Ulcerative colitis – a disease characterised by the abnormal colonic epithelial cell?

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SUMMARY The leakiness of the cell membranes of colonic epithelial cells isolated by the collagenase/Dispase technique from normal or diseased colons was assessed in a 4 h ^{51}Cr release assay. Cells from normal, adenoma bearing or cancer bearing colons showed ^{51}Cr release of 8% or less in almost all of 46 cell populations tested. In contrast, cells from mucosa affected by ulcerative colitis [11.9 (4.3%) $n=23$] or Crohn's disease [8.4 (2.7%) $n=18$] released significantly more ^{51}Cr than the non-inflamed groups. Values are expressed as mean (SD). Overall, release values were greater in ulcerative colitis than Crohn's disease ($p<0.01$). In Crohn's disease, cells obtained from histologically inflamed mucosa released significantly more ^{51}Cr [9.7 (2.5%) $n=11$] than those from non-inflamed mucosa [6.4 (1.5%) $n=7$, $p<0.02$] whereas, in ulcerative colitis, abnormal release values were found in 8 of 13 cell populations isolated from mucosa showing no histological evidence of active disease. In five patients with distal ulcerative colitis, cells from mucosa not apparently involved demonstrated normal ^{51}Cr release in four of five studies despite abnormal release from cells from involved mucosa suggesting that a diffuse abnormality of the colonic epithelial cell is not usually present. These data indicate that chronic mucosal inflammation *per se* is associated with abnormalities of the colonic epithelial cell but that, in ulcerative colitis, the abnormality remains in many patients with quiescent disease. Identification of the local factors responsible for such an abnormality may contribute to an understanding of the pathogenesis of ulcerative colitis.

The colonic epithelial cell has an increased turnover in patients with ulcerative colitis.¹⁻⁴ The most marked increase is seen in actively inflamed mucosa but increased turnover is also found when the disease is clinically and histopathologically quiescent.⁴ The underlying pathogenetic mechanisms for these observations are uncertain. Increased cell turnover implies that colonic epithelial cells are being lost from the mucosa at an increased rate. Four possible mechanisms may be involved. First, proliferation *per se* may, by the pressure of cells migrating up the crypt, force cells from the surface of the mucosa. Second, colonic epithelial cells may be detached from the basement membrane as may occur in the bronchial epithelium in asthma.⁵ The mechanism may be through the action of mucosal proteases such

as plasmin⁶ and it has been shown that urokinase type plasminogen activator is present in markedly increased concentrations in the mucosa of active ulcerative colitis.⁷ Third, injury to the colonic epithelial cell may cause premature cell death leading to secondary detachment from the basement membrane. Postulated mechanisms for such epithelial cell injury include the action of cytotoxic cells,^{8,9} antibodies,^{10,11} lymphotoxin¹² or products of acute inflammatory cells or the metabolic effect of inhibition of fatty acid oxidation.^{13,14} Fourth, the colonic epithelial cell may be inherently abnormal as suggested by studies of mucin subspecies¹⁵ with a possible shortened life span.

Whether abnormality of colonic epithelial cell turnover is specific to ulcerative colitis or just a reflection of mucosal inflammation is uncertain but a recent report suggests that crypt cell proliferation is normal in Crohn's colitis.¹⁶ The rate of crypt replica-

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tion (as measured by the proportion of branched crypts) is increased in Crohn's disease and ulcerative colitis¹⁷ but the relationship of this to colonic epithelial cell turnover is not clear.

The aims of this study were to determine whether the colonic epithelial cell exhibits evidence of abnormal behaviour in inflammatory diseases of the colon and to examine the relationship of this to mucosal inflammation and the underlying disease. This has been assessed by isolating colonic epithelial cells from their natural microenvironment and by measuring the leakiness of their cell membranes *in vitro*. The findings in cells from mucosa affected by ulcerative colitis were compared with those from mucosa affected by Crohn's disease or other types of colitis and from histologically normal mucosa.

Methods

PATIENTS AND SPECIMENS

Colonic mucosa was obtained from two sources, surgically resected colon and colonoscopic biopsies. Patient details including the source of mucosa are shown in the Table. The diagnoses were made on standard clinical, radiological, and histopathological criteria. The protocol for the study was approved by the Medical Ethics Committee of the ACT Health Authority in September, 1986.

HISTOPATHOLOGICAL ASSESSMENT

Mucosa adjacent to that studied was examined histopathologically by one pathologist (PJB) without knowledge of the results of other studies. The degree of inflammation was graded on a scale of 0 to 3 according to the method of Savarymuttu *et al.*¹⁸ Mucosa affected by ulcerative colitis was graded 0 in 12, 1 in 1, 2 in 5, and 3 in 3. Gradings for Crohn's disease were 0 in 7, 1 in 3, 2 in 1, and 3 in 6.

ISOLATION OF COLONIC EPITHELIAL CELLS

The epithelial cells were isolated using the collagenase Dispase method as previously described.¹⁹ Briefly, mucosa was prepared from surgical specimens by scraping it from the submucosa with a glass slide. The mucosa was then minced using crossed scalpels. Biopsy specimens were cut into smaller pieces with a scalpel blade. The minced mucosa was then digested for 1.5 h in culture medium (see below) containing 2.4 U/ml Dispase I (Boehringer Mannheim) and 50 U/ml collagenase (Type IV, Worthington) with gentle rotation. The digest was then triturated through a 21 gauge needle, washed in Hanks Balanced Salt Solution (HBSS) by centrifuging at 75 g for two to three minutes and passed through a stainless steel mesh to remove mucus and poorly digested lamina propria matrix. The cells were

then resuspended in HBSS and washed two more times, centrifuging at 75 g for two to three minutes to remove single cells by differential sedimentation. The final cell population was resuspended in culture medium which comprised DMEM containing 20% Nu serum (Collaborative Research), 2% luria broth (bactotryptone 10 g, yeast extract 5 g, NaCl 5 g in 11 water), 4mM L-glutamine, 50 µg/ml gentamicin, and 50 U/ml penicillin.

The cells were counted after ⁵¹Cr labelling and viability assessed by 0.1% trypan blue exclusion. Greater than 97% of cells just before assay and >95% at the end of 4 h culture were viable irrespective of the underlying disease. Cell differential counts were assessed both by phase contrast microscopy of fresh cell preparations and by microscopy of cytocentrifuge preparations stained with Wright's stain. As previously reported in detail,¹⁹ epithelial cell purity (excluding red cells) was 92–96% (mean 94.5%) with no disease related differences. Of the non-epithelial cells, no disease differences in composition or morphology were seen apart from the presence of polymorphonuclear leucocytes in populations from inflamed mucosa but these comprised <1% of the total cell population. Red cells comprised a variable component with 4–30 red cells seen per 100 epithelial cells.

⁵¹Cr-RELEASE ASSAY

One to two million epithelial cells from surgical specimens or the entire isolated epithelial cell population from colonoscopic biopsies were incubated in 200–500 µl culture medium containing 100 uCi sodium ⁵¹chromate for one hour. They were then washed three times in culture medium, being resuspended between washes by gentle tipping of the test tubes to avoid cell damage. The cells were then counted in a haemocytometer (Neubauer chamber) and resuspended in culture medium at a concentration of 50 000 cells/ml.

Assays were carried out in U-bottomed 96 well

Table 1 Patient details and source of colonic mucosa

Disease	Source of colonic mucosa		Age range (mean) (years)	Men/ women
	Resection	Biopsies		
Normal	0	16	16–84 (50)	10/6
Miscellaneous*	5	0	37–84 (53)	3/2
Adenoma	0	10	46–76 (63)	7/3
Carcinoma†	15	1	54–84 (65)	8/8
Ulcerative colitis	2	21	22–71 (42)	11/12
Crohn's disease	3	14‡	16–75 (39)	8/9

*Diverticulosis coli (2), redundant sigmoid loop (1), caecal lipoma (1), Meckel's diverticulum (1); †Dukes' grading: 4-A, 4-B, 8-C/D; ‡One patient studied on two different occasions.

microculture plates with 100 μ l epithelial cells made up to a total volume of 200 μ l/well with culture medium (spontaneous release) or 5% Triton-X-100 in medium (maximal release). Spontaneous release of ^{51}Cr was assessed at the beginning of the assay (SR0) in four to six replicate wells by centrifuging the plate immediately at 200 g for three minutes, and aspirating 100 μ l cell free supernatant for counting. The four hour spontaneous release (SR4) and maximal release (M) were assessed in 8–12 replicate wells in a separate plate. After a four hour incubation at 37°C 5% CO_2 95% air, 100 μ l cell free supernatant were aspirated from each well and counted in a gamma counter (Packard Auto-Gamma 500). The four hour ^{51}Cr release was calculated by the following formula:

$$4 \text{ h } ^{51}\text{Cr release} = (\text{SR4} - \text{SR0}) / \text{M} \times 100\%.$$

SR0 was 8.2 (2.9%) with no disease related differences. The SD of variation between replicate wells was always less than 10% of the mean.

STATISTICAL EVALUATION

Parametric data have been compared using Student's *t* test while, for non-parametric data, Wilcoxon's rank-sum test was used. *p* Values in the results have been determined with parametric testing unless otherwise stated.

Results

As shown in Figure 1, all but one epithelial cell population from histologically normal mucosa from

Fig. 1 Four hour ^{51}Cr release in vitro from colonic epithelial cells isolated from colons which were normal, adenoma- or carcinoma bearing, or affected by ulcerative or Crohn's colitis. Broken line is the upper limit of normal (8%).

Fig. 2 Hourly ^{51}Cr release in vitro over four hours from colonic epithelial cells isolated from three patients with ulcerative colitis (■) and nine patients with non-inflammatory bowel disease (○) expressed as mean (SD).

adenoma bearing or normal colon demonstrated 8% or less release over four hours. Cells from cancer bearing colons showed normal or, in the minority, slightly increased release values. In contrast, epithelial cells from mucosa affected by colitis showed widely varying release values. Overall, ^{51}Cr release from cells from mucosa affected by ulcerative colitis was significantly greater than that from mucosa affected by Crohn's disease ($p < 0.01$) or from the three groups with histologically normal mucosa ($p < 0.001$). Release from cells from mucosa affected by Crohn's colitis was also significantly greater than that from the normal ($p < 0.05$), adenoma ($p < 0.01$), and carcinoma ($p < 0.05$) groups. The ^{51}Cr release from epithelial cells isolated from mucosa affected by ulcerative colitis occurred in a linear fashion over the four hour period, a pattern similar to that of cells from normal mucosa as previously reported (Fig. 2).

The relationship between the degree of histopathologically evident mucosal inflammation and ^{51}Cr release was examined in both Crohn's disease and ulcerative colitis groups. No difference between release values of cells from mucosa with grades 1, 2, or 3 inflammation was evident and the results have therefore been expressed as either non-inflamed (grade 0) or inflamed (grades 1, 2, or 3). In Crohn's disease, ^{51}Cr release of cells from mucosa not currently inflamed was on all but one occasion within normal limits whereas nine of 11 cell populations from inflamed mucosa exhibited increased release (Fig. 3). Overall, the presence of inflammation was associated with significantly greater ^{51}Cr than that from non-inflamed mucosa in patients with Crohn's

Fig. 3 Relationship of four hour ⁵¹Cr release in vitro from colonic epithelial cells to the presence or absence of histopathologically evident mucosal inflammation in patients with ulcerative colitis or Crohn's disease. Broken line represents the upper limit of normal.

disease involving the colon ($p < 0.01$). Epithelial cells from inflamed mucosa in ulcerative colitis were always associated with release greater than 9% and cells from mucosa previously involved but not inflamed at the time of study (confirmed histologically) showed normal release values in only five of 13

Fig. 4 Four hour ⁵¹Cr release in vitro from colonic epithelial cells isolated from mucosa involved and not apparently involved with ulcerative colitis in the same patient. Shaded circles represent results obtained from cells isolated from inflamed mucosa. Broken line represents the upper limit of normal.

populations (Fig. 3). There was no significant difference ($0.1 > p > 0.05$, Wilcoxon's rank-sum test) between the release from cells derived from mucosa actively inflamed and that from mucosa showing evidence of past inflammation only.

Additional studies were done to determine whether epithelial cells throughout the colon showed increased ⁵¹Cr leakage in patients with ulcerative colitis confined to the distal colon or rectum. Epithelial cells were assessed from mucosa not apparently involved with ulcerative colitis as judged by normal histology and by the findings of past investigations. These cells exhibited an abnormal release value in only one of five studies. In contrast, cells from mucosa affected by ulcerative colitis in these patients showed abnormal release values in all five studies (Fig. 4). This occurred whether the mucosa from which the cells were obtained was inflamed or previously involved but not inflamed at the time of study.

Discussion

The degree of release of ⁵¹Cr from cells previously labelled with sodium ⁵¹chromate provides a quantitative and dynamic measure of the leakiness of cell membranes during short term culture. The release values obtained in this study reflect the state of the cell membranes of the enriched colonic epithelial cell component of the isolated population, as although the contaminating non-epithelial cells also take up ⁵¹Cr, their proportions were low, few disease related differences were found, and their ⁵¹Cr content per cell is much lower than that for epithelial cells (unpublished observations). Thus their numerical contribution to ⁵¹Cr release would be minimal. Colonic epithelial cells isolated from histologically normal mucosa by the collagenase-Dispase method show the maintenance of intact cell membranes on this criterion and continuing high viability on other criteria for at least 16 hours *in vitro*.¹⁴ This occurs whether the mucosa from which the cells are isolated is from normal or adenoma/cancer bearing colons. By marked contrast, epithelial cells from mucosa affected by ulcerative colitis exhibit increased leakiness of their cell membranes. This indicates that the colonic epithelial cell is abnormal in ulcerative colitis and may be a reflection of cell injury occurring *in vivo*. A contribution of cell injury, however, secondary to enhanced susceptibility to mechanical or enzymatic damage during the isolation process is also possible. The significance of such a finding to the pathogenesis of ulcerative colitis depends upon defining its relationship to mucosal inflammation *per se* and its disease specificity.

Epithelial cells from mucosa affected by Crohn's

disease showed abnormal leakiness of cell membranes in only 56% of the populations assessed. The level of ^{51}Cr release was significantly less than that from ulcerative colitis derived cells though still greater than that of the control groups. Moreover, in Crohn's disease, the presence of mucosal inflammation was associated with significantly increased release values suggesting that mucosal inflammation is associated with epithelial cell injury *in vivo*. Such a finding is not surprising as inflammation *per se* is associated with many potentially cell damaging factors. These include secreted factors from inflammatory phagocytes and antibody production which may lead to cellular damage either directly or through antibody dependent cellular cytotoxicity. Epithelial cell directed antibodies have been found in the mucosa of patients with ulcerative colitis²⁰ and Crohn's disease²¹ but the role of these are not known. In addition, cytotoxic or potentially cytotoxic cells – for example, natural killer and lymphokine activated killer cells, are present in the intestinal mucosa^{8,9,22,23} and augmentation of lysis of autologous colonic epithelial cells by intestinal mononuclear cells has been reported in inflammatory bowel disease.²⁴ Activation of intestinal mononuclear cells may also induce the release of lymphotoxin¹² and/or tumour necrosis factor which may injure epithelial cells as innocent bystanders.^{12,25}

In contrast, the increased ^{51}Cr release of colonic epithelial cells from mucosa affected by ulcerative colitis is less clearly related to the degree of inflammation present as assessed by histopathological criteria. In many studies, mucosa showing evidence of inactive disease when taken from patients with a typical clinical history of relapsing diffuse colitis exhibited abnormal release values of similar magnitude to those of colonic epithelial cells from mucosa that was actively inflamed. This abnormality was not usually evident when cells from apparently uninvolved mucosa were examined indicating that a diffuse primary defect of colonic epithelial cells (as suggested by studies of mucin subspecies)¹⁵ is not responsible for the observation. The persistence of this abnormality even when the disease is in apparent remission may indicate a possible mechanism favouring the development of chronicity of mucosal inflammation. Thus, an understanding of the pathogenetic mechanisms involved may contribute greatly by elucidating the pathogenesis of chronicity in ulcerative colitis.

Despite the lack of histopathological evidence of mucosal inflammation, inflammatory mechanisms may still be involved. There is evidence that patients with ulcerative colitis in clinical and histopathological remission have continuing mucosal inflammation with – for example, raised release of inflammatory

mediators from rectal mucosa in untreated patients.²⁶ It is possible that the minority of studies in which epithelial cells from non-inflamed mucosa demonstrated normal ^{51}Cr release may reflect truly 'burnt out' ulcerative colitis and these patients may no longer be at risk of further relapse of mucosal inflammation. Metabolic mechanisms may also be involved as the colonic epithelial cell in ulcerative colitis demonstrates alterations in its metabolic profile with evidence for inhibition of β -oxidation of fatty acids by the cells.¹³ In addition, nitrate anions are detectable in the colonic lumen only in ulcerative colitis.²⁷ Nitrite anions stimulate fatty acid oxidation in colonic epithelial cells²⁸ and may contribute to intracellular energy deficiency with consequent cell breakdown especially in view of the overall inhibition of such pathways.¹³

In conclusion, increased leakiness of the cell membranes of colonic epithelial cells is found for most populations derived from mucosa affected by ulcerative colitis whereas, in disease controls, it was an inconstant finding and only associated with histopathologically evident mucosal inflammation. The epithelial cell abnormality in ulcerative colitis bore little relationship to the degree of mucosal inflammation but was not usually present in apparently uninvolved mucosa. These findings suggest that the raised epithelial cell turnover is at least in part caused by a sick colonic epithelial cell and that local micro-environmental cell injuring factors present in active and quiescent disease are responsible. Identification of such factors would contribute to an understanding of the pathogenesis of this disease.

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