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Early acquisition of bowel segment-specific Bcl-2 homolog expression profiles during development of the human ileum and colon

P.H. Vachon[1,3], É. Cardin[1], C. Harnois[1], J.C. Reed[2], A. Plourde[1] and A. Vézina[1]

[1]CIHR Group on the Functional Development and Physiopathology of the Digestive Tract, Department of Anatomy and Cell Biology, Faculty of Medecine, University of Sherbrooke, Sherbrooke, QC, Canada,

[2]The Burnham Institute, La Jolla Cancer Research Center, La Jolla, CA, USA, and

[3]Research Group on Digestive Physiopathology of the Center of Clinical Research of the CHUS, Fleurimont, QC, Canada

Summary. The adult small and large intestines display distinct expression profiles of Bcl-2 homologs, known regulators of apoptosis. This is thought to indicate that control mechanisms of intestinal apoptosis are gut segment-specific. Little is known on the expression of Bcl-2 homologs during gut development. In man, intestinal features and functions are acquired largely by mid-gestation (18-20 wks); the question whether segment-specific controls of intestinal apoptosis are also acquired early during development remains open. In the present study, we approached this by investigating the expression of six Bcl-2 homologs (Bcl-2, Bcl-XL, Mcl-1, Bax, Bak, Bad), and one nonhomologous associated molecule (Bag-1), during development of the human ileum and colon (12-20 wks of gestation). Beginning at 18 wks, we found that the epithelial localization of Bcl-2 homologs displayed differential patterns (or gradients) in both the ileum and colon; however, the patterns of some of the homologs differed between the two segments. For instance, Bag-1 and Bcl-2 exhibited crypt-villus decreasing gradients of expression in the ileum but not in the colon, whereas Mcl-1 displayed differing compartimentalizations between the two segments. Further analyses indicated that the steady-state expression levels of Bcl-2 homologs underwent modulations between 12 and 20 wks; however, the observed developmental profiles contrasted significantly between the two segments. For example, Bcl-2, Bag-1 and Bak levels increased in the colon, but the levels of these same homologs decreased in the ileum. Furthermore, by 18-20 wks, we found that the expression levels of each Bcl-2 homolog analyzed differed greatly between the ileum and colon. Altogether, these data indicate that the expression of Bcl-2 homologs is modulated differentially during human gut development in order to establish, by mid-gestation, distinct expression profiles for the small and large intestines. This in turn suggests that gut segment-specific control mechanisms of human intestinal apoptosis are acquired early during fetal life.

Key words: Apoptosis, Crypt-villus axis, Enterocyte, Gut, Programmed cell death

Offprint requests to: Pierre H. Vachon, PhD, Département d'anatomie et de biologie cellulaire, Faculté de Médecine, Université de Sherbrooke, Sherbrooke (QC), J1H5N4 Canada. Fax: 819-564-5320. e-mail: phvachon@courrier.usherb.ca

Introduction

Although originating from the same tube-like primitive gut, the adult human small and large intestinal mucosae differ in organization and physiological functions. The small intestine is characterized by its crypt-villus axis, where epithelial cell production occurs in small crypts and differentiated cells line along lumen-projecting villi, whereas the colon is characterized by gland-like crypts and a lack of villi, the differentiated cells constituting instead a 'surface' epithelium (Leblond, 1981; Ménard, 1989; Cobb and Williamson, 1991; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994; Jones and Gores, 1997; Potten, 1997). The predominant physiological role of the colon epithelium is to regulate luminal fluid and electrolyte contents, whereas that of the small intestine's lie in the digestive process and absorption of nutrients (Ménard, 1989; Binder et al., 1991; Cobb and Williamson, 1991; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994).

Within the context of the rapid, continuous cell renewal of the intestinal epithelium, apoptosis is the normal fate of villus-tip cells in the small intestine, and of surface cells in the colon (Moss and Holt, 1996; Jones and Gores, 1997; Potten, 1997; Barkla and Gibson, 1999). In the gut, as in other tissues, it is necessary for apoptosis to be highly regulated, in order to ensure proper tissue homeostasis (Potten, 1992; Thompson,

1995; White, 1996; Jacobson et al., 1997). Among the genes regulating apoptosis, the Bcl-2 family of proteins governs a critical checkpoint regulating the common downstream effector pathway of programmed cell death (Reed, 1994; White, 1996; Adams and Cory, 1998; Saini and Walker, 1998). At least 15 family members have been identified so far, functioning either as anti-apoptotic or pro-apoptotic regulators. Bcl-2 homologs are well known to interact among themselves, as well with an expanding repertoire of associated molecules (e.g. Bag-1), allowing for the titration of pro- and anti-apoptotic functions (Farrow and Brown, 1996; Reed et al., 1996a,b; Adams and Cory, 1998).

A role for some Bcl-2 homologs (namely Bcl-2, Bcl-X_L, Mcl-1 and Bak) has been proposed in the regulation of apoptosis in the epithelium of the human adult small and large intestines, a potential function well illustrated by their differential patterns (or gradients) of expression between the undifferentiated (crypt) and differentiated cell compartments (Hockenbery et al., 1991; Lu et al., 1993; Krajewski et al., 1994c, 1995, 1996; Krajewska et al., 1996; Moss et al., 1996; Liu et al., 1999). Intestinal physiopathologies that involve dysregulation of apoptosis further underlie distinctions between the small intestine and colon. Indeed, numerous such disorders exhibit epidemiological characteristics that appear to be bowel segment-specific (Ahnen, 1991; Janowitz and Mauer, 1991; Mendeloff, 1991; Kagnoff, 1995; Stenson, 1995). An example of this is cancer, which is extremely rare in the small intestine as opposed to the colon (Ahnen, 1991; Mendeloff, 1995). To this effect, there is accumulating evidence which indicates that the epithelial expression profiles of Bcl-2 homologs differ between the adult small intestine and colon (Hockenbery et al., 1991; Lu et al., 1993; Krajewski et al., 1994a-c, 1995, 1996; Merritt et al., 1995; Krajewska et al., 1996; Wilson and Potten, 1996; Hirose et al., 1997; Wilson et al., 2000). Such differences are thought to reflect differential segment-specific control mechanisms of enterocytic apoptosis and may also account (at least in part) for the apparent segmental preferences of bowel disorders that involve apoptosis dysregulation (Potten, 1992, 1997; Jones and Gores, 1997).

However, little is known on the epithelial expression of Bcl-2 homologs during human gut development. The morphogenesis of the intestinal mucosa has been the subject of many reviews (Ménard, 1989; Cobb and Williamson, 1991; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). In man, intestinal architectural features and functions are acquired early during fetal life (Ménard, 1989; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). Villus formation by mesenchymal infiltration of the stratified epithelium begins around 8-9 wks of gestation, proceeding distally until around 12 wks when the entire intestine is lined by short villi covered by a simple columnar epithelium. By mid-gestation (18-20 wks), all of the gut mucosa, from duodenum to colon, is organized into a functional crypt-villus axis which is highly similar to that of the adult small intestine, including digestive capacities and dynamic epithelial cell renewal (Ménard, 1989; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). To this effect, we have previously shown that intestinal epithelial apoptosis is absent between 12 and 17 wks, whereas by mid-gestation villus-tip apoptosis has emerged and is consistently observed thereafter (Vachon et al., 2000). Finally, between 30 wks and birth, the colon loses its villi and its digestive enzyme activities diminish, or disappear (Ménard, 1989; Cobb and Williamson, 1991; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). Therefore, the question remains open as to whether segment-specific control mechanisms of enterocytic apoptosis are also acquired early during human gut development.

In the present study, we approached this question by examining the epithelial expression and localization of six Bcl-2 homologs (Bcl-2, Bcl-X_L, Mcl-1, Bax, Bak, Bad), and one nonhomologous associated molecule (Bag-1), during the development of the human ileum and colon between 12 and 20 wks of gestation. Herein, we find that all homologs analyzed are expressed throughout morphogenesis of the two adjacent bowel segments; however, the developmental expression profiles of some of the homologs studied contrasted significantly between the ileum and colon. Furthermore, by 18-20 wks, differences are observed between the two segments in the crypt-villus localization of Bcl-2 homologs, as well as in their relative epithelial expression levels. Hence, these data altogether indicate that the expression of Bcl-2 homologs is modulated differentially during human gut development in order to establish, by mid-gestation, distinct epithelial Bcl-2 homolog expression profiles for the small and large intestines.

Materials and methods

Tissue processing

Human fetal ileum and colon (proximal, median, distal) specimens from 42 fetuses ranging from 12 to 20 weeks of age (post-fertilization) were obtained after therapeutic termination of pregnancy. Only specimens obtained rapidely (60 min or less) were used. The present study was in accordance with a protocol approved by the institutional Human Research Ethical Review Committee for the use of human biological materials.

For immunolocalization studies, tissues were washed in PBS (pH 7.4) and embedded in OCT (Optimum Cutting Temperature) compound (Tissue Tek, Miles Laboratories, Elkhart, IN), as previously described (Beaulieu et al., 1991; Perreault et al., 1995; Vachon et al., 2000). For analyses of protein expression levels, mucosal scrappings (Beaulieu et al., 1993; Beaulieu and Vachon, 1994; Vachon et al., 2000) of samples were washed in PBS (pH 7.4) and homogenized in 20 mM Tris-HCl (pH 6.8) containing 0.1 mM phenyl-methylsulfonyl fluoride (PMSF), 50 μg/ml leupeptin, 50

μg/ml antipain, and 0.1 mg/ml aprotinin. Note that mucosal scrappings separate all of the mucosa from the presumptive/developing outer muscular layers (Beaulieu et al., 1993; Beaulieu and Vachon, 1994; Vachon et al., 2000). Total proteins were measured using the BioRad (Hercules, CA) protein assay. Aliquots of homogenates were directly solubilized in 2x solubilization buffer (2.3% [w/v] SDS, 10% [v/v] glycerol, and 0.001% [w/v] bromophenol blue in 62.5 mM Tris-HCl [pH 6.8] containing 5% [v/v] ß-mercaptoethanol), boiled (105 °C, 5 min), cleared by centrifugation (13000g, 5 min, room temperature), and processed for storage as described (Vachon et al., 1995, 1996, 2000).

Antibodies

Primary rabbit polyclonal antibodies used in the present study were Ab 1682, directed against human Mcl-1 (Krajewski et al., 1994a, 1995); Ab 1695, directed to human/mouse Bcl-XL (Krajewski et al., 1994c); Ab 1701 (Krajewski et al., 1994a) and Ab PC68 (Calbiochem, San Diego, CA), both directed against human Bcl-2; Ab 1712 (Krajewski et al., 1994b) and Ab PC66 (Calbiochem), both directed to human Bax; Ab 1764, directed against human Bak (Krajewska et al., 1996; Krajewski et al., 1996); Ab I-19 (Santa Cruz Biotech., Santa Cruz, CA), directed to human/mouse Bak; Ab PC67 (Calbiochem), directed to human Bcl-XL; Ab K-20 (Santa Cruz Biotech.), directed against human/mouse Mcl-1; and Ab 9292 (New England Biolabs, Beverly, MA) and Ab R-20 (Santa Cruz Biotech.), both directed to human Bad. Primary mouse monoclonals used were mAb K56C8 (Takayama et al., 1995; Wang et al., 1996), directed against human Bag-1; mAb 32 and mAb 48 (both from Transduction Labs./Biocan Scientific, Mississauga, ON, Canada), directed to human Bad; and mAb CY-90 (Sigma-Aldrich Canada Ltd., Oakville, ON), directed against human cytokeratin 18 (K18). Note that antibodies Ab1682, Ab1695, Ab1701, Ab1712, Ab1764 and mAb K56C8 were developed in the laboratory of one of the authors (J.C.R.) of the present study and have been characterized extensively in previous studies (Krajewski et al., 1994a-c, 1995, 1996; Takayama et al., 1995; Krajewska et al., 1996; Moss et al., 1996; Wang et al., 1996; Liu et al., 1999; Boucher et al., 2000; Vachon et al., 2000).

Immunolocalization analyses

Cryosections (4-6 μm thick) of human fetal ileum and colon samples were fixed and stained by indirect immunofluorescence as described previously (Beaulieu et al., 1991; Beaulieu and Vachon, 1994; Perreault et al., 1995; Vachon et al., 2000). Rabbit antisera were used at 1:100-1:1000 dilutions, and mouse monoclonals were used at 1:50-1:1000 dilutions. All dilutions were made in PBS (pH 7.4) containing 5% (w/v) non fat powdered milk. FITC-conjugated goat anti-rabbit or anti-mouse IgG (Roche Diagnostics/Boehringer Mannheim, Laval, QC, Canada) were used as secondary antibodies. Sections were counterstained with 0.01% (w/v) Evans blue in PBS (pH 7.4), mounted in glycerol-PBS (9:1) containing 0.1% (w/v) paraphenylenediamine, and viewed with a Reichart Polyvar 2 microscope (Leica, St-Laurent, QC, Canada) equipped for epifluorescence. In all cases, no specific immunofluorescent staining was observed when primary antibodies were omitted or replaced by nonimmune (rabbit or mouse) serum (not shown). All immunofluorescent micrographs shown herein are representative of at least three ($n \geq 3$) different specimens for each developmental stage analyzed.

Analyses of protein expression levels

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% (w/v) acrylamide Tris-HCl gels (Biorad) was performed as described previously (Vachon et al., 1996, 1997, 2000). Broad range molecular mass markers (6.8-209 kD range; Biorad) were used as standards. Total proteins (50 μg/well) were separated by electrophoresis and then electrotransferred to nitrocellulose membranes (Supported NitroCellulose-1; Life Technologies/Gibco-BRL, Burlington, ON, Canada) for subsequent immunoblotting (Vachon et al., 1996, 1997, 2000). Rabbit antisera were used at 1:200-1:2000 dilutions, and mouse monoclonals were used at 1:100-1:5000 dilutions. Immunoreactive bands were visualized by the enhanced chemiluminescence method (ECL system; Amersham/Pharmacia Biotech., Baie D'Urfé, QC, Canada) according to the manufacturer's instructions.

To characterize the developmental epithelial expression profiles of Bcl-2 homologs in relation to morphogenesis of the ileum and colon, relative epithelial expression levels were evaluated by comparison with a reference protein, cytokeratin 18 (K18). This epithelial cytoskeletal component is expressed in the intestinal epithelium at constant levels throughout the development of the gut (Beaulieu et al., 1993; Vachon and Beaulieu, 1995; Vachon et al., 1995, 2000). Band intensities were quantified by laser densitometry using an Alpha Imager 1200 Documentation and Analysis system (Alpha Innotech Corp., San Leondro, CA). Total peak areas (AU x mm) were determined at 12-14, 15-16, 17-18 and 19-20 weeks of gestation in order to establish the ratios homolog/K18 for each molecule studied. Values shown represent mean ± SEM of at least three different specimens ($n \geq 3$) per developmental age group for each gut segment analyzed; statistically significant ($0.001 \leq p \leq 0.01$) differences were determined with the Student *t* test.

Results

To ascertain whether segment-specific control mechanisms of enterocytic apoptosis are acquired early during human gut development, we examined the epithelial expression and localization of Bcl-2 homologs,

acknowledged central regulators of programmed cell death, during morphogenesis of the ileum and colon.

Epithelial localization of Bcl-2 homologs during development of the ileum and colon: similarities and differences

Using specific antibodies, we first investigated by immunofluorescence the presence and localization of six Bcl-2 homologs and one associated molecule (Bag-1) in cryosections of human fetal ileum and colon specimens, aged between 12 and 20 wks of gestation. Of the Bcl-2 homologs studied, three are known for their anti-apoptotic functions (Bcl-2, Bcl-X_L, Mcl-1) and three others are pro-apoptotic regulators (Bax, Bak, Bad); also, the Bag-1 protein functions as an anti-apoptotic molecule (Wang et al., 1994, 1996; Takayama et al., 1995; Reed et al., 1996a,b; Adams and Cory, 1998). To our knowledge, there has been no report of Bag-1, Bax or Bad expression in the human ileum, or of Bag-1 and Bad expression in the human colon, prior to the present study.

Herein, all molecules analyzed were strongly detected throughout the epithelium of both the ileum and colon beginning at 12 wks (not shown; see also Fig. 3), when villogenesis is already underway (Ménard, 1989; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994). Between 15 and 17 wks of gestation, we observed in both gut segments that the epithelial localization of some of the Bcl-2 homologs underwent a gradual process of compartimentalization, which was found to culminate by mid-gestation (as illustrated in Figs. 1, 2). We have previously shown that this compartimentalization of epithelial Bcl-2 homolog expression coincides with the mid-gestation emergence of villus-tip apoptotic cells, whereas apoptotic enterocytes are not evidenced in prior developmental stages (Vachon et al., 2000).

For the ileum (Fig. 1), epithelial stainings for Bcl-X_L (Fig. 1A), Bag-1 (Fig. 1C), Bcl-2 (Fig. 1D) and Bak (Fig. 1E) were detected as decreasing crypt-to-villus gradients. In contrast to these, Mcl-1 staining was found concentrated in villus cells, but poorly detectable in crypt cells (Fig. 1B). Bax remained detectable throughout the epithelium (Fig. 1F). Note that, by 18 wks, staining for Bcl-2 had become very weak (Fig. 1D), whereas that for Bad was below detectable levels (not shown; see next section).

For the colon (Fig. 2), epithelial stainings for Bcl-X_L (Fig. 2A) and Bak (Fig. 2E) were detected as slightly decreasing crypt-to-villus gradients, whereas those for Bag-1 (Fig. 2C), Bcl-2 (Fig. 2D) and Bax (Fig. 2F) were observed throughout the epithelium. Mcl-1 staining was found weak at the apex of villi and base of crypts, but otherwise strongly expressed in the rest of the epithelium (Fig. 2B). Note that, beginning around 15 wks, staining for Bad had become below detectable levels (not shown, see next section).

The differential crypt-villus patterns of Bcl-2 homolog expression remained unchanged from 18 to 20 wks in both the ileum and colon. However, the comparison of the stainings between the two segments at mid-gestation revealed sharp differences in the case of some homologs. For instance, the relative staining intensity for Bcl-X_L (Fig. 1A, 2A), Bag-1 (Figs. 1C, 2C), Bcl-2 (Figs. 1D, 2D) and Bak (Figs. 1E, 2E) was clearly stronger in the colon than in the ileum. Conversely, Bag-1 (Figs. 1C, 2C) and Bcl-2 (Figs. 1D, 2D) exhibited crypt-villus decreasing gradients of expression in the ileum but not in the colon, whereas Mcl-1 (Fisg. 1B, 2B) displayed differing compartimentalizations between the two segments. In contrast to such differences between the two gut segments, we found that the staining patterns for Bcl-2 homologs (and the Bag-1 protein) in the proximal, median and distal portions of the mid-gestation colon were highly similar, if not identical (not shown).

Finally, it is of note that staining for all Bcl-2 homologs analyzed herein (including the non-homologous molecule Bag-1) was predominantly detected in the intestinal epithelium at all developmental stages studied (9-20 wks), mesenchymal cellular elements in both segments being weakly and/or sparsely stained depending on the molecule analyzed (Figs. 1, 2).

Epithelial expression levels of Bcl-2 homologs during morphogenesis of the ileum and colon

To further characterize the differences in staining intensities and crypt-villus axis localization of Bcl-2 homologs between the ileum and colon, we then investigated the developmental protein expression levels of these same homologs. Immunoblot analyses of lysates from mucosal scrappings (12 to 20 wks) demonstrated, in both gut segments, the protein expression of all molecules analyzed (Fig. 3). Thus, Bcl-2 (~26 kDa), Bcl-X_L (~28-30 kDa), Bag-1 (~32-34 kDa), Mcl-1 (~39-42), Bax (~21 kDa), Bak (~25-28 kDa) and Bad (~28-32 kDa) were detected at all developmental stages studied as protein bands migrating at their previously reported relative molecular weights (Krajewski et al., 1994a-c, 1995, 1996; Takayama et al., 1995; Krajewska et al., 1996; Packham et al., 1997; Adams and Cory, 1998; Metcalfe et al., 1999; Boucher et al., 2000; Vachon et al., 2000). To characterize the developmental epithelial expression profiles of each molecule studied in relation to the morphogenesis of the ileum and colon, their relative epithelial expression levels were evaluated by comparison with a reference protein, cytokeratin 18 (K18). The densitometric data presented in Figures 4-8 show that the relative epithelial expression levels of all Bcl-2 homologs analyzed (including the Bag-1 protein) are differentially modulated in parallel to the morphogenesis of both the ileum and colon.

In the case of the ileum (Figs. 3A, 4-5), Bcl-2 levels remained stable between 12 and 17 weeks, but decreased sharply around 18-19 wks (Figs. 3A, 4A), thus resulting in a significant ~36% overall reduction (Fig. 8A). Similarly, Bag-1 expression levels gradually decreased

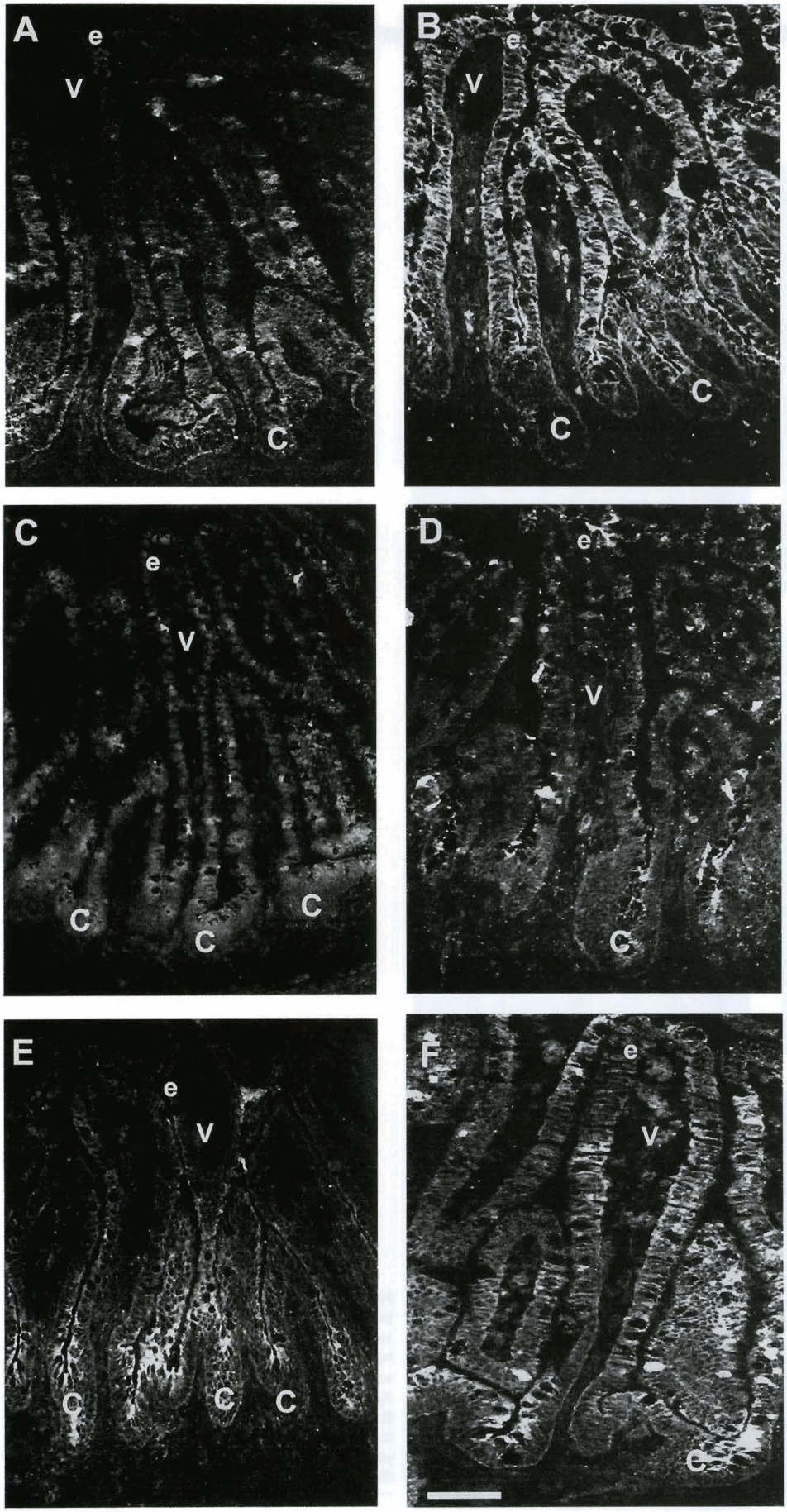


Fig. 1. Epithelial localization of Bcl-2 homologs in the human ileum at mid-gestation. Representative immunofluorescence micrographs of cryosections of human fetal ileums at 18 **(B, F)**, 19 **(A, D)** or 20 weeks of gestation stained for the detection of Bcl-X_L **(A)**, Mcl-1 **(B)**, Bag-1 **(C)**, Bcl-2 **(D)**, Bak **(E)** and Bax **(F)**. Note in (A), (D) and (F) that the primary antibodies cross-reacted with goblet/mucus cells of specimens used, as evidenced by bright spots sparsely distributed within the epithelium. C: crypt; e: simple columnar epithelium; V: villus. Bars: B, F, 90 μm; A, D, 100 μm; E, F, 125 μm.

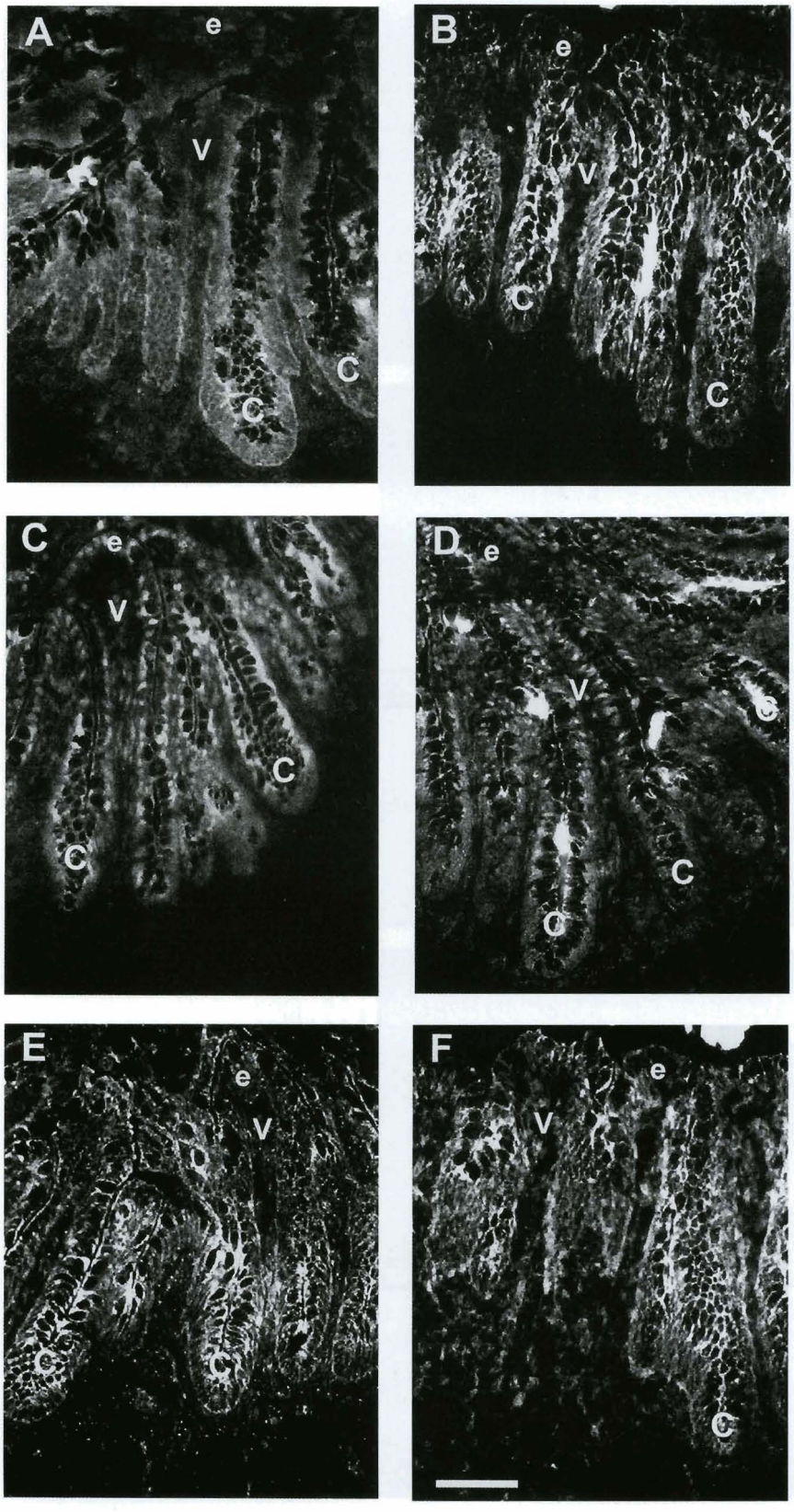


Fig. 2. Epithelial localization of Bcl-2 homologs in the human colon at mid-gestation. Representative immunofluorescence micrographs of cryosections of human fetal colons at 18 **(A,B)**, 19 **(C, D)** or 20 **(E,F)** weeks of gestation stained for the detection of Bcl-X_L **(A)**, Mcl-1 **(B)**, Bag-1 **(C)**, Bcl-2 **(D)**, Bak **(E)** and Bax **(F)**. Note in (D) that the primary antibodies cross-reacted with goblet/mucus cells of specimens used, as evidenced by bright spots sparsely distributed within the epithelium. C, crypt; e, simple columnar epithelium; V: villus. Bar: 100 μm.

between 12 and 20 wks (Figs. 3A, 4C), resulting in a significant ~36% overall reduction (Fig. 8A). Although Bcl-X_L increased between 12 and 16 wks, its levels decreased in subsequent stages (Figs. 3A, 4B), thus resulting in a slight (and non-significant) ~18% overall decrease (Fig. 8A). Mcl-1 steadily increased between 12 and 20 wks (Figs. 3A, 4D), resulting in a significant ~230% overall increment (Fig. 8A). Finally, Bax levels (Figs. 3A, 5A) underwent an overall non-significant decrease of ~20%, whereas Bak (Fig. 3A, 5B) and Bad (Figs. 3A, 5C) decreased significantly throughout ileum development, resulting in overall decreases of ~70% and ~90% respectively (Fig. 8A). Note that the weak detection of the Bad protein by mid-gestation (Fig. 3A) correlated with our failure to visualize its epithelial localization by immunofluorescence at the same stages (see previous section).

In the case of the colon (Figs. 3B, 6-7), the expression levels of Bcl-2 (Figs. 3B, 6A), Bag-1 (Figs. 3B, 6C), Mcl-1 (Figs. 3B, 6D) and Bak (Figs. 3B, 7B) gradually increased between 12 and 20 wks, resulting in significant overall increments of ~194%, ~79%, ~80% and ~110% respectively (Fig. 8B). Bcl-X_L (Figs. 3B, 6B) decreased sharply between 14 and 15 wks in order to increase again around 18-19 wks, resulting nonetheless in a significant overall decrease of ~48% (Fig. 8A). Similarly, Bad (Figs. 3B, 7C) decreased sharply around 14 and 15 wks in order to remain at low levels throughout subsequent stages, resulting in a significant overall decrease of ~83% (Fig. 8B). Note that the weak detection of the Bad protein by mid-gestation (Fig. 3B) correlated with our failure to visualize its epithelial localization by immunofluorescence at the same stages (see previous section). Finally, Bax levels decreased slightly between 12 and 20 wks (Figs. 3B, 7A), representing a non-significant ~25% overall decrease (Fig. 8B).

Establishment by mid-gestation of distinct epithelial Bcl-2 homolog expression profiles for the ileum and colon

Although the observed developmental modulations of Bcl-2 homolog expression appeared to parallel the morphogenesis of both the ileum and colon, we found

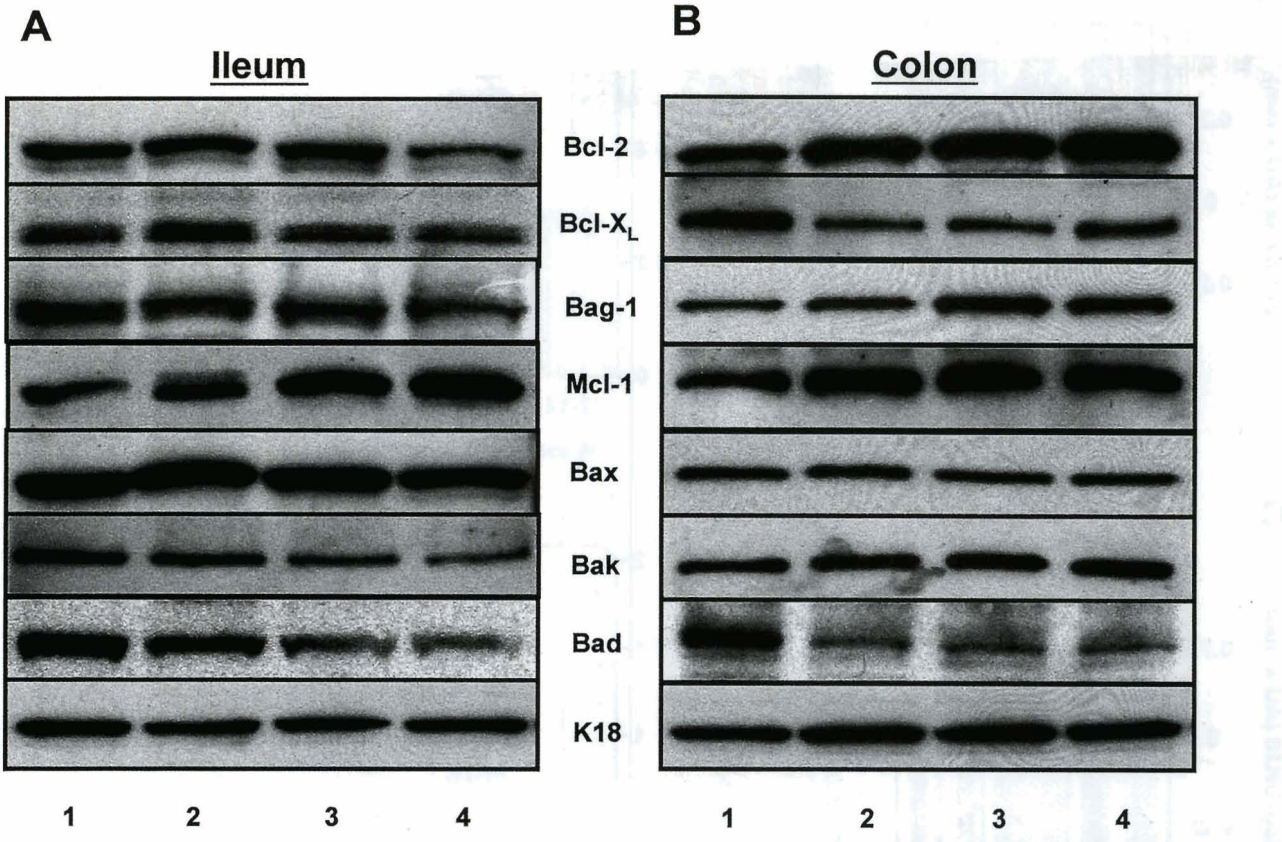


Fig. 3. Protein expression levels of Bcl-2 homologs during development of the human ileum and colon. Representative Western blot analyses of Bcl-2, Bcl-X_L, Bag-1, Mcl-1, Bax, Bak, Bad and cytokeratin 18 (K18) expression levels from mucosal scrappings of human fetal ileums **(A)** and colons **(B)** at 12-14 (lane 1), 15-16 (lane 2), 17-18 (lane 3) and 19-20 (lane 4) weeks of gestation. Total proteins (50 μg/well) were separated by SDS-PAGE under reducing conditions, electrotransferred onto nitrocellulose membranes, and then probed with specific antibodies for the detection of Bcl-2 (~26 kDa), Bcl-X_L (~28-30 kDa), Bag-1 (~32-34 kDa), Mcl-1 (~39-42 kDa), Bax (~21 kDa), Bak (~25-28 kDa), or Bad (~28-32 kDa). Detection of K18 (~50-55 kDa) was used as control of protein quantities analyzed per each developmental age group.

Table 1. Distinct relative epithelial Bcl-2 homolog expression profiles between the ileum and colon at mid-gestation.

HOMOLOG	ILEUM (ratio homolog/K18)	COLON (ratio homolog/K18)
Bcl-2	0.4885±0.0352	2.1143±0.2765*
Bcl-X_L	0.2144±0.0249	0.4033±0.0253*
Bag-1	0.4936±0.0705	1.0361±0.0567*
Mcl-1	1.3476±0.0953	1.5582±0.1138
Bax	1.9340±0.1149	0.7533±0.1614*
Bak	0.2920±0.0058	1.1667±0.2159*
Bad	0.0391±0.0067	0.1867±0.0087*

Data represent means±SEM for at least three ($n \geq 3$) specimens per gut segment. Total proteins from mucosal scrappings of ileum or colon specimens, aged between 18 and 20 wks of gestation, were separated and probed as represented in Figure 3, and then scanned by laser densitometry. Total peak areas (AU x mm) were determined for anti-apoptotic (Bcl-2, Bcl-X_L, Bag-1, Mcl-1) and pro-apoptotic (Bax, Bak and Bad) proteins in order to establish their relative epithelial expression levels in comparison to those of cytokeratin 18 (K18), as expressed by the ratios "homolog/K18". Asterisks (*) indicate statistically significant ($0.001 \leq p \leq 0.01$) differences between the colon and ileum.

that such modulations could differ sharply in tendencies (i.e. up or down-regulation) between the two gut segments (Figs. 3, 8). For instance, Bcl-2, Bag-1 and Bak levels increased between 12 and 20 wks in the colon (Figs. 3B, 8B), but the levels of these same homologs decreased in the ileum throughout the same developmental period (Figs. 3A, 8A). However, not all homologs behaved in such a differential fashion: the expression levels of Bcl-X_L, Mcl-1, Bax and Bad underwent developmental modulations with similar tendencies in both gut segments, albeit with differing orders of magnitude (Figs. 3A, 8A vs Figs. 3B, 8B). Nonetheless, the differential tendencies and/or orders of magnitude of these developmental modulations resulted by mid-gestation in the establishment of distinct Bcl-2 homolog expression profiles for the ileum and colon (Table 1; Fig. 3A, lane 4 vs Fig. 3B, lane 4). Indeed, the relative epithelial expression levels of nearly all molecules analyzed at 18-20 wks contrasted significantly between the two segments (Table 1).

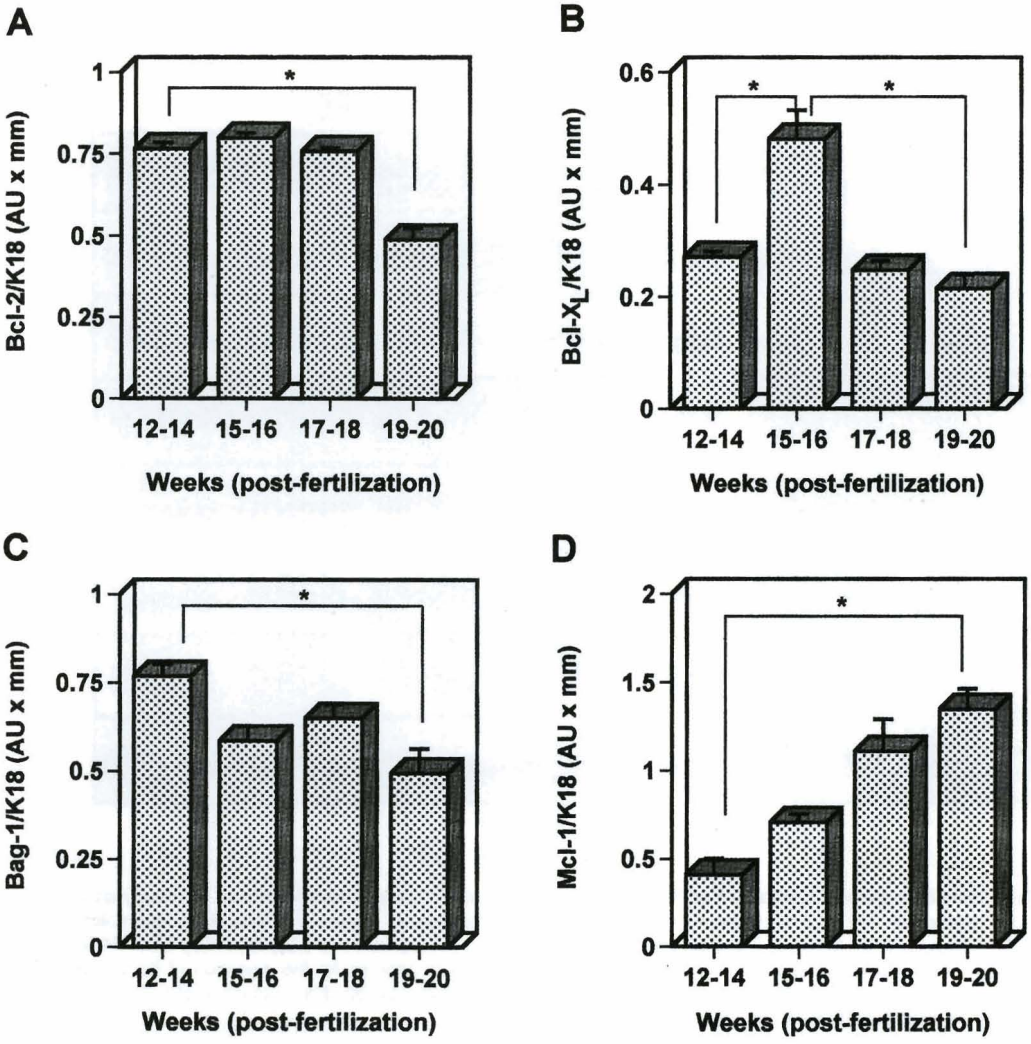
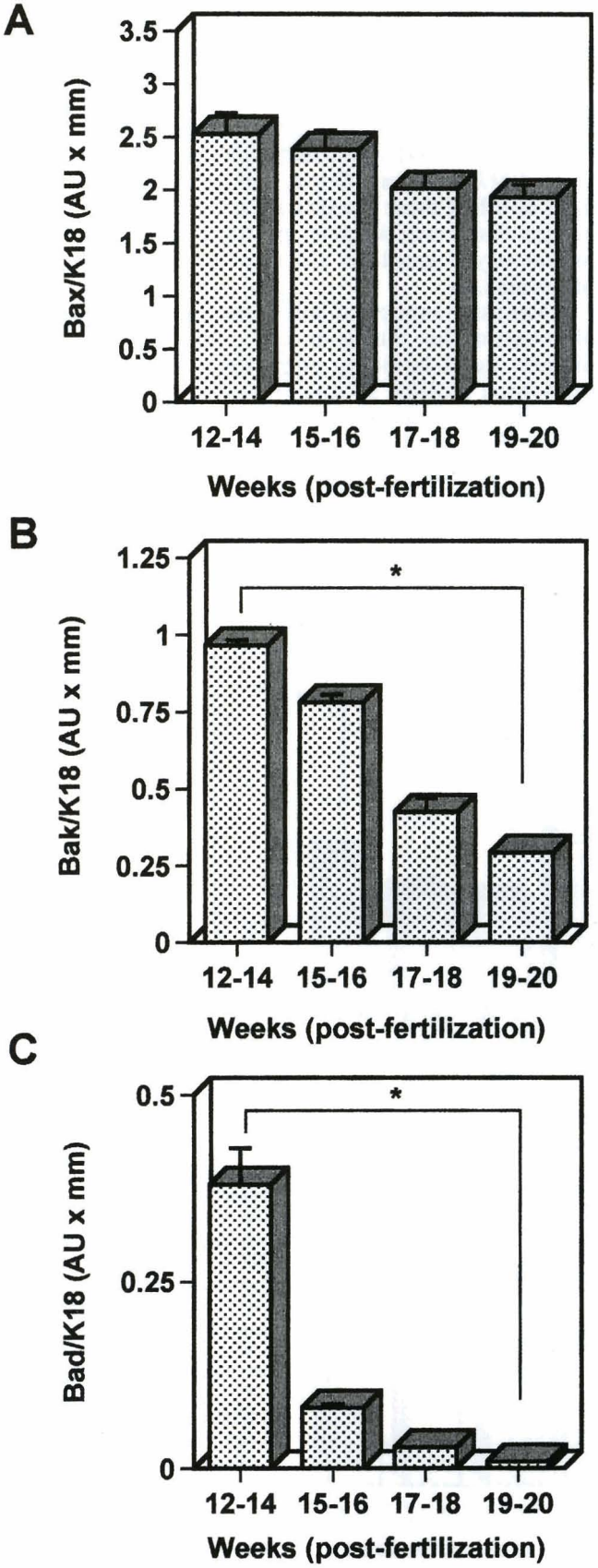


Fig. 4. Relative epithelial expression levels of anti-apoptotic Bcl-2 homologs during development of the human ileum. Relative expression levels of Bcl-2 **(A)**, Bcl-X_L **(B)**, Bag-1 **(C)** and Mcl-1 **(D)** during development of the human ileum, in comparison to cytokeratin 18 (K18). Total proteins from mucosal scrappings were separated and probed as represented in Fig. 3, and then scanned by laser densitometry. Total peak areas (AU x mm) were determined for each protein analyzed at 12-14, 15-16, 17-18 and 19-20 weeks of gestation (post-fertilization), in order to establish the ratios Bcl-2/K18 **(A)**, Bcl-XL/K18 **(B)**, Bag-1/K18 **(C)** and Mcl-1/K18 **(D)**. Each column represents the mean±SEM of at least three different specimens ($n \geq 3$) analyzed per developmental age group; statistically significant ($0.001 \leq p \leq 0.01$) differences are indicated by an asterisk (*).



Discussion

In this study, we examined the question whether segment-specific control mechanisms of enterocytic apoptosis are acquired early during human gut development. To do so, we investigated the localization and expression of six Bcl-2 homologs (Bcl-2, Bcl-X_L, Mcl-1, Bax, Bak, Bad), and one nonhomologous associated molecule (Bag-1), in the developing human ileum and colon between 12 and 20 wks of gestation. We found that the relative epithelial expression levels of Bcl-2 homologs undergo modulations during morphogenesis of the ileum and colon; however, the developmental profiles observed contrast significantly between the two gut segments. We also found that, beginning at 18 wks, the epithelial expression of Bcl-2 homologs exhibits differential patterns (or gradients) along the crypt-villus axis of both the ileum and colon; however, such patterns of expression differed between the two segments for some of the homologs studied. Finally, we found that the relative epithelial expression levels of Bcl-2 homologs differed greatly between the ileum and colon at 18-20 wks. Hence, by mid-gestation, the small intestine and colon exhibit distinct patterns of epithelial Bcl-2 homolog localization along their crypt-villus axis, as well as distinct epithelial Bcl-2 homolog expression profiles.

Previous studies in rodents (Krajewski et al., 1994a-c, 1995, 1996; Merritt et al., 1995; Wilson and Potten, 1996; Hirose et al., 1997; Aschoff et al., 1999; Pritchard et al., 1999) and man (Hockenbery et al., 1991; LeBrun et al., 1993; Lu et al., 1993, Hague et al., 1994; Krajewski et al., 1994c, 1995, 1996; Moss et al., 1996; Wilson et al., 2000) have shown differences in the expression of Bcl-2 homologs between the adult small and large intestines. The present study provides further documentation on segment-specific patterns of epithelial localization and expression of Bcl-2 homologs for the human small intestine and colon. Furthermore, our data show that the expression of Bcl-2 homologs is modulated differentially during gut development, resulting in the establishment by mid-gestation of distinct epithelial Bcl-2 homolog expression profiles between the two adjacent gut segments. Because Bcl-2 homologs constitute a critical checkpoint in the regulation of apoptosis, it has been suggested that

Fig. 5. Relative epithelial expression levels of pro-apoptotic Bcl-2 homologs during development of the human ileum. Relative expression levels of Bax **(A)**, Bak **(B)** and Bad **(C)** during development of the human ileum, in comparison to cytokeratin 18 (K18). Total proteins from mucosal scrappings were separated and probed as represented in Fig. 3, and then scanned by laser densitometry. Total peak areas (AU x mm) were determined for each protein analyzed at 12-14, 15-16, 17-18 and 19-20 weeks of gestation (post-fertilization), in order to establish the ratios Bax/K18 **(A)**, Bak/K18 **(B)** and Bad/K18 **(C)**. Each column represents the mean ± SEM of at least three different specimens ($n \geq 3$) analyzed per developmental age group; statistically significant ($0.001 \leq p \leq 0.01$) differences are indicated by an asterisk (*).

differential enterocytic Bcl-2 expression profiles may reflect the existence of segment-specific controls of intestinal epithelial apoptosis (Potten, 1992, 1997; Jones and Gores, 1997). Indeed, the balance of pro-versus anti-apoptotic Bcl-2 homologs, as well as their activities, determines in good part how cells respond to death and/or survival signals (Gajewski and Thompson, 1996; Reed et al., 1996a; Adams and Cory, 1998; Evan and Littlewood, 1998; Saini and Walker, 1998). To this effect, the observations in rodents that small intestinal cells display greater sensitivity to chemotherapeutic drug- or irradiation-induced apoptosis than colonic cells (Hall et al., 1994; Kiefer et al., 1995; Merritt et al., 1995; Hirose et al., 1997; Pritchard et al., 1999) strenghten the hypothesis that differences in Bcl-2 homolog expression correlate with distinct control mechanisms of apoptosis for the small intestine and colon (Potten, 1992, 1997; Jones and Gores, 1997). The need for such segment-specific distinctiveness in the regulation of cell survival is thought to be related to the nature of the luminal contents to which the intestinal epithelium is exposed to, along the proximal-distal axis of the gut: indeed, the colonic epithelium is exposed to an environment more pro-apoptotic than the small intestinal epithelium and thus would require greater resistance to apoptosis (Binder et al., 1991; Cobb and Williamson, 1991; Potten, 1992, 1997; Jones and Gores, 1997; Liu et al., 1999). In light of all of these considerations, our data altogether suggest that bowel segment-specific control mechanisms of enterocytic apoptosis are acquired early during fetal life in man.

Another aspect of our findings concerns our observation that the ileum and colon already display distinct Bcl-2 homolog crypt-villus localization and expression profiles by mid-gestation, even though the colon has yet to acquire its adult-like properties. On the one hand, the Bcl-2 homolog epithelial staining patterns observed herein for the mid-gestation ileum correlate well with (or confirm) those previously reported in the fetal (LeBrun et al., 1993; Lu et al., 1993; Vachon et al.,

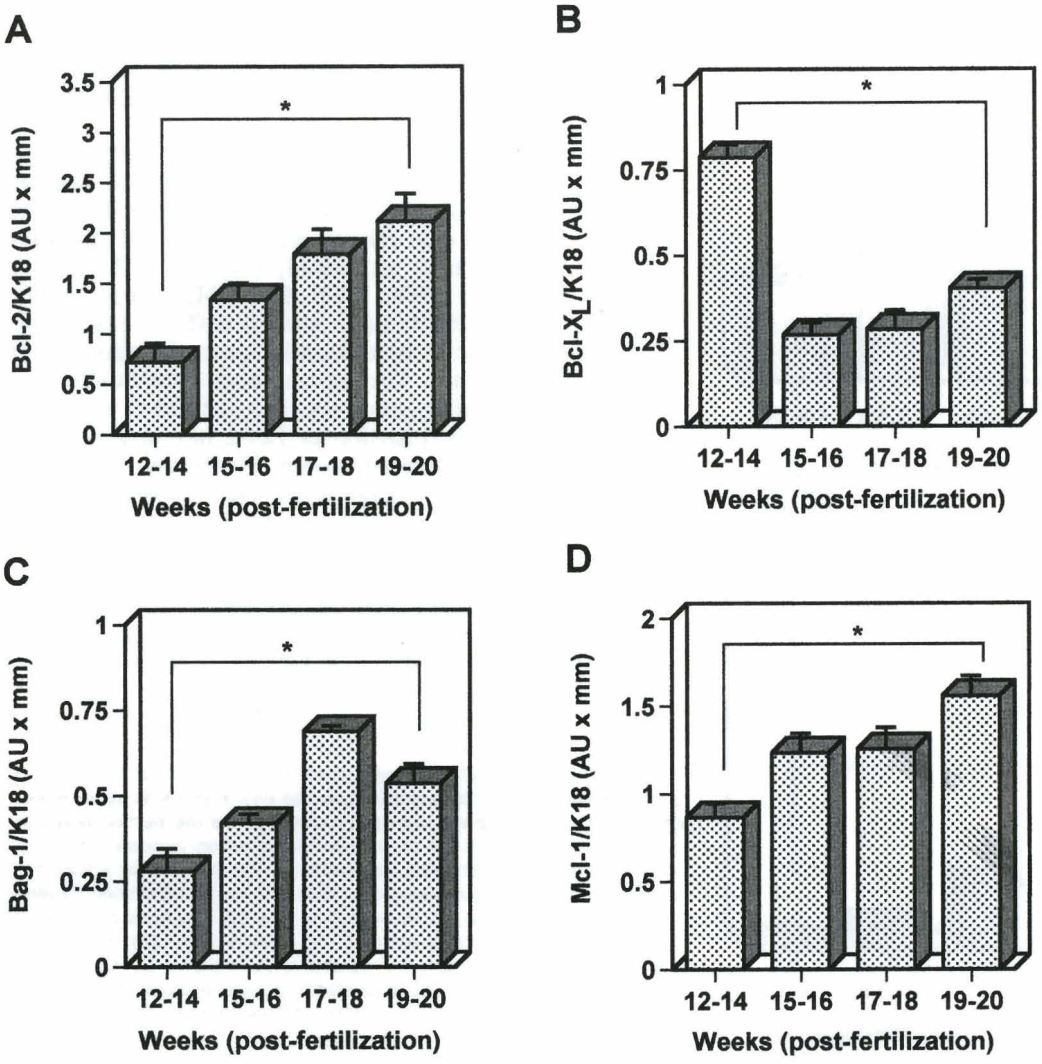
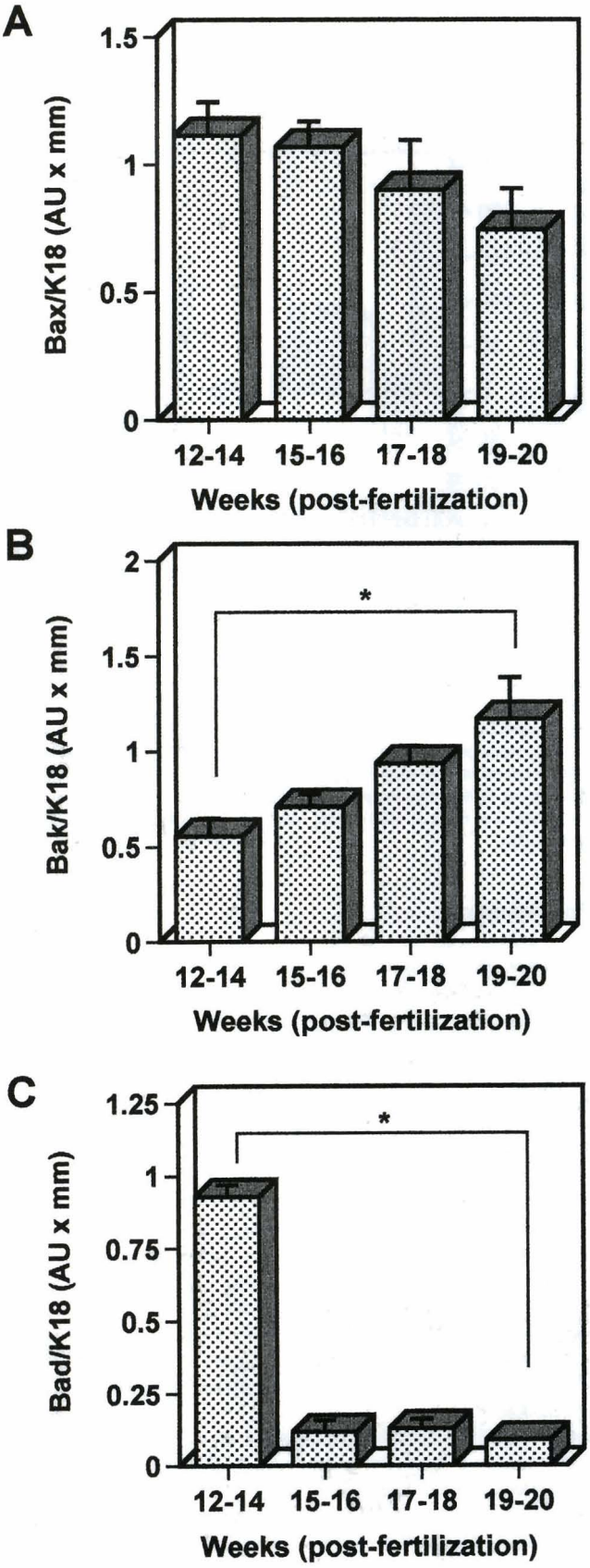


Fig. 6. Relative epithelial expression levels of anti-apoptotic Bcl-2 homologs during development of the human colon. Relative expression levels of Bcl-2 **(A)**, Bcl-X_L **(B)**, Bag-1 **(C)** and Mcl-1 **(D)** during development of the human colon, in comparison to cytokeratin 18 (K18). Total proteins from mucosal scrappings were separated and probed as represented in Fig. 3, and then scanned by laser densitometry. Total peak areas (AU x mm) were determined for each protein analyzed at 12-14, 15-16, 17-18 and 19-20 weeks of gestation (post-fertilization), in order to establish the ratios Bcl-2/K18 **(A)**, Bcl-XL/K18 **(B)**, Bag-1/K18 **(C)** and Mcl-1/K18 (D). Each column represents the mean ± SEM of at least three different specimens ($n \geq 3$) analyzed per developmental age group; statistically significant ($0.001 \leq p \leq 0.01$) differences are indicated by an asterisk (*).



2000) and adult human small intestine (Hockenbery et al., 1991; Lu et al., 1993; Krajewski et al., 1994c, 1995, 1996; Moss et al., 1996), indicating that adult-like small intestinal Bcl-2 homolog expression profiles are acquired early in man, as is the case for other enterocytic cellular processes and functions (Ménard, 1989; Cobb and Williamson, 1991; Ménard and Calvert, 1991; Ménard and Beaulieu, 1994; Vachon et al., 2000). On the other hand, the stainings shown herein for Bcl-2, Bcl-X_L, Mcl-1, Bax and Bak in the mid-gestation colon differ from those already observed in the adult counterpart (Lu et al., 1993; Krajewski et al., 1994c, 1995, 1996; Krajewska et al., 1996; Moss et al., 1996; Liu et al., 1999). For example, Bcl-2 staining was observed throughout the epithelium of the 18-20 wks old fetal colon (Fig. 2D), whereas it exhibits a decreasing crypt-to-surface gradient of expression in the adult (Lu et al., 1993; Krajewski et al., 1995; Krajewska et al., 1996). Similarly, Bak epithelial localization was found as a decreasing gradient along the crypt-villus axis of the mid-gestation colon (Fig. 2E), whereas it is compartimentalized to surface and upper mid-crypt cells in the adult (Krajewski et al., 1996; Moss et al., 1996; Liu et al., 1999). Hence, such apparent differences between the mid-gestation and adult colon suggest that further developmental changes in colonic Bcl-2 homolog expression profiles and apoptotic control mechanisms may occur between 30 wks and birth, as the large intestine undergoes its final maturation to acquire its adult morphological and functional features. In support of this, it is noteworthy that a recent study has provided evidence for differences in epithelial sensitivity to apoptosis and Bak expression between the right and left adult human colon (Liu et al., 1999), whereas we observed no differences in the staining patterns of Bcl-2 homologs between the proximal, median and distal portions of the mid-gestation colon.

In conclusion, the present findings provide new insights into the developmental establishment of putative segment-specific control mechanisms of epithelial cell apoptosis in the human gut, whereby the expression of Bcl-2 homologs is modulated differentially in order to establish, by mid-gestation, distinct Bcl-2 homolog epithelial localization and expression profiles for the small intestine and colon. The developmental processes responsible for such early acquirement of segmental distinctions in intestinal Bcl-2 homolog expression in

Fig. 7. Relative epithelial expression levels of pro-apoptotic Bcl-2 homologs during development of the human colon. Relative expression levels of Bax **(A)**, Bak **(B)** and Bad **(C)** during development of the human colon, in comparison to cytokeratin 18 (K18). Total proteins from mucosal scrappings were separated and probed as represented in Fig. 3, and then scanned by laser densitometry. Total peak areas (AU x mm) were determined for each protein analyzed at 12-14, 15-16, 17-18 and 19-20 weeks of gestation (post-fertilization), in order to establish the ratios Bax/K18 **(A)**, Bak/K18 **(B)** and Bad/K18 **(C)**. Each column represents the mean ± SEM of at least three different specimens ($n \geq 3$) analyzed per developmental age group; statistically significant ($0.001 \leq p \leq 0.01$) differences are indicated by an asterisk (*).

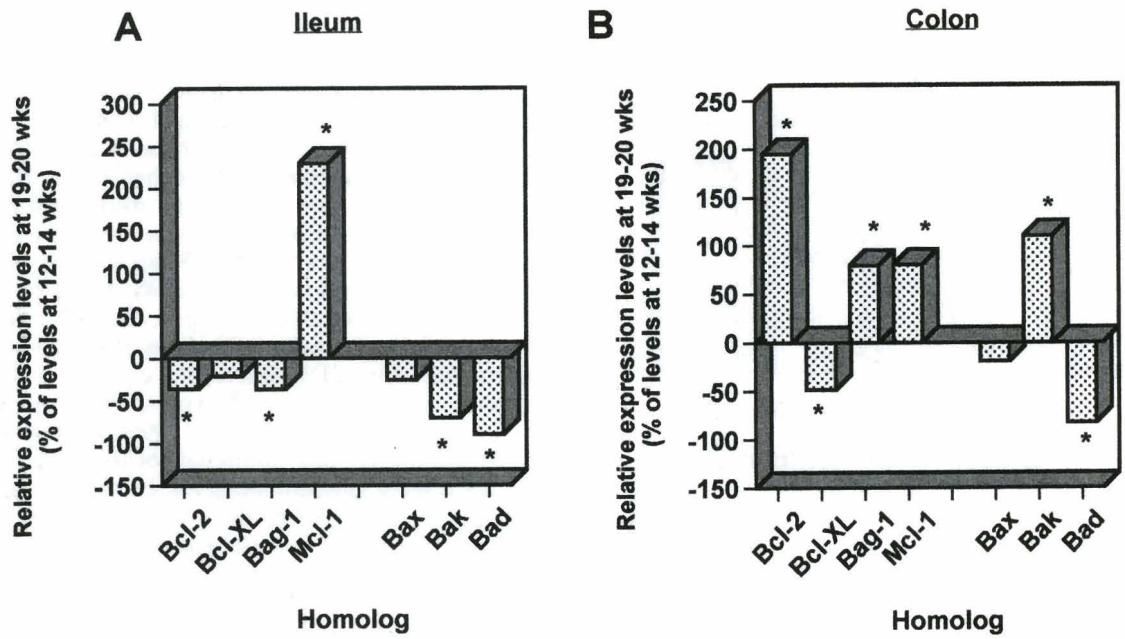


Fig. 8. Differential modulations of epithelial Bcl-2 homolog expression levels during development of the human ileum and colon. Total proteins (50 μg/well) from mucosal scrappings of human fetal ileums **(A)** and colons **(B)** at 12-14 and 19-20 weeks (wks) were separated by SDS-PAGE under reducing conditions, electrotransferred onto nitrocellulose membranes, probed with specific antibodies for the detection of Bcl-2, Bcl-X_L, Bag-1, Mcl-1, Bax, Bak or Bad, and then scanned by laser densitometry. Total peak areas (AU x mm) were determined for each protein analyzed at 12-14 and 19-20 wks of gestation (post-fertilization), in order to establish the relative increase or decrease of protein expression levels at 19-20 wks, in comparison to 12-14 wks. Values are expressed as "% of levels at 12-14 wks", where "0%" indicates no differences in protein expression levels between 19-20 and 12-14 wks. Each column represents the mean ± SEM of at least six different specimens (i.e. n≥ 3 at 12-14 wks, n≥ 3 at 19-20 wks) per gut segment analyzed; statistically significant (0.001≤ p≤ 0.01) differences are indicated by asterisks (*).

man remain to be understood. For instance, do the distinct segmental localization patterns of some of the Bcl-2 homologs studied herein reflect upon differential functions, for these same homologs, in the precise control of epithelial cell survival and/or apoptosis between the small intestine and colon? Further analyses, using in vitro model systems, will be required to dissect at the molecular level the mechanisms which influence the functions of Bcl-2 homologs in epithelial cells, including their reciprocal homo-/heterodimerization patterns along the crypt-villus axis, as well as to identify the exact roles enacted by Bcl-2 homologs themselves in the regulation of epithelial programmed cell death and in the pathogenesis of disorders that involve dysregulation of apoptosis.

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