Diet Restriction Increases Apoptosis in the Gut of Aging Rats

Peter R. Holt,1 Steven F. Moss,1 Ahmad R. Heydari,2 and Arlan Richardson2

1The Gastrointestinal Division, Department of Medicine, St. Luke’s/Roosevelt Hospital Center and Columbia University, New York.
2Audie L. Murphy Memorial Veterans Hospital, San Antonio, Texas.

Previous studies have shown that epithelial cell production rates are increased throughout the gastrointestinal tract in aging rats. We tested the hypothesis that alteration in cell death (apoptosis) might be involved. Fischer 344 rats aged 4–5 months and 24–25 months fed ad libitum (AL) or calorie restricted (CR) to 60% of the AL intake were studied. Epithelial cell apoptosis was determined by a terminal deoxyuridine nucleotidyl nick end labeling (TUNEL) technique validated in our laboratory, and the expression of four members of the Bcl-2 family was evaluated by Western blotting in the small intestine and colon. The apoptotic index was low in young and aging AL and young CR rats. However, CR in aging rats was associated with a significantly higher apoptotic index in the jejunum and colon. The expression of the Bcl-2 family of genes was unchanged. Enhanced apoptosis in CR may protect the gastrointestinal tract from accumulation of DNA-altered cells during the aging process.

HOMEOSTATIC regulation of cell numbers in proliferating normal tissues reflects a well-regulated balance between cell production (proliferation) and cell death. An increase in cell production has been emphasized as a factor in carcinogenesis (1). Apoptotic cell death is a protective mechanism to remove cells with DNA damage or diseased cells that might interfere with normal functioning, or result in neoplastic transformation as well as a physiologic event to eliminate cells at the end of their functional life span (2). The relative balance and efficiency of this cell death program, therefore, may have an impact on the risk of susceptibility to degenerative and neoplastic diseases. Abnormalities in cell death and proliferation can promote cancer development by permitting inappropriate survival of DNA damage in mutated cells, or by permitting cell production rates to exceed cell loss, which could result in expansion of preneoplastic cells (3).

Our previous studies have suggested that the aging process is associated with an excessive rate of cell production in the small and large intestine in normal rodents (4,5). Calorie restriction appears to delay this abnormally high rate of cell production to a later time period in the life of the rat (6). Furthermore, many studies have demonstrated that calorie restriction reduces cancer risk in experimental animals (7). Because changes in cell death (apoptosis) could be a factor in stimulating excessive cell production, therefore making the gastrointestinal tract more susceptible to neoplasia (8), we have investigated the rate of apoptosis as well as the content of several pro- and anti-apoptotic gene products in the small and large intestine of young and aging rats who were either ad libitum fed or were calorie restricted for much of their lives.

METHODS

Male Fischer 344 rats (specific pathogen-free) were obtained at 3 weeks of age from Harlan Sprague-Dawley (Indianapolis, IN). All procedures for handling the rats were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and the Subcommittee for Animal Studies at Audie L. Murphy Memorial Veterans Hospital. The rats were caged individually in a barrier facility on a 12 h light/dark cycle and were fed a semi-synthetic soy protein diet (5770M-S Vitamin Fortified RP 101 Purified Diet; Purina Mills, St. Louis, MO), which consisted of the following ingredients: 21% RP101 soy protein isolate, 15% sucrose, 46.0% dextran, 6% corn oil, 5% mineral mix with reduced sodium, 3% solka floc, 0.35% DL-methionine, 0.33% choline chloride, and 3.33% RP vitamin mix. At 6 weeks of age, the rats were randomly assigned to two groups. The control, ad libitum group was given free access to the diet; the CR group received 60% of the diet consumed by the rats fed ad libitum beginning at 6 weeks of age as previously described (9,10). The soy protein diet has been shown to prevent the progression of chronic nephropathy, which has been reported in this strain of rats on other protein diets (11). The median survival of the CR rats was 938 days compared to a median survival of 813 days for the control rats that had free access to the diet. Both young and old rats were carefully examined for the presence of disease and major pathological lesions.

The rats were housed under barrier-reared conditions, individually caged with a 12 h lighting cycle (light cycle 7 am–7 pm) with constant temperature and humidity. Animals in the colony were rigorously culled to minimize the transmission of disease. Animals showing any sign of disease were separated from the rest of the colony and observed. Groups of control and CR animals were studied at 4–5 months and 24–25 months of age.

Animals were killed by decapitation, and the small in-
testinal contents were gently washed out with cold saline. Segments of duodenum, jejunum starting approximately 10 cm beyond the ligament of Treitz, and ileum starting 3–5 cm above the ileocecal valve, were removed. The intestinal contents in the colon also were gently washed out, and segments of proximal colon (1 cm above the cecum) and descending colon (approximately 1 cm above the pelvic rim) were removed. All segments were rapidly processed as described previously (12). Briefly, the intestine was cut open and placed serosal side down onto dental wax, rapidly fixed in 10% buffered formalin, and blocked in paraffin within 24 hours.

In addition, for immunoblotting, a portion of the jejunum and transverse colon were opened, and the mucosa scraped. Mucosal scrapings were lysed on ice in 150 ml of ice-cold lysis buffer [containing 150 mM NaCl, 1% Triton X100, 10 mM Tris (pH 7.4), 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 50 μg/ml leupeptin, 0.3 trypsin inhibitor unit/ml aprotonin, 1 mM benzamidine and 1 μg/ml pepstatin, 1 mM Na-p-tosyl-L-lysine chloromethyl ketone, 1 mM N-tosyl-L-phenylalanine chloromethyl ketone, 50 mM sodium fluoride, 1 mM sodium vanadate, 25 mM β-glycerophosphate and 2 mM sodium pyrophosphate] for 20 min at 4°C [all purchased from Sigma, St. Louis, MO]. Triton X100 insoluble materials were removed by centrifugation at 14,000 rpm for 15 min. Protein concentrations were measured by the Bradford method and 50–100 μg samples were mixed with 2X Laemmli buffer, boiled for 5 min, and subjected to 12% SDS-PAGE. Western blot analyses were then performed as described previously, using specific polyclonal antibodies to Bcl-2, Bcl-X, Bax, and Bak, which were raised to sequence specific peptides (13,14,15) at a concentration of 1:1500 (v/v). Detection of antibody binding was by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

TUNEL Histochemistry

Cells containing DNA strand breaks, a marker of apoptosis, were detected by terminal deoxyuridine nucleotidyl nick end labeling (TUNEL) histochemistry using a method modified from Gavrieli et al., as described previously (16). Briefly, deparaffinized sections were digested with proteinase K 20 μg/ml (Sigma) for 15 minutes at room temperature, washed, and then incubated with terminal transferase and digoxigenin-11-deoxyuridine triphosphate (dUTP) (both from Boehringer Mannheim, Indianapolis, IN) in a buffer containing 200 mM potassium cacodylate, 0.2 mM EDTA, 25 mM TRIS-HCl, bovine serum albumin 0.25 mg/ml, and 1 mM cobalt chloride. The reaction was terminated with 300 mM sodium chloride and 30 mM sodium citrate. Incorporated digoxigenin-11-dUTP was detected with peroxidase conjugated Fab fragments of antidigoxigenin (Boehringer) and nickel-diamino-benzene and counterstained with 0.5% methyl green.

The identification of the appropriate conditions for TUNEL was established in our laboratory in human and rodent intestinal tissue as well as the presence of apoptotic bodies in serially sectioned tissue blocks stained by hematoxylin and eosin. Validation of the method has been previously reported (16).

The total numbers of epithelial cells and apoptotic epithelial cells in small intestinal segments were determined by counting the number of cells present on the left side of 12–16 well-oriented crypts and villi as previously described (17). A cell was considered apoptotic if the nucleus, either intact or fragmented, showed a distinct, dense brown stain following TUNEL histochemistry. Cell numbers in colonic crypts also were determined by counting cells on one side of 12–15 well-oriented crypts, as previously described (18). Apoptotic colonic crypt cells were determined as described above. The results were reported as apoptotic index, and expressed as a percentage of TUNEL-positive epithelial cells in each segment as numerator, and mean total epithelial cells as denominator. In the small intestine, the apoptotic index was calculated for each portion of the villus-crypt column divided into quintiles in the colon as previously described (19).

Statistics.—Differences between different dietary conditions and young and aging animals were analyzed by two-way analysis of variance and significance was evaluated by multiple comparison of means (Newman and Keuls method).

RESULTS

The numbers of epithelial cells in the jejunal crypt-villus column were measured in the four groups of animals. Calorie restriction of 60% of the calories of ad libitum-fed rats did not alter the numbers of jejunal epithelial cells significantly (Table 1).

The apoptotic index in the jejunal crypt-villus column was significantly increased in aging rats that had been calorie restricted to a level of 10.2% of total cells, whereas the remaining groups showed only between 1.8 and 3.6% apoptotic cells. The apoptotic cells in all groups of rats were almost entirely confined to the upper 20% of the villus-crypt column (Table 1). In the colon, crypt cell numbers were slightly more in distal than proximal colon (p < .05) in each group, but did not differ between the young and aging ad libitum and calorie-restricted groups of animals (Table 2). The apoptotic index was significantly greater in the total colonic crypt than in the jejunal crypt-villus column in all groups of animals. However, because there are more epithelial cells in the small intestine than the colon, the number of apoptotic cells per crypt or villus was similar. The number of apoptotic cells in the proximal colon of calorie-restricted aging rats was significantly greater than in any other group (Table 2). Furthermore, the colon of aging CR animals overall showed greater apoptosis than the colon of young CR animals.

We used Western blotting to study the effect of age and calorie restriction upon the expression of the proapoptotic gene products Bax, Bak and Bclx, as well as the antiapoptotic gene products Bcl-2 and Bclx (Figure 1). The expression of Bcl-2 was quite low, but the remaining gene products were expressed in reasonable quantities. No significant differences in the expression of Bcl-2, Bclx, or Bclx, Bax or Bak with age or with diet were detected.

DISCUSSION

The present experiments confirm previous studies from this laboratory (20,21) and others (22,23) that apoptotic
cells can be detected by the TUNEL method in both small and large intestine. Furthermore, most apoptotic cells were found in the upper 20–30% of the small bowel crypt-villus column and in the upper portion of colonic crypts. Jejunal and colonic cell numbers in both young and aging rats were very similar; distal colonic crypts, however, had significantly more cells than were measured in the proximal colon without any difference as a result of age or of dietary intake. The percentage of apoptotic cells also did not differ in young and aging ad libitum-fed animals or in the group of calorie-restricted young rats. However, calorie restriction in the aging animals resulted in a significant increase in apoptotic epithelial cells in the small intestine as well as proximal and distal colon.

The Bcl-2 family comprises a group of largely homologous proteins which, through interactions with themselves and with other molecules intimately involved in the regulation of intracellular signal transduction, regulates a common downstream checkpoint in the effector pathway of apoptosis. Bcl-2 is the prototype molecule of the class; in most circumstances it inhibits apoptosis and, therefore, promotes growth without directly increasing proliferation. Recent advances in our understanding of this growing class of molecules have emphasized the complex nature and effects of the interactions of Bcl-2 and its related molecules (24). Exactly which members of the Bcl-2 family are important functional consequences. Calorie restriction is a well accepted method of reducing the incidence of cancer, as well as of cells in culture (29). The rate of apoptosis may have important functional consequences. Calorie restriction is a well-accepted method of reducing the incidence of cancer, whether induced by carcinogens (30) or occurring sponta-

---

Table 1. Crypt-Villus Distribution of Apoptotic Cells in the Small Intestine of Young and Aging Rats

<table>
<thead>
<tr>
<th></th>
<th>Ad Libitum Mean ± SE</th>
<th>Calorie Restricted Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Aging</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Number CV cells</td>
<td>86.2 ± 1.8</td>
<td>91.4 ± 2.4</td>
</tr>
<tr>
<td>Apoptotic Index CV Portion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper 20%</td>
<td>13.4 ± 3.1</td>
<td>9.7 ± 2.0</td>
</tr>
<tr>
<td>Mid 60%</td>
<td>1.6 ± 0.6</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Lower 20%</td>
<td>0.8 ± 0.2</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Total CV Portion</td>
<td>3.62 ± 2.5</td>
<td>2.41 ± 0.9</td>
</tr>
</tbody>
</table>

Notes: Apoptotic index represents apoptotic epithelial cells as a percentage of total epithelial cells in each portion and in the whole crypt-villus (CV) column. Mean ± SE in 6–7 animals.
*2-way ANOVA: F between groups = 44.2, p < .0001. Aging calorie-restricted significantly higher than each other group (p = .0002 in each case) by multiple comparison of means (Newman and Keuls method).
†2-way ANOVA: F between groups = 17.6, p = .0004. Aging calorie-restricted significantly higher than each other group (p < .001 in each case) by multiple comparison of means (Newman and Keuls method).

Table 2. Effect of Calorie Intake Upon Apoptosis in the Proximal and Distal Colonic Crypt of Young and Aging Rats

<table>
<thead>
<tr>
<th></th>
<th>Young Mean ± SE</th>
<th>Aging Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ad Libitum</td>
<td>Calorie Restricted</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Number CV column</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>33.6 ± 0.3</td>
<td>33.6 ± 0.6</td>
</tr>
<tr>
<td>Distal</td>
<td>37.6 ± 1.0</td>
<td>38.3 ± 0.3</td>
</tr>
<tr>
<td>Apoptotic Index CV Portion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>9.8 ± 1.5</td>
<td>10.6 ± 1.0</td>
</tr>
<tr>
<td>Distal</td>
<td>10.9 ± 2.1</td>
<td>9.3 ± 1.0</td>
</tr>
</tbody>
</table>

Notes: Mean ± SE of 6–7 animals in each group. Apoptotic index represents apoptotic crypt epithelial cells as a percent of total crypt epithelial cells. Total crypt epithelial cell numbers were not significantly different between the groups shown, but proximal versus distal colon differences were significant within each group (p < .05) in each case.
*2-way ANOVA between groups F = 9.86, p = .003, p < .005 comparing aging calorie-restricted with all other groups by multiple comparison of means (Newman and Keuls method).
†2-way ANOVA between groups F = 2.39, p < .04, but combining colon segments F = 8.53, p = .0007. Aging calorie-restricted significantly (p < .005) higher than each group by multiple comparison of means (Newman and Keuls method).
neously as the rodent ages (31). The balance between apoptosis and cell production (proliferation) also is accepted as being crucial in the maintenance of organ cellular homeostasis (32). Apoptosis may provide a protective mechanism whereby DNA damaged or potentially neoplastic cells are selectively eliminated. Previous studies have shown that such DNA damaged cells occur in greater numbers in the gut of aging animals than in the young (33). Thus, interventions that increase the sensitivity of gut epithelial cells to apoptotic death might protect cellular stability with age. Reduced apoptosis occurs in a number of epithelial cancers, including the colon, which is a common site of spontaneous human cancer (20,34). Lower rates of apoptosis also have been detected in precancerous colonic lesions such as adenomatous polyps (21).

A previous study from this laboratory showed that the small and large intestines of aging rats had increased numbers of crypt cells (17), more proliferating cells, an increase in cell production rate (4,5), and a major expansion of the proliferative zone during the stress of a refeeding regimen (18). Calorie restriction retarded the onset of these changes to a later period in the life span of the rat (6). A delay in small bowel epithelial differentiation also was reversed by calorie restriction (12). Thus, calorie restriction not only may reduce cancer occurrence by lowering the rate of proliferation as we have argued (35), but also by enhancing the rate of apoptosis as shown in the present study.

ACKNOWLEDGMENTS

We appreciate the technical expertise of Dr. AbdelRahman Abdalla, Shaobai Wang, and Ms. Anne Washington, who contributed substantially to this project.

Address correspondence to Dr. Peter R. Holt, Chief, Gastroenterology Division, St. Luke's Roosevelt Hospital Center, Amsterdam Avenue at 114th Street, New York, NY 10025.

REFERENCES

20. Moss SF, Scholes JW, Holt PR. Abnormalities of epithelial apoptosis in multistep colorectal neoplasia demonstrated by terminal deoxynu-


Received August 4, 1997
Accepted November 25, 1997

---

**Erratum**

In Table 1 of “Changes in Hepatic DNA Binding Proteins as a Function of Age in Rats,” by Robin Walter and Felipe Sierra, which appeared in the March issue (Vol. 53A, No. 2, p. B105), there was an error in the title. It should have read: “Sequences of Synthetic Oligonucleotides.”