Increase in dietary protein content exacerbates colonic inflammation and tumorigenesis in azoxymethane-induced mouse colon carcinogenesis

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BACKGROUND/OBJECTIVE: The incidence of colorectal cancer (CRC) has been attributed to higher intake of fat and protein. However, reports on the relationship between protein intake and CRC are inconsistent, possibly due to the complexity of diet composition. In this study, we addressed a question whether alteration of protein intake is independently associated with colonic inflammation and colon carcinogenesis.

MATERIALS/METHODS: Balb/c mice were randomly divided into 4 experimental groups: 20% protein (control, 20P, 20% casein/kg diet), 10% protein (10P, 10% casein/kg diet), 30% protein (30P, 30% casein/kg diet), and 50% protein (50P, 50% casein/kg diet) diet groups and were subjected to azoxymethane-dextran sodium sulfate induced colon carcinogenesis.

RESULTS: As the protein content of the diet increased, clinical signs of colitis including loss of body weight, rectal bleeding, change in stool consistency, and shortening of the colon were worsened. This was associated with a significant decrease in the survival rate of the mice, an increase in proinflammatory protein expression in the colon, and an increase in mucosal cell proliferation. Further, colon tumor multiplicity was dramatically increased in the 30P (318%) and 50P (438%) groups compared with the control (20P) group.

CONCLUSIONS: These results suggest that a high protein diet stimulates colon tumor formation by increasing colonic inflammation and proliferation.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in both men and women and the fourth most common cause of death from cancer worldwide [1,2]. The incidence of CRC shows, however, a large geographical variation [3]. The highest incidence is reported in Australia, New Zealand, Canada, the United States, and Europe, whereas the lowest incidence is reported in China, India, and parts of Africa. This variation appears to be largely attributable to cultural differences in diet composition [4].

Many epidemiological studies have shown that the consumption of a typical Western diet high in fat and protein significantly increases the risk of CRC [5], whereas high intake of fruits, vegetables, and whole grains is protective against CRC [6]. In fact, over 63% of all CRC cases have been identified in developed countries with a Western-type diet [3]. Because of the complexity of diet composition, however, the direct influence of dietary protein intake on CRC risk is still under intense scientific debate [7].

High protein diet (HPD, > 20% of total energy intake) appears to be closely associated with the risk of CRC in animal studies. When rats were fed HPD with protein from casein (25%), soya (25%), or red meat (25%), genetic damage in colon cells was significantly increased compared to rats fed a normal protein diet (15% casein) [8-10]. In another animal study comparing a HPD and normal protein diet (NPD, 53% vs. 14% whole milk proteins), protease activities in the colon were nearly 3-fold higher (when expressed per gram content) and the heights of brush-border membranes were drastically reduced in HPD fed rats [11].

Chronic inflammation in the colon and rectum is often associated with an increased risk of CRC development [12]. It has been reported that after 10 years of chronic inflammation, the risk of colitis-associated cancer (CAC) increases by 0.5~1% every year [13]. Molecules that have been implicated in the CAC includes pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6, and chemokine CCL2 [14]. The mRNA and protein levels of these molecules are increased in the inflamed colonic mucosa of human and rodents [15-17]. Activation

of NF-κB is also associated with colon cancer. It controls the expression of inflammatory genes such as IL-6, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) [18]. However, reports on the connection between dietary protein and colorectal inflammation are scarce [19]. In this study, we therefore investigated the effect of different contents of protein in the diet on the development of CRC in a colitis-associated colon carcinogenesis mouse model.

MATERIALS AND METHODS

Animals and diet

Twenty nine female Balb/c mice were obtained from Koatech Bio Inc. (Busan, Korea) at 4 weeks of age. Animals were housed 2 mice per cage and acclimated for 1 week and divided into four diet groups: control (20P; 20% casein/kg diet, n = 5), 10P (10% casein/kg diet, n = 8), 30P (30% casein/kg diet, n = 8), and 50P (50% casein/kg diet, n = 8) group. Diets were prepared based on AIN-76A diet formulation as shown in Table 1. Casein was commercially achieved from local company, Dongmyung Inc. (Chungdogun, Korea). All animals were kept in controlled conditions of humidity (50 ± 10%), light (12-hour light/dark cycle), and temperature (23 ± 2℃). Food intake was recorded daily and body weight was measured once a week. Animal protocols employed in this study were approved by the Animal Care and Use Committee at Catholic University of Daegu (IACUC 2014-042, Gyeongsan, Korea)

Inflammation-induced colon carcinogenesis

The mice of four groups were initiated with a single i.p. injection of 12.5 mg/kg body weight azoxymethane (AOM, Sigma-Aldrich, St, Louis, MO, USA) at 5 weeks of age and promoted with 3 cycles of dextran sodium sulfate (DSS, MP Biomedicals, Irvine, CA, USA) in drinking water after 1 week of AOM injection (Fig. 1). The animals received 2% DSS in their drinking water for one cycle and then switched to 1% DSS drinking water for remaining two cycles due to the severity of

Table 1. Composition of experimental diet

Ingredients	Groups[1]			
	20P (Control)	10P	30P	50P
Corn starch	150	150	150	150
Casein	200	100	300	500
D,L-methionine	3	3	3	3
Sucrose	500	600	400	200
Cellulose powder	50	50	50	50
Corn oil	50	50	50	50
Mineral mixture[2]	35	35	35	35
Vitamin mixture[3]	10	10	10	10
Choline bitartrate	2	2	2	2
t-BHQ[4]	0.01	0.01	0.01	0.01
Total (g)	1,000	1,000	1,000	1,000
Protein, kcal/kg (% energy)	800 (20.8%)	400 (10.4%)	1,200 (31.2%)	2,000 (51.9%)
Carbohydrate, kcal/kg (% energy)	2,600 (67.5%)	3,000 (77.9%)	2,200 (57.1%)	1,400 (36.4%)
Fat, kcal/kg (% energy)	450 (11.7%)	450 (11.7%)	450 (11.7%)	450 (11.7%)
Total energy, kcal/kg	3850	3850	3850	3850

[1] 20P, 20% casein; 10P, 10% casein; 30P, 30% casein; 50P, 50% casein contents of diet.
[2] Composition of AIN-76A mineral Mix (g/kg): Calcium phosphate, dibasic 500; Sodium chloride 74; Potassium citrate, monohydrate 220; potassium sulfate 52; Magnesium oxide 24; Manganous carbonate (43-48% Mn) 3.5; Ferric citrate (16-17% Fe) 6; Zinc carbonate (70% ZnO) 1.6; Cupric carbonate (53-55% Cu) 0.3; Potassium iodate 0.01; Sodium selenite 0.01; Chromium potassium sulfate 0.55; Sucrose, finely powdered 118.03
[3] Composition of AIN-76A vitamin Mix (g/kg): Thiamin hydrochloride 0.6; Riboflavin 0.6; Pyridoxine hydrochloride 0.7; Nicotinic acid 3.0; D-calcium pantothenate 1.6; Folic acid 0.2; D-biotin 0.02; Cyanocobalamine 0.001; Cholecalciferol (400,000IU/g) 0.25; Manaquinone 0.005; Ascorbic acid 0.2; Sucrose, finely powdered 992.824
[4] t-BHQ: tert-butylhydroquinone

the disease. Each cycle was consisted of five days and separated by 16 days. Experimental diet was fed to the mice with AOM initiation and continued for 10 weeks. When experiment was
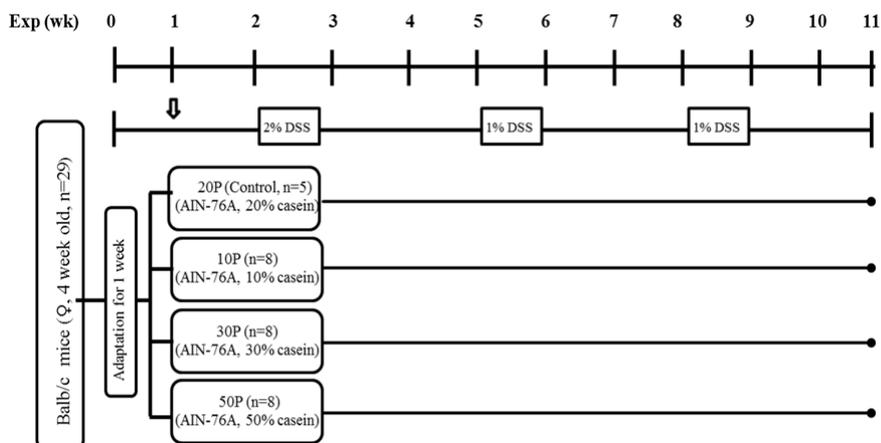

Fig. 1. Schematic representation of the experiment. Twenty-nine 4-week-old female Balb/c mice were acclimated for 1 week and then randomly divided into four diet groups based on AIN-76A diet composition: control (20P; 20% casein, n = 5), 10P (10% casein, n = 8), 30P (30% casein, n = 8), and 50P (50% casein, n = 8). Carcinogenesis was initiated with a single intraperitoneal injection of 12.5 mg/kg body weight AOM and was promoted with 3 cycles of DSS in drinking water 1 week after AOM injection in the mice of all four groups. The animals were received 2% DSS in their drinking water for one cycle and were then switched to 1% DSS drinking water for the remaining two cycles because of the severity of the disease. Each cycle lasted five days and the cycles were separated by 16 days. Experimental diet was fed to the mice after AOM initiation and was continued for 11 weeks. AOM, azoxymethane; DSS, dextran sodium sulfate.

terminated, animals were sacrificed after 12 h fasting. The entire large intestine from cecum to rectum was taken out and the length of large intestine was measured with a ruler. The large intestine was weighed, flushed out luminal contents with phosphate buffered saline, and cut open longitudinally. We divided the large intestine into 3 parts (proximal, middle, and distal part) and counted tumors macroscopically categorized on size (< 1 mm, 1 to 3 mm, or > 3 mm). The tumor diameter was measured by a caliper.

Assessment of DSS-induced colitis

The mice were checked daily for colitis development by monitoring body weight, gross rectal bleeding, stool consistency and survival. The overall disease severity was assessed by a clinical scoring system on a scale of 0-4 [20]. In brief, scoring was as follows: 0, no weight loss, no gross blood in the stool or anus, and normal stool consistency; 1, weight loss of 1-5%, no gross blood in the stool or anus and normal stool consistency; 2, 5-10% weight loss, positive for gross blood in the stool or anus and loose stools; 3, 10-20% weight loss, positive for gross blood in the stool or anus and loose stools; and 4, greater than 20% weight loss, gross blood in the stool or anus and diarrhea.

Western analysis

Since the tumor multiplicity of each group was significantly different in the distal part of large intestine, we homogenized these regions in RIPA buffer (0.1% SDS, 1% Triton X-100, 0.5% Sodium deoxycholate, 50 mM Tris [pH 7.5], 150 mM Nacl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin) for western analysis. Samples were resolved by SDS-PAGE and transferred to polyvinylidenedifluoride membrane. The blot was blocked with 5% non-fat dried milk in 0.1% Tween-20-Tris-buffered saline (TTBS) and probed overnight at 4°C with antibody against cyclooxygenase-2 (COX-2, Cell signaling Technology, Danvers, CO, USA), inducible nitric oxide synthase (iNOS, Abcam, Cambridge, MA, USA), and actin (Santa Cruz biotechnolgy, Santa Cruz, CA, USA) at 1:1000 dilution. After washing three times with 0.1% TTBS, the blot was then probed with horseradish peroxidase- conjugated secondary antibody (Santa Cruz biotechnolgy, Santa Cruz, CA, USA) at 1:5000 dilution for 1 hr at room temperature. After washing three times with 0.1% TTBS, the specific bands were detected by an enhanced chemiluminescence kit (Bio Science Technology, Pohang, Korea) and quantified by GelDoc-It TS imaging system (UVP, Upland, CA, USA) .

Histological analysis

Distal part of large intestine was removed and fixed in 10% formalin. Tissue sections were stained with hematoxylin and eosin (H&E) solution and immunostained with antibodies against proliferating cell nuclear antigen (PCNA, Abcam, Cambridge, MA, USA). The number of total cells in the colon epithelium was counted in four random tissue sections in each mouse. The labeling index was calculated as the percentage of stained cells to total number of cells in the colon epithelium. The height of the highest hyperplastic region of mucosa at 100X magnification was measured by Leica Application Suite program (ver. 4.7.1). We measured 8 different regions in each mouse and used the average height as the mucosal thickness (µm).

Statistical analysis

The data are presented as means ± standard error (SE). All statistical analyses were performed by the SPSS program (Ver.19). The data were analyzed by one-way analysis of variance (ANOVA) and differences between experimental groups were evaluated at the $P < 0.05$ by Duncan's multiple range test.

RESULTS

Food intake, DSS intake, and body weight change in the experimental groups during colon carcinogenesis

We first evaluated the daily food intake, the amount of DSS ingested during each DSS cycle, and body weight changes of the mice throughout the experiment to assess whether there were differences in these parameters among the different diet groups. Mean food intake was lower in the 30P and 50P groups than in the 20P (Control) and 10P groups (Table 2). The mean

Table 2. Daily food intake and DSS intake

Groups[1]	Food intake (g/day)	DSS drink (mL/day)
20P (Control, n = 5)	3.03 ± 0.09[a]	4.83 ± 0.13[b]
10P (n = 8)	2.97 ± 0.07[a]	5.68 ± 0.25[a]
30P (n = 8)	2.63 ± 0.65[b]	5.12 ± 0.19[ab]
50P (n = 8)	2.70 ± 0.11[b]	4.89 ± 0.29[b]

[1] 20P, 20% casein; 10P, 10% casein; 30P, 30% casein; 50P, 50% casein contents of diet.
Fresh food was provided to the mice in each group everyday and daily food intake was calculated as the amount of food provided - the amount of food remaining the next day. The animals received 2% DSS in their drinking water for one cycle and then switched to 1% DSS drinking water for remaining two cycles. Each cycle was consisted of five days. The amount of DSS drink was measured everyday during each DSS cycle as the amount of fresh DSS drinking water provided - the amount of DSS drinking water remaining the next day. Means with different letters within a column are significantly different from each other at $P < 0.05$ as determined by Duncan's multiple range test. DSS; dextran sodium sulfate.
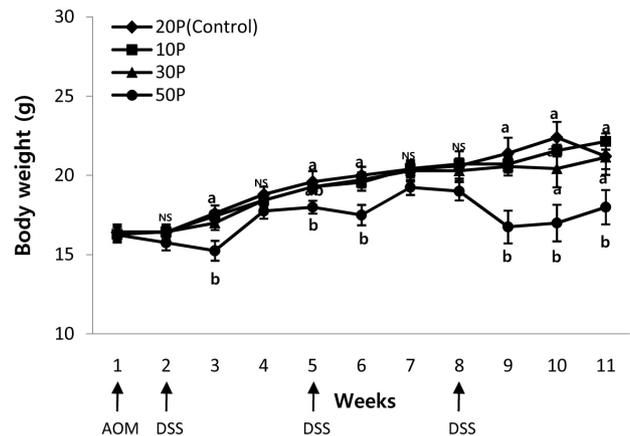

Fig. 2. Changes in body weight. Body weight was measured once a week during the experimental period. Time points of AOM injection and DSS supplementation are indicated by arrows. Values are presented as the mean ± SE. Means with different letters are significantly different at $P < 0.05$ by Duncan's multiple range test. NS, not significant; AOM, azoxymethane; DSS, dextran sodium sulfate; 20P, 20% casein; 10P, 10% casein; 30P, 30% casein; 50P, 50% casein contents of diet.

amount of DSS drunk was significantly increased in the 10P group but the amount drunk did not differ significantly between the 20P, 30P, and 50P groups. There was no significant difference in body weight between the 10P, 20P, and 30P groups throughout the experimental period (Fig. 2). However, the mean body weight of the 50P group was significantly lower than that of other 3 groups showing a consistent pattern of weight loss within 1 week after each DSS cycle. At the end of week 11, the mean body weights of the 20P, 10P, 30P, and 50P groups were 21.20 ± 0.80, 22.14 ± 0.50, 21.14 ± 1.10, and 18.00 ± 1.08 g, respectively. Taken together, 50P group showed the lowest food intake and body weight compared to those of 20P control group.

Increase in protein content in the diet exacerbated disease severity and reduced survival rate

Cyclic administration of DSS in the mouse colon carcinogenesis protocol results in a condition of chronic colitis that resembles human CAC [21-23]. To measure the symptomatic parameters of colitis, we monitored body weight loss, rectal bleeding, and change in stool consistency on a daily basis and scored them in aggregate as the disease activity index (DAI) on a scale of 0-4. As shown in Fig. 3A, the DAI of the 20P group did not change throughout the experiment. However, it was significantly increased in the 10P, 30P, and 50P groups compared to the 20P group. At 11 weeks of the experiment, the DAI of the 20P, 10P, 30P, and 50P groups were 0.00 ± 0.00, 0.85 ± 0.40, 2.42 ± 0.20, and 4.00 ± 0.00, respectively. As compared to other reports in which DSS administration induced colitis in the normal diet group as well, the reason why the DAI of the 20P group maintained a score of 0 throughout the experimental period is probably that we used 1% DSS, which is a lower concentration than normally used for inducing colitis (3-5% DSS) for 2 cycles of DSS administration due to the severe DAI of the high protein diet group. In accordance with the DAI, the survival rate of the mice was dramatically reduced in the 50P group and only 50% of the animals in the 50P group were alive at 11 weeks (Fig. 3B). The survival rates in the 20P, 10P, and 30P groups at 11weeks were 100%, 87.5%, and 87.5%, respectively.

As the degree of inflammation increases, the colon length is significantly shortened whereas the colon weight is increased in the DSS-induced colitis model [24-26]. To assess these parameters in the present study, the length and weight of the entire colorectum were measured when the experiment was terminated. We found that the weight of the colon was significantly increased but the colon length was shortened in the 30P and 50P groups compared to the 20P group (Fig. 4A, 4B). Taken together, this suggests that HPD causes more severe inflammation and disease activity in the mouse colon, which leads to reductions in mouse survival.

HPD increased proinflammatory protein expression and cell proliferation in the colon

Since clinical signs of colitis were exacerbated in HPD-fed groups, we next measured the effects of HPD on the expression of iNOS and COX-2, the main enzymes involved in the inflammatory process in the colon [27].

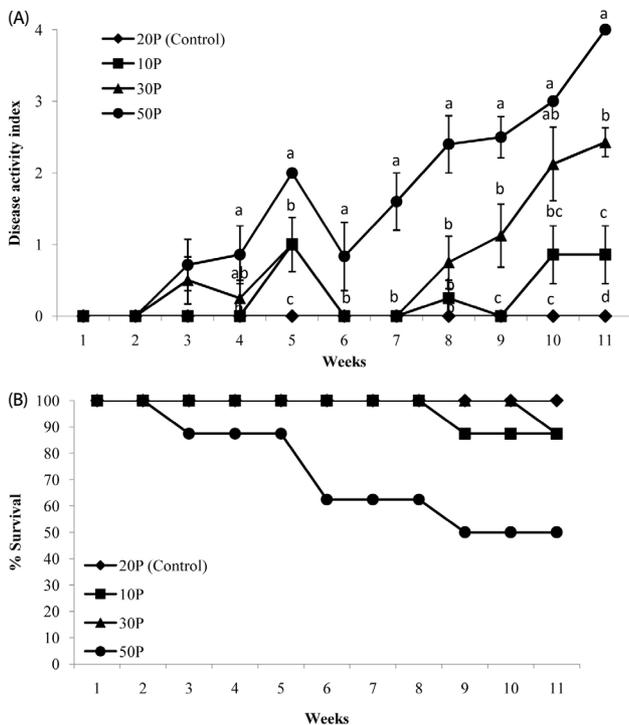COX-2 expression was significantly increased in 30P and 50P

Fig. 3. Disease activity index and the survival rate of mice. (A) Clinical scores of mice in each experimental group were monitored every day. 0, no weight loss, no occult blood in the stools, and normal stool consistency; 1, weight loss of 1-5%, no occult blood, and normal stool consistency; 2, 5-10% weight loss, positive for fecal occult blood, and loose stools; 3, 10-20% weight loss, positive for fecal occult blood, and loose stools; 4, greater than 20% weight loss, gross rectal bleeding, and diarrhea. Means with different letters are significantly different at $P < 0.05$ by Duncan's multiple range test. (B) The survival rate of mice is expressed as a percentage of live mice in each group every week. 20P, 20% casein; 10P, 10% casein; 30P, 30% casein; 50P, 50% casein contents of diet.
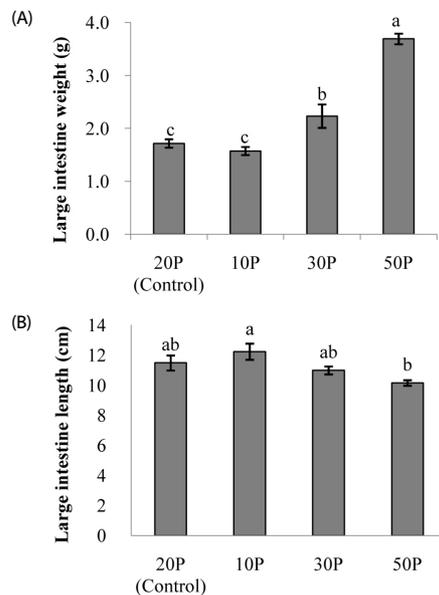

Fig. 4. Weight and length of large intestine. Colons were obtained 10 days after the last DSS cycle and their weight (A) and length (B) were measured as described in materials and methods. Values are presented as the mean ± SE. Means with different letters are significantly different at $P < 0.05$ by Duncan's multiple range test. 20P, 20% casein; 10P, 10% casein; 30P, 30% casein; 50P, 50% casein contents of diet.

Fig. 5. Expression of inflammatory proteins. The large intestine from cecum to rectum was divided into 3 parts and the distal parts were removed and homogenized. The inflammatory proteins COX-2 (A) and iNOS (B) levels were determined by immunoblotting with the appropriate antibodies. Values are presented as the mean ± SE. Means with different letters are significantly different at $P < 0.05$ by Duncan's multiple range test. 20P, 20% casein; 10P, 1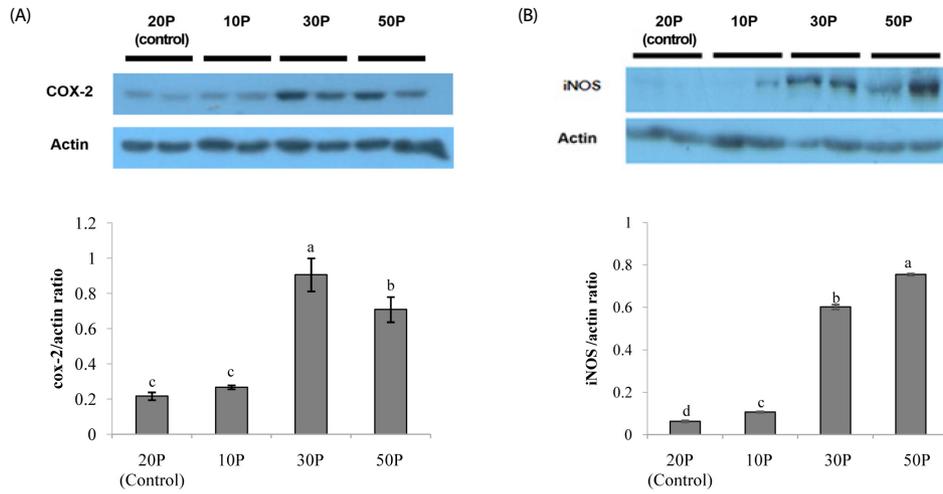0% casein; 30P, 30% casein; 50P, 50% casein contents of diet; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase.
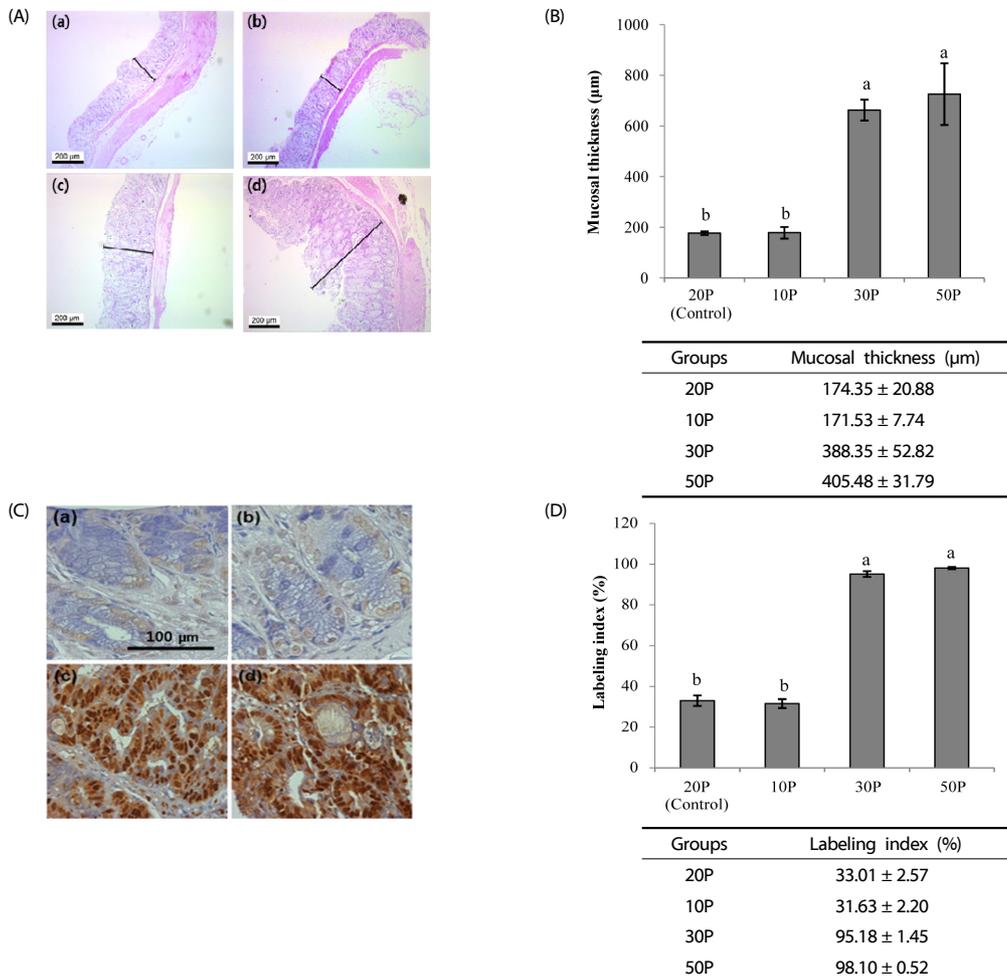

Groups	Mucosal thickness (μm)
20P	174.35 ± 20.88
10P	171.53 ± 7.74
30P	388.35 ± 52.82
50P	405.48 ± 31.79

Groups	Labeling index (%)
20P	33.01 ± 2.57
10P	31.63 ± 2.20
30P	95.18 ± 1.45
50P	98.10 ± 0.52

Fig. 6. Effect of HPD on intestinal epithelial hyperplasia and proliferation. The distal part of large intestine was removed and fixed in 10% formalin. (A) Tissue sections were stained with hematoxylin and eosin and photographed at 100X: (a) 20P (control), (b) 10P, (c) 30P, and (d) 50P groups. (B) Mucosal thickness was measured microscopically. (C) Tissue sections were immunostained for PCNA and photographed at 400X: (a) 20P (control), (b) 10P, (c) 30P, and (d) 50P groups. (D) The labeling index was calculated as the percentage of stained cells to the total number of cells in the colon epithelium. Means with different letters are significantly different at $P < 0.05$ by Duncan's multiple range test. PCNA: proliferating cell nuclear antigen. 20P, 20% casein; 10P, 10% casein; 30P, 30% casein; 50P, 50% casein contents of diet.

Table 3. Number of tumors by location

Region of large intestine	Tumor size (mm)	Groups[1]			
		20P (Control, n = 5)	10P (n = 8)	30P (n = 8)	50P (n = 8)
Proximal	< 1	1.80 ± 0.08[NS]	2.00 ± 0.93	2.86 ± 0.55	2.25 ± 0.95
	1-3	1.40 ± 0.24[NS]	2.00 ± 0.44	2.14 ± 0.67	2.00 ± 0.71
	> 3	2.20 ± 0.49[b]	2.29 ± 0.61[b]	3.29 ± 0.71[ab]	4.75 ± 0.75[a]
Middle	< 1	0.40 ± 0.24[NS]	1.14 ± 0.46	1.00 ± 0.49	0.75 ± 0.75
	1-3	0.60 ± 0.24[b]	1.00 ± 0.58[b]	3.14 ± 0.63[a]	2.25 ± 0.48[ab]
	> 3	1.20 ± 0.58[c]	1.43 ± 0.53[c]	4.86 ± 0.94[b]	9.25 ± 0.85[a]
Distal	< 1	0.00 ± 0.00[b]	0.00 ± 0.00[b]	1.57 ± 0.72[a]	0.50 ± 0.50[a]
	1-3	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.86 ± 0.40[b]	3.50 ± 1.89[a]
	> 3	0.00 ± 0.00[c]	0.43 ± 0.30[c]	4.43 ± 0.95[b]	8.00 ± 1.78[a]
Total number of tumors		7.60 ± 0.28[c]	10.29 ± 0.29[c]	24.14 ± 0.47[b]	33.25 ± 1.03[a]

[1] 20P, 20% casein; 10P, 10% casein; 30P, 30% casein; 50P, 50% casein contents of diet.
When experiment was terminated, mice in each group were sacrificed and the large intestine from cecum to rectum was taken. The intestine was divided into 3 parts (proximal, middle, and distal part) and the number of tumors by location was counted macroscopically categorized on size (< 1 mm, 1 to 3 mm, or > 3 mm) in either control or experimental diet groups. The tumor diameter was measured by a caliper. Values are Mean ± SE. Means not sharing a common letter in a row are significantly different among groups at $P < 0.05$. NS; not significant.

groups compared to the 10P and 20P groups (Fig. 5). The iNOS expression was the highest in the 50P group, followed by the 30P, 10P, and 20P groups.

Inflammation may also contribute to cell proliferation by producing cytokines that act as growth factors, providing proliferation signals and preventing apoptosis [28]. As shown in Fig. 6A and B, mucosal hyperplasia was apparent in the 30P and 50P groups. The percentage of cells positive for PCNA, an indicator of cell proliferation, was also significantly increased in the 30P and 50P groups by 287% and 296%, respectively, compared to the 20P group (Fig. 6C & 6D). These results suggest that HPD-induced inflammation may lead to more colonic mucosal cell proliferation and hyperplasia.

Colon carcinogenesis was exacerbated by HPD

An association between chronic inflammation and tumorigenesis of CRC has been well established [29]. We therefore examined the impact of HPD on colon tumor formation. The number and size of tumors was grossly observed in the proximal, middle, and distal part of the large intestine. As shown in Table 3, there was no difference between groups in the number of tumors smaller than 3 mm in diameter in the proximal part; however, the number of tumors larger than 3 mm was increased in the 30P and 50P groups. In the middle part, tumors larger than 1 mm in diameter were found more in the 30P and 50P groups than in the 20P and 10P groups. In the distal part, the number of tumors, especially those larger than 1 mm in diameter, was drastically increased in the 30P and 50P groups compared to the 20P and 10P groups. The total number of tumors was highest in the 50P group (33.25 ± 1.03), followed by the 30P group (24.14 ± 0.47). This represented a 318% and 438% increase in tumor multiplicity in the 30P and 50P groups, respectively, compared to the 20P group. Taken together, more and larger tumors were found in the 50P group than in other groups, and this effect was most prominent in the middle and distal part of the large intestine.

DISCUSSION

The effect of dietary protein on colonic mucosal inflammation and CAC has remained controversial. In the present study, we established for the first time a causal role for a high protein (casein) diet in exacerbating colonic inflammation and tumor formation in an inflammation-based murine model of tumorigenesis. An increase of just 10% in protein content (30P) compared to the protein content of a normal diet (20P) significantly increased colitis symptoms as well as inflammatory protein expression, mucosal hyperplasia, and tumor multiplicity in the colon of the mouse.

The effects of a 50% casein protein content diet were even greater. Tumor multiplicity and mucosal hyperplasia of 50P group were increased by 438% and 600%, respectively, compared to the 20P group. Moreover, only 50% of the animals in the 50P group survived to the end of experiment due to severe disease activity and tumor burden. This clearly shows that HPD accelerates inflammation-associated colonic tumorigenesis.

Of note is that, however, the 10P group also showed significantly increased DAI and colonic iNOS expression as compared to the 20P group in our study. Although mice in the 10P group drank significantly more DSS than the 20P group, this does not seem to be a reason for the higher DAI in the 10P group than the 20P group since mice in the 10P group drank even more DSS than the 50P group. Tatsuta *et al.* has reported that a low-protein diet enhanced AOM-induced colon carcinogenesis [30]. When rats were given isocaloric diet containing 25% casein (normal-protein diet), 10% casein (low-protein diet) or 5% casein (very-low-protein diet), 10% and 5% casein diet resulted in significant increases in the incidence and multiplicity of colon tumors compared to the 25% casein diet. They found significant increases in norepinephrine concentration in the colon of 10% and 5% casein diet group and this was suggested to be related to the stimulation of colon epithelial cell proliferation. In the study of Fleming *et al*, when intestinal epithelial cell proliferation in F344 rats was measured by [3H] thymidine technique, 20% casein diet resulted in modestly lower cellular proliferation

in all intestinal segments as compared to the 5% casein diet [31]. It will be important to address a question in the future why both low- and high-protein diet are not beneficial to intestine health.

In this study, we used casein as the source of protein in the diet based on the AIN-76A formula. Previous experimental studies with animal models of chemically induced colon carcinogenesis showed varied influences of dietary proteins on cancer risk depending on the source of protein. For example, whey protein and white meat such as poultry and fish appear to have a preventive effect relative to an arbitrarily established standard, while casein, red and processed meat, and soybean protein were found to have a promotional influence on the development of CRC [32,33]. However, regardless of protein source (casein, soybean protein, or white or red meat), the increase in protein content from 15% to 25% significantly increased genetic damage in colon cells. It seems that the total protein content is more important to CRC risk than the source of protein (animal-derived vs. plant-derived protein). As a possible explanation for this, Andriamihaja *et al.* found that the colonic luminal contents of ammonia, short chain fatty acids (SCFAs), and branched-chain fatty acids (BCFAs) were significantly increased on consumption of a HPD [11]. Thus, it is thought that a HPD may increase the amount of protein entering the colon, which is followed by protein fermentation (putrefaction) by colonic bacteria. Protein fermentation produces potentially toxic bacterial metabolites such as ammonia, phenolic and indolic compounds, BCFAs, and hydrogen sulfide [7,34]. These products may promote DNA damage and metabolic alterations in colonic epithelial cells, which could hamper the normal renewal of the epithelium and its homeostasis. Although whether this contributes to colorectal carcinogenesis has not been elucidated, approximately 60% of cases of CRC is found in the distal colon or rectum, where protein fermentation mainly occurs [5]. Our results also showed that tumor multiplicity became significantly higher in the distal part of large intestine as the protein content of the diet increased.

Although we provided an isocaloric diet to each experimental group, we cannot exclude the possibility that the observed effects resulted from a reduced intake of carbohydrates (sucrose) in the HPD group. However, it is believed that normally sucrose is digested and absorbed in the small intestine and does not reach the colon. Therefore, it is unlikely that undigested sucrose used by the colon microbiota affect colon tumorigenesis. In healthy rats, short-chain fatty acid concentrations in the colonic luminal content and colonocyte oxidative capacity were not different between rats on a HP lower-carbohydrate diet and isocaloric NP diet [35,36].

Chronic damage to the colon and rectum has known to increase the risk of CRC [12]. Several molecules are reported to play a critical role in contributing to CAC including COX-2 and iNOS [14,37]. Abundant evidence shows that the expression and activities of COX-2 and iNOS are increased at sites of inflammation and in neoplastic colonic epithelial cells whereas it is not detected in normal epithelium in rodents or in humans [37-42]. COX-2 is thought to contribute to tumor development by providing resistance to apoptosis [43], activation of metalloproteinase-2 [44], and stimulation of endothelial vascular

formation [45]. NO can directly damage DNA, inhibit DNA repair, enhance oncogene expression, modulate transcriptional factors, block apoptosis, and contribute to angiogenesis [46].

The effect of HPDs on inflammatory gene expression in mouse colon cancer development has not been investigated thoroughly. In this study, we showed the expression of both COX-2 and iNOS was significantly increased in the colon of 30P and 50P groups compared to the 20P control. Recently, Lan *et al.* reported that HPD (53% as whole milk protein) ingestion increased plasma concentrations of the acute-phase inflammatory proteins serum amyloid A (SAA) and IL-6 compared to the NPD (14% as whole milk protein) group in a DSS-induced acute colitis mouse model [36]. The clinical inflammatory symptoms based on the fecal consistency and blood of DSS-treated animals were also maintained higher in the HPD group than in the NPD group. Moreover, the intensity of intestinal inflammation was clearly enhanced by HPD after the DSS challenge. Although they showed that HPD was helpful in post-colitis epithelial repair, repeated cycles of DSS treatment in our AOM-DSS colon carcinogenesis protocol maintained a chronic inflammation state in which increased inflammatory proteins and gene expression due to HPD ingestion may lead to more colonic epithelial cell proliferation and tumor development as indicated by the increased mucosal hyperplasia and PCNA positive cells in 30P and 50P groups.

In conclusion, this study showed that a high level of dietary proteins in mice promotes intestinal inflammation and tumor development as evidenced by (i) severity of clinical and pathological colitis symptoms, (ii) increase of inflammatory protein (COX-2 and iNOS) expression in the colon, and (iii) enhanced mucosal hyperplasia and cell proliferation. HPD consumption is thought to cause changes of colonic microbiota composition and produces various toxic metabolites including ammonia, SCFAs, and BCFAs. How this change affects the colonic inflammatory process and tumorigenesis needs to be further investigated in future studies.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interests.

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