Production of epithelial cell growth factors by lamina propria mononuclear cells

J R Lowes, J D Priddle, D J Jewell

Abstract

The effects of lamina propria mononuclear cell culture supernatant on epithelial cell DNA synthesis were studied using cells isolated from patients with inflammatory bowel disease and normal controls. Supernatants from resting and phytohaemagglutinin stimulated cells were studied and supernatants that strongly promoted DNA synthesis were pooled, and growth factor activity partially characterised. The effects of recombinant interleukins-1β, 2, 3, interferon-γ, and granulocyte macrophage colony stimulating factor were tested in the same system. Resting lamina propria mononuclear cells produce factors that increase DNA synthesis. Production of these factors is increased by phytohaemagglutinin stimulation. No significant differences were found in production of these factors between patients with inflammatory bowel disease and normal controls. The molecular weight of the active factor(s) lies in the region 31-48 kD. Chromatofocusing produced two peaks of activity, one in the region pk 5-5 and one around pk 6-4. The activity was heat and acid pH labile. Activity was not destroyed, however, by 0-05% trypsin. Recombinant granulocyte macrophage colony stimulating factor was a weak stimulus to epithelial DNA synthesis, interleukin-1β was weakly inhibitory but other cytokines tested did not have any effect. Granulocyte macrophage colony stimulating factor is probably important in controlling epithelial cell growth.

Gastrointestinal epithelium is in a constant state of orderly renewal. In inflammatory conditions affecting large and small bowel, the crypt cell production rate (CCPR) is increased. In ulcerative colitis CCPR is increased when the disease is in relapse and to a lesser extent it is also increased when the disease is in histological remission. The net result of an increased numbers of cells synthesising DNA, expansion of the proliferative zone, and increased cell turnover rate. There is an increased risk of malignant change in these diseases and the risk, particularly in ulcerative colitis, appears to be related to the extent and the duration of the disease.

The control of cell growth is complex and multifactorial. The roles of hormones, peptide regulatory factors such as epidermal growth factor and insulin like growth factors have been extensively investigated. The relationship between epithelial cells and immune cells of the lamina propria is attracting increased attention. It has been suggested that cytokines released from intestinal mononuclear cells are responsible for the increased expression of class II major histocompatibility proteins on epithelial cells and soluble factors released from T cells are responsible for this increase in cell turnover. Continued exposure to such growth factors may be responsible for the increased risk of malignant change.

This study measures the effects on epithelial cell turnover by measuring incorporation of bromodeoxyuridine into the epithelial cell line HT29 after exposure to supernatant from culture of lamina propria mononuclear cells, or known as cytokines. Attempts have been made to characterise growth promoting factors produced after culture of intestinal lamina propria mononuclear cells from mucosal tissue affected by ulcerative colitis, Crohn's disease, or histologically normal.

Methods

Patients

Fresh surgical specimens were obtained from patients undergoing intestinal resection for ulcerative colitis (11), Crohn's disease (three), colonic neoplasia or chronic constipation (9). Where possible mucosa from inflamed and non-inflamed sites were compared in specimens from patients with distal ulcerative colitis. In patients with colon cancer, mucosa was taken >5 cm from a tumour. Patient details are listed in the Table.

Lamina Propria Mononuclear Cells

Lamina propria mononuclear cells were obtained by the method of Bull and Bookman. Briefly, surgical resected intestine was scraped free of excess mucus and the mucosa dissected from the muscularis propria. Approximately 30 mm×5 mm mucosal strips were washed once in 1 mM dithiothreitol (Sigma, Poole, UK) for 20 minutes at room temperature and then epithelial cells were removed by shaking at 37°C in 5 mM ethylene diamine tetra-acetic acid for 30 minutes. This was repeated three times with intermediate washes in calcium and magnesium free Hank's balanced salt solution. The resulting fragments were then minced and digested in RPMI 1640 containing 10% fetal calf serum (Gibco, Paisley, Scotland) and collagenase from

Patient details

<table>
<thead>
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<th>Disease group</th>
<th>Normal</th>
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<th>Crohn's</th>
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<tr>
<td>n</td>
<td>9</td>
<td>11</td>
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<td>Drug therapy</td>
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<td>iv steroids</td>
<td>sulphasalazine (5)</td>
</tr>
</tbody>
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clostridium histolyticum (BCL, Boehringer Manheim GmbH, W Germany). Digestion continued for three hours (1 mg/ml collagenase (15)) or 16 hours (0-3 mg/ml collagenase (eight)). The results from long and short digestion times were comparable and have therefore been combined. The digest was then passed through a nylon mesh (200 μm) and the mononuclear cells separated by density centrifugation on Ficoll-paque (Pharmacia, Milton Keynes, UK). The resulting preparation of mononuclear cells were resuspended at 10⁶ viable cells/ml in RPMI 1640, with 10% fetal calf serum, supplemented with gentamicin 1 μg/ml. Viability was always >95%.

Mononuclear cells were cultured for 72 hours in 24 well plates (Linbro, Flow Laboratories, Rickmansworth, UK), in 1 ml aliquots (10 μg/m, phytohaemagglutinin (Wellcome Laboratories, Beckenham, UK). Pilot experiments had shown that growth factor production was maximal after 72 hours growth in the presence of lectin. Supernatants were subsequently harvested, spun and filtered through a 22 μm filter, frozen and stored at −20°C for future testing. Freezing and storing at −20°C had no detectable effect on stimulation index for up to six months.

**EPITHELIAL CELL LINE**

The human colonic adenocarcinoma cell line HT29 was obtained from Dr P Brandtzæg, Oslo, Norway. The cells were grown in 25 cm² T-Flasks (Linbro, UK), the cells were seeded at 10⁵/flask and passaged weekly in Leibowitz-15 medium (L-15) (Flow Laboratories, UK), supplemented by 10% fetal calf serum, 2 mM glutamine, and antibiotics.

**EPITHELIAL CELL PROLIFERATION ASSAY**

This was adapted from an ELISA technique developed for the measurement of leucocyte proliferation.

HT29 cells were seeded 5 × 10⁴/ml, 200 μl/well in 96 well plates in L-15 medium. After 48 hours the medium was removed and 50 μl mononuclear cell culture supernatant together with 150 μl medium was added to the wells. Control wells received 50 μl RPMI 1640 and 10% fetal calf serum from the same batch used in the culture of the mononuclear cells. After a further 24 hours, 50 μl 5 × 10⁻⁸ M bromodeoxyuridine was added to the wells. After another 24 hours the plates were washed four times with Hank’s balanced salt solution and air dried at 37°C for three to six hours. The cells were then fixed by the addition of 200 μl 95% ethanol (BDH, Poole, UK) for 10 minutes, emptying the wells and air drying at room temperature for 20 minutes. The DNA was denatured by heating to 70°C for 45 minutes in 95% formamide in 0-15M sodium salt citrate buffer (Sigma, Poole, UK). The wells were then washed five times in pH 7-4, 0-13 M phosphate buffered saline (PBS) 0-1% Tween 20 (Sigma, UK). The monoclonal antibody Bu20a, directed towards the bromodeoxyuridine incorporated into single stranded DNA, was a generous gift from Dr D Mason, John Radcliffe Hospital, Oxford. This was used as a culture supernatant diluted 1:50 in PBS Tween. Two hundred microlitres of antibody was added to each well and incubated at room temperature for 30 minutes. The plate was then washed four times with PBS Tween and a second layer antibody of peroxidase conjugated rabbit antimouse serum (Dako, High Wycombe, UK) was used. After washing four times with PBS Tween the peroxidase substrate orthophenylene diamine (OPD, Dako UK) was added at a concentration of 0-2 mg/ml. The plate was then incubated for 20 minutes in the dark at room temperature and the reaction terminated by the addition of 100 μl 2 M sulphuric acid. The coloured reaction product was read in a Multiscan plate reader at 492 nm. The amount of cellular protein/well was quantified by a modification of the Bradford technique. The plates were washed four times with methanol, air dried for four hours at 37°C, 30 μl Bradford reagent (Biorad Laboratories, Watford, UK) added and the plates shaken for 20 minutes, 120 μl water added and the plates shaken for a further 20 minutes. The optical density at 595 nm was then measured in the Multiscan plate reader.

**GROWTH FACTOR ACTIVITY**

Growth factor activity was expressed as increment in optical density at 492 nm against control.

**CYTOKINES**

Human interferon-γ was a generous gift of Dr G Scott, Wellcome Biotechnology, Kent, and was a culture supernatant from a Chinese hamster ovary cell line transfected with recombinant human interferon-γ gene. Recombinant interleukin-1β (IL-1) and interleukin-2 (IL-2) were purchased from Koch-Light UK. Recombinant interleukin-3 (IL-3) and granulocyte macrophage colony stimulating factor (GM-CSF) were the generous gift of Glaxo Research, Geneva, Switzerland.

**GEL CHROMATOGRAPHY**

Supernatants that produced an increment of 0-2 OD₄₉₂ units were considered to be ‘active.’ Active supernatants were pooled, desalted on a Sephadex G25 column and lyophilised. The resulting powder was redissolved in a minimum volume of PBS and fractionated on a TSK G2000SW column (Pharmacia-LKB, UK) using an LKB HPLC pump 2150 and fraction collector.

Active fractions in the DNA synthesis assay were pooled, desalted, lyophilised and passed onto a chromatofocusing column (Pharmacia-LKB) and eluted with polybuffer 74 (Pharmacia-LKB).

**PHYSICAL TREATMENTS**

Active supernatants were placed in boiling water for 30 minutes. Five millilitres of active supernatant was passed onto a sephadex G-25 column equilibrated with 0-1 M glycine buffer pH 2 and eluted at five minutes, six hours, and 24 hours.
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Three millilitres of active supernatant was incubated with 0·05% Trypsin for 30 minutes at 37°C.

STATISTICAL ANALYSIS

Unless stated otherwise experiments were carried out in quintuplicate and points represent means (SD). Comparisons between groups of non-parametric data were made using two-tailed Mann–Whitney U test and paired t-tests were used for parametric data.

Results

The supernatants of resting lamina propria mononuclear cells (LPMNC) produced a significant increase in DNA synthesis in the epithelial cell line. Stimulation of the LPMNC with phytohaemagglutinin produced a further significant rise in DNA synthesis. There was no difference, however, between supernatants produced from culture of cells obtained from diseased or control mucosa either in the resting state or after lectin stimulation (Fig 1). Phytohaemagglutinin alone had no effect on the rate of DNA synthesis (Fig 2). After concentration of 212 ml of active supernatant by desalting and lyophilisation, the lyophilate was redissolved in 1 ml PBS and fractionated on a TSK G2000SW column, 35×0·45 ml fraction were collected. The column was calibrated using proteins of known molecular weight. Active fractions were found in the 31–48kD range and fractions of molecular weight less than 20 kD were inhibitory to DNA synthesis (Fig 3).

Active fractions from the high performance liquid chromatography gel column were concentrated as previously and applied to a chromatofocusing column. The active fractions were found to elute around pH 6·4 and 5·5 as seen in Figure 4. Biological activity was destroyed by exposure to heat (p<0·05), pH 2 (p<0·05) but not 0·05% trypsin as shown in Figure 5.

RECOMBINANT CYTOKINES

Interferon-γ, interleukin-2, interleukin-3 produced no significant effect on DNA synthesis over a wide range of concentrations. Interleukin-1β produced a slight decrease in the rate of DNA synthesis that only achieved statistical significance at a dose of 50 IU/ml (p<0·05). Granulocyte-macrophage colony stimulating factor was a stimulator of DNA synthesis producing a maximal increase in DNA synthesis at a dose of 100 U/ml. Higher doses did not produce any greater effect suggesting that the stimulus was saturated at about this level (Fig 6).

Discussion

These results show that human lamina propria lymphocytes produce factor(s) that increase DNA synthesis in a colonic epithelial cell line. We have not shown any difference in the production of these factors by cells obtained from either inflammatory bowel diseases or control tissue from normal mucosa (albeit from colonic mucosa that had neoplastic change at a distant site). Production of active supernatants markedly increased after lectin stimulation of the mononuclear cell preparation, although in two cases of actively inflamed ulcerative colitis, net production decreased after lectin stimulation. The molecular weight of the growth factor activity lies in the range 31–48 kD and activity was found to lie in two peaks with pK of 5·5 and 6·4 approximately. The biological activity of the supernatant could be destroyed by exposure to acid pH and to high temperature, but not 0·05% trypsin. This is consistent with activity lying in a native polypeptide. Using recombinant cytokines this effect was partially reproduced by recombinant granulocyte-macrophage colony stimulating factor.

Increased quantities of colony stimulating factors including granulocyte-macrophage colony stimulating factor and interleukin-3 have been found in lamina propria mononuclear cell isolated from patients with ulcerative colitis and Crohn’s disease. Synthesis by lamina propria mononuclear cell culture supernatant. Gel chromatography showed that the supernatants
contain both growth inhibitory and growth promoting activities. No significant differences were detected in the activity of supernatants from control subjects and either Crohn's disease or ulcerative colitis subjects.

Although differences in epithelial growth factor production between the different populations were not detectable using this in vitro technique, small in vivo differences may be important in controlling crypt cell production rate. In the experiments carried out here we are not comparing like with like as the lamina propria lymphocyte subsets are altered in inflammatory bowel disease. We know that in vitro isolation selectively enriches certain populations and alters the function of isolated cells. Therefore conclusions about differences between control and inflammatory bowel disease subjects must be made with caution.

The observation that phytohaemagglutinin stimulation of the cells increases growth factor production suggests that activation of the lamina propria mononuclear cell population leads to epithelial cell growth factor release, we have not further characterised the cell type producing growth factor, but the observation that phytohaemagglutinin leads to an increase in production suggests that T cells may be important.

Culture supernatants will contain a wide variety of biologically active substances. Precise characterisation of the stimulatory factor has not been achieved but, of the recombinant growth factors tested, only granulocyte-macrophage colony stimulating factor showed a proliferative effect. The cytokines used, however, were produced by recombinant DNA technology in bacterial plasmids. Thus they had not undergone eukaryotic glycosylation and have potentially different biological activities. Using the same cell line in an assay of class II HLA antigen induction, we have shown that it is approximately 100-fold more sensitive to interferon-γ produced in a eukaryotic system than other workers have found using an E coli derived product. Interferon-γ, interleukin-2, and interleukin-3 did not have any effect on DNA synthesis. These cytokines were used across a wide range of concentrations and in the case of interferon-γ at least, HT29 cells have been shown to be sensitive to induction of class II HLA molecules and is therefore likely to express interferon-γ receptors. It has been suggested that interferon-γ may be important in increasing epithelial cell growth rate in the murine model of intestinal damage, and that monoclonal antibodies to interferon-γ may abrogate the proliferative phase of an intestinal graft versus host reaction. Interferon-γ is usually considered to have growth inhibitory properties. From the in vitro data presented here it would seem unlikely that interferon-γ has a primary role in controlling

Figure 3: Stimulation indices of high performance liquid chromatography fractions generated from pooled and concentrated supernatant.

Figure 4: Stimulation indices of fractions generated by a chromatofocusing column.
epithelial cell DNA synthesis although it may have an effect on crypt cell production rate by other mechanisms or be important in synergising with or controlling the release of other potent cytokines in vivo. This has parallels in the study of keratinocyte growth regulation. Direct intra-
dermal injection of interferon-γ results in an increase in keratinocyte proliferation, but used in isolation in vitro it inhibits growth. Granulocyte-macrophage colony stimulating factor and interleukin-3, however, have been found to stimulate keratinocyte growth in vitro. An attempt was made to abrogate the increase in DNA synthesis observed by lamina propria mononuclear cell supernatant by titrated incubation with anti-interferon-γ antiserum and anti-
granulocyte-macrophage colony stimulating factor antibody, but it was found that sheep antiserum or murine monoclonal antibody alone was a potent stimulus to epithelial DNA synthesis (data not shown).

Granulocyte-macrophage colony stimulating factor has been identified initially as important in the control of haematopoietic stem cell expansion. It has not previously been shown to have an effect on the growth of epithelial cells. The physical characteristics of the factor produced in vitro by the lamina propria cells are consistent with those of granulocyte-macrophage colony stimulating factor, which has a great degree of variability in its degree of glycosylation producing quite a wide range in its observed molecular weight. The observation that lower molecular weight fractions of the supernatant inhibited DNA synthesis suggest that the increase observed with crude supernatants may be the net effect of opposing actions of several cytokines.

These observations have implications for the control growth of the epithelium in physiological and pathological states. It may relate epithelial growth control to the amount of antigenic stimulation of the immune cells in the underlying lamina propria.

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6 Bull DM, Bookman MA. Isolation and functional characteri-
7 Magaud J-P, Sargent I, Mason DY. Detection of human white cell proliferative responses by immunoenzymatic measure-
15 Kaplan G, Nusrat A, Sarro EN, Job CK, McElrath JI, Porto JA, et al. Local and systemic effects of intradermal injec-
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