Characterisation of humoral trophic factors involved in intestinal adaptation using a 3T3 cell growth factor assay

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SUMMARY Indirect evidence has suggested that circulating trophic factor(s) are involved in intestinal adaptation. A 3T3 fibroblast cell culture system was used to more directly delineate the presence of such factors. Rats were divided into four groups; C-unop, those undergoing no surgery, S, those in which a dorsal slit was made; C-op, those in which the peritoneum was incised; and R, those undergoing 80% intestinal resection. At the time of death at 24 hours, one week and two weeks postoperative, stimulation of DNA synthesis in the 3T3 cells was noted after incubation with platelet free plasma from the C-op and R groups, and simultaneously an increase in ileal DNA specific activity occurred. Characterisation of the plasma fraction with growth factor activity revealed it to have a MW of greater than 6000 and less than 14000 d. The factor(s) was resistant to reduction with DTT, and was partially inactivated by heating to 60 °C. The use of 3T3 cell growth factor assay system allows further characterisation of circulating factors involved in intestinal adaptation.

The small intestine undergoes a series of complex histological and functional changes after limited surgical resection, enabling the organism to adapt to the reduced absorptive surface.1-3 Enteral nutrition plays a major trophic role in intestinal adaptation which occurs distal to the site of the resection.4-7 Luminal factors cannot, however, explain all of the changes occurring in the adaptation process, and experimental evidence indirectly suggests the presence of circulating humoral factors with trophic effects on small intestinal mucosa.

The difficulty in documenting and characterising the presence of humoral growth factors after intestinal resection has, in part, been related to a lack of a reproducible in vitro assay for growth factor activity. The 3T3 cell culture system used in the present study has been shown previously to be a reproducible assay for growth factor activity for epidermal growth factor,8 platelet derived growth factor9 and fibroblast growth factor.10

In this study we present evidence supporting the usefulness of the 3T3 cell growth factor assay for detecting and further characterising such circulating trophic factors.

Methods

These experiments were conducted using male Sprague-Dawley derived rats (151–175 g). One hundred twenty rats were divided equally among three control groups and one resected group as follows:

(1) The unoperated controls (C-unop), which included those rats anaesthetised, but in which no operation was performed;

(2) The dorsal slit group (S), comprised those rats in which a 3 cm incision was made on the back of the animal, with the subcutaneous tissue torn using a clamp;

(3) The operated control group (C-op) which included those rats in which a 3 cm abdominal incision was made and the peritoneum incised, but the bowel was not manipulated;
(4) The resected group (R) comprised rats that underwent an 80% proximal small intestinal resection with a primary anastomosis of the remaining segments. Ten rats in each group were killed at either 24 hours, one week, or two weeks after start of the experiment. One hour before death, rats were injected intraperitoneally with 50 μCi of ³H thymidine. At the time of death, blood was collected for the in vitro 3T3 growth factor assay (see below). In addition, a 5 cm segment of ileum was removed, and mucosa was obtained and stored at −40 °C until used to measure the incorporation of ³H thymidine into DNA (see below).

**IN VITRO GROWTH FACTOR ASSAY**

Mouse BALB/c 3T3 embryo fibroblasts (clone A 31) were used in the in vitro growth factor assay as previously reported.¹³ The 3T3 cells were incubated for seven to 10 days without a medium change in order to yield confluent monolayers of nondividing cells. Platelet free plasma was obtained¹⁴ and after a dose response curve was calculated, aliquots were added to the 3T3 microtiter wells, together with ³H thymidine. After incubation for 48 hours, incorporation of ³H thymidine into DNA (CPM/μg DNA) was determined¹⁵¹⁶ and expressed as units of activity per ml of platelet free plasma, with one unit of activity equalling the volume necessary to give half maximal stimulation.

**IN VIVO DNA SYNTHESIS**

Extraction of ileal DNA was carried out as previously reported¹⁷ and results expressed as mg of DNA per 5 cm segment. Aliquots of the pure DNA solution were counted and DNA specific activity (SA) expressed as counts per minute per μg DNA was calculated.

**CHARACTERISATION OF PLASMA GROWTH FACTOR**

To establish a range for the molecular weight of the plasma fraction containing in vitro growth factor activity, platelet free plasma was sequentially dialysed against Ringer’s solution using membranes with pore sizes 3 500 d, 6 000–8 000 d and then 12 000–14 000 d. The growth factor activity of the dialysed sample was measured in the 3T3 cell system as described above. In a separate experiment, plasma samples were assayed before and after heating at 60 °C for 30 minutes and pre- and post-treatment with 5 mmol dithiothreitol (DTT) for one hour.

**AUTORADIOGRAPHIC AND HISTOLOGIC STUDIES**

Autoradiographic¹⁸ and histological studies of ileal mucosa were prepared from a randomised group of animals killed at two weeks postoperatively. Villous height and crypt depth were calculated as well as the number of labelled crypt cells and labelling index (percentage of the crypt cells labelled).

**Results**

**MUCOSAL DNA EXTRACTION**

As noted in Figure 1, there was no significant difference for either mucosal weight or DNA per segment (mg/5 cm) at any of the time points among the unoperated animals (C-unop), those undergoing either a dorsal skin incision (S) or those undergoing peritoneal incision (C-op). There was, however, a significant increase in both mucosal weight and DNA content per 5 cm segment for those undergoing an intestinal resection at each time point compared to controls (p < 0.005).

**24 HOURS POSTOPERATIVE**

At 24 hours (Fig. 2), the 3T3 assay revealed significant stimulation by plasma from both the operated control animals (C-op) and those undergoing intestinal resection (R) as compared with the unoperated control (p < 0.025, p < 0.005, respectively). At the same time, there was significant stimulation of DNA
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16
14
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16

SA for those two groups as compared with the unoperated animals (p < 0.005 for each).

ONE WEEK POSTOPERATIVE
At one week after operation (Fig. 3), stimulation of 3T3 cell proliferation by plasma from the resected group was significantly greater than that of the unoperated animals (p < 0.025). Stimulation by the plasma from the group undergoing peritoneal incision (C-op) increased but did not reach statistical significance. The DNA SA increased significantly for both those undergoing peritoneal incision alone (C-op), as well as those with intestinal resection (R) (p < 0.01, p < 0.005 respectively).

TWO WEEKS POSTOPERATIVE
At two weeks after operation (Fig. 4) the plasma of rats undergoing peritoneal incision (C-op) and intestinal resection (R) significantly stimulated 3T3 cell proliferation as compared with the unoperated controls (C-unop) (p < 0.005 for each). The DNA SA was also significantly increased for both groups (p < 0.025, p < 0.005 respectively).

CHARACTERISATION OF PLASMA GROWTH FACTOR
As shown in the Table, growth factor activity was retained after dialysis with membrane pore sizes of

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Treatment</th>
<th>Residual activity (%) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>Dialysis: pore size</td>
<td>Residual activity (%) ± SE</td>
</tr>
<tr>
<td></td>
<td>3500</td>
<td>89.5 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>6000-8000</td>
<td>101 ± 15.5</td>
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<tr>
<td></td>
<td>12000-14000</td>
<td>&lt; 1</td>
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<tr>
<td>Heat</td>
<td></td>
<td>50.4 ± 7.3</td>
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<tr>
<td>DTT</td>
<td></td>
<td>90.9 ± 9.4</td>
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</tbody>
</table>

Retention of growth factor activity of plasma from R and C-op groups after dialysis, heating and treatment with DTT utilising the 3T3 growth factor assay.
Fig. 4  Degree of 3T3 cell stimulation (units of activity/ml plasma) by platelet free plasma, and DNA SA (cpm/μg DNA) for each group at 2 weeks postoperative. Shown are the means ± SE and statistics reflect comparison to C-unop group using Student’s t test.

3500 d as well as 6000–8000 d. Virtually all activity was lost after dialysis with a membrane pore size 12000–14000 d. Heating the sample resulted in a 50% decrease in activity. Treatment with the reducing agent DTT resulted in retention of 90% of initial activity.

AUTORADIOGRAPHIC AND HISTOLOGICAL STUDIES

As shown in Figure 5, villus height and crypt depth was increased only for those animals undergoing resection (R) (p = 0.0006). The number of labelled crypt cells was increased for the sham operated group C-op (p = < 0.0004) and for the resected group R (p = 0.0006), with the latter (R) significantly greater than C-op (p = 0.0006). Likewise, the labelling index was increased for C-op (p = < 0.0004), and R (p = 0.0006) with the latter (R) significantly greater than C-op (p = 0.0006).

Discussion

In this study, we have shown the presence of a plasma factor(s) that stimulates incorporation of 3H thymidine into a 3T3 fibroblast cell line, concurrent with an increase in DNA synthesis in the remaining ileum after abdominal surgery. Ileal DNA specific activity, crypt cell labelling index, and 3T3 cell stimulation were increased for the operated controls (C-op) and the resected animals (R) as compared with the unoperated controls (C-unop). This effect appears specific for those animals undergoing abdominal surgery because a dorsal incision produced results similar to those of the unoperated control group. The fact that peritoneal incision without intestinal manipulation can stimulate intestinal DNA production at 24 hours, one week, and two weeks postoperative strongly suggests that a humoral growth factor is involved in the adaptation process. While there was stimulation of DNA specific activity and crypt cell labelling index for both the sham operated animals (C-op) and those undergoing resection (R) (Figs 2–4), only the latter group manifested an increase in total DNA in the ileal segment (Fig. 1). Clearly, factors stimulating 3T3 and crypt cell proliferation are not always reflected in an increase in villous height. Consequently, other factors must be involved in this adaptation process.

In summary, the use of the 3T3 growth factor assay now makes it possible to more directly define and characterise the trophic humoral factor(s) involved in intestinal adaptation. Future studies should be...
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directed at further isolation of this factor(s) and examination of the mechanism of action.

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