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Inhibition of Azoxymethane-initiated Colon Tumor by Bovine Lactoferrin Administration in F344 Rats

Kazunori Sekine,[1,5] Emiko Watanabe,[1] Joe Nakamura,[1] Nobuo Takasuka,[1] Dae Joong Kim,[1] Makoto Asamoto,[1] Vladimir Krutovskikh,[1] Hiroyasu Baba-Toriyama,[1] Tomonori Ota,[1] Malcolm A. Moore,[1] Mitsuharu Masuda,[2] Hajime Sugimoto,[2] Hoyoku Nishino,[4] Tadao Kakizoe[3] and Hiroyuki Tsuda[1]

[1]Chemotherapy Division, [2]Cancer Prevention Division, National Cancer Center Research Institute, [3]National Cancer Center Central Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104 and [4]Department of Biochemistry, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602

The influence of bovine lactoferrin (bLF) on colon carcinogenesis was investigated in male F344 rats treated with azoxymethane (AOM). Following three weekly injections of AOM, the animals received 2 or 0.2% bLF for 36 weeks. No effects indicative of toxicity were noted, but significant reduction in both the incidence and number of adenocarcinomas of the large intestine was observed with both doses. Thus, the incidences of adenocarcinomas in the groups receiving 2% and 0.2% bLF were 15% and 25%, respectively, in contrast to the 57.5% control value ($P<0.01$ and $P<0.05$, respectively). The results indicate that bLF might find application for chemoprevention of colon cancer.

Key words: Lactoferrin — Colon cancer — Azoxymethane

Neoplasia of the colon is a leading cause of cancer death in the United States and other developed countries and numerous epidemiological studies and a plethora of experimental evidence point to a role for dietary factors in the development of the disease.[1)] The recent drastic increase in the incidence of colon cancer patients in Japan is also thought to be related to the adoption of a more western style diet over the last three decades.[2)] This has generated a great deal of interest in which environmental agents might exert an influence, and the possibility of employing chemicals which can prevent neoplasia is now attracting increasing attention.[3)]

Milk and its products are major dietary constituents in the west but because of the range of included ingredients the question of what role they might play remains to be determined. However, it has recently been established that the whey fraction of bovine milk can significantly inhibit the development of tumors in the colons of rats given dimethylhydrazine (DMH).[4)] This fraction contains five major proteins, α-lactalbumin, β-lactoglobulin, immunoglobulin, bovine serum albumin and lactoferrin (bLF). This last, an 80 kDa siderophilic protein which has two iron-binding sites per molecule, is well known to have bacteriostatic properties.[5)] In addition to sequestration of the ferric ion necessary for microbes to grow, it activates natural killer (NK) cells[6,7)] and neutrophils,[8)] induces colony-stimulating activity,[9)] stimulates lymphokine-activated killer (LAK) cells[7)] and augments macrophage cytotoxicity.[10)] It is present in large amounts in mammalian secretions such as tears, saliva and seminal fluid, as well as being particularly abundant in colostrum.[11)] However, despite extensive studies of the antimicrobial properties, little information is available regarding the influence of lactoferrin on disease processes. This is unfortunate, since Bezault *et al.* have pointed out a protective influence against growth of solid tumors and development of experimental metastases in mice.[12)] They argued that the action of lactoferrin might have been mediated by NK cells, in line with its stimulation of NK and LAK cell activity *in vitro* and *in vivo*.[7)]

In the present study, in view of the high priority now accorded to finding effective chemopreventors of colon cancer in man, we conducted an investigation of the influence of lactoferrin on experimental colon carcinogenesis. For this purpose we chose an established model utilizing azoxymethane (AOM) administration to F344 rats,[13)] to assess the post-initiation effects of bLF on histopathologically diagnosed tumor development. The carcinogen AOM (CAS: 25843-45-2) was obtained from Sigma (St. Louis, MO). bLF[14)] was kindly provided by Drs. Tomita and Shimamura of Morinaga Milk Industry Co., Ltd. (Zama). Male F344 rats were purchased from Charles River Japan (Atsugi) at 5 weeks of age and maintained in plastic cages in an air-conditioned room under constant conditions of temperature (22±2°C) and humidity (55±10%). The animals were allowed free

[5] To whom correspondence should be addressed.

access to basal diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) and tap water and were used in the experiment after a 1 week acclimation period. A total of 215 male rats were divided into 3 groups (see Fig. 1). Starting at 6 weeks of age, those in groups 1 and 2 were administered AOM (15 mg/kg body weight s.c.) once a week for three weeks. One week after the last injection of AOM, animals in group 1 were fed diet containing 2% (40 rats) or 0.2% bLF (20). Animals in group 2 received the basal diet alone (40). In group 3, rats were fed the basal (5), 2% bLF (5), or 0.2% bLF (5) diet without prior carcinogen exposure. All animals were weighed weekly and their condition was checked daily throughout the experiment. Rats demonstrating loss of condition and marked weight reduction (more than 20 g in one week) during weeks 26–39, mainly due to development of duodenal adenocarcinomas, were killed and autopsied (basal diet, 8 animals; 2% bLF, 2; 0.2% bLF, 1). At week 40 all surviving animals were killed under ether anesthesia and carefully autopsied. The major organs, with especial attention to the intestines, were inspected before fixation of tissue samples, including all lesions, in 10% buffered formalin. The tissues were then routinely processed for embedding in paraffin and histopathological examination of hematoxylin and eosin-stained sections. Carcinomas were classified into invasive and non-invasive categories depending on the presence or absence of tumor elements within the submucosa. The significance of differences between group means was assessed with Dunnet's t test. The data for tumor incidence were analyzed using Fisher's exact probability test. The analyses were all carried out with the JMP software package (Version 3.1, SAS Institute Japan) on a Macintosh.

Data for body and organ (liver, right and left kidney) weights of the animals killed at 40 weeks are summarized in Table I. No significant intergroup variation was observed for the groups of animals given AOM. Slight increases were detected in group 3 without AOM injection compared with groups 1 and 2, but no statistically significant influence of bLF was apparent.

Incidences and multiplicities of small and large intestinal tumors are given in Table II. In the small intestines, no adenomas were found and all the malignant lesions detected were of invasive adenocarcinoma type. A tendency for reduction was observed in group 1 as compared with group 2 regarding the incidence and the multiplicity, but this was without significance.

In the large intestine, while the development of adenomas was not altered by any of the treatments, both doses of bLF significantly reduced the incidence and the multiplicity of carcinomas. In addition, 2% bLF caused significant decreases of total tumor incidences (adenomas

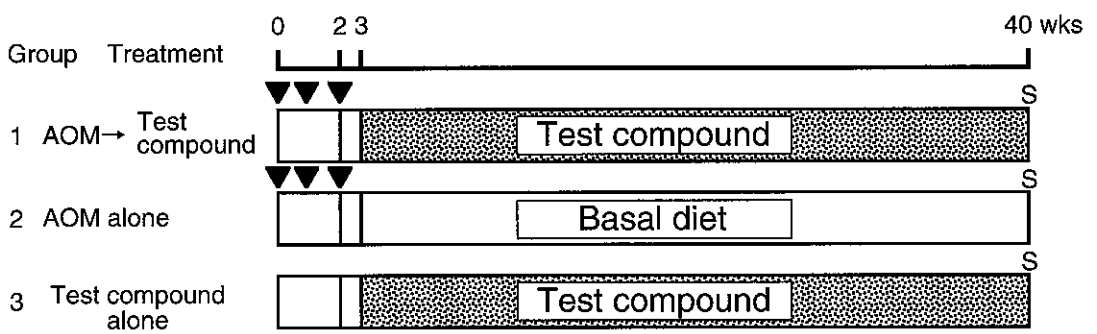

Fig. 1. Experimental protocol. ▼, AOM 15 mg/kg body weight subcutaneous injection; S, animals were killed.

Table I. Body and Organ Weights of AOM-treated Male F344 Rats at Week 40

Group	Test compound	No. of animals	Body (g)	Liver (g)	Kidney (g)	
					right	left
1	2% bLF	38	391.7±27.9[a)]	9.41±1.14	1.04±0.12	1.06±0.12
	0.2% bLF	19	392.4±31.6	9.32±1.07	1.05±0.10	1.06±0.09
2	Basal diet	32	391.2±33.9	9.82±0.87	1.07±0.08	1.10±0.15
3	2% bLF	5	409.2±27.4	10.45±1.30	1.03±0.08	1.02±0.07
	0.2% bLF	5	399.5±17.4	9.01±0.95	0.95±0.08	1.03±0.09
	Basal diet	5	410.2±32.6	10.15±0.80	1.08±0.06	1.09±0.04

a) Mean±SD.

Table II. Effects of bLF on the Incidences and Multiplicities of Intestinal Tumors in AOM-initiated Male F344 Rats

Group	Test compound	No. of animals	Incidence			Multiplicity		
			Adenoma	Carcinoma	Adenoma +carcinoma	Adenoma	Carcinoma	Adenoma +carcinoma
Small intestine								
1	2% bLF	40[a]	0 (0.0)[b]	5 (12.5)	5 (12.5)	0	0.13±0.33	0.13±0.33
	0.2% bLF	20[a]	0 (0.0)	4 (20.0)	4 (20.0)	0	0.20±0.41	0.20±0.41
2	Basal diet	40[a]	0 (0.0)	9 (22.5)	9 (22.5)	0	0.28±0.55	0.28±0.55
Large intestine								
1	2% bLF	40[a]	17 (42.5)	6 (15.0)[d]	21 (52.5)[c]	0.55±0.75	0.18±0.45[d]	0.73±0.88[c]
	0.2% bLF	20[a]	7 (35.0)	5 (25.0)[c]	11 (55.0)	0.50±0.76	0.35±0.67[c]	0.85±0.93
2	Basal diet	40[a]	14 (35.0)	23 (57.5)	31 (77.5)	0.43±0.75	0.83±0.90	1.25±0.76

a) Effective numbers include rats during weeks 26–39.
b) Values in parenthesis are percentages.
c) $P<0.05$ compared with group 2.
d) $P<0.01$ compared with group 2.

+ carcinomas) and also reduced the multiplicities. Both invasive and non-invasive carcinomas appeared to be equally affected. No obvious intergroup differences were evident in the histopathological appearance of tumors. There was no clear dose dependence for the inhibitory potential of bLF. No evidence of toxicity in the organs of animals treated with bovine lactoferrin was found and none of the rats in group 3 (not administered AOM) developed any proliferative lesions in either the small or the large intestines.

The results of the present study provide clear evidence of an inhibitory potential of bLF against development of malignant tumors in the rat colon when given in the post-initiation stage. With regard to lactoferrin effects on neoplasia, there has so far been only one publication, documenting reduction of fibrosarcoma development and metastasis from a transplantable melanoma line in mice.[12] However, population studies in man have indicated a protective influence of milk and other dairy foods against various tumors, including colorectal cancer,[15,16] although there is a lack of any consensus about this point, presumably reflecting the variety of ingredients which are included in milk preparations. However, experimental studies in rats and mice have generated much support for the idea that some proteinaceous component of milk exerts a beneficial influence. Thus tumor growth was found to be inhibited by dairy products.[17] Inhibition of the initiation stage of colon cancer in rats has been shown with skim milk using ACF as endpoint lesions.[18] Attention has been concentrated on whey protein in mice by the group of Bounous,[19] who established purified preparations to be more efficacious than casein against DMH-induced colon carcinogenesis. Papenburg *et al.*[20] subsequently demonstrated marked retardation in terms of both number and size of DMH-induced tumors as well as enhanced activity of the immune system. Whey protein was also confirmed to exert stronger inhibitory potential than casein in the rat intestine by McIntosh *et al.*[4] Interestingly, administration of whey protein was further found to reduce tumor growth in patients with metastatic carcinomas.[21] Therefore the results of the present study, viewed together with the information available for whey protein in the literature, strongly suggest that bLF holds great promise as a natural chemopreventive agent very suitable for practical use in the human situation. While bLF is present in raw milk at concentrations ranging from 0.02–0.2 mg/ml in the bovine case to over 2 mg/ml in man, it is denatured by the heat treatment applied for pasteurization or UHT processing (personal communication, Dr. Kawase, Morinaga Milk Industry) and therefore it is not contained in commercially available milk beverages. However, it is found at relatively high concentrations in colostrum (approximately 10 mg/ml), and may therefore be related to anti-bacterial and immunoprotective actions. Indeed, lactoferrin is well known to act against bacteria and stimulate the immune response.[11,22] The latter findings might be relevant to the mechanisms underlying the observed inhibition. Another possibility which also warrants attention is the effect on proliferation. Since in this case a post-initiation model was applied, any influence of bLF on carcinogen metabolism could be ignored.

In conclusion, the present findings indicate that bLF may be a promising chemopreventor of colon carcinogenesis. Confirmatory studies and investigations of the possible mechanisms of action are under way.

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REFERENCES

1) Giovannucci, E. and Willett, W. C. Dietary factors and risk of colon cancer. *Ann. Med.,* **26,** 443–452 (1994).
2) Wynder, E. L., Fujita, Y., Harris, R. E., Hirayama, T. and Hiyama, T. Comparative epidemiology of cancer between the United States and Japan. A second look. *Cancer,* **67,** 746–763 (1991).
3) Wattenberg, L. W. Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res.* (Suppl.), **52,** 2085s–2091s (1992).
4) McIntosh, G. H., Regester, G. O., Le, L. R., Royle, P. J. and Smithers, G. W. Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. *J. Nutr.,* **125,** 809–816 (1995).
5) Lonnerdal, B. and Iyer, S. Lactoferrin: molecular structure and biological function. A review. *Ann. Rev. Nutr.,* **15,** 93–110 (1995).
6) Nishiya, K. and Horwitz, D. A. Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody-dependent cell-mediated cytotoxicity. *J. Immunol.,* **129,** 2519–2523 (1982).
7) Shau, H., Kim, A. and Golub, S. H. Modulation of natural killer and lymphokine-activated killer cell cytotoxicity by lactoferrin. *J. Leukocyte Biol.,* **51,** 343–349 (1992).
8) Gahr, M., Speer, C. P., Damerau, B. and Sawatzki, G. Influence of lactoferrin on the function of human polymorphonuclear leukocytes and monocytes. *J. Leukocyte Biol.,* **49,** 427–433 (1991).
9) Sawatzki, G. and Rich, I. N. Lactoferrin stimulates colony stimulating factor production *in vitro* and *in vivo*. *Blood Cells,* **15,** 371–385 (1989).
10) McCormick, J. A., Markey, G. M. and Morris, T. C. Lactoferrin-inducible monocyte cytotoxicity for K562 cells and decay of natural killer lymphocyte cytotoxicity. *Clin. Exp. Immunol.,* **83,** 154–156 (1991).
11) Levay, P. F. and Viljoen, M. Lactoferrin: a general review. *Haematologica,* **80,** 252–267 (1995).
12) Bezault, J., Bhimani, R., Wiprovnick, J. and Furmanski, P. Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. *Cancer Res.,* **54,** 2310–2312 (1994).
13) Reddy, B. S., Simi, B., Patel, N., Aliaga, C. and Rao, C. V. Effect of amount and types of dietary fat on intestinal bacterial 7 α-dehydroxylase and phosphatidylinositol-specific phospholipase C and colonic mucosal diacylglycerol kinase and PKC activities during stages of colon tumor promotion. *Cancer Res.,* **56,** 2314–2320 (1996).
14) Tomita, M., Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H. and Kawase, K. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J. Dairy Sci.,* **74,** 4137–4142 (1991).
15) Kampman, E., Goldbohm, R. A., Van den Brandt, P. A. and Van't Veer, P. Fermented dairy products, calcium, and colorectal cancer in the Netherlands cohort study. *Cancer Res.,* **54,** 3186–3190 (1994).
16) Sorenson, A. W., Slattery, M. L. and Ford, M. H. Calcium and colon cancer: a review. *Nutr. Cancer,* **11,** 135–145 (1988).
17) Tsuru, S., Shinomiya, N., Taniguchi, M., Shimazaki, H., Tanigawa, K. and Nomoto, K. Inhibition of tumor growth by dairy products. *J. Clin. Lab. Immunol.,* **25,** 177–183 (1988).
18) Abdelali, H., Cassand, P., Soussotte, V., Daubeze, M., Bouley, C. and Narbonne, J. F. Effect of dairy products on initiation of precursor lesions of colon cancer in rats. *Nutr. Cancer,* **24,** 121–132 (1995).
19) Bounous, G., Papenburg, R., Kongshavn, P. A., Gold, P. and Fleiszer, D. Dietary whey protein inhibits the development of dimethylhydrazine induced malignancy. *Clin. Invest. Med.,* **11,** 213–217 (1988).
20) Papenburg, R., Bounous, G., Fleiszer, D. and Gold, P. Dietary milk proteins inhibit the development of dimethylhydrazine-induced malignancy. *Tumour Biol.,* **11,** 129–136 (1990).
21) Kennedy, R. S., Konok, G. P., Bounous, G., Baruchel, S. and Lee, T. D. The use of a whey protein concentrate in the treatment of patients with metastatic carcinoma: a phase I–II clinical study. *Anticancer Res.,* **15,** 2643–2649 (1995).
22) Brock, J. Lactoferrin: a multifunctional immunoregulatory protein? *Immunol. Today,* **16,** 417–419 (1995).