Increased small intestinal apoptosis in coeliac disease

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Abstract

Background—Coeliac disease (CD) mucosa is flattened despite epithelial hyperproliferation.

Aims—To establish mechanisms of cell loss in CD.

Patients—14 controls, 17 active CD patients, and 16 maintained with gluten free diet.

Methods—Programmed cell death was examined in small intestinal biopsy specimens by staining fragmented DNA using terminal uridine deoxynucleotidyl nick end labelling (TUNEL), in comparison with haematoxylin and eosin stained adjacent sections. Double staining with anti-CD45 antibodies determined the origin of apoptotic cells. Apoptosis was graded from 1-3 (<5, 5-20, >20% respectively). Proliferating cells, immunostained by Ki-67 (MIB-1) antibody, were counted. **Results**—Apoptotic cells were seen rarely by haematoxylin and eosin but more readily by TUNEL. In controls, $1.4 \pm 0.2\%$ of epithelial cells were apoptotic (mean grade 1.1), mainly located in the upper villus. In active CD, frequent apoptotic cells were distributed throughout the crypt-villus unit (mean grade 2.4), decreasing after treatment to 1.1 ($p < 0.001$) even when still histologically abnormal. CD45 antibodies rarely stained apoptotic cells in active CD. The number of TUNEL positive cells correlated with proliferating cell number ($p < 0.001$).

Conclusion—Enterocyte apoptosis is greatly increased in untreated CD, correlates with proliferation, and falls to normal with a gluten free diet, before histological improvement. Increased apoptosis may be responsible for villous atrophy in CD.

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disorder may represent an atrophic mucosal process accompanying a fall in cell proliferation. However, others recognised that in the elongated crypts in CD, mitoses were frequent in active CD.^{4,5} Furthermore, direct measures of small intestinal proliferation demonstrated increased numbers of crypt proliferating cells in CD,^{6,7} subsequently confirmed in more recent studies using quantitative metaphase arrest⁸ and confocal microscopy.⁹ The flat mucosal surface in CD was therefore thought not to be caused by decreased proliferation but to excessive cell loss, 'exfoliative enteropathy'.¹⁰ In recent years, it has been appreciated that normally cells are lost from the intestinal surface not by passive exfoliation but instead by programmed cell death or apoptosis,¹¹⁻¹³ thus raising the possibility that the increased cell loss in CD is caused by increased apoptosis.

Apoptosis, programmed cell death or 'cell suicide' is, in distinction to passive cell death or necrosis, an active, physiological process. In tissues that undergo continual cellular turnover, such as the gut, homeostasis is dependent upon the balance between cell proliferation and apoptosis.¹⁴ Cellular changes that occur in the late stages of apoptosis include intense nuclear shrinkage or pyknosis, resulting in an 'apoptotic body', occasionally seen by haematoxylin and eosin.¹⁵ However, because such apoptotic bodies are very difficult to see by routine microscopy, cells undergoing programmed cell death can be more easily identified by in situ staining of fragmented DNA, which occurs at an earlier step in apoptosis.¹⁶ We therefore used this more sensitive technique of terminal uridine deoxynucleotide nick end labelling (TUNEL) to examine whether apoptosis is increased in the mucosa of patients with CD, whether it returns to normal after treatment, and whether changes in apoptosis are associated with changes in proliferation in human small intestinal mucosa.

Methods

Patients and specimens

Patients with a diagnosis of coeliac disease and controls with no evidence of small intestinal disease who had undergone small bowel biopsy for the investigation of gastrointestinal symptoms were identified from the pathology records and gastroenterology clinic records of St Luke's-Roosevelt Hospital Center, NY and Hammersmith Hospital, London, UK. Patients who had any known gastrointestinal disease other than coeliac disease, were taking

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Coeliac disease (CD) is characterised histologically by active inflammation and cell damage in the small bowel mucosa, resulting in a loss of villous height, increased crypt depth, more crypt mitotic figures, and infiltration of enterocytes and the lamina propria by mononuclear cells.¹ These changes are thought to be initiated by a T cell mediated reaction to dietary gluten in genetically susceptible people.²

As an explanation for the 'flat mucosa' in CD patients, Creamer³ proposed that the

non-steroidal anti-inflammatory medication or had positive antibodies to HIV were excluded from this study. The study was approved by the Investigational Review Board of St Luke's-Roosevelt Hospital Center.

Sections from the small bowel biopsy specimens taken from distal duodenum or jejunum, which had been fixed in formalin and embedded in paraffin wax for routine clinical histopathology, were then cut, stained with haematoxylin and eosin, and examined by light microscopy by an experienced histopathologist to exclude tissues with poor orientation, containing fixation artefacts or in which there was any doubt about the clinical diagnosis. Suitable cases were then stained by TUNEL to evaluate apoptosis in situ and by Ki-67 (MIB-1) immunohistochemistry to detect proliferating cells.

In total, 47 small bowel biopsy specimens were obtained from 28 CD patients and 17 controls. Table I lists some of the characteristics of these patients. Five patients with active CD also had biopsy specimens taken after less than one year of a gluten free diet (GFD) (median duration seven months) (short-term GFD). These patients all had achieved a clinical remission. A further 11 CD patients had biopsy specimens taken after more than one year of treatment (longterm GFD).

TUNEL histochemistry

Cells containing DNA strand breaks, a marker of apoptosis, were detected by TUNEL histochemistry using a method modified from Gavrieli *et al*¹⁷ as described previously.¹⁸ De-

paraffinised sections were digested with proteinase K 20 µg/ml (Sigma, St Louis, MO) for 15 minutes at room temperature, washed, and then incubated with 0.5 U/µl terminal transferase and 0.4 nM digoxigenin-11-deoxyuridine triphosphate (dUTP) (both from Boehringer Mannheim, Indianapolis, IN) in a buffer containing 200 mM potassium cacodylate, 0.2 mM EDTA, 25 mM TRIS-HCl, bovine serum albumin 0.25 mg/ml, and 1 mM cobalt chloride (Sigma) at 37°C for 90 minutes. The reaction was terminated with 300 mM sodium chloride and 30 mM sodium citrate. Incorporated digoxigenin-11-dUTP was detected with peroxidase conjugated Fab fragments of anti-digoxigenin (Boehringer) and nickel-diaminobenzene and counterstained with 0.5% methyl green.

The identification of the appropriate conditions for TUNEL on these sections were established in our laboratory in human intestinal tissue and with reference to the presence of apoptotic bodies in serially sectioned tissue blocks stained by haematoxylin and eosin. As one positive control, human colonic and small intestinal tissue was treated in vitro with DNAase (Sigma) at concentrations from 10 µg/ml and this resulted in positive staining in all cells in a dose dependent manner.¹⁷ As a further and biological positive control, we also used colonic tissue from a mouse six hours after treatment with azoxymethane 30 mg/kg subcutaneously, containing numerous apoptotic epithelial cells¹⁹ as well as a surgical specimen of small intestine that was included in every run. For negative controls, the omission of either terminal deoxynucleotidyl transferase, cobalt chloride or of digoxigenin-dUTP resulted in uniformly negative staining. The numbers of positively stained cells and the intensity of TUNEL staining was dependent upon several factors summarised in Table II. In particular, substituting other cations for cobalt in the reaction mixture influenced staining intensity. Manganese chloride (0.4 mM) increased background stain and the numbers of positive cells while magnesium sulphate (1–8 mM) reduced the number of positive cells. Staining intensity was reduced by decreasing the concentration of either terminal transferase or digoxigenin-dUTP although the number of cells that were stained was not affected by shortening protease K pretreatment to less than 10 minutes or increasing salt concentration.²⁰ In marked contrast with other tissues such as the tonsil, in which the protease concentration was critical, similar numbers of cells stained for TUNEL over the range of protease concentration from 5–20 µg/ml, but the staining intensity in each positively stained cell increased with protease concentration. Pepsin 0.5% in 0.1M TRIS-HCl for 15 minutes showed identical results to protease K. Using 2 µm or 3 µm thin sections resulted in increased numbers of positive stained cells, compared with 4 µm sections. As described previously, for detection of apoptotic cells by Klenow DNA polymerase I,¹² a delay in placing fresh tissue into the formalin fixative, increased the numbers of apparently apoptotic

TABLE I Patient characteristics and histological architectural features of small intestinal biopsy specimens

	Controls	Active coeliac disease	Short-term gluten free diet	Longterm gluten free diet
Number	14	17	5	11
Age (y) (mean, range)	44.4 (20–71)	57.4 (27–82)	46.6 (37–62)	50.0 (24–61)
Male/female	8/6	10/7	2/3	2/9
Villous atrophy:				
None	14	0	0	11
Subtotal	0	7	4	0
Total	0	10	1	0

Short-term gluten free diet is defined as less than 12 months treatment. Longterm gluten free diet is defined as over 12 months treatment.

TABLE II Important methodological determinants of TUNEL staining intensity in human gastrointestinal tissues

Variable	Effect on TUNEL staining
Fixation	
Time to fixation	Delay of >60 seconds → false positive staining at tissue edges
Time in fixation	Over 48 hours in formalin → non-specific positive staining
Fixative	10% neutral buffered formalin.
	Alcohol or Bouin's fixative → non-specific positive staining
Section thickness	4 µm sections used
	Thin sections → non-specific positive staining
Tissue permeabilisation	No protease: very faint positive staining, reduced positive cell numbers
	Increasing protease K concentration from 5–20 µg/ml → increased staining intensity per positive cell, no change in numbers of positive cells
	Time in protease (10–20 minutes): no difference
	Pepsin 0.5% – similar to protease K in 0.1 M TRIS
	Microwave pretreatment → non-specific positive staining
TUNEL reaction cation	1 mM cobalt used
	Manganese (0.4 mM) → non-specific positive staining
	Magnesium (1–8 mM) no positive staining
Tdt enzyme	Concentration <0.1 U/µl → fewer and less intense staining of positive cells
dig-dUTP	concentration >0.8 nM → increased numbers of positive cells

cells. In addition, prolonged formalin fixation (more than 48 hours) resulted in similar changes. Non-specific staining was seen if the section was folded and at the cut edge of a section, particularly where the tissue contained crush artefact.

Double staining with anti-CD45 antibody and TUNEL

Representative cases of all clinical groups were double stained by TUNEL and by antibodies against the CD45 leucocyte common antigen to determine whether the TUNEL positive cells were of white cell origin. For double staining, the TUNEL procedure was modified, so that after exposure to proteinase K, sections were blocked with normal mouse serum (Vector Laboratories, Burlingame, CA) diluted 1:10 in phosphate buffered saline, washed, and then incubated with anti-CD45 antibody (clones 2B11 and PD7/26, Dako, Carpinteria CA) overnight at 4°C. Anti-CD45 immunoreactivity was detected with biotinylated horse antimouse IgG (Vector), and a red colour was developed using 3-amino-9-ethylcarbazole as substrate with the ABC and AEC kits (Vector). After thorough washing of the sections, the TUNEL methodology was continued as described above, starting at the step after protease K treatment but using an aqueous mounting medium (Aqua Perm, Lipshaw Immunon, Pittsburgh, PA).

Proliferative cell immunostaining with Ki-67

Four micron thick sections were cut, placed on 'Superfrost' coated slides, and deparaffinised. They were then exposed to microwave pretreatment (in 10 mM citrate buffer, pH 6 at 850 W for two periods of five minutes) to improve antigenicity. Non-specific binding was blocked with 1:10 normal horse serum (Vector Laboratories, Burlingame, CA) in phosphate buffered saline and sections were then incubated with the primary antibody overnight at a concentration of 1:200 at 4°C for 18 hours. Detection of binding of primary antibody was achieved using a biotinylated antimouse IgG (Vector) and the streptavidin-peroxidase complex (ABC kit, Vector) with nickel-diaminobenzene as chromogen.²¹ Finally sections were lightly counterstained with 0.5% methyl green (Sigma, St Louis, MO) or 2% haematoxylin.

Quantitation and statistical analysis

For each specimen, the haematoxylin and eosin stained section was examined without knowledge of the clinical details of the case and the villous architecture classified as being either normal or having total or subtotal villous atrophy, and the presence of increased intra-epithelial lymphocytes (more than four per villus-crypt column) recorded. Where the crypt architecture appeared normal, the number of TUNEL positive cells in the epithelium were counted in at least eight half crypt-villus units and the number of positive cells expressed per

100 total epithelial cells as the apoptotic index. For all sections, a minimum of 500 total epithelial cells were counted. In sprue, as the crypt-villus column often is obscured and cell damage is variable, the percentage of TUNEL positive cells also was expressed as a grade of 1 (<5% positive cells), 2 (between 5 and 20%), and 3 (greater than 20%). Proliferating cells were counted in all small intestinal sections as the number of MIB-1 immunostained cells per crypt.

The Mann-Whitney U test was used to compare scores between clinicopathological groups. Differences between apoptotic scores in the same patients before and after gluten free diet were compared by the Wilcoxon signed rank test and the relation between numbers of apoptotic and proliferating cells was determined by Spearman's rank correlation. All statistical tests were two sided.

Results

Apoptosis in normal mucosa

In normal small intestinal biopsy specimens, occasional apoptotic epithelial cells consisting of dark, basophilic, condensed or fragmented pyknotic nuclei (apoptotic bodies), could be detected by an experienced pathologist using standard microscopy in sections stained with haematoxylin and eosin. These apoptotic nuclei were found in or beneath the basal pole of luminal villus surface epithelial cells but occasionally near the apical pole (Fig 1). By haematoxylin and eosin, nuclei of epithelial cells located at the villus tip always show nuclear chromatin condensation and cell

Figure 1: Normal small intestinal mucosa. Apoptotic bodies at villus tip (arrows). Haematoxylin and eosin, original magnification $\times 200$.

shrinkage when compared with epithelial cells located in the mid-villous region, but frank apoptotic bodies are infrequent. Although the distribution of TUNEL positive cells was similar to the distribution of apoptotic bodies, many TUNEL positive cells had comparatively normal nuclear morphology. The brown positive TUNEL stain was localised to the nucleus but rarely also involved the perinuclear cytoplasm.

In the jejunum of control patients without intestinal disease, TUNEL positive cells were infrequent. The median number of TUNEL positive cells detected in each half villus-crypt axis in normal subjects was 2.2 (range 0 to 5) and the mean number of total epithelial cells per villus-crypt axis was 126 (range 120–138) with a mean overall apoptotic index of 1.4% (range 0.3–2.8%). Most TUNEL positive cells (mean 65%, range 25–100%) in the normal biopsy sections were found in the uppermost one third of the villus column (Fig 2). Of the rest, a mean of 21% (range 0–34%) of total apoptotic cells were found in the lower half of crypts and the remainder between the villus tip and crypt base (mean 14%, range 0–21%). Double staining showed that TUNEL stained epithelial cells did not stain with the CD45 antibody that detects white cells.

Apoptosis in coeliac disease

In untreated CD, apoptotic cells were found more readily by conventional microscopy in sections stained with haematoxylin and eosin. TUNEL stained cells were abundant and were scored by giving each biopsy a grade ranging

from 1 to 3 (for details see Methods). In control subjects without intestinal disease, the median apoptotic grade was 1 (mean 1.14, range 1–2). In untreated CD, 10 of 17 patients had a score of 3 and the remainder had a score of 2 (Fig 3). TUNEL positive epithelial cells were distributed along the surface of the blunted and flattened villi and throughout the whole crypt. Double staining with TUNEL and the CD45 antibody showed that the epithelial cells undergoing apoptosis were mainly CD45 negative although occasional doubly positive (TUNEL and CD45) cells were seen in the epithelium consistent with apoptotic lymphocytes (Fig 4). Occasional TUNEL stained cells were seen in the lamina propria; most of these apoptotic cells were also CD45 positive.

After 'short-term' gluten free diets of three to six months, the apoptotic grade fell to a mean of 1.2 (range 1 to 2, $p < .005$) and for patients treated for over 12 months to a mean of 1.0 ($p < .001$) (Fig 5). The five subjects whose biopsy specimens were examined before and after the GFD also showed a fall in the apoptotic score ($p < .05$) (Fig 6). There were no difference in apoptotic grade between the biopsy samples showing subtotal or total villous atrophy ($p = 0.36$).

Changes in the degree of apoptosis were then compared with standard histological features of intestinal damage, as judged by light microscopy. Treatment with a GFD resulted in some improvement in overall villous architecture in all of the CD patients. After short-term gluten exclusion, the biopsy specimens from four patients were classified as subtotal villous atrophy and from one as total villous

Figure 2: Normal small intestinal mucosa. Apoptotic cells are evident at the villus tip (arrows) with staining of occasional crypt epithelial cells. TUNEL, original magnification $\times 200$.

Figure 3: Active coeliac disease. Numerous positively stained epithelial cells are shown at the luminal surface of the abnormal mucosa and scattered throughout the depth of the elongated crypts. TUNEL, original magnification $\times 200$.

Figure 4: Active coeliac disease mucosa showing apoptotic epithelial cells with black columnar shaped nuclei, CD45 positive cells with red cytoplasmic staining and apoptotic lymphocytes which exhibit double staining for CD45 and apoptosis (arrows). TUNEL and CD45 staining, original magnification $\times 400$.

atrophy. There was still evidence for increased numbers of intraepithelial lymphocytes in all of these patients usually near the luminal villous surface. Lamina propria inflammation also was present. Biopsy specimens from patients treated 'longterm' with a GFD all returned their villous-crypt architecture to normal, but most still demonstrated a modest increase in intraepithelial mononuclear cells. Overall, cases of subtotal villous atrophy had a mean apoptotic score of 1.9, (range 1–3) and cases of total villous atrophy a mean score of 2.3 (range 1–3), both of which were significantly higher than normal mucosa (mean score 1.1, range 1–2), $p < 0.001$ and $p < 0.003$ compared with normal respectively. However, there was no significant difference in apoptotic score between the cases of subtotal and total villous atrophy ($p = 0.24$).

Cell proliferation and apoptosis

In small intestinal biopsy specimens from 13 normal subjects, epithelial cells expressing the Ki-67 antigen were detected only within crypts. The mean (SD) number of these Mib-1

Figure 5: Apoptotic scores of TUNEL stained sections of small intestinal tissues from patients with untreated CD, CD patients after short and after longterm gluten free diets (GFD) and normal controls. Data represent the mean (SD) for each group. * $p < 0.005$ compared with each of the other groups.

Figure 6: Changes in apoptotic scores in five patients with active CD treated with a gluten free diet (GFD) for between three and 12 months.

labelled proliferating cells was 4.8 (1.9) per crypt. In active CD ($n = 14$ cases), there were greatly increased numbers of proliferating cells in the hyperplastic crypts. A mean (SD) of 20.0 (9.8) proliferating cells per crypt were found in CD mucosa ($p < 0.001$ compared with normal). In the five CD patients treated with a GFD for three months, 18.9 (7.8) proliferating cells per crypt were detected, which was not significantly different to the active CD patients ($p = 0.43$). However, after a prolonged GFD (11 subjects) there was a significant fall in proliferating cell number to 9.5 (3.9) cells per crypt ($p < 0.05$ compared with all other groups), although in these patients the number of proliferating cells per crypt was still significantly higher than normal ($p < 0.01$). Overall, in the total group of subjects, the number of proliferating cells per crypt showed a close correlation with apoptosis ($p < 0.0001$).

Discussion

This study shows that in the normal upper small intestine, apoptotic nuclei are seen rarely by light microscopy – usually only in the region of the villus tip. Using the more sensitive TUNEL staining technique, apoptotic cells also were seen quite uncommonly representing less than 3% of epithelial cells in the crypt-villus axis. These TUNEL positive nuclei were localised mainly to the epithelial cell layer and were shown to be in enterocytes and not mononuclear cells as they were not immunoreactive to the universal white cell membrane antigen, CD45. Approximately two thirds of TUNEL stained apoptotic cells were present near the villus tip, about 20% near the crypt base, and the remainder were scattered between these regions.

Biopsy specimens from patients with active CD showed more apoptotic cells, mainly located in the flattened mucosal surface epithelium but also deep in crypts. Over 90% of the positively stained nuclei were in non-CD45 staining epithelial cells. Apoptotic cells were also present in the adjacent lamina propria. Most of these TUNEL positive cells and also rare epithelial apoptotic cells stained with the CD45 antibody, consistent with apoptotic lamina propria and intraepithelial lympho-

cytes. We also observed occasional TUNEL positive but CD45 negative cells in the lamina propria, presumably representing apoptotic parenchymal cells. The GFD reduced the apoptotic index close to normal even after short-term GFD at which time the villous-crypt architecture remained greatly altered, greater proliferation was evident, and increased lymphocytes still were present in the epithelium and lamina propria. After long-term (more than 12 months) GFDs, both crypt-villous architecture and apoptotic index had returned essentially to normal and the numbers of proliferating cells were greatly reduced.

Recent evidence from our group²² and others using TUNEL staining^{12, 17} has demonstrated that the majority of DNA fragmentation, and by implication apoptosis, occurs normally in effete small intestinal enterocytes mainly near the tips of normal villi. This confirms previous findings using standard histology in rats,²³ and electron microscopy in the small intestine of rodents^{11, 24} and in humans.²⁵ Indeed, epithelial cells near the lumen in normal small intestine are well known to show cell shrinkage and nuclear condensation when compared with mid-villous epithelium suggesting that they may be initiating changes that lead to apoptosis. In contrast, when the gut is exposed to genotoxic damage, apoptotic cells are found principally in the crypt base.²⁶ This finding has led to the idea that apoptosis in the crypt prevents intestinal cells bearing mutations from continuing to replicate. As, after carcinogen treatment, apoptosis in small intestinal crypts occurs very close to the position of the stem cell, whereas in the large bowel apoptosis occurs several positions away from the stem cell, Potten and coworkers have suggested that effective crypt apoptosis was responsible for the low incidence of small bowel epithelial cancers.²⁷ Indeed, we found that although most TUNEL positive cells were located near the normal villous tip, there were also a small number of positively stained epithelial cells in the crypts.

The demonstration of increased epithelial apoptosis in CD may explain the paradox that although CD is typified by increased cell proliferation,⁶⁻⁹ the mucosa is flat. In the steady state, normal mucosal mass is thought to be maintained by the balance between new cell production (proliferation) and cell loss (apoptosis, or necrosis, or 'shedding', or all three). In the flat mucosa of CD, the rate of overall cell loss must therefore exceed the rate of new cell formation, despite the numbers of proliferating cells being higher than normal. Our data suggests that increased apoptosis is a major factor in the increased cell loss of CD although it does not exclude the possibility that these other forms of cell loss may also be increased in this condition.

Histological studies such as ours cannot tackle the relative mathematical contributions of cell proliferation and loss to tissue mass because although they may identify specific time points in the cell cycle they cannot measure rate of progression through these time points. Thus, we have demonstrated that there

is a strong correlation between the numbers of TUNEL positive and Mib-1 positive cells although the absolute numbers of TUNEL positive and Mib-1 positive cells are not the same, even in normal mucosa. Indeed, in the normal mucosa there were approximately four times as many Mib-1 labelled cells as TUNEL positive cells – perhaps an enterocyte is only TUNEL positive for a period comparable to 25% of the time during that in which proliferating cells express the Ki67 antigen.

The ultimate fate of cellular fragments resulting from apoptosis within the intestinal mucosa is unclear but the nuclear material probably is engulfed by macrophages and perhaps reutilised.^{11, 13} Such reutilisation of nuclear material may be useful for the gut of CD patients because hyperproliferation demands increased supplies of precursors for new cellular formation. If enterocyte loss in CD occurred solely into the lumen, then the malabsorbing mucosa might become deficient in the availability of such important precursors.

From our data it is not possible to conclude whether increased apoptosis in CD is the primary event, stimulating a hyperproliferative response or whether increased apoptosis is secondary to a hyperproliferative state. Following the time course of apoptosis and proliferation after gluten challenge in patients with CD should help resolve this question. The finding of a 'normal' number of apoptotic cells in the patients taking longterm GFD despite a mildly hyperproliferative mucosa may suggest that hyperproliferation is the primary event. However, there is experimental evidence to support the concept that enterocyte apoptosis is a direct response to local cytokine release. For example, increased mRNA expression of several pro-inflammatory cytokines has been found in the mucosa of patients with CD,^{28, 29} and two of these mediators, interferon γ and tissue necrosis factor α , are capable of inducing enterocyte apoptosis in vitro.³⁰ In the case of tissue necrosis factor α , the induction of enterocyte apoptosis is probably via the Fas ligand.³¹ It is also possible that rather than immune mediated apoptosis, gluten or a fragment of gliadin may directly induce enterocyte apoptosis. Finally, if apoptosis is the stimulus for increased proliferation in CD, an intervention that reduces apoptosis in this condition may decrease proliferation and improve gut function. If this were the case then perhaps an antiapoptotic treatment³² could one day be used to treat CD patients whose disease is poorly responsive to a GFD.

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