Short Communication

Dietary Whey Protein Protects against Azoxymethane-induced Colon Tumors in Male Rats[1]

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Abstract

Epidemiological studies have suggested a relationship between diet and colon cancer incidence. Results from animal studies suggest that whey protein, but not casein protein, may provide protective effects against experimentally induced breast cancer in animals. In the current study, we investigated the effects of casein and whey diets on chemically induced colon cancer in male rats. Pregnant female Sprague Dawley rats (days 3–4 of gestation) were maintained on modified AIN-93G diets formulated with a single protein source of either casein or whey. Life-time exposure to these diets was studied in the F_1 generation (experiment A) or the F_2 generation (experiment B). Male offspring were weaned to the same diets as the dams and were maintained on these diets throughout the study. At age 90 days, all rats received azoxymethane once a week for 2 weeks (s.c., 15 mg/kg). Forty weeks after the last azoxymethane injection, all rats were euthanized, the colon was examined visually for tumors, and each tumor was histologically evaluated. The weights and distribution of all of the tumors were recorded. In experiment A, rats fed the casein diet had a 56% incidence of colon tumors compared with 30% of the rats on whey-based diets ($P < 0.05$). In experiment B, rats fed the casein diet had 50% incidence of colon tumors compared with 29% in the whey group ($P < 0.05$). There were no significant effects of diet on tumor multiplicity or mass. These results suggest that consumption of whey protein-containing diets may reduce the risk of developing colon tumors.

Introduction

Colorectal cancer is the second leading cause of cancer deaths in the United States. The American Cancer Society estimated that during 2000, almost 94,000 people would be diagnosed with colon cancer and that ~48,000 would eventually die of the disease (1). Advances in early detection and surgery have been largely responsible for reducing mortality and morbidity of colon cancer, and our understanding of prevention is increasing.

Epidemiological data suggest that diet is a major factor in the etiology of cancer. Metabolic phenotype, the Western-style diet (low dietary fiber and high levels of fat and red meat), and cooking techniques (*e.g.,* charbroiling or overcooking) are risk factors for developing colon cancer (2, 3). For example, people who consume relatively high levels of well-cooked pan-fried or charred meats and who also have rapid metabolic phenotypes for cytochrome P4501A2 and slow metabolic phenotypes for acetyltransferase may be at increased risk for colon cancer (4). Thus, reduced consumption of charred meats, especially in people who may be genetically predisposed to greater colorectal cancer risk because of their metabolic phenotype, may be important in lowering risk of colon cancer.

Moreover, epidemiological and animal studies suggest that diets low in animal fat and high in fruits, vegetables, grains, and legumes may protect against colon cancer. For example, diets containing soybeans and soybean-based products may reduce the risk of certain types of cancer, including breast, prostate, and colon cancer (5, 6). Data obtained from studies of Japanese subjects point to lower colon cancer incidence in areas with high tofu consumption (7). Furthermore, several animal studies have suggested that diets containing certain vegetables, grains, or specific phytochemicals reduce the risks of experimentally induced colon cancer (8–10).

In addition to the protective effects of certain phytochemicals, bovine milk products may exert inhibitory effects on the growth of several tumor types (11). An antitumor activity of these dairy products has been attributed to a class of proteins that represent 20% of the total milk protein, the whey fraction (12). Recently, Tsuda *et al.* (13) reported that the major whey protein component, bovine lactoferrin, reduced the incidence and multiplicity of colon carcinoma in male rats. GSH[3] concentrations in a number of tissues have been reported to increase in rats fed whey protein, and this is thought to be attributable to relatively high levels of γ-glutamylcysteine groups, which serve as substrate for glutathione synthetase (11). γ-Glutamylcysteine groups are considered extremely rare in edible proteins, with whey protein being one of the few such proteins containing the glutamylcysteine disulfide link (14). Increased tissue concentrations of GSH would be predicted to have a protective effect because elevated antioxidant capacity would favor decreased mutagenicity.

Recently, our laboratory has demonstrated that AIN-93G diets, which are rich in whey protein, reduced the incidence of chemically induced mammary tumors by 38–46% compared with casein in female Sprague Dawley rats (6). The present study was conducted to determine the possible preventive effects of lifetime exposure to whey proteins on AOM-induced colon tumors in male Sprague Dawley rats.

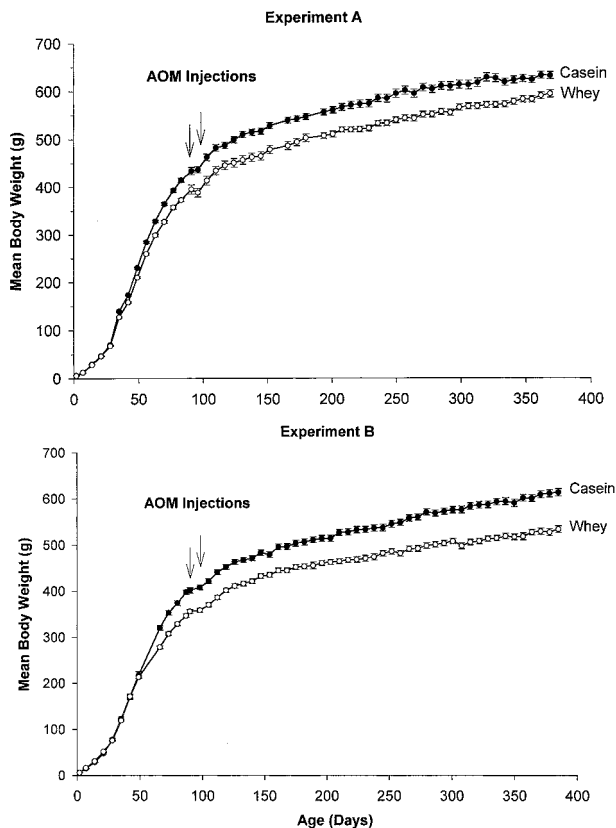[3] The abbreviations used are: GSH, glutathione; AOM, azoxymethane.

Fig. 1. Average body weights of male rats during the study. At age 90 days, rats received two s.c. injections of AOM (15 mg/kg) as indicated by the *arrows*. In experiment A (*top panel*), 32 rats were fed casein and 42 rats were fed whey diets. In experiment B (*bottom panel*), 42 rats were fed casein and 49 rats were fed whey diets. Data are presented as means; *bars*, ± SE.

Table 1 Incidence and histology of AOM-induced colonic tumors in male rats

	Experiment A				Experiment B			
	Casein		Whey		Casein		Whey	
	n^a	$\%^b$	n	$\%$	n	$\%$	n	$\%$
Rats per group	32	100	47	100	42	100	49	100
Rats with tumors								
Entire colon	18	56	14^c	30	21	50	14^c	29
Proximal	8	25	5	11	13	31	6^c	12
Distal	12	38	9^d	21	13	31	8	16
Tumor type								
Invasive adenocarcinoma								
Entire colon	7	22	7	15	13	31	9	18
Proximal	5	16	3	6	8	19	4	8
Distal	3	9	4	9	7	17	5	10
Benign								
Entire colon	11	35	7	15	8	19	5	10
Proximal	3	9	2	4	5	12	2	4
Distal	9	29	5	11	6	14	3	6

^{a} Number of rats per group per experiment.

^{b} $\dfrac{\text{Number of rats}}{\text{Number of rats per group}} \times 100$.

^{c,d} Compared with casein within the same experiment: $^c P < 0.05$; $^d P < 0.01$.

Materials and Methods

Adult breeder female and male Sprague Dawley rats, purchased from Harlan Industries (Indianapolis, IN), were housed individually in polycarbonate cages and allowed *ad libitum* access to water and pelleted food. All rats were housed in an American Association for Accreditation of Laboratory Animal Care–approved animal facility.

Two experiments were performed. In experiment A, pregnant female Sprague Dawley rats (gestation day 4) were randomly assigned to one of two groups and fed a modified AIN-93G diet (15) in which corn oil was substituted for soybean oil and the protein source of either casein or whey (New Zealand Milk Products, Santa Rosa, CA). Amino acids were added to both diets to equalize the essential amino acids. Male offspring (F_1) were weaned to the same diets as their dams and were maintained on these diets throughout the study.

In experiment B, female rats were maintained on the diets described above for 4 weeks prior to breeding, and the offspring from these dams were weaned to the same diet as their mothers. Male and female offspring from different parents within a diet group were selected at random and mated to form the F_2 generation. The F_2 generation was studied to simulate people consuming the same basic diet for generations.

At age 90 days, all male offspring from experiments A and B received s.c. injections of 15 mg/kg AOM (Ash Stevens, Detroit, MI) in saline once a week for 2 weeks. All procedures

were approved by the Institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences, and AOM handling was in accordance with manufacturing and institutional guidelines. Rats were weighed weekly and observed daily during the first 30 days for signs of toxicity (*i.e.,* fecal blood, altered fur coat appearance, anemia, and body weight gains). Forty weeks after the last AOM injection, all rats were euthanized, and the colon (cecum to anus) was divided into two equal segments (proximal and distal), opened longitudinally, washed free of contents with ice-cold saline, and examined visually for tumors. The locations, weights, and distribution of all tumors were recorded. A representative section of each tumor was fixed in 10% neutral-buffered formalin. Sections (5 μm) of the paraffin-embedded tumors were stained with H&E for histological analysis.

Pathology. All tumors were evaluated in a blinded protocol by an American College of Pathology-certified pathologist (S. K.) and classified.

GSH Concentrations. After the lifetime feeding of casein or whey, male (age, 65 days; $n = 5$) and female (age, 50 days; $n = 5$) rats were euthanized. The livers were removed, and cytosols were prepared by using the method of Chipman and Walker (16). Soluble protein was assessed using the Coomassie Brilliant Blue assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, and cytosolic GSH concentrations were determined using a colorimetric kit (Oxis Int., Inc., Minneapolis, MN) according to the manufacturer's instructions.

Statistical Analysis. Fisher's exact test was used to compare the percentage of rats with tumors in each treatment group. The nonparametric Mann-Whitney U test was used for comparing tumor multiplicity and weight of tumors. GSH levels were compared by t test. Statistical significance was set at $P < 0.05$. The technique described by Fisher (17) was used to combine the probabilities from the two experiments.

Results

Body Weight. Fig. 1 demonstrates that rats fed both diets had excellent body weight gains throughout the study. Consistent

Fig. 2. Photomicrographs demonstrating invasive adenocarcinoma of the colon. Magnification: ×40 in *A* and ×400 in *B*.
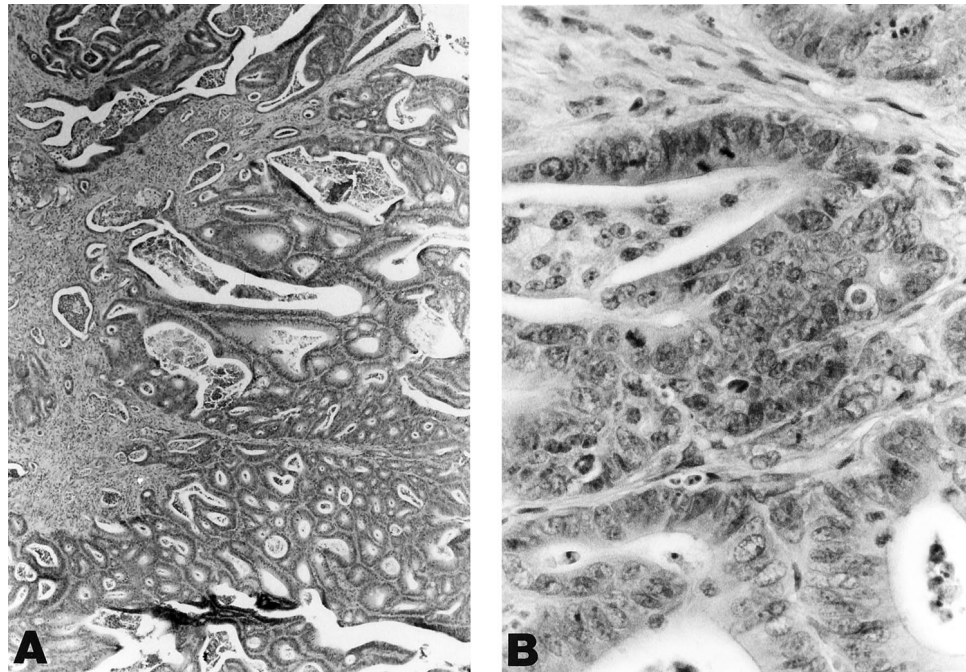
with previous reports from our laboratory (6), however, whey-fed rats weighed slightly less than casein-fed rats at the end of the study ($P < 0.05$). This body weight difference occurred because the weight gain of whey-fed rats was lower ($P < 0.05$) between 50 and 90 days of age. Using the crude method of measuring the food and water remaining in the hoppers or bottles as a measure of the previous day's intake, we noted no significant differences in food or water intake between casein-fed and whey-fed rats (data not shown).

Tumor Incidence, Histology, Weight, and Multiplicity. Approximately one-half of the rats that ate casein diets developed colonic tumors (Table 1). The median incidence in total tumors found in the entire colon was lower ($P < 0.05$) in rats fed whey diets compared with casein-fed rats in both experiments.

The median incidence of tumors in the proximal region was lower for whey-fed rats than for casein-fed rats only in experiment B ($P < 0.040$). However, the overall P calculated from the data of both experiments demonstrated a significant ($P < 0.03$) reduction in proximal colon tumor incidence in whey-fed rats. Similar results were found with the tumor incidence in the distal colon, where a lower incidence ($P < 0.01$) could be detected in whey-fed rats only in experiment A, but the overall P calculated from both experiments was $P < 0.03$.

Although there was a clear trend toward reduced incidence (32–42%) of tumors histologically identified as invasive adenocarcinoma (Fig. 2), the numbers of rats per group was too low to reach statistical significance (Table 1).

No statistical differences were noted in mean or median tumor weights between groups (0.08–0.22 g). Multiplicity (the number of tumors per tumor-bearing rat) varied between 1 and 4, but neither the mean nor the median differed significantly between groups.

Although GSH levels were not determined in the experiment reported above, they were assessed in another set of male and female rats treated with the same diets. The GSH concentrations (μmol/mg of protein) did not differ significantly between diet or gender. The concentrations in male ($n = 5$) and

female ($n = 5$) rats fed casein were 0.029 ± 0.002 and 0.031 ± 0.003 μmol/mg of protein, respectively, and concentrations in male ($n = 5$) and female ($n = 5$) rats fed the whey diet were 0.026 ± 0.002 and 0.026 ± 0.002 μmol/mg of protein, respectively.

Discussion

Dietary influences on cancer risks have become an increasingly important area of research. The prevention of colon cancer by dietary whey proteins has been studied in mice and rats, but the results are contradictory (18, 19). McIntosh *et al.* (20) reported that 1,2-dimethylhydrazine-induced colon tumor incidence was reduced in rats fed diets made with either casein or whey protein compared with diets made with red meat or soy protein. Although there was a tendency toward a lower tumor incidence in whey-fed than casein-fed rats, it was not significant in these studies, and data on tumor mass were not consistent.

We recently demonstrated that female rats fed diets made with whey protein had significantly reduced incidence of chemically induced (dimethylbenz[*a*]anthracene) mammary tumors compared with rats fed casein diets (6). These data were consistent with the study of Papenburg *et al.* (18), who reported lower 1,2-dimethylhydrazine-induced colon tumor incidence and mass in mice fed whey protein than in mice fed either casein or a commercially available mouse diet. A recent report by Tsuda *et al.* (13) demonstrated that bovine lactoferrin, a major whey protein component, inhibits colon carcinogenesis in male rats. The major aim of this study was to evaluate the chemopreventive effects of the whey protein diet against AOM-induced colon tumors in male Sprague Dawley rats. Our results indicate that rats fed a whey-containing diet had an ~40% lower tumor incidence rate, but no significant differences were noted in the tumor mass and multiplicity.

The current study also histologically characterized the tumor type and segmented the colon into the proximal and distal sections to determine whether protective effects occurred

equally for benign and invasive adenocarcinoma and to determine whether any regional differences existed. We found that the tumor incidence of invasive adenocarcinoma in the colon was 32–42% lower in whey-fed rats than in casein-fed rats. Although the total number of rats studied in each group was substantial (32–49 rats/group), the absolute numbers of rats that developed tumors for individual experiments were too small to permit detection of statistically significant effects of diet (whey *versus* casein) on tumor type (invasive adenocarcinoma *versus* benign) or colon location (proximal *versus* distal) within individual experiments. Analysis of combined treatment effects of experiments A and B revealed lower incidences of invasive adenocarcinoma tumors of the colon ($P < 0.05$), but it failed to detect differences between proximal and distal sites.

One possible explanation for lower percentages of rat with tumors in the present study could be the length of the post-AOM treatment in our experiments. Other investigators studying this colon cancer model used two s.c. doses of AOM and sacrificed rats 52 weeks post-AOM injection compared with the 40 weeks post-AOM that we used in this experiment (21, 22). This longer period would tend to increase the number of tumors detected.

The rats fed a whey protein diet had slightly lower weight gain than rats fed a casein diet for both experiments. Previous results from our laboratory have demonstrated that rats fed whey protein or soy had a tendency to gain less body weight than casein-fed rats and that food intake could not account for these differences (6). The rate of body weight gain was lower in whey-fed rats between 50 and 90 days of age ($P < 0.05$), which led to a significant difference in absolute weight at end of the experiment ($P < 0.05$). However, the rate of body weight gain following administration of AOM did not differ significantly between groups. Because the whey-fed rats had excellent body weight gains within the normal range for rats fed standard rat feed, it is unlikely that the protective effects were linked to the slight differences in absolute body weight between groups.

One suggested mechanism for whey protection has been the reported increases in tissue GSH concentrations. One expected effect of increased tissue GSH levels would be increased detoxification of the free radicals produced by metabolism of carcinogenic and xenobiotic compounds (23). However, the hepatic GSH levels of rats fed whey protein- or casein protein-containing diets did not differ. Another possible mechanism involves altered procarcinogen activation/deactivation via phase I and phase II enzymes. In this regard, we have reported significant increases in dimethylbenz[*a*]anthracene-induced hepatic GST-α activities in female rats fed whey protein diets (24).

In summary, we studied the effects of whey protein on AOM-induced colon tumors in male rats. Rats fed a diet made from whey protein demonstrated protection against AOM-induced colonic tumor incidence compared with rats fed a casein-containing diet. These results suggest that long-term consumption of whey protein may reduce the risk of colon cancer. Further studies are under way in our laboratory to understand the mechanisms responsible for this protection.

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