Crypt cell proliferation and HLA-DR expression in pelvic ileal pouches

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Abstract

To investigate the nature of the morphological changes that occur in ileal pouches, 26 biopsy specimens from patients with functioning ileo-anal pouches (eight with pouchitis) were studied. Normal ileum ($n = 10$) was used as a control. Mucosal morphometry (using linear measurements), crypt cell proliferation (CCP) (using the monoclonal antibody Ki67), and epithelial HLA-DR expression (monoclonal antibody CR3/43) were assessed. CCP (expressed as the percentage of Ki67 positive nuclei for each crypt) was significantly higher in pouches with pouchitis, compared with those without, and in pouches without pouchitis compared with normal ileum. CCP values in some pouches without pouchitis approached values found in those with pouchitis. CCP was related inversely to villous height and an index of villous atrophy (VH/TMT), and directly to crypt depth. In the presence of pouchitis there was intense epithelial HLA-DR expression that extended into the crypts. In some pouches with high CCP values, but without clinically important inflammation, surface epithelial HLA-DR expression was weak and patchy.

It is concluded that villous atrophy and crypt hyperplasia in ileal pouches are associated with high CCP values. These may be increased even in the absence of active inflammation, and this increase may occur as a response to the new luminal environment.

or chronic mucosal inflammation.^{5,6} Furthermore, subtotal villous atrophy and crypt hyperplasia can also occur in pouches without evidence of inflammation.^{1,3,5} Mucosal transformation from a villous morphology not only gives the pouch mucosa a strong resemblance to colonic mucosa,^{1,3} but is associated with the presence of colorectal type sulphomucin^{1,4,5} and the expression of colonic antigens.⁵ These changes, which are independent of the original diagnosis,^{1,5} are thought to occur in response to the new luminal environment,^{1,4,6,7} and it has been suggested that they may predispose to pouchitis.^{1,8} The major histocompatibility complex (MHC) class 2 molecules are normally expressed only by fully differentiated small intestinal enterocytes in the villi.⁹ Therefore, the intensity of the expression of these molecules may also be affected by the morphological changes in functioning ileal pouches, which in turn may affect antigen presentation.⁹

The aims of this study were to investigate the association between the morphological changes occurring in functioning pouches, crypt cell proliferation (CCP), and epithelial HLA-DR expression.

Methods

Mucosal biopsy specimens were obtained from the ileo-anal pouches of 26 patients (18 men, eight women; median age 30.8 years, range 18–56 years). Twenty three of them originally had ulcerative colitis and three had familial adenomatous polyposis. Eight (all with previous ulcerative colitis) had pouchitis at the time of biopsy. Pouchitis was defined as the occurrence of diarrhoea with pouch inflammation on sigmoidoscopy, in the absence of specific pathogenic organisms in the pouch stool.^{8,10} The pouches had been functioning for a median of 28.3 months (range eight to 68 months).

Two endoscopic biopsy specimens were obtained from adjacent areas of the pouch mucosa, at least 10 cm from the anal verge. Control tissues comprised normal ileal mucosa ($n = 10$) obtained at colonoscopy or from surgical resected specimens (at least 5 cm distant to tumour), and normal large bowel mucosa ($n = 3$) obtained at endoscopy on patients with the irritable bowel syndrome (all histologically normal). One biopsy specimen was fixed in formalin and embedded in paraffin wax. The other was snap frozen in isopentane in liquid nitrogen and stored in liquid nitrogen until sectioning.

Restorative proctocolectomy and ileal pouch-anal anastomosis is now a well established alternative to an ileostomy in the surgical management of ulcerative colitis and familial polyposis coli. The mucosa of functioning pouches, both pelvic and intra-abdominal (Kock), undergo morphological changes—namely, villous atrophy and crypt hyperplasia.^{1–4} Severe degrees of villous atrophy (subtotal villous atrophy) and crypt hyperplasia are features of pouchitis, an acute, macroscopic inflammation of the pouch mucosa of unknown aetiology.^{1,5} Histologically, mild to moderate degrees of chronic mucosal inflammation can occur even in pouches without pouchitis.¹ Two recent studies, however, have shown no correlation between the degree of villous atrophy and scores for either acute

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HISTOLOGY AND MUCOSAL MORPHOMETRY

Sections of formalin fixed, paraffin wax embedded tissue (4 μ m) were stained with haematoxylin and eosin for routine histological examination and mucosal morphometry. Mucosal morphometry (linear measurements) was performed using a calibrated eye piece (Leitz) at a magnification of $\times 100$. For each section, at least five measurements of total mucosal thickness (TMT) and crypt depth (CD) were made as described previously,^{11,12} and their mean values taken. Villous height (VH = TMT - CD), and an index of villous atrophy (VH/TMT)^{6,11,12} were then calculated.

IMMUNOPEROXIDASE STAINING

Monoclonal antibodies

Ki67 is an IgG1 mouse anti-human antibody, directed towards an antigen in the nuclear matrix of cells in the late G1, S, G2 and M phases of the cell cycle, but not those in G0 or early G1—that is, only the nuclei of proliferating and not of resting cells.^{13,14} CR3/43 is a mouse anti-human antibody, directed towards the β chain of the human HLA-DR molecule.^{15,16}

Immunohistology

Cryostat sections (5 μ m) of snap frozen tissue were stained with monoclonal antibodies using a three stage immunoperoxidase technique. Briefly, the sections were fixed in acetone and then incubated with the respective monoclonal antibodies for 30 minutes. After washes with TRIS buffered saline they were in turn incubated with peroxidase conjugated rabbit anti-mouse immunoglobulin and peroxidase conjugated swine anti-rabbit immunoglobulin for 30 minutes each, with washes in between. The peroxidase reaction was then developed with diaminobenzidine (0.6 mg/ml) and H₂O₂ (0.01%). The sections were counterstained with haematoxylin and mounted in DPX.

ESTIMATION OF CRYPT CELL PROLIFERATION

The degree of proliferative activity was estimated as the percentage of Ki67 positive epithelial cell nuclei per total number of crypt epithelial cell nuclei, in a slight modification of the method previously described by Franklin *et al.*¹⁷ Ideally, only crypts that were cut along their entire length were counted. Some sections, however, had few or no full length crypts due to tissue distortion, and in these the upper and lower halves of different crypts were counted.¹⁷ A minimum of three crypts (usually five) were counted per section and their mean value was taken.¹⁷

The intensity of HLA-DR expression was graded from negative (0) to strongly positive (+++) in the surface epithelium and crypts. All sections were coded, and the observer had no knowledge of the clinical diagnosis.

Grouped data were expressed as median and range. Differences between measurements were assessed by the Mann-Whitney U test. The significance of correlation was determined by the Spearman rank correlation coefficient. Significance was taken as $p < 0.05$.

Results

The clinical diagnosis of pouchitis was confirmed histologically in all eight cases.¹ Biopsy specimens from these cases all showed subtotal villous atrophy and crypt hyperplasia. Biopsy specimens obtained from pouches without pouchitis showed varying degrees of villous atrophy and crypt hyperplasia. Subtotal villous atrophy and crypt hyperplasia occurred in four of these, in the absence of clinically important mucosal inflammation on histological examination. In normal ileum and in pouch biopsy specimens showing well preserved villous

Figure 1 Section of pouch mucosa with subtotal villous atrophy and crypt hyperplasia (without pouchitis), showing numerous dark staining Ki67 positive nuclei extending into the upper and lower thirds of the crypt (immunoperoxidase).

Figure 2 Percentages of epithelial cell nuclei labelled with Ki67 per crypt in biopsy specimens obtained from normal ileum, pouches without pouchitis, and pouches with pouchitis. Middle horizontal bars denote the median.

Figure 3 Association between the percentages of Ki67 positive epithelial cell nuclei per crypt and the index of villous atrophy (Spearman rank correlation coefficient).

architecture Ki67 positive epithelial cells were mainly seen in about the middle third of the crypts. In biopsy specimens with more severe degrees of villous atrophy, however (with or without pouchitis), the population of positively staining cells was increased, and these were then seen in the lower third and sometimes even in the upper third of the crypts (fig 1).

Crypt cell proliferation (fig 2) was significantly higher in biopsy specimens from pouches with pouchitis 51.7% (45.9–65.6%) compared with those without pouchitis 33.6% (20.3–47.5%) ($p < 0.001$), and in pouch biopsy specimens without pouchitis compared with normal ileum 19.8% (16.4–24.3%) ($p < 0.001$). CCP values in some pouch biopsy specimens without pouchitis, however, which also showed little or no inflammation on histological examination, approached CCP values found in those with pouchitis. CCP values were related inversely to villous height $rs = -0.79$ ($p < 0.001$) and the index of villous atrophy (VH/TMT) $rs = -0.81$ ($p < 0.001$) (fig 3), and directly to crypt depth $rs = -0.77$ ($p < 0.001$).

In biopsy specimens obtained from pouches with pouchitis there was intense HLA-DR expression (+++) in the surface epithelium extending into the crypts. Its expression in biopsy specimens from pouches without pouchitis ($n = 18$) was variable. Of these, biopsy specimens from five pouches, which showed no clinically important microscopic inflammation but had low VH/TMT and high CCP values, the HLA-DR expression on the surface epithelium was weak or patchy (+) compared with its expression on the villi of normal ileum (++). In the pouch biopsy specimens which had less severe degrees of villous atrophy the HLA-DR expression (++) was similar to that of normal ileum. There was no HLA-DR expression in the normal colonic controls or in the crypts of biopsy specimens from either normal ileum or pouches without pouchitis.

Discussion

The monoclonal antibody Ki67 has previously been used in the assessment of CCP values.¹⁷ The results obtained with this antibody have been found to correlate very well with the results of other more sophisticated methods used to quantitate cell proliferation.^{13 17 18} Al-

though only a small number of crypts were counted (three to five) per section, this study has shown that villous atrophy and crypt hyperplasia in ileal pouches are associated with increased CCP values. In normal controls linear measurements of villous height correlate well with absolute counts of villous cells which are a precise and accurate method of estimating the villous cell population.¹⁹ This correlation becomes weaker in disturbed systems where the villi are abnormally shaped, and linear measurements may then fail to reflect accurately the size of the villous cell population.^{19 20} Linear measurements of villous height and crypt depth, however, have been found to correlate very well with other more sophisticated morphometric methods such as stereology and computer aided microscopy.¹¹ An index of villous atrophy (VH/TMT) was used in addition to measurements of villous height (VH), as the value of villous height alone as an indicator of morphological change has been questioned,^{6 21} because of a tendency for mucosal stretching during the processing of biopsy specimens.²¹

In an earlier study of six patients with continent ileostomies Philipson *et al* also found an increase in CCP values, shown by increased numbers of mitoses in each crypt,²² which was associated with a reduction in villous height and an increase in the total number of cells in each crypt. This pattern of change in the ileal pouch mucosal morphology—mucosal transformation of the hyperregenerative type—is one of three possible ways in which the small bowel mucosa responds to various types of stress.²³ It is thought to be brought about by damage and increased exfoliation of cells in the surface epithelium, together with hyperproliferation of crypt cells in the germinal zone.^{23 24}

Mucosal transformation is often seen in functioning pouches.^{1 2 5} This change is patchy¹ and may therefore be underestimated when multiple biopsy specimens are not studied. Mucosal transformation is also seen in a variety of other small bowel diseases, such as coeliac disease, parasitic infestations, and graft versus host disease, where it is associated with a considerable degree of mucosal inflammation.^{23 25–27} In these conditions there is growing evidence that the morphological changes are related to the presence in the mucosa of activated T lymphocytes.^{23 26 27} Our results show that although mucosal transformation is a feature of pouchitis, it can occur, sometimes to a similar degree of severity, in non-inflamed pouches. This may suggest that non-immune factors stimulate CCP in non-inflamed pouches.

In pouch biopsy specimens without clinically important inflammation but with severe degrees of villous atrophy and crypt hyperplasia (low VH/TMT and high CCP), HLA-DR expression on the surface epithelium was weak or patchy, or both. This probably reflects the immaturity of the cells lining the surface as a result of the high crypt cell turnover, as MHC class 2 molecules are only expressed by mature, fully differentiated small intestinal enter-

ocytes.⁹ This, together with the lack of HLA-DR expression in the crypt cells of any of the biopsy specimens of pouches without pouchitis, may again indirectly suggest that the rise in CCP values in these pouches occurred in the absence of immune activation. If immune activation was present it would be expected that HLA-DR expression would have been induced, as was the case in pouchitis.

The regulation of small intestinal crypt cell proliferation is still not well understood. Although a role for immunological factors cannot be completely ruled out, there are several other possible factors which may play a part in the regulation of CCP in ileal pouches. It has frequently been suggested that the morphological changes that occur in pouches are a response to the new luminal environment.¹⁴⁻¹⁷ Faecal stasis, bacterial overgrowth, and increased amounts of potentially toxic deconjugated bile acids are found in pouches.²⁸⁻³¹ These are said to cause damage and increased exfoliation of surface epithelial cells, together with increased crypt cell turnover, in experimental, small intestinal self-filling blind loops.²⁴ They may give rise to similar changes in pouches. Factors present in the faecal stream, which include products of fermentable dietary fibre, such as short chain fatty acids, stimulate CCP in the distal small intestine and colon.³²⁻³³ This is supported by the finding of considerably reduced CCP with crypt hypoplasia in the dysfunctional rectum³⁴ and in the colon of animals fed on elemental diets,³⁵ where this can be prevented by adding fibre to their diet.³⁵ This may be of relevance in ileal pouches where there is faecal stasis and bacterial overgrowth.²⁸⁻²⁹ Nasmyth *et al* found that short chain fatty acid concentrations in pouch stool specimens were significantly higher than those found in ileostomy effluent, but similar to concentrations found in normal faeces.⁶ They suggested that this was, indeed, a reflection of faecal stasis and bacterial overgrowth.⁶ Ambrose *et al*, however, found that short chain fatty acid concentrations in pouch stool specimens were significantly lower than in normal faeces, with the exception of acetate.³⁶ Nevertheless, even poorly fermentable fibre may by itself stimulate an increase in CCP by abrading the colonic mucosa,³²⁻³⁷ a mechanism by which faecal stasis may induce CCP.

The presence of immature cells lining the surface epithelium may have implications for the mucosal defence barrier. Interepithelial cell tight junctions are poorly organised between immature cells.³⁸ As these tight junctions influence transepithelial permeability³⁹ permeability to macromolecules may be increased in areas where the pouch mucosa is lined by immature cells. Furthermore, there is increasing evidence that small intestinal enterocytes expressing MHC class 2 molecules may be directly involved in antigen presentation⁹ and may also act as an immunological "sieve", permitting access only to selected antigens.⁹ Both of these functions may also be impaired in immature epithelial cells with weak or no class 2 expression. Although mere speculation at present, it remains an interesting possibility

that these changes may have a role in the pathogenesis of pouchitis, which is thought to have an immunological basis.¹⁷⁻⁸

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