Chemopreventive Effect of Bovine Lactoferrin on 4-Nitroquinoline 1-Oxide-induced Tongue Carcinogenesis in Male F344 Rats

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The modifying effects of dietary feeding of bovine lactoferrin (bLF) on tongue carcinogenesis initiated with 4-nitroquinoline 1-oxide (4-NQO) were investigated in male F344 rats. The activities of phase II detoxifying enzymes, glutathione S-transferase (GST) and quinone reductase (QR), polyamine content and ornithine decarboxylase (ODC) activity in the tongue were also examined for mechanistic analysis of possible modifying effects of bLF on carcinogenesis. At 7 weeks of age, all animals except those treated with bLF alone and untreated rats were given 20 ppm 4-NQO in drinking water for 8 weeks to induce tongue neoplasms. Starting 7 days before 4-NQO exposure, experimental groups were fed experimental diets containing bLF (0.2% and 2%) for 10 weeks (“initiation feeding”). Starting 1 week after the cessation of exposure to 4-NQO, the other experimental groups given 4-NQO and a basal diet were fed the experimental diets for 22 weeks (“post-initiation feeding”). At week 32, the incidence and multiplicity of tongue neoplasms in the “initiation feeding” groups of 0.2% and 2% bLF and the “post-initiation feeding” group of 0.2% bLF were lower than those of the 4-NQO alone group, but without statistical significance. However, “post-initiation feeding” of 2% bLF caused a significant reduction in the incidence (20% vs. 55%, \(P=0.02418\)) and multiplicity (0.25±±±± 0.54 vs. 0.70±±±± 0.71, \(P<0.05\)) of tongue squamous cell carcinoma (by 64%, \(P=0.02418\). bLF treatment elevated liver and tongue GST activities and liver QR activity. The “post-initiation feeding” with 2% bLF significantly decreased QR activity, proliferating cell nuclear antigen-positive index and ODC activity in the tongue. In addition, feeding with bLF decreased tongue polyamine content. These results suggest that bLF, when given at the 2% dose level during the post-initiation phase, exerts chemopreventive action against tongue tumorigenesis through modification of cell proliferation activity and/or the activities of detoxifying enzymes.

Key words: Bovine lactoferrin — Chemoprevention — 4-Nitroquinoline 1-oxide — Tongue cancer — Rats

Oral carcinoma progresses from hyperplastic lesions through dysplasia to invasive carcinoma and the concept of “field cancerization” with molecular alterations has been suggested for oral cavity tumorigenesis.1,2) Such oral malignancy is a common neoplasm in certain regions, such as Asia, the Pacific Islands, parts of Europe, and parts of Brazil.3) The survival of patients with oral carcinoma remains poor despite recent surgical advances. About 30–40% of patients with oral carcinoma survive for 5 years.4) The short survival might be mainly due to late detection. Public awareness of oral carcinoma as compared with other cancers is low and this contributes to delays in diagnosis.5) Oral carcinoma is estimated to be the sixth most common cancer in the world, its prevalence being highest in India.6) An increase in the incidence has been reported in central and eastern Europe, especially among younger men.7–9) Thus, it is important to prevent this malignancy. Tobacco use is a major cause of oral carcinoma,10) including tongue carcinoma.11) Tobacco in all forms, including the tobacco in snuff and betel quid, is carcinogenic in the upper aerodigestive tract, which includes the mouth. It is also known that alcohol acts synergistically with tobacco.12)

Active primary prevention, including chemoprevention of carcinoma development, is important and many studies are currently directed at identifying possible chemopreventive agents.13–17) A multifunctional iron-binding glycoprotein, lactoferrin (LF), is present in large amounts in mammalian secretions.18) LF has several important biological functions, such as anti-bacterial and immunoprotective effects.19,20) Other functions might relate to host primary defense mechanisms.21–23) Among them, the anti-bacterial action of LF is considered to be caused through sequestra-
tion of iron required for microbial growth. Recently, chemopreventive ability of bovine LF (bLF) was demonstrated in chemically induced carcinogenesis in colon, esophagus and lung. bLF could also inhibit intestinal polyposis in ApcMin mice. Moreover, bLF could inhibit tumor metastasis. More recently bLF was reported to inhibit hepatitis C virus viremia, suggesting that it might inhibit human liver tumorigenesis. Thus, bLF may have chemopreventive ability against carcinogenesis in addition to other important biological properties, such as antibacterial and immunoprotective effects.

In our laboratories, possible chemopreventive agents have been found in edible plants, including fruits and vegetables, that are active against tongue carcinogenesis. Such chemopreventive agents possess anti-proliferation, anti-inflammatory and anti-oxidant effects. LF or bLF inhibits cell proliferation by blocking the cell cycle progression, acts as an anti-inflammatory molecule and has an anti-oxidant effect. These findings led us to investigate whether bLF exerts inhibitory effects on tongue carcinogenesis.

In the present study, we investigated the modifying (possibly inhibiting) effects of dietary bLF on tongue carcinogenesis in rats initiated with 4-nitroquinoline 1-oxide (4-NQO). The effects of dietary bLF on the cell proliferation activity of tongue epithelium were also assessed by measuring proliferating cell nuclear antigen (PCNA)-positive index, ornithine decarboxylase (ODC) activity and polyamine level. In addition, the activities of glutathione S-transferase (GST) and quinone reductase (QR) were assayed in the liver and tongue, since certain chemopreventive compounds are known to increase activities of detoxifying enzymes, such as GST and QR.

**MATERIALS AND METHODS**

**Animals, diets, and carcinogen** Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka), 4 weeks old, were used. All animals were housed in wire cages (3 or 4 rats/cage) with free access to drinking water and basal diet, CE-2 (CLEA Inc., Tokyo), under controlled conditions of humidity (50±10%), light (12-h light/dark cycle) and temperature (23±2°C). They were quarantined for 14 days and randomized into experimental and control groups. Powdered CE-2 diet (345.2 Cal) was used as a basal diet throughout the study. 4-NQO (CAS, 56-57-5; 98% pure) was obtained from Wako Pure Chemical Ind. (Osaka). bLF, obtained from bovine milk by the method described previously, was kindly provided by Drs. Tomita and Shimanura (Morinaga Milk Ind., Zama). Experimental diets were prepared by mixing bLF at a concentration of 0.2% or 2%. The 4-NQO solution (20 ppm) was prepared on a weekly basis. The experimental diets and 4-NQO solution were stored in a cold room until used. They were freely available during the study.

**Experimental procedures** A total of 124 male F344 rats were divided into 7 groups as shown in Fig. 1. Groups 1 through 5 were given 20 ppm 4-NQO in drinking water for 8 weeks. Groups 2 and 3 were fed diets containing bLF at 0.2% and 2%, respectively, starting at 6 weeks of age until 1 week after the end of the exposure to the carcinogen. They were then returned to the basal diet and maintained on this diet for 22 weeks. Groups 4 and 5 were fed diets mixed with 0.2% and 2% bLF, respectively, starting 1 week after cessation of 4-NQO treatment and maintained on these diets for 22 weeks. Group 6 was given 2% bLF-containing diet alone. Group 7 served as an untreated control. The experiment was terminated at 32 weeks after the start and all animals were killed to assess the incidences of neoplastic and preneoplastic lesions in the tongue. At the termination of the study, all organs including tongue in the rats were carefully inspected for pathological lesions. The tongues from 6 randomly selected rats from group 1, 4 rats each from groups 2–6 and 3 rats from group 7 were rapidly removed, rinsed with saline and cut into halves: one portion was used for histological examination and PCNA-immunohistochemistry, and the other (without macroscopic lesions) for assays of ODC activity, polyamine content, and GST and QR activities. For histological examination, tissues and gross lesions were fixed in 10% buffered formalin, embedded in paraffin blocks and stained with hematoxylin and eosin. Tongue lesions (hyperplasia, dysplasia and neoplasms) were diagnosed according to the criteria described by Kramer et al.
PCNA immunohistochemistry  PCNA-positive cell nuclei were counted in the tongue mucosa without tumors of rats (6 from group 1, 4 each from groups 2–6 and 3 from group 7). Anti-PCNA antibody (Dako, Kyoto) was used with the avidin-biotin complex method. Tissue sections were deparaffinized with xylene, hydrated through a graded ethanol series and incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxide activity. They were then incubated with 10% normal horse serum at room temperature for 30 min to block background staining, and stained with PCNA antibody. The number of nuclei positively stained for PCNA in the tongue epithelium was counted and divided by the total number of nuclei of the tongue epithelium to obtain the PCNA-positive index (%).

Measurement of ODC activity  Tongue mucosa was scraped with a stainless steel disposable microtome bladed knife (S35, Feather Safety Razor Co., Ltd., Osaka), pooled and homogenized in 1.5 ml of homogenizing buffer (250 mmol sucrose, 50 mmol Tris-HCl, pH 7.4, containing 1 mmol dithiothreitol, 1 mmol EDTA and 0.4 mmol pyridoxal 5′-phosphate) using a Polytron. The homogenates were centrifuged at 15000 rpm for 30 min at 4°C. The resulting cytosol fraction was used for determination of ODC activity and protein. ODC activity in tongue mucosa was determined by a modification of the method described previously.49

Assay of polyamines  The tongue mucosa scraped immediately after sample collection was used for the measurement of polyamine contents. Tissue polyamine contents were determined using the method described by Koide et al.40

Measurement of GST and QR activities in the liver and tongue epithelium  GST and QR activities in liver and tongue epithelium were also determined. When the animals were killed, the livers were perfused with saline for 10 min to remove blood, excised immediately and minced. Aliquots of minced liver and the mucosal scrapings of the tongue were processed to obtain the cytosolic fraction.41 The activities of GST with 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) as substrates and those of QR with NADH and menadione as substrates were determined.41 All spectroscopic assays were based on measurement of absorption at 340 nm, and all samples were measured in triplicate. One unit of enzyme activity is the amount of enzyme catalyzing the conversion of 1 mmol of substrate to product per min at 25°C. Cytosolic protein concentrations were determined by the Bradford method,42 using bovine serum albumin as the standard.

Statistical analysis  Where applicable, the data were analyzed using the Fisher’s exact probability test, Student’s t-test or Welch’s t-test (software “DA Stats”), taking \(P<0.05\) as the level of significance.

RESULTS

General observations  The rats tolerated well the oral administration of 4-NQO and/or bLF feeding. During the study, no clinical signs of toxicity were seen in any group. Histologically, there were no pathological alterations suggesting toxicity of bLF in the liver, kidneys, heart, and lungs.

The data on mean body, liver, and relative liver weights (g liver weight/100 g body weight) in all groups at the termination are given in Table I. The mean body weight of rats in group 6 (2% bLF alone) was significantly lower than that of group 7 (untreated) \(P<0.05\). The mean liver and relative liver weights in group 1 (4-NQO alone) were significantly larger than those of group 7 \(P<0.05\) and \(P<0.01\), respectively. The mean liver and relative liver weights of groups 2 (4-NQO+0.2% bLF) and 3 (4-NQO+0.4% bLF) were significantly larger than those of group 1 \(P<0.05\) and \(P<0.01\), respectively.

Table I. Body, Liver, and Relative Liver Weights of Rats at the End of the Study

| Group no. | Treatment       | No. of rats examined | Body wt. (g) | Liver wt. (g) | Relative liver wt. (g/100 g body wt.) |
|-----------|-----------------|---------------------|-------------|--------------|-------------------------------------|
| 1         | 4-NQO alone     | 20                  | 336±27\(^a\) | 14.4±2.1\(^b\) | 4.26±0.45\(^c\)                      |
| 2         | 4-NQO+0.2% bLF  | 20                  | 336±24      | 12.5±1.4\(^d\) | 3.70±0.32\(^e\)                      |
| 3         | 4-NQO+0.4% bLF  | 20                  | 336±18      | 12.9±1.3\(^f\) | 3.82±0.27\(^g\)                      |
| 4         | 4-NQO→0.2% bLF  | 20                  | 332±22      | 14.0±1.3     | 4.17±0.21                           |
| 5         | 4-NQO→2% bLF    | 20                  | 326±18      | 14.1±0.6     | 4.33±0.15                           |
| 6         | 2% bLF          | 12                  | 315±9\(^h\) | 14.0±1.2     | 4.44±0.29\(^i\)                     |
| 7         | No treatment    | 12                  | 336±28      | 12.9±1.7     | 3.84±0.31                           |

\(^{a}\) Mean±SD.

\(^{b}\), \(^{c}\), \(^{g}\) Significantly different from group 7 by Student’s t-test or Welch’s t-test (\(b\), \(c\), \(g\)\(P<0.05\), \(e\), \(f\)\(P<0.01\) and \(g\)\(P<0.001\)).

\(^{d}\), \(^{e}\), \(^{f}\) Significantly different from group 1 by Student’s t-test or Welch’s t-test (\(d\), \(e\), \(f\)\(P<0.02\)).
NQO+2% bLF) were significantly lower than those of group 1 ($P<0.001$, $P<0.001$ or $P<0.01$). The mean relative liver weight in group 6 was significantly greater than that of group 7 ($P<0.001$).

**Incidence of tongue neoplasms and preneoplastic lesions**

Tongue tumors developed in the posterior tongue (dorsal region) of rats in groups 1–5. Histologically, they were well differentiated squamous cell carcinoma or papilloma. Dysplastic and hyperplastic lesions also developed in the tongues of rats in these groups. No preneoplastic or neoplastic lesions in any other organs including tongue were observed in groups 6 and 7.

The incidences of tongue tumors (squamous cell papilloma and carcinoma) and preneoplasia (hyperplasia and dysplasia) in each group are shown in Table II. In group 1 (4-NQO alone), the incidences of tongue squamous cell carcinoma and papilloma were 55% and 20%, respectively. On the other hand, the incidences of tongue carcinoma in rats given bLF together with 4-NQO administration (groups 2 and 3) or after 4-NQO exposure (groups 4 and 5) were lower than that of group 1. Similarly, the incidences of tongue papilloma of these groups except for group 2 were smaller than that of group 1. Statistical analysis revealed a significant reduction (64%) in the incidence of tongue carcinoma in rats fed the bLF-containing diet (2%) during the post-initiation stage (group 5), compared with that in group 1 ($P=0.02418$). The multiplicities (number of tumors/rat) of papilloma and carcinoma in groups 2 through 5 were smaller than those in group 1 and a statistically significant difference was found between groups 1 ($0.70\pm0.71$) and 5 ($0.25\pm0.54$) ($P<0.05$).

As shown in Tables II and IV, various degrees of hyperplasia or dysplasia with or without neoplasms were also observed in the tongue of rats in groups 1–5. The incidences of tongue squamous hyperplasia and dysplasia in rats in groups 1–5 were comparable (Table II). Among the various types of hyperplasia and dysplasia, the incidences of papillary hyperplasia of rats in groups 3, 4 and 5 were significantly lower than that of group 1 ($P=0.02037$, $P<0.02037$ and $P<0.00053$, respectively), as indicated in

### Table II. Incidences of Tongue Preneoplasia and Neoplasia of Rats Treated with 4-NQO and/or bLF

| Group no. | Treatment | No. of rats examined | Hyperplasia (%) | Dysplasia (%) | No. of rats with tongue tumors | Squamous cell papilloma (%) | Squamous cell carcinoma (%) |
|-----------|-----------|----------------------|-----------------|---------------|------------------------------|-----------------------------|-----------------------------|
| 1         | 4-NQO alone | 20                   | 20 (100)        | 18 (90)       | 11 (55)                      | 4 (20)                      | 11 (55)                     |
| 2         | 4-NQO+0.2% bLF | 20                   | 20 (100)        | 15 (75)       | 8 (40)                       | 5 (25)                      | 7 (35)                      |
| 3         | 4-NQO+2% bLF | 20                   | 20 (100)        | 16 (80)       | 6 (30)                       | 3 (15)                      | 6 (30)                      |
| 4         | 4-NQO→0.2% bLF | 20                   | 20 (100)        | 18 (90)       | 6 (30)                       | 2 (10)                      | 5 (25)                      |
| 5         | 4-NQO→2% bLF | 20                   | 20 (100)        | 17 (85)       | 4a) (20)                     | 3 (15)                      | 4a) (20)                    |
| 6         | 2% bLF | 12                   | 0                | 0             | 0                            | 0                           | 0                           |
| 7         | No treatment | 12                  | 0                | 0             | 0                            | 0                           | 0                           |

a) Significantly different from group 1 by Fisher’s exact probability test ($P=0.02418$).

### Table III. Multiplicity of Tongue Tumors in Rats Treated with 4-NQO and/or bLF

| Group no. | Treatment | No. of rats with tongue tumors | Multiplicity (no. of tumors/rat) of tongue tumors |
|-----------|-----------|-------------------------------|-----------------------------------------------|
|           |           |                               | Total Squamous cell papilloma Squamous cell carcinoma |
| 1         | 4-NQO alone | 11 | 1.05±1.20a) | 0.35±0.79 | 0.70±0.71 |
| 2         | 4-NQO+0.2% bLF | 8 | 0.65±0.91 | 0.25±0.43 | 0.40±0.58 |
| 3         | 4-NQO+2% bLF | 6 | 0.55±0.92 | 0.20±0.51 | 0.35±0.57 |
| 4         | 4-NQO→0.2% bLF | 6 | 0.45±0.74 | 0.10±0.30 | 0.35±0.65 |
| 5         | 4-NQO→2% bLF | 4 | 0.40±0.80 | 0.15±0.36 | 0.25±0.54a) |
| 6         | 2% bLF | 0 | 0 | 0 | 0 |
| 7         | No treatment | 0 | 0 | 0 | 0 |

a) Mean±SD.
b) Significantly different from group 1 by Student’s $t$-test ($P<0.05$).
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Table IV. Incidence of Various Types of Preneoplasia in the Tongue

| Group no. | Treatment           | No. of rats examined | Hyperplasia (%) | Dysplasia (%) |
|-----------|---------------------|----------------------|-----------------|---------------|
|           |                     |                      | Total           | Simple        | Papillary     | Total | Mild | Moderate | Severe |
| 1         | 4-NQO alone         | 20                   | 20 (100)        | 17 (85)       | 18 (90)       | 12 (60) | 10 (50) |
| 2         | 4-NQO+0.2% bLF      | 20                   | 20 (100)        | 17 (85)       | 15 (75)       | 12 (60) | 8 (40)  |
| 3         | 4-NQO+2% bLF        | 20                   | 20 (100)        | 18 (90)       | 16 (80)       | 6 (30)  | 7 (35)  |
| 4         | 4-NQO→0.2% bLF      | 20                   | 20 (100)        | 10<sup>o</sup> (50) | 18 (90)       | 8 (40)  | 6 (30)  |
| 5         | 4-NQO→2% bLF        | 20                   | 20 (100)        | 6<sup>b</sup> (30) | 17 (85)       | 4<sup>d</sup> (20) | 4<sup>d</sup> (20) |
| 6         | 2% bLF              | 12                   | 0               | 0             | 0             | 0      | 0      |
| 7         | No treatment        | 12                   | 0               | 0             | 0             | 0      | 0      |

<sup>a), b), c), d)</sup> Significantly different from group 1 by Fisher’s exact probability test (<sup>a</sup>P<0.02037, <sup>b</sup>P<0.00053, <sup>c</sup>P<0.01123 and <sup>d</sup>P<0.04792).<sup>29</sup>

Table IV. The frequencies of moderate and severe dysplasias in group 5 were also significantly smaller than that in group 1 (<sup>P</sup>=0.01123 and <sup>P</sup>=0.04792).

PCNA-labeling index The results of measurement of the PCNA-labeling index in the tongue epithelium are shown in Table V. The PCNA-labeling index in group 1 was significantly greater than that in group 7 (<sup>P</sup><0.005). The values in groups 2–4 were smaller than that in group 1, but without statistical significance. The PCNA index in group 5 was significantly smaller than that of group 1 (<sup>P</sup><0.02). The value in group 6 was slightly higher than that of group 7.

Polymine in tongue epithelium The data on tongue polyamine levels are presented in Table VI. Exposure to 4-NQO significantly elevated the total polyamine levels (the sum of diamine, spermidine and spermine) in tongue epithelium (<sup>P</sup><0.02). This elevation was caused by the significant increase in spermidine and spermine contents (<sup>P</sup><0.005 and <sup>P</sup><0.05, respectively). bLF feeding (groups 2–5) reduced the total polyamine amounts in tongue epithelium, as compared with group 1. Total tissue polyamine levels in groups 3, 4 and 5 were significantly lower than in group 1 (<sup>P</sup><0.01, <sup>P</sup><0.05 and <sup>P</sup><0.005, respectively). The amounts of spermidine in these groups were significantly higher than that in group 7.

Table V. PCNA-labeling Index in the Tongue Epithelium

| Group no. | Treatment           | No. of rats examined | PCNA-positive nuclei (%) |
|-----------|---------------------|----------------------|--------------------------|
| 1         | 4-NQO alone         | 6                    | 28±5<sup>a,b</sup>       |
| 2         | 4-NQO+0.2% bLF      | 4                    | 21±6                     |
| 3         | 4-NQO+2% bLF        | 4                    | 20±7                     |
| 4         | 4-NQO→0.2% bLF      | 4                    | 20±11                    |
| 5         | 4-NQO→2% bLF        | 4                    | 16±7<sup>a</sup>         |
| 6         | 2% bLF              | 4                    | 18±5                     |
| 7         | No treatment        | 3                    | 14±3                     |

<sup>a)</sup> Mean±SD (pmol 14CO<sub>2</sub>/h/mg protein), <sup>b)</sup> Significantly different from group 7 by Student’s <sup>t</sup>-test (<sup>P</sup><0.005). <sup>c)</sup> Significantly different from group 1 by Student’s <sup>t</sup>-test (<sup>P</sup><0.02).

Table VI. Polyamine Levels in the Tongue Epithelium

| Group no. | Treatment           | No. of rats examined | Polyamine content (mmol/mg protein) |
|-----------|---------------------|----------------------|-------------------------------------|
|           |                     |                      | Diamine | Spermidine | Spermine | Total    |
| 1         | 4-NQO alone         | 6                    | 0.08±0.11<sup>a,b</sup> | 1.73±0.27<sup>c</sup> | 1.90±0.27<sup>d</sup> | 3.71±0.38<sup>e</sup> |
| 2         | 4-NQO+0.2% bLF      | 4                    | 0       | 1.40±0.40  | 1.64±0.20  | 3.04±0.57 |
| 3         | 4-NQO+2% bLF        | 4                    | 0.02±0.04 | 1.25±0.16  | 1.78±0.25  | 2.78±0.44<sup>f</sup> |
| 4         | 4-NQO→0.2% bLF      | 4                    | 0.13±0.21 | 1.20±0.27  | 1.78±0.24  | 3.11±0.41<sup>g</sup> |
| 5         | 4-NQO→2% bLF        | 4                    | 0.09±0.07 | 1.02±0.07  | 1.65±0.30  | 2.76±0.36<sup>h</sup> |
| 6         | 2% bLF              | 4                    | 0.11±0.15 | 1.22±0.25  | 1.43±0.43  | 2.76±0.62 |
| 7         | No treatment        | 3                    | 0.36±0.08 | 0.85±0.38  | 1.41±0.21  | 2.61±0.66 |

<sup>a)</sup> Mean±SD. <sup>b)</sup> Significantly different from group 7 by Student’s <sup>t</sup>-test (<sup>b</sup>P<0.01), <sup>c)</sup>P<0.005, <sup>d)</sup>P<0.05 and <sup>e)</sup>P<0.02. <sup>f)</sup> Significantly different from group 1 by Student’s <sup>t</sup>-test or Welch’s <sup>t</sup>-test (<sup>f</sup>P<0.02, <sup>g</sup>P<0.01, <sup>h</sup>P<0.05, <sup>i</sup>P<0.001 and <sup>j</sup>P<0.005).
lower than that in group 1 ($P<0.02$, $P<0.02$ and $P<0.001$, respectively).

**ODC activity in the tongue** The data on tongue ODC activity are listed in Table VII. 4-NQO exposure significantly increased tongue ODC activity ($P<0.005$). The mean tongue ODC activities in groups 2–5 were lower than that of group 1 and the difference between groups 1 and 6 was statistically significant ($P<0.05$).

**GST and QR activities in the liver and tongue** The results of GST and QR assays in the liver and tongue are summarized in Tables VIII and IX, respectively. As shown in Table VIII, the liver GST-DCNB in group 1 was significantly higher than that in group 7 ($P<0.05$). The liver GST-CDNB activities in groups 2 and 3 were significantly higher than that of group 1 ($P<0.02$ and $P<0.001$, respectively). The liver QR activity in group 1 was significantly smaller than that in group 7 ($P<0.001$). The liver QR activity in group 6 was significantly higher than that of group 7 ($P<0.005$). In the tongue epithelium, 4-NQO reduced GST and QR activities and the decrease in GST-CDNB activity was greater than that in QR activity (Table IX). bLF feeding significantly increased the tongue GST-DCNB activities in groups 2, 3 and 5, compared with that of group 1 ($P<0.05$).

### Table VII. ODC Activity in the Tongue Epithelium

| Group no. | Treatment        | No. of rats examined | ODC activity |
|-----------|------------------|----------------------|--------------|
| 1         | 4-NQO alone      | 6                    | 37.3±15.8 ab  |
| 2         | 4-NQO+0.2% bLF   | 4                    | 25.8±9.8     |
| 3         | 4-NQO+2% bLF     | 4                    | 21.7±6.4     |
| 4         | 4-NQO→0.2% bLF   | 4                    | 19.1±8.6     |
| 5         | 4-NQO→2% bLF     | 4                    | 15.4±7.4 c  |
| 6         | 2% bLF           | 4                    | 9.8±3.4     |
| 7         | No treatment     | 3                    | 3.6±1.8     |

a) Mean±SD (pmol $^{14}$CO$_2$/h/mg protein).
b) Significantly different from group 7 by Welch $t$-test ($P<0.05$).
c) Significantly different from group 1 by Student’s $t$-test ($P<0.05$).

d) $P<0.02$, e) $P<0.01$.

### Table VIII. GST and QR Activities in the Liver

| Group no. | Treatment        | No. of rats examined | GST-CDNB | GST-DCNB | QR   |
|-----------|------------------|----------------------|----------|----------|------|
| 1         | 4-NQO alone      | 6                    | 511±37 ab | 16±2 ab  | 43±9 ab|
| 2         | 4-NQO+0.2% bLF   | 4                    | 610±68 d  | 19±2     | 49±1  |
| 3         | 4-NQO+2% bLF     | 4                    | 683±55 e  | 16±2     | 64±16  |
| 4         | 4-NQO→0.2% bLF   | 4                    | 587±90    | 16±2     | 85±10 c|
| 5         | 4-NQO→2% bLF     | 4                    | 599±54    | 14±1     | 100±10 a|
| 6         | 2% bLF           | 4                    | 595±41    | 16±2     | 185±20 a|
| 7         | No treatment     | 3                    | 54±14     | 13±1     | 115±15 |

a) Mean±SD (mU/mg protein).
b), c), g) Significantly different from group 7 by Student’s $t$-test ($b) P<0.05$, c) $P<0.001$ and g) $P<0.005$).
d), e), f) Significantly different from group 1 by Student’s $t$-test (d) $P<0.02$, e) $P<0.01$ and f) $P<0.05$).
DISCUSSION

Our results indicate that dietary administration of 2% bLF during the post-initiation phase significantly lowered the incidence and multiplicity of the tongue squamous cell carcinoma induced by 4-NQO. Although bLF feeding at two dose levels (0.2% and 2%) during the initiation phase and at a low dose level (0.2%) during the post-initiation phase decreased the incidence and multiplicity of tongue carcinoma, the reduction was not statistically significant. Similar results were reported using the multi-organ carcinogenesis model,28) in which dietary bLF at the doses of 0.2% and 0.02% significantly inhibited the multiplicities of esophageal and lung tumors, but did not significantly affect the incidences of these tumors and those in other organs, such as liver, kidney and thyroid. These results suggest that the inhibitory effect of bLF on carcinogenesis was relatively weak in tongue, esophagus and lung.

In the present study, feeding with bLF-containing diet at the two dose levels (0.2% and 2%) did not cause any delay in body weight gain. Also, there were no significant pathological alterations in the major organs in rats fed the bLF-containing diets during the study. These findings indicate no toxicity of bLF, as reported in previous studies.25–28) This is important for the possible application of this cancer chemopreventive compound in human clinical trials, because such trials require long-term oral administration of tested compounds.

The tongue is known to metabolize 4-NQO.43) Defense against carcinogenic events is provided by phase II enzymes such as GST and QR.36) These enzymes are of importance because they can conjugate electrophiles and protect the host from the carcinogenic effects of chemical carcinogens. Unlike other chemical carcinogens, 4-NQO requires metabolic activation, not by cytochrome P450 enzymes, but by a phase II enzyme QR (DT-diaphorase) to exert its carcinogenic activity.44, 45) In the present study, dietary bLF at 2% during the post-initiation phase significantly decreased QR activity in the tongue. Feeding of bLF significantly elevated the activity of GST in the liver (by “initiation feeding”) and tongue (by “initiation feeding” and “post-initiation feeding”), and increased QR activity in the liver (by “initiation feeding” and “post-initiation feeding”). Similar results have been obtained in our previous studies showing that an organoselenium compound, 1,4-phenylenebis(methylene)selenocyanate,41) a citrus coumarin derivative, auraptene33) and a flavonoid, morin,46) inhibit 4-NQO-induced rat tongue tumorigenesis. These enzymatic alterations caused by bLF feeding may contribute to its chemopreventive activity, although other mechanisms, including immunomodulation,25, 26, 28, 29) by which bLF might inhibit tumor formation should also be considered.

Cell proliferation plays an important role in multistage carcinogenesis and involves multiple genetic changes.47, 48) Polyamines and polyamine synthetic enzyme activities have been associated with cell proliferation. A decrease in the numbers of PCNA-positive cells reflects a decrease in S phase cells and thus a reduced proliferative activity. Our previous study demonstrated that an ornithine decarboxylase inhibitor, α-difluoromethylornithine, effectively suppressed 4-NQO-induced tongue carcinogenesis in rats.49) Therefore, lowering the polyamine levels and ODC activity in the target organ might suppress the high proliferative activity of cells initiated with a carcinogen and inhibit carcinogenesis. Also, most of the possible chemopreventive agents against carcinogen-induced oral carcinogenesis suppress cell proliferation activity.14) In the current study, bLF administration during either the initiation or post-initiation phase significantly decreased polyamine levels in the target organ and bLF feeding at the 2% dose level during the post-initiation stage significantly decreased PCNA-labeling index and ODC activity. Thus, one of the mechanisms by which bLF exerts chemopreventive ability might be related to suppression of cell proliferation in the target tissue, especially when bLF is given during the post-initiation phase.

In conclusion, our results suggest that dietary bLF feeding exerts a chemopreventive effect on 4-NQO-induced rat tongue carcinogenesis, when fed during the post-initiation phase. This protective effect of bLF may be related to activation of phase II enzymes (GST and QR) and/or the control of carcinogen-induced hyper-cell proliferation.

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