Lactoferrin enhances Fas expression and apoptosis in the colon mucosa of azoxymethane-treated rats

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Bovine lactoferrin, a multifunctional glycoprotein, has been shown to strongly inhibit development of azoxymethane (AOM)-induced rat colon tumors. Little, however, is known about the inhibitory mechanisms. We have demonstrated recently that lactoferrin enhances the expression of a member of the tumor necrosis factor receptor family, Fas, in the colon mucosa during both early and late stages of carcinogenesis. Thus, Fas could be involved in bovine lactoferrin-mediated inhibition of tumor development. To investigate this possibility, we studied the influence of bovine lactoferrin on Fas-mediated apoptosis with regard to expression of Fas, activation of caspase-8 and caspase-3, and DNA fragmentation in the colon mucosa of AOM-treated rats. Western blot analysis demonstrated a >2.5-fold increase in Fas protein expression, as well as elevation of the active forms of both caspase-8 and caspase-3. Immunohistochemical analysis revealed Fas-positive cells and apoptotic cells preferentially within the proximal colon region, clearly at the site of bovine lactoferrin-mediated tumor inhibition. These results suggest that apoptosis caused by elevated expression of Fas is involved in chemoprevention by lactoferrin of colon carcinogenesis.

Introduction

Lactoferrin is an iron-binding glycoprotein, which is present at high concentrations in mammalian milk colostrum. It also exists in lower concentrations in a variety of other secretions from epithelial cells such as the salivary, lacrimal and prostate glands (1). Another source of lactoferrin is the secondary granules of neutrophils (2). The primary functions of lactoferrin seem to be in immunological responses, as well as iron transport, storage and chelation. For example it activates natural killer (NK) cells (3,4), polymorphonuclear leukocytes and monocytes (5), induces colony-stimulating activity (6), enhances antibody-dependent cell cytotoxicity (7), stimulates lymphokine-activated killer cell activity (3) and regulates myelopoiesis (8). However, there is no consensus view on the biological roles of lactoferrin.

It has been reported that injections of human lactoferrin reduce the growth of transplanted solid tumors in mice (9). Recently, we have demonstrated that dietary supplementation with bovine lactoferrin inhibits development of azoxymethane (AOM)-induced aberrant crypt foci (10), putative precursor lesions for colon tumors (11,12), as well as the tumors themselves in the rat (13). Similar preventive effects of bovine lactoferrin on carcinogenesis in other organs have also been demonstrated (14-18). The severe side effects observed with other anticancer drugs, however, have not been observed, even after long-term administration of bovine lactoferrin (13-16). Thus, lactoferrin can be considered a good candidate chemopreventive agent for human cancers.

Apoptosis is essential not only for development and maintenance of tissue homeostasis but also for the elimination of damaged cells, such as cells damaged by microbial infections and cells found in neoplasms (19,20). Many triggers can lead to apoptosis induction, including stimulation of tumor necrosis factor (TNF) receptor family members, such as Fas (also known as CD95 or APO-1), TNF-R1, TNF-related apoptosis-inducing ligand (TRAIL)-R1/DR4 and TRAIL-R2/DR5 (21,22). These ‘death’ receptors contain structurally and functionally conserved death domains in their intracellular regions, which recruit certain adapter molecules to the death-inducing signaling complex, ultimately activating the apoptotic caspase cascade (23). Recently, we have demonstrated elevated mRNA expression of Fas, but not of TNF-R1, in colon mucosa after bovine lactoferrin administration (24). Since, to date, TRAIL-R1/DR4 and TRAIL-R2/DR5 have not been identified in the rat, involvement of specifically induced Fas expression in bovine lactoferrin-mediated chemoprevention of tumor development is plausible. Bovine lactoferrin induction of Fas expression would increase the sensitivity of the enhanced Fas-expressing target cells to attack by NK cells.

The present study was conducted to characterize the involvement of Fas in lactoferrin-mediated chemoprevention of AOM-induced tumors in the rat colon. We found that in lactoferrin-treated rats, expression of Fas, but not TNF-R1, was enhanced at both the mRNA and protein levels. Also, activation of both caspase-8 and caspase-3, constituents of the intracellular signaling pathway from Fas, was observed. Coincident with the increase in Fas signaling, apoptotic cells in the colon mucosa were also increased in number. Importantly, reduction of the incidence of tumors closely correlated with the location of enhanced Fas expression and apoptosis. The results thus suggest that apoptosis caused by elevated expression of Fas is involved in the mechanisms underlying chemoprevention of colon carcinogenesis by lactoferrin.

Materials and methods

Animal treatments and tissue harvesting

Fischer 344 male rats (Charles River Japan Co., Atsugi, Japan), 6 weeks old at the commencement of the experiments, were maintained in plastic cages in an
air-conditioned room with a 12-h light/12-h dark cycle. A total of 40 rats were injected subcutaneously with AOM (Sigma Chemical, St Louis, MO) at a dose of 15 mg/kg body wt once a week for 3 weeks, as described previously (13,24). One week after the last injection, the animals received a powdered basal diet MF (Oriental Yeast Co., Tokyo, Japan) alone and the other half a diet containing 2% bovine lactoferrin (Moringa Milk Industry Co., Ltd, Zama, Japan) for 27 weeks. Food and water were available ad libitum, and body weights and food consumption were measured weekly. At week 30, all animals were killed under deep ether anesthesia. Immediately after death, colons were removed, cut open along the longitudinal axis from cecum to anus and spread on filter papers. Colon tumors were identified visually, and the location and size of each were recorded. A portion of each tumor was fixed with 10% formalin, embedded in paraffin, and stained with H&E. For RNA and protein analyses, total colon mucosa (free of visual tumors) was scraped off with a slide glass, frozen in liquid nitrogen, and stored at −80°C until analysis. For immunohistochemical analyses, colons (free of visual tumors) were fixed in ice-cold acetone, divided into proximal (2–4 cm from the cecum), middle (10–12 cm from the anus) and distal (2–4 cm from the anus) segments and embedded in paraffin. The experiments were conducted according to the ‘Guidelines for Animal Experiments in National Cancer Center’ of the Committee for Ethics of Animal Experimentation of the National Cancer Center, Japan.

RNA analysis
Total RNAs were isolated from scraped colon mucosa (free of visual tumors) using Isogen (Nippon Gene, Tokyo, Japan). The integrity of 18S and 28S rRNA was determined by ethidium bromide staining after electrophoresis in agarose gels. RT–PCR amplification was performed as previously described (24). A portion of each PCR product was electrophoresed through a 1.0% agarose gel transferred onto a Hybond-N (Amersham, Arlington Heights, IL) and immobilized by UV cross-linking. Fas, TNF-R1, Fas ligand (Fasl), TNF-α and β-actin cDNAs were cloned as described previously using RT–PCR (24); the sequences of the cDNAs completely matched those described in previous reports (25–29). Membranes were hybridized with 32P-labeled probe at 42°C for 16 h in ultrahyb hybridization buffer (Ambion, St Austin, TX), and then washed twice in 2× SSC/0.1% SDS at 65°C for 15 min. Autoradiograms were analyzed on a BAS2000 (Fuji Film, Tokyo, Japan) and quantification of each mRNA was normalized to β-actin mRNA levels.

Western blot analysis
Total scraped colon mucosa (free of visual tumors) was lysed in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 250 mM sucrose, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and protease inhibitors (complete (50 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 250 mM sucrose, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and protease inhibitors (complete), Roche Molecular Biochemicals, Indianapolis, IN) for 30 min on ice. The insoluble material was removed by centrifugation at 14 000g for 20 min at 4°C and the protein concentration was measured with a modified Bradford assay (Bio-Rad, Hercules, CA) using BSA as the standard. Protein (100 μg) was subjected to SDS–PAGE using 8.0% gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blotted with polyclonal antibodies specific for Fas, FasL, TNF-R1, TNF-α, caspase-8, caspase-3, poly(ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA), and a monoclonal antibody specific for β-actin (Sigma Chemical). After application of a horseradish peroxidase-conjugated secondary antibody, reactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) and immobilized by UV cross-linking. Fas, TNF-R1, FasL and TNF-α were detected using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and counterstaining with Mayer’s hematoxylin. Apoptotic reactivity was detected using an Apoptosis detection kit (30) using an Apoptosis detection kit (30) using an Apoptosis detection kit (30) using an Apoptosis detection kit (30) using an Apoptosis detection kit (30) using an Apoptosis detection kit (30) using an Apoptosis detection kit (30) using an Apoptosis detection kit (30) using an Apoptosis detection kit (30) using an Apoptosis detection kit (30) using an Apoptosis detection kit (30). Band intensity was quantified by densitometric scanning and values calculated using NIH Image (Version 1.58) software. The level of expression of individual proteins was normalized to the β-actin level in each sample.

Immunohistochemical analysis
Five micrometer sections of colonic epithelium in proximal, middle and distal segments were deparaffinized, re-hydrated and treated with 3% H2O2 in methanol. After blocking non-specific binding with 1% skim milk in PBS, the tissue sections were incubated with a primary antibody to Fas, FasL, TNF-R1 or TNF-α (1:100 dilution in PBS containing 1% BSA) for 2 h. Immunoreactivity was detected using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and counterstaining with Mayer’s hematoxylin. Apoptotic cells were identified with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique (30) using an Apoptosis detection kit (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer’s instructions, followed by counterstaining with 0.5% methyl green solution.

Statistical analysis
Relative expression values are given as mean ± SD, expressed as fold of expression in colon mucosa of rats treated by AOM alone. Quantitative differences between group values were statistically analyzed by Dunnett’s multiple comparison t-test. P values < 0.05 were considered to be significant.

Results

**Bovine lactoferrin increases expression of Fas mRNA in colon mucosa**

The relative mRNA levels for Fas, TNF-R1, FasL and TNF-α in colon mucosa of AOM-treated rats at 30 weeks are shown in Figure 1. Although two bands were detected, except for FasL, the upper bands were regarded as the objective products because they were the expected size and sequence. The level of Fas mRNA in the colon mucosa of rats administered bovine lactoferrin was significantly increased relative to animals fed the basal diet alone (2.5-fold, \(P < 0.01\), Figure 1B). In contrast, TNF-R1, FasL and TNF-α mRNA levels were not markedly altered (Figure 1B), consistent with a previous report (24).

**Bovine lactoferrin increases expression of Fas protein in colon mucosa**

We further examined the level of Fas, TNF-R1, FasL and TNF-α protein expression in colon mucosa of AOM-treated...
rats by western blot analysis (Figure 2A). Dominant bands with apparent molecular weights of 48, 55, 40 and 26 kDa for Fas, TNF-R1, FasL and TNF-α, respectively, were detected, consistent with the predicted size of each protein. The Fas protein level was significantly increased, ~2-fold ($P < 0.05$), to a level commensurate with the increase in mRNA expression of Fas in the colon mucosa of rats administered bovine lactoferrin (Figure 2B). In contrast, the level of TNF-α protein was significantly decreased ($P < 0.05$), whereas TNF-R1 and FasL protein levels were not markedly altered (Figure 2B).

**Lactoferrin increases activation of caspase-8 and caspase-3 in colon mucosa**

Death-inducing ligands such as FasL, TNF-α and TRAIL can induce apoptosis via activation of caspases, including caspase-8 and caspase-3 (21,22). Thus, we investigated whether caspase-8 and caspase-3 are affected by bovine lactoferrin in the colon mucosa of AOM-treated rats. Bovine lactoferrin slightly, but significantly, increased the cleavage of the p55 proform of caspase-8 into its p18 active fragment ($P < 0.05$) and the p32 proform of caspase-3 into its p17 active fragment ($P < 0.05$) in the colon mucosa (Figure 3B).

It has been reported that caspase-3 cleaves the death substrate PARP to a specific 85-kDa form during apoptosis (31,32). As shown in Figure 3B, increased cleavage of PARP from a native 116-kDa protein into a p85 fragment was observed in colon mucosa of rats fed bovine lactoferrin.

**Immunohistochemical detection of Fas and TUNEL-positive cells**

It has been reported that Fas is broadly expressed throughout the entire length of the human colon and constitutively present on the basolateral surfaces of colon epithelial cells (33,34). To examine the distribution of Fas in rat colon mucosa, immunohistochemical detection was performed on epithelium from the proximal, middle, and distal colon of AOM-injected rats (Figure 4). As in humans (33,34), Fas was found to be expressed on the basolateral surface of colon epithelial cells. However, a major difference was observed regarding its distribution; Fas-positive cells were only detected within the...
proximal region of the colon (Figure 4), the site where the number of immunoreactive cells was clearly increased by bovine lactoferrin (Figure 4D). In contrast, TNF-R1 was expressed throughout the epithelium of the entire colon, and the numbers of TNF-R1-positive cells and their distribution were not altered by bovine lactoferrin (data not shown). Examination of the location of apoptotic cells by TUNEL staining revealed that positive epithelial cells were largely confined to the proximal colon of rats fed bovine lactoferrin (Figure 5D); a distribution strikingly similar to that of Fas and Fas induction. In contrast, stromal cells were observed as TUNEL-positive cells only in the middle and distal colon of AOM-injected rats (Figure 5).

Inhibitory effects of bovine lactoferrin on development of colon tumors

We demonstrated previously that oral administration of bovine lactoferrin strongly inhibits development of AOM-induced tumors in rat colon (13,24). In the present study, AOM-treated rats fed 2% bovine lactoferrin also exhibited a lower incidence (2 of 20 rats, 10%; multiplicity: 0.25 ± 0.31/rat) of tumors than rats treated by AOM alone (incidence: 8 of 20 rats, 40%; multiplicity: 0.40 ± 0.50/rat). In contrast, tumor development in the middle and distal colon was not affected by bovine lactoferrin administration: incidence of tumor development in AOM-treated rats fed 2% bovine lactoferrin was 30% in the middle colon and 30% in the distal colon; multiplicity of tumors in AOM-treated rats fed 2% bovine lactoferrin was 0.25 ± 0.47/rat in the middle colon and 0.25 ± 0.47/rat in the distal colon; multiplicity of tumors in AOM-treated rats was 0.30 ± 0.47/rat in the middle colon and 0.30 ± 0.55/rat in the distal colon.

Discussion

In the present study, enhanced expression of Fas was specifically evident in the proximal colon mucosa after administration
of bovine lactoferrin; the same site where tumor development was significantly inhibited by administration of bovine lactoferrin. Activation of both caspase-8 and caspase-3, along with an increased number of apoptotic cells, was also observed specifically in the proximal colon mucosa after administration of bovine lactoferrin. These results provide the first concrete indication that apoptosis caused by elevated expression of Fas is plausibly involved in chemoprevention of colon carcinogenesis by lactoferrin.

A previous study demonstrated that diverse anticancer drugs induce Fas expression in cultured cell lines, thereby strongly increasing their sensitivity towards Fas-mediated apoptosis (35). Recently, we have reported up-regulation of Fas mRNA expression by bovine lactoferrin in the colon mucosa in both the early and late stages of colon carcinogenesis (24). As there was no alteration in expression of TNF-R1 in the present investigation, and as TRAIL-R1/DR4 and TRAIL-R2/DR5 have not been identified in the rat to date, we hypothesize that the specifically enhanced expression of Fas increases the capacity of target cells to bind with FasL expressed on the cell surfaces of NK cells. Interaction of activated Fas with Fas-associated death domain (36), results in recruitment of caspase-8, which in turn induces self-activation of the protease domain and triggers the effector protease cascade through caspase-3 (37,38). Activated caspase-3 and downstream elements then cleave various substrates, such as PARP (31,32), and cause DNA fragmentation and apoptosis.

It is well known that NK cells responsible for innate immunity have diverse biological functions, including recognition and destruction of certain microbes (39,40). NK cells can kill tumor cells by causing apoptosis mediated by TNF family members, such as FasL, TNF-α and TRAIL, expressed on the cell surfaces of the NK cells (41,42). Previous studies have shown that lactoferrin can activate NK cells (3,4) and the antitumor activity of lactoferrin is diminished by elimination of NK cell activity (9). Furthermore, we have reported recently that oral administration of bovine lactoferrin markedly induced the number of NK cells in the white blood cell fraction and lymphoid tissues and lamina propria of the small intestine (43,44).

A very interesting finding of the present study was increased Fas expression within the proximal region of the colon after administration of bovine lactoferrin, where the TUNEL-positive epithelial cells were detected and tumor development was inhibited. Unfortunately, we were not able to observe NK cells or cytotoxic T lymphocytes in this proximal region, or any other region, by immunohistochemistry using antibodies against asialoGM1 and CD8, respectively (data not shown). Further studies are thus needed to determine site-specific lactoferrin actions on effector cells.

In conclusion, our data suggest that apoptosis caused by elevated expression of Fas is involved in the chemopreventive mechanisms of lactoferrin against the development of colon cancer. Further studies are now under way to address the molecular basis of its actions. Because no severe side effects are seen in experimental animals (18) or in human (our unpublished data), bovine lactoferrin has promise for human clinical application.

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