Biometals (2010) 23:399–409
DOI 10.1007/s10534-010-9331-3

Cancer prevention by bovine lactoferrin: from animal studies to human trial

Hiroyuki Tsuda · Takahiro Kozu · Gen Iinuma · Yasuo Ohashi · Yutaka Saito · Daizo Saito · Takayuki Akasu · David B. Alexander · Mitsuru Futakuchi · Katsumi Fukamachi · Jiegou Xu · Tadao Kakizoe · Masaaki Iigo

Received: 5 February 2010 / Accepted: 23 March 2010 / Published online: 21 April 2010

Abstract Colorectal cancer (CRC) is one of the most frequently diagnosed cancers and, despite improved colonoscopic screening, CRC is a leading cause of death from cancer. Administration of bovine lactoferrin (bLF) suppresses carcinogenesis in the colon and other organs of test animals, and recently it was shown that ingestion of bLF inhibits the growth of adenomatous polyps in human patients. Here we review work which established bLF as an anti-carcinogenic agent in laboratory animals and the results of a clinical trial which demonstrated that bLF can reduce the risk of colon carcinogenesis in humans.

Keywords Bovine lactoferrin · Chemoprevention · Colorectal cancer

Introduction

Neoplasia of the colon is a major cause of cancer death in developed and, recently, in developing countries (Rehman et al. 2009). Numerous epidemiological and experimental findings indicate a role for dietary factors in the development of colon cancer (Freiburghaus et al. 2009). The recent dramatic increase in the incidence of colon cancer patients in Japan is also thought to be related to diet; specifically, the adoption of a more western style diet over the last three decades (Rehman et al. 2009).

Lactoferrin, an 80 kDa siderophilic protein which has two iron binding sites per molecule, is well known to have anti-bacterial properties (Lonnerdal

H. Tsuda · D. B. Alexander (✉) · M. Iigo
Nanotoxicology Project, Graduate School of Medical Sciences, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
e-mail: dalexand@med.nagoya-cu.ac.jp

T. Kozu
Cancer Screening Division, National Cancer Center, Research Center for Cancer Prevention and Screening, Tokyo, Japan

G. Iinuma
Diagnostic Radiology Division, National Cancer Center Hospital, Tokyo, Japan

Y. Ohashi
Department of Biostatistics, School of Health Sciences and Nursing, The University of Tokyo, Tokyo, Japan

Y. Saito · D. Saito
Endoscopy Division, National Cancer Center Hospital, Tokyo, Japan

T. Akasu
Colorectal Surgery Division, National Cancer Center Hospital, Tokyo, Japan

H. Tsuda · D. B. Alexander · M. Futakuchi · K. Fukamachi · J. Xu · M. Iigo
Department of Molecular Toxicology, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan

T. Kakizoe
National Cancer Center, Tokyo, Japan

and Iyer 1995). It is present in mammalian secretions like tears, saliva and seminal fluid as well as being particularly abundant in colostrum (Levay and Viljoen 1995). One of the initial findings that suggested that ingestion of bovine lactoferrin (bLF) could inhibit tumorigenesis was published in 1995: In this study it was established that the whey fraction of bovine milk could significantly inhibit the development of colon tumors in rats given dimethylhydrazine (DMH) (McIntosh et al. 1995). The whey fraction is composed of five major proteins: α-lactalbumin, β-lactoglobulin, immunoglobulin, bovine serum albumin and lactoferrin. At the time the study was published, it was known that lactoferrin was present in the specific granules of neutrophils (Cramer et al. 1985), and that it stimulated neutrophil motility and superoxide production, activated NK cells, and strongly augmented LAK cell activity and macrophage cytotoxicity (Gahr et al. 1991; McCormick et al. 1991; Nishiya and Horwitz 1982; Shau et al. 1992). A year before McIntosh et al. published their results, another group demonstrated that intraperitoneal injection of human LF inhibited the growth of solid tumors and the development of experimental metastases in mice (Bezault et al. 1994). Thus, in the mid 1990s, it was determined that lactoferrin was present in a milk fraction that inhibited the development of colon tumors, it was able to activate immune responses that would be expected to kill tumor cells, and it was able, at least under certain conditions, to inhibit carcinogenesis and metastasis in animal models.

Here, we review work which elaborated the preventive effects of bovine lactoferrin (bLF), bovine lactoferrin-hydrolysates (bLFH) and bovine lactoferricin (bLFcin) on colon and other organ carcinogenesis and metastasis and the findings of a human trial on the effects of bLF on the growth of adenomatous polyps.

Experiments which provided key insights into bLF mediated suppression of carcinogenesis

Suppression of colon carcinogenesis

To evaluate possible suppression by bLF on the post-initiation stage of colon carcinogenesis, a post-initiation model using 6 week old Fischer 344 male rats was utilized (Sekine et al. 1997; Tsuda and Sekine 2000). Rats were administered a known colon carcinogen, azoxymethane (AOM), for initiation of colon carcinogenesis, and 1 week after the last AOM treatment animals were fed basal diet alone (control group) or diet containing 0.2 or 2% bLF. At week 40, all surviving animals were killed and major organs were histopathologically examined. The incidence of adenocarcinomas in the large intestine in the animals receiving 2 and 0.2% bLF were 26.0% ($P < 0.01$) and 43.5% ($P < 0.05$) of the control. The multiplicity (numbers of tumors per animal) was also significantly reduced in the bLF fed groups. Cell proliferation in the carcinoma lesions, as assessed by 5-bromo-2′-deoxyuridine (BrdU) labeling indices, was significantly decreased in the 2 and 0.2% bLF fed rats compared to the control rats. In addition to bLF, both bLFH and bLFcin inhibited AOM initiated colon carcinogenesis (Tsuda et al. 1998). These results provided clear evidence of an inhibitory potential of bLF against rat colon tumor development when given in the post-initiation stage of carcinogenesis.

Suppression of bladder carcinogenesis

In a similar model, the suppressive effects of bLF on rat bladder carcinogenesis were investigated (Masuda et al. 2000). Six week old F344 male rats were treated with 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), a bladder carcinogen, in their drinking water for 8 weeks and after a 1-week interval received basal diet alone (control group) or diet containing 0.2 or 2% bLF. All rats were killed at the end of week 36. Animals receiving 2% bLF demonstrated a significant decrease in the multiplicity of tumors (carcinomas and papillomas) in the bladder compared with the control group (66.0% of the control value). These findings indicated that bLF could inhibit BBN-induced rat bladder carcinogenesis.

Suppression of carcinogenesis in other organs

To determine if lactoferrin had inhibitory effects on carcinogenesis in other organs, an experiment was performed using a multi-organ carcinogenesis model (Ushida et al. 1999). Male F344 rats, at 6 weeks of age, were treated sequentially with diethylnitrosamine by intraperitoneal injection, *N*,*N*′-bis(2-hydroxypropyl)nitrosamine in the drinking water and *N*-nitrosomethylbenzylamine by subcutaneous injection over the course

of 8 weeks. Then the animals were fed basal diet alone (control group) or diet containing bLF at doses of 0.002–2% (20 rats in each group) for 41 weeks. In the esophagus, a significant decrease in large sized papillomas (more than 50 mm^3 volume) in the 0.2% bLF fed group (11% of the control) was evident. In addition, the multiplicity of tumors (adenomas and carcinomas) in the lung was decreased in animals fed 0.02% bLF (56.9% of the control). These results indicated that bLF exerted preventive effects on esophagus and lung carcinogenesis.

The modifying effects of dietary bLF on tongue carcinogenesis initiated with 4-nitroquinoline 1-oxide (4-NQO) were investigated in male F344 rats (Tanaka et al. 2000). At 7 weeks of age, rats were given 20 ppm 4-NQO in their drinking water for 8 weeks. Starting 1 week after cessation of carcinogen exposure, the animals were fed basal diet alone (control group) or diet containing 0.02 or 2% bLF for 22 weeks. The incidence and multiplicity of tongue squamous cell carcinomas in the 2% bLF fed group were significantly reduced (36.4 and 35.7% of the control values, respectively). Furthermore, 2% bLF feeding resulted in a significant decrease in polyamine content and ornithine decarboxylase activity and deceased proliferating cell nuclear antigen (PCNA) labeling index. These results indicated that bLF exerted preventive effects on tongue carcinogenesis.

The findings presented above showed that bLF was a chemopreventive agent (suppressing agent) of colon, esophagus, lung, bladder and tongue carcinogenesis when applied during the post-initiation phase of carcinogenesis (Table 1), and that one of the mechanisms by which bLF suppressed carcinogenesis was by inhibiting tumor cell proliferation (Fig. 1, arrow #1).

Inhibition of colon carcinogenesis by bLF in the initiation stage (blocking effects)

To ascertain whether bLF protects against the initiation stage of colon carcinogenesis, a two part experiment was performed (Tsuda et al. 2002). In one part, protection against 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) induced colon carcinogenesis was determined, and in the other part, protection against caffeine enhancement of PhIP induced colon carcinogenesis was determined. PhIP is a carcinogenic heterocyclic amine (HCA) found in cooked food which requires metabolic conversion by P450 enzymes into an active form before it is able to react with DNA and form DNA adducts (Weisburger 1993). The endpoint lesion in this set of experiments was the presence of aberrant crypt foci (ACF), a well known marker for colon carcinogenesis. The primary P450 enzyme which metabolically activates PhIP in humans is the phase I enzyme CYP1A2 (Turesky et al. 1999), and caffeine induces CYP1A2 expression in PhIP-initiated ACF (Tsuda et al. 1999). Six week old F344 males received PhIP concurrently with caffeine mixed in the basal diet for 10 weeks: Group 1, 400 ppm PhIP; Group 2, PhIP + 2% bLF; Group 3, PhIP + 1,000 ppm caffeine; Group 4, PhIP + bLF + caffeine. The number of ACF in Group 2 (PhIP + bLF) was significantly reduced, 61.8%, compared to Group 1 (PhIP alone); ACF in Group 3 (PhIP + caffeine) was significantly increased,

Table 1 Inhibitory effect of bLF on chemically-induced carcinogenesis in rats

Target organ	Type of inhibition	
	Initiation phase (blocking effect)	Post-initiation phase (suppressing effect)
Colon	•	•
Lung		•
Esophagus		•
Tongue		•
Bladder		•

• Effective

Please refer to the text for experimental details and references

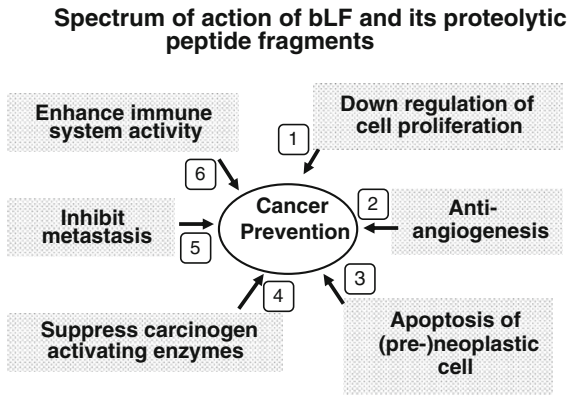

Fig. 1 bLF, and its proteolytic peptide fragments, has a wide range of actions by which it mediates its anti-carcinogenic effects. Please refer to the text for a discussion of the rational behind each arrow

153%, compared to Group 1 (PhIP alone); and ACF in Group 4 (PhIP + caffeine + bLF) was significantly reduced, 64.2%, compared to Group 3 (PhIP + caffeine). Thus, caffeine enhanced PhIP-induced ACF, and bLF inhibited both PhIP-induced ACF and PhIP-induced, caffeine enhanced ACF. Therefore, bLF inhibited colon tumor development when given in the initiation stage of carcinogenesis, and was effective even when carcinogenesis was enhanced by ingestion of caffeine (Table 1; Fig. 1, arrow #4). Importantly, since many people ingest both heterocyclic amines and caffeine-containing beverages and drugs, the results indicated that bLF could be an important chemopreventive agent to reduce the risk of colon carcinogenesis in human populations.

Inhibition of intestinal polyposis in the Apc^{Min} mouse

Chemopreventive effects of bovine lactoferrin (bLF) on spontaneous intestinal polyp development were assessed in the Apc^{Min} mouse (Ushida et al. 1998), a model for both familial adenomatous polyposis and sporadic colon cancers. In this experiment, mice at 6 weeks of age were given AIN-93G diet without bLF (24 mice) (control) or diet supplemented with 0.2% bLF (15 mice) or 2% bLF (15 mice) for 8 weeks. A significant suppression in spontaneous jejunum polyp formation in the 2% bLF group was observed ($P < 0.05$, 68% of the control). These data supported the concept that bLF could be a useful chemopreventive agent of intestinal polyposis (Table 1).

Toxicology studies in rats

Male F344 rats were fed a diet containing 0 or 0.2% bLF for 40 weeks. Serum triglyceride levels were significantly decreased (72%) compared to the basal diet group; blood glucose levels were not affected by bLF. No toxicity was observed. In a chronic feeding study, male and female F344 rats were fed diets containing 0.02, 0.2, 2, or 5.0% bLF; 2.0% bLF hydrolysate (bLF-H); or 0.1% bovine lactoferricin (bLFcin) for 60 weeks (males) or 65 weeks (females) (Tamano et al. 2008). No toxicity was observed in either sex. These results provided strong support for the safety of bLF.

Analysis of mechanisms by which bLF mediates its effects

Anti-angiogenesis

One possible mechanism by which carcinogenesis can be inhibited is by inhibiting angiogenesis. Adult male Sprague–Dawley rats were injected intraperitoneally with VEGF twice per day for 4 1/2 days. Beginning the day before VEGF injection, bLF was administered by oral gavage twice a day for 5 1/2 days. Animals were sacrificed 4 days after the end of the treatment and the mesenteric membrane examined for angiogenesis (Norrby et al. 2001). bLF significantly suppressed VEGF-mediated angiogenesis.

In another set of experiments, bLF induced dose-dependent inhibition of angiogenesis using 4–6-day-old chick embryo chorioallantoic membranes (CAMs) (Shimamura et al. 2004). This inhibition was reversed by basic fibroblast growth factor (bFGF). In a second set of experiments performed in this study, administration of bLF, either by intraperitoneal injection or oral gavage, inhibited Lewis lung carcinoma cell induced angiogenesis using a mouse dorsal air sac assay. In addition, bLF inhibited capillary-like tube formation by KOP2.16 cells in vitro. Finally, bLF inhibited bFGF- or VEGF-induced proliferation of KOP2.16 cells, but not bFGF- or VEGF-induced proliferation of mouse fibroblast A31 cells or Lewis lung carcinoma cells. These results suggested that the anti-tumor activity of bLF may be partly mediated by inhibition of angiogenesis (Fig. 1, arrow #2).

Apoptosis

Another mechanism by which transformed cells are thought to be eliminated from the body is by apoptosis. We investigated the possibility that bLF inhibits development of AOM-induced colon tumors in rats by enhancing apoptosis (Fujita et al. 2004a, b). Ingestion of bLF resulted in inhibition of tumorigenesis and induction of Fas expression, activation of caspase-8 and caspase-3, and induction of DNA fragmentation in the colon mucosa of rats treated with AOM. These results suggested that apoptosis caused by elevated expression of Fas is involved in bLF-mediated suppression of colon carcinogenesis in this animal model (Fig. 1, arrow #3).

Prevention of metastasis

Highly metastatic Co26Lu cells (1×10^5 cells/mouse) were implanted subcutaneously into male BALB/c mice. Three days after implantation, the mice were administered bLF by oral gavage 6 days a week for 3 weeks. The animals were sacrificed on Day 27 and the lungs examined for Co26Lu cell colonization (Iigo et al. 1999). Administration of 30 mg/kg bLF resulted in a significant decrease in colonization of the lung by Co26Lu cells (43% of the control value).

In another set of experiments, BALB/c and BALB/c athymic (*nu*/*nu*) mice were injected intravenously with Co26Lu cells (5×10^4). bLF or bLFH was administered by oral gavage for 7 consecutive days either from Day 7 before Co26Lu cell injection or beginning at Day 1 after injection. Mice were sacrificed on Day 11 and the lungs examined for Co26Lu cell colonization (Kuhara et al. 2000). Administration of bLF or bLFH, before or after Co26Lu cell injection, resulted in a significant decrease in colonization of the lung by Co26Lu cells: less than 50% of the control value at the higher concentrations of bLF and bLFH. In these experiments, administration of bLF and bLFH was more effective in inhibiting lung colonization when administered after injection of Co26Lu cells.

The results presented here indicated that bLF inhibits metastasis (Fig. 1, arrow #5).

Modulation of immune system activity—cytokine production

Using orthotopic murine models for squamous cell carcinoma and fibrosarcoma of the floor of the mouth, administration of human or murine recombinant lactoferrin inhibited the growth of tumors in vivo. In these experiments, lactoferrin-mediated inhibition of tumorigenesis was diminished in immunodeficient mice, and lactoferrin itself had no effect on the growth of tumor cells in vitro (Wolf et al. 2003). These results suggested that immunomodulation is an important mechanism of action for lactoferrin.

The small intestine contains a well developed immune network that is involved in the protection of the host from commensal bacteria and invading pathogens. In healthy mice, IL-18 is believed to be important for normal mucosal physiology (Takeuchi et al. 1997). In organ culture experiments using mouse intestine, addition of bLF or bLFcin induces expression of IL-18 and this induction is dependent upon (1) bLF/bLFcin induction of IFNγ and caspase-1 expression, (2) IFNγ dependent induction of proIL-18 expression and IFNγ dependent activation of caspase-1, and (3) proIL-18 cleavage by activated caspase-1 to generate mature IL-18 (Iigo et al. 2009). Numerous in vivo experiments support these results: Mice treated with bLF, bLFH, or bLFcin have increased IFNγ expression, caspase-1 activity, and mature IL-18 levels in the mucosa of the small intestine (Iigo et al. 2004; Kuhara et al. 2000; Wang et al. 2000), indicating that both intact bLF and proteolyzed bLF are able to interact with cells in the intestinal mucosa and stimulate the generation of IFNγ and mature IL-18. IFNγ and mature IL-18 are well known activators of a variety of immune cell types including neutrophils, macrophages, natural killer (NK) cells, and T cells (MHC restricted $CD4^+$ and $CD8^+$ T cells, CD1d restricted T cells [also known as natural killer T cells], and $\gamma\delta$ T cells). Moreover, IL-18 is reported to inhibit proliferation of capillary endothelial cells and suppress corneal neovascularization (Cao et al. 1999; Iigo et al. 2005).

Another cytokine important for normal mucosal physiology in the mouse is IL-12 (Hessle et al. 1999). In mice, bLF stimulates expression of IL-12 and decreases expression of IL-10 (Hwang et al. 2007; Wakabayashi et al. 2006). IL-12 promotes the activity of Th1 type $CD4^+$ helper T cells and IL-10 depresses the activity of these cells; therefore, an increase in the IL-12:IL-10 ratio would promote a Th1 response, potentially leading to enhancement of neutrophil, macrophage, NK cell, and cytotoxic T cell activities.

Oral administration of bLF also induces expression of type I interferons (IFNα and IFNβ) in the mouse small intestine (Kuhara et al. 2006; Wakabayashi et al. 2006). All tissues contain cells which respond to type I IFNs; in particular type I IFNs are well known activators of NK cells, macrophages, and T cells.

As noted above, bLF interacts with the intestinal mucosa to induce expression of IFNγ and generate mature IL-18. In IFNγ knock-out mice (GKO) bLF does not induce expression of either IFNγ or IL-18. In addition, in GKO mice bLF administration results in induction of caspase-1 expression but not in enhanced activation of caspase-1 (Iigo et al. 2009). However, in these mice bLF is still able to induce cytokine production in the small intestine and provide protection against tumor cell metastasis: In GKO mice, administration of bLF results in induction of IFNα and IL-7 (Iigo et al. 2009).

The results presented here indicate that ingested bLF is able to induce expression of various cytokines in the intestinal mucosa. Since, bLF is not detected in the blood after oral administration, the effect of bLF is, in all probability, due to interaction with the intestinal mucosal immune network. The exact pattern of cytokine expression induced by bLF likely depends on the physiological state of the intestine and the activity of bystander immune cells.

Modulation of immune system activity—effector cell activation

As described above, bLF enhances production of various cytokines in the intestinal mucosa. These cytokines include IFNγ and IL-18, IL-12, type I IFNs and IL-7. These cytokines are known to be involved in activation of immune cells, including dendritic cells (DCs), macrophages, neutrophils, natural killer (NK) cells, and various T cell subsets: MHC restricted $CD4^+$ and $CD8^+$ T cells, CD1d restricted T cells (natural killer T cells), and $\gamma\delta$ T cells. In addition, the LF released by neutrophils is known to be an alarmin (de la Rosa et al. 2008; Yang et al. 2009); alarmins recruit leukocytes and activate neutrophils, monocytes/macrophages, dendritic cells, and NK cells.

In an experiment using BALB/cByJ Jcl mice, administration of 2.5 g/kg body weight bLF by oral gavage resulted in an apparent mobilization of leukocytes from the spleen into the blood (Wakabayashi et al. 2006). In another set of experiments, recombinant human LF was able to induce the maturation of human DCs in vitro (Spadaro et al. 2008). In a third set of experiments, using C3h/HeJ mice implanted with SCCVII (squamous cell carcinoma cell line) tumors, oral administration of recombinant human LF inhibited tumor growth by 75% compared with control mice and there was up to a 20-fold increase of lymphocytes within the tumors of LF treated animals; and when mice were depleted of $CD3^+$ cells (T cells), all lactoferrin-induced tumor inhibition was abrogated (Wolf et al. 2007). Finally, oral administration of recombinant human LF to *neu* (*Erb2*) transgenic mice or to wild-type BALB/c mice implanted with *neu*$^+$ mammary adenocarcinoma resulted in a significant delay in tumorigenesis, and this effect was dependent on IFNγ-dependent enhancement of $CD8^+$ T cell and natural killer T cell activity initiated within the intestinal mucosa (Spadaro et al. 2007). These results suggest a possible mode of action of LF: (1) induction of cytokine release from immune cells of the intestinal mucosa, (2) maturation of mucosal DCs, (3) mobilization of T cells, (4) interaction between the mobilized T cells and DCs in the presence of mucosal cytokines, (5) activation of T cells, and (6) T cell mediated cytotoxic response against tumor cells and consequent inhibition of tumorigenesis.

In addition to T cells, NK cells have been implicated in the inhibition of carcinogenesis and/or metastasis mediated by bLF. Several studies have shown that in addition to $CD4^+$ and $CD8^+$ T cells, LF administration results in enhanced activity of NK cells (Iigo et al. 1999; Kuhara et al. 2000; Varadhachary et al. 2004; Wang et al. 2000), and in the experiments performed by Iigo et al. (1999), leukocytes isolated from the bLF treated rats exhibited dramatically enhanced cytotoxicity toward Co26Lu cells in vitro compared to leukocytes isolated from control rats, but when the leukocytes were treated with anti-asialoGM1 Ab (which recognizes NK cells) or anti-CD8 mAb (which recognizes cytotoxic T cells) and complement (to kill the antibody-bound cells) the cytotoxic activity was significantly lessened.

Other experiments support a role for NK cells in bLF-mediated inhibition of metastasis. Using the B16-F10 experimental metastasis model in C57BL16 mice, intraperitoneal administration of human LF dramatically inhibited lung metastasis, and this effect was abolished by deletion of the NK cell function using anti-asialo GM1 antibodies (Bezault et al. 1994). Oral administration of bLF also inhibited metastasis to the lung of Co26Lu cells implanted subcutaneously in athymic nude mice or in SCID mice, and this inhibition was also markedly reduced by deletion of NK cell function using anti-asialo GM1 antibodies (Iigo et al. 1999, 2005).

The results presented in these last two sections indicate that bLF modulates immune system function and this modulation is involved in bLF inhibition of carcinogenesis (Fig. 1, arrow #6).

Suppressive effect on the growth of colorectal polyps in a clinical trial

Introduction

Accumulating evidence from animal experiments has demonstrated that oral administration of bovine

lactoferrin (bLF) exerts anti-carcinogenesis effects in the colon and other organs of test animals. In 2002, Morinaga Milk Industry Co Ltd. supported a human clinical trial, conducted by the National Cancer Center Hospital, Tokyo, Japan, to determine if oral intake of bLF would inhibit the growth of adenomatous colorectal polyps in human patients; the results of this trail were published in 2009 (Kozu et al. 2009). It was found that a 1-year oral intake of 3.0 g of bLF per day induced statistically significant retardation of colorectal adenomatous polyp size in participants 63 years-old or younger.

The trial

Over the course of 3 years, from February 2002 until January 2005, patients scheduled for colonoscopic examination at the National Cancer Center Hospital, Tokyo, Japan, were approached prior to their examinations and invited to join the trial. Trial participation for each individual patient began within 30 days of the patient's colonoscopic examination. Participants took six tablets every day for 12 months; each tablet contained 0, 250, or 500 mg bLF, resulting in trial participants ingesting 0, 1.5, or 3.0 g of bLF daily for 12 months. Intake of any other product containing lactoferrin was prohibited throughout the entire study period. Treatment assignments and participant assessments were not revealed to investigators, participants, or the sponsor over the study period.

Polyp assessment and handling

Target polyps were polyps less than 5 mm in diameter with a pit pattern III (Kudo et al. 1996). These polyps were likely to be adenomas (Kudo et al. 2001; Su et al. 2004; Togashi et al. 1999) and increase in size during the study period (Hofstad et al. 1996), however, these small adenomas were unlikely to develop malignant characteristics during the course of the trial. During the initial colonoscopic examination, target polyps were identified and their locations marked. Polyps with a pit pattern III larger than 5 mm in diameter and any other polyp likely to develop into a malignant growth were removed at this time. At the end of the trial period, the pit pattern of the target polyps was noted and their sizes measured, and target polyps and all other premalignant and malignant growths were removed.

Results of the trail

89% of the polyps identified by colonoscopy as target polyps were histologically diagnosed as adenomas at the end of the trial, verifying pit pattern III as a reliable predictor of adenoma. Importantly, no serious adverse effects associated with bLF ingestion occurred during the trial period, verifying the safety of bLF ingestion as a treatment regimen.

Ingestion of bLF had two significant effects, and both of these effects were age dependent. Participants 63 years of age or younger ingesting 3.0 g bLF had a significant reduction in target polyp size compared to the placebo group, and this group also had a significant increase in their levels of serum human lactoferrin (hLF), but ingestion of bLF did not have a significant effect on either polyp size or serum hLF in participants 64 years of age and older. (bLF was undetectable in the serum of any of the trail participants.) Overall, the study found a correlation between induction of serum hLF and decreased target polyp size. The study also found that in the participants ingesting 3.0 g bLF, induction of serum hLF decreased with age.

Another finding of the study was a negative correlation between induction of serum hLF and infiltration of polymorphonuclear leukocytes (PMNs) into target polyps and a positive correlation between infiltration of PMNs into target polyps and target polyp size. In other words, participants with increased serum hLF had fewer infiltrating PMNs in their target polyps and target polyps with fewer infiltrating PMNs grew more slowly than target polyps with more infiltrating PMNs.

Overall, participants with higher levels of NK cell activity had smaller polyps, but the effect of bLF ingestion on serum natural killer (NK) cell activity was inconclusive. A significant increase in NK cell activity was seen in the participants in the 1.5 g bLF group but not in the 3.0 g bLF group. A larger study is needed to resolve this issue.

Ingestion of bLF had no effect on the levels of lymphocytes expressing CD4 (Th1 and Th2 T cells, Th17 cells, and regulatory T cells), CD8 (cytotoxic T cells), CD16 (NK cells, macrophages, and PMNs), or CD56 (NK cells and natural killer T cells) in the serum, or on serum levels of IL-18 or IFNγ.

Conclusions of the trail

Perhaps, the most interesting findings of this study was the correlation between polyp growth and induction of serum hLF levels and the dependence of these effects on age. The authors reasoned that induction of serum hLF indicated that bLF was impacting the immune system. If this was indeed the case, then the increase in serum hLF could reflect enhancement of immune system function. This enhancement would very likely lead to enhanced immune surveillance and containment of neoplastic growth. Moreover, if bLF did enhance immune function, it would be likely that this effect would weaken as immune function weakened with age. These postulates are all reflected in the results of the study.

The other correlation found in the study was that induction of serum hLF was associated with lower infiltration of PMNs into target polyps and lower infiltration of PMNs into target polyps was associated with decreased polyp growth. It is known that (1) inflammatory responses by immune effector cells can result in release of cytokines that not only signal other immune effector cells but can also enhance neoplastic growth and (2) infiltration of PMNs into a tumor site can enhance tumor growth (Queen et al. 2005; van den Tol et al. 2007; Wada et al. 2007; Wislez et al. 2007). Therefore, removal of chronic inflammatory signals can result in suppression of neoplastic growth. However, while there was a correlation between ingestion of bLF, induction of serum hLF, and neutrophil infiltration into target polyps, the study was not able to draw a conclusion as to whether ingestion of bLF affected neutrophil infiltration into the target polyps.

Finally, participants with higher NK cell activity had less target polyp growth than participants with lower NK cell activity. The study, however, was unable to find a significant correlation between ingestion of bLF and NK cell activity.

Two possible general mechanisms of bLF-mediated inhibition of target polyp growth can be envisioned from the study. In patients with immune responses which tended to depress target polyp growth, such as low inflammatory activity at the polyp site and higher NK cell activity, bLF contributed a third signal which tipped the balance and led to suppression of polyp growth. In the second mechanism, bLF modulation of immune system function included enhancement of NK activity and/or suppression of inflammation at the target site (but these effects were not statically demonstrated in this relatively small trial) and led to suppression of polyp growth. Another possible contributing factor was serum hLF; however, the effect of serum LF on neoplastic development is unknown at this time. Whichever mechanism is correct, the study does strongly suggest that ingestion of bLF modulated immune system function and this in turn led to suppression of neoplastic growth.

Importantly, the purpose of the study was not to prove that ingestion of bLF could cure colorectal cancer (CRC), but that ingestion of bLF could suppress the progression of CRC. Colonoscopy with clearing of neoplasms by polypectomy significantly reduces CRC, however, CRC incidence after clearing colonoscopy is appreciable (Levin et al. 2008). Factors considered to be involved in CRC which arise after clearing colonoscopy include detection failures during colonoscopy and incomplete polyp extraction (Levin et al. 2008; van Rijn et al. 2006). Therefore, suppression of adenomatous polyp growth between colonoscopic examinations by a safe well-tolerated regimen can be effective in suppression of CRC incidence.

Summary and conclusions

The data reviewed here establishes bLF as a natural compound which has anti-carcinogenesis activity (Table 1; Fig. 1). Ingestion of bLF is both effective and safe. Experimental animal models suggest that a likely mode of action of ingested bLF is enhancement of immune function, in particular the immune function of the gut-associated lymphoid tissue (GALT). For example, bLF, or more likely its proteolytic peptide fragments, potentially interacts with the GALT and induces expression of various cytokines. These cytokines would in turn function in immune physiology: Interferons inhibit cell proliferation and induce the expression of numerous apoptotic effector proteins (Chawla-Sarkar et al. 2003; Clemens 2003), resulting in inhibition of tumor growth and enhanced sensitivity of transformed cells to apoptotic stimuli; interferons and IL-18 inhibit angiogenesis (Cao et al. 1999; Iigo et al. 2005; McCarty et al. 2002; Sidky and Borden 1987; von

Marschall et al. 2003), resulting in inhibition of tumorigenesis; and other bLF induced cytokines are involved in several of the signaling pathways associated with the activation of immune effector cells, resulting in an enhanced immune response against transformed cells.

In humans, ingested bLF appears to act in a similar manner, i.e., bLF (or its proteolytic peptide fragments) potentially interacts with the GALT to modulate immune responsiveness. The most compelling evidence for immune modulation by ingestion of bLF is the increase in serum human LF seen in the clinical trial participants who ingested 3.0 g bLF per day. It is now well accepted that lactoferrin is an important component of the mammalian innate immune system (Legrand et al. 2008) and, consequently, the induction of serum LF observed in the trial participants very likely reflected modulation of immune system activity.

If ingested bLF modulates immune activity, its effectiveness would depend on the immune status of the individual. For example, in elderly people bLF would not be expected to be as effective as in younger people with a more robust immune system; and this was seen in the bLF clinical trial. The major advantages of bLF are that it is easy to use, it is safe, and it is effective in suppressing carcinogenesis.

Acknowledgments We thank Shigeru Nawano, Atsushi Otsu, Tomotaka Sobue, and Chikuma Hamada as members of the Independent Data Monitoring Committee; Takahisa Matsuda, Ryuzo Sekiguichi, and Kunihisa Miyakawa for their valuable contributions to the bLF trial; and finally, Takuji Gotoda at the Endoscopic Data Adjudication Committee, Tadakazu Shimoda, a pathologist, Takahiro Fujii, an endoscopist (until June 2003), and Morinaga Milk Industry Ltd., the bLF trial sponsor. We thank Morinaga Milk Industry for providing the bovine lactoferrin and placebo tablets.

References

Bezault J, Bhimani R, Wiprovnick J et al (1994) Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. Cancer Res 54:2310–2312

Cao R, Farnebo J, Kurimoto M et al (1999) Interleukin-18 acts as an angiogenesis and tumor suppressor. FASEB J 13:2195–2202

Chawla-Sarkar M, Lindner DJ, Liu YF et al (2003) Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. Apoptosis 8:237–249

Clemens MJ (2003) Interferons and apoptosis. J Interferon Cytokine Res 23:277–292

Cramer E, Pryzwansky KB, Villeval JL et al (1985) Ultrastructural localization of lactoferrin and myeloperoxidase in human neutrophils by immunogold. Blood 65:423–432

de la Rosa G, Yang D, Tewary P et al (2008) Lactoferrin acts as an alarmin to promote the recruitment and activation of APCs and antigen-specific immune responses. J Immunol 180:6868–6876

Freiburghaus C, Janicke B, Lindmark-Mansson H et al (2009) Lactoferricin treatment decreases the rate of cell proliferation of a human colon cancer cell line. J Dairy Sci 92:2477–2484

Fujita K, Matsuda E, Sekine K et al (2004a) Lactoferrin enhances Fas expression and apoptosis in the colon mucosa of azoxymethane-treated rats. Carcinogenesis 25:1961–1966

Fujita K, Matsuda E, Sekine K et al (2004b) Lactoferrin modifies apoptosis-related gene expression in the colon of the azoxymethane-treated rat. Cancer Lett 213:21–29

Gahr M, Speer CP, Damerau B et al (1991) Influence of lactoferrin on the function of human polymorphonuclear leukocytes and monocytes. J Leukoc Biol 49:427–433

Hessle C, Hanson LA, Wold AE (1999) Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clin Exp Immunol 116:276–282

Hofstad B, Vatn MH, Andersen SN et al (1996) Growth of colorectal polyps: redetection and evaluation of unresected polyps for a period of three years. Gut 39:449–456

Hwang SA, Wilk KM, Bangale YA et al (2007) Lactoferrin modulation of IL-12 and IL-10 response from activated murine leukocytes. Med Microbiol Immunol 196:171–180

Iigo M, Kuhara T, Ushida Y et al (1999) Inhibitory effects of bovine lactoferrin on colon carcinoma 26 lung metastasis in mice. Clin Exp Metastasis 17:35–40

Iigo M, Shimamura M, Matsuda E et al (2004) Orally administered bovine lactoferrin induces caspase-1 and interleukin-18 in the mouse intestinal mucosa: a possible explanation for inhibition of carcinogenesis and metastasis. Cytokine 25:36–44

Iigo M, Shimamura M, Hirano S et al (2005) Cancer prevention and anti-metastatic effects by oral administration of bovine lactoferrin. In: Takuji T, Hiroyuki T (eds) Carcinogenesis and modification of carcinogenesis. Research Signpost, Kerala, India, pp 229–242

Iigo M, Alexander DB, Long N et al (2009) Anticarcinogenesis pathways activated by bovine lactoferrin in the murine small intestine. Biochimie 91:86–101

Kozu T, Iinuma G, Ohashi Y et al (2009) Effect of orally administered bovine lactoferrin on the growth of adenomatous colorectal polyps in a randomized, placebo-controlled clinical trial. Cancer Prev Res (Phila Pa) 2:975–983

Kudo S, Tamura S, Nakajima T et al (1996) Diagnosis of colorectal tumorous lesions by magnifying endoscopy. Gastrointest Endosc 44:8–14

Kudo S, Rubio CA, Teixeira CR et al (2001) Pit pattern in colorectal neoplasia: endoscopic magnifying view. Endoscopy 33:367–373

Kuhara T, Iigo M, Itoh T et al (2000) Orally administered lactoferrin exerts an antimetastatic effect and enhances production of IL-18 in the intestinal epithelium. Nutr Cancer 38:192–199

Kuhara T, Yamauchi K, Tamura Y et al (2006) Oral administration of lactoferrin increases NK cell activity in mice via increased production of IL-18 and type I IFN in the small intestine. J Interferon Cytokine Res 26:489–499

Legrand D, Pierce A, Elass E et al (2008) Lactoferrin structure and functions. Adv Exp Med Biol 606:163–194

Levay PF, Viljoen M (1995) Lactoferrin: a general review. Haematologica 80:252–267

Levin B, Lieberman DA, McFarland B et al (2008) Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. CA Cancer J Clin 58:130–160

Lonnerdal B, Iyer S (1995) Lactoferrin: molecular structure and biological function. Annu Rev Nutr 15:93–110

Masuda C, Wanibuchi H, Sekine K et al (2000) Chemopreventive effects of bovine lactoferrin on N-butyl-N-(4-hydroxybutyl)nitrosamine-induced rat bladder carcinogenesis. Jpn J Cancer Res 91:582–588

McCarty MF, Bielenberg D, Donawho C et al (2002) Evidence for the causal role of endogenous interferon-alpha/beta in the regulation of angiogenesis, tumorigenicity, and metastasis of cutaneous neoplasms. Clin Exp Metastasis 19:609–615

McCormick JA, Markey GM, Morris TC et al (1991) Lactoferrin inducible monocyte cytotoxicity defective in esterase deficient monocytes. Br J Haematol 77:287–290

McIntosh GH, Regester GO, Le Leu RK et al (1995) Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. J Nutr 125:809–816

Nishiya K, Horwitz DA (1982) Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody-dependent cell-mediated cytotoxicity. J Immunol 129:2519–2523

Norrby K, Mattsby-Baltzer I, Innocenti M et al (2001) Orally administered bovine lactoferrin systemically inhibits VEGF(165)-mediated angiogenesis in the rat. Int J Cancer 91:236–240

Queen MM, Ryan RE, Holzer RG et al (2005) Breast cancer cells stimulate neutrophils to produce oncostatin M: potential implications for tumor progression. Cancer Res 65:8896–8904

Rehman MU, Buttar QM, Khawaja MI et al (2009) An impending cancer crisis in developing countries: are we ready for the challenge? Asian Pac J Cancer Prev 10:719–720

Sekine K, Watanabe E, Nakamura J et al (1997) Inhibition of azoxymethane-initiated colon tumor by bovine lactoferrin administration in F344 rats. Jpn J Cancer Res 88:523–526

Shau H, Kim A, Golub SH (1992) Modulation of natural killer and lymphokine-activated killer cell cytotoxicity by lactoferrin. J Leukoc Biol 51:343–349

Shimamura M, Yamamoto Y, Ashino H et al (2004) Bovine lactoferrin inhibits tumor-induced angiogenesis. Int J Cancer 111:111–116

Sidky YA, Borden EC (1987) Inhibition of angiogenesis by interferons: effects on tumor- and lymphocyte-induced vascular responses. Cancer Res 47:5155–5161

Spadaro M, Curcio C, Varadhachary A et al (2007) Requirement for IFN-gamma, $CD8^+$ T lymphocytes, and NKT cells in talactoferrin-induced inhibition of neu^+ tumors. Cancer Res 67:6425–6432

Spadaro M, Caorsi C, Ceruti P et al (2008) Lactoferrin, a major defense protein of innate immunity, is a novel maturation factor for human dendritic cells. FASEB J 22:2747–2757

Su MY, Ho YP, Chen PC et al (2004) Magnifying endoscopy with indigo carmine contrast for differential diagnosis of neoplastic and nonneoplastic colonic polyps. Dig Dis Sci 49:1123–1127

Takeuchi M, Nishizaki Y, Sano O et al (1997) Immunohistochemical and immuno-electron-microscopic detection of interferon-gamma-inducing factor ("interleukin-18") in mouse intestinal epithelial cells. Cell Tissue Res 289: 499–503

Tamano S, Sekine K, Takase M et al (2008) Lack of chronic oral toxicity of chemopreventive bovine lactoferrin in F344/DuCrj rats. Asian Pac J Cancer Prev 9:313–316

Tanaka T, Kawabata K, Kohno H et al (2000) Chemopreventive effect of bovine lactoferrin on 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in male F344 rats. Jpn J Cancer Res 91:25–33

Togashi K, Konishi F, Ishizuka T et al (1999) Efficacy of magnifying endoscopy in the differential diagnosis of neoplastic and non-neoplastic polyps of the large bowel. Dis Colon Rectum 42:1602–1608

Tsuda H, Sekine K (2000) Milk components as cancer chemopreventive agents. Asian Pac J Cancer Prev 1:277–282

Tsuda H, Sekine K, Nakamura J et al (1998) Inhibition of azoxymethane initiated colon tumor and aberrant crypt foci development by bovine lactoferrin administration in F344 rats. Adv Exp Med Biol 443:273–284

Tsuda H, Sekine K, Uehara N et al (1999) Heterocyclic amine mixture carcinogenesis and its enhancement by caffeine in F344 rats. Cancer Lett 143:229–234

Tsuda H, Sekine K, Fujita K et al (2002) Cancer prevention by bovine lactoferrin and underlying mechanisms—a review of experimental and clinical studies. Biochem Cell Biol 80:131–136

Turesky RJ, Constable A, Fay LB et al (1999) Interspecies differences in metabolism of heterocyclic aromatic amines by rat and human P450 1A2. Cancer Lett 143:109–112

Ushida Y, Sekine K, Kuhara T et al (1998) Inhibitory effects of bovine lactoferrin on intestinal polyposis in the Apc(Min) mouse. Cancer Lett 134:141–145

Ushida Y, Sekine K, Kuhara T et al (1999) Possible chemopreventive effects of bovine lactoferrin on esophagus and lung carcinogenesis in the rat. Jpn J Cancer Res 90:262–267

van den Tol MP, ten Raa S, van Grevenstein WM et al (2007) The post-surgical inflammatory response provokes enhanced tumour recurrence: a crucial role for neutrophils. Dig Surg 24:388–394

van Rijn JC, Reitsma JB, Stoker J et al (2006) Polyp miss rate determined by tandem colonoscopy: a systematic review. Am J Gastroenterol 101:343–350

Varadhachary A, Wolf JS, Petrak K et al (2004) Oral lactoferrin inhibits growth of established tumors and potentiates conventional chemotherapy. Int J Cancer 111:398–403

von Marschall Z, Scholz A, Cramer T et al (2003) Effects of interferon alpha on vascular endothelial growth factor gene transcription and tumor angiogenesis. J Natl Cancer Inst 95:437–448

Wada Y, Yoshida K, Tsutani Y et al (2007) Neutrophil elastase induces cell proliferation and migration by the release of

TGF-alpha, PDGF and VEGF in esophageal cell lines. Oncol Rep 17:161–167

Wakabayashi H, Takakura N, Yamauchi K et al (2006) Modulation of immunity-related gene expression in small intestines of mice by oral administration of lactoferrin. Clin Vaccine Immunol 13:239–245

Wang WP, Iigo M, Sato J et al (2000) Activation of intestinal mucosal immunity in tumor-bearing mice by lactoferrin. Jpn J Cancer Res 91:1022–1027

Weisburger JH (1993) Heterocyclic amines in cooked foods: possible human carcinogens. Cancer Res 53:2422–2424

Wislez M, Antoine M, Rabbe N et al (2007) Neutrophils promote aerogenous spread of lung adenocarcinoma with bronchioloalveolar carcinoma features. Clin Cancer Res 13:3518–3527

Wolf JS, Li D, Taylor RJ et al (2003) Lactoferrin inhibits growth of malignant tumors of the head and neck. ORL J Otorhinolaryngol Relat Spec 65:245–249

Wolf JS, Li G, Varadhachary A et al (2007) Oral lactoferrin results in T cell-dependent tumor inhibition of head and neck squamous cell carcinoma in vivo. Clin Cancer Res 13:1601–1610

Yang D, de la Rosa G, Tewary P et al (2009) Alarmins link neutrophils and dendritic cells. Trends Immunol 11:531–537