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Methionine Restriction Inhibits Colon Carcinogenesis

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Abstract: *Previously, we demonstrated that life-long methionine restriction (MR) in rats increases life span and inhibits aging-related disease processes. The present study examines the effects of MR on the formation of preneoplastic aberrant crypt foci (ACF) in the colon of azoxymethane (AOM)-treated rats. Six-week-old male F344 rats were placed on essential amino acid-defined diets containing either 0.86% Met (control diet) or 0.17% Met (MR diet) and 1 wk later were given AOM (15 mg/kg/wk, s.c.) for 2 consecutive wk. Ten weeks after the final AOM treatment, ACF formation was markedly reduced in rats fed the MR diet with ACF containing ≥4 crypts/focus being reduced by over 80% compared to controls (*$P < 0.001$*). A similar 83% reduction in ACF containing ≥4 crypts/focus was observed in rats fed the MR diet only during the post-initiation period (after the final dose of AOM;* $P < 0.001$*). Five weeks after AOM administration, a 12% reduction in colonic cell proliferation was observed in MR rats compared to controls (*$P < 0.05$*). These results show that MR inhibits colonic tumor development in the rat, an effect that occurs primarily during post-initiation phases of carcinogenesis and may be due, in part, to an inhibition of colonic cell proliferation.*

Introduction

Colon cancer, the second leading cause of cancer death in the United States, is progressively increased with age in humans (1) and experimental animals (2). The cause, however, of colon cancer and the increased incidence in old age are unknown. Although it has been suggested that the underlying mechanisms in aging and carcinogenesis are closely related, the nature of the relationship between the biological aging process and the development of neoplasia remains unclear (3,4). There is a need for well-designed studies with proper animal models to investigate and determine the mechanisms involved in carcinogenesis during aging (5).

Environmental factors such as nutrition are strongly related with cancer risk and are clearly involved in both initiation and post-initiation phases of the disease (6–9). Caloric restriction (CR), a well-accepted dietary means of increasing life span and delaying the aging process, reduces spontaneous as well as chemically induced tumors in rodents (10). Thus, CR represents an important experimental tool to study the role of potential age-related factors in cancer development. In studies on colon cancer development, CR prevented age-associated crypt hyperplasia (11) and inhibited the formation of carcinogen-induced preneoplastic lesions and tumors in the colon (12).

Previous studies demonstrate that another dietary regimen, methionine restriction (MR), substantially enhances longevity in Fischer 344 (F344) rats (13–16). Lifelong feeding of a diet containing only 0.17% Met, as the sole source of sulfur amino acid, compared to 0.86% Met in the control diets, resulted in increases in both mean (42%) and maximum (44%) longevity while maintaining a reduced body weight. Recently, a similar effect of MR was observed in (BALB/cJ × C57BL/6J)F1 mice (17). In F344 rats, MR was a potent inhibitor of chronic progressive nephropathy, the major cause of aging-related mortality, and testicular cancer, a common neoplasia in aged rats supporting the hypothesis that MR causes a delay in the biological aging process itself (18). Although these effects are similar to those reported for CR (19), it does not appear that MR is accompanied by a reduction in caloric intake, which could explain its anti-aging properties. Only minor decreases in energy intake per animal were observed in MR rats and overall intake per gram of body mass was substantially greater in MR animals (13,14). Pair-feeding experiments demonstrated that the increase in longevity could not be attributed to the slight decrease in calories consumed in MR rats (16). Thus, the effects of MR do not appear to be driven by a decrease in energy intake.

In the present study, we examined whether MR could also inhibit colon carcinogenesis by determining its effects on the formation of preneoplastic aberrant crypt foci (ACF) in the azoxymethane (AOM)-induced colon carcinogenesis rat model. This is a well-validated animal model for human colon cancer with the ACF formed in 8–10 wk and the subsequently developed tumors (34 wk) having close similarity in morphology, regional distribution, and histochemistry to those of humans (20–22). In addition, markers of cell proliferation were assessed as a possible mechanism of action for MR.

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Materials and Methods

Animals

Five-week-old male F344 rats were obtained from Taconic Farms (Germantown, NY) and maintained in quarantine for 1 wk prior to the initiation of the experimental protocol. Throughout the quarantine and experimental periods, all animals were housed in a conventional animal facility in groups of three in solid bottomed cages lined with wood chips, given free access to food (standard laboratory rodent chow) and acidified water, and maintained on a 12 h light/dark cycle, 50% humidity, and 21°C temperature. These animals were cared for according to the guidelines of the American Council on Animal Care. Body weights were recorded weekly.

Diets

Two experimental diets were used (control and MR). Both diets were chemically defined, based on the AIN-76 formulation with the protein replaced by an essential amino acid mixture as previously described (Table 1) (14). In the control diet methionine was supplied at the level of 0.86% (wt/wt) as the only source of sulfur amino acid, a level consistent with the Met content of AIN-76. The methionine content of the MR was 0.17% (wt/wt), a level known to enhance lifespan in these animals.

Carcinogen Bioassay

The experimental protocol is described in Fig. 1. One week after the acclimation period, at 6 wk of age, animals were randomly allocated into four diet groups as follows: 1) Control group (n = 12), which received the control diet throughout the entire experimental period; 2) MR group (n = 12), which received the MR diet throughout the entire experimental period; 3) MR-initiation group (n = 6), which received the MR diet during the carcinogen initiation phase of carcinogenesis (ages 6–8 wk) and control diet during the post-initiation phase (ages 8–18 wk beginning 3 days after the final AOM injection); 4) MR-post-initiation group (n = 6), which received control diet during the carcinogen initiation phase and MR diet during post-initiation phase (ages 8–18 wk beginning 3 days after the final AOM injection). Beginning at 7 wk of age, AOM (Ash Stevens, Detroit, MI) dissolved in sterile saline was administered to all rats in two weekly doses of 15 mg/kg body weight (*s.c.*; total dose of 30 mg/kg body weight). Five weeks after the final AOM treatment (at 13 wk of age), 6 rats from both the control and MR groups were sacrificed for analysis of colonic cell proliferation (described subsequently). The remaining 6 rats from each group were sacrificed 10 wk after the final AOM injection (at 18 wk of age) for analysis of colonic ACF (described subsequently).

Table 1. Composition of the Methionine-Restricted Diet

Ingredient	Concentration in Diet (%)	Ingredient	Concentration in Diet (%)
L-Arginine	1.12	L-Phenylalanine	1.16
L-Lysine	1.44	Glycine	2.33
L-Histidine	0.33	Dextrin	5.00
L-Leucine	1.11	Corn starch	43.61
L-Isoleucine	0.82	Sucrose	20.00
L-Valine	0.82	Solca floc	5.00
L-Threonine	0.82	Choline bitartrate	0.20
L-Tryptophan	0.18	Vitamin mix-AIN	1.00
L-Methionine	0.17 (0.86)[a]	Mineral mix-AIN	3.50
Glutamic acid	3.39 (2.70)[a]	Corn oil	8.00

a: Amino acid concentrations of control diet are given in parenthesis.

ACF Analysis

For ACF analysis, rats from each group (6/group) were sacrificed 18 wk of age by CO_2 euthanasia. The colons were removed and flushed with Kreb's Ringer salt solution. Visualization and quantification of the number and crypt multiplicity of ACF in the entire colon were conducted as described previously (23) with some minor modifications. Colons were cut open along the longitudinal median axis, placed on a piece of filter paper, flattened and stretched, covered with a second piece of filter paper, and fixed in 10% phosphate buffered formalin for at least 24 h. The colons were cut into 2 cm segments, stained with 0.5% methylene blue solution for 5 min, washed with Kreb's Ringer solution (Sigma-Aldrich, St. Louis, MO), and placed on microscope slides with the mucosa side up. Foci of aberrant crypts were scored under a light microscope and distinguished from the surrounding normal crypts by their increased size. To determine crypt multiplicity, the number of crypts in each focus was recorded. Particular emphasis was given to those that formed 4 or more crypts per focus due to their high correlation to tumor formation potential.

Analysis of Colonic Cell Proliferation Rates

Five weeks after the final AOM treatment (at 13 wk of age) 6 rats from the control and MR groups were removed for analysis of colonic cell proliferation rates. To this end, BrdU (Boehringer Mannheim, Indianapolis, IN) was administered at a dose of 20 mg/kg bw (*i.p.*) and one hour later rats were sacrificed by carbon dioxide euthanasia. The colons were removed, fixed in 80% ethanol, and embedded in paraffin.

BrdU incorporation was analyzed on sections of paraffin embedded colons by immunohistochemistry as previously described (24). Tissue sections were incubated for 30 min in 0.3% H_2O_2 in methanol to quench the endogenous peroxidase activity and for 20 min in HCl to denature DNA. The BrdU taken up by the nucleus was detected using monoclonal anti-BrdU (Boehringer Mannheim). The sections were incubated with biotinylated antibody for 1 hr at room temperature and then with peroxidase avidin-biotin complex. The antibody peroxidase complex was visualized by incubating the sections with peroxidase substrate solution

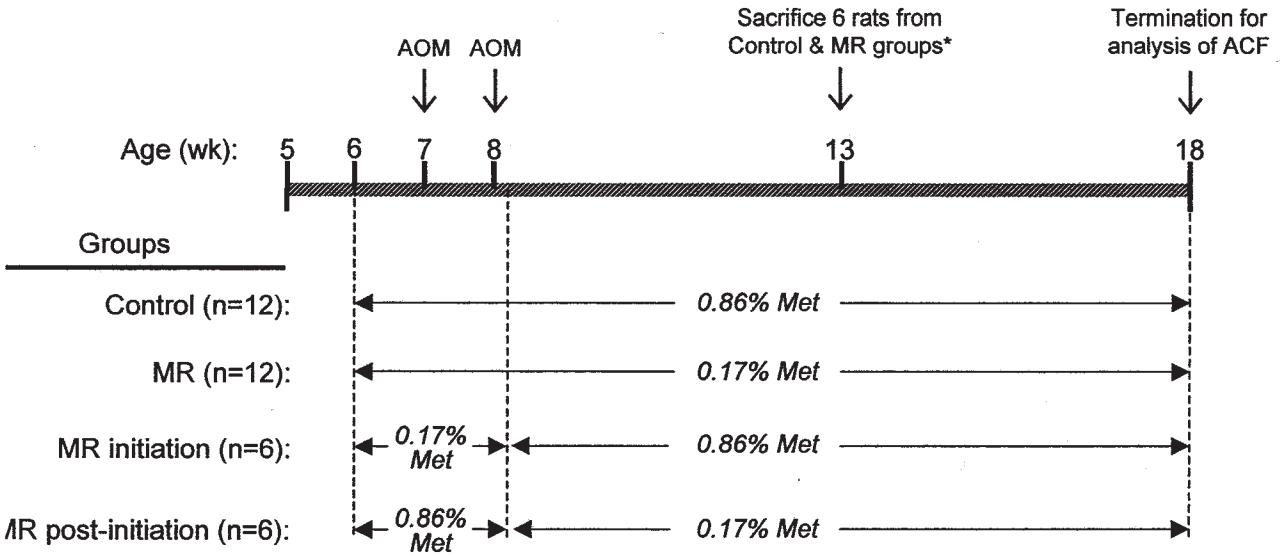

Figure 1. Experimental design. *, analysis of cell proliferation.

containing an equal volume of 0.02% H_2O_2 and 0.1% 3,3′diaminobenzidine tetrahydrochloride (Sigma-Aldrich) in 0.1 M tris-HCl buffer, pH 7.2. The slides were then counterstained with hematoxylin, dehydrated, clarified, mounted, and examined using a standard light microscope. Approximately 30–35 crypts were observed for each animal in both distal and proximal portions of the colon. Attention was confined to the well-oriented crypts in which the base, lumen, and top of the crypt could clearly be seen. The number of the labeled cells in each crypt column was recorded. The percentage of the labeled cells to total number of cells (labeling index) was determined for the whole crypt by calculating the ratio of labeled cells to total number of cells × 100.

Proliferating cellular nuclear antigen (PCNA) incorporation was analysed on sections of paraffin embedded colons by immunohistochemistry as previously described (25). Tissue paraffin sections were deparaffinized, rehydrated, and then incubated with 3% hydrogen peroxide for 10 min at 37°C. Tissue sections were incubated with the anti-PCNA monoclonal antibody (DAKO, Santa Barbara, CA) optimally diluted 1:20 in phosphate-buffered saline (PBS) for 30 min at 37°C. A biotinylated goat anti-mouse immunoglobulin was used as secondary antibody at a dilution 1:200 in PBS, in which sections were incubated for 30 min at 37°C. This was followed by incubation to streptavidin at a dilution of 1:400 in PBS for 30 min at 37°C. The slides were incubated with 0.05% diaminobenzidine solution for 10 min at room temperature. Each step was followed by three washes in PBS. The sections were lightly counterstained with Lillie-Mayer's alum hematoxylin (Sigma-Aldrich). PBS replaced the PCNA monoclonal antibody as a negative control for immunohistochemical staining experiments. PCNA positivity was expressed as the ratio of PCNA-positive nuclei to total nuclei ×100 (labeling index) in each well-oriented crypt. A minimum of 20 crypts per section were analyzed.

Statistical Analysis

Data were analyzed by Student's *t*-test or analysis of variance where applicable. Differences were considered statistically significant at $P < 0.05$.

Results

To examine whether MR has any inhibitory effects on colon carcinogenesis, four groups of 6-wk-old male F344 rats (n = 6/group) were placed into four diet groups as follow: Control, MR, MR during initiation only, and MR during post-initiation only. AOM was given at a dose of 15 mg/kg bw/wk for 2 wk as described in **Materials and Methods.** Body weight data demonstrate that growth was significantly delayed in the MR groups, particularly during the periods of feeding the MR diet (Fig. 2). By 10 wk after the carcinogen administration, when sacrificed, animals in MR and MR post-initiation groups weighed about 50% and 40% less, respectively, than those in the control group. In the MR-initiation group, body weights were approximately 50 g lower than in control rats after only 3 wk of feeding (9-wk-old rats). After switching back to the control diet, the rats in this group grew more rapidly than control animals, but still remained 20 g lighter at termination.

When colons were examined for ACF formation, an over 80% inhibition of ACF containing 4 or more crypts per focus was observed in the MR diet group (Fig. 3). In particular, the distribution of crypt multiplicity was significantly shifted to smaller sized ACF in MR groups compared to controls (Table 2). The MR diet administered during the post-initiation period alone had the full effect of inhibiting the number and crypt multiplicity of ACF formation reaching a remarkable 98% inhibition of large ACF. MR treatment given only during initiation resulted in decreased numbers of crypts, how-

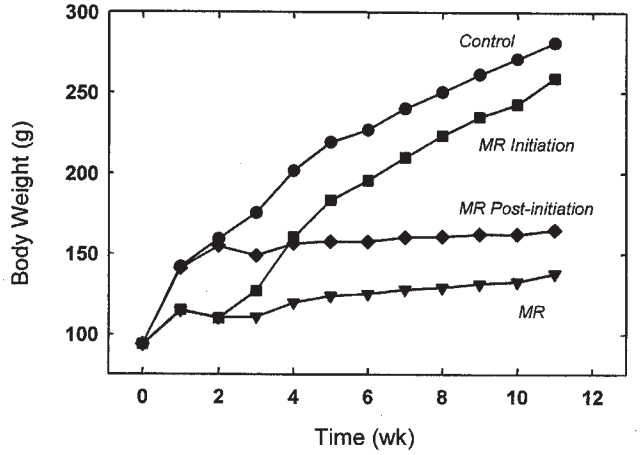

Figure 2. Body weight changes of azoxymethane (AOM)-treated rats on control and methionine restricted diets. Young male F344 rats were placed on their respective diets, control (0.86% Met), MR (methionine restriction, 0.17% Met), MR during initiation only, and MR during post-initiation only. The initiation agent, AOM, was given at 15 mg/kg bw/wk for 2 wk. Body weight was recorded weekly throughout the experiment. Data represent means of six animals per group; bars representing standard error of the mean were omitted because they were smaller than the point size for each time per group.

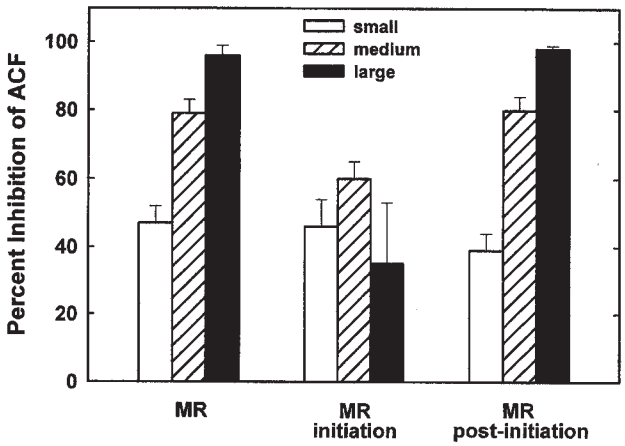

Figure 3. Mean potency of methionine restriction to reduce aberrant crypt foci (ACF) multiplicity in azoxymethane-treated F344 rats. Bars represent mean percent inhibition of ACF (small, medium, and large with 1–3, 4–6, and more than 6 crypts per focus, respectively) in methionine restricted (MR) groups. Error bars represent SEM values (n = 6/group).

ever, the crypt multiplicity showed a similar pattern to the control group. These data suggest that the major inhibitory effects of MR occur primarily on the post-initiation phases of colon carcinogenesis.

To determine whether MR exerts its effects by altering colonic cell proliferation, MR and control rats were administered BrdU (20 mg/kg ip) 5 wk after the AOM treatment, and sacrificed 1 h later. BrdU and PCNA labeling indexes of colonic epithelium were determined by immunohistochemistry as described in **Materials and Methods.** The values for both indexes are decreased by 12% with results being significant for PCNA only (Table 3).

Discussion

The present study demonstrates that MR, a dietary treatment that appears to delay the aging process, also inhibits AOM-induced ACF formation primarily during the post-initiation phase of colon carcinogenesis. Of particular interest, MR completely inhibited the formation of large ACF, which have been shown to be well correlated with tumor formation (26,27), suggesting that MR effectively inhibits colon carcinogenesis. Indeed, based on a previous ranking of promising chemopreventive agents against colon carcinogenesis (28), MR is of outstanding potency because it reduces the most significant ACF by more than fivefold. The MR diet administered after carcinogen initiation had the same inhibitory effect on the formation of large ACF as when MR was administered throughout the entire experiment. Thus, it appears that the MR-specific mechanisms responsible for this inhibition involve primarily the post-initiation phases of colon carcinogenesis. This is important because the initiating agents and events involved in colon cancer development in humans are unknown. In addition, post-initiation phases apparently occur over a much longer period of time and represent a more likely target for the development of preventive strategies.

Although the mechanisms by which MR inhibits colon carcinogenesis are unknown, the present results suggest that a reduction in cell proliferation could be involved. Using both cell proliferation markers, BrdU and PCNA, a 12% inhibition in the proportion of proliferating cells was observed after only 5 wk of MR (Table 3). PCNA data were statistically significant ($P < 0.01$) whereas BrdU measurements were just over the significance cut-off ($P < 0.06$) due to the greater degree of variability in the BrdU measurements. Lower absolute values of BrdU compared to PCNA reflect the fact that only S phase cells are stained for BrdU, whereas PCNA stains cells in G1, S, and G2 phases of the cell cycle. The greater variability observed in BrdU measurements resulted in a substantially reduced statistical power than that for PCNA.

Cell replication is commonly thought to be an important factor regulating cancer risk. Decreased proliferation has been linked to reductions in colon carcinogenesis, possibly by decreasing the likelihood that DNA-damaged or mutated cells can be subjected to clonal expansion prior to being repaired. Many of the most effective chemopreventive agents such as polyamine synthesis inhibitors depress cell proliferation rates within the colon (29). Calorie restriction also leads to decreases in colonic cell proliferation as well as ACF formation in the AOM-induced rat model (12). Although the presently observed differences in cell proliferation indexes are small, they are similar to those observed for the well-established chemopreventive agent difluoromethylornithine (30). Further, one could expect that the inhibitory effects of MR to be magnified if measurements were conducted after

Table 2. Effect of Methionine Restriction on AOM-Induced ACF and Colon Crypt Multiplicity[a]

Group	No. of Animals	Small (1–3 crypts/focus)	Medium (4–6 crypts/focus)	Large (>6 crypts/focus)	Total ACF
Control	6	246 ± 14[b] (66%)	114 ± 13 (30.5%)	13 ± 3.0 (3.5%)	373 ± 28 (100%)
MR	6	132 ± 12[c] (84%)	24.5 ± 5[c] (15.6%)	0.5 ± 0.3[d] (0.4%)	157 ± 16[c] (100%)
MR initiation	6	134 ± 20[c] (71%)	45.5 ± 6[c] (24.4%)	8.5 ± 2.0 (4.6%)	188 ± 26[c] (100%)
MR post-initiation	6	152 ± 13[c] (87%)	22.5 ± 5[c] (12.9%)	0.2 ± 0.2[d] (0.1%)	175 ± 18[c] (100%)

a: Abbreviations are as follows: AOM, azoxymethane; ACF, aberrant crypt foci; MR, methionine restriction.
b: Values are mean ± SEM.
c: Significantly different from controls, $P < 0.001$.
d: Significantly different from controls, $P < 0.01$

Table 3. Effect of Methionine Restriction on Colonic Epithelium Proliferation[a]

Diet	No. of rats	BrdU Labeling Index (%)	PCNA Labeling Index (%)
Control	6	13.5 ± 1.2[b] (100%)	39.2 ± 1.3 (100%)
MR	6	11.7 ± 1.1 (87%)	34.4 ± 0.6[c] (88%)

a: Abbreviations are as follows: PCNA, proliferating cellular nuclear antigen; MR, methionine restriction.
b: Mean ± SEM.
c: Significantly different from control group, $P < 0.01$.

20 or 30 weeks when cell proliferation rates are normally greatly increased in AOM-treated rats.

The beneficial effects of MR may also be due, in part, to decreases in Met availability due to its critical role in cancer development. Proliferation of many human and rodent cancer cells depends on Met in contrast to normal cells, which are Met-independent (31–33). A possible explanation for Met dependence is the increased rate of transmethylation in cancer compared to normal cells (34,35). Indeed, a late S/G2 cell cycle arrest in Met-dependent tumor cell lines growing under conditions of limiting Met source was reported (33). Furthermore, diets in which Met was either the only amino acid excluded from the protein composition or replaced by homocysteine resulted in regression of animal tumors and inhibition of metastasis in animal models (36–38). In our present results, it is not known if the beneficial effects of MR are specific for Met alone or are a more general result of insufficient amino acid intake. However, in previous studies, dietary restriction of other essential amino acids in tumor-bearing animals resulted either in no antitumor effect or in life-threatening toxicity (39). Methioninase, an enzyme that specifically degrades Met, inhibited the growth of solid tumors and leukemia in animals (40–42). In a clinical trial, when Met-free parenteral nutrition was combined with chemotherapy, response rates in gastric cancer were improved in comparison to those from only chemotherapy (43). A phase I clinical study showed that dietary methionine restriction is safe and tolerable in adults with metastatic tumors for as long as 39 mo with some preliminary evidence of antitumor activity (44).

A common mechanism for the regulation of both aging and colon carcinogenesis may involve oxidative damage. Aging is characterized by changes in those factors, such as increased cell proliferation together with accumulation of oxidative (45) and non-oxidative DNA damage over time; decreased repair capacity (46); and depletion of a major regulator of oxidative stress, glutathione (GSH) (47). Decreased blood levels of GSH are found in the elderly (48) and have been associated with the pathogenesis of cancer (49). Administration of specific GSH enhancing agents, such as N-acetylcysteine or 2-oxothiazolidine-4-carboxylic acid, significantly increased life span in mosquitoes (50,51) and inhibited the AOM-induced ACF formation in the rat colon (52). Furthermore, CR, which like MR (14) corrects the GSH depletion during aging, is also associated with decreased oxidative damage, enhanced DNA repair capacity, and reduced age-related accumulation of certain types of DNA damage (53).

Another possible link between colon cancer and aging is insulin resistance (54), a common condition in older animals and humans (55,56), which elicits many of the signs of early aging, suggesting that insulin-signaling pathways mediate aging processes. It seems plausible that MR exerts its anti-proliferative effects through specific metabolic and cellular changes providing protection against colon carcinogenesis by interfering with the insulin-signaling pathways and inhibiting the oxidative/inflammatory processes.

It has been reported that aging is a major contributor to hypermethylation in cancer, including colorectal cancer (57). There is also evidence for a potential link between aberrant methylation and genetic instability due to deficient DNA repair (58). In particular, promoter hypermethylation, an alternative mechanism of tumor suppressor gene inactivation, was found to be an age-related as well as gene and tissue specific phenomenon that involves genes important in colorectal cancer (59). In that study, four out of seven hypermethylated genes in colorectal cancer showed significant age-related methylation in normal colon. MR might prevent this hypermethylation by tightly regulating the distribution of limited methyl groups due to methionine deficiency.

MR appears to have a similar effect on growth and life span as CR, but the mechanisms involved are apparently different. MR results in reduced growth, even though food is provided ad lib and consumption is only 10% below that of controls. Actually in MR caloric intake expressed as per gram of body weight is 62–193% greater than that of control. Also, pair-feeding the control diet to match the intake of the methionine-restricted animals did not inhibit growth and methionine-restricted animals did not resume growth when their diet was calorically enriched to match the intake of control rats (13). In the present study growth reduction does not appear to be responsible for the MR-induced inhibition of ACF formation. Indeed, in the MR-initiation group body weights were only 10% less than controls, while this group showed a 58% reduction in crypt formation (Fig. 3). Although there are differences between MR and CR, their effects on life span, aging, and colon carcinogenesis may be through some common final pathway that regulates factors such as cell proliferation, oxidative damage, insulin-signaling, age-related GSH loss, and aberrant methylation.

MR represents an important tool for exploring the underlying mechanisms responsible for both the biological process of aging and carcinogenesis. The unique combination of MR with the AOM-induced colon carcinogenesis animal model is used to study mechanistic aspects involved primarily during promotion/progression phases after AOM metabolism and initiation have occurred. Altogether our results provide additional information for the mechanisms involved in aging and carcinogenesis and may explain in part the enhanced colon cancinogenicity in aging rats (discussed previously). These findings and those from further studies on this model system will provide important information for the understanding of the mechanisms of aging and carcinogenesis. Based on this information, the development of preventive strategies and effective interventions could be applicable in older individuals, particularly because the promotional phase is arguably the most critical in human colon cancer development.

Acknowledgments and Notes

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