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

P R Gibson, E van de Pol, W F Doe

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-β-naphthylamide 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) vs 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. 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 pro-collagenase (to activate collagenase). uPA has also been shown to have direct effects on cell biology in vitro such as a mitogenic effect. Some of the postulated physiological functions of uPA include pivotal roles in fibroblast motility, trophoblast implantation, and tissue involution. In pathological conditions uPA may also have important pathogenetic roles. For example, it is found in increased levels in inflammatory lesions 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.

uPA is secreted by a variety of cells and binds to specific cell receptors which are often expressed by the same cell. 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 may be fastacting inhibitors of uPA would be less likely to occur.

In the human colon uPA activity is present in normal mucosa, but its cellular origin remains uncertain. In the inflamed colon levels of uPA activity in mucosal homogenates are increased but the source also remains undetermined. High levels of uPA activity are also found in colonic neoplasms. The level correlating with the apparent degree of invasiveness of the neoplasm. Thus, less uPA activity is found in adenomas than carcinomas (though more than in normal mucosa) and the measured uPA activity correlates with the Duke'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. 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-
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, 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. 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, 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 coculture with optimal concentrations of mitogen free interleukin-2 enriched supernatants of phytohaemagglutinin stimulated lymphocytes as previously described 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, colon 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-ethane-sulphonic 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. 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. 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 ml of tissue homogenate, 20 ml (2 μg) of affinity purified human plasminogen, and 20 ml of assay glycine buffer containing 6-aminoacproic 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 proteases 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.

To determine organelle distribution in the subcellular fractions obtained (as above), activities of enzymes enriched in specific organelles were measured. The activities of Mn-stimulated leucine-β-naphthylamidase, 5'-nucleotidase, monoamine oxidase, ariysulphatase C, galactosyl transferase, and β-glucuronidase were measured as previously described. 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.](http://gut.bmj.com/Downloadedfrom)
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 of 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 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).

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


nuclear cell experiments of 1 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-β-naphthylamide, 5'-nucleotidase), mitochondria (monooamine oxidase), endoplasmic reticulum (arylsulphatase C), Golgi apparatus (galactosyl transferase), lysosomes (β-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-β-naphthylamide 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. 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. 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-
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Gut 1991 32: 191-195
doi: 10.1136/gut.32.2.191

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