt fission are essential components of gut defence, excess proliferation has been associated with increased cancer risk [15], and crypt fission is increased in crypts isolated from human adenomas and hyperplastic polyps [16]. This has led to the idea that sporadic human colorectal adenomas and hyperplastic polyps grow not only by altered proliferation, but also by the process of crypt fission. There is therefore concern that administration of recombinant growth factors may accelerate the progression of premalignant lesions by increasing cell production and influencing crypt fission [17].

We therefore examined the effect of recombinant EGF on proliferation, crypt fission and polyp development in multiple intestinal neoplasia (Min) mice, who have spontaneous polyp formation due to disruption of the adenomatous polyposis coli (APC) gene, associated with familial adenomatous polyposis (FAP) in humans.

**METHODS**

**Ethics**

All procedures, including mutant and transgenic breeding and breeding of Min mice, were approved by the Cancer Research UK and Imperial College School of Medicine Animal Ethics Committees and covered by the appropriate licences under the Home Office Animal Procedures Act, 1986.

**EGF**

EGF used for the study was obtained from Heber Biotec S.A. (Havana, Cuba) and consisted of human recombinant EGF that had been expressed in *Saccharomyces cerevisiae*. The resulting EGF consists of a 60:40 mixture of EGF1–52 and EGF1–51, and has equivalent bioactivity to the full-length EGF1–53 form [18].

**Mice**

The C57BL/6j-Apc MIN/+ Apc heterozygous mice were originally obtained as a gift from Dr Amy R. Moser (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI, U.S.A.). Male mice were back-crossed to female C57BL/6j and the resultant embryos were transferred by aseptic hysterectomy to foster mothers in specific pathogen-free isolators. All breeding was subsequently performed in specific pathogen-free units by brother–(C57BL/6j-Apc MIN/+ Apc) sister (C57BL/6j) mating. Genotyping was carried out by a PCR based method, using three primers including an internal control for normal mouse DNA.

**Study protocol**

Min mice (*n* = 26) and wild-type littermates (*n* = 26), 4–6 weeks old (weight 17–21 g), were randomly divided into two groups. All animals had a mini-osmotic pump (Alzet Model 2004, Alza Corporation, Palo Alto, CA, U.S.A.) implanted subcutaneously in the back of the neck. This system delivered 0.25 µl/h continuously for 28 days. Half of the pumps contained EGF, so that each mouse received 6.7 µg of EGF/day, equating to a mean dose of 223 µg of EGF/kg per day. The pumps in the other half of the animals contained saline alone. Animals were allowed access to standard mouse diet (GR3EK-R20; Special Diets Services, Witham, Essex, U.K.) and water *ad libitum* throughout. At the end of the 28 days, all animals were killed and autopsied.

**Autopsy**

At autopsy, the wet weight of the stomachs, small intestines, caecae, colons, pancreata and spleens were recorded. The small intestines and colons were isolated, rinsed and weighed. The small bowel was divided into three equal sections, proximal, middle and distal, dissected longitudinally and spread on to filter paper, as was the entire colon. The gut preparations were then fixed in Carnoy’s fixative for 3 h and stored in 70% ethanol.

The intestines were later assessed, under 20× magnification, for polyp number and size (mean of two largest diameters measured with digital callipers). The tumour burden was calculated as the product of polyp number and polyp volume. After evaluation the small bowel sections were rolled up into a ‘Swiss-roll’, embedded in paraffin wax and sectioned at 4 µm for subsequent histological and immunohistochemical evaluation. Histological examination was performed in a blinded fashion by a qualified histopathologist.

**Assessment of proliferation/fission**

Assessment of proliferation and fission was performed using previously well-validated methods [19]. Briefly, at
each site, representative samples of tissue were hydrated, hydrolysed and stained with the Feulgen reaction. The mucosal crypts were gently teased apart under a dissection microscope. The numbers of mitoses per crypt (mean of 20 crypts) and crypt fission events per 200 crypts were then determined in this ‘microdissected’ tissue. All samples were counted in a blinded fashion.

Statistics
Results are presented as the means ± S.E.M. Data were tested by a two-way ANOVA using a general linear model (GLM) using Minitab Statistical Software (release 10.5 Xtra; Minitab Ltd, Coventry, U.K.). Two factors, Min and EGF, classified the data. If the presence of one factor alters the effect of the other, this is indicated by a significant interaction effect.

RESULTS
Wet weights of tissues
In animals not given EGF, Min mice had 33% heavier small intestines, 16% heavier caeca and 20% heavier colons compared with the wild type (Figure 1).
Table 1  Effects of Min in the absence and presence of EGF treatment on polyp number and polyp burden

Values are means ± S.E.M. *P = 0.016 compared with Min. The two-way ANOVA demonstrated a significant effect (P < 0.001) of site on polyp burden in the small intestine.

<table>
<thead>
<tr>
<th>Site</th>
<th>Polyp number (n)</th>
<th>Polyp burden (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Min + EGF</td>
</tr>
<tr>
<td>Small bowel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>3.85 ± 0.80</td>
<td>3.29 ± 0.72</td>
</tr>
<tr>
<td>Middle</td>
<td>23.62 ± 2.96</td>
<td>20.50 ± 3.88</td>
</tr>
<tr>
<td>Distal</td>
<td>47.08 ± 8.25</td>
<td>40.79 ± 7.61</td>
</tr>
<tr>
<td>All</td>
<td>74.54 ± 10.42</td>
<td>64.57 ± 11.28</td>
</tr>
<tr>
<td>Colon</td>
<td>2.92 ± 0.47</td>
<td>2.64 ± 0.65</td>
</tr>
</tbody>
</table>

Figure 2  Effects of the various treatments on cell proliferation in the proximal small intestine and colon

10% small intestine (SI) and 10% colon indicate that the sites sampled were defined by their percentage length. The results of two-way ANOVA are shown on the right.

Polyp formation

No polyps were found in the wild-type mice. Min mice not given EGF had an average of 75 ± 10 polyps in the small intestine and 2.9 ± 0.5 polyps in colon (Table 1). EGF-treated Min mice had 65 ± 11 polyps in the small intestine and 2.6 ± 0.7 in the colon. Sub-analyses of different segments of the small intestine showed no effect of EGF at any site.

The diameter of the polyps was used to calculate tumour volume or burden. There was a 3-fold increase in tumour burden in the proximal third of the small intestine (Table 1), but no significant effect of EGF on tumour burden was seen in the other sites.

Proliferation and fission

In animals not given EGF, there were no significant differences in proliferation rates between wild-type and Min mice in either the small or large intestine (Figure 2). EGF significantly increased proliferation by an average of 25% in both the small intestine and colon of the wild-type and Min mice. There was no interaction between the presence of Min and EGF on proliferation rates, showing that the effect of EGF was not affected by the co-presence of the Min allele.

In animals not given EGF, crypt fission in the proximal small intestine and colon was slightly increased in the Min group compared with wild-type animals, but this difference was not statistically significant (Figure 3).
Epidermal growth factor and the intestine of Min mice

Figure 4  Haematoxylin- and eosin-stained section of tissue from Min mice
Upper panel: a microadenoma consisting of a single adenomatous crypt lined by atypical epithelial cells with hyperchromatic nuclei. Lower panel: a 'Swiss roll' with a larger adenoma comprising many abnormal adenomatous acini covered with normal epithelium. Scale bars, 100 µm.

Administration of EGF did not affect the fission index in the small intestine of wild-type or Min mice. In contrast, administration of EGF caused an approx. 45% reduction in the crypt fission index of the colons of both wild-type and Min mice.

Histology and immunohistochemistry
Stained sections of rolled intestinal tract were examined by a pathologist and were found to be the typical intravillous adenomas described previously [20,21]. Administration of EGF did not affect the degree of dysplasia (Figure 4).

DISCUSSION
In the present study, we have shown that chronic systemic administration of EGF increased the polyp size, but not the number or degree of dysplasia, in the proximal small intestine of Min mice. EGF also stimulated the growth of both the small and large intestine and decreased colonic fission index.

Although recombinant peptides are proving of clinical value in a variety of medical conditions (e.g. human insulin), it is only relatively recently that powerful pro-mitogenic peptides have been studied in a clinical setting. There is therefore concern about potential risks of stimulating cancer development or progression when such peptides are given. This has particular relevance to the gastrointestinal tract when peptide therapy is being suggested as potential treatment for colitis, because these patients are already at increased risk of spontaneous colonic cancer development [22].

There are several animal models for gastrointestinal cancer, including administration of pro-carcinogenic compounds such as dimethylhydrazine [23] or using genetically modified animals who have increased spontaneous tumour development. We chose to study the effect of EGF on Min mice [24]. The Min mouse is generally agreed to be a useful model for the study of various factors on the early stages of intestinal cancer, as Min mice are highly susceptible to spontaneous intestinal adenoma formation due to a mutation in the APC tumour suppressor gene [25]. The APC gene is usually inactivated early in the carcinogenic process in man, both in sporadic colorectal cancer and in FAP [26]. Although this is a useful model, it is important to remember the limitations of the use of such animals as polyp formation in APC-deficient humans is virtually always confined to the colon, whereas the predominant site in the murine model is the small intestine. The number of polyps exhibited varies according to the background strain used, reflecting the presence of other genetic modifiers such as the modifier of Min (Mom1) locus [27].

We administered the recombinant peptide for 4 weeks, as peptide therapy is likely to be given over several days or weeks and prolonged treatment was used in an attempt to establish a new steady state for proliferation and fission. Prolongation of administration beyond 4 weeks is possible, but would have required replacing the infusion pumps in the animals. The dose of 223 µg of EGF/kg per day was chosen as it is similar to that used in animal models studying the potential benefit of EGF to treat/prevent injury. This dose is higher than that used in many human studies (e.g. 6 µg of EGF intravenously twice a week [28], and 50 µg of EGF 3 times a day for duodenal ulcer repair [6]).

There is no one optimal method of quantifying gut growth, but wet weight remains an important easily measurable parameter. We also determined proliferation rate on a per crypt basis, which we have shown previously [16,19,29] provides robust reproducible results. However, this method is also limited by the fact that if the test factor influences the denominator, i.e. number of
crypts, and this may make interpretation of the data more difficult. In order to attempt to address this, we therefore also examined the crypt fission index; a measurement often omitted in other studies.

The results of the present study confirm the powerful actions of EGF on gut growth (as demonstrated by wet weight and proliferation indices) reported previously by us [1] and other groups [29a], although we are not aware of any previous reports showing increased splenic weight in response to EGF. The pro-proliferative effects of EGF in the present mouse study were, however, less pronounced than those seen by us previously [30] using a similar dose in rats, where a doubling of proliferation rate was seen, suggesting differences in species sensitivity. Our present finding that EGF increased splenic weight is also a novel result.

In the present chronic study, crypt fission in the colon, but not the small intestine, was decreased in response to EGF, supporting our previous findings [30] that EGF causes an initial transient increase followed by reduced values. Crypt fission is an uncommon event in normal human colonic mucosa, but common in crypts isolated from adenomas and hyperplastic polyps, leading to the suggestion that sporadic human colorectal adenomas and hyperplastic polyps grow by crypt fission [16].

Administration of EGF did not induce new polyp formation in wild-type or Min mice, suggesting that it is not involved in polyp initiation in this model. In the normal → polyp → cancer sequence suggested by Fearon and Vogelstein [31], abnormalities of the APC gene are considered to be a 'gatekeeper' defect, increasing the possibility of accruing subsequent genetic and epigenetic abnormalities and leading to cancer development [32]. Our finding that the degree of dysplasia in the polyps was unaffected by EGF argues against it being a major progression factor in this model. However, it is also important to note that EGF did increase polyp size, which is thought to be relevant to subsequent cancer development in humans [33].

Increase in polyp size was only found in the proximal small intestine. The molecular mechanisms underlying this difference are unclear, but we [29] and others [29b] have demonstrated previously that different peptides have different site specificity for the various regions of the intestinal tract and may also be dependent on whether the bowel is damaged. In the normal non-damaged gut, infusion studies of EGF tend to result in its major trophic effect being seen in the stomach and colon, rather than the small intestine [29]. However, a similar regional effect focussing on the proximal small bowel was seen in mice with a triple null mutation of EGF, amphiregulin and transforming growth factor α (TGFα), where frank ulceration of the small bowel was seen in the proximal, but not the distal, small intestine [34]. This may reflect regional differences in the number of c-erbB1 receptors expressed in different parts of the small bowel, although no marked difference was seen in c-erbB1 receptor expression between the proximal and distal small bowel in triple null mice [34]. Alternatively, the more marked proximal distribution of effect in the triple null mutation mice and in the present study may reflect an interaction between the effect of c-erbB ligands, food and/or pancreatic bile secretions. In contrast with the findings with EGF, the main proliferative actions of keratinocyte growth factor are on the stomach and small intestine with only modest actions on the colon [34a], whereas glucagon-like peptide II is trophic throughout the gut [35].

There have been relatively few studies examining the effect of EGF on experimental carcinogenesis. In mice given the carcinogen dimethylhydrazine an increased number of anal squamous cell carcinomas were seen if 5 µg of EGF was co-administered on alternate days for 2 weeks; however, no change in the number or size of colonic tumours was observed [36]. An antagonistic relationship between EGF and azoxymethane on proliferation and tumour growth was reported following intra-colonic administration of EGF [37], suggesting that, although luminal EGF can increase normal colonic epithelial growth, it does not potentiate carcinogenesis. Nevertheless, EGF and its receptor may have relevance to tumour development and therapy, as the EGF receptor (EGFR) and its ligands (EGF, TGFα etc.) are overexpressed in many tumours and EGFR blockade has been shown to be therapeutic [38]. Furthermore, in the Min mouse, impaired EGFR signalling or pharmacological inhibition of EGFR can result in a large reduction in polyp number, but not apparently of polyp progression, suggesting that normal EGFR activity is required for establishment of intestinal tumours [39]. In addition, combination of a chemopreventative drug (sulindac) and an irreversible inhibitor of the EGFR dramatically reduced tumour number in these Min mice [40].

EGF is one of the most extensively studied peptides for the treatment of gastroenterological disease and is normally produced by the salivary glands and Brunner's glands of the duodenum and also by the ulcer-associated cell lineage; a glandular structure that develops locally at sites of injury and secretes pro-healing peptides on to the wounded area [41]. The pathophysiological role of EGF in humans continues to be a matter of debate, although most recent studies suggest that the EGFR is restricted to basolateral (but not apical) membranes in the adult non-damaged bowel [42]. This has led us to suggest that its major role is to act as luminal surveillance peptide [43], having little effect in the normal adult bowel, but being readily available to stimulate repair when basolateral receptors are exposed at sites of injury due to local damage or increased permeability. Luminal EGF may play a more important role in the growth of the normal neonatal gut due to its increased permeability. EGF reduces injury and/or stimulates repair in multiple animal models,
including gastric injury [5], colitis [7], liver damage caused by carbon tetrachloride [44] or thioacetamide [45], and reduces intestinal injury caused by ischaemia reperfusion as well as the risk of multi-organ failure [45,46]. Preliminary clinical studies also suggest it may be of value for the treatment of conditions such as necrotizing enteritis and ulcerative colitis [47], where marked rapid improvements have been reported, and it is therefore likely that its use will increase over the next decade.

The actions of EGF on the repair response are multiple. Stimulation of restitution re-establishes epithelial continuity and this may have particular importance in reducing the antigenic load of luminal contents in conditions such as ulcerative colitis, where the secondary inflammatory response exacerbates the injury. Loss of cell number requires replenishment and the pro-proliferative effect of EGF will enhance the rate of replacement. The effect of EGF on crypt fission is more complex as there is temporal variation with an initial increase followed by a reduction. The initial early enhancement of fission may allow the production of new crypts at the recently damaged area, whereas the later reduction in fission rate may facilitate repopulation of the newly formed crypt/villus complexes. With regards to tumour production, the early stages of cancer development of the colon include clonal expansion with crypt fission probably playing a role in this process. Although growth factors, by definition, increase proliferation and are therefore considered as a potential concern in tumour progression, the effect of EGF in reducing crypt fission may paradoxically provide a potential mechanism in reducing this expansive process. Further work is clearly required to address these points.

In summary, our studies do not support a major cancer-promoting activity of EGF on the gut in this model. However, further studies are clearly required, including other animal models and surveillance examinations of patients being given enema EGF preparation for conditions such as colitis. In addition, particular caution must be shown before giving factors such as EGF via the intravenous route, exposing multiple end organs to this mitogen.

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