

Urokinase and the intestinal mucosa: evidence for a role in epithelial cell turnover

P R Gibson, I Birchall, O Rosella, V Albert, C F Finch, D H Barkla, G P Young

Abstract

Background—The functions of urokinase in intestinal epithelia are unknown.

Aims—To determine the relation of urokinase expressed by intestinal epithelial cells to their position in the crypt-villus/surface axis and of mucosal urokinase activity to epithelial proliferative kinetics in the distal colon.

Methods—Urokinase expression was examined immunohistochemically in human intestinal mucosa. Urokinase activity was measured colorimetrically in epithelial cells isolated sequentially from the crypt-villus axis of the rat small intestine. In separate experiments, urokinase activity and epithelial kinetics (measured stathmokineticly) were measured in homogenates of distal colonic mucosa of 14 groups of eight rats fed diets known to alter epithelial turnover.

Results—From the crypt base, an ascending gradient of expression and activity of urokinase was associated with the epithelial cells. Median mucosal urokinase activities in each of the dietary groups of rats correlated positively with autologous median number of metaphase arrests per crypt ($r=0.68$; $p<0.005$) and per 100 crypt cells ($r=0.75$; $p<0.001$), but not with crypt column height.

Conclusions—Localisation of an enzyme capable of leading to digestion of cell substratum in the region where cells are loosely attached to their basement membrane, and the association of its activity with indexes of cell turnover, suggest a role for urokinase in facilitating epithelial cell loss in the intestine.

(Gut 1998;43:656-663)

Keywords: urokinase; intestinal epithelium; colon; epithelial proliferation

Urokinase (urokinase type plasminogen activator, uPA) is a neutral proteinase that is secreted by a wide variety of cells, including monocytes, fibroblasts, keratinocytes, cancer cells,¹ and intestinal epithelial cells.² Most cells that secrete uPA also express specific receptors for uPA on their external surface.¹ Urokinase exerts its main physiological action when receptor bound.³⁻⁵ Its range of protein substrates is limited but its ultimate effects are broad: it activates precursor molecules of other proteinases (such as plasmin and metalloproteinases) but it can also act on fibronectin directly.⁶ With such a substrate spectrum and its action predominantly at the cell surface, it is

not surprising that uPA is strongly implicated in cellular functions that involve control of cell-substratum adhesion, such as cell migration associated with epithelial restitution.^{7,8}

The maintenance of an efficient barrier is essential to the normal function of the intestinal epithelium. Tight cell-cell and cell-substratum adhesion is critical to the barrier. However, intestinal epithelium consists of a mobile population of cells that must migrate from their site of birth in the crypt to the functional surface before dying. Sites of adhesion to the substratum must, therefore, be broken and reformed in a carefully controlled manner. Furthermore, epithelial cells are only loosely bound to the basement membrane in the surface compartment⁹ and this may contribute to the shedding of cells to the lumen, the induction of apoptosis, or both. The uPA-plasmin system may be involved in this process, especially as a similar role has been identified in the migration of epithelial cells at other sites such as the skin and cornea.^{7,8} Urokinase activity is associated with epithelial cells from the colon,¹⁰ which secrete uPA² and express its receptor¹¹ in vitro. However, the distribution of uPA expression along the crypt-villus or crypt-surface axis is unknown due to the apparently poor sensitivity of immunohistological detection of uPA in tissue sections.¹²⁻¹⁹

The aims of the present study were twofold. Firstly, as the distribution of functionally important molecules may give clues to their roles, the distribution of uPA protein and activity within the epithelium of small and large intestine was determined. Immunohistological assessment of uPA expression was restricted to human tissue because of species specificity of the antibodies, while the examination of the distribution of uPA activity required the use of rat small intestine due to the well established technique of differentially isolating enterocytes from along the crypt-villus axis. Secondly, the relation of mucosal uPA activity to indexes of epithelial cell turnover was examined in rats fed a variety of diets previously shown to alter epithelial turnover in the distal colon. This approach was based on the notion that the loss of cell-substratum adhesion in the surface epithelial compartment may be a key event in the death of colonic epithelial cells and on the putative role of uPA in the control of cell-substratum adhesion.

Methods

HUMAN COLONIC AND SMALL INTESTINAL MUCOSA

Macroscopically normal colonic mucosa was obtained from human bowel surgically resected for treatment of colorectal cancer ($n=6$). Two

Department of
Medicine, The Royal
Melbourne Hospital

P R Gibson
O Rosella
V Albert
C F Finch
G P Young

Department of
Pathology, University
of Melbourne
I Birchall

Department of
Anatomy, Monash
University, Victoria,
Australia
D H Barkla

Correspondence to:
Associate Prof. P R Gibson,
Department of Medicine,
Royal Melbourne Hospital,
Victoria 3050, Australia.

Accepted for publication
4 June 1998

Table 1 Dietary details, urokinase activities and proliferative kinetic indexes in the distal colonic mucosa of normal rats

Experiment no	Dietary supplement†	Urokinase activity (mIU/mg protein)	Crypt column height (cells)	No of metaphase arrests/crypt column	Metaphase index
1	10% uncooked potato starch	10.8 (1.3–40.2)*	34.4 (32.3–38.4)	3.9 (2.7–7.1)**	11.7 (7.8–19.1)**
	10% cooked potato starch	0.7 (0.2–2.5)	42.4 (25.0–35.6)	2.0 (1.4–3.4)	6.4 (4.8–9.6)
2	10% precooked corn flour	2.2 (1.2–5.5)**	30.3 (25.9–32.3)**	1.8 (1.1–2.5)	6.1 (4.3–7.7)
	10% All Bran plus 10% cooked potato starch	0.8 (0.4–1.7)	35.4 (31.3–37.8)	2.7 (2.1–5.4)	7.8 (6.1–14.3)
3	10% All Bran plus 10% uncooked potato starch	1.6 (1.1–3.9)	34.8 (32.1–40.4)	3.1 (1.2–4.2)	9.0 (3.4–10.4)
	10% guar gum plus 10% cooked potato starch	9.7 (2.4–24.6)**	31.0 (28.3–36.2)	2.7 (0.9–4.9)	8.8 (2.9–16.1)
	10% wheat bran plus 10% cooked potato starch	4.9 (1.3–9.9)	30.5 (26.6–34.3)	2.1 (1.3–3.4)	6.9 (4.2–10.5)
	10% wheat bran plus 10% uncooked potato starch	1.4 (0.7–4.3)	32.7 (29.4–35.6)	2.0 (1.2–16.0)	5.4 (3.6–10.4)
4	10% methylcellulose	0.4 (0.3–0.6)**	33.3 (27.8–38.6)	0.9 (0.4–1.9)	2.7 (1.2–5.4)
	10% wheat bran	0.7 (0.2–1.2)	33.3 (32.8–30.4)	1.2 (1.0–1.7)	3.3 (2.9–5.1)
	10% oat bran	1.0 (0.6–1.7)	32.7 (31.4–35.0)	1.0 (0.6–2.3)	3.2 (1.8–7.0)
	10% guar gum	2.01.1 (0.5–1.5)	36.3 (32.8–40.5)	1.3 (0.6–2.7)	3.8 (1.6–7.1)
5	Nil	1.7 (0.8–2.9)	28.0 (26.2–30.1)**	1.5 (0.8–2.5)**	5.1 (3.1–9.1)*
	15% methylcellulose	2.0 (1.2–3.3)	35.6 (30.1–38.9)	2.6 (1.7–3.9)	7.0 (5.6–11.3)

Results are expressed as median (range).

†Baseline diet same for all studies (see text for details).

* $p < 0.05$ for non-parametric ANOVA across diets within the experiment.

** $p < 0.01$ for non-parametric ANOVA across diets within the experiment.

specimens of terminal ileum were also obtained from these. Tissue was taken at least 10 cm from the margin of the tumour. In two patients, histologically normal biopsy specimens were obtained from the second part of the duodenum at endoscopy, being performed to exclude small intestinal mucosal disease. Mucosa from the terminal ileum of four patients having resection because of Crohn's ileitis and two with ulcerative colitis were also examined.

ISOLATION OF EPITHELIAL CELLS FROM RAT SMALL INTESTINE

Six Sprague-Dawley rats, weighing approximately 250 g each, were fed rat chow prior to sacrifice by carbon dioxide narcosis and cervical dislocation. Three 10 cm segments of small intestine were removed immediately: a duodeno-jejunal segment (denoted "proximal jejunum"), a mid-jejunal segment, and an ileal segment 1 cm proximal to the ileocaecal junction.

Epithelial cells were isolated from the intestinal segments using a modification of the techniques described by Weiser²⁰ and Gratecos and colleagues²¹ as previously reported in detail.²² Briefly, a flanged plastic catheter was passed into the lumen of the intestinal segment and tied into position at the proximal end. A series of sequential steps involved distending the lumen with a buffer and shaking the segment in an orbital shaking water bath at 37°C for varying lengths of time, after which the luminal buffer was retrieved and the lumen flushed. The first cycle aimed to wash the mucosal surface and comprised 10 minutes at 75 rpm with a citrate buffer (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH₂PO₄, 5.6 mM Na₂PO₄). The next 12 cycles used 10 mM phosphate buffered saline (pH 7.3) containing 1.5 mM EDTA and 0.5 mM dithiothreitol and were shaken at 100 rpm for 2, 2, 2, 3, 4, 5, 7, 9, 10, and 10 minutes and then at 150 rpm for 10 and 10 minutes respectively, making a total of 12 fractions. These time points had previously been validated¹⁸ to achieve multiple small fractions towards the top of the villus. The cell suspensions were collected into plastic centrifuge tubes and CaCl₂ was added to a final concentration of 5 mM. The cells were washed twice, then suspended in 1 ml mannitol buffer (50 mM D-mannitol

and 2 mM trizma base in dH₂O, pH 7.4) and mechanically homogenised over ice. The homogenates were stored at -20°C until assay.

DIETARY EXPERIMENTAL PROTOCOL AND RAT DISTAL COLONIC MUCOSA

Sprague-Dawley rats (150–200 g, 24 weeks old) were randomly divided into groups of eight. The rats had free access to water and were housed in dropped bottom wire cages to inhibit coprophagy and to avoid ingestion of sawdust. The rats were fed one of 14 different diets in a series of five consecutive experiments (see table 1). All diets comprised a basic, low fibre diet (plain white wheat flour, skimmed milk powder, and low salt butter with standard vitamin and mineral mixes added) with or without one or more supplements. Supplements used comprised the following: unprocessed wheat bran (Purina Health Foods, Wahgunyah, Victoria), processed wheat bran (All Bran, Kellogg, Sydney, NSW), methylcellulose (Dow, Michigan, USA), oat bran (Anchor Foods, West Footscray, Victoria), guar gum (Pro-col, Rydalmere, NSW), precooked corn flour (Kellogg), and potato starch (Avebe, Veendam, The Netherlands). The potato starch was used either raw or precooked for 15 minutes in sufficient water to ensure gelatinisation of the starch. All supplements comprised 10% of the total weight of the diet, except in the fifth experiment where methylcellulose was added at a ratio of 15%. The diets were standardised so that the protein:carbohydrate:fat ratio was approximately 20:70:10 by weight. The rats were fed the diets for four weeks. The rats were of similar weights (measured weekly) across the dietary groups during the study, confirming the maintenance of general health and nutritional status.

Three hours before death, rats were injected intraperitoneally with 1 mg/kg vincristine. The rats were killed by CO₂ narcosis and cervical dislocation, the peritoneal cavity was rapidly opened, and the entire large bowel removed. Two 1 cm segments were removed from the distal colon (about 2 cm proximal to the rectum). In one segment, the mucosa (epithelium and lamina propria) was scraped from the submucosa using a glass slide. It was placed immediately into ice cold mannitol buffer and was mechanically homogenised at 4°C. Triton

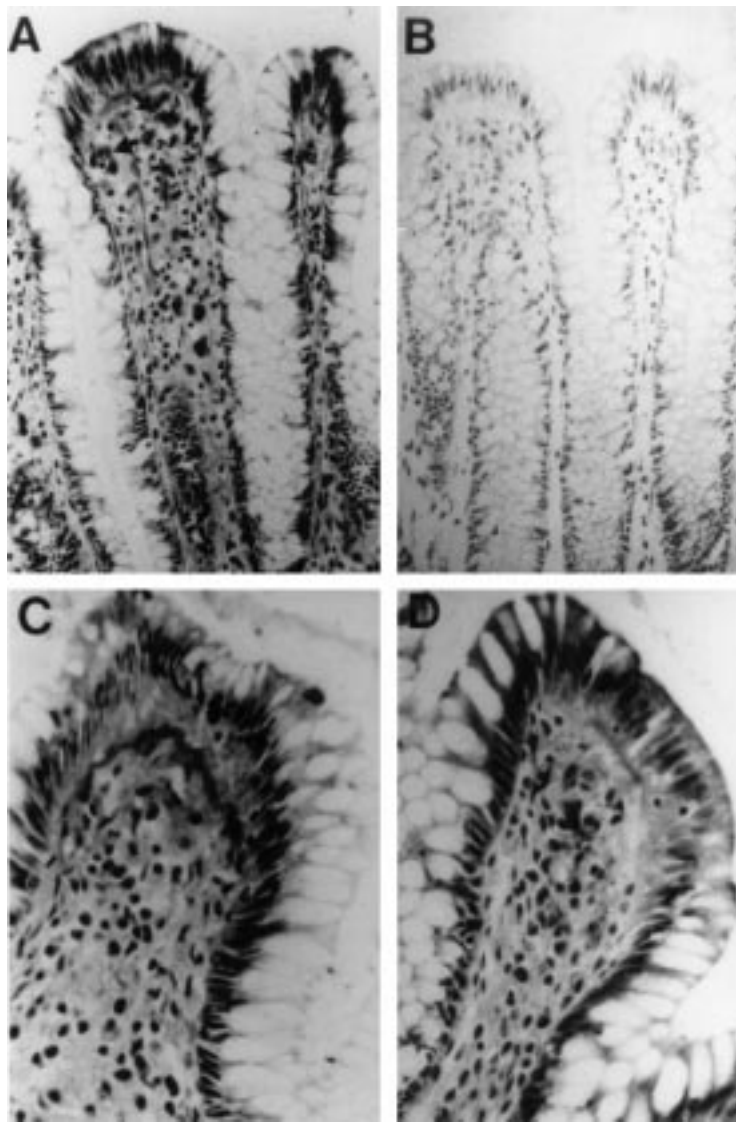


Figure 1 Immunohistochemical staining of sections of histologically normal colonic mucosa from patients with colorectal cancer. (A) Urokinase staining of upper crypt and surface epithelium (original magnification $\times 400$). Arrows show specific staining of cells with morphology consistent with macrophages. (B) Negative control for (A). (D) and (C) Higher power magnification (original magnification $\times 800$) of colonic sections from two other patients showing staining of cytoplasm of surface colonic epithelial cells, and of the basal and underlying basement membranes.

X-100 was added to a final concentration of 0.1%. The homogenates were then stored at -20°C until assay. The other segment was immediately fixed in Bouin's fixative for histological examination.

IMMUNOHISTOCHEMISTRY

Intestinal mucosal biopsy specimens were fixed in a non-formaldehyde based fixative, STF (Streck Laboratories, no. 2656000), for periods ranging from two to 36 hours. Tissue was routinely processed to paraffin wax, sectioned at 4 μm , and the slides dried overnight at 37°C . Sections were deparaffinised, rehydrated, and endogenous peroxidase was blocked using 5% hydrogen peroxide for five minutes. The sections were then washed in 0.5 mol/l Tris buffer (pH 7.6) prior to application of the primary antibody.

A monoclonal antibody²³ that binds to all known forms of human uPA, including recep-

tor bound uPA (American Diagnostica, no. 394), was diluted in Tris buffer (1/50, 1/100, and 1/200) and applied to the sections for one hour at room temperature. Primary antibody binding was then detected with a peroxidase labelled streptavidin-biotin (LSAB) method (Dako K0677) and visualised with diaminobenzidine tetrahydrochloride (Pierce, no. 34065). Controls consisted of replacing the primary antibody with Tris buffer and non-immune serum from the same species as the primary antibody.

MEASUREMENT OF ENZYME ACTIVITIES

Alkaline phosphatase and uPA activities were assayed in aliquots of the cell or mucosal homogenates. Alkaline phosphatase activity was measured spectrophotometrically using *p*-nitrophenol phosphate as substrate as previously described.²⁴ Urokinase activity was measured in mucosal homogenates by the colorimetric method of Coleman and Green.²⁵ Enzyme activities were expressed relative to the mucosal protein content that was measured using bovine γ globulin as standard.²⁶ The DNA content of small intestinal enterocyte populations was also measured fluorometrically²⁷ using calf thymus (Sigma) as standard, and enzyme activities associated with those cells were expressed relative to DNA content.

ASSESSMENT OF EPITHELIAL KINETICS

Paraffin wax sections (2–3 μm) stained with haematoxylin and eosin (H&E) were prepared from Bouin's fixed distal colonic tissue. Twenty crypt columns sectioned down the entire crypt-lumen axis were examined from the distal and proximal colon in each rat. The observer was blinded to the origin of the sections. The total number of epithelial cell nuclei, and position and number of cells arrested in metaphase, were noted for each crypt column and averaged for each animal.^{28, 29} Crypt column height was defined as the number of epithelial nuclei per crypt column. Cell proliferation was expressed as the number of cells arrested in metaphase per crypt column. Cell turnover was determined by calculating the metaphase index—the number of metaphase arrests divided by the total number of cells per crypt column, expressed as a percentage.

Sections of mucosa stained with H&E were also examined; the observer was blinded to the nature of the experimental conditions. Histological assessment was directed specifically towards signs of mucosal inflammation (including the presence of mucosal oedema, polymorphonuclear leucocytes in the lamina propria and intraepithelial compartment, increased chronic inflammatory infiltrate, or crypt abscesses). In addition, the sections were examined for morphologically evident epithelial injury (including the presence of distortion of crypt architecture, goblet cell depletion, or epithelial flattening, erosions, or ulceration).

ETHICAL CONSIDERATIONS

The Board of Medical Research and Human Ethics Committee approved the use of human

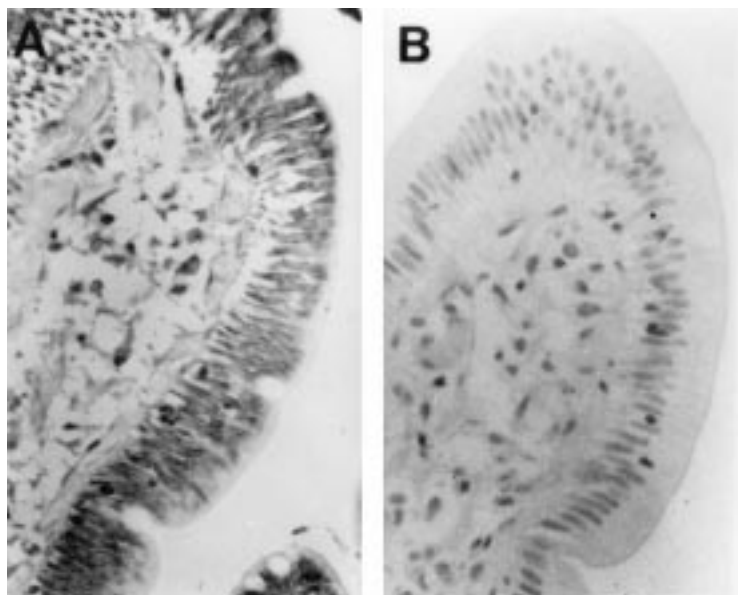


Figure 2 Immunohistochemical staining for urokinase of a section of normal human duodenal mucosa. (A) High power magnification (original magnification $\times 800$) of the tip of a duodenal villus showing cytoplasmic staining for urokinase. Enterocytes in the lower villus and crypt showed minimal staining for urokinase (not shown). (B) Negative control of (A).

tissue and the experimental protocols for the studies on rats were approved by the Board of Medical Research and Animal Ethics Committee of The Royal Melbourne Hospital.

STATISTICAL EVALUATION

All data were analysed with the SPSS for Windows statistical package. A value of $p \leq 0.05$ was considered statistically significant. For small intestinal enterocyte experiments, data from individual cell fractions approximated a normal distribution. Differences in values across the fractions were assessed by one way analysis of variance (ANOVA) while values in the cell fractions across regions were assessed by two way ANOVA. Mean alkaline phosphatase and uPA activities in each fraction were compared by linear regression analysis and the Pearson correlation coefficient (r) was calculated.

For the statistical comparisons of specific diets, analyses were conducted within but not across experiments. As uPA activities did not approximate a normal distribution, median levels of the uPA activity were compared by non-parametric ANOVA techniques. Relations between the mucosal uPA activities and epithelial kinetic indexes across experiments were assessed by linear regression. For these analyses, median levels of the mucosal uPA activities were logarithm transformed to approximate more closely a normal distribution and correlated against median levels of the epithelial kinetic indexes for each diet.

Results

IMMUNOHISTOCHEMISTRY OF HUMAN INTESTINE
Specific staining for uPA was found in the cytoplasm and in the basal membrane of colonic epithelial cells and in the basement membrane beneath the surface epithelium of the colon in all tissues examined (fig 1). There

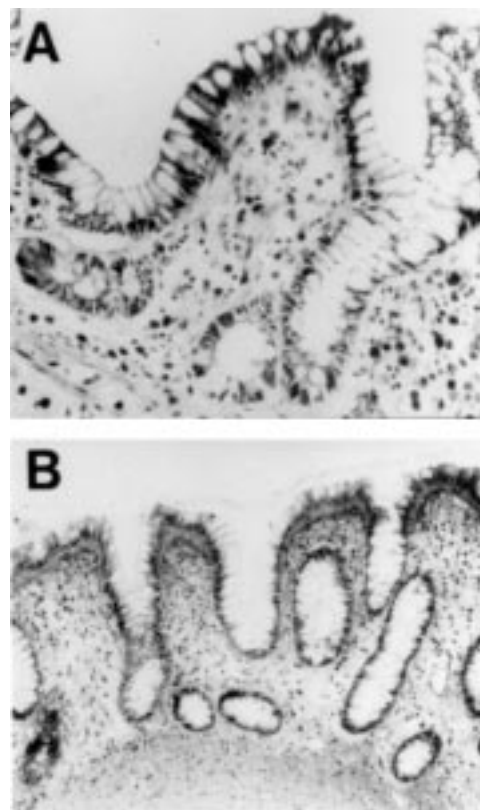


Figure 3 Immunohistochemical staining for urokinase in terminal ileum and colon affected by inflammatory bowel disease. (A) Terminal ileum of a patient with Crohn's disease showing cytoplasmic staining of villous but not crypt epithelium (original magnification $\times 400$). (B) Colonic mucosa of a patient with ulcerative colitis, showing distorted crypt architecture but heavy staining of surface epithelial cells and underlying basement membrane (original magnification $\times 400$).

was a gradient of positive staining for uPA from slight or not detectable in the crypt base to notable in the surface epithelium (fig 1). Villous enterocytes of the ileum and duodenum exhibited specific cytoplasmic staining for uPA but basement membrane staining was less prominent or absent (fig 2). An ascending gradient towards the villous tip of staining of enterocytes for uPA was found, this being strongest at the upper third of the villus. All control sections displayed negative staining (figs 1 and 2). In the lamina propria, cells with large, rounded morphology, consistent with macrophages, also stained positively for uPA (fig 1). These cells were located predominantly beneath the surface epithelium in the colon and the villus tips in small intestine. In tissue affected by inflammatory bowel disease, the pattern of staining was similar to that seen in histologically normal tissue. Examples are shown in fig 3.

UROKINASE ACTIVITIES ASSOCIATED WITH RAT SMALL INTESTINAL EPITHELIAL CELLS

In order to confirm the ascending crypt-villus gradient of uPA expression, subpopulations of enterocytes from rat small intestine were sequentially isolated from proximal jejunum, mid-jejunum, and ileum into 12 fractions and their cell associated uPA and alkaline phosphatase activities measured. As shown in

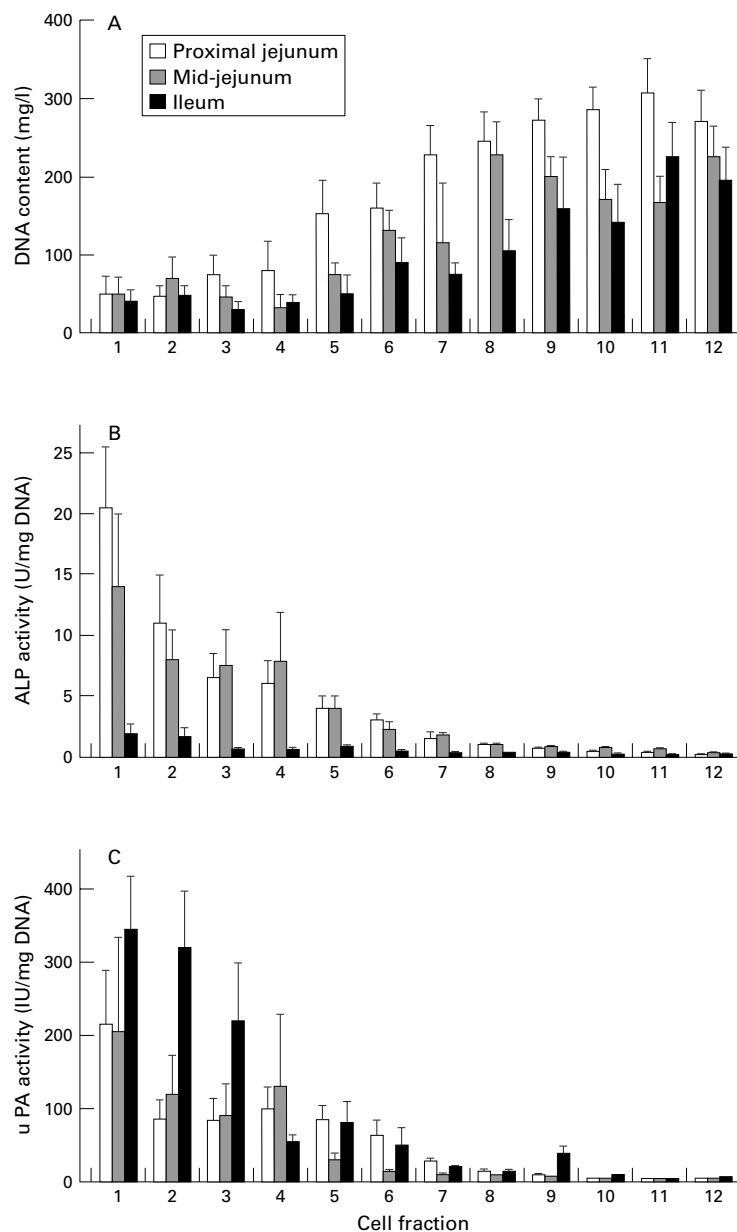


Figure 4 (A) DNA content, (B) alkaline phosphatase activities (ALP), and (C) urokinase (uPA) activities in enterocyte cell fractions from proximal jejunum, mid-jejunum, and ileum of normal rats. Results are shown as mean (SEM). Statistically significant differences (ANOVA) were found across cell fractions for DNA content (all $p < 0.001$), alkaline phosphatase activities ($p < 0.001$ for proximal and mid-jejunum, $p = 0.009$ for ileum), and urokinase activities for proximal jejunum and ileum only ($p < 0.001$ for both).

fig 4, the majority of cells (as determined by total DNA content) were obtained from fractions 5–12 whereas the majority of the activities of alkaline phosphatase and uPA were found in fractions 1–6. Urokinase and alkaline phosphatase activities were highest for the first cell fraction for all sites. Significant differences were found across the fractions for DNA content and for both alkaline phosphatase and uPA activities for cells isolated from all regions except for uPA activities in the mid-jejunal segment (fig 4). The mean alkaline phosphatase activity for each cell fraction was compared with that of the autologous mean uPA activity by linear regression and, as shown in fig 5, highly significant correlations ($r \geq 0.90$, $p < 0.0001$) were found for all regions. Alkaline

phosphatase activities differed across the small intestinal segments ($p = 0.014$; two way ANOVA), with activities being highest in the proximal jejunum and lowest in the ileum. Urokinase activities also differed across the regions ($p = 0.014$) but the difference seemed to be due to higher uPA activities in the ileal cell fractions. No regional difference in the pattern of DNA content across the cell fractions was evident ($p = 0.78$).

THE RELATION OF MUCOSAL uPA ACTIVITY AND EPITHELIAL KINETICS

Because of the putative role of uPA in control of cell-substratum adhesion and because of the relative compartmentalisation of uPA to the major region of epithelial cell death in the mucosa (that is, the surface epithelium), the possible association between epithelial turnover and mucosal uPA activity was examined in the distal colon of rats. Diets varying in their carbohydrate content and amount were used in a series of five experiments to induce changes in epithelial kinetics. The results of some of these experiments have been reported in detail elsewhere,³⁰ but for clarity, medians and ranges for these indexes are shown in table 1. Urokinase activity was measured in homogenates of mucosa taken from sites immediately adjacent to those used for kinetic measurements. Table 1 shows medians and ranges for mucosal uPA activities for all diets. Relative to their respective comparators, resistant starch (as uncooked potato starch) and soluble non-starch polysaccharides were associated with higher uPA activities, while wheat bran and methylcellulose were generally associated with reduced uPA activities. Concurrent ingestion of wheat bran with resistant starch reduced or abolished the notable elevation of uPA activities associated with resistant starch alone. Diet associated alterations in proliferative kinetics or uPA activities were not due to the development of mucosal inflammation or to morphologically evident epithelial injury, as the colonic mucosa from rats in all dietary groups had a normal histological appearance.

The relations between median uPA activities and median proliferative indexes for each dietary group were assessed across all groups of rats by linear regression analysis. Urokinase activity did not correlate with crypt column height ($r = -0.25$; fig 6A). However, a significant positive correlation was observed between uPA activity and both the metaphase index ($r = 0.75$, $p < 0.001$; fig 6B) and the number of metaphase arrests per crypt ($r = 0.68$, $p < 0.005$; fig 6C).

Discussion

The immunohistochemical survey of small and large intestinal mucosa in the present study indicates that the major site at which uPA is found is the epithelium and its associated basement membrane. Furthermore, striking ascending gradients of expression of uPA from crypt to villus tip in the small intestine or from crypt to surface epithelium in the colon were evident. Both the cytoplasm and the cell membrane were stained positively for uPA, suggesting that uPA was being synthesised and

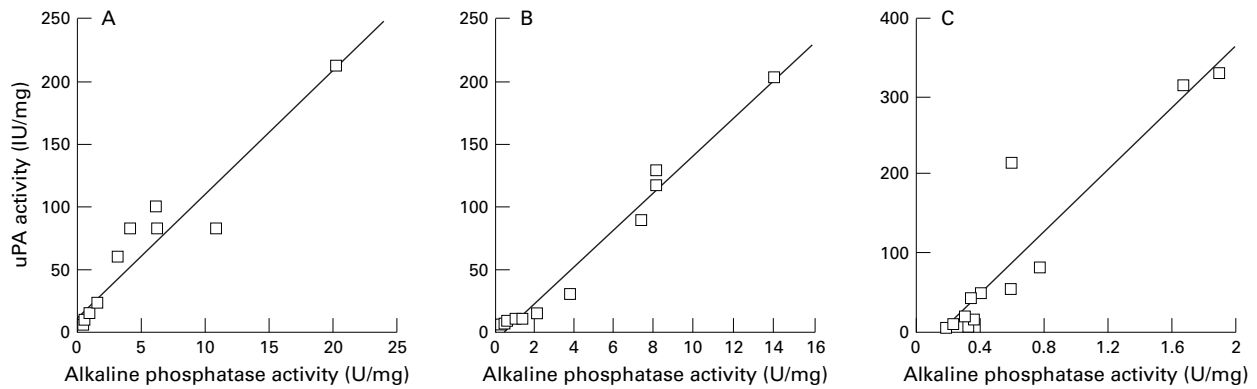


Figure 5 Comparison of mean alkaline phosphatase activities with mean urokinase (uPA) activities across 12 cell fractions from (A) proximal jejunum ($r=0.95$), (B) mid-jejunum ($r=0.99$), and (C) ileum ($r=0.93$). Correlations were all highly significant ($p<0.0001$; linear regression analysis).

secreted by the epithelial cells themselves and not by adjacent macrophages with subsequent binding to epithelial membrane receptors. Whether these findings reflect those occurring in truly normal mucosa of human ileum and colon has not been determined. Despite its normal histological appearance, the colonic epithelium in patients with colorectal carcinoma has reported diffuse abnormalities of epithelial proliferation³¹ and brush border hydrolase expression.³² In inflamed mucosa, epithelial turnover³³ and urokinase activities are elevated.³⁴ Nevertheless, the same patterns of staining were observed. Extending this study to biopsy specimens from individuals without colonic disease is warranted.

An alternative approach is to examine the activity of uPA associated with intestinal epithelial cells isolated from specific regions of the crypt-villus/surface axis. The only validated method of doing this is restricted to the small intestine of experimental animals.^{20, 21} However, a similar ascending gradient of uPA activity along the crypt-villus axis was observed. This supports the notion that the distribution of uPA found immunohistochemically in human intestinal epithelium does reflect that occurring normally.

Previous surveys of the rodent or human intestine for the distribution of either uPA protein or uPA transcripts have not been successful in localising uPA to the epithelium.¹²⁻¹⁹ These have, however, been at odds with the

discovery of uPA protein and activity associated with colonic epithelial cells in vitro and the presence of uPA transcripts within those cells.^{2, 10} Two factors have probably enabled this apparent lack of sensitivity of immunohistochemistry to be overcome in the present study: the binding characteristics of the antibody, and the fixative. The antibody used binds to receptor bound human uPA in addition to uPA that is free or associated with inhibitors.²³ It is possible that the affinity of previously studied antibodies for uPA was unduly affected by the binding in vivo of uPA to its multiple ligands, including uPA receptors,¹ specific and non-specific inhibitors,³⁵ and components of the cell matrix.³⁶ However, we found that staining was not consistent using conventional fixatives, even with protease digestion, but only became so when the non-formalin containing STF fixative was used.

The epithelial compartments in which uPA is most strongly expressed (that is, the surface epithelium of the colon and top half of the villus in the small intestine) have characteristic features. Firstly, mature, differentiated cells that play a major role in the absorptive/digestive function of the intestinal epithelium line them. Secondly, they are the compartments where most epithelial cells are lost from the epithelium, either by being shed into the lumen, or by undergoing apoptosis and subsequently being shed into the lumen and/or phagocytosis by subepithelial macrophages.³⁷

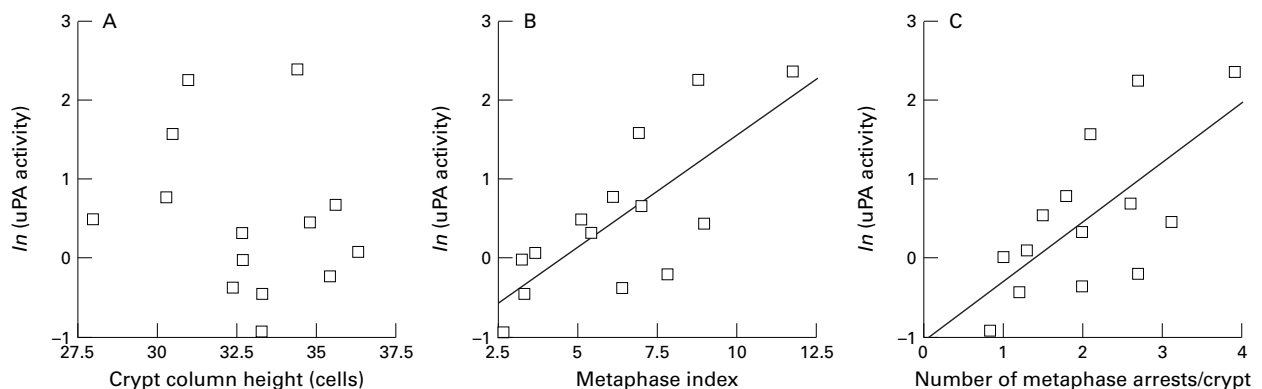


Figure 6 Correlation of (A) median crypt column height, (B) metaphase index, and (C) number of metaphase arrests per crypt column of distal colonic epithelium with median uPA activities (logarithm transformed) in homogenates of adjacent colonic mucosa in normal rats ingesting a variety of diets. Pearson correlation coefficients were -0.25 for crypt column height ($p>0.1$), 0.68 ($p<0.005$) for the number of arrests per crypt, and 0.75 ($p<0.001$) for the metaphase index.

Thirdly, cells in these regions are relatively loosely bound to their basement membrane and can be easily dislodged by mild mechanical trauma.⁹ Evidence would suggest that at least some of these features might be related to the presence of uPA in their immediate microenvironment. The proteolytic action of uPA *in vivo* seems to be localised to receptor bound enzyme. Urokinase receptors are located around cell-cell and cell-substratum contact points³⁸⁻⁴⁰ and are intimately associated with integrins.⁴¹ The major activation product of uPA, plasmin, can digest laminin, fibronectin, and proteoglycans, molecules which bind to specific receptors on the basal membrane of the epithelial cells. Thus, the concomitant presence of elevated levels and activity of uPA and of loosely adherent cells may be more than coincidence and suggests that uPA/plasmin activation may be acting to loosen those cells from their basement membrane in order to facilitate shedding. Alternatively, such loosening may be inducing apoptosis, as loss of cell adhesion is a potent stimulus for apoptosis of colonic epithelial cells.⁴²⁻⁴⁴ Whatever the case, a link between cell death in the intestinal epithelium and uPA is evident.

In order to support this contention further, a possible relation between uPA activity in colonic mucosa and indexes of epithelial proliferative kinetics and turnover was sought. We have previously observed that varying the content and type of undigestible carbohydrate in the diet of rats alters epithelial kinetics³⁰ and mucosal uPA activity in the distal colon.⁴⁵ We therefore took advantage of this by feeding 14 groups of rats differing diets and compared the median uPA activity of distal colonic mucosa in each of the dietary groups with indexes of epithelial population kinetics measured in adjacent tissue. Mucosal uPA activity directly and significantly correlated with an index of the rate of proliferation of colonic epithelial cells (the number of metaphase arrests per crypt) and an index of epithelial cell turnover (the metaphase index), but not with an index of the total cell population (the number of cells per crypt column) in adjacent mucosa. Interpretation of these data must be guarded due to two methodological limitations. Firstly, uPA activity in the whole mucosa rather than that specifically associated with the epithelium was measured. Diet induced changes are assumed to have been epithelium associated as no reason for elevation of lamina propria uPA activity, such as the presence of inflammation, was histologically identified. Secondly, the uPA activity assay itself cannot distinguish pro-uPA from active uPA as all pro-uPA in the homogenates is converted to active uPA by plasmin generated within the assay. However, the ratio of pro-uPA to uPA in mucosal homogenates of normal colonic tissue seems to fall within a fairly narrow range,⁴⁶ and it is unlikely that imbalances in the ratio would significantly alter the findings. Despite these limitations, the direct relation between uPA activity and epithelial proliferation and turnover may indeed represent a real biological relation.

Three possible mechanisms may explain why uPA content and activity increase as cells migrate from crypt to surface epithelium or villous tip. Firstly, increased uPA expression, via enhanced production and secretion of uPA and/or via expression of receptors for uPA, might be a direct consequence of the increased differentiation of intestinal epithelial cells. Urokinase activity closely correlated with autologous alkaline phosphatase activity, an established marker of differentiation. However, in contrast to alkaline phosphatase activity, which progressively decreased more distally, uPA activity was highest in the ileum. Secondly, rather than being part of the differentiation process, the increased expression of uPA may be a cellular response to increasing hypoxia and to luminal conditions which adversely affect enterocytes. Evidence in support of this possibility includes the higher uPA activities in the ileal cell fractions where the luminal bacterial cell population is larger. In addition, during the isolation and culture of colonic epithelial cells, the transcription, production, and secretion of uPA are induced independently of soluble factors in the medium.² Though cellular alkaline phosphatase activity also increases under these conditions,⁴⁷ the magnitude of the response is far less than that for uPA. The third possibility is that uPA associated with the enterocytes may be predominantly derived from adjacent non-epithelial cells rather than from the enterocytes themselves, as has been suggested in colon cancer.¹⁵ Intestinal macrophages are concentrated near the tips of the villi⁴⁸ and beneath the surface epithelium. This and other studies indicate that cells with dendritic morphology stain positively for both uPA and its transcripts.^{15, 18} However, our demonstration of intracellular staining of intestinal epithelial cells for uPA points to an epithelial origin.

The mechanisms by which diets that vary in their content and type of undigestible carbohydrates alter mucosal uPA activity are uncertain, but are likely to reflect changes in the luminal environment. Butyrate, a major product of the bacterial fermentation of carbohydrate, suppresses uPA expression by isolated normal colonic epithelial cells.⁴⁹ Butyrate has been previously postulated to cause the reduction in mucosal uPA activity in distal colon of wheat bran fed rats,⁴⁵ as wheat bran is fermented throughout the colon and delivers high concentrations of butyrate to the distal colonic epithelium.⁵⁰ However, the factors responsible for elevation of uPA activity with resistant starch or soluble fibre are unclear. It may relate to their rapid fermentation in the caecum since the addition of wheat bran, which shifts fermentation of resistant starch more distally in the colon,⁵¹ reduces or abolishes the effect of uncooked potato starch alone. Even though dietary methylcellulose is not fermented in the colon and results in low luminal butyrate levels, it tended to be associated with low uPA activity in the mucosa. This may represent a roughage effect but how this might be achieved is uncertain.

In conclusion, an ascending crypt-villus and crypt-surface gradient of uPA content of the epithelium is present in the small intestine and colon of humans and at least in the small intestine of rats. Whether this gradient is a reflection of differentiation of the cells or of their exposure to changed luminal conditions has not been established. However, the finding of the greatest uPA content and activity associated with cells in the upper part of the villus or surface epithelium suggests roles for this cell surface acting protease in loosening cell-substratum adhesion and in facilitating cell shedding. The significant correlation of mucosal uPA activity with indexes of epithelial proliferation and turnover further supports a causal relation between the uPA/plasmin system and cell loss from the epithelium in the small intestine and colon.

Part of this work has been published abstract form (*Gastroenterology* 1996;100:A915; *J Gastroenterol Hepatol* 1994;9:A108). This study was supported in part by grants from the National Health and Medical Research Council of Australia and Anti-Cancer Council of Victoria.

- 1 Blasi F. Surface receptors for urokinase plasminogen activator. *Fibrinolysis* 1988;2:73-84.
- 2 Gibson PR, Rosella O, Rosella G, et al. Secretion of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 by normal colonic epithelium in vitro. *Gut* 1994;35:969-75.
- 3 Stephens RW, Pollanen J, Tapiovaara H, et al. Activation of pro-urokinase and plasminogen on human sarcoma cells: a proteolytic system with surface-bound reactants. *J Cell Biol* 1989;108:1987-95.
- 4 Kirchheimer JC, Binder BR. Function of receptor-bound urokinase. *Semin Thromb Hemost* 1991;17:246-50.
- 5 Hollas W, Boyd D. Urokinase-dependent proteolysis in cultured cancer is directed by its receptor. *Semin Thromb Hemost* 1991;17:225-30.
- 6 Gold LL, Schwimmer R, Quigley JP. Human plasma fibronectin as a substrate for human urokinase. *Biochem J* 1989;262:529-34.
- 7 Lazarus GS, Jensen PJ. Plasminogen activators in epithelial biology. *Semin Thromb Hemost* 1991;17:210-16.
- 8 Morimoto K, Mishima H, Nishida T, et al. Role of urokinase type plasminogen activator (u-PA) in corneal epithelial migration. *Thromb Haemost* 1993;69:387-91.
- 9 Buck RC. Ultrastructural features of rectal epithelium of the mouse during the early phases of migration to repair a defect. *Virchows Arch* 1986;51:331-40.
- 10 Gibson PR, Van de Pol E, Doe WF. Cell associated urokinase activity and colonic epithelial cells in health and disease. *Gut* 1991;32:191-5.
- 11 Gibson PR, Rosella O. Abnormalities in the urokinase system in colonic crypts from patients with ulcerative colitis. *Inflammatory Bowel Diseases* 1996;2:105-14.
- 12 Kohga S, Harvey SR, Weaver RM, et al. Localization of plasminogen activators in human colon cancer by immunoperoxidase staining. *Cancer Res* 1985;45:1787-96.
- 13 Grondahl-Hansen J, Ralfkiaer E, Kirkeby LT, et al. Localization of urokinase-type plasminogen activator in stromal cells in adenocarcinomas of the colon in humans. *Am J Pathol* 1991;138:111-17.
- 14 Sier CF, Fellbom C, Verspaget HW. Immunolocalization of urokinase-type plasminogen activator in adenomas and carcinomas of the colorectum. *Histopathology* 1991;19:231-7.
- 15 Tanaka N, Fukao H, Ueshima S, et al. Plasminogen activator inhibitor 1 in human carcinoma tissues. *Int J Cancer* 1991;48:481-4.
- 16 Pyke C, Kristensen P, Ralfkiaer E. Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. *Am J Pathol* 1991;138:1059-67.
- 17 Buo L, Lyberg T, Jorgensen HT, et al. Location of plasminogen activator (PA) and PA inhibitor in human colorectal adenocarcinomas. *APMIS* 1993;101:235-41.
- 18 Koretz K, Moller P, Schwartz-Albiez R. Plasminogen activators and plasminogen activator inhibitors in human colorectal carcinoma tissues are not expressed by the tumour cells. *Eur J Cancer* 1993;29A:1184-9.
- 19 Kristensen P, Eriksen J, Dano K. Localization of urokinase-type plasminogen activator messenger RNA in the normal mouse by in situ hybridization. *J Histochem Cytochem* 1991;9:341-9.
- 20 Weiser MM. Intestinal epithelial cell surface membrane glycoprotein synthesis. I. An indicator of cellular differentiation. *J Biol Chem* 1973;248:2536-41.
- 21 Gratecos M, Kniebehler M, Benoit V, et al. Plasma membranes from rat intestinal epithelial cells at different stages of maturation. I. Preparation and characterization of plasma membrane fractions originating from crypt cells and from villous cells. *Biochim Biophys Acta* 1978;512:508-24.
- 22 Albert V, Young GP. Differentiation status of rat enterocytes after intestinal adaptation to jejunoileal bypass. *Gut* 1992;33:1628-43.
- 23 Grondahl-Hansen J, Agerlin N, Munkholm-Larsen P, et al. Sensitivity and specificity of an enzyme-linked immunosorbent assay for urokinase-type plasminogen activator and its application to plasma from patients with breast cancer. *J Clin Lab Med* 1988;111:42-51.
- 24 Young GP, Rose IS, Cropper S, et al. Hepatic clearance of rat plasma intestinal alkaline phosphatase. *Am J Physiol* 1984;247:G419-26.
- 25 Coleman PL, Green GDJ. A sensitive coupled assay for plasminogen activator using a thiol ester substrate for plasmin. *Ann NY Acad Sci* 1981;370:617-26.
- 26 Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-51.
- 27 Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* 1980;102:344-52.
- 28 Albert V, Young GP, Morton CL, et al. Systemic factors are trophic in bypassed rat small intestine in the absence of luminal contents. *Gut* 1990;31:311-16.
- 29 Cameron IL, Ord VA, Hunter KE, et al. Suppression of a carcinogen (1,2-dimethylhydrazine dihydrochloride)-induced increase in mitotic activity in the colonic crypts of rats by addition of dietary cellulose. *Cancer Res* 1989;49:991-5.
- 30 Folino M, McIntyre A, Young GP. Dietary fibres differ in their effects on large bowel epithelial proliferation and fecal fermentation-dependent events in rats. *J Nutr* 1995;125:1521-8.
- 31 Terpstra OT, van Balnkenstein M, Dees J, et al. Abnormal pattern of cell proliferation in the entire colonic mucosa in patients with colon adenoma or cancer. *Gastroenterology* 1987;93:704-8.
- 32 Gibson PR, Folino M, Rosella O, et al. Neoplasia and hyperplasia of the large bowel—focal lesions in an abnormal epithelium. *Gastroenterology* 1992;103:452-9.
- 33 Serafini EP, Kirk AP, Chambers TJ. Rate and pattern of cell proliferation in ulcerative colitis. *Gut* 1981;22:648-52.
- 34 Elliott R, Stephens RW, Doe WF. Expression of urokinase-type plasminogen activator in the mucosal lesions of inflammatory bowel disease. *J Hepatol Gastroenterol* 1987;2:517-23.
- 35 Hart D, Rehemtulla A. Plasminogen activators and their inhibitors: regulators of extracellular proteolysis and cell function. *Comp Biochem Physiol (B)* 1988;90:691-708.
- 36 Stephens RW, Aumailley M, Timpl R, et al. Urokinase binding to laminin-nidogen. Structural requirements and interactions with heparin. *Eur J Biochem* 1992;207:937-42.
- 37 Hall PA, Coates PJ, Ansari B, et al. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J Cell Biol* 1992;119:493-501.
- 38 Pollanen J, Saksela O, Salonen EM, et al. Distinct localizations of urokinase-type plasminogen activator and its type I inhibitor under cultured human fibroblasts and sarcoma cells. *J Cell Biol* 1987;104:1085-96.
- 39 Takahashi K, Ikee K, Gojorbori T, et al. Local function of urokinase receptor at the adhesion contact sites of a metastatic tumour cell. *Thromb Res* 1990;10(suppl):55-61.
- 40 Schmitt M, Chucholowski N, Busch E, et al. Fluorescent probes as tools to assess the receptor for the urokinase-type plasminogen activator on tumor cells. *Semin Thromb Hemost* 1991;17:291-302.
- 41 Wei Y, Lukashev DI, Simon DI, et al. Regulation of integrin function by the urokinase receptor. *Science* 1996;273:1551-5.
- 42 Bates RC, Buret A, van Helden DF, et al. Apoptosis induced by inhibition of intercellular contact. *J Cell Biol* 1994;125:403-15.
- 43 Hermiston ML, Gordon JI. In vivo analysis of cadherin function in the mouse intestinal epithelium: essential roles in adhesion, maintenance of differentiation, and regulation of programmed cell death. *J Cell Biol* 1995;129:489-506.
- 44 Strater J, Wedding U, Barth TFE, et al. Rapid onset of apoptosis in vitro follows disruption of β_1 -integrin/matrix interactions in human colonic crypt cells. *Gastroenterology* 1996;110:1776-84.
- 45 Gibson P, Folino M, McIntyre A, et al. Dietary modulation of colonic mucosal urokinase activity in rats. *J Gastroenterol Hepatol* 1995;10:324-30.
- 46 Sim PS, Stephens RW, Fayle DRH, et al. Urokinase-type plasminogen activator in colorectal carcinomas and adenomatous polyps: quantitative expression of active and proenzyme. *Int J Cancer* 1984;42:483-8.
- 47 Gibson PR, Rosella O, Young GP. Serum-free medium increases expression of markers of differentiation in human colonic crypt cells. *Gut* 1994;35:791-7.
- 48 Hume DA, Allan W, Hogan PG, et al. Immunohistochemical characterization of macrophages in human liver and gastrointestinal tract: Expression of CD4, HLA-DR, OKM1 and the mature macrophage marker, 25F9, in normal and diseased tissue. *J Leuk Biol* 1987;42:74-84.
- 49 Gibson PR, Rosella O, Rosella G, et al. Butyrate is a potent inhibitor of urokinase secretion by normal colonic epithelium in vitro. *Gastroenterology* 1994;107:410-19.
- 50 McIntyre A, Young GP, Taranto T, et al. Different fibers have different regional effects on luminal contents of rat colon. *Gastroenterology* 1991;101:1274-81.
- 51 Young GP, McIntyre A, Albert V, et al. Wheat bran suppresses potato-starch potentiated colorectal tumorigenesis at the aberrant crypt stage in a rat model. *Gastroenterology* 1996;110:508-14.