

## Cell associated urokinase activity and colonic epithelial cells in health and disease

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### Abstract

It is not known if urokinase-type plasminogen activator (uPA) is associated with normal colonic epithelial cells. The aims of this study were to determine if normal colonic epithelial cells have uPA activity and whether this is concentrated at the cell membrane. In addition, the contribution of colonic epithelial cell associated uPA activity to disease related perturbations of mucosal uPA activity were examined. A highly enriched population of colonic epithelial cells was isolated from resected colon or biopsy specimens by an enzymatic technique. uPA activity was measured in cell homogenates by a specific and sensitive colorimetric method and expressed relative to cellular DNA. In two experiments subcellular fractionation of colonic epithelial cells was performed by nitrogen cavitation followed by ultracentrifugation over a linear sucrose gradient. The fractions collected were analysed for uPA and organelle-specific enzyme activities. Normal colonic epithelial cells have cell associated uPA activity (mean (SEM) 5.6 (1.1) IU/mg, n=18). This co-localised with fractions enriched for leucine- $\beta$ -naphthylamidase and 5'-nucleotidase, markers of plasma membrane. uPA activities in epithelial cells from cancerous colons (9.8 (3.1) n=7) or from mucosa affected by inflammatory bowel disease (3.8 (0.7) n=15) were not significantly different from normal (paired *t* test), while that in epithelial cells from greatly inflamed mucosa was similar to that from autologous normal or mildly inflamed areas (4.4 (1.2) *v* 5.9 (3.6), n=9). Thus normal colonic epithelial cells have cell associated uPA activity which is concentrated on the plasma membranes, suggesting the presence of uPA receptors. Increased mucosal levels of uPA previously reported in patients with inflammatory bowel disease are not due to increased colonic epithelial cell associated uPA.

Urokinase-type plasminogen activator (uPA) is a neutral proteinase which seems to have one major physiological substrate, plasminogen, from which it generates plasmin.<sup>1</sup> Plasmin is also a neutral proteinase which has a broad spectrum of substrates that include fibrin polymers (associated with intravascular thrombosis and extravascular inflammatory lesions), important components of the extracellular matrix and basement membranes (such as laminin, fibronectin, proteoglycans), and procollagenase (to activate collagenase).<sup>1,2</sup> uPA has also been shown to have direct effects on cell biology in vitro such as a mitogenic effect.<sup>3</sup> Some

of the postulated physiological functions of uPA include pivotal roles in fibroblast motility,<sup>4</sup> trophoblast implantation,<sup>5</sup> and tissue involution.<sup>6</sup> In pathological conditions uPA may also have important pathogenetic roles. For example, it is found in increased levels in inflammatory lesions<sup>7,8</sup> where it may contribute to tissue injury as well as to repair processes, and may be an important mechanism in invasiveness and metastasis formation by neoplastic cells.<sup>9</sup>

uPA is secreted by a variety of cells and binds to specific cell receptors which are often expressed by the same cell.<sup>1</sup> Bound uPA is not internalised or degraded but retains its proteolytic activity. The cell surface may indeed be the most physiologically relevant site of action of uPA, as not only is there a concentration of uPA and plasminogen at that site due to specific receptor binding but also chance inhibition by fast-acting inhibitors of uPA would be less likely to occur.<sup>1</sup>

In the human colon uPA activity is present in normal mucosa,<sup>8,10,11</sup> but its cellular origin remains uncertain. In the inflamed colon levels of uPA activity in mucosal homogenates are increased<sup>7,8</sup> but the source also remains undetermined. High levels of uPA activity are also found in colonic neoplasms,<sup>10-15</sup> the level correlating with the apparent degree of invasiveness of the neoplasm.<sup>10,11</sup> Thus, less uPA activity is found in adenomas than carcinomas (though more than in normal mucosa) and the measured uPA activity correlates with the Dukes's staging. The source of uPA is likely to be the neoplastic cells. Moreover, studies of colon cancer cell lines have shown cell associated and secreted uPA as well as uPA receptor expression.<sup>16</sup> There have, however, been no reports of whether normal colonic epithelial cells contain or produce uPA or express uPA receptors. The aims of the present study were twofold: (i) to determine whether normal colonic epithelial cells have cell associated uPA and whether this is concentrated in the cell membrane fraction suggesting the presence of receptors; (ii) to examine whether perturbation of uPA content of epithelial cells from cancer-bearing colons occurs and whether increased colonic epithelial cells-associated uPA may contribute to the increased mucosal uPA content found in inflammatory bowel disease.

### Methods

#### SPECIMENS

Colonic mucosa was obtained from either surgically resected colon or biopsy specimens taken at colonoscopy. The underlying diagnosis was determined by a combination of standard clinical, endoscopic, radiological, and patho-

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logical criteria. The colons studied comprised 18 normal, seven cancerous, and 15 affected by inflammatory bowel disease (nine Crohn's colitis, six ulcerative colitis). Histopathology was assessed in sections of mucosa taken adjacent to those from which colonic epithelial cells were isolated. In patients with inflammatory bowel disease the mucosa was assessed as being non-, mildly, moderately, or severely inflamed, using histopathological criteria as previously described,<sup>17</sup> without knowledge of the uPA results. The study was approved by the relevant ethics committee.

#### CELL ISOLATION

Colonic epithelial cells were isolated from resected mucosa or biopsy material by the collagenase/Dispase technique previously described and characterised.<sup>18</sup> Briefly, mucosa was digested for 1.5 hours in media containing collagenase 50 U/ml and Dispase 1.2 U/ml followed by trituration through a needle and sieving through a stainless steel mesh. Colonic epithelial cells were enriched by three differential sedimentation steps comprising three minute centrifugation at 75 *g*. The resulting population was 92–95% colonic epithelial cells as previously shown,<sup>18</sup> and more than 98% of the cells were viable on trypan blue exclusion.

For some control experiments peripheral blood was obtained from healthy volunteers and leucocyte subpopulations isolated by centrifugation over a discontinuous Ficoll-Paque gradient. The interface cells (mononuclear cells) were harvested and polymorphonuclear leucocytes were separated from the red cells in the pellet by hypotonic lysis of the red cells. Mononuclear cells were activated in some experiments by co-culture with optimal concentrations of mitogen free interleukin-2 enriched supernatants of phytohaemagglutinin stimulated lymphocytes as previously described<sup>19</sup> or phorbol-12-myristate-13-acetate (40 ng/ml) for three days at 37°C.

In addition, COLO 397 cells, a colon cancer cell line (American Type Culture Collection), were used for a control experiment to determine the effect of collagenase and Dispase treatment on uPA activity of epithelial cells. This cell line grows as a monolayer and a single cell suspension was obtained by trypsinisation of the monolayer. Before use, the cells were cultured with constant rotation overnight to allow recovery after trypsinisation while remaining in suspension.

#### SUBCELLULAR FRACTIONATION

As previously described in detail,<sup>20</sup> colonic epithelial cells were first disrupted by nitrogen cavitation using a Parr cell disruption bomb. The homogenate was centrifuged at 1000 *g* for 10 minutes at 4°C and the supernatant collected. Subcellular fractionation was then performed by isopycnic centrifugation of the supernatant over a linear 55–20% sucrose density gradient containing 10 mM hydroxyethylpiperazine-ethanesulphonic acid (HEPES), pH 7.0. The gradient was allowed to equilibrate overnight at 4°C and then 10 ml of cell homogenate was layered onto the gradient. After centrifugation for at least five

hours at 108 000 *g*, 19 equivolume fractions were collected from the bottom of the gradient. These fractions were frozen at –20°C until further assessment.

#### ENZYME ASSAYS

uPA activity was measured by the colorimetric method of Coleman and Green.<sup>21</sup> The assay was shown to be specific for uPA and results were unaffected by the addition of antibody specific for tissue plasminogen activator as previously described.<sup>15</sup> Freshly isolated cells were placed in a 50 mM glycine buffer (pH 7.8) containing 0.1% Triton X-100 and 0.1% gelatine and homogenised. To 20 µl of tissue homogenate, 20 µl (2 µg) of affinity purified human plasminogen,<sup>22</sup> and 20 µl of assay glycine buffer containing 6-aminocaproic acid (5 mM) were added and incubated for 45 minutes at 37°C. The plasmin generated was then assayed by the addition of 200 µM of *z*-lysine-thiobenzyl ester (Peninsula Laboratories, San Carlos CA, USA) and 1 ml of reagent containing 0.2 M phosphate pH 7.5, 0.2 M KCl, 220 µM 5,5'-dithiobis[2 nitrobenzoic acid], and Triton X-100, and further incubated for 30 minutes at 37°C. The colour development was terminated by the addition of Trasylol (15 µg in 20 µl) and the absorbance read at 412 nm using a spectrophotometer. Activity found was corrected for the presence of plasminogen-independent proteinases in the tissue homogenates by subtraction of absorbances obtained in a system identical to that above except plasminogen was not added. Because the assay was carried out in the presence of plasmin, the uPA activity measured comprises both pro-uPA and active uPA and, therefore, represents total uPA activity. This was expressed as IU/mg DNA. The DNA content of cell homogenates was determined fluorometrically by complexing DNA with 4,6'-diamidino-2-phenylindole 2 HCl using calf thymus DNA as standard.<sup>23</sup>

To determine organelle distribution in the subcellular fractions obtained (as above), activities of enzymes enriched in specific organelles were measured. The activities of Mn<sup>2+</sup> stimulated leucine-β-naphthylamidase, 5'-nucleotidase, monoamine oxidase, arylsulphatase C, galactosyl transferase, and β-glucuronidase were measured as previously described.<sup>20</sup> Lactate

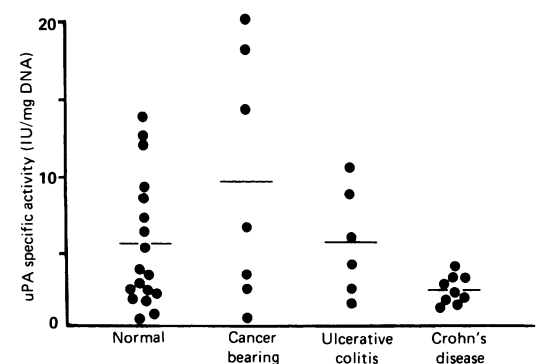


Figure 1: Urokinase activity of homogenates of colonic epithelial cells from macroscopically normal mucosa of normal or cancer bearing colons or from colons affected by ulcerative colitis or Crohn's disease. The bars represent mean values. uPA = urokinase-type plasminogen activator.

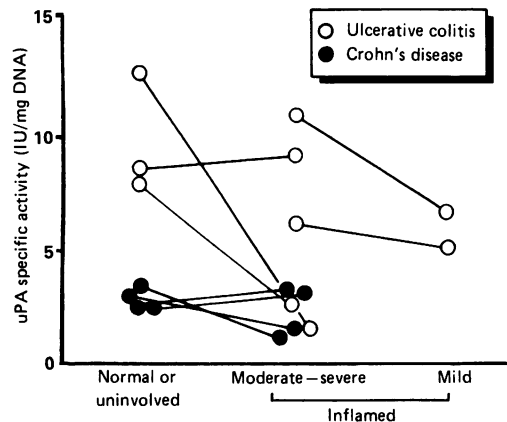


Figure 2: The effect of mucosal inflammation on the urokinase (uPa) activity of homogenates of colonic epithelial cells from colons affected by ulcerative colitis or Crohn's disease.

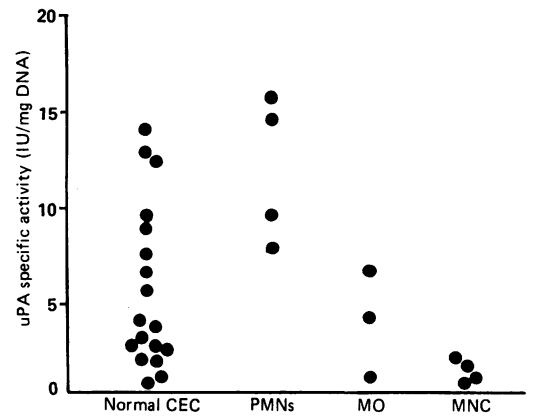


Figure 3: Urokinase (uPa) content of normal colonic epithelial cells (CEC), polymorphonuclear leucocytes (PMNs), and peripheral blood monocytes (MO) or mononuclear cells (MNC).

dehydrogenase activity was determined spectrophotometrically using pyruvate and nicotinamide adenine dinucleotide (reduced form).

STATISTICAL EVALUATION

Quantitative data are expressed as mean (SEM). The activities of uPA in the differing cell populations were compared using Student's *t* test. A *p* value of <0.05 was considered significant.

Results

Homogenates of normal colonic epithelial cells had mean detectable uPA activities of 5.6 (1.1) IU/mg DNA in 18 populations (Fig 1). Levels in epithelial cells isolated from macroscopically normal mucosa from cancerous colons (9.8 (3.1) IU/mg, n=7) or from colons affected by inflammatory bowel disease (3.8 (0.7) IU/mg, n=15) were not significantly different from normal. For patients with inflammatory bowel disease no difference was seen between activities in epithelial cells from ulcerative colitis and Crohn's disease patients (Fig 1). The severity of the

inflammation had no influence on the measured uPA levels. Thus, as shown in Figure 2, colonic epithelial cells from moderately to severely inflamed mucosa had similar activity to those from autologous areas of colon which were only mildly inflamed. In addition, activity in epithelial cells from inflamed mucosa in patients with distal ulcerative colitis was similar to that in autologous apparently normal mucosa proximal to the involved colon. There were no differences in uPA specific activity in epithelial cells isolated from different regions of the large bowel and from biopsy specimens *v* resected colon (data not shown).

The possibility that the uPA activity in the colonic epithelial cell population was due to contaminating non-epithelial cells was examined by measuring the activity associated with normal cells which are not only known to secrete or express uPA receptors, or both, but also to comprise a proportion of the contaminating cell subpopulation. The uPA activity of unseparated or plastic adherent mononuclear cells and of polymorphonuclear leucocytes was similar to or less than the activity of the whole isolated cell population (Fig 3). Activation of mononuclear cells by co-culture for three days with phorbol myristic acid or interleukin-2 rich supernatants increased DNA content two to four fold but decreased cell associated uPA activities (data not shown).

The effect of the colonic epithelial cell isolation process on cell associated uPA activity was assessed in two ways. Firstly, collagenase and Dispase were assessed for intrinsic uPA-like activity in the colorimetric assay. No activity was found. Thus, inadequate washing of the enzymes from the cell suspensions was not responsible for the measured activity. Secondly, to determine whether the enzyme digestion was stripping urokinase or its receptors, or both, from the cell membrane, mononuclear or COLO 397 cells were co-cultured with collagenase and Dispase at the same concentration and for the same concentration and for the same duration used in the extraction procedure and then washed in a similar fashion. The uPA activity in the treated population was compared with that in untreated cells. Enzyme treatment tended to increase rather than decrease cell associated uPA activity. Thus, uPA activity changed from 1.1

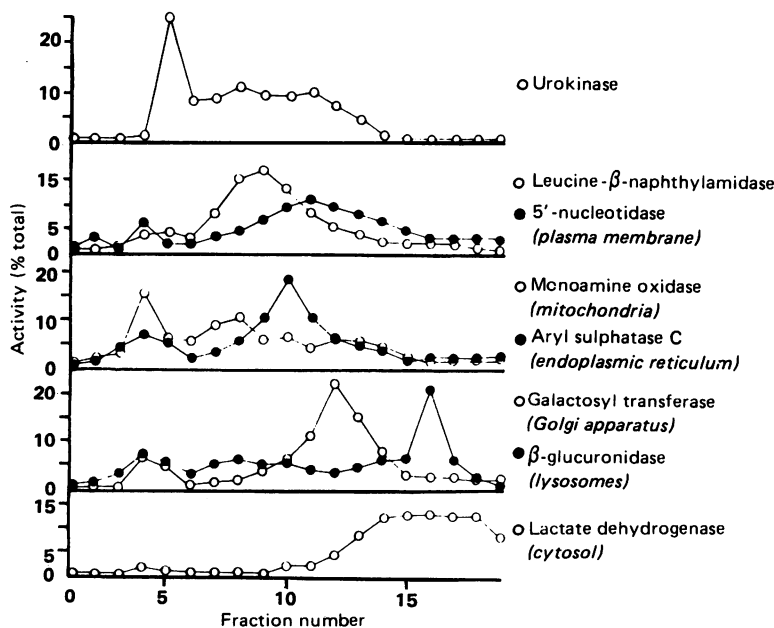


Figure 4: The localisation of organelle enriched enzymes in subcellular fractions of colonic epithelial cells.

(0.4) IU/mg to 3.2 (1.3) IU/mg in four mononuclear cell experiments and 1.1 IU/mg to 1.6 IU/mg in one COLO 397 experiment.

Two colonic epithelial cell populations from mucosa of macroscopically normal colon were subjected to subcellular fractionation. Nineteen fractions were obtained and the activity of uPA as well as that of enzymes enriched in plasma membrane (leucine- $\beta$ -naphthylamidase, 5'-nucleotidase), mitochondria (monoamine oxidase), endoplasmic reticulum (arylsulphatase C), Golgi apparatus (galactosyl transferase), lysosomes ( $\beta$ -glucuronidase), and cytosol (lactate dehydrogenase) were measured in all the fractions. Both experiments showed similar results and those from one experiment are shown in Figure 4. In fractions 4-6 poor separation of enzyme activities was seen but good separation of enzyme activities and, therefore, organelles was seen in the other fractions. uPA activity most closely followed the distribution of the plasma membrane markers, leucine- $\beta$ -naphthylamidase and 5'-nucleotidase, and did not follow the elution profile of any other marker. These findings suggest that uPA is located on the plasma membrane.

### Discussion

The present study shows that the colonic epithelial cell enriched population obtained by the collagenase/Dispase isolation technique has uPA activity which seems to be concentrated at the plasma membrane. This suggests that cell membrane receptors for uPA may also be found on colonic epithelial cells as have been shown to reside on other cell types.<sup>16</sup> Whether the uPA is produced and secreted by colonic epithelial cells or by other cells in the colonic mucosa before subsequent binding to surface receptors on colonic epithelial cells, however, has not been examined. It is likely that uPA activity is predominantly associated with epithelial cells, since non-epithelial contaminating cells comprise a minority of the total nucleated cell population. Moreover, cells known to secrete uPA or carry uPA receptors (peripheral blood mononuclear cells and polymorphonuclear leucocytes), or both, contain cell associated activities similar to those found for the total isolated cell population. Previous immunohistological studies of normal human colonic mucosa using antibodies to active uPA have generally failed to detect any uPA protein associated with colonic epithelial cells.<sup>24,25</sup> This apparently contradictory finding may reflect the low sensitivity of detection of the proenzyme of uPA in tissue sections by these methods (unpublished observations) or shielding of receptor bound uPA from the antibodies.

The digestion phase of the isolation process may potentially alter uPA content by, for example, removing cell surface receptors. Collagenase and Dispase treatment, however, tended to increase rather than decrease uPA activity of mononuclear or COLO 397 cells. Since these proteinases have no intrinsic uPA-like activity, residual enzyme cannot be responsible for the changes found. Likewise, the brief duration of exposure to the enzymes (1.5 hours) is too short for quantitatively significant induc-

tion of uPA production or receptor display. The proteinases may be unmasking uPA by dissociating complexes of plasminogen activator inhibitors and uPA on the cell surface with the resultant apparent increase in measured activity. Studies using assays of uPA protein rather than activity may resolve this question.

Neoplasms of the colon and rectum have increased levels of uPA activity.<sup>10-15</sup> Immunohistochemical studies of tissue sections<sup>9</sup> as well as investigation of colon cancer cell lines<sup>16</sup> suggest that the neoplastic cell is the likely source of such increased activity. The demonstration of uPA activity associated with normal colonic epithelial indicates that the ability of neoplastic cells to express uPA is not a neoplasia-specific phenomenon but probably represents an exaggeration of normal. Colonic epithelial cells from histologically normal mucosa in cancerous colons have been shown to exhibit some abnormal features such as increased proliferative activity,<sup>26</sup> but in the present study no perturbation of colonic epithelial cell associated uPA activity was evident.

The biology of colonic epithelial cells is perturbed by colonic inflammation *per se*<sup>17</sup> and additional specific abnormalities have been documented in colonic epithelial cells from patients with ulcerative colitis.<sup>17,27,28</sup> The normal colonic epithelial cell associated uPA activity in patients with Crohn's disease and ulcerative colitis shown in this study indicates that perturbation of uPA may not be part of these abnormalities. Moreover, such normal activity indicates that the increased uPA activity of homogenates of inflamed colonic mucosa<sup>7,8</sup> is not due to increased uPA associated with the colonic epithelial cells. It is more likely to relate to inflammatory cells, such as polymorphonuclear leucocytes and newly arrived monocytes, which probably actively secrete uPA into the extracellular fluid. Plasminogen activator inhibitors, however, also seem to be present in increased concentrations in the inflamed colonic mucosa.<sup>8</sup> The possibility that inhibitors bound to receptor associated uPA on colonic epithelial cells may be masking an increased expression has not been excluded and, as suggested above, further studies using an assay of uPA protein rather than activity are needed.

This study has provided the first evidence that colonic epithelial cells have cell associated uPA activity and has indirectly suggested that uPA receptors may indeed be present on their cell membrane. Future studies need to assess directly receptor expression and to examine whether colonic epithelial cells themselves produce and secrete uPA. Perturbations of colonic epithelial cell biology which occur secondary to mucosal inflammation *per se* and specifically in ulcerative colitis do not seem to be reflected in perturbed colonic epithelial cell associated uPA activity. Moreover, the increased mucosal activities of uPA in inflamed colon are unlikely to be secondary to that associated with colonic epithelial cells.

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