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Supplemental Silk Protein, Sericin, Suppresses Colon Tumorigenesis in 1,2-Dimethylhydrazine-Treated Mice by Reducing Oxidative Stress and Cell Proliferation

Siqin ZHAORIGETU,* Masahiro SASAKI,# Hiromitsu WATANABE,** and Norihisa KATO*

**Faculty of Applied Biochemistry, Hiroshima University, Higashi-Hiroshima 739-8528, Japan*
***Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima 734-8553, Japan*
#Seiren Co., Ltd., 10-1 Keya 1-Chome, Fukui 918-8560, Japan

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This study was done to discover the underlying mechanism of the inhibitory effect of sericin against colon tumorigenesis. Mice were fed a diet with 30 g/kg sericin for 115 d, and given a weekly injection of 1,2-dimethylhydrazine (10 mg/kg body weight) for the initial 10 wk. Dietary supplemental sericin caused a 62% reduction in the incidence of colonic adenoma ($P<0.05$), but did not affect the incidence of colonic adenocarcinoma. Sericin intake significantly reduced the number of colon adenomas. Consumption of sericin significantly reduced the BrdU labeling index of colonic proliferating cells and the expression of colonic c-*myc* and c-*fos*. The levels of colonic 8-hydroxydeoxyguanosine, 4-hydroxynonenal, and inducible nitric oxide synthase protein were significantly suppressed by sericin. The results suggest that dietary sericin suppresses the development of colon tumors by reducing oxidative stress, cell proliferation, and nitric oxide production.

Key words: sericin; colon tumorigenesis; cell proliferation; oxidative stress; nitric oxide

The silk protein, sericin, is the main constituent of silk (20–30% of the total cocoon weight), enveloping the fibroin with successive sticky layers.[1] When the cocoon is used for silk textiles, the sericin is mostly removed from the cocoon and disposed of without any use. Recently, we have found an antioxidant action of sericin.[2] This characteristic effect of sericin make this protein a valuable natural ingredient for cosmetic and food industries.[2] Moreover, we have shown that sericin intake has a colon tumor-preventive effect,[3] although the underlying mechanism is unknown. Since sericin is protease-resistant,[4] we postulated that the antioxidant action of undigested sericin may play a role in the mechanism of the inhibitory effect of sericin against colon tumorigenesis. To test this possibility, our study was done to investigate the influence of dietary sericin on cell proliferation, the expression of colonic c-*myc* and c-*fos* proteins being relating to cell proliferation, and colonic oxidative stress markers including 8-hydroxydeoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE). In addition, recent studies suggest that oxidative stress increases nitric oxide (NO) production and inducible NO synthase (iNOS), being considered to cause carcinogenesis.[5–7] Thus, we further attempted to analyze the expression of iNOS protein in colonic mucosa of mice treated with 1,2-dimethylhydrazine (DMH).

Materials and Methods

Animals and diets. Male CD-1 (ICR): Crj mice (5 wk old, Charles River Inc., Hino, Japan) were housed three or four to a metal cage in a room with controlled temperature (24±1°C) and a 12-h light:dark cycle (lights on, 0800–2000 h). They had free access to food and deionized water. The animals were maintained according to the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University. After feeding a commercial stock diet (MF, Oriental Yeast, Tokyo, Japan) for 1 wk, the animals (average 26 g) were divided into two groups (control and sericin). The composition of experimental diets is shown in Table 1. Sericin was added to the diet at the level of 30 g/kg. Adjustment of the dietary protein level (230 g/kg) was made by reducing dietary casein. The preparation of sericin was described in our previous study.[3] The amino acid composition of casein and sericin was measured by amino acid analyzer (LC- 10A, Shimadzu, Kyoto, Japan) (Table 2). The protein sericin has a very high content of serine (30~33%). The feeding experimental

To whom correspondence should be addressed. Norihisa KATO, PhD, TEL: +81-824-24-7980; FAX: +81-824-22-7067; E-mail: *nkato@hiroshima-u.ac.jp*

Abbreviations: DMH, 1,2-dimethylhydrazine; BrdU, 5-bromo-2′-deoxyuridine; 8-OHdG, 8-hydroxydeoxyguanosine; 4-HNE, 4-hydroxynonenal; NO, nitric oxide; iNOS, inducible nitric oxide synthase

Table 1. Composition of Experimental Diets

Ingredients	Control	Sericin
	(g/kg)	
Casein[1]	230	200
Sericin[2]	0	30
L-cystine	3	3
Corn oil	100	100
Cellulose powder	50	50
Vitamin mixture[3]	10	10
Salt mixture[4]	35	35
Sucrose	200	200
Corn starch	372	372

[1] Casein (nitrogen × 6.25), 870 g/kg.
[2] Sericin (nitrogen × 6.25), 929 g/kg.
[3] AIN-93
[4] AIN-93 G

Table 2. Composition of Amino Acids of Casein and Sericin

Amino Acid	Casein	Sericin
	Molar Percent	
Asp	6.4	19.1
Thr	4.9	6.0
Ser	5.3	30.4
Glu	19.7	4.1
Pro	11.6	0.8
Gly	3.3	12.2
Ala	4.6	4.6
Cys	0.2	<0.05
Val	7.1	2.6
Met	2.6	<0.05
Ile	5.6	1.4
Leu	8.7	0.6
Tyr	4.1	3.8
Phe	4.2	0.4
His	2.6	0.9
Lys	6.4	10.2
Arg	2.6	2.8

Values are means by triplicate analysis.

period was 115 d. Food intake and body weight were measured every day. Mice were given DMH (Nacalai Tesque, Kyoto, Japan, 10 g/L of 0.1 mol/L phosphate buffer, pH 6.8; 10 mg/kg body weight) by s.c injection once a week for the initial 10 wk of the experimental feeding. One hour before termination (1300–1600 h), 5-bromo-2′-deoxyuridine (BrdU, Sigma Chemical, St. Louis, MO, U.S.A.) was given i.p.injection (100 mg/kg body weight) for immunohistochemical analysis of cell proliferation.

Visualization and histological examination. At the end of the studies, the colon was removed, slit open longitudinally from cecum to anus, placed on a paper towel, and fixed in 10% neutral formalin for 24 h. Tumor-bearing areas and volumes were observed with a microscope and embedded in paraffin. In addition to tumor-bearing areas, the areas of flat mucosa with no visible tumors were embedded in paraffin. The colon was examined histologically after staining

Table 3. Effects of Dietary Sericin on the Incidence and Number of Colon Tumors in 1,2-Dimethylhydrazine-Treated Mice[1]

	Control	Sericin
Mice with colon tumors, n (%)		
Total	15 (37.5)	5 (12.5)*
Adenomas	13	5*
Adenocarcinomas	3	0
Tumors/mouse, n		
Total	0.55 ± 0.13	0.13 ± 0.05#
Adenomas	0.48 ± 0.12	0.13 ± 0.05#
Adenocarcinomas	0.08 ± 0.04	0

[1] Values are means ± SE (n = 40).
* Significantly different from control by X^2 test ($P < 0.05$).
# Significantly different from control by Student's *t*-test ($P < 0.05$).

with hematoxylin and eosin, and tumors in the colon were classified into two types: adenomas and adenocarcinomas. Adenomas were tumors with no evidence of invasion of muscularis mucosa. Adenocarcinomas were tumors with evidence of invasion of muscularis mucosa.

The BrdU staining method was described elsewhere.[8] BrdU-positive cells in the colonic mucosal epithelium were counted under a microscope at a magnification of ×200 in the rectum, distal colon, and proximal colon, and at least 15 crypts were observed in each area. Immunohistochemical analysis of apoptosis labeling was examined by the TUNEL method. This method is based on TdT-mediated dUTP-biotin nick end labeling of fragmented DNA.[9] After deparaffinization, they were stained by an "Apoptosis in situ Detection Kit" (Wako Pure Chemicals, Osaka, Japan). Apoptosis-positive cells in the normal colonic mucosal epithelium were counted under a microscope at a magnification of × 200.

Immunohistochemical analysis of c-*myc* and c-*fos* proteins was done for the normal colonic mucosa. The c-*myc* and c-*fos* staining was as follows. After deparaffinization, rabbit polyclonal anti-c-*myc* antibody (Santa Cruz Biochemistry, Santa Cruz, CA, U.S.A.) and rabbit polyclonal anti-c-*fos* antibody (Oncogene Research Product, Cambridge, MA, U.S.A.) were reacted with the specimens, and they were stained by "Vectastain Elite ABC Kit" (Vector Laboratories, Burlingame, CA, U.S.A.). The c-*myc* and c-*fos*-positive cells in all area of colonic mucosal epithelium were counted under the microscope at a magnification of ×200. For each area, at least 15 crypts were observed for the analysis of c-*myc* and c-*fos*-positive cells. Immunohistochemical analyses of 8-hydroxydeoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE) labeling were done for colonic mucosa. The 8-OHdG and 4-HNE staining methods were as follows. After deparaffinization, monoclonal anti-8-OHdG antibody (Nippon Yushi, Tokyo, Japan) and monoclonal anti-4-HNE antibody

(Nippon Yushi, Tokyo, Japan) were put on the specimens, and they were stained by a "Vectastain Universal quick Kit" (Vector Laboratories, Burlingame, CA, U.S.A.). The 8-OHdG and 4-HNE positive cells in the colonic mucosal epithelium were counted under the microscope at a magnification of ×200. For each area of colonic mucosal epithelium, at least 15 crypts were observed for the analysis of 8-OHdG and 4-HNE positive cells.

Immunohistochemical analysis of iNOS protein was done for the normal colonic mucosa. The iNOS staining was as follows. After deparaffinization, rabbit polyclonal anti-iNOS antibody (Santa Cruz Biochemistry, Santa Cruz, CA) was put on the specimens, and they were stained by "Vectastain Elite ABC Kit" (Vector Laboratories, Burlingame, CA). The iNOS-positive cells in the colonic mucosal epithelium were counted under the microscope at a magnification of ×200.

Values are presented as means ± SEM. To evaluate the differences among groups, the data except for tumor incidence were analyzed by Student's *t*-test. Tumor incidence was analyzed by the x^2 test. Some data were analyzed by regression analysis and the correlation coefficient was calculated. Differences with $P<0.05$ were considered significant.

Results

Body weight and food intake

Final body weight and food intake for 115 d were not significantly different between the two groups (data not shown).

Colonic tumors

The incidence of colon tumors (adenomas and adenocarcinomas) was significantly reduced by sericin intake ($P<0.05$, Table 3). The volumes of tumors were unaffected by dietary manipulation (data not shown). Colonic tumors were located mainly in the distal colon of all the groups. The majority of the tumors were identified as adenomas and only three of the 40 mice fed the control diet developed adenocarcinomas. No adenocarcinomas were observed in the mice fed the sericin diet. Only one mouse fed the control diet had both an adenoma and an adenocarcinoma in the colon. Most adenomas and adenocarcinomas were well differentiated, and the histological type was not affected by supplemental sericin. The number of the total tumors in the sericin group was also significantly lower than that in the control group ($P<0.01$, Table 3)

Cell proliferation and apoptosis

Dietary sericin significantly suppressed the labeling index of proliferation cells in all area of the normal-like colonic epithelium ($P<0.05$, Table 4). The position of the highest BrdU labeled cell counted from the bottom of the crypt was also significantly reduced by supplemental sericin ($P<0.01$, Table 4). The number of cells per crypt column was unaffected by supplemental sericin ($P>0.05$, Table 4). The labeling index of apoptosis cells was unaffected by supplemental sericin ($P>0.05$, Table 4).

Table 4. Effects of Dietary Sericin on the Colonic Cell Proliferation and Apoptosis in 1,2-Dimethylhydrazine-Treated Mice[1]

Group	Control	Sericin
Labeling index of Proliferative cells, %		
Rectum	8.03 ± 0.42	6.15 ± 0.34*
Distal colon	8.19 ± 0.40	6.56 ± 0.31*
Proximal colon	7.45 ± 0.26	4.98 ± 0.35*
All colon[2]	7.92 ± 0.29	5.90 ± 0.32*
Position of the highest BrdU[3] labeled cell, n		
Rectum	4.59 ± 0.33	3.20 ± 0.20*
Distal colon	4.62 ± 0.35	3.42 ± 0.19*
Proximal colon	3.14 ± 0.27	2.34 ± 0.18*
Labeling index of Apoptosis cells, %	1.15 ± 0.28	1.07 ± 0.24
Cells/crypt column, n	21.2 ± 0.2	21.5 ± 0.2

[1] Values are means ± SE (n = 20). *Significantly different from control by Student's *t*-test ($P<0.01$).
[2] Rectum, distal colon, and proximal colon. [3] 5-bromo-2′-deoxyuridine.

Table 5. Effects of Dietary Sericin on Colonic Oncogene Protein Expression in 1,2-Dimethylhydrazine-Treated Mice[1]

Group	Control	Sericin
Labeling index of c-*myc* expression cells, %		
Rectum	8.24 ± 0.30	6.10 ± 0.20*
Distal colon	8.65 ± 0.41	5.85 ± 0.26*
Proximal colon	6.01 ± 0.30	4.42 ± 0.27*
All colon[2]	7.63 ± 0.24	5.46 ± 0.24*
Labeling index of c-*fos* expression cells, %		
Rectum	11.65 ± 1.05	7.89 ± 0.43*
Distal colon	11.63 ± 1.06	7.99 ± 0.49*
Proximal colon	8.68 ± 0.71	6.27 ± 0.48*
All colon	10.66 ± 0.93	7.39 ± 0.46*

[1] Values are means ± SE (n = 20). *Significantly different from control by Student's *t*-test ($P<0.01$).
[2] Rectum, distal colon, and proximal colon.

c-Myc and c-fos expression

The sericin diet significantly reduced the expression of c-*myc* and c-*fos* proteins in all area of the colonic crypt compared to the control diet ($P<0.01$, Table 5).

Oxidative stress markers

Labeling index of 8-OHdG in all areas of the colonic crypt was significantly reduced by supplemental sericin ($P<0.01$, Table 6). The Labeling index of 8-OHdG observed in all the colon epithelium was correlated with the labeling index of c-*myc* and c-*fos*

Table 6. Effects of Dietary Sericin on Colonic Oxidative Stress Markers in 1,2-Dimethylhydrazine-Treated Mice[1]

Group	Control	Sericin
Labeling index of 8-OHdG[2] expression cells, %		
Rectum	7.10±0.51	4.56±0.30*
Distal colon	7.19±0.55	4.83±0.30*
Proximal colon	5.50±0.50	3.32±0.21*
All colon[3]	6.58±0.51	4.24±0.24*
Labeling index of 4-HNE[2] expression cells, %		
Rectum	8.68±0.18	5.84±0.19*
Distal colon	8.60±0.24	5.89±0.22*
Proximal colon	7.33±0.19	4.23±0.11*
All colon	8.16±0.16	5.32±0.12*

[1] Values are means ± SE (n = 20). *Significantly different from control by Student's *t*-test ($P < 0.01$).
[2] 8-OHdG: 8-hydroxy-deoxyguanosine, 4-HNE: 4-hydroxynonenal.
[3] Rectum, distal colon, and proximal colon.

proteins in all the colon epithelium (c-*myc*: $r = 0.70$, $P < 0.01$, c-*fos*: $r = 0.84$, $P < 0.01$, respectively). Similarly, the sericin diet significantly lowered the 4-HNE levels in all areas of the colonic crypt compared to the control diet ($P < 0.01$, Table 6). The labeling index of 4-HNE observed in all the colon epithelium was correlated with the labeling index of c-*myc* and c-*fos* proteins in all the colon epithelium (c-*myc*: $r = 0.62$, $P < 0.01$, c-*fos*: $r = 0.75$, $P < 0.01$, respectively).

iNOS expression

Labeling index of iNOS protein in the all colonic crypt was significantly reduced by supplemental sericin ($P < 0.05$, Table 7). In all the colon epithelium, the labeling index of iNOS observed was correlated with the labeling index of 8-OHdG and 4-HNE (8-OHdG: $r = 0.57$, $P < 0.01$, 4-HNE: $r = 0.52$, $P < 0.01$, respectively).

Discussion

Recently, we have shown that dietary supplemental sericin protects against colon tumorigenesis in mice treated with DMH.[3] This study further examined the underlying mechanism of the inhibitory effect of sericin against colon tumorigenesis. Consumption of sericin was found to reduce cell proliferation in colon epithelium, although the supplemental sericin had no significant effect on colon epithelium apoptosis. The results suggest that reduced cell proliferation is, at least in part, responsible for the protective effect of sericin against colon tumorigenesis. The position of the highest BrdU labeled cell was also significantly lower in the sericin group compared to control group in all area of the colonic crypt, implying that supplemental sericin reduced the region of cell proliferation occurring. We further found a suppression in the protein expression of proliferation-related genes, c-*myc* and c-*fos* by sericin. c-*Myc* and c-*fos* are well known to be associated with a variety of carcinogeneses.[10] In addition, some studies showed that the expression of c-*myc* and c-*fos* protein was elevated in normal and neoplastic tissues of the rat colon by the colon tumorigenesis inducer.[11,12] As expected, the c-*myc* and c-*fos* expression in normal colonic crypt was significantly reduced by supplemental sericin. In view of these results, the inhibitory effect of sericin on colon cell proliferation might be mediated through alteration of these oncogene expressions.

Table 7. Effects of Dietary Sericin on Colonic Expression of Inducible Nitric Oxide Synthase (iNOS) Protein in 1,2-Dimethylhydrazine-Treated Mice[1]

Group	Control	Sericin
Labeling index of iNOS Expression cells, %		
Rectum	10.61±0.72	9.06±0.16
Distal colon	11.06±0.65	9.10±0.24*
Proximal colon	8.08±0.62	7.36±0.16
All colon[2]	9.92±0.62	8.37±0.15*

[1] Values are means ± SE (n = 20). *Significantly different from control by Student's *t*-test ($P < 0.05$).
[2] Rectum, distal colon, and proximal colon.

This study further indicated that supplemental sericin suppressed colonic oxidative stress markers including 8-OHdG and 4-HNE. Oxidative stress and lipid peroxidation have been suggested to play a role in tumorigenesis. Free radical lipid peroxidation might be involved in tumor promotion and progression of tumorigenesis.[13,14] This study indicated that the labeling index of 8-OHdG and 4-HNE observed in all the colon epithelium were correlated with the labeling index of c-*myc* and c-*fos* proteins in all the colon epithelium. c-*Myc* and c-*fos* have been reported be elevated by oxidative stress.[15,16] 8-OHdG is a product of DNA damage by oxygen radicals. Production of 8-OHdG induces mutagenesis and tumorigenesis by causing misreading of DNA bases.[17,18] In addition, 4-HNE is produced as a major product of the peroxidation of $\omega 6$ polyunsaturated fatty acids and possesses cytotoxic, hepatotoxic, mutagenic, and genotoxic properties.[19,20] Elevated levels of 4-HNE were found in plasma and various organs under conditions of oxidative stress.[21,22] Elevated levels of the products of lipid peroxidation including 4-HNE were found in colon tumorigenesis.[21,23] In this study, dietary sericin significantly reduced the levels of these oxidative stress markers in the colon, being associated with the alterations in c-*myc* and c-*fos* proteins. Thus, the reduced oxidative stress in the colon of mice fed sericin might at least in part lead to the reduction of hyper-cell proliferation.

This study further demonstrated that supplemental sericin suppressed the expression of colonic iNOS protein, being associated with the alterations in oxidative stress markers. Recently, it has been suggested that higher NO or higher activity of iNOS in-

creases colon carcinogenesis.[24-26] Such an effect of NO appears to be at least in part mediated through higher angiogenesis and lower apoptosis.[5,27-29] iNOS has also been shown to be involved in the up-regulation of cyclooxygenase-2, which plays a pivotal role in colon tumorigenesis.[30] From these facts, the reduced expression of iNOS by supplemental sericin may also be partially responsile for the preventive effect of dietary sericin against colon tumorigenesis.

This study provided evidence that dietary sericin caused a reduction in colonic cell proliferation, oxidative stress markers and iNOS protein. Sericin is resistant to several proteases, and has some physiological functions such as strong water-holding capacity and antioxidant activity.[4,31] The antioxidant activity of sericin appears to be at least in part due to its strong affinity for copper and other metal ions because of its high contents of hydroxyl and carboxyl groups (Kato *et al.* unpublished data). Undigested sericin may suppress colonic oxidative stress, leading to a reduction in cell proliferation, NO production and tumor development. Further study is in progress in our laboratory to examine if the contents of the large intestine in mice fed a sericin diet has an antioxidant effect on colonic mucosa. Since our study was done with mice that received DMH for the initial 10 wk, the possibility that sericin affects the initiation stage in colon carcinogenesis remains to be studied.

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