

Food Restriction Inhibits the Growth of Intestinal Polyps in Multiple Intestinal Neoplasia Mouse

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The effect of food restriction (FR) on spontaneous intestinal carcinogenesis in multiple intestinal neoplasia (Min) mice was examined. Thirty male Min mice were allotted to *ad libitum* feeding control and 20% FR groups from six weeks of age until the end of the 13-week experimental period. Although the total number of visible intestinal polyps in the FR group was not significantly different from the control group value, a significant decrease in large-sized polyps (>2 mm) and an increase in small-sized polyps (≤2 mm) were observed in the distal small intestine. In this segment, the percentage of apoptotic cells counted in intestinal polyps in the FR group was significantly higher than in the control group, the percentage of proliferating cell nuclear antigen (PCNA)-positive cells not being significantly different. These results indicate that the FR may inhibit the growth of intestinal polyps in the Min mouse, and that apoptosis contributed in part to the inhibitory effect.

Key words: Food restriction — Min mouse — Colorectal carcinogenesis — FAP

Lifestyle encompasses many factors related to cancer.¹⁾ Many researchers have revealed that external factors, such as smoking, excessive alcohol consumption, and a high energy intake increase the risk of certain kinds of cancers.^{2–5)} In particular, the influence of the dietary food intake is of paramount importance.⁶⁾ An appropriate diet predisposes to a healthy life⁷⁾ while an imbalanced diet may lead to cancer development.⁸⁾

Restriction of food and/or calorie intake, accompanied by a decrease in body weight gain, generally exerts inhibitory effects on carcinogenesis in experimental animals,^{9–13)} reducing tumor incidence or tumor growth^{9–13)} by influencing cell division and/or apoptosis.^{10, 13)} Although the reports suggest that food and/or calorie restriction affects cancer development in most organs, there is little information available with regard to carcinogenesis in the gastrointestinal tract.

A preliminary study (unpublished data), which was conducted to analyze the effects of a colon carcinogen, the food-derived heterocyclic amine PhIP, on intestinal carcinogenesis of Min mouse, revealed the values for food intake and body weight to be decreased in the PhIP-fed group, and induction of intestinal polyps was significantly

inhibited as compared with the control group. These results suggested to us that decreased food intake might have an inhibitory effect on intestinal carcinogenesis in Min mouse. In this study, to test whether the link was causal we examined the effects of FR on polyp development in the Min mouse. In addition, we examined the cell kinetics, including apoptosis.

MATERIALS AND METHODS

Animal experiment Thirty male C57BL/6J-APC <Min>/+ mice were obtained from Jackson Laboratory (Bar Harbor, ME) at five weeks of age. These Min mice have a heterozygous nonsense mutation in codon 850 of the murine APC gene.¹⁴⁾ Five animals were housed per polycarbonate cage in an animal room controlled at 24±2°C with a 12 h light/dark cycle (lights on at 8:00 a.m.). The experiment started at six weeks of age, when the animals were allotted randomly to the *ad libitum* feeding control group and the FR group (15 mice each).

Twenty percent FR was employed as a modest and possibly effective dose,¹³⁾ and the treatment was continued throughout the 13-week experimental period. The diet was CE-2 pellets purchased from Clea Japan Inc. (Tokyo). Food consumption by the control group, with *ad libitum* access, was estimated by weekly weighing, from which we calculated the average daily food consumption. This average was multiplied by 0.8 and the resultant amount of food was fed to the 20% FR group throughout the next week. Tap water was given *ad libitum* to animals in both groups. Body weights were measured once a week.

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Abbreviations: ACF, aberrant crypt foci; APC, adenomatous polyposis coli; COX-2, cyclooxygenase-2; FAP, familial adenomatous polyposis; FR, food restriction; H&E, hematoxylin and eosin; IGF, insulin-like growth factor; Min, multiple intestinal neoplasia; PCNA, proliferating cell nuclear antigen; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.

Necropsy was carried out at the end of experimental week 13. Mice were anesthetized with diethyl ether and euthanized after blood samples had been collected for the determination of serum gastrin and IGF-I levels. Individual small and large intestines were removed, inflated and fixed with 10% neutral-buffered formalin.

Macroscopic and microscopic findings Macroscopically, the number of ACF, a marker for colorectal tumorigenesis, was estimated with methylene blue staining. The counting of visible intestinal polyps and the measurement of polyp diameter were performed separately for the proximal small intestine, distal small intestine, proximal large intestine and distal large intestine. The intestinal polyps were classified into two groups according to their diameter, ≤ 2 mm and > 2 mm.¹⁵⁾

Microscopic studies were performed with sagittal sections of formalin-fixed and paraffin-embedded tissues obtained from the four intestinal areas. These sections were routinely processed for paraffin embedding and sectioned at 3 μ m thickness. Histological diagnoses were determined in H&E-stained sections.

Immunohistochemical study Proliferating cells and apoptotic cells were detected with anti-PCNA antibody (DAKO Japan Co., Ltd., Kyoto) staining and an "ApopTag" detection kit (Intergen Co., New York, NY), respectively. PCNA immunohistochemical staining was done with a "VECTASTAIN" ABC kit (Vector Laboratories, Inc., Burlingame, CA). Since a significant difference in lesion numbers was only observed in the distal segment of the small intestine on macroscopic assessment, PCNA and "ApopTag"-positive cells were only counted in all of the adenomatous proliferative lesions in this segment. The numbers of total tumors available for PCNA-positive cell counting were 92 from the control and 114 from the 20% FR group, respectively, and the numbers of tumors for "ApopTag"-positive cell counting were 96 from the control and 117 from the 20% FR group, respectively. With PCNA-stained sections, the numbers of positive cells and negative cells were counted in a total of 100–200 cells. Since apoptotic cells were few in number, positive cells were counted in a total of 1000–2000 cells. In both cases, percentage values were calculated for comparisons.

Serum gastrin and IGF-I level Serum was obtained from blood collected at necropsy and frozen in -80°C . Ten randomly selected samples in both groups were used for the determination of serum gastrin and IGF-I levels by means of immunoradiometric assay in Mitsubishi-Kagaku Bio-clinical Labs, Inc. (Tokyo).

Statistical analysis All values were expressed as mean \pm SD. The statistical significance of differences in values for body weight, number of ACFs and intestinal polyps, and serum gastrin and IGF-I levels was determined using Student's *t* test with "Stat View-J" 4.02 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Body weight Average body weight data are shown in Fig. 1. The values for the FR group were significantly lower than those for the control group throughout the experimental period and showed almost 15% body weight gain inhibition at the end of the experiment (FR: 24.0 ± 1.7 g, control: 28.4 ± 2.7 g, as compared to initial value of FR: 22.3 ± 1.1 g, control: 22.4 ± 2.0 g).

No deaths occurred during the experimental period. However, because one animal in the control group showed a body weight decrease from the start of the experiment, it was not included in the analysis. The general condition of the other animals was healthy throughout the experimental period.

Intestinal carcinogenesis Regarding ACFs in the large intestine, the value for the FR group (0.1 ± 0.4) was lower than that for the control (0.4 ± 0.5), albeit without statistical significance.

Table I summarizes average number values of intestinal polyps in the small and large intestines. In the former, the incidence of intestinal polyps was 100% in both groups, and the total numbers of lesions were almost equal in the two groups for all segments. However, there was a significant decrease ($P < 0.01$) in the number of large-sized polyps (> 2 mm) with a concomitant increase in small-sized polyps (≤ 2 mm) in the distal segment, where polyps were most frequent. A similar but non-significant trend was apparent in the proximal small intestine. All the polyps seen on H&E staining were microscopically diagnosed as adenomas.

In the large intestine, the incidences of intestinal polyps in the FR and control groups were 53% (8/15) and 71% (10/14), respectively. While the total numbers of polyps were small as compared to those in the small intestine, the

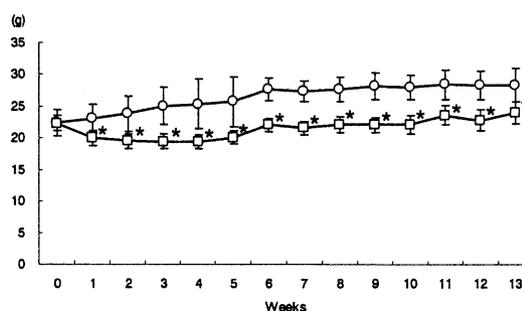


Fig. 1. Body weight changes. The mean values of body weight in the food-restricted group showed significant decreases compared with the control group throughout the experimental period, but the final mean value did not fall below the initial one. Open circles: control group. Open squares: FR group. *: $P < 0.01$ compared to the control group.

Table I. Numbers of Polyps in Small and Large Intestines

	Control (n=14)			FR (n=15)		
	Total	≤2 mm	>2 mm	Total	≤2 mm	>2 mm
Small intestine	55.2±28.4			51.5±13.8		
Proximal	11.1±6.3	2.5±1.7	8.6±5.6	8.5±4.1	3.1±2.0	5.4±3.9
Distal	44.1±25.2	11.4±4.1	32.7±22.7	43.0±11.0	25.9±7.6*	17.1±12.7*
Large intestine	2.3±2.6			1.1±1.6		
Proximal	0.9±1.0	0.1±0.3	0.8±0.9	0.3±0.6	0.1±0.3	0.3±0.5
Distal	1.4±2.3	0.5±0.9	0.9±1.4	0.7±1.4	0.2±0.6	0.5±0.9

Values are average±SD.

* P<0.01 compared with the control group.

Table II. Immunohistochemical Results, Serum Gastrin and IGF-I Levels

	Control (n)	FR (n)
PCNA ^{a)} (%)	89.4±9.7 (92)	88.2±10.0 (114)
Apoptotic cells ^{a)} (%)	0.5±0.8 (96)	1.0±1.5* (117)
Serum gastrin ^{b)} (pg/ml)	194.0±101.0 (10)	162.0±98.0 (10)
Serum IGF-I ^{b)} (ng/ml)	4.0±1.3 (10)	4.1±1.3 (10)

Values are average±SD.

a) Values are represented per tumors.

b) Values are represented per animals.

* P<0.01 compared to control group.

value for the FR group was again lower than that for the control group, although without statistical significance. Polyps were distributed almost uniformly in the proximal and distal segments and were all microscopically diagnosed as adenomas in H&E-stained sections.

PCNA and apoptosis analysis Table II summarizes indices for PCNA and “ApopTag”-positive cells in intestinal polyps of distal small intestine. The PCNA-positive indices showed no significant difference between the FR and control group. On the other hand, the “ApopTag”-positive index was significantly higher (P<0.01) in the FR group.

Serum gastrin and IGF-I levels The serum gastrin level was decreased in the FR group, but without statistical significance, while values for serum IGF-I level were almost equal in the two groups (Table II).

DISCUSSION

The present study demonstrated that FR inhibits the growth of polyps of the small intestine in Min mice, without any effect on multiplicity. A significant decrease in large-sized intestinal polyps was noted in the distal segment of the small intestine in the FR group, and a similar tendency was apparent in the other segments. Interestingly, the index of apoptotic cells was significantly increased in

polyps of the distal segment of the small intestine in the FR group, with no significant variation in PCNA indices. The results thus indicate that FR could inhibit the growth of intestinal lesions in the Min mouse, and that apoptosis might contribute in part to this inhibitory effect.

FR was earlier reported to reduce both the multiplicity and the incidence of colorectal lesions in F344 rats treated with azoxymethane,¹⁶⁾ and suppression of neoplasia in many organs has been documented.⁹⁻¹³⁾ Although these results suggest that FR might inhibit the growth, namely promotion as well as initiation, of colorectal cancer in azoxymethane-treated rat, the animal model is not sufficient for evaluation of the effect of FR on spontaneous colorectal carcinogenesis. Our present study indicates that the FR is effective for growth inhibition of spontaneous intestinal carcinogenesis in APC gene-mutated Min mouse.

An understanding of the pathogenesis of APC mutation-induced intestinal carcinogenesis in Min mouse is critical for interpretation of the results in the present study. The genetic characteristic of the Min mouse is a heterozygous nonsense mutation in the APC gene,^{14,17)} with additional mutation or loss of heterozygosity in the allelic APC gene readily leading to abnormalities of APC protein expression and/or function. Since APC plays an essential role in the degradation of β-catenin in the Wnt signal pathway,¹⁸⁾ abnormalities result in accumulation of β-catenin in the cell cytoplasm and translocation into the nucleus.¹⁹⁻²¹⁾ Many studies have revealed that this is critical for intestinal carcinogenesis in this model.^{17, 22-24)} Since the onset of intestinal carcinogenesis is genetically determined in Min mouse, it is plausible that the multiplicity of intestinal polyps was not significantly affected in the present study.

Numerous polyp growths in the small intestine compared with the other intestinal lesions are another characteristic of intestinal carcinogenesis of Min mouse.²⁵⁾ Although the mechanism remains unclear, this higher multiplicity may partly explain the observation of significant

growth inhibition in the distal segment of the small intestine of the FR group.

Indeed, there have been a number of reports suggesting that the inhibitory mechanisms of FR are related to the promotion phase of tumor development. Recently, Watson and Smith²⁶⁾ revealed a correlation between increase of serum gastrin levels and promotion of intestinal carcinogenesis in the Min mouse, this hormone acting as a growth factor for colorectal carcinogenesis,^{27,28)} with anti-apoptotic effects having been demonstrated *in vitro*.²⁹⁾ It is known that fasting can decrease the serum gastrin level,^{30,31)} in line with our present results.

Dunn and his collaborators¹³⁾ reported that FR modulates apoptosis and cell proliferation via a reduction of serum IGF-I level, suggesting that FR might be a potent inhibitor of neoplastic progression. However, the serum IGF-I level in the FR group was similar to that in control animals in the present study. Furthermore, no appreciable effects on cell proliferation were evident in polyps. However, since the effects of IGFs are mediated through the IGF-I receptor, and modulated by IGF-binding proteins,³²⁾ the situation is complex and no firm conclusion regarding the relationship between FR-induced inhibition of intestinal carcinogenesis in the Min mouse and serum IGF-I levels can be drawn at this stage. Further study is needed for clarification.

FR could exert an inhibitory effect on intestinal carcinogenesis of Min mouse via influence on the arachidonic acid cascade. Recently FR was proven to attenuate chemical-induced inflammatory reactions via an increase in the diurnal plasma glucocorticoid level.³³⁾ Colorectal cancers are widely accepted to be inflammation-associated,^{34–36)} and nonsteroidal anti-inflammatory drugs or specific COX-2 inhibitors have been shown to inhibit colorectal carcinogenesis in both humans^{37–40)} and APC gene-mutated animals.^{36,41–43)} The evidence suggests that actual growth

inhibition in the Min mouse might be effected by glucocorticoid physiological anti-arachidonic acid cascade factors.

Since clinical reports have revealed that genetic alteration in the APC gene may be a key factor in the human colorectal adenoma-carcinoma sequence,^{44,45)} non-polypoid colorectal tumorigenesis⁴⁶⁾ and FAP,⁴⁷⁾ the genetic abnormalities of the Min mouse make this model suitable for analysis of human colorectal carcinogenesis. Whether growth inhibition by FR is reproducible in human beings at high risk of colorectal cancer development is a possibility that warrants further consideration, especially as other benefits would be expected from a balanced diet, without any detrimental side effects. The key finding in the present study is that the modest 20% FR was enough to inhibit the growth of intestinal polyps in Min mouse without severe body weight decrease or abnormalities in general condition. Whether a further increase of FR dosage would be more effective is unclear, since severe FR might increase the risks for undesirable side effects.

In conclusion, FR may inhibit the growth of intestinal polyps in the Min mouse by a mechanism partly dependent on increase in apoptosis within polyps. We suggest that FR may have potential for inhibition of human colorectal carcinogenesis.

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