Review

Prevention of carcinogenesis and cancer metastasis by bovine lactoferrin

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Abstract: Increasing attention is being paid to chemopreventive agents for individuals at high risk of cancer. We have concentrated on bovine lactoferrin (bLF), an 80 kDa iron-binding glycoprotein known to have anti-microbial and immunoprotective effects. Lactoferrin is particularly abundant in colostrum, and is also present in tears, saliva and seminal and uterine secretions. However, only little is known regarding its influence on carcinogenesis. We have shown preventive effects of bLF and its fragment peptide, lactoferricin (bLFcin), consisting of a 25 amino acid sequence without iron binding capacity, on chemically-induced colon carcinogenesis in the rat and transplanted carcinoma cell metastasis in the mouse. The mechanisms are wide-spectrum, including elevation of caspase-1 and IL-18 in the small intestine, enhancement of the cell killing activity of cytotoxic T and natural killer (NK) cells, and anti-inflammatory and anti-angiogenic effects. It also inhibits the induction of liver CYP1A2, a carcinogen activating enzyme, and induces apoptosis in the colon epithelium of carcinogen treated rats. Thus, bLF possesses multi-functional potential to suppress carcinogenesis and is a good candidate for practical application in humans.

Key words: Cancer chemoprevention; lactoferrin; lactoferricin; multi-function; IL-18.

Introduction. The purpose of cancer prevention is to cause a delay in onset of carcinogenesis and to suppress promotion from precancerous lesions to cancer. Cancer can be prevented either by avoiding risk factors such as smoking, a high caloric diet, physical inactivity and exposure to environmental carcinogens,1 or by increasing intake of chemopreventive agents from foods.2 The latter method may be particularly practical because the agents can be taken simply by changing the current dietary style or as supplements.3 As target subjects for chemoprevention, two groups should be considered, one with precancerous lesions and the other with lifestyles related to elevated cancer risk. Whichever of the two, it is necessary that exposure be chronic, possibly for life. In this regard, there are advantages to using natural compounds because adverse effects are generally known and therefore the required procedures for forwarding to clinical trials can be reduced as compared to newly developed compounds. Since carcinogenic processes are multistage and complex,4 agents possessing multiple mechanisms of action offer the most promising candidates. In this review, basic studies on cancer chemoprevention by bovine lactoferrin (bLF) and its peptide fragment lactoferricin (bLFcin) are discussed, focusing on multi-functional mechanistic aspects.

Stages in neoplastic development and inhibition of carcinogenesis. In the prevailing paradigm, three stages of neoplasia can be recognized, “initiation”, “promotion” and “progression”, as illustrated in Fig.1. In the initiation stage, carcinogens such as heterocyclic amines, polycyclic aro-
matic hydrocarbons and nitrosamines are metabolized to proximate and ultimate forms by drug-metabolizing enzymes such as phase I xenobiotic-metabolizing enzyme species, including CYP 450s and also some phase II enzymes, such as sulfotransferases. Generally, however, phase II enzymes such as glutathione transferases protect cells from activated carcinogens by converting them to more readily excreted forms. They may also act as oxygen radical scavengers. Accordingly, initiation is a process which is dependent on a balance of enzyme activity between activation and detoxification. Initiated cells eventually undergo clonal growth to become preneoplastic lesions from which malignant lesions are thought most likely to arise.

Chemopreventive agents which interfere with the initiation process by blocking metabolic activation of carcinogens are defined as “blocking agents”. Compounds acting to reduce cancer development in the post-initiation stage are defined as “suppressing agents”. For the analysis of mechanisms, chemopreventive agents can act during initiation, post-initiation or both.

Since human beings are continuously exposed to various environmental carcinogens, "initiated cells” can always be expected to exist, awaiting stimuli to commit to neoplastic development (promotion) and further malignant conversion (progression). Practical application of chemopreventive agents should therefore be focused on both initiation and promotion stages.

**Mechanisms of inhibition of carcinogenesis.** The following actions are proposed as possible mechanisms for cancer chemoprevention.

1) **Anti-oxidant action**

Reactive oxygen species and/or nitrogen oxide species-induced stress (ROS/NOSS) and downstream events are clearly important for carcinogenesis. ROS/NOSS can induce DNA adducts and their excision, which may cause DNA damage leading to impaired gene regulation. Anti-oxidants, such as polyphenols, carotenoids and curcumin are therefore expected to inhibit carcinogenesis. Chronic inflammation and associated elevated levels of cell proliferation appear to predispose to cancer. Non-steroidal anti-inflammatory drugs (NSAIDs) act to strongly inhibit cyclooxygenase activity resulting in reduction of prostaglandin formation and effects on neoplasia. For example, COX2 inhibitors, nimesulide and celecoxib, have been found to markedly inhibit colon carcinogenesis in the rat.

2) **Anti-inflammatory action**

Enhanced cell proliferation through hormone-receptor signaling is a risk factor of cancer development. For example, estrogen is a growth factor in the absence of progesterone and is the major causative agent for breast and ovarian cancer. Thus the estrogen antagonist tamoxifen citrate has been used for subjects at high risk of breast cancer.

3) **Anti-hormone action**

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4) **Modulation of immune activity**

Since the immune system can influence inflammatory cell reactions through the function of various cytokines, it is important in host defense during the early stages of carcinogenesis. Compounds which influence immune status and suppress carcinogenesis include alpha-tocopherol, unsaturated fatty acids and proanthocyanidins.

5) **Modulation of xenobiotic-metabolizing enzyme activity**

The balance between the phase I activating and phase II detoxifying enzymes plays important roles in determining initiation of carcinogenesis. Thus, intervention in carcinogen metabolism, for example 8-methoxypsoralen inhibition of tobacco-specific 4-(methylnitrosamino)-l-(3-pyridyl)-l-butane (NNK) activation in the lung, causes inhibition of carcinogenesis.

6) **Inhibition of angiogenesis**

The question of whether neoplasias or early stage tumors can continue to grow is dependent to a large extent on the availability of nutrients; consequently, anti-angiogenic agents are clearly of importance. Anti-angiogenic effects have been shown for both NSAIDs and retinoids.

7) **Regulation of signal transduction**

Interference with signaling pathways downstream of receptors responsible for cell growth, dif-
differentiation and apoptosis is clearly important for cancer prevention. For example, d-limonene may act by inhibiting Ha-ras oncogene activation through reduction of H-ras p21 isoprenylation.27)

Milk components and lactoferrin. The whey fraction of milk is actually composed of a large number of ingredients such as alpha-lactoalbumin, beta-lactoalbumin, immunoglobulin, bovine serum albumin and lactoferrin. Very little is known about their individual effects on carcinogenesis, although alpha-lactoalbumin has been shown to be a potent Ca^{2+} elevating and apoptosis-inducing agent.28), 29)

We have concentrated attention on lactoferrin, an 80-kD member of the transferrin family with two iron-binding sites per molecule, which is well known to have antimicrobial properties against bacteria, fungi and viruses.30)–32) The complete amino acid sequence of bLF has been determined and the protein comprises 689 amino acids, folding into N- and C- globular lobes (Fig. 2), each capable of binding up to 1.4 mg iron per 1 g of protein. In its native state in milk, iron saturation is 20~30%. Lactoferrin (bLFcin), a peptide fragment composed of 15 amino acids produced by pepsin hydrolysis of bLF has the sequence NH$_2$-Phe-Lys-Cys-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Ala-Phe-COOH, with Cys-Cys cyclic formation. bLFcin does not have any iron binding site.33), 34)

In humans, lactoferrin also exists in various secretions, for example, tears, saliva and seminal fluid. Levels of lactoferrin in colostrum are particularly high (5–10 mg/ml) and newborn infants receiving mother’s milk ingest lactoferrin at 1–2 g/day. Concentrations of lactoferrin in different body fluids are listed in Table I. Lactoferrin is well known to act against bacteria and stimulate immune responses, activating NK cells and neutrophils, enhancing lymphokine-activated killer (LAK) activity and augmenting macrophage cytotoxicity.35), 36)

bLF and prevention of chemical carcinogenesis. We have generated data on the preventive effects of bLF on carcinogenesis in the colon and other organs (see below). Another focus of interest is its influence on metastasis. Therefore, we have studied effects on prevention of cancer and metastases by oral administration of bLF and bLFcin.

1) Inhibition of colon and liver carcinogenesis by concurrent administration with carcinogen (blocking effects)

The inhibitory influence of bLF on colon carcinogenesis by a food carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), was investigated. Rats were treated with PhIP alone or concurrently with 0.2% bLF (approximately 100 mg/kg/day) for 10 weeks. Effects were evaluated by counting the numbers of colon aberrant crypt foci (ACF), a postulated preneoplastic lesion for colon carcinomas. bLF caused a significant decrease in ACF. Similar effects were observed for preneoplastic lesion development in the liver induced by 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). One possible mechanism is down-regulation of CYP1A2 expression, associated with a decrease in metabolic activation of the carcinogen and DNA adduct formation, resulting in reduction of preneoplastic development.37) bLF also inhibited ACF development induced by another colon carcinogen, 1,2-dimethylhydrazine. This inhibition

| Organ         | Concentration (µg/ml) |
|---------------|-----------------------|
| Human Colostrum | 5–10                 |
| Ordinary milk  | 2                     |
| Tears          | 0.4–1.2               |
| Nasal secretion| 0.1                   |
| Uterine secretion | 0.5–1.0            |
| Salivary juice | 5–10                  |
| Urine          | 1                     |
| Bovine Colostrum | 0.5–1.0             |
| Ordinary milk  | 0.4                   |

Fig. 2. Structure of bovine lactoferrin (courtesy of Professor Keiichi Shimazaki of Graduate School of Agriculture, Hokkaido University).
2) Inhibition of colon carcinogenesis in the post-initiation stage (suppressing effects)

To evaluate preventive activity of bLF and bLFcin in the post-initiation stage of colon carcinogenesis, male rats were initially administered a colon carcinogen, azoxymethane (AOM), and then fed a diet containing bLF or bLFcin from weeks 3 to 40. Control rats received the basal diet alone after AOM treatment (Fig. 3A). Both the incidences and multiplicity (number of tumors/rat) of adenocarcinomas in animals receiving bLF and bLFcin were clearly reduced as compared to the control group (Fig. 3B). No obvious toxicity was noted in major organs. The results provided clear evidence of an inhibitory effect of bLF against colon tumor development when given in the post-initiation stage.\(^ {38}\) Furthermore, carcinogenesis in rats treated with different carcinogens inducing tumors in the tongue, liver, esophagus, lung, bladder and thyroid was inhibited by administration of bLF during the post-initiation period.\(^ {39} \)
Fig. 4. Prevention of metastasis of transplanted cancer cells to the lung (A) and assay of IL-18 in the small intestine in mice (B). 3LL (mouse Lewis Lung carcinoma) cells were subcutaneously inoculated into C57BL/6 mice; then bLF and bLFcin were administered for days 3-7 and 10-14 at 30, 300 and 1000 mg/kg/day by gavage. Lung metastatic colony counts were clearly decreased in both bLF and bLFcin treated groups (Mann-Whitney U test). For the IL-18 protein level assay, mice were given bLF or bLFcin, both at 30 and 300 mg/kg/day, by gavage for 7 days and killed. Levels of IL-18 in the mucosal epithelium and mucosa propria tissue were assayed. bLFcin treatment induced a clear increase in IL-18 protein levels (Dunnet’s t test). bLF treated animals showed tendency of increased IL-18 protein levels. *, P < 0.05, **, P < 0.01.

The results of our series of investigations provide clear evidence of the inhibitory potential of bLF against chemically-induced carcinogenesis, especially in the colon, with actions as both a blocking and a suppressing agent. In addition, bLF reduces metastasis of transplanted tumors. Thus bLF is a promising chemopreventive agent for human colon carcinogenesis. It is likely to also be active against metastasis of colon and lung tumor cells, as well as inhibiting development of neoplastic lesions in the tongue, esophagus, lung and bladder. Furthermore, its mechanisms of action have been partly clarified and include induction of IL-18 and increases in the numbers of interferon gamma-positive cells and cytotoxic T-cells and augmentation of intestinal immunity.

Neoplastic development of the human endocervix has been shown to be associated with down-regulation of lactoferrin mRNA expression in early carcinomas, accompanied by a pronounced elevation in cell proliferation. The mechanisms of action of cancer prevention by bLF is summarized in Fig. 7. bLF exhibits six out of ten classic chemopreventive functions. Furthermore, bLF possesses unique characteristics regarding the immune system, not always shared by compounds such as carotenoids and polyphenols. Increases in IL-18 and enhancement of cytotoxic T cell and NK cell activities may share common functions

Discussion. The objective of cancer chemoprevention is to cause a delay in the onset of clinical cancers so that they do not cause suffering but rather occur at the natural termination of life, as conceived in the “Natural-end cancer” concept. The great interest in developing chemicals suitable for chemopreventive agents has generated a massive literature. However, in spite of the large number of compounds for which efficacy has been proven in experimental models, few clinical trials have been performed and none has, so far, proven a net benefit conferred by natural compounds.

Compounds such as anti-estrogens and NSAIDs actually have been used for high risk patients, but they require careful observation for adverse effects because they are used for far longer periods than originally envisaged as medicines. Thus, usage should be based on thorough examinations regarding beneficial (preventive) and adverse (promotion of carcinogenesis or toxic) effects. Unfortunately for most micronutrients only limited data are available on toxicity. In this regard, use of bLF, a protein with high homology to human lactoferrin, is advantageous because human lactoferrin is ingested by neonates with mother’s milk and is already permitted for use as a food additive. Furthermore, ingestion of bLF for up to 75 weeks did not show any adverse effects in any of the experiments so far conducted (manuscript in preparation).
for both cancer prevention and inhibition of metastasis. Further studies on the responsiveness of pre-neoplastic cells and carcinoma cells to such cytotoxic factors are obviously required.

In conclusion, bLF and bLFcin are promising chemopreventive agents of colon and less markedly the tongue, esophagus, lung, bladder and thyroid carcinogenesis (in rats) and carcinogenesis of the small intestine/jejunum (Apc\textsuperscript{Min} mice). They also prevent metastasis of transplanted carcinomas. Their mechanisms of action include: 1) inhibition of induction of CYP1A2, an activating enzyme for carcinogenic heterocyclic amines; 2) induction of cytotoxic CD8\textsuperscript{+}, CD4\textsuperscript{+}, IFN\gamma\textsuperscript{+} and NK cells in the small intestine and blood; 3) induction of IL18 and caspase 1 in small intestine epithelium; 4) induction of apoptosis in carcinogen-initiated colon epithelium by activation of Fas, caspase-8 and caspase-3; and 5) inhibition of angiogenesis. Based on these observations, bLF has been forwarded for a clinical trial in patients with small colon polyps at the National Cancer Center Hospital, Japan.

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Fig. 6. Inhibition of angiogenesis by bLF. Millipore chambers filled with 3LL (Lewis lung carcinoma) cells \((4 \times 10^6\) cells) were subcutaneously implanted into ICR mice. The mice were given bLF or bLFcin by gavage from day 2 to 6 and killed on day 7. The skin adjacent to the chamber was removed for observation of angiogenesis. The length of the blood vessels was scored as 0 for no induction and 3 for maximal induction (Mann-Whitney U test). bLF dose-dependently inhibited angiogenesis. **, P < 0.01.

Fig. 7. Summary of the mechanism of action of cancer chemoprevention. bLF, by itself, possesses six out of ten well known chemopreventive functions.

preparation of the manuscript. Experiments by the authors were conducted according to the “Guidelines for Animal Experiments of the National Cancer Center” of the Committees for Ethics of Animal Experimentation of the respective institutes.

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