

Calorie Restriction and Diet Composition Modulate Spontaneous Intestinal Tumorigenesis in *Apc*^{Min} Mice through Different Mechanisms¹

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Abstract

We evaluated the effects of diet on intestinal tumorigenesis in male *Apc*^{Min} mice by comparing AIN-76A diet fed *ad libitum* (CON); calorie intake restricted by 40% of the CON (CR); diet high in olive oil and supplemented with freeze-dried fruit and vegetable extracts (OFV); and diet high in total fat (HF). Compared with CON, the frequency of intestinal polyps was reduced by 57% by CR ($P < 0.001$) and by 33% OFV diet ($P = 0.04$). Both effective interventions reduced total body weight, lean mass, and fat mass and increased daily urinary corticosterone output, but only CR reduced serum insulin-like growth factor I and leptin. We conclude that dietary interventions can partially offset genetic susceptibility to intestinal carcinogenesis.

Introduction

Several diet-related factors, including obesity, low fruit and vegetable intake, and high red meat intake, have been associated with an increased risk of developing cancers of the colon and/or rectum in humans (1). However, it remains unclear why a reduction in caloric intake or a change in diet composition to a regimen that includes more fruits and vegetables can decrease colorectal carcinogenesis. One experimental approach for developing and characterizing colorectal cancer prevention strategies is to use relevant animal models such as *Apc*^{Min} mice to identify interventions that lower intestinal polyp burden and slow the process of intestinal carcinogenesis. *Apc*^{Min} mice have a mutation in the murine homologue of the *APC*³ gene (2) that is also mutated in humans with a genetic predisposition to develop multiple intestinal adenomas such as familial adenomatous polyposis syndrome; *APC* mutations also frequently occur early in many spontaneously arising colorectal cancers (3). Excessive total caloric intake, often because of consumption of a high-fat diet, results in increased body weight and concomitant changes in hormone levels, including IGF-I, leptin, and corticosterone, that might also be involved in carcinogenesis (4–6). Thus, we tested the effects of CR and diet composition on intestinal polyp burden in *Apc*^{Min} mice and investigated the associations between intestinal carcinogenesis, body composition, and levels of IGF-I, leptin, and corticosterone.

Materials and Methods

Animals and Treatment Regimens. Six-week-old male C57BL/6J-*Apc*^{Min} mice (*Apc*^{Min} mice) were obtained from the Jackson Laboratory (Bar Harbor, ME). Upon receipt, mice were randomized and housed individually in quarantine for 3 weeks, after which the animals were transferred to the main National Cancer Institute-Frederick specific pathogen-free animal facility. Animal care was provided in accordance with the procedures outlined in the “Guide for the Care and Use of Laboratory Animals.” Two batches of 60 animals from the same breeding colony (4 weeks apart) were divided randomly into four dietary treatment groups, 30 mice/group: (a) CON; (b) 40% CR; (c) OFV, and (d) HF. Pelleted AIN-76A diet was used as the CON diet or was modified for the other dietary regimens (Table 1). The CR diet was formulated such that the reduction in calories was entirely from carbohydrates, with all other components isonutrient relative to the CON group when administered in daily aliquots equivalent to 60% of the average daily intake of the CON mice. CON, OFV, and HF mice were fed their respective diets for a total of 9 weeks, including the time in quarantine. CR mice were fed the CON diet during quarantine, then switched to the CR diet and restricted by 40% after week 3 of the study. Diets were manufactured by Bio-Serv, Inc. (AIN-76 CON and CR; Frenchtown, NJ), and Research Diets, Inc. (OFV, HF; New Brunswick, NJ). The freeze-dried fruit and vegetable mix was supplied by Van Drunen Farms, Inc. (Mokena, IL).

The 15 HF mice from the first batch that were administered a diet containing (w/w) 10% lard and 5% corn oil became moribund and had to be sacrificed after 4 weeks on the diet. This was not a gene-diet interaction, as 4 wild-type C57BL/6J mice (included in the study as a sentinel group) on the same diet exhibited similar toxicity symptoms. Thus, for the second batch of mice ($n = 15$), we modified the HF diet to make corn oil the sole source of fat (15% w/w, Table 1). One mouse in the second batch of the HF group and 2 mice in the CR group that became moribund after 4 weeks on the intervention did not exhibit any apparent intestinal lesions and were excluded from the analysis. Mice were kept on a reverse 12-h dark (10:00–22:00)/light (22:00–10:00) cycle and provided with access to acidified distilled water *ad libitum*. Food intake and body weights were monitored weekly, and mice were observed daily for signs of ill health. Daily urine output was collected for 2 days from each mouse in metabolic cages during week 5 of the intervention.

Necropsy and Tumor Enumeration. Mice were sacrificed after 9 weeks on the intervention by CO₂ inhalation in accordance with current NIH guidelines. Blood was collected, and serum was immediately frozen at -70°C . The entire gastrointestinal tract was removed for dissection, but the stomach and cecum were omitted from the analysis because of their low tumor incidence. The small intestine was divided into four segments of approximately equal lengths, and the colon was left intact. All four intestinal segments were completely dissected using filter paper as a support. Each segment was opened longitudinally using scissors and then washed with Dulbecco’s PBS without calcium and magnesium (pH = 7) to remove and collect intestinal contents. Tumor enumeration was performed using a dissecting microscope. The smallest tumors scored by this method were 0.5 mm in diameter. Tumors were scored by size (<2 mm, 2–4 mm, and >4 mm) and location (duodenum, proximal jejunum, distal jejunum, ileum, proximal colon, and distal colon).

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³The abbreviations used are: APC, adenomatous polyposis coli; IGF-I, insulin-like growth factor I; CR, calorie restriction; CON, *ad libitum*-fed control diet; OFV, olive oil, fruit, and vegetable; HF, high fat.

Table 1 Composition of control and intervention diets

Values are in grams, except for kcal from fat, which is given in percentages.

| Ingredient (g) | CON | CR ^a | OFV | HF |
|----------------------------------|------|-----------------|-------|------|
| Casein, 80 Mesh | 200 | 335 | 200 | 200 |
| DL-methionine | 3 | 5 | 3 | 3 |
| Cornstarch | 150 | 83 | 10.6 | 50 |
| Maltodextrin 10 | 0 | 0 | 100 | 100 |
| Dextrose | 320 | 223 | 0 | 0 |
| Sucrose | 175 | 113 | 275 | 275 |
| Cellulose | 45 | 79 | 30.7 | 50 |
| Corn oil | 50 | 84 | 50 | 150 |
| Olive oil | 0 | 0 | 97.4 | 0 |
| Mineral mix AIN-76A | 35 | 58.5 | 35 | 35 |
| Vitamin mix AIN-76A | 10 | 16.5 | 10 | 10 |
| Choline | 2 | 3.5 | 2 | 2 |
| Fruit/Vegetable mix ^b | 0 | 0 | 87.5 | 0 |
| % kcal from fat | 11.8 | 19.9 | 35.0 | 35.0 |
| Total | 1000 | 1000 | 883.6 | 875 |

^a Diet was administered daily at 60% of the mean daily intake of the control group during the previous week.

^b Fruit/vegetable mix contains per 100 g: 12.5 g carrots; 12.5 g broccoli; 12.5 g spinach; 10 g tomato; 10 g kale; 10 g strawberry fiber; 10 g blueberry fiber; 7.5 g onions; 5 g brussel sprouts; 5 g garlic; 2.5 g parsley; and 2.5 g green tea extract.

Body Composition Analysis. Mouse carcasses were scanned with a GE Lunar PIXImus Dual-energy X-ray Absorptiometer (7). Three replicate scans of each mouse (heads excluded) were made on thawed carcasses ($n = 13$ – 15 /group; all from batch 2 of the experiment).

IGF-I, Leptin, and Corticosterone Analysis. Serum levels of IGF-I were analyzed with a RIA kit from Diagnostic Systems Laboratories, Inc. (Webster, TX), serum leptin levels with an ELISA kit from R&D Systems (Minneapolis, MN), and urinary corticosterone levels with an RIA kit from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Two separate aliquots of serum or urine from each mouse were each analyzed in duplicate.

Statistical Analysis. All analyses were conducted using SAS, Inc. (Cary, NC). We first assessed the influence of CR and the two dietary interventions, as well as batch on the number of polyps using ANOVA performed with PROC GLM, after applying log transformations to the observed polyp counts. We also compared the treatment groups to the CON group based on Wilcoxon's rank-sum tests and the Kolmogorov-Smirnov tests that do not require normality of the observations with PROC NPAR1WAY. We also tested for interactions between batch and treatment assignment using both methods. The differences between the groups in body weight, lean and fat mass, bone mineral density, serum leptin levels, and urinary corticosterone output (log-transformed) were assessed using one-way ANOVA and Tukey-Kramer multiple comparison analysis. Because the serum IGF-I data did not meet the assumption of equal SDs required for ANOVA, even after log transformation, differences in serum IGF-I levels were evaluated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test.

Results

Relative to the CON group, mean body weights at the end of the study were significantly lower in the CR (75% of CON) and the OFV groups (83% of CON) and insignificantly higher in the HF group (108% of CON, Table 2). Mice in the CON and HF groups gained weight throughout the study. Mice in the CR group began to lose weight when they were restricted during week 3 of the study, whereas mice in the OFV group maintained their weight throughout the study. The differences in body weight in the CR and OFV groups, relative to

the CON group, were attributable to lower fat and lean mass and were associated with a reduction in calorie intake, which for the CR group was reduced by 40% by design, whereas the *ad libitum* calorie intake of the OFV mice was ~90% of CON intake (data not shown). Conversely, HF mice had marginally increased calorie intake, resulting in nonsignificantly higher lean and fat mass than CON mice (Table 2). No effect of treatment was observed for total bone mineral content, but bone mineral density was slightly (5.4%) lower in the OFV group compared with the CON group.

We observed significant effects with CR and, to a lesser extent, the OFV regimen on the numbers of intestinal polyps (Fig. 1). The CR group (3.8 polyps/mouse), relative to the CON group (8.8 polyps/mouse), displayed a ~60% reduction in polyp numbers ($P < 0.0001$; Wilcoxon's rank-sum test), whereas the OFV group (5.9 polyps/mouse) demonstrated a 33% reduction ($P = 0.04$; Wilcoxon's rank-sum test). No differences in the distribution of tumor numbers across the treatment groups were observed. The small increase in polyp numbers (10.4 polyps/mouse) in the HF group compared with the CON group was not statistically significant. ANOVA results indicated no interaction between batch and treatment effect. All of the above results were confirmed using the Kolmogorov-Smirnov test.

We also analyzed polyp frequencies by size and location in the intestine (Fig. 1). The average numbers of polyps >2 mm was 57% lower in the CR group ($P < 0.001$) and 33% lower in the OFV group ($P = 0.08$) compared with the CON. The average number of polyps in the colon, however, did not differ among the treatment groups (Fig. 1).

We analyzed urinary corticosterone output and serum IGF-I and leptin levels to determine their correlation with the observed differences in intestinal polyp formation. The CR and OFV groups exhibited significantly increased (~360% of CON) urinary corticosterone output levels (Table 3). However, relative to CON mice, only the CR group exhibited significantly decreased serum levels of IGF-I. A similar pattern was observed for leptin (Table 3); relative to CON mice, levels were decreased by 71% in the CR mice ($P < 0.05$) and by 25% in the OFV mice ($P = 0.46$), whereas in the HF group, leptin levels were significantly elevated (243% of CON; $P < 0.05$).

It should be noted that the OFV and HF groups contained the same levels of dietary fat and caloric density but differed in the type of fat and the fruit and vegetable content of the diet. The lack of a statistically significant difference in polyp numbers between the two groups may be partially attributable to reduced statistical power, resulting from the smaller sample size in the HF group ($n = 15$). However, other measures differed significantly between these two groups, including total body weight, lean mass, and fat mass (all $P < 0.05$, Table 2). In addition, relative to the HF group, the OFV group displayed lower serum leptin levels ($P < 0.01$, Table 3) and higher urinary corticosterone output ($P < 0.01$, Table 3).

Discussion

A majority of the effective interventions in *Apc*^{Min} mice currently described in the literature have focused on pharmacological agents or

Table 2 Body composition and bone characteristics of mice at necropsy

Values are mean (SD) total body weights, lean and fat mass, bone density, and bone mineral content for control and treated *Apc*^{Min} mice from batch 2 of the study. Values within a column that are statistically different ($P < 0.05$) are indicated by different superscripts.

| Treatment | <i>n</i> | Body Weight (g) | Lean mass (g) | Fat mass (g) | Bone Density (g/cm ³) | Bone Content (g) |
|-----------|----------|-------------------------|-------------------------|--------------------------|-----------------------------------|---------------------------|
| CON | 15 | 25.9 ^a (2.7) | 18.2 ^a (2.1) | 7.7 ^{a,b} (1.0) | 0.0498 ^a (0.0013) | 0.48 ^a (0.033) |
| CR | 13 | 19.4 ^b (1.0) | 13.6 ^b (0.9) | 5.8 ^c (0.7) | 0.0486 ^{a,b} (0.0010) | 0.46 ^a (0.042) |
| OFV | 15 | 21.5 ^b (3.5) | 15.2 ^b (2.4) | 6.3 ^{b,c} (1.3) | 0.0471 ^c (0.0016) | 0.45 ^a (0.024) |
| HF | 14 | 28.1 ^a (3.0) | 18.7 ^a (1.1) | 9.4 ^a (2.1) | 0.0482 ^{b,c} (0.0017) | 0.46 ^a (0.039) |

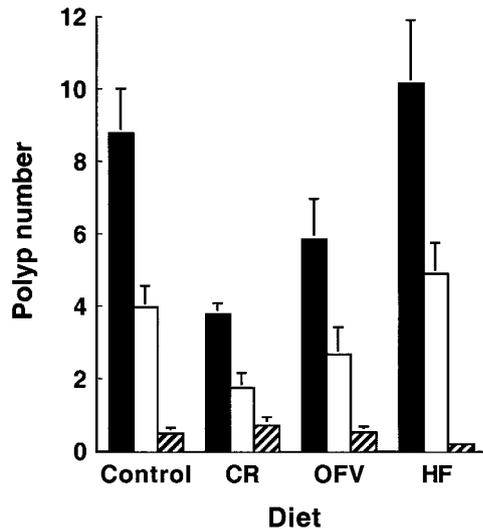


Fig. 1. Mean (\pm SE) polyp numbers in *Apc^{Min}* mice after 9 weeks of dietary treatment. Total polyps (■), total polyps > 2 mm (□), colon polyps (▨). Control ($n = 30$); CR [$n = 28$; P versus control (total polyps) <0.0001]; OFV [$n = 30$; P versus control (total polyps) = 0.04]; and HF = high corn oil [$n = 14$; P versus control (total polyps) = 0.5].

specific dietary compounds administered in hyperphysiologic dosages. This report adds CR, as well as a healthy diet, high in olive oil and a fruit and vegetable concentrate, to the growing list of effective interventions that significantly reduce polyp burden in *Apc^{Min}* mice (8). Previous studies have established the potential for a reduction of intestinal carcinogenesis in *Apc^{Min}* mice with various interventions, including nonsteroidal anti-inflammatory drugs (9), increased n -3 polyunsaturated fatty acid intake (10), and increased intake of plant polyphenolics (11) and (+)-catechins (12). Our findings of a protective effect of a diet high in fruits and vegetables are in contrast to an earlier observation of enhancement of polyp burden by the addition to a HF diet of a vegetable-fruit mixture in male and female *Apc^{Min}* mice (13). In that same study, increased fat content alone did not affect tumor multiplicity, which is in contrast to the findings of Wasan *et al.* (14). In our hands, a HF (corn oil) diet exerted a nonsignificant increase in polyp number. Differences in the composition of the vegetable-fruit mixture and/or the main fat source (lard/sunflower oil versus corn oil/olive oil) may have contributed to the different outcomes observed in these studies. Our vegetable-fruit mixture was designed to mimic a potential human diet, and the percent calories from fat in the OFV diet was matched to that of the HF diet, both representing the fat intake (\sim 35% of calories) that is commonly observed in human populations. Olive oil was included in the OFV diet because of its proposed beneficial health effects as a functional food (15).

The total numbers of polyps observed in our study are low compared with many other published studies with *Apc^{Min}* mice. We detected polyps 0.5 mm in diameter or larger; other studies have scored even smaller polyps, resulting in higher total polyp numbers. Recently, a modifier of the *Apc^{Min}* phenotype that results in significantly decreased polyp numbers (*Mom2*) has been described (16). However, any such modifier would not have affected the validity of our results because we randomized mice into the intervention groups upon arrival. Our finding that the distributions of polyp numbers across the treatment groups did not statistically differ (based on two-sample Kolmogorov-Smirnov tests) suggests that our randomization scheme equally distributed any putative differences in genotype among the groups. Another possible explanation for our apparent reduction in polyp numbers relative to other reports using *Apc^{Min}* mice is the fact that our mice were housed under specific pathogen-

free conditions. Bacterial infection has previously been shown to promote intestinal carcinogenesis in *Apc^{Min}* mice (17), but it is speculative to propose that the specific pathogen-free maintenance of our animals might have contributed to the lower polyp numbers.

Excessive body weight and the hormonal changes associated with it are increasingly appreciated as causes for human disease, including various cancers (18). CR, which prevents such excessive gain in body weight, has been consistently shown to reduce the number and severity of tumors in many cancer models (18). However, only one small study of the effects of CR in *Apc^{Min}* mice has been published and that study of moderate (20%) CR failed to show a reduction in the total number of intestinal polyps (19). In this study, 40% CR and, to a lesser extent, OFV diet significantly reduced the frequency of intestinal polyps in *Apc^{Min}* mice. Serum levels of IGF-I and leptin were significantly reduced in the CR group but not in the OFV group. However, both of the effective interventions were associated with lower body weights and higher production of corticosterone, an adrenal steroid hormone involved in regulating the effects of diet on body composition. Conversely, increased body weight and decreased corticosterone levels were observed in the HF group. Serum leptin levels, but not IGF-I levels, were also increased in response to the HF regimen. The complex interactions of corticosterone with other energy balance-related hormones and growth factors such as IGF-I and leptin in the anticancer effects of CR and other dietary regimens require further study (4, 18).

Although interventions that are effective in animal studies do not always translate into effective interventions in humans, various lines of evidence support the hypothesis that limiting excess body weight by reducing the amount of calories consumed and/or modulating the composition of the diet might offer realistic opportunities for prevention of human cancers. Colorectal cancers are the most frequent intestinal cancers in humans and often develop through precancerous polyps that are similar to the polyps that we observed in *Apc^{Min}* mice. Obesity is associated with a variety of human cancers, including colorectal cancer. Furthermore, it has been suggested that elevated serum levels of IGF-I and leptin are associated with increased risk of developing cancer (5, 6), whereas elevated corticosterone levels in experimental models are associated with decreased tumor development (4, 18). Thus, interventions aimed at lowering body weight, increasing adrenal steroid levels, and/or decreasing serum IGF-I and leptin levels may hold promise in slowing human colon carcinogenesis.

Importantly, lower serum IGF-I levels in the CR group were not associated with a significant reduction in bone mineral density, a potential problem for interventions that result in reduced IGF-I levels. Previous studies have found that CR, which consistently reduces serum IGF-I, is associated with reduced bone mineral density (20).

Table 3 Effect of diet on serum IGF-I and leptin levels and total daily urinary corticosterone output in male *APC^{Min}* mice

Mean values for each treatment group that are statistically different from each other ($P < 0.05$) are indicated by different superscripts. The samples analyzed for leptin and corticosterone were from batch 2 of the study.

| Hormone | Treatment group | | | |
|-------------------------|-------------------|--------------------|--------------------|--------------------|
| | CON | CR | OFV | HF |
| IGF-I (ng/ml) | 377 ^a | 291 ^b | 389 ^a | 366 ^a |
| \pm SD | ± 98 | ± 67 | ± 140 | ± 48 |
| (n) | (21) | (20) | (20) | (9) |
| Leptin (ng/ml) | 5.93 ^a | 1.73 ^b | 4.43 ^a | 14.39 ^c |
| \pm SD | ± 5.90 | ± 0.83 | ± 2.19 | ± 9.37 |
| (n) | (10) | (9) | (10) | (9) |
| Corticosterone (ng/day) | 29.7 ^a | 108.0 ^b | 108.4 ^b | 24.6 ^a |
| \pm SD | ± 16.6 | ± 26.8 | ± 46.9 | ± 8.1 |
| (n) | (6) | (6) | (6) | (6) |

The duration of the CR regimen in this study (6 weeks) may not have been long enough for the changes in bone density to occur. The OFV diet resulted in a slight (5.4%) but statistically significant decrease in bone mineral density, a surprising observation that needs to be confirmed in follow-up studies.

Our data extend the spectrum of malignant diseases inhibited by CR at a level that does not represent malnutrition. The CR mice in our study lost weight initially before stabilizing at a lower body weight than CON mice for the remainder of the study. CR in our experiments does not deprive the animals of calories needed for development but restricts overfeeding and excessive weight gain. Throughout the study, the CR mice appeared more active and alert than mice in the other groups, and their hematocrit levels at the time they were euthanized were higher than those in the other groups (data not shown). It is interesting to note that mice in the OFV voluntarily decreased calorie intake, albeit by only 10%, after 5 weeks on the diet, implying potential effects of the diet on satiety.

The effective interventions presented here are based simply on reducing the total calorie intake or changing the type of fat and fruit and vegetable content of the diet. Although it is possible that the effects seen in the OFV and the HF group are to a large extent attributable to changes in calorie intake, it is likely that antioxidants, fatty acids, and other active ingredients present in the diets also contributed. Although it would be appealing to establish single dietary agents that inhibit carcinogenesis such a magic bullet approach to the nutritional prevention of cancer is unrealistic given the complexity of dietary interactions. It is also unlikely that consumption of a single nutrient at pharmacologically effective levels can achieve positive anticancer effects without causing significant side effects. In contrast, the diet regimens used here, including a reduced calorie diet and a diet high in olive oil plus a variety of fruits and vegetables, are unlikely to cause side effects even in the long term. Furthermore, because of their simplicity, these no-cost or low-cost interventions could be readily applied to human populations, an important prerequisite for any effective population-based prevention regimen. Human intervention studies need to be designed to confirm the potential anticancer effects of energy balance modulating interventions and to characterize the potential mediating roles of diet-responsive hormones/growth factors such as IGF-I, leptin, and corticosterone, in cancer prevention.

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