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

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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 stathmokinetically) 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.

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.3 Urokinase exerts its main physiological action when receptor bound.3,4 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.10 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 13

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

<table>
<thead>
<tr>
<th>Experiment no</th>
<th>Dietary supplement‡</th>
<th>Urokinase activity (mU/mg protein)</th>
<th>Crypt column height (cells)</th>
<th>No of metaphase arrests/crypt column</th>
<th>Metaphase index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% uncooked potato starch</td>
<td>10.8 (1.3-40.2)*</td>
<td>34.4 (32.3-38.4)</td>
<td>3.9 (2.7-7.1)**</td>
<td>11.7 (7.8-19.1)**</td>
</tr>
<tr>
<td>2</td>
<td>10% cooked potato starch</td>
<td>0.7 (0.2-2.5)</td>
<td>42.4 (25.0-35.6)</td>
<td>2.0 (1.4-3.4)</td>
<td>6.4 (4.9-9.6)</td>
</tr>
<tr>
<td>3</td>
<td>10% precooked corn flour</td>
<td>2.2 (1.2-5.5)**</td>
<td>30.3 (25.9-32.3)**</td>
<td>1.8 (1.1-2.5)</td>
<td>6.1 (4.3-7.7)</td>
</tr>
<tr>
<td>4</td>
<td>All Bran plus 10% cooked potato starch</td>
<td>0.8 (0.4-1.7)</td>
<td>35.4 (31.3-37.8)</td>
<td>2.7 (2.1-5.4)</td>
<td>7.6 (6.1-14.3)</td>
</tr>
<tr>
<td>5</td>
<td>10% All Bran plus 10% uncooked potato starch</td>
<td>1.6 (1.1-3.0)</td>
<td>34.8 (32.1-40.4)</td>
<td>3.1 (1.2-4.2)</td>
<td>9.0 (3.4-10.4)</td>
</tr>
<tr>
<td>6</td>
<td>10% guar gum plus 10% cooked potato starch</td>
<td>9.7 (2.4-24.6)**</td>
<td>31.0 (28.3-36.2)</td>
<td>2.7 (0.9-4.9)</td>
<td>8.8 (2.9-16.1)</td>
</tr>
<tr>
<td>7</td>
<td>10% wheat bran plus 10% cooked potato starch</td>
<td>4.9 (1.3-9.9)</td>
<td>30.5 (26.6-34.3)</td>
<td>2.1 (1.3-3.4)</td>
<td>6.9 (4.2-10.5)</td>
</tr>
<tr>
<td>8</td>
<td>10% wheat bran plus 10% uncooked potato starch</td>
<td>1.4 (0.7-4.3)</td>
<td>32.7 (29.4-35.6)</td>
<td>2.0 (1.2-16.0)</td>
<td>5.4 (3.6-10.4)</td>
</tr>
</tbody>
</table>

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.

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isolates 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 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 duodenal segment, which was mechanically homogenised 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.

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...continued...
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 antibody that binds to all known forms of human uPA, including receptor 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. Urokinase activity was measured in mucosal homogenates by the colorimetric method of Coleman and Green. Enzyme activities were expressed relative to the mucosal protein content that was measured using bovine γ globulin as standard. The DNA content of small intestinal enterocyte populations was also measured fluorometrically 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. 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
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 \( p < 0.05 \) 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 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
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, 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...
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 proliferation and brush border hydrolase expression. In inflamed mucosa, epithelial turnover and urokinase activities are elevated. 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. 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. 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. 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. 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, specific and non-specific inhibitors, and components of the cell matrix. 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.

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).

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. 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 points and are intimately associated with integrins. 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. 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 and protein of the diet alters epithelial cell kinetics and turnover. 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, 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 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. Though cellular alkaline phosphatase activity also increases under these conditions, 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 adenocarcinoma. Intestinal macrophages are concentrated near the tips of the villi and beneath the surface epithelium. This and other studies indicate that cells with dendritic morphology stain positively for both uPA and its transcripts. 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. Butyrate has been previously postulated to cause the reduction in mucosal uPA activity in distal colon of wheat bran fed rats, as wheat bran is fermented throughout the colon and delivers high concentrations of butyrate to the distal colonic epithelium. 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, 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 lower 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 the concept of a gradient in uPA/plasmin system and cell loss from the epithelium in the small intestine and colon.

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