Short-Chain Fatty Acid Metabolism, Apoptosis, and *Apc*-initiated Tumorigenesis in the Mouse Gastrointestinal Mucosa[1]

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ABSTRACT

Short-chain fatty acids (SCFAs) are physiological regulators of growth and differentiation in the gastrointestinal tract, and we have previously shown that apoptosis induced in colonic cell lines by these compounds is dependent on their metabolism by *B*-oxidation in the mitochondria (B. G. Heerdt *et al.*, J. Biol. Chem., *266:* 19120–19126, 1991; Cancer Res., *54:* 3288–3293, 1994). Because tumors initiated by an inherited *Apc* mutation have been reported to be linked to decreases in apoptosis in the flat mucosa of the gastrointestinal tract, the aims were to determine whether elimination of efficient metabolism of SCFAs affected apoptosis in the gastrointestinal mucosa of the mouse, and whether this altered tumorigenesis initiated by an inherited *Apc* mutation. We, therefore, generated mice that have a chain-terminating mutation in the *Apc* gene and that were either wild-type for SCFA metabolism, or deficient, due to homozygous deletion of the gene (*Scad*) that encodes the enzyme short-chain acyl dehydrogenase, which catalyzes the first step in SCFA *B*-oxidation. $Scad^{+/+}$ mice maintained on a wheat bran-fiber-supplemented diet gained significantly more weight than mice maintained on AIN76A, but this was eliminated by the *Scad* mutation, demonstrating that uptake and metabolism of SCFAs in the gastrointestinal tract can be a significant energy source. As predicted, on either AIN76A or wheat bran diet, the *Scad* mutation almost completely eliminated apoptosis in the flat mucosa of the proximal colon and reduced apoptosis by 50% in the distal colon compared with littermates that were wild-type for *Scad*. The mutation also reduced apoptosis by approximately 50% in the duodenum in AIN76A-fed mice. These reductions in apoptosis had no effect on incidence, frequency, or site specificity of tumors initiated by the *Apc* mutation. Therefore, the metabolism of SCFAs by the gastrointestinal mucosa plays a role in modulating apoptosis, but a general decrease in apoptosis in the mucosa of the gastrointestinal tract is not linked to gastrointestinal tumorigenesis initiated by an inherited *Apc* mutation.

INTRODUCTION

SCFAs,[3] principally produced by the fermentation of dietary fiber, are present at concentrations over 100 mM in the lumen of the gastrointestinal tract (1, 2). They serve as the primary energy source for colonic epithelial cells, metabolized by *B*-oxidation in the mitochondria (3). SCFAs can induce differentiation, growth arrest, and apoptosis of colonic epithelial cells *in vitro* (4–10). They are also physiological modulators of these pathways *in vivo* in humans and rodents. This is illustrated by two observations: (*a*) in patients with diversion colitis, in which the absence of a fecal stream deprives the mucosa of exposure to SCFAs and other colonic contents, instillation of SCFAs into the colon by enema stimulates differentiation of

mucosal epithelial cells (11); and (*b*) total parental nutrition after intestinal resection is associated with intestinal atrophy and impaired function, but supplementation of parental nutrition in rats with SCFAs promotes intestinal adaptation by increasing basolateral intestinal nutrient transport (12).

Reports from our laboratory have established that the induction of differentiation, growth arrest, and apoptosis of colonic cells in culture by SCFAs is linked to their efficient metabolism (6, 7). Thus, we hypothesized that the elimination of efficient SCFA metabolism would interfere with this pathway and reduce apoptosis in the gastrotintestinal tract. Moreover, because it has been reported that such reductions in apoptosis characterize the mucosa in mice and in humans that inherit a mutant *Apc* allele (13, 14), we further hypothesized that this would provide a means of determining the contribution of altered apoptosis in the gastrointestinal mucosa to *Apc*-initiated tumorigenesis.

To test this hypothesis, we generated mice in which tumors were initiated by the inheritance of a mutant *Apc* allele but that were inefficient in their metabolism of SCFAs due to a homozygous deletion of the gene that encodes *Scad*, the first enzyme in the mitochondrial *B*-oxidation of SCFAs. Although the deletion of the *Scad* gene indeed had dramatic affects on the utilization of SCFAs as an energy source and apoptosis in the duodenum and proximal and distal colon, this had no effect on tumorigenesis. Moreover, although there were modest alterations in tumor formation in the jejunum in mice that were fed a normal or fiber-supplemented diet, there was no alteration in the frequency of apoptotic cells at this site. Therefore, the data strongly support the hypothesis that SCFAs, through their role as an energy source, play a role as modulators of apoptosis in the gastrointestinal tract. However, coupled with reports from our laboratory and others that there are no alterations in apoptosis in the $Apc1638N^{+/-}$ mouse in which an inherited *Apc* mutation leads to gastrointestinal tumorigenesis, the data do not support a role for a general decrease in apoptosis in the mucosa in initiating tumorigenesis by a mutant *Apc* allele.

MATERIALS AND METHODS

Mice. Apc1638N mice have a targeted, chain-terminating mutation in the *Apc* gene at codon 1638 and have been bred onto a constant B6 background (15, 16). To breed the *Apc*1638N mice, each cage consisted of two female $Apc1638N^{+/-}$ mice and a male $Apc1638N^{+/-}$ mouse. Genotyping was done on tail DNA by a PCR assay as described previously (15).

The BALB/cByJ strain, obtained from The Jackson Laboratory, has a homozygous deletion of the gene that encodes short-chain acyl dehydrogenase (17). This locus was termed *Bcd-1*, and the gene has more recently been referred to as *Scad* (18). $Scad^{-/-}$ mice are deficient in SCFA metabolism (18, 19). These mice were mated with $Apc1638N^{+/-}$ mice to generate offspring that were $Apc^{+/-}$, $Scad^{+/-}$. Crosses between these animals then generated litter mates that were $Apc^{+/-}$ and either $Scad^{+/+}$ or $Scad^{-/-}$. DNA from tail clips were analyzed for the presence of the *Apc* and *Scad* mutations, as described (15, 20), and the mice were randomized to different dietary groups. Mice were killed after 36 weeks on the AIN76A diet or on this diet supplemented with 120 g/Kg hard red wheat bran (Harlan-Teklad, Madison, WI). The wheat bran

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was added at the expense of cellulose in the diet. The entire gastrointestinal tract was analyzed for tumors as described previously (15, 16).

Mice were maintained under the supervision of a veterinarian, and experimental protocols were approved by the Institutional Animal Care and Use Committee in accord with NIH guidelines for the use and care of animals.

Apoptosis and Proliferation. Animals were killed by CO_2 inhalation, and the GI tract was rapidly dissected. Portions of the intestinal tract were placed in 10% formalin for no longer than 24 h. The tissues were then transferred to 80% and then 95% ethanol, each for 18–24 h, before embedding in paraffin. Sections were cut at 4-μm thickness. Apoptotic cells were evaluated by TUNEL using the kit from Trevigen (Gaithersburg, MD). Briefly, we adhered to the following protocol: formalin fixation was strictly limited to no more than 24 h; after deparaffinization and rehydration of the sections, proteinase K digestion was limited to 10 min; the sections were immersed in 3% H_2O_2 in 40% methanol for no more than 5 min to quench endogenous peroxidase activity. After incorporation of biotin-labeled dUTP into 3′ ends of fragmented DNA, and complex formation with streptavidin-horseradish peroxidase, reaction with freshly prepared diaminobenzidine for color development was done in the presence of 0.4 mM $MnCl_2$ for no more than 10 min. Sections were finally counterstained lightly with methyl green. For the detection of PCNA, a kit from Zymed (South San Francisco, CA) was used, according to the manufacturer's protocol. The scoring of both TUNEL- and PCNA-positive cells was done at ×400.

For each animal, at least 500 cells were scored in well-oriented crypts and villi of the proximal and distal colon and of the duodenum. For colonic sections, data are expressed as percent of crypt cells positive, whereas for duodenum (and jejunum), it is the percent of total crypt and villus cells that are positive.

RESULTS

In BALB/cByJ mice, the homozygous deletion of the *Scad* gene eliminates efficient mitochondrial B-oxidation of SCFAs (17–19). This mutation was introduced into *Apc*1638N$^{+/-}$ mice, which have a targeted, chain-terminating mutation in the *Apc* gene. On AIN76A diet, weight gain for the *Scad* $^{-/-}$ mice was initially slightly faster than for *Scad* $^{+/+}$ mice, but by 24 weeks of age, body weights in the two groups were equivalent and remained so until the mice were killed at 36 weeks (Fig. 1). In contrast, *Scad* $^{+/+}$ mice fed a diet high in wheat bran fiber, which provides a potential energy source from fermentation of this fiber to SCFAs, gained weight at a significantly faster rate and reached a higher body weight (Fig. 1; $P < 0.003$). However, animals that were *Scad* $^{-/-}$ (*i.e.,* deficient in SCFA metab-

Fig. 2. An example of PCNA and TUNEL staining to detect proliferating and apoptotic cells, respectively. Tissue was taken from the proximal colon of an *Apc*$^{+/-}$, *Scad*$^{+/+}$ mouse after 36 weeks on AIN76A diet, fixed in formalin, embedded in paraffin, and sectioned at 4 microns. *Arrows*, TUNEL-positive cells. Magnification, ×400.

olism) did not exhibit this additional weight gain on the high fiber diet. Thus, fermentation of dietary fiber to SCFAs by intestinal microflora, which are taken up and used by intestinal epithelial cells, can be a significant energy source, but this can be eliminated by a block to mitochondrial metabolism of these SCFAs.

TUNEL and PCNA staining were used to assay levels of apoptotic and proliferating cells in the mucosa of these mice at 36 weeks on diet. Fig. 2, an example of stained sections from the proximal colon of an *Apc* $^{+/-}$, *Scad* $^{+/+}$ mouse, clearly shows that the incidence of apoptotic cells in the mucosa is low, in agreement with published work on completely wild type animals (21–25). Fig. 3 (*top*) demonstrates the effect of homozygous deletion of the *Scad* gene on apoptosis in the gastrointestinal tract for the animals fed the AIN76A diet. The percent of apoptotic cells was reduced by 98% in the proximal colon ($P < 0.006$), and by approximately 50% in the duodenum and distal colon of *Scad* $^{-/-}$ mice compared with littermates which were wild-type for *Scad*. The greater effect of the elimination of SCFA metabolism on apoptosis in the proximal colon than in either the duodenum or distal colon was consistent with the fact that fiber fermentation to SCFAs takes place in the colon, and that SCFAs levels are significantly higher in the proximal colon than at these other sites (1, 2). Hence, intestinal epithelial cells in different regions of the gastrointestinal tract have adapted differently to environments in which they function.

Fig. 3 (*middle*) illustrates that there were no significant alterations in proliferation in the mucosa to compensate for the decreases in apoptosis. Thus, as shown in Fig. 3 (*bottom*), in mice fed the AIN76A diet, the ratio of apoptotic to proliferating cells was reduced in all regions of the gastrointestinal tract by the *Scad* mutation, with apoptotic cells essentially absent from the proximal colon.

Fig. 1 illustrated that mice fed the high fiber diet could use the SCFAs generated in the colon as an additional energy source, and that this was eliminated by the *Scad* mutation. Similar to the mice fed the basal AIN76A diet, mice fed the wheat bran diet showed the same pattern of reduction of apoptosis in the colon in the absence of ability to metabolize SCFAs. Apoptosis was again reduced by >80% ($P < 0.03$) in the proximal colon, with a smaller reduction that was not significant ($P < 0.10$) in the distal colon (Fig. 4). In contrast to the basal diet, there was no reduction in apoptosis in the duodenum for the mice on the wheat bran diet (Fig. 4).
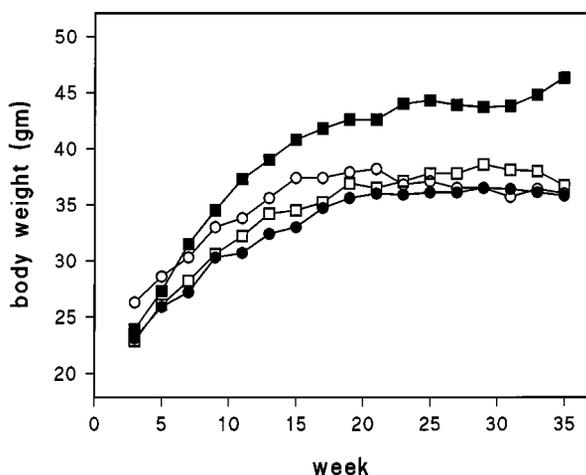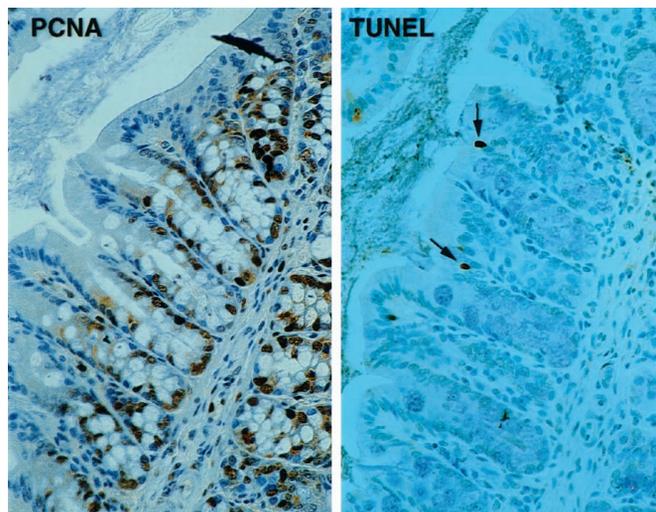

Fig. 1. Weight gain of *Scad*$^{+/+}$ and *Scad*$^{-/-}$ mice. Mice of the two genotypes were fed AIN76A, or diet supplemented with wheat bran, *ad libitum* after weaning, and weights were recorded weekly. ●, ■, *Scad*$^{+/+}$, AIN76A or wheat-bran-supplemented diet, respectively; ○, □, *Scad*$^{-/-}$, AIN76A or wheat-bran-supplemented diet, respectively. Values shown are means of all of the animals (approximately 16) in each group.

The inheritance of a mutant *Apc* allele in the mouse has been reported to decrease apoptosis in the gastrointestinal tract, and this is postulated as a mechanism by which *Apc* initiates tumor formation. Therefore, we asked whether the reduction in apoptosis associated with the homozygous deletion of the *Scad* gene altered tumorigenesis in these mice, which were all $Apc^{+/-}$.

The data in Table 1 demonstrate that for the mice on the AIN76A diet, the elimination of SCFA metabolism, and hence significant reduction in apoptosis in the mucosa, did not have an effect on tumor incidence or number. Further, Fig. 5 (*top*) illustrates that the homozygous deletion of the *Scad* gene did not change the site specificity of tumor formation in the gastrointestinal tract initiated by the *Apc* mutation. As previously reported, the majority of tumors in the $Apc1638N^{+/-}$ mice were in the small intestine, principally in the duodenum (15, 16), and tumor incidence was not affected by the decrease in apoptosis in the duodenum associated with the homozy-
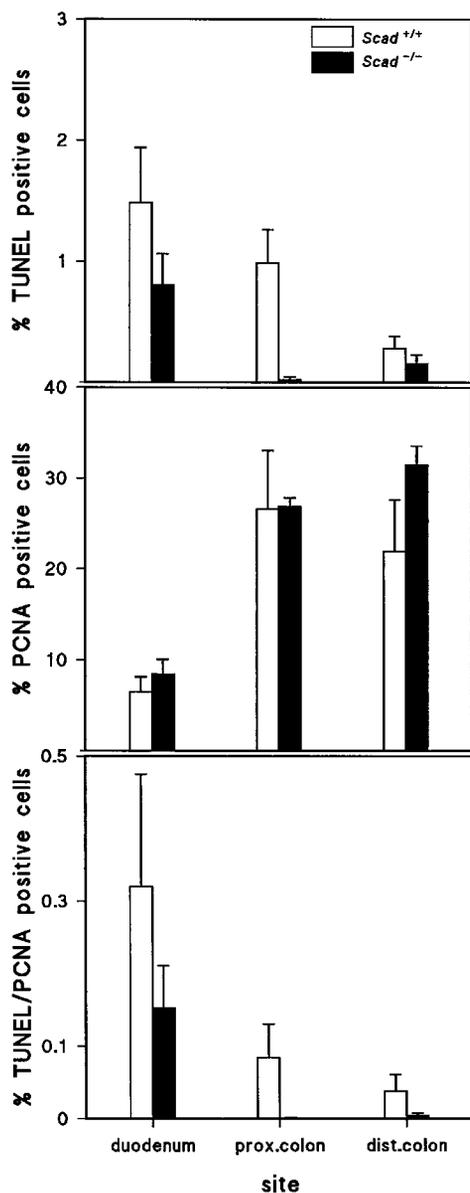

Fig. 4. Apoptosis in the gastrointestinal tract of $Apc1638^{+/-}$, $Scad^{+/+}$ or $^{-/-}$ mice at 36 weeks of age maintained on wheat-bran-supplemented diet. Data were generated as for Fig. 3.

Table 1 *Tumor incidence and number*

	AIN76A		AIN76A + wheat bran	
	$Scad^{+/+}$	$Scad^{-/-}$	$Scad^{+/+}$	$Scad^{-/-}$
Mice with GI[a] tumors	71% (10/14)	75% (9/12)	86% (12/14)	93% (14/15)
Tumors per mouse	2.0 ± 3.1	2.1 ± 2.7	2.6 ± 2.1	5.5 ± 5.9

[a] GI, gastrointestinal.

gous deletion of the *Scad* gene. Most important, however, was that the nearly complete elimination of apoptotic cells in the proximal colon by the *Scad* mutation (Fig. 3), did not cause tumors to form at this site, although there was an initiating *Apc* mutation (Fig. 5 (*top*)).

For animals fed the wheat bran supplemented diet, there was an increase in the number of tumors per mice for those with the $Scad^{-/-}$ mutation compared with wild-type *Scad*, although this was not statistically significant (Table 1). Whereas there were some differences in tumor distribution between the wheat bran (5, *bottom*) and basal diets (5, *top*), the distributions were similar. As for the basal diet, for wheat bran, the majority of the tumors were in the small intestine. However, in the wheat bran group a few tumors appeared in the cecum and a single tumor in the colon.

For both diets, there were somewhat more tumors in the jejunum for the $Scad^{-/-}$ group compared with the wild type *Scad* mice. We therefore investigated whether there were alterations in apoptosis at this site. Fig. 6 illustrates that the percent apoptotic cells in the jejunum was not altered by the *Scad* mutation in either diet group.

It should be noted that the mice were derived from crosses between $Apc1638N^{+/-}$ (B6) × $Scad^{+/-}$ (BALB/cByJ). Littermates which were $Scad^{+/+}$ or $Scad^{-/-}$ were used to equalize the genetic background contributed by the B6 × BALB/cByJ mating. Further, both *Scad* groups on the AIN76A diet exhibited the same incidence, frequency and distribution of tumors as reported previously for the $Apc1638N^{+/-}$ mice on a homogeneous B6 genetic background (16). There are no reported data regarding tumorigenesis in the *Apc*1638N mice on the wheat bran diet. However, in other experiments using a diet high in fat and phosphate and low in calcium and vitamin D, our $Apc^{+/-}$ mice that were either $Scad^{+/+}$ or $Scad^{-/-}$ again exhibited the same tumor formation as the inbred *Apc*1638N mice on the same diet (26).[4] There is, therefore, no evidence for contribution of an unlinked modifier locus to these results.

Fig. 3. Proliferation and apoptosis in the gastrointestinal tract of $Apc1638^{+/-}$, $Scad^{+/+}$ or $^{-/-}$ mice at 36 weeks of age. Mice were genotyped at the *Apc* and the *Scad* loci and, after weaning, were maintained on AIN76A diet. Proliferation and apoptosis were determined by staining for PCNA and by use of the TUNEL assay, respectively (*top, middle,* and *bottom*). Data are the mean ± SE of 6 mice per group.
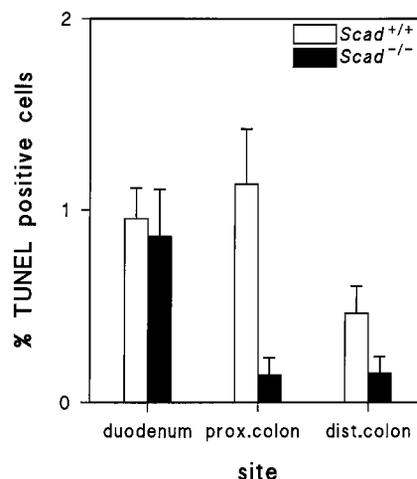
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[4] Unpublished data.

DISCUSSION

There are three important conclusions from the data presented:

(*a*) the data demonstrate that SCFAs derived from fermentation of fiber can be a significant energy source. This is concluded from the increased weight gain in the mice fed the wheat-bran-supplemented diet and the fact that the weight gain was eliminated by the *Scad* mutation, which prevents efficient SCFA metabolism;

(*b*) frequency of apoptosis in the gastrointestinal tract is influenced by SCFA metabolism. The elimination of efficient *B*-oxidation of SCFAs in the gastrointestinal tract of mice by homozygous deletion of the *Scad* gene (17–19) almost completely eliminated apoptosis in the proximal colon and decreased apoptosis in the distal colon in mice fed either AIN76A or a wheat-bran supplemented diet. Decreased apoptosis in the duodenum was also seen in the mice that were fed AIN76A. This supports our hypothesis, based on extensive work we have published elsewhere (6, 7), that the efficient metabolism of SCFAs is necessary for their stimulation of an apoptotic pathway in colonic epithelial cells *in vitro*. It also supports the conclusion from the work of others that pathways induced by SCFAs *in vitro* also function *in vivo*, through the role of SCFAs as the principal energy source for colonic epithelial cells (3–12). Thus, metabolic activity of the cell can have an important influence on frequency with which cells enter and complete an apoptotic pathway;

(*c*) the frequency of apoptosis in the mucosa of the gastrointestinal tract is not directly related to the frequency of tumor formation due to an inherited mutation of the *Apc* gene. This is demonstrated by the fact that the reduction of apoptotic cells in the duodenum of mice on the AIN76A diet did not increase tumor formation at this site and, most
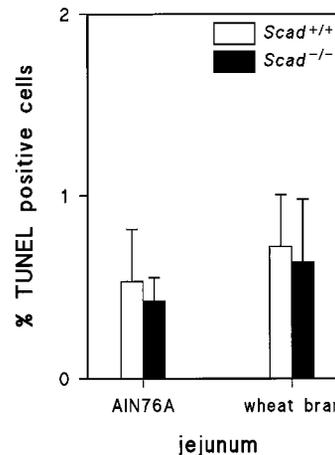

Fig. 6. Apoptosis in the jejunum of *Apc*1638$^{+/-}$, *Scad*$^{+/+}$ or $^{-/-}$ mice at 36 weeks of age maintained on either AIN76A or wheat-bran-supplemented diet. Data were generated as for Fig. 3.

important, that the almost complete elimination of apoptotic cells in the proximal colon in mice fed AIN76A or wheat-bran diet was insufficient to produce tumors at that site. Moreover, although there were modest alterations in tumor formation in the jejunum in the *Apc*-initiated *Scad* mutant mice on either diet, there was no change in the frequency of apoptotic cells at this site.

There are conflicting data in the literature regarding whether an inherited *Apc* mutation leads to tumor formation through an alteration in frequency of apoptosis. Consistent with the work of others (21–23), we have reported that the percentage of apoptotic cells in the gastrointestinal mucosa of the mouse is low, as illustrated in Figs. 2–4. At 15 weeks of age, approximately 4% of the cells in the duodenum, 2% in the proximal colon, and 1% in the distal colon are detected as apoptotic when scored by TUNEL assay done as described in "Materials and Methods." These values decrease with age and are not altered by the presence of the *Apc* mutation in *Apc*1638N mice (24, 25). This low incidence of apoptotic cells is similar to values reported using morphological criteria in the mouse (21) and rat (23) and using TUNEL in the rat (22). Moreover, in Apc*1638* mice, we score a similar incidence of apoptosis, and lack of change in apoptosis, in the gastrointestinal tract compared with control, whether apoptotic cells are recognized by TUNEL assay (24, 25) or by morphology in H&E-stained sections (27).

It was recently confirmed that the inherited *Apc* mutation in *Apc*1638N does not alter apoptosis in the small intestine (28). However, that report also reiterated previous work from the same group that, in contrast, *ApcMin*$^{+/-}$ mice do exhibit a decrease in apoptosis (14, 29, 30). This was attributed to a dominant-negative effect of the *Apc* mutation in *ApcMin* and the absence of such an effect of the different mutation in *Apc*1638N(28). Moreover, it was suggested that this dominant-negative effect on reducing apoptosis in the mucosa of *ApcMin* was the cause of the much greater number of tumors that form in *ApcMin* compared with *Apc*1638N (28). There are three critical points, however, that should be noted regarding this conclusion. First, the data presented here demonstrate that reduction of apoptosis in the duodenum and nearly complete elimination of apoptosis in the proximal colon were insufficient to increase tumor formation in the mouse gastrointestinal tract. Therefore, because *ApcMin* and *Apc*1638N differ by more than an order of magnitude in frequency of tumor formation, the two strains must differ in fundamental aspects affecting tumorigenesis other than affects on apoptosis. Second, the *Apc*Δ^{716} mouse has a mutation in codon 716 of *Apc*, similar to the position of the mutation at codon 850 of *Apc* in *ApcMin* (31, 32), and both *Apc*Δ^{716} and *ApcMin* exhibit much higher levels of tumor formation than does
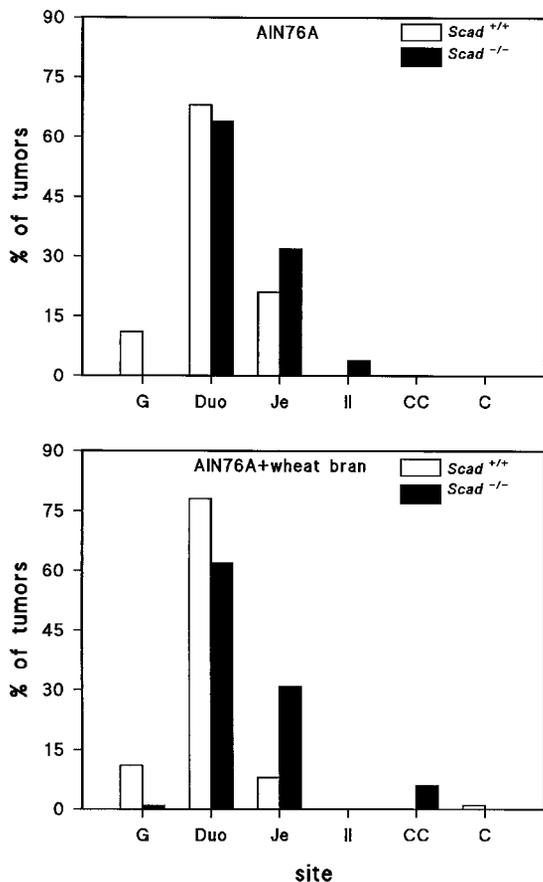

Fig. 5. Site distribution of tumors in the gastrointestinal tract of *Apc*1638$^{+/-}$, *Scad*$^{+/+}$ or $^{-/-}$ mice at 36 weeks on AIN76A (*top*) or wheat-bran-supplemented diet (*bottom*). G, gastric; *Duo*, duodenum; *Je*, jejunum; *Il*, ileum; *CC*, cecum; *C*, colon.

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*Apc*1638N. However, evidence has been reported that tumor formation in *Apc*Δ^{716} always involves loss of the wild-type *Apc* allele in even the smallest microdissected tumors, and that the Δ^{716} mutation has no dominant-negative effect (31, 32). Thus, the data suggesting that there is a decrease in apoptosis in the flat mucosa of *ApcMin* mice due to a dominant-negative effect of the inherited mutant allele should be examined. In this regard, publications from the group that suggest a dominant-negative affect in *ApcMin* on apoptosis reported levels of apoptosis in control animals of 35–50% (14, 29, 30). This is an order of magnitude higher than that reported by others and is in contrast to lower levels that they have recently reported (28). Moreover, such extensive apoptosis is not a feature of the histology of the gastrointestinal tract and is difficult to reconcile with the normal function of the mucosa and with the growth and development of the animals. This discrepancy has not been addressed.

The data presented raise one final point. A recent large epidemiological study has demonstrated that individuals consuming higher amounts of vegetable fiber exhibited a statistically significant increase in gastrointestinal tumors (33). It is interesting that in our study, wheat-bran fiber increased tumor formation in these genetically initiated mice but only in those animals incapable of *B*-oxidation of SCFAs (*i.e., Scad* mutant mice). We have found that levels of mitochondrial gene expression in rectal biopsies vary over a wide range in the human population, but that the level is stable for each patient when assayed in serial biopsies taken over six months.[5] If this reflects altered mitochondrial function, then one might speculate that the epidemiological linkage of dietary fibers and, potentially, other dietary constituents to tumorigenesis would be stronger if one factored in mitochondrial function as a variable.

In summary, we conclude that SCFAs derived from fiber fermentation in the gastrointestinal tract are an additional energy source, and that alterations in SCFA metabolism can substantially modulate levels of apoptosis in the gastrointestinal tract, especially in the proximal colon, the site at which these compounds are produced by microbial fermentation and at which they are at highest concentration. However, these reductions in apoptosis did not alter tumorigenesis initiated by an inherited *Apc* mutation. This finding and the lack of alteration in apoptosis in the gastrointestinal mucosa in *Apc*1638N$^{+/-}$ mice compared with control do not support the hypothesis that *Apc* initiates tumorigenesis by causing a general decrease of apoptosis in the mucosa.

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[5] L. H. Augenlicht and P. Holt, unpublished data.

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Short-Chain Fatty Acid Metabolism, Apoptosis, and *Apc* -initiated Tumorigenesis in the Mouse Gastrointestinal Mucosa

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