Journal of Cellular Biochemistry 82:339–355 (2001)

Differential Sensitivity to Apoptosis Between the Human Small and Large Intestinal Mucosae: Linkage With Segment-Specific Regulation of BCL-2 Homologs and Involvement of Signaling Pathways

Rémy Gauthier,[1] Patrick Laprise,[1] Éric Cardin,[1] Charlène Harnois,[1] Annie Plourde,[1] John C. Reed,[2] Anne Vézina,[1] and Pierre H. Vachon[1,3]*

[1]CIHR Group on the Functional Development and Physiopathology of the Digestive Tract, département d'anatomie et de biologie cellulaire, Faculté de médecine, Université de Sherbrooke, Sherbrooke, QC, Canada
[2]The Burnham Institute, La Jolla Cancer Research Center, La Jolla, CA, USA
[3]Thématique de recherche en physiopathololgie digestive du Centre de recherches cliniques du CHUS, Fleurimont, QC, Canada

Abstract The small and large intestines differ in their expression profiles of Bcl-2 homologs. Intestinal segment-specific Bcl-2 homolog expression profiles are acquired as early as by mid-gestation (18–20 weeks) in man. In the present study, we examined the question whether such distinctions underlie segment-specific control mechanisms of intestinal cell survival. Using mid-gestation human jejunum and colon organotypic cultures, we analyzed the impact of growth factors (namely insulin; 10 μg/ml) and pharmacological compounds that inhibit signal transduction molecules/pathways (namely tyrosine kinases, Fak, PI3-K/Akt, and MEK/Erk) on cell survival and Bcl-2 homolog expression (anti-apoptotic: Bcl-2, Bcl-X_L, Mcl-1; pro-apoptotic: Bax, Bak, Bad). The relative activation levels of $p125^{Fak}$, $p42^{Erk-2}$,and $p57^{Akt}$ were analyzed as well. Herein, we report that (1) the inhibition of signal transduction molecules/pathways revealed striking differences in their impact on cell survival in the jejunum and colon (e.g., the inhibition of $p125^{Fak}$ induced apoptosis with a significantly greater extent in the jejunum [~43%] than in the colon [~24%]); (2) sharp distinctions between the two segments were noted in the modulatory effects of the various treatments on Bcl-2 homolog steady-state levels (e.g., inhibition of tyrosine kinase activities in the jejunum down-regulated all anti-apoptotics analyzed while increasing Bax, whereas the same treatment in the colon down-regulated Bcl-X_L only and increased all pro-apoptotics); and (3) in addition to their differential impact on cell survival and Bcl-2 homolog expression, the MEK/Erk and PI3-K/Akt pathways were found to be distinctively regulated in the jejunum and colon mucosae (e.g., insulin in the jejunum increased $p42^{Erk-2}$ activation without affecting that of $p57^{Akt}$, whereas the same treatment in the colon decreased $p42^{Erk-2}$ activation while increasing that of $p57^{Akt}$). Altogether, these data show that intestinal cell survival is characterized by segment-specific susceptibilities to apoptosis, which are in turn linked with segmental distinctions in the involvement of signaling pathways and the regulation of Bcl-2 homolog steady-state levels. Therefore, these indicate that cell survival is subject to segment-specific control mechanisms along the proximal-distal axis of the intestine. J. Cell. Biochem. 82: 339–355, 2001.

Key words: cell survival; enterocyte; Fak; gut; MAP kinases; PI3-K; programmed cell death; signal transduction

Apoptosis, or programmed cell death, is a highly regulated process which plays a crucial role in tissue morphogenesis, repair and homeostasis [White, 1996; Jacobson et al., 1997]. Dysregulated apoptosis can contribute to cancer, autoimmunity, immunodeficiency, infertility, and degenerative disorders [Thompson, 1995; Reed et al., 1996a; White, 1996]. Developmental or environmental cues deliver complex signals that promote cell death or survival.

Rémy Gauthier and Patrick Laprise contributed equally to this work.

Grant sponsor: The Canadian Institutes of Health Research (CIHR); Grant number: MGC-15186.

*Correspondence to: Pierre H. Vachon, Département d'anatomie et de biologie cellulaire, Faculté de médecine, Université de Sherbrooke, Sherbrooke (QC) J1H5N4, Canada. E-mail: phvachon@courrier.usherb.ca

Received 28 November 2000; Accepted 8 February 2001

In this respect, dissection of the cell death pathway has led to the identification of a large number of both pro- and anti-apoptotic molecules [Reed et al., 1996a,b; White, 1996; Adams and Cory, 1998]. Such molecules are expressed to varying degrees in tissues, with differential "subsets" and/or levels expressed according to the cell type studied. This in turn is thought to confer distinct susceptibilities to apoptosis between the various tissues and organs, even for those closely related developmentally such as the small and large intestines [Reed et al., 1996a; Jones and Gores, 1997; Potten, 1997; Adams and Cory, 1998].

The Bcl-2 family of proteins constitutes a critical decisional checkpoint in apoptosis, regulating the common downstream effector pathway of programmed cell death [Reed et al., 1996a,b; Adams and Cory, 1998]. At least 15 family members have been identified so far, functioning either as anti-apoptotic (e.g., Bcl-2, Bcl-X_L, Mcl-1) or pro-apoptotic (e.g., Bax, Bad, Bak) 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 [Gajewski and Thompson, 1996; Reed et al., 1996a,b; Adams and Cory, 1998]. To this effect, it is now well acknowledged that suppression or induction of apoptosis in different cell types does not depend on the activity of single Bcl-2 homologs, but rather on a balance of anti- and pro-apoptotic activities from multiple homologs [Reed et al., 1996a,b; Adams and Cory, 1998].

Incoming extracellular signals determine in large part if a cell lives or die, and involve signaling events that ultimately affect the expression and/or functions of multiple anti- and pro-apoptotic Bcl-2 homologs [Gajewski and Thompson, 1996; Anderson, 1997; Frisch and Ruoslahti, 1997; Adams and Cory, 1998; Datta et al., 1999; Cross et al., 2000]. Such survival signals originate from growth factors (e.g., insulin) and cell adhesion [Anderson, 1997; Frisch and Ruoslahti, 1997; Datta et al., 1999; Giancotti and Ruoslahti, 1999; Cross et al., 2000; Whitehead et al., 2000] often implicating common signaling pathways [Anderson, 1997; Parsons and Parsons, 1997; Datta et al., 1999; Giancotti and Ruoslahti, 1999; Cross et al., 2000]. Among the molecules/pathways known to intervene in cell survival, the roles of focal adhesion kinase ($p125^{Fak}$), of the PI3-K (phosphatidylinositol 3′-kinase)/Akt (PKB; $p57^{Akt}$) pathway and of the MEK (mitogen-activated protein kinase [MAPK] kinase)/Erk (extracellular regulated kinases I and 2; $p42^{Erk-2}$/$p44^{Erk-1}$ MAPK) pathway, have received considerable attention [Anderson, 1997; Parsons and Parsons, 1997; Datta et al., 1999; Giancotti and Ruoslahti, 1999; Cross et al., 2000]. However, it is becoming increasingly evident that the involvement of signaling molecules and pathways in cell survival greatly varies depending on the cell type that is receiving such signals, thus underlying the complexity in the regulation of apoptosis among various tissues [Anderson, 1997; Adams and Cory, 1998; Datta et al., 1999; Coppolino and Dedhar, 2000; Cross et al., 2000; Whitehead et al., 2000].

Although originating from the same tube-like primitive gut, the adult small and large intestinal mucosae differ in organization and physiological functions. The predominant physiological role of the colon is to regulate luminal fluid and electrolyte contents, whereas that of the small intestines lie in the digestive process and absorption of nutrients [Ménard, 1989; Cobb and Williamson, 1991; Ménard and Beaulieu, 1994]. Intestinal pathologies 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; Mendeloff, 1991; Ménard and Beaulieu, 1994]. An example of this is cancer, which is extremely rare in the small intestine as opposed to the colon [Ahnen, 1991; Mendeloff, 1991]. To this effect, there is accumulating evidence in both rodents and man which indicates that the expression profiles of Bcl-2 homologs differ between the adult small and large intestines [Hockenbery et al., 1991; Krajewski et al., 1994a,b,c, 1995, and 1996; Merritt et al., 1995; Krajewska et al., 1996; Wilson and Potten, 1996; Potten, 1997; Wilson et al., 2000]. In our laboratory, we have shown that such segment-specific expression profiles are acquired as early as by mid-gestation (18–20 weeks) in man [Vachon et al., 2001], a stage when adult-like intestinal functional and physiological processes, including apoptosis [Vachon et al., 2000] are already established [Grand et al., 1976; Ménard, 1989; Ménard and Beaulieu, 1994]. It has been suggested that such distinct Bcl-2 homolog expression profiles along with the

proximal-distal gradient of the gut may underlie segment-specific control mechanisms of intestinal cell survival [Potten, 1992; Jones and Gores, 1997; Potten, 1997].

In the present study, we examined this hypothesis by maintaining mid-gestation human fetal jejunum and colon explants in the presence of growth factors or pharmacological compounds that inhibit signal transduction molecules, in order to comparatively evaluate their impact on intestinal cell survival and Bcl-2 homolog expression. We find that jejunum and colon enterocytes, as well as mesenchymal cells, display striking differences in their susceptibility to undergo apoptosis when exposed to the various treatments. We also find that exposure to growth factors, or inhibition of signaling pathways, modulate the expression of Bcl-2 homologs in the jejunum and colon mucosae; however, the modulatory effects and homologs affected vary between the two segments. Furthermore, we find that the PI3-K/Akt and MEK/Erk pathways display a distinct involvement in the survival of jejunum and colon cells. Altogether, these data indicate that the differential sensitivity to apoptosis between the small and large intestinal mucosae is linked with segment-specific regulation of Bcl-2 homologs and involvement of signaling molecules/pathways.

MATERIALS AND METHODS

Tissue Processing and Organotypic Culture

Human fetal jejunum and colon specimens from 24 fetuses aged 18–20 weeks (post-fertilization) were obtained after therapeutic termination of pregnancy. Only specimens obtained rapidly (30 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.

The organotypic culture of jejunum and colon explants was performed according to the method of Ménard and Arsenault [1985, 1987]. Specimens were cleansed of mesentery, washed at room temperature in Leibovitz L-15 culture medium (Gibco BRL, Burlington, ON, Canada) supplemented with garamycin (40 μg/ml) and cut into explants (3 mm × 7 mm). The explants were then transferred onto lense paper (Canadian Laboratory Supplies Ltd., Montrèal, QC, Canada) with the mucosal side up. The lense paper covered a stainless steel grid lying over the central well of a Falcon organ culture dish (Falcon Plastics, Los Angeles, CA). Sufficient medium was present to just wet the paper; the medium used was Leibovitz L-15 supplemented with garamycin (40 μg/ml) and mycostatin (40 μg/ml). Explants were stabilized for 4 h at 37°C in a water-saturated atmosphere composed of 5% CO_2-95% air, at which point mucus was carefully removed and the medium renewed. Explants were thereafter maintained for 48 h under the same conditions and medium (with or without further additions, see below), with removal of mucus and renewal of medium (with or without additions) after 24 h.

For each gut segment, 48-h treatments of explants (4–12 explants/treatment) were performed by adding the following to the medium: 10 μg/ml human recombinant insulin (Gibco/BRL) [Ménard, 1989]; 150–300 μM genistein (Sigma, St-Louis, MO), a wide-specter tyrosine kinase activity inhibitor [Anderson, 1997]; 0.3–1 μM cytochalasin D (Sigma), which inhibits Fak specifically without affecting other cell adhesion components within the concentration range used [Lipfert et al., 1992; Oktay et al., 1999; Wary et al., 1999; Barberis et al., 2000; Gauthier et al., 2001]; 20–60 μM PD98059 (Calbiochem, San Diego, CA), a specific inhibitor of MEK, the upstream activator of Erk-1/Erk-2 [Allesi et al., 1995; Boucher et al., 2000; Le Gall et al., 2000; Gauthier et al., 2001]; or 5–30 μM Ly294002 (Calbiochem), a specific inhibitor of PI3-K [Vlahos et al., 1994; Le Gall et al., 2000; Gauthier et al., 2001]. For both the jejunum and colon, determination of the working concentrations of the compounds used led us to settle with the following: genistein, 150 μM; cytochalasin D, 1 μM; PD98059, 20 μM; and Ly294002, 15 μM. In any event, necrosis was not observed throughout the treatments, including in the control cultures (not shown), as expected [Ménard and Arsenault, 1985, 1987].

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-X_L [Krajewski et al., 1994c]; Ab 1701 [Krajewski et al., 1994a] and Ab PC68 (Calbiochem), 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-X_L; 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 1505 (Chemicon Intl., Harrow, ON, Canada), directed against human actin.

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,b,c, 1995, and 1996; Takayama et al., 1995; Krajewska et al., 1996; Wang et al., 1996; Vachon et al., 2000; Gauthier et al., 2001; Vachon et al., 2001].

In Situ Detection of Apoptosis-Associated DNA Strand Breaks

After treatments (see above), explants were washed in PBS (pH 7.4) to remove mucus and medium, then embedded in OCT (Optimum Cutting Temperature) compound (Tissue Tek, Miles Laboratories, Elkhart, IN) as previously described [Beaulieu et al., 1991; Vachon et al., 2000, 2001]. In situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (ISEL) [Gavrieli et al., 1992] was carried out as already described [Vachon et al., 1996a, 2000; Gauthier et al., 2001] on 4–6 μm thick cryosections, using the ApopTag apoptosis detection kit (Oncor, Gaithersburg, MD). Preparations where then counterstained with Evans blue, mounted and viewed with a Reichart Polyvar 2 microscope (Leica, St-Laurent, QC, Canada) equipped for epifluorescence.

Evaluation of ISEL-positive (i.e., apoptotic) cells was performed as previously described [Vachon et al., 1996a,b, 2000; Gauthier et al., 2001]. For the evaluation of the total proportion of apoptotic intestinal cells (i.e., enterocytes and mesenchymal cells combined), a minimum of 600 cells (300 epithelial cells and 300 mesenchymal cells) was counted in at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment). For the specific evaluation of the proportion of apoptotic enterocytes and mesenchymal cells, a minimum of 300 cells of either type (epithelial or mesenchymal) was counted in at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment). In all cases, the apoptotic indices were expressed as the percentage (%) of apoptotic cells over the total number of cells counted (±SEM); statistically significant ($0.001 \leq P \leq 0.01$) differences were determined with the Student's t test.

Protein Expression Levels

For analyses of protein expression levels of Bcl-2 homologs, explants were washed in PBS (pH 7.4) to remove mucus and medium, carefully stripped of their outer muscle walls, then homogenized in 20 mM Tris–HCl (pH 6.8) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 50 μg/ml leupeptin, 50 μg/ml antipain, and 0.1 mg/ml aprotinin. Total proteins were measured using the BioRad (Hercules, CA) protein assay. Aliquots of homogenates were directly solubilized in 2 × 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] β-mercaptoethanol), boiled (105°C, for 5 min), cleared by centrifugation (13000*g*, for 5 min, at room temperature), and processed for storage as described [Vachon et al., 1996a, 2000, 2001].

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., 1997, 2000, 2001; Gauthier et al., 2001]. Broad range molecular mass markers (6.8–209 kDa 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., 1997, 2000, 2001]. 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. Band intensities were quantified by laser densitometry using an Alpha Imager 1,200 Documentation and Analysis system

(Alpha Innotech Corp., San Leondro, CA). For each treatment of explants, relative expression levels of molecules analyzed were evaluated in comparison with that of control cultures, by determining total peak areas (AU × mm) in order to establish the ratios treatment/control × 100 (expressed as "% of control"). Values shown represent mean±SEM for at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment); statistically significant ($0.001 \leq P \leq 0.01$) differences were determined with the Student's *t* test.

Assay of Fak, Erk-1/Erk-2 and Akt Relative Activation Levels by Immunoblotting

Total proteins were resolved by SDS-PAGE and electrotransferred as described above. Assays of $p125^{Fak}$, $p42^{Erk\text{-}2}/p44^{Erk\text{-}1}$, or $p57^{Akt}$ relative activation levels were performed as described elsewhere [Aliaga et al., 1999; Boucher et al., 2000; Le Gall et al., 2000; Gauthier et al., 2001]. For Fak assays, membranes were first probed with the rabbit polyclonal Ab07–012 (Upstate Biotechnology, Lake Placid, NY) directed to the activated phospho-Tyr397 form of Fak [Giancotti and Ruoslahti, 1999; Coppolino and Dedhar, 2000], then reprobed with the rabbit polyclonal Ab C-903 (Santa Cruz Biotech) directed to total Fak for normalization purposes. Similarly, for Erk-1/Erk-2 assays, membranes were probed with the rabbit polyclonal Ab9101s (New England Biolabs) directed to the doubly-phosphorylated (activated) forms of Erk-1/Erk-2 [Anderson, 1997; Cross et al., 2000], then reprobed with the rabbit polyclonal Ab9102 (New England Biolabs) directed to total Erk-1/Erk-2. Finally, assays of $p57^{Akt}$ relative activation levels were performed by first probing membranes with the rabbit polyclonal Ab9271s (New England Biolabs) directed to the activated phospho-Ser473-Akt form [Anderson, 1997; Datta et al., 1999; Vanhaesebroeck and Alessi, 2000], then reprobed with the rabbit polyclonal Ab9272 (New England Biolabs) directed to total Akt.

In all cases, immunoreactive bands were visualized by the enhanced chemiluminescence method (ECL system; Amersham/Pharmacia Biotech.) according to the manufacturer's instructions. Band intensities were then quantified by laser densitometry using an Alpha Imager 1,200 Documentation and Analysis system (Alpha Innotech Corp.). The relative activation levels of $p125^{Fak}$, $p42^{Erk\text{-}2}/p44^{Erk\text{-}1}$, or $p57^{Akt}$ were evaluated by first determining the total peak areas (AU × mm) for the phosphorylated forms and for the corresponding total protein, in order to establish the ratios $pp125^{Fak}/p125^{Fak}$, $pp42^{Erk\text{-}2}/p42^{Erk\text{-}2}$, and $pp57^{Akt}/p57^{Akt}$. In the case of Erk-1/Erk-2, analyses focused on $p42^{Erk\text{-}2}$ since this MAPK is predominantly expressed and activated in intestinal cells, as compared to $p44^{Erk\text{-}1}$ (see Fig. 5; Aliaga et al., 1999; Gauthier et al., 2001). Ratios were in turn compared to those of control cultures, × 100 (expressed as "% of control"). Values shown represent mean±SEM for at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment); statistically significant ($0.001 \leq P \leq 0.01$) differences were determined with the Student's *t* test.

RESULTS

To ascertain whether human intestinal cell survival is subject to bowel segment-specific regulatory mechanisms, we analyzed the in vitro effects of survival-promoting growth factors and pharmacological inhibitors of signal transduction molecules/pathways on intestinal cell apoptosis and Bcl-2 homolog expression, in cultured human jejunum and colon explants. The relative activation levels of Fak, Erk-1/Erk-2, and Akt were specifically analyzed as well.

Differential Susceptibilities to Apoptosis Between the Jejunum and Colon Mucosae

Using the ISEL method, we first determined the total apoptotic indices (i.e., of both epithelial and mesenchymal cells) in jejunum and colon explants maintained under normal conditions (i.e., L-15 medium without further additions) as controls, as well as in the presence of insulin or pharmacological inhibitors of signal transduction molecules/pathways. Exposure to insulin had no significant influence on cell survival in either the jejunum or colon mucosae after 48 h of treatment (Table I), most likely due to the fact that the intestinal epithelium and mesenchyme are normally well preserved after two days of organotypic culture even in the absence of growth factors (Table II; Ménard and Arsenault, 1985, 1987; Ménard, 1989). In contrast, the inhibition of tyrosine kinase activities greatly impacted on cell survival in both gut segments (~65% apoptosis in the jejunum and ~54% apoptosis in the colon; Table I).

TABLE I. Segment-Specific Control Mechanisms of Intestinal Cell Survival in the Human Jejunum and Colon Mucosae

Treatment[a]		Apoptotic index[c]	Homolog[d]						
			Bcl-2	Bcl-X_L	Mcl-1	Bag-1	Bax	Bak	Bad
Control	J[b]	5.8±1.7							
	C	3.9±1.5							
+I (10 μg/ml)	J	3.7±1.4	↓	↑		↓	↓		↓
	C	2.9±0.9		↓		↓			
+G (150 μM)	J	64.5±10.4*	↓	↓	↓	↓	↑		↓
	C	53.8±8.1*		↓			↑	↑	↑
+PD (20 μM)	J	21.9±4.5*#	↓	↓					↓
	C	9.1±2.1*	↓				↑		
+Ly (15 μM)	J	37.3±6.7*#	↓		↓		↑		↓
	C	11.7±2.2*	↓		↓				
+CD (1 μM)	J	43.4±8.0*#	↓	↓		↓	↓	↑	
	C	23.5±4.7*		↓				↑	↑

a:Human jejunum or colon explants were maintained 48 h in L-15 medium (Control) or in the presence of growth factors (insulin) or inhibitors of signaling molecules/pathways (+G, genistein; +PD, PD98059; +Ly, Ly294002; +CD, cytochalasin D) as specified. b: Human intestinal segment analyzed; J, jejunum; C, colon. c: ISEL was performed on cryosections of treated explants to establish the total intestinal apoptotic indices. A minimum of 600 cells (300 epithelial cells and 300 mesenchymal cells) was counted in at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment). The apoptotic indices are expressed as the percentage (%) of apoptotic cells over the total number of cells counted (±Sem); (*) indicates statistically significant ($0.001 \leq P \leq 0.01$) differences between treatments and their respective control cultures; (#) indicates statistically significant ($0.001 \leq P \leq 0.01$) differences between the jejunum and colon. d: qualitative assessment of significant modulations in the expression of Bcl-2 homologs (Bcl-2, Bcl-X_L, Mcl-1, Bax, Bak, Bad) and one associated molecule (Bag-1) in treated explants, in comparison to control cultures, as observed from Western blot analyses (see Figure 4–6); ↓: down-modulation; ↑: up-regulation; lack of symbol indicates no significant modulation or control steady-state levels.

Likewise, the specific inhibition of Fak, MEK/Erk, and PI3-K resulted in significant increases in intestinal cell apoptosis in the two segments (Table I). However, stark differences were noted on the respective impacts of these treatments on cell survival between the jejunum and colon (Table I). For instance, the inhibition of Fak induced apoptosis with a significantly greater extent in the jejunum than in the colon (~43 vs. ~24%; Table I). Similarly, the impacts of the inhibition of MEK/Erk (~22 vs. ~9%; Table I) and PI3-K (~37 vs. ~12%; Table I) differed significantly between the two segments.

To better characterize this differential susceptibility to apoptosis between the jejunum and colon mucosae, we then determined the specific apoptotic indices for epithelial and mesenchymal cells in both segments, following the same treatments. As expected from comparing two different cell types [Anderson, 1997; Jacobson et al., 1997; Adams and Cory, 1998; Giancotti and Ruoslahti, 1999; Cross et al., 2000], we found for each gut segment that the treatments had varying effects on the survival of epithelial cells in comparison to mesenchymal cells (Table II). However, the cell type-specific comparison between jejunum and colon epithelial cells, as well as between jejunum and colon mesenchymal cells, revealed further differences in susceptibilities to apoptosis between the two segments. Thus, although no significant variations were observed between the two segments while examining the total apoptotic indices after inhibiting tyrosine kinase activities (see above; Table I), we found that the same treatment produced a significantly greater impact on the survival of jejunum epithelial cells than that of colonic ones (~69 vs. ~49%; Table II). However, no significant differences were noted between jejunum mesenchymal cells and those of the colon (~55 vs. ~58%; Table II). Similarly, the inhibition of the MEK/Erk pathway was found to have a significantly greater effect on the survival of jejunum epithelial cells than that of colonic ones (~27 vs. ~10%; Table II), whereas no significant differences were noted in mesenchymal cells of the two segments (~11 vs. ~9%; Table II). On the other hand, the inhibition of PI3-K had a significantly greater impact on the survival of both epithelial and mesenchymal cells of the jejunum, as compared to those of the colon (~47 and ~26% vs. ~9 and ~14%, respectively; Table II). Likewise, the inhibition of Fak produced significantly greater impacts on the survival of epithelial and mesenchymal cells of the jejunum, than those of the colon (~52 and ~34% vs. ~27 and ~19%, respectively; Table II).

Hence, these results altogether indicate that the jejunum and colon mucosae display segment-specific susceptibilities to apoptosis, and

TABLE II. Differential Susceptibilities to Apoptosis Between the Human Jejunum and Colon Mucosae

	Jejunum (% apoptotic cells±SEM)		Colon (% apoptotic cells±SEM)	
Treatment[a]	Epithelium	Mesenchyme	Epithelium	Mesenchyme
Control (L-15 Medium)	3.6±1.8	8.8±2.6	1.4±0.6	6.0±1.3
Insulin (10 μg/ml)	1.3±0.9	5.7±2.0	1.1±0.4	4.4±2.5
Genistein (150 μM)	68.5±8.3*#	54.9±11.2*	49.0±7.2*	57.7±9.1*
PD98059 (20 μM)	27.2±5.4*#	10.6±3.3	9.5±1.7*	8.6±2.0
Ly294002 (15 μM)	47.3±7.8*#	26.4±6.1*†	8.9±2.2*	13.9±2.7*
Cytochalasin D (1 μM)	51.6±10.5*#	33.5±4.9*†	26.9±4.8*	19.1±1.7*

a: Explants of human jejunum or colon were maintained 48 h in minimal L-15 medium only (Control), or in the presence of growth factors (insulin) or inhibitors of signaling molecules/pathways (genistein-tyrosine kinase activities; PD98059-MEK/Erk pathway; Ly294002-Pl3-K; cytochalasin D-Fak) as specified. Cryosections of explants were then stained by the ISEL method in order to evaluate the proportion of ISEL-positive (i.e., apoptotic) epithelial and mesenchymal cells. A minimum of 300 epithelial or mesenchymal cells was counted in at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment). The apoptotic indices are expressed as the percentage (%) of apoptotic cells over the total number of cells counted (±SEM); (*) indicates statistically significant ($0.001 \leq P \leq 0.01$) differences between treatments and their respective control cultures; (#) indicates statistically significant ($0.001 \leq P \leq 0.01$) differences between jejunum and colon epithelial cells; (†) indicates statistically significant (($0.001 \leq P \leq 0.01$) differences between jejunum and colon mesenchymal cells.

that such distinct susceptibilities are further reflected upon the epithelial and mesenchymal compartments of the two gut segments. It is noteworthy that an additional level of distinction in apoptosis susceptibility was observed within the epithelia of both the jejunum and colon. While exposure to genistein, cytochalasin D, and Ly294002 resulted in apoptosis throughout the crypt-villus axis of both segments, apoptosis induced by exposure to PD98059 was almost exclusively concentrated in villus cells (not shown). This confirms in organ culture our previous observations in intestinal cell lines, which indicate that the regulation of survival in undifferentiated (crypt) cells exhibits distinctions from that of differentiated (villus) cells [Gauthier et al., 2001].

Differential Involvement of Fak in Cell Survival of the Jejunum and Colon Mucosae

In order to verify whether the observed segmental differences in intestinal apoptosis susceptibility were linked to a differential involvement of signaling pathways, we first investigated specifically the role of cell adhesion signaling in intestinal cell survival, focusing on $p125^{Fak}$ activation (Fig. 1) [Frisch and Ruoslahti, 1997; Giancotti and Ruoslahti, 1999; Coppolino and Dedhar, 2000]. We found that the relative activation levels of $p125^{Fak}$ were equally inhibited in the two gut segments when either genistein (Fig. 1A, B, lane 3, and Fig. 1C) or cytochalasin D (Fig. 1A, B, lane 6, and Fig. 1C) were used as treatments. The non-specific inhibition of $p125^{Fak}$ activation by genistein, a wide-specter inhibitor of tyrosine kinases, was expected [Lipfert et al., 1992; Wary et al., 1999], whereas cytochalasin D acts as a specific inhibitor of $p125^{Fak}$ at the concentration range used [Lipfert et al., 1992; Oktay et al., 1999; Wary et al., 1999; Barberis et al., 2000; Gauthier et al., 2001]. We also observed that the inhibition of the downstream MEK/Erk pathway or PI3-K (Fig. 1A, B, lanes 4 and 6, respectively, and Fig. 1C), as well as exposure to insulin (Fig. 1A, B, lane 2, and Fig. 1C), did not impact on the relative activation levels of $p125^{Fak}$ itself in either the jejunum or colon, as expected from previous studies in other cell types and tissues [Lipfert et al., 1992; Anderson, 1997; Wary et al., 1999; Danilkovitch et al., 2000; Le Gall et al., 2000].

Although $p125^{Fak}$ activation was equally inhibited in both the jejunum and colon, its inhibition nonetheless induced apoptosis significantly to a much lesser extent in the colon mucosa than in the jejunum's (Tables I, II). Hence, these data suggest that Fak-mediated cell adhesion signaling is differentially involved in intestinal cell survival between the jejunum and colon.

Segment-Specific Regulation of MEK/Erk Signaling and Differential Involvement in Intestinal Cell Survival

To characterize further the observed segmental differences in intestinal apoptotis susceptibility between the jejunum and colon mucosae,

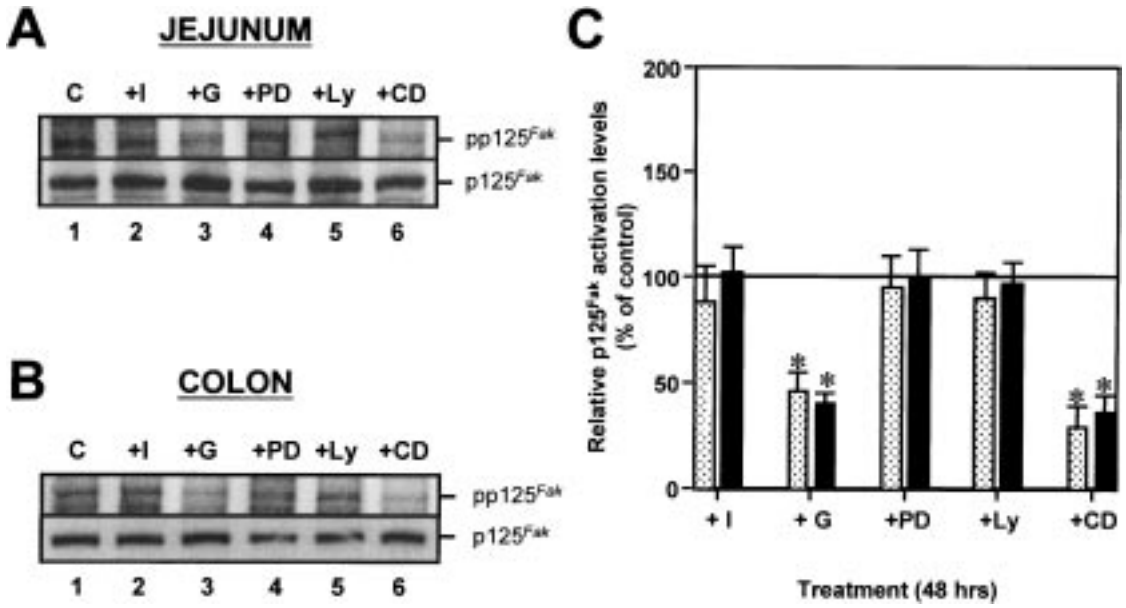

Fig. 1. Modulations of Fak activation in the jejunum and colon mucosae. **(A, B)** Representative immunoblot assays of $p125^{Fak}$ activation in human jejunum (A) or colon (B) explants, maintained 48 h in minimal L-15 medium only (control; **C**; **lane 1**) or with the addition of 10 μg/ml insulin (+I; **lane 2**), 150 μM genistein (+G; **lane 3**), 20 μM PD98059 (+PD; **lane 4**), 15 μM Ly294002 (+Ly; **lane 5**), or 1 μM cytochalasin D (+CD; **lane 6**). Total proteins were resolved by SDS-PAGE and electrotransferred, probed for the detection of the phosphorylated (activated) form of Fak ($pp125^{Fak}$), then reprobed for total Fak ($p125^{Fak}$). (C) Relative activation levels of $p125^{Fak}$ in explants of jejunum (light columns) or colon (dark columns) maintained as in (A, B). Total proteins were separated and probed as described above, then scanned by laser densitometry. For each treatment of explants, the relative activation levels of $p125^{Fak}$ were evaluated by first determining the total peak areas (AU × mm) for the phosphorylated form and the corresponding total protein, for the normalization of activation levels. Normalized values were then compared to those of control cultures, ×100 (expressed as ''% of control''). Columns represent mean±SEM for at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment). For jejunum and colon explants, statistically significant ($0.001 \leq P \leq 0.01$) differences between treatments and their respective controls are indicated by an asterisk (*).

we then examined the regulation of the MEK/Erk pathway by focusing on $p42^{Erk\text{-}2}$ activation (Fig. 2). As expected, the inhibition of upstream MEK with PD98059 lowered the activation levels of $p42^{Erk\text{-}2}$ in the two segments (Fig. 2A, B, lane 4, and Fig. 2C), while the inhibition of PI3-K had no effect (Fig. 2A, B, lane 5, and Fig. 2C). Also as expected [Anderson, 1997; Giancotti and Ruoslahti, 1999; Coppolino and Dedhar, 2000; Cross et al., 2000; Le Gall et al., 2000], the inhibition of $p125^{Fak}$ (Fig. 2A, B, lane 6, and Fig. 2C) and of tyrosine kinase activities (Fig. 2A, B, lane 3, and Fig. 2C) significantly lowered the relative activation levels of $p42^{Erk\text{-}2}$ in both the jejunum and colon. However, the exposure of explants to insulin revealed segment-specific distinctions in the regulation of the stimulation of MEK/Erk. Indeed, insulin significantly increased the relative activation levels of $p42^{Erk\text{-}2}$ in the jejunum (Fig. 2A, lane 2, and Fig. 2C) while lowering them in the colon (Fig. 2B, lane 2, and Fig. 2C).

In addition to the regulation of the stimulation of the MEK/Erk pathway displaying both common as well as segment-specific characteristics between the jejunum and the colon mucosae, we also found that the inhibition of this pathway had a significantly greater impact on cell survival in the jejunum than in the colon (Tables I, II). Hence, these data altogether indicate that the MEK/Erk pathway is not only regulated according to segmental distinctions in the jejunum and colon mucosae, but is also involved differentially in intestinal cell survival between the two segments.

Segment-Specific Regulation of the PI3-K/Akt Pathway and Differential Involvement in Intestinal Cell Survival

Since the MEK/Erk pathway displayed segmental distinctions in both its regulation of activation and its involvement in intestinal survival, we then verified whether this was not also the case for the PI3-K/Akt pathway. By

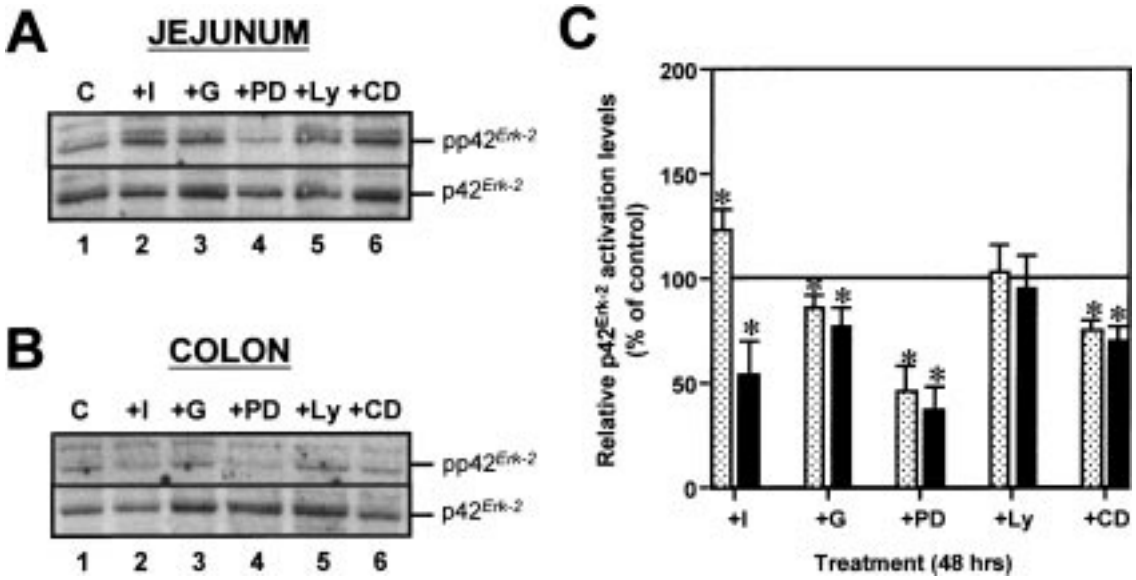

Fig. 2. Segment-specific modulations of Erk-2 activation in the jejunum and colon mucosae. **(A, B)** Representative immunoblot assays of $p42^{Erk\text{-}2}$ activation in explants of human jejunum (A) or colon (B) maintained 48 h in minimal L-15 medium only (control; **C**; **lane 1**) or with the addition of 10 μg/ml insulin (+I; **lane 2**), 150 μM genistein (+G; **lane 3**), 20 μM PD98059 (+PD; **lane 4**), 15 μM Ly294002 (+Ly; **lane 5**), or 1 μM cytochalasin D (+CD; **lane 6**). Total proteins were resolved by SDS-PAGE and electrotransferred, probed for the detection of the doubly-phosphorylated (activated) form of Erk-2 ($pp42^{Erk\text{-}2}$), then reprobed for total Erk-2 ($p42^{Erk\text{-}2}$). (C) Relative activation levels of $p42^{Erk\text{-}2}$ in explants of jejunum (light columns) or colon (dark columns) maintained as in (A, B). Total proteins were separated and probed as described above, then scanned by laser densitometry. For each treatment of explants, the relative activation levels of $p42^{Erk\text{-}2}$ were evaluated by first determining the total peak areas (AU × mm) for the phosphorylated form and the corresponding total protein, for the normalization of activation levels. Normalized values were then compared to those of control cultures, × 100 (expressed as ''% of control''). Columns represent mean±SEM for at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment). For jejunum and colon explants, statistically significant ($0.001 \leq P \leq 0.01$) differences between treatments and their respective controls are indicated by an asterisk (*).

focusing on $p57^{Akt}$ activation (Fig. 3), we found that the inhibition of $p125^{Fak}$ (Fig. 3A, B, lane 6, and Fig. 3C) and of tyrosine kinase activities (Fig. 3A, B, lane 3, and Fig. 3C) lowered $p57^{Akt}$ relative activation levels in both the jejunum and colon, as observed for $p42^{Erk\text{-}2}$ (see above). Also as noted for $p42^{Erk\text{-}2}$, insulin exerted differential influences on $p57^{Akt}$ activation in the two gut segments. In this case, however, exposure to insulin increased the activation levels of $p57^{Akt}$ in the colon (Fig. 3B, lane 2, and Fig. 3C) without affecting those in the jejunum (Fig. 3A, lane 2, and Fig. 3C).

Further distinctions were noted in the regulation of the PI3-K/Akt pathway between the jejunum and colon mucosae. Indeed, the specific inhibition of PI3-K with Ly294002 resulted in a significant decrease of downstream $p57^{Akt}$ activation in the jejunum (Fig. 3A, lane 5, and Fig. 3C), as expected [Vlahos et al., 1994; Datta et al., 1999; Le Gall et al., 2000], but did not affect $p57^{Akt}$ activation levels in the colon (Fig. 3B, lane 5, and Fig. 3C). To this effect, it is noteworthy that there is increasing evidence which indicates that Akt activation can be PI3-K-independent [Datta et al., 1999; Vanhaesebroeck and Alessi, 2000]. Furthermore, we noted in the colon, but not in the jejunum, that a decrease of $p42^{Erk\text{-}2}$ activation can be accompanied by an increase of $p57^{Akt}$ activation. For instance, exposure of colon explants to insulin resulted in a decreased $p42^{Erk\text{-}2}$ activation levels (Fig. 2B, lane 2, and Fig. 2C), while at the same time caused an increase in $p57^{Akt}$ activation (Fig. 3B, lane 2, and Fig. 3C). Likewise, the specific inhibition of MEK in the colon caused an expected decrease in $p42^{Erk\text{-}2}$ activation (Fig. 2B, lane 4, and Fig. 2C), but also resulted in an increase of $p57^{Akt}$ activation levels (Fig. 3B, lane 4, and Fig. 3C). Exceptions to this seeming inverse relationship between MEK/Erk and Akt in the colon were noted when tyrosine kinase activities were inhibited non-specifically (Fig. 2B and Fig. 3B, lane 3, and

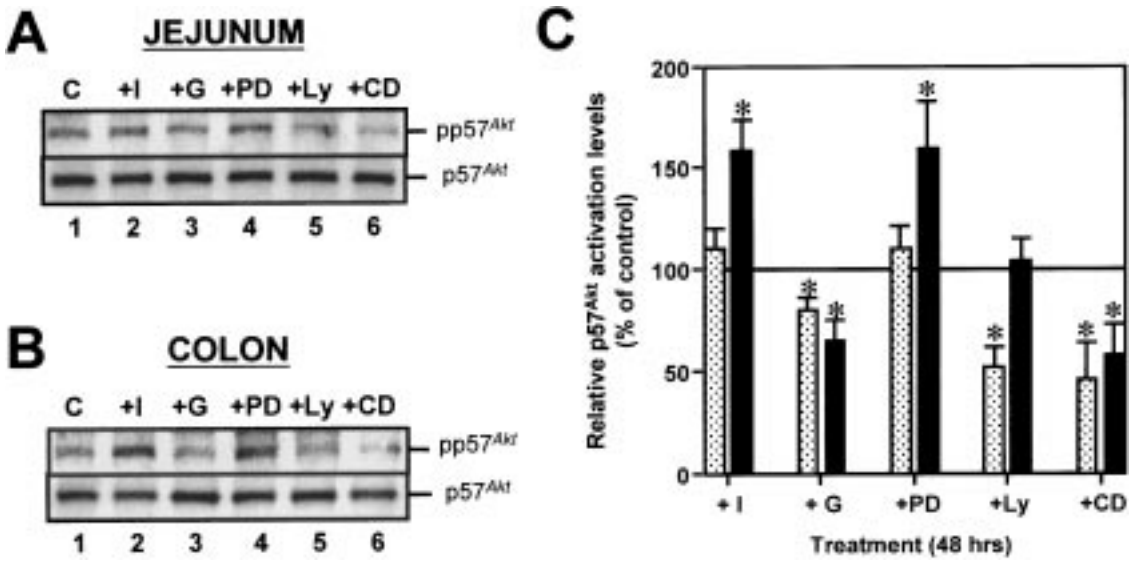

Fig. 3. Segment-specific modulations of Akt activation in the jejunum and colon mucosae. (**A**, **B**) Representative immunoblot assays of $p57^{Akt}$ activation in explants of human jejunum (A) or colon (B), maintained 48 h in minimal L-15 medium only (control; **C**; **lane 1**) or with the addition of 10 μg/ml insulin (+I; **lane 2**), 150 μM genistein (+G; **lane 3**), 20 μM PD98059 (+PD; **lane 4**), 15 μM Ly294002 (+Ly; **lane 5**), or 1 μM cytochalasin D (+CD; **lane 6**). Total proteins were resolved by SDS-PAGE and electrotransferred, probed for the detection of the phosphorylated (activated) form of Akt ($pp57^{Akt}$), then reprobed for total Akt ($p57^{Akt}$). (C) Relative activation levels of $p57^{Akt}$ in explants of jejunum (light columns) or colon (dark columns) maintained as in (A, B). Total proteins were separated and probed as described above, then scanned by laser densitometry. For each treatment of explants, the relative activation levels of $p57^{Akt}$ were evaluated by first determining the total peak areas (AU × mm) for the phosphorylated form and the corresponding total protein, for the normalization of activation levels. Normalized values were then compared to those of control cultures, × 100 (expressed as ''% of control''). Columns represent mean±SEM for at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment). For jejunum and colon explants, statistically significant ($0.001 \leq P \leq 0.01$) differences between treatments and their respective controls are indicated by an asterisk (*).

Fig. 2C, 3C) or when upstream $p125^{Fak}$ activation was inhibited (Fig. 2B and Fig. 3B, lane 6, and Fig. 2C, Fig. 3C).

In addition to such complex but segment-specific regulation of PI3-K/Akt pathway activation between the jejunum and colon, we also found that the inhibition of PI3-K had a significantly greater impact on cell survival in the jejunum than in the colon, as already noted (Tables I, II). Hence, these data altogether indicate that the PI3-K/Akt pathway is not only distinctively regulated in the jejunum and colon mucosae, but is also involved differentially in intestinal cell survival between the two segments.

Distinct, Segment-Specific Regulation of Intestinal Bcl-2 Homolog Steady-State Levels

To ascertain whether the differences in susceptibility to apoptosis between the jejunum and colon mucosae are linked with segment-specific regulatory mechanisms of Bcl-2 homolog expression, we then investigated the steady-state expression levels of six Bcl-2 homologs (Bcl-2, Bcl-X_L, Mcl-1, Bax, Bak, Bad), and one nonhomologous associated molecule (Bag-1). Immunoblot analyses of lysates from non-treated explants demonstrated the protein expression of all molecules analyzed in both the jejunum (Fig. 4A, lane 1) and colon (Fig. 4B, lane 1). Thus, Bcl-2 (~26 kDa), Bcl-X_L (~28–30 kDa), Mcl-1 (~39–42), Bag-1 (~32–34 kDa), Bax (~21kDa), Bak (~25–28 kDa) and Bad (~28–32 kDa) were detected as protein bands migrating at their previously reported relative molecular weights [Krajewski et al., 1994a,b, c, 1995, 1996; Takayama et al., 1995; Krajewska et al., 1996; Boucher et al., 2000; Vachon et al., 2000, 2001; Gauthier et al., 2001]. The densitometric analyses presented in Figures 5 and 6 show that the relative expression levels of all Bcl-2 homologs analyzed (including the Bag-1 protein) were differentially modulated following the various treatments.

In the jejunum mucosa, inhibition of tyrosine kinase activities (Fig. 4A, lane 3) resulted in a

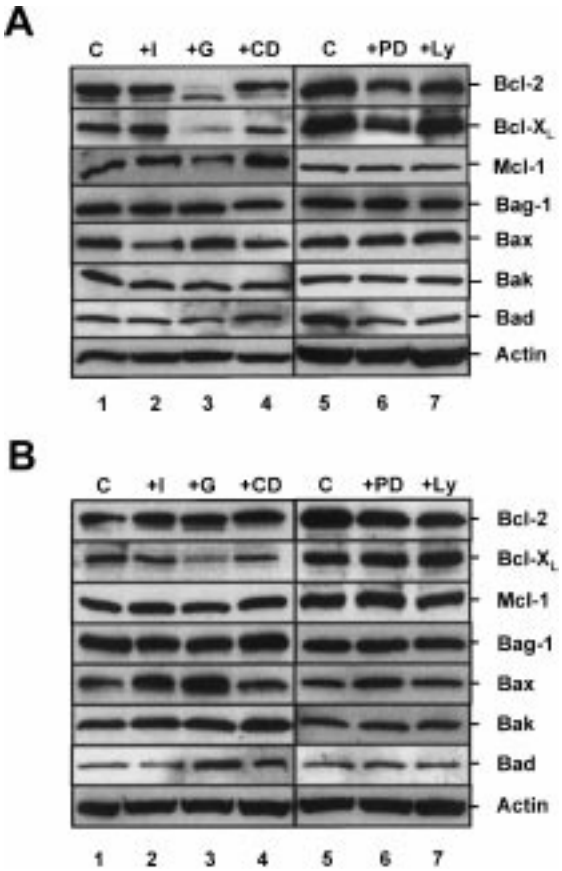

Fig. 4. Differential modulation of Bcl-2 homolog expression levels in the jejunum and colon. **(A, B)** Representative Western blot analyses of Bcl-2, Bcl-X_L, Mcl-1, Bag-1, Bax, Bak, Bad and actin expression levels from explants of human jejunum (A) and colon (B), maintained 48 h in minimal L-15 medium only (control; C; **lanes 1** and **5**) or with the addition of 10 μg/ml insulin (+I; **lane 2**), 150 μM genistein (+G; **lane 3**), 1 μM cytochalasin D (+CD; **lane 4**), 20 μM PD98059 (+PD; **lane 6**), or 15 μM Ly294002 (+Ly; **lane 7**). 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 actin was used as standard of protein quantities analyzed.

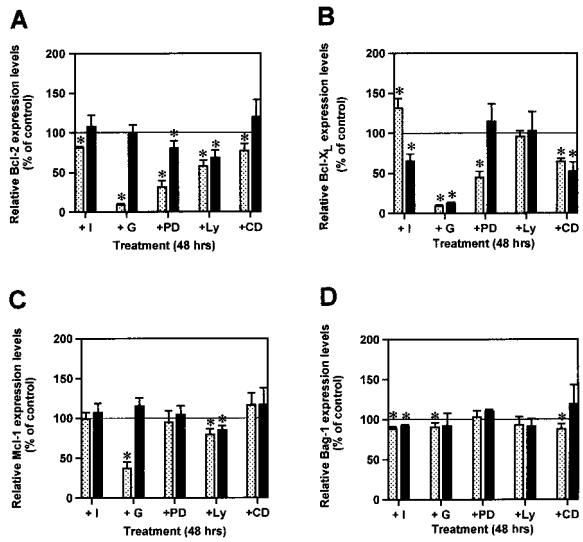

Fig. 5. Segment-specific modulations of anti-apoptotic Bcl-2 homologs in the jejunum and colon mucosae. Relative expression levels of Bcl-2 (**A**), Bcl-X_L **(B)**, Mcl-1 **(C)** and Bag-1 **(D)** in explants of human jejunum (light columns) or colon (dark columns), maintained 48 h in minimal L-15 medium only (control) or with the addition of 10 μg/ml insulin (+I), 150 μM genistein (+G), 20 μM PD98059 (+PD), 15 μM Ly294002 (+Ly), or 1 μM cytochalasin D (+CD). Total proteins were separated and probed as represented in Figure 1, and then scanned by laser densitometry. For each treatment of explants, relative expression levels of molecules analyzed were evaluated in comparison with that control cultures, by determining total peak areas (AU × mm) in order to establish the ratios treatment/control × 100 (expressed as ''% of control''). Columns represent mean±SEM for at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment), with the 100% value corresponding to expression levels observed in control cultures. For jejunum and colon explants, statistically significant ($0.001 \leq P \leq 0.01$) differences between treatments and their respective controls are indicated by an asterisk (*).

significant down-regulation of all anti-apoptotic homologs (Fig. 5A–D) and of Bad (Fig. 6C), while only Bax expression was increased (Fig. 6A). The inhibition of Fak (Fig. 4A, lane 4) lead to a significant down-modulation of Bcl-2 (Fig. 5A), Bcl-X_L (Fig. 5B), Bag-1 (Fig. 5D), and Bax (Fig. 6A), whereas only Bak was up-regulated (Fig. 6B). The inhibition of the MEK/Erk pathway (Fig. 4A, lane 6) decreased the expression of Bcl-2 (Fig. 5A), Bcl-X_L (Fig. 5B), and Bad (Fig. 6C) without affecting the other homologs studied. Lastly, the inhibition of PI3-K (Fig. 4A, lane 7) resulted in a significant decrease of Bcl-2 (Fig. 5A), Mcl-1 (Fig. 5C) and Bad (Fig. 6C), while up-regulating the expression of Bax only (Fig. 6A).

In the case of the colon, the inhibition of tyrosine kinase activities (Fig. 4B, lane 3) lead to a significant down-regulation of Bcl-X_L (Fig. 5B) and to an increase of all pro-apoptotic homologs analyzed (Fig. 6A–C). The inhibition of Fak (Fig. 4B, lane 4) also resulted in a significant decrease of Bcl-X_L (Fig. 5B), but in an increase of Bak (Fig. 6B), and Bad (Fig. 6C) only. The inhibition of the MEK/Erk pathway (Fig. 4B, lane 6) led to a significant decrease of Bcl-2 (Fig. 5A) while up-regulating Bax (Fig. 6A). Lastly, the inhibition of PI3-K (Fig. 4B, lane 7) resulted in a decrease of Bcl-2

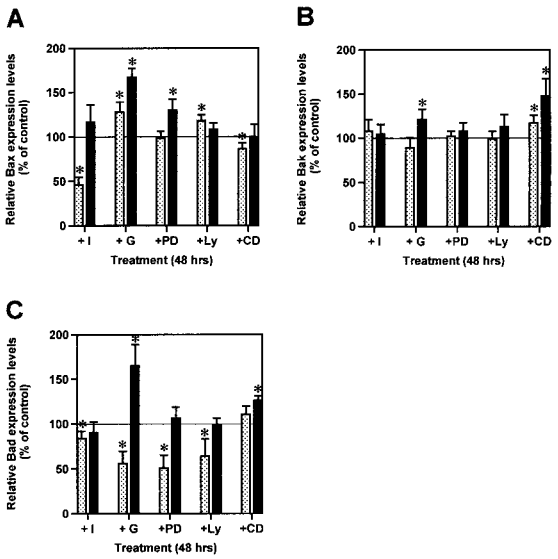

Fig. 6. Segment-specific modulations of pro-apoptotic Bcl-2 homologs in the jejunum and colon mucosae. Relative expression levels of Bax **(A)**, Bak **(B)** and Bad **(C)** in explants of human jejunum (light columns) or colon (dark columns), maintained 48 h in minimal L-15 medium only (control) or with the addition of 10 μg/ml insulin (+I), 150 μM genistein (+G), 20 μM PD98059 (+PD), 15 μM Ly294002 (+Ly) or 1 μM cytochalasin D (+CD). Total proteins were separated and probed as represented in Figure 1, and then scanned by laser densitometry. For each treatment of explants, relative expression levels of molecules analyzed were evaluated in comparison with that of control cultures, by determining total peak areas (AU × mm) in order to establish the ratios treatment/control × 100 (expressed as ''% of control''). Columns represent mean±SEM for at least three ($n \geq 3$) separate experiments (each: 4–12 explants/treatment/gut segment), with the 100% value corresponding to expression levels in control cultures. For jejunum and colon explants, statistically significant ($0.001 \leq P \leq 0.01$) differences between treatments and their respective controls are indicated by an asterisk (*).

(Fig. 5A) and Mcl-1 (Fig. 5C), without affecting any of the pro-apoptotics analyzed (Fig. 6A–C).

In addition to such complex modulations of Bcl-2 homolog expression in both the jejunum and colon, we also observed stark differences between the two gut segments (summarized in Table I). For instance, insulin had opposing effects on Bcl-X_L expression in the jejunum and the colon (Table I; Fig. 5B), while affecting Bcl-2, Bax and Bad in the jejunum only (Table I; Figs. 5A, 6A, C). Similarly, the inhibition of tyrosine kinase activities decreased the expression of Bcl-2, Mcl-1, and Bag-1 in the jejunum only (Table I; Fig. 5A, C, D), while at the same time increasing Bak in the colon only (Table I; Fig. 6B) and displaying opposing effects on Bad expression in the two segments (Table I; Fig. 6C). Further examples of segment-distinct modulations of individual Bcl-2 homologs were noted as well when Fak, MEK/Erk, or PI3-K were inhibited (Table I; Figs. 4–6).

It is noteworthy that the relationship between the balance of anti- vs. pro-apoptotic homologs and the impact on intestinal cell survival was not always clear from the experiments performed herein (Table I). For instance, insulin exposure in the colon mucosa resulted in a balance seemingly in favor of pro-apoptotic homologs (Table I) even if the treatment had no significant effects on cell survival overall (Table I), or more specifically on epithelial and/or mesenchymal cell survival (Table II). Considering that cell survival implicates not just one homolog but rather complex interactions between numerous Bcl-2 homologs, as well as post-translational modifications to further modulate their functions [Gajewski and Thompson, 1996; Reed et al., 1996a,b; Anderson, 1997; Adams and Cory, 1998], such apparent discrepancies are likely due to the involvement of additional homologs other than those analyzed herein (e.g., Bcl-w, Bid, etc.) [Adams and Cory, 1998; Wilson et al., 2000] and/or to Bcl-2 homolog phosphorylation events [Gajewski and Thompson, 1996; Reed et al., 1996a,b; Adams and Cory, 1998] not focused upon in the present study. Nonetheless, these data altogether indicate that the steady-state expression levels of various Bcl-2 homologs are not only subject to complex regulatory mechanisms in both the jejunum and colon mucosae, but that such mechanisms differ between the two gut segments.

DISCUSSION

In this study, we examined the question whether human intestinal cell survival is subject to segment-specific control mechanisms. By analyzing the impact of growth factors and inhibitors of signaling molecules/pathways on cell survival and Bcl-2 homolog expression, in human jejunum and colon organ cultures, we found that the inhibition of tyrosine kinase activities, of Fak, of the MEK/Erk pathway, or of PI3-K have distinct impacts on intestinal cell survival between both gut segments. Such striking, segmental differences in apoptosis susceptibility were found to reflect upon epithelial and mesenchymal cells of the two segments. We also observed that exposure to insulin and

the inhibition of these various signaling molecules/pathways modulated differentially the expression of Bcl-2 homologs in both the jejunum and colon. However, sharp distinctions were noted between the two segments in the resulting effects of the same treatments on the expression of Bcl-2 homologs. Furthermore, we found that the stimulation of the MEK/Erk and PI3-K/Akt pathways are distinctively regulated in the jejunum and colon. Finally, the respective involvements of Fak, MEK/Erk, and PI3-K/Akt in intestinal cell survival were found to differ between the two segments. Hence, human intestinal cell survival is characterized by segment-specific susceptibilities to apoptosis that are linked with segmental distinctions in both the regulation of Bcl-2 homologs and the involvement of signaling molecules/pathways.

In contrast to laboratory animals with short gestational periods (e.g., rat and mouse), adult-like intestinal functional and physiological processes, including apoptosis [Vachon et al., 2000], are acquired as early as by mid-gestation (18–20 weeks) in man [Grand et al., 1976; Ménard, 1989; Ménard and Beaulieu, 1994]. In addition, adult-like segmental distinctions in the expression profiles of Bcl-2 homologs [Vachon et al., 2001], as well as differences in the response to hormones and growth factors [Ménard, 1989; Ménard and Beaulieu, 1994], are already established by mid-gestation. Since Bcl-2 homologs constitute a critical checkpoint in the regulation of apoptosis, it has been suggested that differences in Bcl-2 homolog expression along the proximal-distal axis of the gut may underlie segment-specific control mechanisms of intestinal cell survival, as well as account for the apparent segmental preferences of bowel disorders that involve apoptosis dysregulation [Potten, 1992; Jones and Gores, 1997; Potten, 1997]. In support of this, differences in the normal rates of apoptosis between the jejunum and colon have been reported [Hall et al., 1994; Merritt et al., 1995; Jones and Gores, 1997; Potten, 1997], whereas studies in rodents have shown that small intestinal cells display greater sensitivity to chemotherapeutic drug- or irradiation-induced apoptosis than colonic cells [Potten, 1992; Hall et al., 1994; Kiefer et al., 1995; Merritt et al., 1995; Pritchard et al., 1999]. The present study provides further documentation on the differential susceptibilities to apoptosis between the jejunum and colon mucosae. Furthermore, our data show for the first time that such differences are linked with a segment-specific regulation of Bcl-2 homolog expression and involvement of signal transduction pathways. The need for putative segmental differences in the regulation of intestinal cell survival is thought to be related to the nature of the luminal contents to which the intestine is exposed, along the proximal-distal axis of the gut. Indeed, the colon is exposed to an environment considered more pro-apoptotic than that of the small intestine, and thus would require greater resistance to apoptosis [Cobb and Williamson, 1991; Potten, 1992; Jones and Gores, 1997; Potten, 1997]. Our observations that the inhibition of various signaling molecules/pathways generally impacted with greater extent on the survival of jejunum cells than colonic ones, and the fact that cancer occurs at drastically higher rates in the colon than in the jejunum [Ahnen, 1991; Mendeloff, 1991; Potten, 1992, 1997; Jones and Gores, 1997], strongly support this latter assumption. In light of these considerations, our data altogether clearly establish that human intestinal cell survival is subject to segment-specific control mechanisms.

Another aspect of our findings concerns the differential roles of Fak, MEK/Erk, and PI3-K/Akt in the modulation of Bcl-2 homolog expression either within the jejunum or the colon, or between the two segments. In the past few years, it has become increasingly evident that the regulation of individual Bcl-2 homologs can involve numerous pathways acting in synergy or independently, and that the pathways involved in the regulation of a single homolog can differ depending on the cell type or tissue studied [Anderson, 1997; Adams and Cory, 1998; Giancotti and Ruoslahti, 1999; Osada et al., 1999; Scheid et al., 1999; Coppolino and Dedhar, 2000; Cross et al., 2000; Danilkovitch et al., 2000]. For instance, Mcl-1, Bcl-X_L, and Bcl-2 expression can be up-regulated by stimulation of the MEK/Erk pathway in some cell types, by stimulation of the PI3-K/Akt pathway in others, or by the stimulation of both pathways [Anderson, 1997; Adams and Cory, 1998; Townsend et al., 1998; Wang et al., 1999; Cross et al., 2000]. Following on these specific examples, we found herein that the inhibition of the MEK/Erk pathway in the jejunum down-regulated Bcl-2 and Bcl-X_L, but did not affect Mcl-1, whereas the inhibition of PI3-K down-regulated both Bcl-2 and Mcl-1 without affecting Bcl-X_L.

In the colon, inhibition of MEK/Erk affected Bcl-2 only, while the inhibition of PI3-K affected Bcl-2 and Mcl-1, but not Bcl-X_L, as in the jejunum. Additional, similar examples implicating other Bcl-2 homologs have been reported elsewhere [Wang et al., 1994; Moss et al., 1996; Wang et al., 1996; Anderson, 1997; Hague et al., 1997; Adams and Cory, 1998; Scheid et al., 1999; Cross et al., 2000] as well as in the present study, thus underlying the complexity that is required in the regulation of the expression and functions of Bcl-2 homologs in various tissues. This in turn further stresses the concept that cell survival in different tissues is not regulated by the activity of single Bcl-2 homologs, but rather by a balance of activities from numerous homologs that arises from the input of multiple stimuli and pathways [Reed et al., 1996a; Anderson, 1997; Adams and Cory, 1998; Cross et al., 2000].

This brings the question as to how extracellular signals can result in such different outcomes on cell survival and Bcl-2 homolog expression among the various tissues, particularly when such tissues are developmentally related like those of the jejunum and colon mucosae. This is well illustrated by our observations that the inhibition of Fak-mediated cell adhesion signalling caused a decrease in $p42^{Erk-2}$ and $p57^{Akt}$ activation in both the jejunum and colon, and yet resulted in differential impacts on Bcl-2 homolog expression and cell survival between the two segments. It is now well acknowledged that signaling pathways can cross-talk with each other in order to act in tight coordination, although the exact mechanisms involved remain unclear [Anderson, 1997; Parsons and Parsons, 1997; Giancotti and Ruoslahti, 1999; Cross et al., 2000; Vanhaesebroeck and Alessi, 2000]. A close cooperation has been reported between the MEK/Erk and PI3-K pathways in suppressing apoptosis, even to the point where both pathways can be required to effectively sustain cell survival [Anderson, 1997; Giancotti and Ruoslahti, 1999; Scheid et al., 1999; Cross et al., 2000; Danilkovitch et al., 2000; Vanhaesebroeck and Alessi, 2000]. Thus, our data suggest that this is such a situation in both the jejunum and colon.

On the other hand, it is now becoming increasingly evident that cell survival in various tissues is regulated through cell type-specific mechanisms of integration and fine-tuning of survival pathways, mechanisms which remain to be fully understood [Gajewski and Thompson, 1996; Anderson, 1997; Adams and Cory, 1998; Datta et al., 1999; Cross et al., 2000; Vanhaesebroeck and Alessi, 2000]. Hence our observations that although the inhibition of Fak impacted equally on the MEK/Erk and PI3-k/Akt pathways in the jejunum and colon, these two pathways nonetheless displayed striking segmental differences in the regulation of their activation, as well as in their involvement in intestinal cell survival. Recent evidence indicates that the MEK/Erk and PI3-K/Akt pathways can also act co-independently in maintaining cell survival, with one being able to compensate for the inhibition of the other or even antagonize each other to some degree [Datta et al., 1999; Osada et al., 1999; Cross et al., 2000; Danilkovitch et al., 2000; Le Gall et al., 2000; Vanhaesebroeck and Alessi, 2000]. Thus, $p57^{Akt}$ can exert a negative feedback on the MEK/Erk pathway [Rommel et al., 1999; Zimmermann and Moelling, 1999], whereas the MEK/Erk pathway can down-stimulate PI3-K downstream effectors [Osada et al., 1999; Rommel et al., 1999; Cross et al., 2000; Vanhaesebroeck and Alessi, 2000]. To this effect, our data provide indications of an opposing relationship between $p42^{Erk-2}$ and $p57^{Akt}$ activation in the colon mucosa, but not in the jejunum's. In addition, we found that the inhibition of PI3-K did not impact on the activation levels of $p57^{Akt}$ in the colon, an uncoupling situation shown to exist in some instances [Datta et al., 1999; Cross et al., 2000; Vanhaesebroeck and Alessi, 2000]. These considerations altogether, coupled to our observations that the inhibition of MEK and PI3-K impacted significantly less on cell survival in the colon than in the jejunum, suggest that Akt may be able to compensate, at least partially, for the inhibition of MEK/Erk or PI3-K in the survival of colon cells, but not in jejunum cells.

In conclusion, the present findings provide new insight into the complex regulatory mechanisms that are responsible for the survival of human intestinal cells. It is now clear that the jejunum and colon mucosae exhibit a differential susceptibility to apoptosis through segmental distinctions in the involvement of signaling molecules/pathways, such as Fak, MEK/Erk, and PI3-K/Akt, which in turn impact differentially on the regulation of Bcl-2 homolog steady-state levels. However, the exact molecular processes responsible for such segment-specific

control mechanisms of intestinal survival are still poorly understood. For example, the question remains open as to why Akt is seemingly dependent of PI3-K for its activation in the jejunum, but not in the colon. Conversely, further analyses will be required to dissect, at the molecular level, the exact relationships and functions of the MEK/Erk and PI3-K/Akt pathways in the regulation of Bcl-2 homologs, in the jejunum and colon mucosae. The use of human intestinal organotypic culture, which allows for an "in vivo-like" scrutiny of the intestinal mucosa as a whole [Ménard, 1989; Ménard and Beaulieu, 1994], coupled to the use of cell-type specific intestinal epithelial and mesenchymal model systems [Ménard and Beaulieu, 1994; Pageot et al., 2000; Gauthier et al., 2001], should prove helpful in further deciphering the roles of these pathways in intestinal cell survival and in the pathogenesis of intestinal disorders with dysregulation of apoptosis.

ACKNOWLEDGMENTS

The authors would like to thank Drs. J.F. Beaulieu, D. Ménard, and N. Rivard for useful discussions, as well as Drs. A. Bilodeau, C. Poulin, M. Morin, and F. Jacot from the CHUS of Sherbrooke, for their cooperation in providing specimens for this study.

REFERENCES

Adams JM, Cory S. 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281:1322–1326.

Ahnen DJ. 1991. Etiology of large bowel cancer. In: Pemberton JH, Phillips SF, Shorter RG, editors. The large intestine: physiology, pathophysiology and disease. New York: Raven Press. p 501–520.

Aliaga JC, Deschênes C, Beaulieu J-F, Calvo EL, Rivard N. 1999. Requirement of the MAP kinase cascade for cell cycle progression and differentiation of human intestinal cells. Am J Physiol 277:G631–G641.

Allesi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. 1995. PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. J Biol Chem 270:27489–27495.

Anderson P. 1997. Kinase cascades regulating the entry into apoptosis. Microbiol Mol Rev 61:33–46.

Barberis L, Wary KK, Fiucci G, Liu F, Hirsch E, Brancaccio M, Altruda F, Tarone G, Giancotti FG. 2000. Distinct roles of the adaptor protein Shc and focal adhesion kinase in integrin signaling to ERK. J Biol Chem 275:36532–36540.

Beaulieu J-F, Chartrand S, Vachon PH. 1991. Immunolocalization of extracellular matrix components during organogenesis in the human small intestine. Anal Embryol 183:363–369.

Boucher M-J, Morisset J, Vachon PH, Reed JC, Lainé J, Rivard N. 2000. MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X_L, and Mcl-1 and promotes survival of human pancreatic cancer cells. J Cell Biochem 79:355–369.

Cobb RA, Williamson RCN. 1991. Embryology and developmental abnormalities of the large intestine. In: Pemberton JH, Phillips SF, Shorter RG, editors. The large intestine: physiology, pathophysiology and disease. New York: Raven Press. p 3–12.

Coppolino MG, Dedhar S. 2000. Bi-directional signal transduction by integrin receptors. Int J Biochem Cell Biol 32:171–188.

Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, Lord JM. 2000. Serine/threonine protein kinases and apoptosis. Exp Cell Res 256:34–41.

Danilkovitch A, Donley S, Skeel A, Leonard EJ. 2000. Two independent signaling pathways mediate the antiapoptotic action of macrophage-stimulating protein on epithelial cells. Mol Cell Biol 20:2218–2227.

Datta SR, Brunet A, Greenberg ME. 1999. Cellular survival: a play in three Akts. Genes Dev 13:2905–2927.

Frisch SM, Ruoslahti E. 1997. Integrins and anoikis. Curr Opin Cell Biol 9:701–706.

Gajewski TF, Thompson CB. 1996. Apoptosis meets signal transduction: elimination of a BAD influence. Cell 87:589–592.

Gauthier R, Harnois C, Drolet J-F, Reed JC, Vézina A, Vachon PH. 2001. Human intestinal epithelial cell survival; differentiation state-specific control mechanisms. Am J Physiol (In press).

Gavrieli Y, Sherman Y, Ben-Sasson SA. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501.

Giancotti FG, Ruoslahti E. 1999. Integrin signaling. Science 285:1028–1032.

Grand RJ, Watkins JB, Torti FM. 1976. Development of the human gastrointestinal tract. A review. Gastroenterology 70:790–810.

Hague A, Díaz GD, Hicks DJ, Krajewski S, Reed JC, Paraskeva C. 1997. Bcl-2 and Bak may play a pivotal role in sodium butyrate-induced apoptosis in colonic epithelial cells; however, overexpression of Bcl-2 does not protect against Bak-mediated apoptosis. Int J Cancer 72:898–905.

Hall PA, Coates PJ, Ansari B, Hopwood D. 1994. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. J Cell Sci 107:3569–3577.

Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ. 1991. Bcl-2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 88:6961–6965.

Jacobson MD, Weil M, Raff MC. 1997. Programmed cell death in animal development. Cell 88:347–354.

Jones BA, Gores GJ. 1997. Physiology and pathophysiology of apoptosis in epithelial cells of the liver, pancreas, and intestine. Am J Physiol 273:G1174–G1188.

Kiefer MC, Brauer MJ, Powers VC, Wu JJ, Umanski SR, Tomel LD, Barr PJ. 1995. Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. Nature 374:736–739.

Krajewska M, Moss SF, Krajewski S, Song K, Holt PR, Reed JC. 1996. Elevated expression of Bcl-X and reduced

Bak in primary colorectal adenocarcinomas. Cancer Res 56:2422–2427.
Krajewski S, Bodrug S, Gascoyne R, Berean K, Krejewska M, Reed JC. 1994a. Immunohistochemical analysis of Mcl-1 and Bcl-2 proteins in normal and neoplastic lymph nodes. Am J Pathol 145:515–525.
Krajewski S, Krajewska M, Reed JC. 1996. Immunohistochemical analysis of in vivo patterns of Bak expression, a proapoptotic member of the Bcl-2 protein family. Cancer Res 56:2849–2855.
Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC. 1994b. Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. Am J Pathol 145:1323–1336.
Krajewski S, Krajewska M, Shabaik A, Wang H-G, Irie S, Fong L, Reed JC. 1994c. Immunohistochemical analysis of in vivo patterns of Bcl-X expression. Cancer Res 54:5501–5507.
Krajewski S, Bodrug S, Krajewska M, Shabaik A, Gascoyne R, Berean K, Reed JC. 1995. Immunohistochemical analysis of Mcl-1 protein in human tissues: differential regulation of Mcl-1 and Bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death in vivo. Am J Pathol 146:1309–1319.
Le Gall M, Chambard J-C, Breittmayer J-P, Grall D, Poussyégur J, Van Obberghen-Schilling E. 2000. The p42/p44 MAP kinase pathway prevents apoptosis induced by anchorage and serum removal. Mol Biol Cell 11:1103–1112.
Lipfert L, Haimovitch B, Schaller MD, Cobb JS, Parsons JT, Brugge JS. 1992. Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. J Cell Biol 119:905–912.
Ménard D. 1989. Growth-promoting factors and the development of the human gut. In: Lebenthal E, editor. Human gastrointestinal development. New York: Raven Press. p 123–149.
Ménard D, Arsenault P. 1985. Explant culture of human fetal small intestine. Gastroenterology 88:691–700.
Ménard D, Arsenault P. 1987. Human fetal colon in organ culture. Anat Embryol 176:441–448.
Ménard D, Beaulieu J-F. 1994. Human intestinal brush border membrane hydrolases. In: Bkaily G, editor. Membrane Physiopathology. Norwell: Kluwer Academic Press. p 319–341.
Mendeloff AI. 1991. Limitations of epidemiologic methods in the study of colonic disorders. In: Pemberton JH, Phillips SF, Shorter RG, editors. The large intestine: physiology, pathophysiology and disease. New York: Raven Press. p 199–208.
Merritt AJ, Potten CS, Watson AJM, Yoh DY, Nakayama K-I, Nakayama K, Hickman JA. 1995. Differential expression of Bcl-2 in intestinal epithelia. Correlation with attenuation of apoptosis in colonic crypts and the incidence of colonic neoplasia. J Cell Sci 108:2261–2271.
Moss SF, Agarwal B, Arber N, Guan RJ, Krajewska M, Krejewski S, Reed JC, Holt PR. 1996. Increased intestinal Bak expression results in apoptosis. Biochem Biophys Res Commun 223:199–203.
Oktay M, Wary KK, Dans M, Birge RB, Giancotti FP. 1999. Integrin-mediated activation of focal adhesion kinase is required for signaling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle. J Cell Biol 145:1461–1469.
Osada M, Tolkacheva T, Li W, Chan TO, Tsichlis PN, Saez R, Kimmelman AC, Chan AM-L. 1999. Differential roles of Akt, Rac, and Ral in R-Ras-mediated cellular transformation, adhesion, and survival. Mol Cell Biol 19:6333–6344.
Pageot L-P, Perreault N, Basora N, Francoeur C, Magny P, Beaulieu J-F. 2000. Human cell models to study small intestinal functions: recapitulation of the crypt-villus axis. Microsc Res Tech 49:394–406.
Parsons JT, Parsons SJ. 1997. Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. Curr Opin Cell Biol 9:187–192.
Potten CS. 1992. The significance of spontaneous and induced apoptosis in the gastrointestinal tract of mice. Cancer Metast Rev 11:179–195.
Potten CS. 1997. Epithelial cell growth and differentiation II. Intestinal apoptosis. Am J Physiol 273:G253–G257.
Pritchard DM, Potten CS, Korsmeyer SJ, Roberts S, Hickman JA. 1999. Damage-induced apoptosis in intestinal epithelia from Bcl-2-null and bax-null mice: investigations of the mechanistic determinants of epithelial apoptosis in vivo. Oncogene 18:7287–7293.
Reed JC, Miyashita T, Takayama S, Wang H-G, Sato T, Krajewski S, Aimé-Sempé C, Bodrug S, Kitada S, Hanada M. 1996a. Bcl-2 family proteins: Regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem 60:23–32.
Reed JC, Zha H, Aime SC, Takayama S, Wang HG. 1996b. Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Adv Exp Med Biol 406:99–112.
Rommel C, Clarke BA, Zimmermann S, Nuñez L, Rossman R, Reid K, Moelling K, Yancopoulos GD, Glass DJ. 1999. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. Science 286:1738–1741.
Scheid MP, Schubert KM, Duronio V. 1999. Regulation of Bad phosphorylation and association with Bcl-X_L by the MAPK/Erk kinase. J Biol Chem 274:31108–31113.
Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, Reed JC. 1995. Cloning and functional analysis of Bag-1: a novel bcl-2-binding protein with anti-cell death activity. Cell 80:279–284.
Thompson CB. 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267:1456–1462.
Townsend KJ, Trusty JL, Traupman MA, Eastman A, Craig RW. 1998. Expression of the antiapoptotic MCL1 gene product is regulated by a mitogen activated protein kinase-mediated pathway triggered through microtubule disruption and protein kinase C. Oncogene 17:1223–1234.
Vachon PH, Cardin É, Harnois C, Reed JC, Plourde A, Vézina A. 2001. Early acquisition of bowel segment-specific Bcl-2 homolog expression profiles during development of the human ileum and colon. Histol Histopathol 16:497–510.
Vachon PH, Cardin É, Harnois C, Reed JC, Vézina A. 2000. Early establishment of epithelial apoptosis in the developing human small intestine. Int J Dev Biol 44:891–898.
Vachon PH, Loechel F, Xu H, Wewer UM, Engvall E. 1996a. Merosin and laminin in myogenesis; specific requirement for merosin in myotube stability and survival. J Cell Biol 134:1483–1497.
Vachon PH, Perreault N, Magny P, Beaulieu J-F. 1996b. Uncoordinated, transient mosaic patterns of intestinal

hydrolase expression in differentiating human enterocytes. J Cell Physiol 166:198–207.

Vachon PH, Xu H, Liu L, Loechel F, Hayashi Y, Arahata K, Reed JC, Wewer UM, Engvall E. 1997. Integrins ($\alpha7\beta1$) in muscle function and survival. Disrupted expression in merosin-deficient congenital muscular dystrophy. J Clin Invest 100:1870–1881.

Vanhaesebroeck B, Alessi DR. 2000. The PI3K-PDK1 connection: more than just a road to PKB. Biochem J 346:561–576.

Vlahos CJ, Matter WF, Hui KY, Brown RF. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem 269:5241–5248.

Wang H-G, Miyashita T, Takayama S, Sato T, Torigoe T, Krajewski S, Tanaka S, Hovey III F, Troppmair J, Rapp UR, Reed JC. 1994. Apoptosis regulation by interaction of Bcl-2 protein and Raf-1 kinase. Oncogene 9:2751–2756.

Wang H-G, Takayama S, Rapp UR, Reed JC. 1996. Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. Proc Natl Acad Sci USA 93:7063–7068.

Wang J-M, Chao J-R, Chen W, Kuo M-L, Yen JJ-Y, Yang-Yen H-F. 1999. The antiapoptotic gene mcl-1 is up-regulated by the phosphatidylinositol 3-kinase/Akt signaling pathway through a transcription factor complex containing CREB. Mol Cell Biol 19:6195–6206.

Wary KK, Dans M, Mariotti A, Giancotti FG. 1999. Biochemical analysis of integrin-mediated Shc signaling. In: Howlett AR, editor. Methods in molecular biology, vol. 129: Integrin Protocols. Totowa: Humana Press. p 35–49.

White E. 1996. Life, death, and the pursuit of apoptosis. Genes Dev 10:1–15.

Whitehead JP, Clark SF, Ursø B, James DE. 2000. Signalling through the insulin receptor. Curr Opin Cell Biol 12:222–228.

Wilson JW, Nostro MC, Balzi M, Faraoni P, Cianchi F, Becciolini A, Potten CS. 2000. Bcl-w expression in colorectal adenocarcinoma. British J Cancer 82:178–185.

Wilson JW, Potten CS. 1996. Immunohistochemical localization of Bax and Bad in the normal and Bcl-2-null gastrointestinal tract. Apoptosis 1:183–190.

Zimmermann S, Moelling K. 1999. Phosphorylation and regulation of Raf by Akt (Protein Kinase B). Science 286:1741–1744.