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Dietary Regulation and Localization of Apoptosis Cascade Proteins in the Colonic Crypt

Carmel Avivi-Green,[1] **Sylvie Polak-Charcon,**[2] **Zecharia Madar,**[1] **and Betty Schwartz**[1]*

[1]Institute of Biochemistry, Food Science, and Nutrition, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot, Israel
[2]Institute of Pathology, Sheba Medical Center, Tel-Hashomer, Israel

Abstract This study was designed primarily to assess the localization of apoptosis cascade proteins along the rat colonic crypt and secondarily to test whether the activity and/or localization of these proteins are affected by the enrichment of the diet with the soluble fiber pectin. Expression of apoptosis cascade proteins was assessed in isolated colonocytes harvested from the luminal and basal crypt colonocyte populations. Two different dietary regimens were tested: a standard diet (diet A), and a diet enriched in pectin (diet B), a soluble fiber that undergoes fermentation in the cecum and produces high concentrations of intracolonic short-chain fatty acids. Caspase-1 expression was maximal in luminal colonocytes of rats fed diet B, as evidenced by Western blot and immunohistological analyses. Expression of the cleaved poly(ADP-ribose) polymerase product was elevated in both the luminal and basal colonocytes of the pectin-fed group, whereas in rats fed diet A, the expression was lower, especially in basal crypt colonocytes. The highest expression of the antiapoptotic protein Bcl-2 was observed in the lower compartments of the colonic crypt tissue and was maximal in the rat group fed a standard diet. The apoptotic index in colonocytes of rats fed diet B was higher than that measured in rats fed diet A. Cumulatively, our results indicate that apoptosis cascade proteins are differentially localized along the lumen–crypt axis, and their expression and activity may be controlled by dietary components. These results may, at least partially, account for the documented protective effect of butyrogenic fibers on colorectal cancer. J. Cell. Biochem. 77:18–29, 2000.

Key words: apoptosis; caspase; colon; short-chain fatty acids; soluble fiber

The colon provides a unique model of cells at different stages of proliferation and differentiation, aligned in an ordered pattern along the crypt. Mitotic activities are restricted to cells residing in the proliferative lower crypt zones. During migration to the luminal surface, the cells acquire the typical functions of mature colonocytes, with a limited life span [Schwartz et al., 1991]. Terminally differentiated cells [Wang and Friedman, 1998] populate the top of the colonic crypt, and in this compartment, cells are probably eliminated by programmed cell death [Potten, 1996, 1997].

Apoptosis is triggered by external signals, such as soluble molecules, intercellular and external substrate interactions, and others. These stimuli may regulate specific proteins involved in the apoptotic cascade pathways [King and Goodbourn, 1998; Karnes et al., 1998], including apoptosis-promoting and apoptosis-preventing proteins. The caspases are a family of cysteine proteases acting as central mediators of apoptosis [Marcelli et al., 1998; Cory and Adams, 1998]. At least 10 members of this family have been isolated and cloned [Cory and Adams, 1998; Strasser et al., 1997; Psmantur et al., 1997; Imoto et al., 1998; Van de Craen et al., 1998]. These proteases cleave specific protein substrates, such as DNA-dependent kinase, nuclear lamins, and PARP [King and Goodbourn, 1998]. The latter proteolytic cleavage represents one of the critical activators of apoptosis [Marcelli et al., 1998]. Function analyses

Abbreviations used: ICE, interleukin-1β converting enzyme; PARP, poly(ADP-ribose) polymerase; SCFA, short-chain fatty acid; TUNEL, terminal deoxytransferase-mediated deoxyuridine nick end-labeling.

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*Correspondence to: Betty Schwartz, Institute of Biochemistry, Food Science and Nutrition, Faculty of Agricultural, Food, and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel. E-mail: bschwart@agri.huji.ac.il

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have indicated that activation of caspase-1, formerly known as interleukin-1β converting enzyme (ICE), initiates the apoptotic cascade in many systems [Harvey et al., 1998; Takahashi et al., 1997]. The Bcl-2 protein family is known to regulate apoptosis in mammalian cells [Cory and Adams, 1998]. Some of its members are suppressors of cell death, for example, Bcl-2, Bcl-X_L [Strasser et al., 1997; Harvey et al., 1998; Watson, 1995; Newton and Strasser, 1998] and others, such as Bax, Bak [Potten, 1997; Strasser et al., 1997; Newton and Strasser, 1998; Chinnaiyan and Dixit, 1996], promote apoptosis and antagonize Bcl-2 function.

Environmental and nutritional factors play key roles in colonocyte development, differentiation, and probably death [Statland, 1992; Rao et al., 1995; Kawamori et al., 1995; Hague et al., 1993; Cassand et al., 1997]. Carbohydrates escaping enzymatic digestion in the small intestine are delivered to the colon, where they are fermented by the microflora. These include nonstarch polysaccharides, resistant starch, and endogenous polysaccharides from mucus and shed epithelial cells. The most important end products of the bacterial breakdown of carbohydrate are the SCFAs acetate, propionate, and *n*-butyrate. These SCFAs are rapidly and efficiently taken up by the epithelial cells that line the colonic lumen. Butyrate serves as an energy-yielding substrate in the colonocytes and additionally, affects several cellular functions (proliferation, membrane synthesis, and sodium absorption) [Schwartz et al., 1998]. Propionate and acetate are released by the basolateral membrane to the portal circulation and may have effects far from their production site [Scheppach et al., 1995]. Butyrate is the preferred metabolic energy source for colonocytes; it is used over glucose, glutamine, and the other SCFAs. As such, it is especially important for maintenance of normal health and function of the colonic epithelium.

Pectin and other fermentable fibers are known to produce, after microbial fermentation, relatively large intracolonic concentrations of SCFAs [Zoran et al., 1997]. In contrast, cellulose, the fiber added to standard rat diets, is a poorly fermentable fiber, yielding relatively low intracolonic concentrations of SCFAs [Zoran et al., 1997; Stark and Madar, 1993].

The present study compares two dietary regimens differing in their ability to produce intracolonic SCFAs. We evaluated whether these dietary regimens are able to control apoptosis and apoptosis cascade proteins in rat colonocytes at different stages of proliferation and differentiation.

MATERIALS AND METHODS

Materials

All biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. [^{3}H] thymidine (5Ci/mmol) was obtained from the Nuclear Research Center, Dimona, Israel.

AIN-76 mineral mix and vitamin-free casein were purchased from ICN Biomedicals (Aurora, OH). Cellulose-Solkafloc was purchased from James River Corp. (Hackensack, NJ). High methoxyl pectin type 106 was purchased from Citrus Colloids (Hereford, England). Vitamin mix was obtained from Kofolk (Petach-Tikva, Israel).

Animals

Male rats of a Charles-River-derived (inbred) strain were purchased from Levenstein (Yokneam, Israel). The animals were randomly divided into two groups. Diet and water were freely available. Animals were weighed weekly throughout the study. Research protocols and animal care was supervised by the Animal Welfare Committee of the Faculty of Agriculture of the Hebrew University of Jerusalem.

Diets

The animals were daily fed the experimental diets for 16 weeks, then were killed. During the experiment, group A was provided with a standard diet (2% insoluble fiber, cellulose), and group B was provided with a 15% citrus-pectin soluble-fiber diet. The diets were provided in pellet form. The standard diet was prepared according to the recommended AIN-76A semisynthetic diet (Table I).

Tissue Sampling

Histological examinations. Distal colon specimens eight of each group were fixed in 4% formalin for 18 h. The tissues were dehydrated and fixed in paraffin. Eight 6-μm-wide sections of colon tissues from each of the different experimental groups were stained with hematoxylin and eosin and analyzed for general histology [Schwartz et al., 1991].

Colonocyte harvest and isolation. Cells populations were prepared as previously de-

TABLE I. Dietary Composition

Ingredients	Amount (g/kg) Diet A	Diet B
Casein	180	180
Sucrose	340	275
Starch	339.5	274
Soy oil	80	80
Vitamin mix[a]	2.5	2.5
Mineral mix[b]	35	35
Methionine	3	3
Fiber[c]	20	150
Choline chloride	0.67	0.67

[a]Vitamin Mix AIN 76A (Kofolk Ltd., Petach-Tikva, Israel).
[b]Mineral Mix AIN 76A (ICN Biochemical, Cleveland, OH).
[c]The fibers included in diets A and B were cellulose and pectin, respectively.

scribed [Schwartz et al., 1991]. Briefly, the rats were killed by cervical dislocation. The abdomen was opened and the colon was removed and washed to remove fecal content, then washed with 50 ml of saline containing 2 mmol/L dithiothreitol to remove mucus. The length of cecum and colon of each rat colon was measured. The colon was inverted over a pipette and the distal end was closed by tying with surgical thread. The serosal surface, now facing the constructed lumen, was filled with minimal essential medium (Biological Industries, Beith Haemek, Israel) containing 10% fetal calf serum, 2 mmol/L EDTA, and 1% penicillin-streptomycin, and the other end of the colon was tied to form a closed loop. The colon was placed in a flask containing 15 ml of the same medium, and the flask was agitated in a water bath at 37°C. The medium was changed at intervals and the cells were harvested. In this way, five cell populations (C1–C5) were obtained, from the luminal surface to the base of the crypts. The luminal cell populations C1 and C2 were pooled and designated as upper crypt colonocytes (U), the proliferative populations C4 and C5 as the lower crypt colonocytes (L). C3 population was not included in the study because it contains a mixed colonocyte population [Schwartz et al., 1991].

In vivo methyl [^{3}H]thymidine incorporation into colonic DNA. One hour before the rats were killed, tritiated thymidine (100 µCi) was administered IP to both control and pectin-supplemented rats. The colon cell fractions were collected as previously described. Deoxyribonucleic acid extraction was performed as described [Schwartz et al., 1988]. The samples were homogenized in 1 ml of 0.2N perchloric acid, and the precipitate was dissolved in 3 ml 10% perchloric acid.

Samples were counted in a β-scintillation counter, and the DNA content was measured according to the diphenylamine method of Burton [1956], using calf thymus DNA as a standard. Results are expressed as cpm methyl [^{3}H]-thymidine/µg DNA.

Caspase-1/interleukin converting enzyme immunohistology. Immunostaining of rat colonic tissue was performed in formalin-fixed, paraffin-embedded sections using the amplification avidin–biotin–peroxidase complex method. Sections of 6 µm were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was neutralized in methanol/0.3% H_2O_2 and blocked in normal goat serum. A 1:50 dilution of the primary rabbit polyclonal antibody ICE p10 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used. The sections were incubated with ABC reagent (Elite rabbit IgG vectastain kit, Vector Laboratories, Burlingame, CA) and prepared for color development by reacting the peroxidase with a solution of 0.6 mg/ml diaminobenzidine tetrahydrochloride in phosphate-buffered saline. Sections were counterstained with hematoxylin, rinsed, and mounted.

Western immunoblotting analysis. For the immunodetection of caspase-1, PARP, and Bcl-2, upper and lower crypt cells of colonic tissues obtained from different experimental groups were homogenized in lysis buffer: 20 mmol/L Tris pH 7.8, 100 mmol/L NaCl, 50 mmol/L NaF, 10% glycerol, 1%NP-40, and Complete™ protease-inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). The homogenates were centrifuged and supernatants were kept at −70°C for further processing. Protein concentration in the different supernatants was determined by BCA reagent (Pierce, Rockford, IL) [Karnes et al., 1998]. Aliquots of 100 µg protein were separated by 10% sodium dodecyl sulfate and transferred to nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). The filters were stained with Ponceau S to confirm equal protein loading and effective transfer. The following primary antibodies were used: the rabbit polyclonal antibody ICE p20 equivalent to caspase-1 (Upstate, Lake Placid, NY); goat polyclonal PARP antibody (Santa Cruz Biotechnology Inc.); and mouse monoclonal anti-Bcl-2 antibody (Zymed Laboratories, San Fran-

cisco, CA). Detection was performed with horseradish peroxidase–linked anti-rabbit or anti-goat or anti-mouse IgG antibody, depending on the primary antibody. Immunoreactive bands were visualized using the enhanced chemiluminescence detection reagents SuperSignal (Pierce, Rockford, IL) as suggested by the manufacturer.

Densitometric analysis of band intensity. The total intensity of each band obtained in Western blots was detected and quantified with a PhosphorImager system (Fujix, Tokyo, Japan) and corrected for background. The integration values for each band were taken as arbitrary units.

Apoptotic index. The apoptotic index was evaluated using two independent approaches.

1. By identifying apoptotic cells in hematoxylin and eosin–stained preparations. To this end, distal colon specimens were fixed in 4% formalin for 18 h. The tissues were dehydrated and fixed in paraffin. Sections (6 μm) of colon tissue from the different experimental groups were stained with hematoxylin and eosin. Sections containing crypts in longitudinal orientation (with 60–70 colonocytes per section of crypt column) were selected. The criteria used to distinguish apoptotic cells from other colonocytes within the crypt were typical decreased volume and intense nuclear staining. Thirty crypts were scored per section.
2. By the TUNEL method (ApopTag™, Oncor, Gaithersburg, MD) in paraffin sections of rat colonic tissues obtained from the different experimental groups. Briefly, the apoptotic cells were visualized by direct immunoperoxidase detection of digoxigenin-labeled genomic DNA. The labeling target was the multitude of new 3′-OH DNA ends generated by DNA fragmentation and typically localized in morphologically identifiable nuclei and apoptotic bodies. The number of cells reactive to TUNEL-staining [Lifshitz et al., 1998] were counted in colonic sections with longitudinally oriented crypts, containing 60–70 colonocytes per section of crypt column, and in isolated colonocytes.

Transmission electron microscope examinations. Colonic cells were fixed in 2.5% glutaraldehyde and 0.1 mol/L sodium cacodylate buffer pH 7.2. Dehydration was performed with graded ethanol solutions before propylene oxide exchange, and the colonocytes were embedded in Epon. Ultra-thin sections on grids were stained with uranyl acetate and lead citrate and finally examined in a Jeol 1200EX transmission electron microscope [Schwartz et al., 1991].

Statistical analysis. Results were expressed as mean ± SEM. Statistical significance was calculated by Student's *t* test.

RESULTS

Dietary Intake

Both experimental groups were provided with diets that were well accepted by the animals (Table I). Dietary intake was monitored daily, and no significant differences were observed between the amounts of food ingested by each group (data not shown). Weight gain was measured once a week, and was similar in both experimental groups (Fig. 1).

Morphometric Parameters

Table II summarizes the colonic and cecal lengths in rats under the different dietary regimens. Rats fed the pectin-enriched diet had enlarged colons and ceca relative to those fed the standard diet.

Methyl [^{3}H]Thymidine Incorporation Into DNA of Isolated Colonocytes

Figure 2 shows that the colonocyte isolation procedure used resulted in comparable colono-

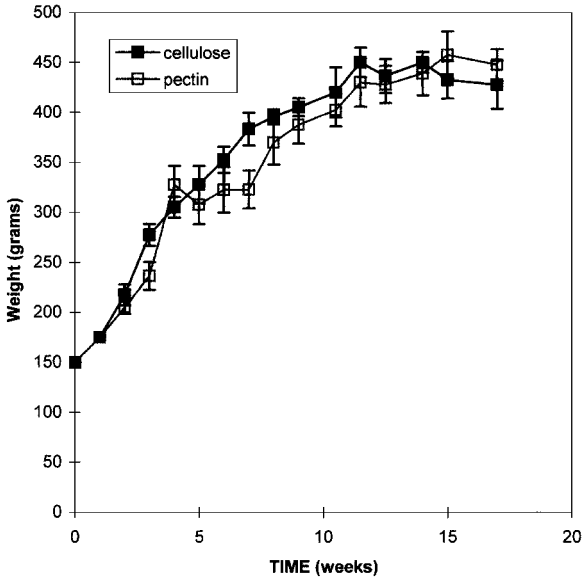

Fig. 1. Weekly weight gain for rats fed control diet as compared to rats fed pectin-supplemented diet (eight rats per group, bars = SEM).

TABLE II. Morphometric Measurements[a]

	Diet A (n = 7)[b]	Diet B (n = 7)
Colon length (cm)	17.6 ± 0.3[c]	19.6 ± 1.0
Cecum length (cm)	4.0 ± 0.2	7.4 ± 0.6[d]

Diet A, control diet; Diet B, pectin-supplemented diet.
[a]Results are from rats killed 16 weeks after the start of the experiment.
[b]n is the number of rats in each group.
[c]Data shown are the mean ± SEM.
[d]Significantly different from Diet A by Student's t test ($P < 0.001$).

cytes populations according to the proliferation parameter (in vivo thymidine incorporation), for both the rats fed control diet and rats fed the pectin-supplemented diet. The selective in vivo uptake of [^{3}H]thymidine into colonic DNA as a function of growth stages is equally observed for colonocytes of both experimental groups.

Caspase-1/ICE Immunohistology

Figure 3 shows representative immunohistological micrographs detecting specific intracellular caspase-1 expression. Rats fed the standard diet (Fig. 3A) showed staining of colonocytes from the upper crypt zones, whereas the antibody did not stain the lower crypt domains. However, rats fed the pectin diet (Fig. 3B) showed stronger caspase-1 expression in the upper crypt and broader immunostaining of other crypt compartments.

Caspase-1 Western Blot Analysis

Expression of caspase-1 uncleaved proenzyme (45 kDa) or its fully processed form (20 kDa) was detected by Western blot in protein extracts of colonocytes isolated from rats of both experimental groups (Fig. 4). Expression of the proenzyme was detected equally and faintly in all colonocyte fractions. In contrast, expression of the 20-kDa processed protein indicative of active enzyme was evident mainly in colonocytes from the upper crypt compartments, whereas only sparse expression was observed in colonocytes from the lower crypt zones. Densitometric analysis of p20 subunit expression revealed that the highest expression was in pectin-fed rats (Table III).

PARP Cleavage Analysis

We used Western immunoblot analysis to examine the expression and activity of the caspase substrate PARP in colonocyte lysates from rats in the different dietary groups. The antibody recognized both the full-length (115-kDa) and cleaved 25-kDa PARP fragment (Fig. 5). Lower crypt colonocytes showed the highest expression of the full-length uncleaved, 115-kDa PARP, whereas in the upper crypt colonocytes its ex-

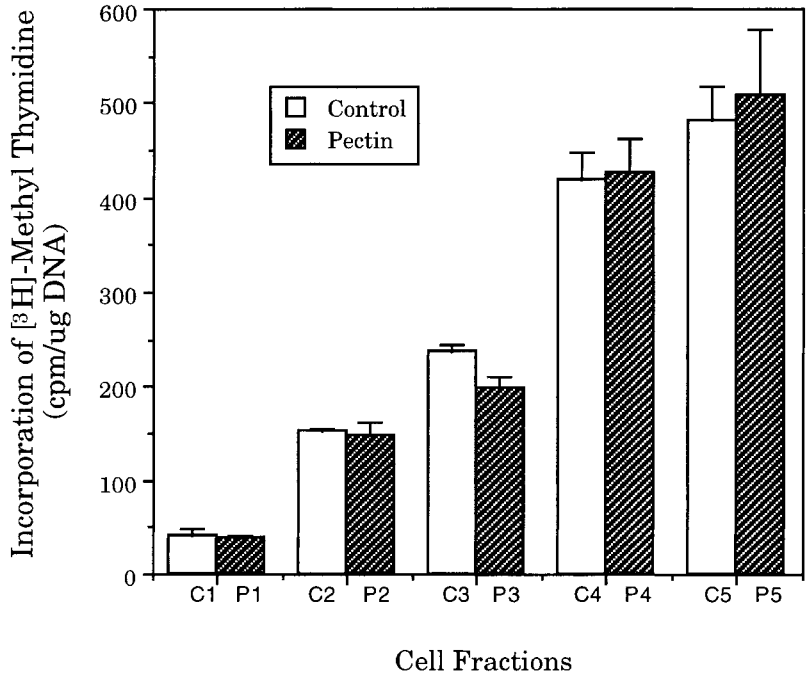

Fig. 2. In vivo selective incorporation of [^{3}H]-thymidine into DNA of isolated colonocytes harvested from colonic crypt axis. The distinct cell populations (C1–C5, for colonocyte populations from rats fed control diet, P1–P5 for colonocytes harvested from rats fed pectin-enriched diet). C1–C2 or P1–P2 represents luminal colonocytes, and C4–C5 or P4–P5, lower crypt colonocytes. Each histogram represents the mean ± SEM of the values obtained in triplicate of eight individual experiments. Bars = SEM.

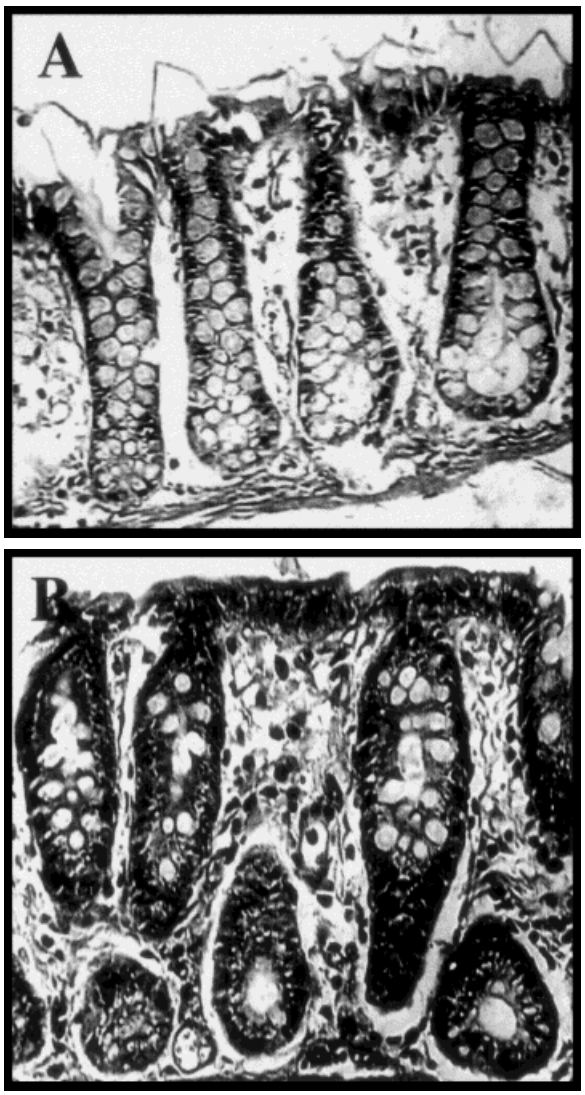

Fig. 3. Detection of caspase-1 expression in colonic tissues from rats killed 16 weeks after the start of the experiment. Caspase-1 epitopes were detected by established immunohistochemical procedures using a rabbit polyclonal caspase-1 antibody (magnification ×200). Dark granularity is mainly detected in upper cells from control rats fed the standard diet (**A**). Dark positive caspase-1 immunostaining was detected in most of the crypt cells from rats fed the pectin-supplemented diet (**B**).

pression was low (Fig. 5, upper panel). On the other hand, the expression of the cleaved 25-kDa fragment generated during active apoptosis was moderately higher in the upper crypt colonocyte harvested from rats fed both dietary regimens (Fig. 5, lower panel). Densitometric analysis revealed pectin-supplemented-diet-induced upregulation of both the uncleaved and cleaved PARP isoforms in lower crypt colonocytes (Table III).

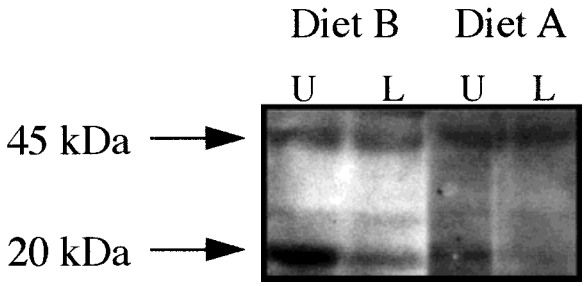

Fig. 4. A representative caspase-1 Western immunoblot analysis performed on lysates of colonocytes from rats fed the standard diet A or the pectin-enriched diet B. U, upper crypt colonocytes; L, lower crypt cell populations. Rats were killed 16 weeks after the start of the experiment. The 45-kDa proenzyme and the processed 20-kDa subunits are evident. Similar results were obtained in seven independent experiments. Their respective densitometric analyses are summarized in Table III.

Bcl-2 Western Blot Analysis

The strongest Bcl-2 expression was observed in colonocytes of rats fed the standard diet (Fig. 6, Table III). Both lower and upper crypt colonocyte fractions were equally reactive to the 26-kDa Bcl-2 antibody. Colonocyte populations from the rats fed pectin supplemented diet exhibited significantly lower Bcl-2 expression than their counterparts from rats fed control diet (Table III) however, the intensity of lower crypt fractions was higher.

Apoptotic Index

Apoptotic index (percent of apoptotic cells) was evaluated in whole colonic tissue in rats fed both diets, and results are summarized in Table IV. The highest apoptotic index, evaluated by hematoxylin and eosin staining or TUNEL, was recorded in rats fed the pectin-enriched diet. The apoptotic index was also evaluated in isolated colonocytes. Again, as for whole tissue, the highest apoptotic index evaluated was from rats fed the pectin-enriched diet (Figs. 7, 8A).

Ultrastructural Examination

Ultrastructural micrographs of colonic tissues obtained from rats under the different dietary regimens were obtained by transmission electron microscope (Fig. 8B,C). Micrograph C in Figure 8 was obtained from a colonic section of a rat that was fed the pectin-enriched diet and was killed 16 weeks after the start of the experiment. It shows a typical apoptotic feature such as condensed chromatin near the

TABLE III. Densitometric Scan Values From the Immunoblots of Apoptosis-Related Molecules

	Diet A		Diet B	
	U[a]	L	U	L
Caspase-1 (7)[b]				
45-kDa	141 ± 11[c]	144 ± 15	127 ± 12	123 ± 10
20-kDa	140 ± 10	110 ± 11	194 ± 18**	142 ± 13
PARP (8)				
115-kDa	139 ± 12	159 ± 16	98 ± 10	217 ± 18*
25-kDa	186 ± 17	208 ± 19	217 ± 14	224 ± 15*
Bcl-2 (8)				
26-kDa	241 ± 18	246 ± 20	163 ± 16**	189 ± 17*

Diet A, control diet; Diet B, pectin-supplemented diet.
[a]U, upper crypt colonocytes; L, lower crypt colonocytes.
[b]The number in brackets represents the number of experiments.
[c]The values represent the mean ± SEM densitometric measurements (arbitrary units) from the various experiments.
*,**Significantly different from Diet A by Student's t test (*$P < 0.05$, **$P < 0.01$).

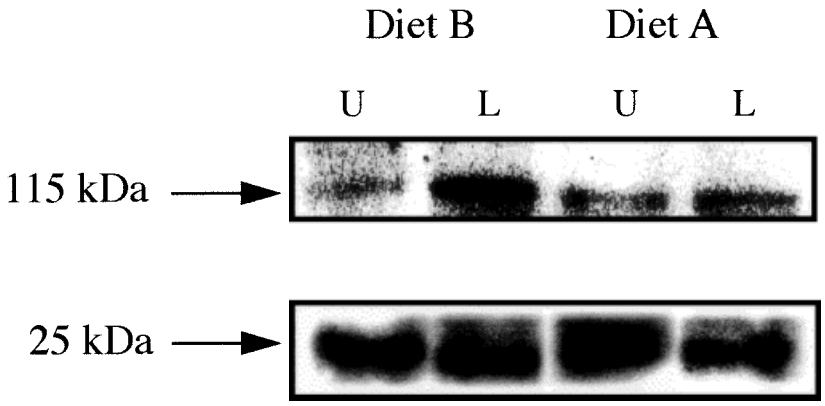

Fig. 5. A representative PARP Western blot analysis. Apoptosis induces cleavage of the "death" substrate PARP. Colonic cell lysates were obtained from rats receiving diets A or B and killed 16 weeks after the start of experiment. PARP cleavage was evidenced by the appearance of 25-kDa product (lower panel), versus the uncleaved 115-kDa protein (upper panel). U, upper crypt colonocytes; L, lower crypt colonocytes. Similar results were obtained in eight additional experiments.

nuclear membrane. In the cells exhibiting this feature, most of the intracellular organelles are not evident. In colonic sections from rats fed the standard diet (Fig. 8B), no such high numbers of apoptotic cells concentrated in one field were ever observed.

DISCUSSION

In this study, we show that several proteins of the apoptosis cascade process are differentially localized along the crypt–lumen axis and that their expression is regulated by different dietary regimens.

The caspase gene family encodes proteins that are translated as inactive proenzymes (zymogens), which are single polypeptide-chain precursors [Strasser et al., 1997; Cory and Adams, 1998; Harvey et al., 1998]. The conversion of the procaspases to active forms marks the cell for death [Cory and Adams, 1998]. This conversion of zymogens into active cysteine proteases appears to be the "point of no return" in apoptosis, and may therefore be the crucial step in this process's regulation [Strasser et al., 1997]. Because the caspase-1 activation is a common integration point for diverse apoptotic stimuli and may serve as a control point [Takahashi et al., 1997], we analyzed the expression of this apoptosis-associated enzyme in the colonic tissue of rats fed different dietary regimens.

Western blot analysis using caspase-1 antibody against the p20 epitope revealed bands of 45 and 20 kDa. The 45-kDa band represents the full-length molecule and includes several domains, which differ in size according to species: the prodomain, the p20 subunit (20-kDa), and the p10 subunit (10 kDa). After enzymatic activation, the prodomain is removed and the subunits are obtained. Cleavage of caspase-1 is always associated with active programmed cell death [Takahashi et al., 1997; Fulda et al., 1997], and we therefore assume active apoptosis wherever the caspase-1 subunit is detected.

Both upper and lower crypt colonocytes expressed the uncleaved proenzyme, i.e., the 45-

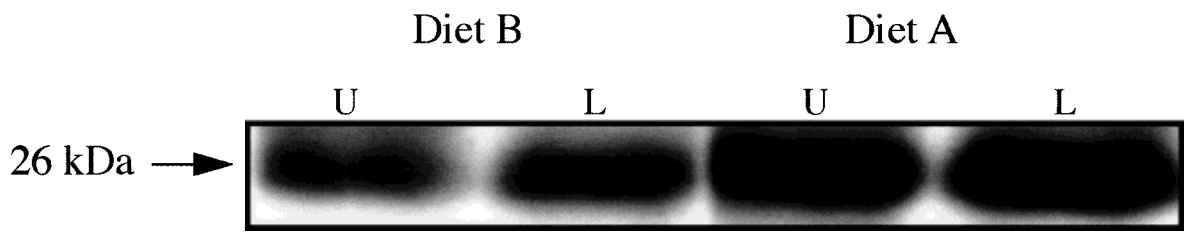

Fig. 6. Representative Bcl-2 Western blot analysis. Expression of 26-kDa Bcl-2 in colonocytes from rats fed diets A or B and killed 16 weeks after the start of experiment. U, upper crypt colonocytes; L, lower crypt colonocytes. Similar results were obtained in eight independent experiments.

TABLE IV. Apoptotic Index[a]

Diet	Hematoxylin and eosin	TUNEL
A	1.38 ± 0.1[b]	1.15 ± 0.1
B	1.58 ± 0.13	1.54 ± 0.14[c]

Diet A, control diet; Diet B, pectin-supplemented diet.
[a]Apoptotic index was calculated as the percentage of apoptotic cells per longitudinal crypt section.
[b]The values are mean ± SEM of 30 crypts scored per rat colonic section, stained with hematoxylin and eosin and reactive to TUNEL, 16 weeks after the start of the experiment.
[c]Significantly different from Diet A by Student's t test ($P < 0.01$).

kDa caspase-1 isoform. However, the cleaved protein, reflecting the active subunit p20, was differentially located within the colonic crypt. The immunoblot intensity of this isoform band was significantly higher in the luminal colonocytes than in proliferative colonocytes harvested from the lower crypt base.

[^{3}H]Thymidine incorporation studies into colonocytes' DNA indicated that in both experimental rats groups (fed the control diet or fed the pectin enriched diet), the cell isolation methodology resulted in five colonocyte populations that differed in their proliferation characteristics. The dietary protocols did not affect either the cell isolation or the proliferation activity of each cell fractions.

In addition, we show that a pectin-rich diet causes enhanced protein expression of the active caspase-1 p20 subunit, both in the upper and lower crypt cells. MacCorkle et al. [1998] and Psmantur et al. [1997] recently demonstrated that caspase overexpression is associated with enhanced apoptotic death. Strasser et al. [1997] showed that transient expression of cysteine proteases causes apoptosis in cell lines, affecting several steps of the apoptotic cascade. Cumulatively, these data indicate that the magnitude of apoptotic death is directly correlated to the extent of caspase activity. Our immunohistological studies completely supported the Western blot analysis, and showed different extents of compartmentalization and expression in colons from each dietary group.

A common feature of the caspase family of enzymes (such as caspase-1,2,3,4,6,7,9) is their ability to cleave the 115-kDa PARP enzyme into an 89-kDa fragment (containing the active site) and a 25-kDa fragment (the DNA-binding domain of the enzyme) [Karnes et al., 1998; Marcelli et al., 1998; Morana et al., 1996; Kaufmann et al., 1993]. PARP cleavage occurs in a conserved amino-acid sequence DVED, which is also recognized by other proteases homologous to caspases [Watson, 1995]. The induction of apoptosis is often indicated by the complete cleavage of PARP into 89- and 25-kDa fragments [Kaufmann et al., 1993; Simbulan-Rosenthal et al., 1998]. Previous studies have suggested a correlation between poly(ADP-ribosyl)ation of nuclear proteins and internucleosomal DNA fragmentation during apoptosis. Poly(ADP-ribosyl)ation of histone H1, for example, during the early stages of apoptosis, has been suggested to facilitate internucleosomal DNA fragmentation by enhancing chromatin susceptibility to cellular endonucleases [Kaufmann et al., 1993].

We assessed the expression of both the uncleaved PARP (115-kDa) and its cleaved product (25-kDa) by Western immunoblotting. The uncleaved PARP protein was detected in all colonocytes from both dietary regimens. The highest expression was detected in lower crypt compartments of rats fed diet B, whereas the expression significantly diminished in the luminal colonocytes. It can be surmised that the dietary regimens plays a role in upregulating the expression of this enzyme in the proliferative compartment, and when the colonocytes

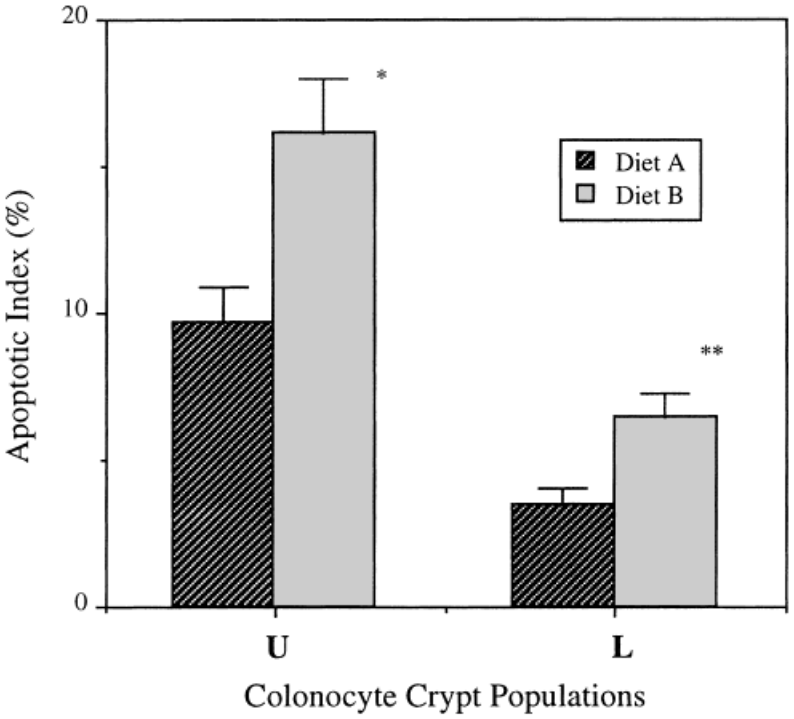

Fig. 7. Apoptotic index of isolated colonocytes along the crypt–lumen axis from rats fed diets A or B. The apoptotic index was measured in rats killed 16 weeks after the start of the experiment using the TUNEL methodology. U and L are as described in legend to Figure 4. *,** Significantly different from diet A by Student's *t* test (*$P < 0.01$, **$P < 0.05$, respectively).

migrate up to the luminal region, this protein substrate is digested by caspase activity and apoptosis can take place. The expression and detection of PARP products completely agree with our findings concerning caspase-1 expression and cleavage.

In rats fed the standard diet, a similar intensity of Bcl-2 expression was detected in both the upper and lower crypt colonocyte populations. In contrast, Bcl-2 expression of this protein was elevated in lower crypt compartments relative to the upper-luminal colonocytes of the rats fed pectin-supplemented diet. This finding supports previous reports that whenever this anti-apoptotic protein is overexpressed, proliferating cells are protected from apoptosis [Strasser et al., 1997; Psmantur et al., 1997; Imoto et al., 1998]. Expression of Bcl-2 protein has been identified in both human and murine intestinal epithelia at the crypt base by Merritt et al. [1995], which is similar to our findings for rats fed a pectin-supplemented diet.

The Bcl-2 protein is involved in a process that can block cell death induced by a variety of stimuli. Bcl-2 seems to function in several subcellular locations, yet lacks any known motifs that would enable predictions of its mechanism of action. Strasser et al. [1997] proposed that there are Bcl-2 pathways that are inhibited by apoptosis, and other that are insensitive to it [Strasser et al., 1997]. Therefore, apoptosis activation pathways can either override the effect of Bcl-2 or bypass the checkpoint at which Bcl-2 acts. A possible explanation for the existence of several pathways may reside in the fact that there are several independent signaling pathways that can trigger apoptosis, and others insensitive to Bcl-2. We show that pectin downregulates the expression of Bcl-2, and this is probably associated with the enhanced apoptotic activity observed in rats fed a high-pectin diet. We assessed the expression of one of the Bcl-2 family proteins. Whether other members of this family are also enhanced and can counteract the effect of Bcl-2 remains to be determined.

Finally, the endpoint after activation of apoptosis cascade enzymes is actual cell death. Apoptosis was evaluated using currently available assays that highlight the typical morphological and biochemical changes of dying cells, such as microscopic, ultrastructural changes or DNA fragmentation assessed by the TUNEL method. The apoptotic index (percent of apoptotic cells) was evaluated in intact colonic tissue and in isolated colonocytes. We found, as expected, a higher apoptotic index in isolated colonocytes than in the colonic tissue as a whole (Fig. 7 vs. Table IV). These results confirm our previous findings [Lifshitz et al., 1998] that isolated colo-

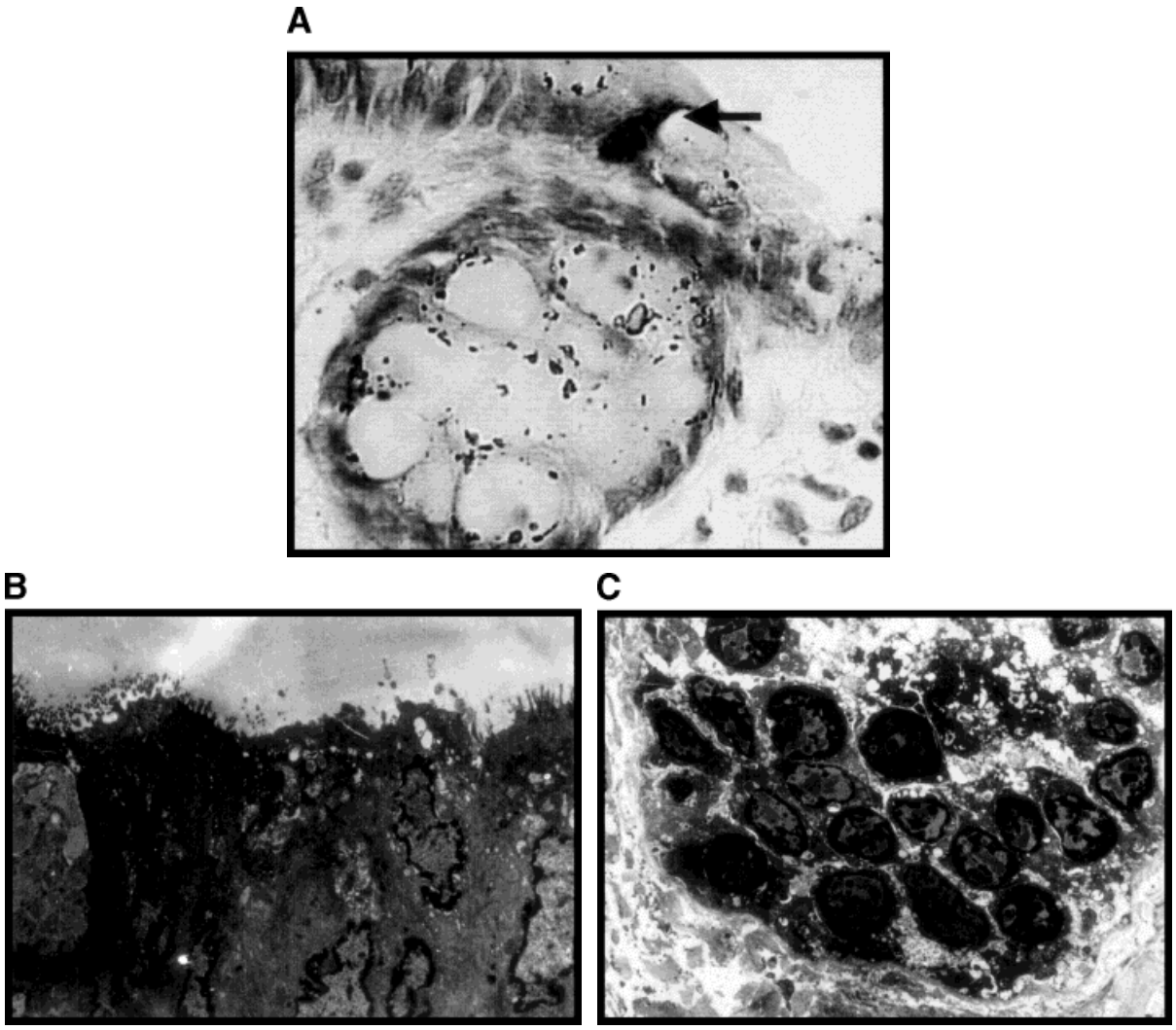

Fig. 8. Apoptosis evaluation. **A:** Representative apoptotic cell detected by TUNEL methodology (magnification ×1,000). Note the intense nuclear stain of luminal epithelial cell (arrow). **B,C:** Representative electron micrographs of colonic tissue from rats fed the different diets. B,C represent diets A and B, respectively (magnification: B, ×3,000; C: ×2,000).

nocytes, devoid of communication with neighboring cells, are triggered to undergo rapid apoptotic death. Apoptotic index evaluation in either system (tissue or isolated cells) showed that TUNEL-positive colonic cells are most frequent in rats fed pectin-supplemented diet.

We have previously shown that in vitro fermentation of pectin with fecal inoculum of rats fed pectin-enriched diet produces 0.32 ± 0.05 mmol butyrate/g substrate dry matter, whereas rats fed a control diet produced 0.12 ± 0.04 mmol butyrate/g substrate dry matter [Stark and Madar, 1993]. SCFA composition of cecal contents in pectin-fed rats resulted in 7.1 µmol butyrate /ml × cecal content (grams); whereas cecal content of rats fed control diet resulted in only 1.7 µmol butyrate/ ml × cecal content (grams) [Stark and Madar, 1993].

In the present study, we show that rats fed a pectin-supplemented diet undergo the expected morphological changes (Table II), such as adaptation of the ceca and large intestine to enhanced fermentation activity. Similar findings were previously reported by Cassand et al. [1997], who showed increased cecal weight in rats fed a diet rich in resistant starch. Parallel morphometric findings were reported in our previous study [Stark et al., 1995], in which 15% pectin induced increased the distal colonic mucosal area.

We assume that rats fed pectin-supplemented diet have high intracolonic butyrate concentra-

tions, providing more energy to the colonocytes and hence tissue growth. Accordingly, the colons of pectin-fed rats were larger than the colons of rats fed a standard diet (Table II). We conclude that the higher the growth rate, the higher the death rate, which needs to be induced to maintain the relative tissue dimensions. Therefore, apoptosis is enhanced as an effective mechanism to eliminate excessive numbers of colonocytes in such actively growing tissue, thereby explaining our findings (Table IV).

Cumulatively, our data suggest that apoptosis in rat colonic tissue takes place through sequential molecular mechanisms of the apoptotic pathway, namely, activation and initiation of the protease cascade, which includes caspase-1 (ICE) and probably other caspase enzymes, ultimately leading to irreversible cleavage of proapoptotic targets, such as PARP. In addition, this study shows that apoptosis-associated proteins are localized along the crypt–lumen axis and that localization is affected by different dietary regimens. Moreover, the expression and activity of apoptotic-associated proteins are also significantly affected by different dietary regimens. We assume that this phenomenon is associated with the enhanced cell turnover of the colonic epithelia in rats fed an enriched butyrogenic diet; as a consequence, the death and shedding of unhealthy cells may occur.

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