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Evidences of the Involvement of Bak, a member of the Bcl-2 Family of Proteins, in Active Coeliac Disease

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Apoptosis of enterocytes is a feature that characterises the development of lesions in coeliac disease (CD). However, the intracellular pathways that lead to apoptosis of enterocytes have not been completely clarified. Bak is a member of the Bcl-2 family of proteins that acts as an endogenous promoter of apoptosis in normal enterocytes. However, its role in coeliac lesions has not been explored. We used small intestinal mucosa from patients with CD to evaluate the differential expression of members of the Bcl-2 family of proteins. Gene expression of Bak was analysed by RT-PCR of biopsies from 14 patients with untreated CD and from 19 controls without CD. In these samples, we also investigated the localisation of the Bak protein by immunohistochemistry and its apoptotic activity. In patients with untreated CD there was a 2.3-fold higher expression of Bak mRNA ($p = 0.026$), without significant differences in the expression of related genes bax or bcl-2. The higher expression of interferon gamma (IFNγ) ($p = 0.036$) and the higher number of apoptotic cells identified by the TUNEL method ($p = 0.032$) confirmed the proapoptotic status in the intestinal mucosa of CD patients. We found a significant positive correlation ($p < 0.0001$) between the expression of IFNγ and Bak mRNA in patients with untreated CD. The expression of Bak protein was higher in patients with CD, and the immunoreactivity was almost restricted to the epithelium. We found that Bak mRNA and its protein were overexpressed in the intestinal lesions of CD patients and that IFNγ confers increased susceptibility for enterocytes to undergo apoptosis via upregulation of Bak.

Keywords: Coeliac disease; Paediatric patients; Enterocytes; Apoptosis; Bcl-2 family of proteins; Interferon gamma

Abbreviations: CD, coeliac disease; PBMCs, peripheral blood mononuclear cells; IELs, intraepithelial lymphocytes; IFNγ, interferon gamma; TNFα, tumour necrosis factor alpha; TUNEL, terminal uridine deoxynucleotidyl nick end labelling

INTRODUCTION

Small intestinal lesions in coeliac disease (CD) are triggered by an abnormal T-cell mediated immune response to gliadin in genetically susceptible individuals.[1,2] Coeliac disease is characterised histologically by total or partial villous atrophy with crypt hyperplasia and the presence of mononuclear infiltrating cells both at the epithelium and lamina propria. Presentation of gliadin by professional antigen-presenting cells drives lamina propria T cell responses toward antibody production and/or inflammation and tissue remodelling.[3] Because the epithelium and the lamina propria are mutually interrelated, local factors generated in the lamina propria by monocytes, lymphocytes, myofibroblasts and endothelial cells, together with those produced in an autocrine manner by enterocytes, might damage epithelial cells and influence enterocyte turnover and intraepithelial lymphocyte (IEL) activation threshold.[4–7]

The mucosa of healthy subjects undergoes a high rate of constitutive epithelial proliferation, which together with the cell loss induced by spontaneous apoptosis, is a central feature in regulating cell numbers in the gastrointestinal tract.[8] *In vitro* studies have shown that cytokines such as IFNγ and tumour necrosis factor α (TNFα) can act as cofactors for epithelial apoptosis by upregulating Fas

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antigen expression on the surface of enterocytes.[9] The small intestinal crypts in patients with CD show an increased number of mitotic figures, and a three-dimensional expansion of this compartment by proliferation is responsible for the crypt hyperplasia.[10] The increase in epithelial apoptosis has also been demonstrated by Moss *et al.*,[11] and it represents an adaptive mechanism responsible for the replacement of damaged epithelial cells. More recently, it has been suggested that IELs might be involved directly in enterocytic apoptosis. It was observed in mucosa affected by CD that activated IELs upregulate Fas ligand expression and have increased numbers of perforin- and granzyme B-containing granules associated with enterocytic apoptosis.[12,13] Regardless of the type and origin of the proapoptotic stimulus, susceptibility of target enterocytes to die might be related to an intracellular imbalance between the expression of members of the Bcl-2 family of proteins that have a differential pattern of expression along the small intestinal epithelium.[14,15] Bak (Bcl-2 homologous antagonist killer) has been shown recently to be functionally involved in apoptosis, indicating that the main role of Bak protein is probably promoting cell death.[16–19] Although Bak is distributed widely in human tissues, its expression is intense in enterocytes (one of the cell types with more prominent Bak expression), negative or weak in Paneth cells, and moderate to strong in muscularis or smooth muscle cells of the small intestine.[14] The aim of this study was to evaluate the involvement of members of the Bcl-2 family of proteins in coeliac lesions of the small intestine.

MATERIALS AND METHODS

Subjects

We evaluated 33 children (median age 3 years, range 1–7 years) admitted for diagnostic purposes to the Gastrointestinal Unit of the "J.P. Garrahan" Paediatric Hospital in Buenos Aires. Fourteen of those patients were diagnosed as having CD.[20] This diagnosis was based on the presence of malabsorption syndrome, positive antiendomysial antibodies and the characteristic histologic features on small intestinal biopsies. The control group consisted of 19 age-matched, nonrelated children who had negative results of an antiendomysial antibodies test and normal intestinal histology.

Small Intestinal Biopsy Samples

Intestinal samples were obtained from distal duodenum or proximal jejunum, below the Treitz region, by means of a paediatric double-opening Crosby–Kugler capsule (TC components, Hampton, UK). For RNA analysis, samples were placed immediately in guanidine thiocyanate buffer until RT-PCR analysis was performed. For detection of *in situ* apoptosis and immunohistochemistry, samples were immersed in 10% buffered formalin solution for 3–4 h. Material was embedded in paraffin wax, and 3 μm sections were stained routinely with haematoxylin and eosin. Biopsy samples were classified according to Marsh criteria.[21] All 14 patients had a flat destructive type 3 lesion. All samples from control individuals had normal histologic features. Patients and control subjects were informed of the aims of the study at the time of biopsy.

cDNA Synthesis

Total RNA was extracted by the method of Chomczynski and Sacchi.[22] Isolated RNAs were subjected to reverse transcription. Briefly, denatured RNA was added to a reaction mixture containing 625 μM dNTPs, 15 units of RNAsin, 30–45 μM of gene-specific reverse primer, and 0.1 unit of MMLV reverse transcriptase (Promega, Madison, WI) and was incubated at 42°C for 1 h. We included the following RNA as positive controls: for bax and bak expression we used small intestinal RNA obtained from healthy individuals; for bcl-2, peripheral blood mononuclear cells (PBMCs) from patients with B-chronic lymphocytic leukaemia; and for IFNγ and β-actin, PBMCs from healthy individuals stimulated with PHA (1 μg/ml) for 36 h. Negative controls without RNA were also included.

PCR Amplification and Southern Blot Analysis

Aliquots of cDNA were added to a mix containing 1.5–2 mM $MgCl_2$, 250 μM dNTPs, 25–50 μM of each primer, and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI). The following forward (F) and reverse (R) primers were located in different exons, so that the amplification product from any contaminating genomic DNA could be distinguished from that of cDNA. For β-actin: F: 5′-TGACGGGGTCACCCACACTGTGCC CTA-3′ and R: 5′-CTAGAAGCATTTGCGGACGATG GAGGG-3′; bax:F: 5′GCTCTGAGCAGATCATGAAG-3′ and R: 5′-AGTAGAAAAGGGCGACAACC-3′; bak:F: 5′-TTACCTCTGCAACCTAGCAG-3′ and R: 5′-ATCT TCGTACCACAAACTGG-3′; bcl-2:F: 5′-AGGATTGT GGCCTTCTTTGA-3′ and R: 5′-AATCAAACAGAGG CCGCATG3′ and IFNγ:F: 5′-AAGATGACCAGAGCA TCCAA-3′ and R: 5′-TCCTTTTTCGCTTCCCTGTT-3′. Oligonucleotide sequences for PCR amplification were checked by GeneBank and synthesised by Operon Technologies (Operon Technologies, Alameda, CA). PCR was done for 5 min at 94°C and 5 min at 57–66°C with *N* cycles of 1 min at 94°C, 1 min at 57–66°C and 1 min at 72°C followed by a final extension period of 10 min at 72°C in a PTC-100™ MJ Research thermocycler (MJ Research, Watertown, USA). The number (*N*) of PCR cycles was dependent on the sensitivity threshold of each set of specific primers. cDNA input for each sample was adjusted *a priori* by normalisation in function of the abundance of β-actin mRNA (expressed constitutively). Annealing temperature was 57°C for bax, 59°C for bak,

60°C for bcl-2 and IFNγ, and 66°C for β-actin. We also verified that in our RT-PCR conditions, the reactions were in a linear range after the established *N* of amplification cycles and not in the plateau and thus were suitable for comparing mRNA amounts (data not shown). In all cases, β-actin transcripts were analysed in identical aliquots of each sample after amplification for 22 cycles. Positive cDNAs synthesised from the sources mentioned above and a sample without RNA or without cDNA were included in each RT-PCR experiment as positive or negative controls, respectively. All due precautions were taken to exclude the possibility of false-positive results.

Samples were electrophoresed in 2% agarose gels and visualised by ethidium bromide staining. Southern blot analysis was followed by overnight hybridisation with 10 pmol of specific [^{32}P] γ (New England Biolabs, Beverly, MA) oligonucleotide in a hybridisation incubator (Robbins Scientific, CA) to validate the specificity of amplification products. The following probes were used: β-actin: 5′-AAAAAAGCTTGGTGATGTCTGG-3′; bax: 5′-GAAGCTGAGCGAGTGTCTCA-3′; bak: 5′-TTGAG AGTGGCATCAATTGG-3′; bcl-2: 5′-ATGTGTGTGGA GAGCGTCAA-3′ and IFNγ: 5′-TCCTTTTTCGCTTCC CTGTT-3′. Hybond™ N^+ membranes (Amersham International, Buckinghamshire, England) were prehybridised for 1 h at 42°C in 6 × SSPE, 5 × Denhardt's solution, 0.25% (m/v) sodium dodecyl sulfate (SDS) and 0.1 mg/ml of sheared salmon sperm DNA (Sigma, St. Louis, MO), hybridised for 12 h at 42°C and washed using tetramethylammonium chloride solution (Fisher Scientific, Pittsburgh, PA).[23]

TUNEL Assay

Apoptosis was assayed on sections mounted on silane-coated slides by using a detection kit according to the manufacturer's instructions (ApopTag® Plus-peroxidase, Oncor, Gaithersburg, MD). In brief, after deparaffinisation with xylene and rehydration with a graded ethanol series, tissues were digested with proteinase K (20 μg/ml) (Sigma, St. Louis, MO) at 37°C for 15 min. Endogenous peroxidase activity was blocked with 3% H_2O_2. Sections were then incubated at room temperature for 1 h with a mixture containing diluted terminal deoxynucleotidyl transferase enzyme (TdT) (as recommended for tissues sensitive to TdT labelling) and digoxigenin-11-deoxiuridine triphosphate (dUTP). dUTP incorporated in DNA strand breaks was detected with peroxidase-conjugated Fab fragments of antidigoxigenin antibody in the presence of nickel-diaminobenzidine. Samples were counterstained with haematoxylin and visualised by light microscopy (Nikon AFX IIA, Japan). Within the epithelial compartment, we analysed 750 enterocytes from 10 contiguous fields, and TUNEL-positive enterocytes were expressed as a percentage of the total number of enterocytes evaluated. The results were interpreted according to criteria previously established.[24] Negative controls were performed without the addition of TdT to tissue sections.

Immunohistochemistry

Staining with anti-Bak antibody was done on formalin-fixed and paraffin-embedded tissues. Sections were deparaffinised, rehydrated (as described in the previous section) and antigen-retrieved by incubation in a microwave oven for 15 min in 10 mM sodium citrate buffer, pH 6.0.[25] Before primary antibody was added, endogenous intestinal alkaline phosphatase was inhibited by incubation for 15 s with cold acetic acid 20%, and sections were blocked with horse serum (Vector Laboratories, Burlingame, CA). Incubation with a polyclonal anti-Bak antibody (Dako, Carpinteria, CA) diluted 1:400 in phosphate-buffered saline was done overnight at 4°C. Anti-Bak immunoreactivity was detected with a biotinylated universal antibody (Vectastain® Universal Elite® Vector Laboratories, Burlingame, CA) and phosphatase alkaline-conjugated ABC (Vectastain® ABC-AP KIT, Vector Laboratories, Burlingame, CA). Blue colour was developed using a phosphatase alkaline developing colour substrate kit (BioRad, CA) for 15 min at room temperature and slightly counterstained with 0.5% methyl green in buffer acetate (pH 4.1) for 1 h at 37°C. Negative controls were incubated with an irrelevant antibody of the same isotype with the primary antibody also omitted.

Data Analysis

Autoradiographs were developed after 1 h of exposure at room temperature. Densitometric analysis and quantification were performed utilising a Shimatzu chromatoscanner (Tokyo, Japan). Steady-state values for each transcript were expressed as arbitrary densitometric units (a.d.u.), and normalised data were expressed without units as the ratio between a.d.u. for the specific transcript and the respective a.d.u. for β-actin. This ratio allowed us to correct for differences in the content of RNA between samples.

The intensity of the immunohistochemical staining was evaluated subjectively on a five-point scale by two independent reviewers as follows: 0, negative; 1, weak staining; 2, obvious staining; 3, strong staining; 4, intense staining. The individuals were masked to the diagnosis of the patients. Discrepancies in scoring (i.e. >2 points) were resolved by re-evaluating the slides together.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Statistical comparisons between mean values were done using the Mann–Whitney *U*-test (two-tailed) for nonparametric data. Correlations were done by Spearman rank correlation test. $p < 0.05$ was considered statistically significant.

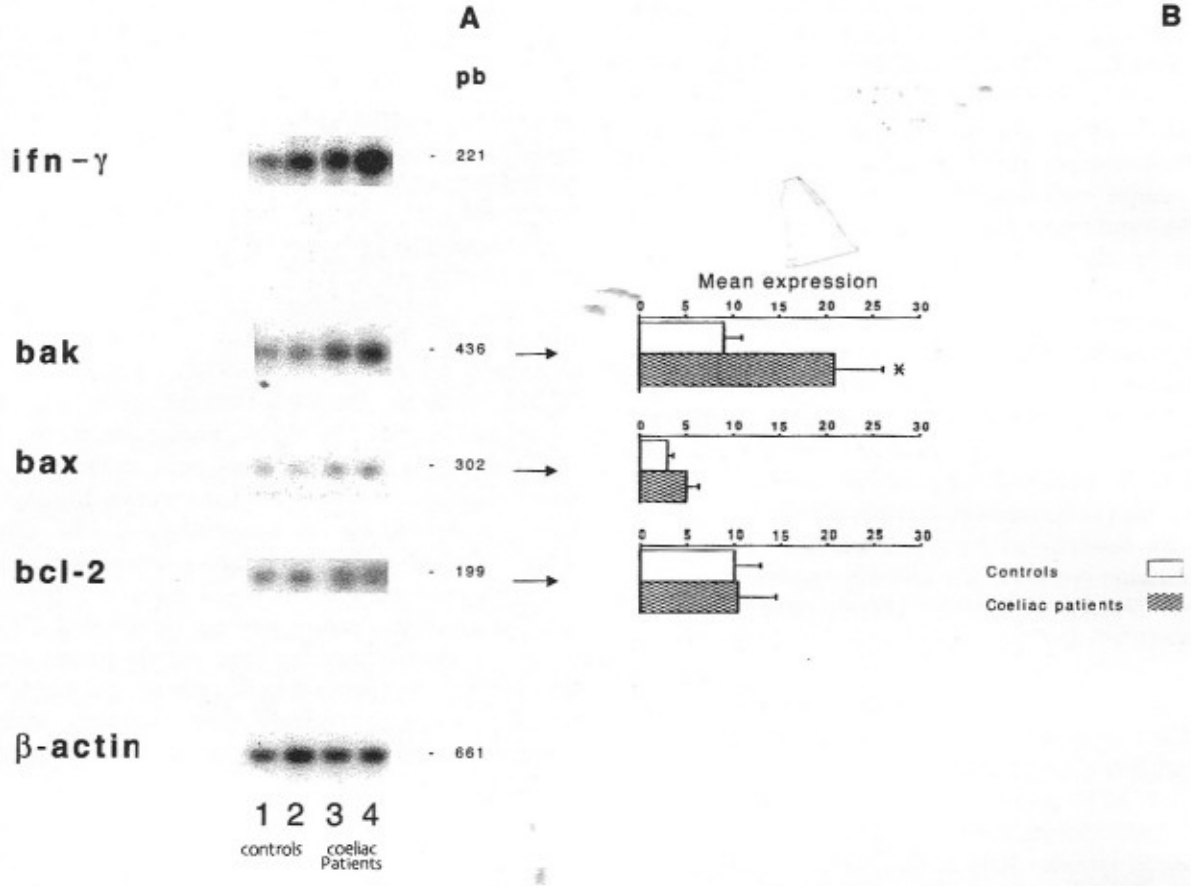

FIGURE 1 (A) Autoradiography showing results of amplification by RT-PCR of mRNAs for IFNγ, bak, bax and bcl-2 in small intestinal biopsies from representative control cases (lanes 1 and 2) and patients with active CD (lanes 3 and 4). Thirty cycles of amplification were performed in all cases except for β-actin, which was amplified for 22 cycles. (B) Comparative levels of mRNA expression for bak, bax, and bcl-2 in small intestinal biopsies from control cases ($n = 19$) and patients with active CD ($n = 14$). Autoradiographs were analysed by densitometry using a chromato-scanner and normalised to β-actin (see "Materials and methods"). Data represent mean ± SEM for each group. *Mean expression value is significantly higher compared with controls ($p = 0.04$, two-tailed Mann–Whitney U test).

RESULTS

Analysis of Gene Expression in Homogenates of Biopsy Samples

We investigated the expression of different genes associated with the induction of apoptosis in homogenates of small intestinal biopsies. The study included RT-PCR analysis of IFNγ and the proapoptotic (bak, bax) or antiapoptotic (bcl-2) members of the Bcl-2 family of proteins. In normal mucosa, we found constitutive expression of those genes. In a comparative study between 19 control individuals and 14 patients with CD, we found that all of the genes evaluated had a frequency of positive samples that was similar in controls and in CD patients (between 80 and 100% of the biopsies were positive).

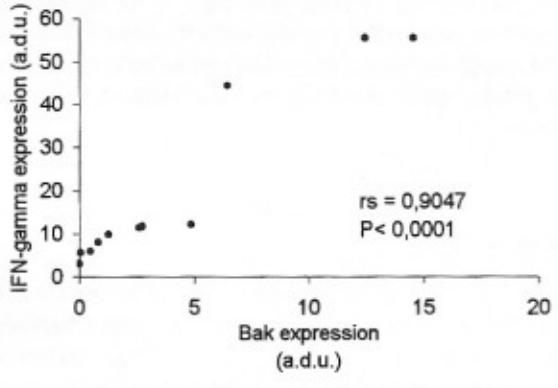

FIGURE 2 Positive correlation between bak and IFNγ mRNA expression in homogenates from small intestinal mucosa of 11 CD patients. Points represent individual normalised values.

We then evaluated differences in the steady-state level of gene expression. IFNγ and bak autoradiographic signals obtained in samples of untreated CD patients were clearly more intense (see two representative controls and CD patients in Fig. 1A). Statistical analysis revealed that IFNγ mRNA expression was 7.6-fold higher in CD patients than in controls (4.21 ± 1.522 vs. 0.55 ± 0.19 in controls, $p = 0.036$). Among the members of the Bcl-2 family studied, bak was the only gene significantly overexpressed in the mucosal samples of CD patients. After normalisation to the β-actin housekeeping gene, we found that expression of bak in patients with CD was higher than the constitutive expression found in controls (20.79 ± 5.23, $n = 14$ vs. 8.98 ± 1.96, $n = 19$ in

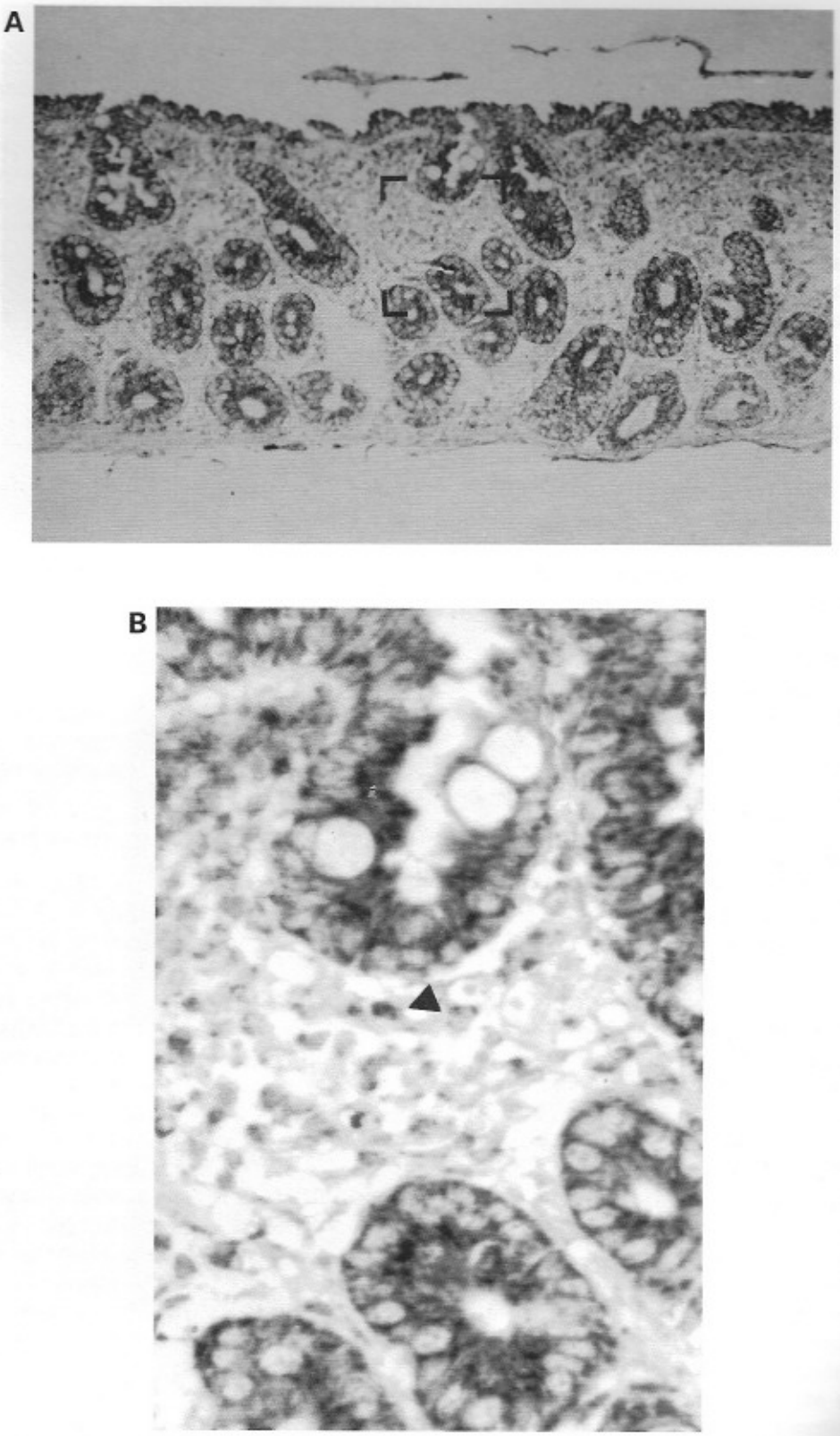

FIGURE 3 Distribution of Bak protein in the small intestinal mucosa from a representative CD patient. (A) Bak immunoreactivity is uniformly distributed all along the epithelial layer with intense superficial and glandular staining. Scanty positive infiltrating lymphocytes are evident in the lamina propria. The staining was developed with blue substrate (alkaline phosphatase ABC, magnification × 125) and nuclei were slightly counterstained with methyl green. The marked area is shown in higher detail in 3B. (B) Cytoplasm staining in superficial and glandular epithelium is observed above and below the nuclei. Arrow points to the bottom of the crypt (magnification × 400).

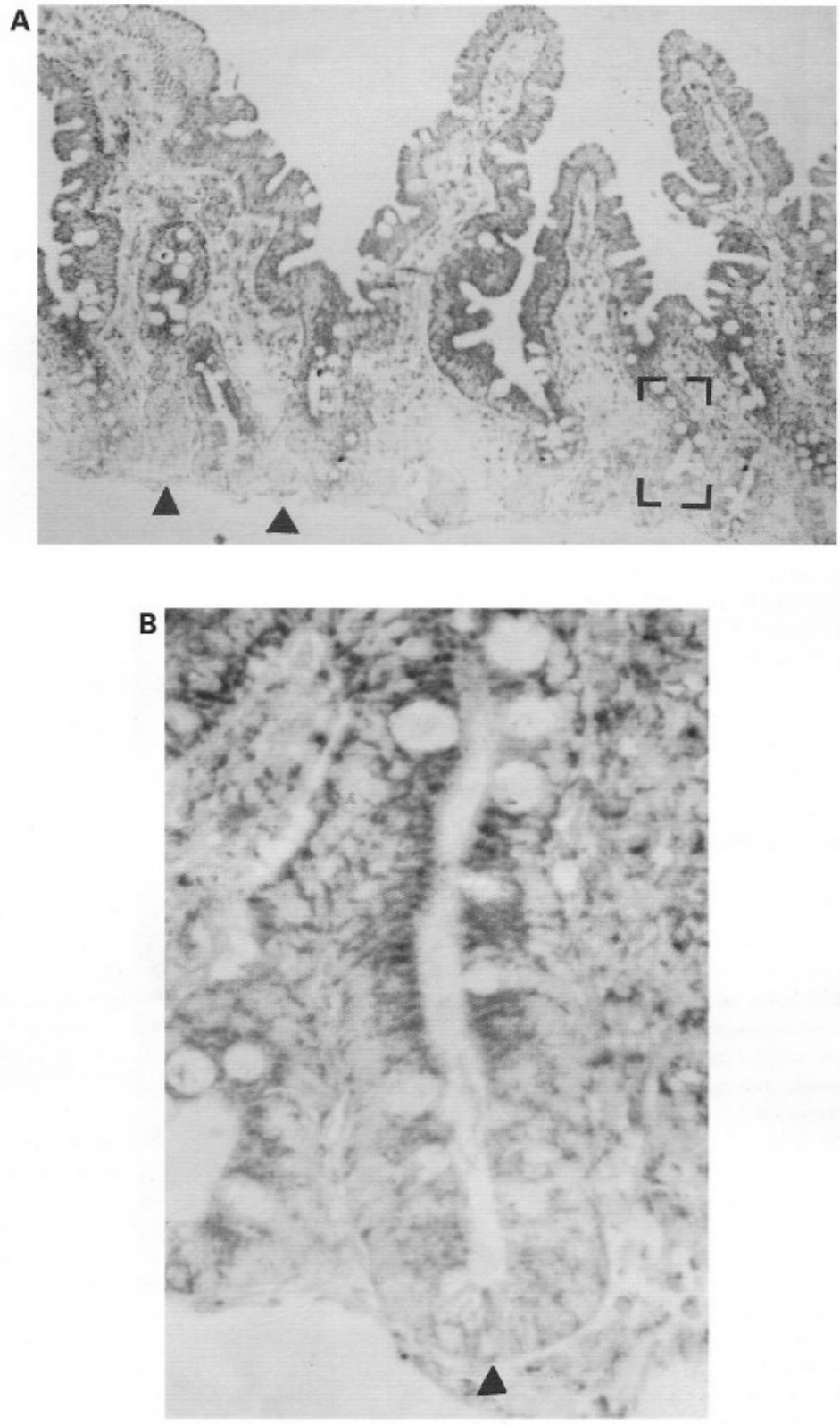

FIGURE 4 Distribution of Bak protein in the small intestinal mucosa from a representative control individual. (A) Weakly stained cells show a gradient of expression from the base to the tip of the villi along the epithelial compartment (magnification × 125). Bak immunoreactivity is absent in epithelial cells from deep glands (arrows). The marked area is shown in detail in (B). (B) Cytoplasm staining is absent at the bottom of the gland but increases toward the tip of the villus. Arrow points to the bottom of the crypt (magnification × 400).

controls, $p = 0.0261$). In contrast, no significant differences in the expression of bax (4.98 ± 1.26 vs. 2.90 ± 0.64, $p > 0.05$) or bcl-2 (10.40 ± 4.12 vs. 10.05 ± 2.78, $p > 0.05$) were found between CD patients and controls (Fig. 1B). Interestingly, the simultaneous analysis of IFNγ and bak mRNA investigated in 11 CD patients showed that the normalised values of IFNγ and bak mRNA were highly positively correlated. High values of bak mRNA expression were always accompanied by a high steady-state expression of IFNγ and, reciprocally, low values of expression of one of these genes were accompanied by a decreased steady-state expression of the other (Fig. 2). Apoptosis was assayed in biopsies from eight CD patients and eight samples obtained from control individuals. The TUNEL method detected an increased number of apoptotic enterocytes mainly in the deeper glands of the small intestinal mucosa of CD patients. The apoptotic events (expressed as apoptotic enterocytes/100 enterocytes) in control samples were only one fifth of the apoptotic events in biopsies from patients with CD (0.44 ± 0.14 vs. 1.86 ± 0.30, $p = 0.032$) (data not shown). This result may be a reflection of a greater susceptibility of enterocytes from CD patients to cell death. The apoptotic activity in enterocytes from small intestinal biopsies of CD patients was higher than the rate of spontaneous enterocytic apoptosis in controls.

Localisation of Bak Protein

To confirm the increased expression of the bak gene detected by RT-PCR, biopsy samples from the same patients were analysed by immunohistochemistry, which also allowed us to examine the distribution of the Bak protein. Epithelial glands and superficial cells from the affected mucosa of CD patients stained intensely for Bak (Fig. 3A), with an overall higher intensity than that of controls (score of 4 points in glands and 3–4 points in surface cells). Bak immunoreactivity was located in the cytoplasm, below and above the enterocyte nuclei. Staining was distributed uniformly along the epithelial layer in the mucosa of patients with CD. Figure 3B shows that the staining was particularly intense at the bottom of the crypt. From these results we conclude that enterocytes are the main cell type contributing to the higher levels of bak mRNA that were detected by RT-PCR in homogenates from biopsies of patients with CD.

A different distribution of Bak staining was found in control samples. As depicted in Fig. 4A, in control biopsies Bak protein was not detected in deep glands (score of 0–1 points), and the expression in superficial enterocytes attained a score of 2–3 points. This gradient of Bak expression from the base to the tip of the villus is shown in Fig. 4B. No epithelial staining was observed in negative controls in which an irrelevant antibody of the same isotype was used or the primary antibody was omitted. RT-PCR experiments described in the previous section show that the frequencies of samples positive for bak expression were similar in CD patients and controls. However, the expression was more intense in CD patients, with a preferential localisation at the deep glands of the epithelial compartment.

DISCUSSION

In the present study, we found constitutive mRNA expression of proapoptotic (bak, bax) and antiapoptotic (bcl-2) members of the Bcl-2 family of proteins in small intestinal mucosal homogenates from control individuals. In order to characterise the proapoptotic status of the small intestinal mucosa of CD patients, we intended to correlate the *ex vivo* mRNA expression of the Bcl-2 family of proteins with the ongoing autoimmune intestinal lesion, as was done during physiologic or pathologic processes in rat or human tissues.[26,27] The results obtained were not unexpected because it was known that some cell types in the mucosa are sources of Bcl-2 family of genes. However, we found that only bak mRNA was significantly overexpressed in the mucosa of CD patients, whereas bax and bcl-2 steady-state transcription levels were not different from those of control mucosa.

It is known that members of the Bcl-2 family of proteins regulate cell death by forming homodimers or heterodimers,[28] but Bak can act as a proapoptotic factor, independent of its heterodimerisation, with antiapoptotic members of the family as reported in *in vitro* chemotherapy or butyrate-induced apoptosis of epithelial cell lines of human and rat origin.[29,16]

We also showed that the main source of Bak protein both in control and CD samples was the epithelial compartment. However, patients with CD showed intense and uniform staining of the entire epithelial layer, whereas in control tissue the staining was much weaker and showed a gradient of expression that increased from the base to the tip of the villus. Krajewski *et al.*.[14] delineated a similar graded pattern of *in vivo* Bak expression in other human tissues, including the colonic epithelium, although those authors did not describe a gradient in the small intestinal epithelium. This discrepancy might be due to the use of different commercial polyclonal antibodies.

Strong Bax immunostaining was shown previously in cells located at the base of the crypts of normal small intestinal mucosa, which is consistent with reports of high rates of spontaneous and inducible apoptosis in this region where Bcl-2 immunostaining was completely absent.[15] The present study demonstrated, in the small intestinal mucosa of CD patients, the possible functional implication of increased Bak expression at the glandular crypts, a site where we and others[11] found enhanced cell apoptosis. Although the importance of the balance between homoproteins of the Bcl-2 family has been suggested to affect Crohn's disease[30] and Sjögren syndrome,[31] the involvement of Bak in CD is reported here for the first time.

It has been suggested that a T cell-mediated reaction against an epitope generated by the transglutaminase-mediated deamidation of dietary gliadin triggers the

characteristic injury of mucosa affected by CD. This process seems to be performed via elevated synthesis of TNFα, IFNγ, matrix metalloproteinases, transforming growth factor β and keratinocyte growth factor by lamina propria T cells and fibroblasts[32–35] and in part interferes with the balanced regulation of enterocytic death. One of those mediators of tissular injury, IFNγ, is known to sensitise cells to be killed by inducing the expression of members of some apoptosis-related gene families, including apoptosis-signalling receptors, the interleukin-1beta-converting enzyme (ICE) family and Bcl-2 family members such as Bak. IFNγ upregulates Bak protein expression in endothelial and epithelial cells.[36,37] Based on those previous studies, we speculate that the increased expression of epithelial Bak observed in our study might be a consequence of the higher local IFNγ expression shown by RT-PCR analysis of the mucosa of CD patients.

The locally higher expression of IFNγ might have a dual role in CD: it has shown harmful cytotoxic effects on epithelial cells, and it regulates enterocytic turnover by promoting the loss of damaged cells through upregulation of the proapoptotic Bak protein. In summary, the epithelial localisation of overexpressed Bak in the mucosa of patients with CD might support a role for this proapoptotic endogenous factor in conferring high enterocytic susceptibility to die, a feature in enterocytes of patients with CD.

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