Purple corn color suppresses Ras protein level and inhibits 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in the rat

Katsumi Fukamachi,[1] Takafumi Imada,[1,2] Yutaka Ohshima,[1,3] Jiegou Xu[1] and Hiroyuki Tsuda[1,4]

[1]Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601; [2]San-Ei Gen F.F.I. Corporation, 1-1-11 Sanwa-cho, Toyonaka, Osaka 561-8588; [3]Chemicals Evaluation and Research Institute, Hita Laboratory, 3-822 Ishii-machi, Hita, Oita 877-0061, Japan

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Anthocyanins belong to the class of phenolic compounds collectively named flavonoids. Many anthocyanins are reported to have inhibitory effects on carcinogenesis. Purple corn color (PCC), an anthocyanin containing extract of purple corn seeds, is used as a food colorant. The major anthocyanin in PCC is cyanidin 3-*O*-β-D-glucoside (C3-G). The present study was conducted to assess the influence of dietary PCC on 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis in rats. PCC significantly inhibited DMBA-induced mammary carcinogenesis in human c-Ha-*ras* proto-oncogene transgenic (Hras128) rats and in their non-transgenic counterparts. PCC and C3-G also inhibited cell viability and induced apoptosis in mammary tumor cells derived from Hras128 rat mammary carcinomas. At the molecular level, PCC and C3-G treatment resulted in a preferential activation of caspase-3 and reduction of Ras protein levels in tumor cells. It is proposed that C3-G could act as a chemopreventive and possibly chemotherapeutic agent for cancers with mutations in *ras*. Secondly, the *in vitro–in vivo* system used in this study can be utilized for screening for cancer preventive compounds that act via Ras down-regulation. (*Cancer Sci* 2008; 99: 1841–1846)

Anthocyanins are found throughout the plant kingdom and impart purple, blue and red color to fruits and vegetables. They belong to the class of phenolic compounds collectively named flavonoids. Anthocyanins are naturally present as glycosides having glucose, galactose, rhamnose, xylose or arabinose attached to the aglycon nucleus; the sugar-free aglycon nucleus is known as anthocyanidin. Several hundred anthocyanin species exist depending on the glycoside structure. It is generally accepted that anthocyanin food colors do not exert obvious toxicity, teratogenicity or mutagenicity and, indeed, anthocyanins may inhibit mutagenesis in the Ames test.[1–3] In studies testing the effects of anthocyanins on carcinogenesis, inhibitory effects of anthocyanins have been reported.[4–9]

PCC (Maize morado color) is extracted from the seeds of purple corn, *Zea mays* L., and is used as a beverage colorant (as Chica Morada) in Latin America, especially in Peru. PCC has been shown to inhibit azoxymethane-induced colon tumors in rats.[4] PCC contains six anthocyanins; the major anthocyanin found in PCC is cyanidin 3-*O*-β-D-glucoside (C3-G). *In vitro*, C3-G reacts with peroxyradicals and is converted into the oxidation products 4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran and protocatechuic acid (PC); PC is also a radical scavanger.[10] Thus, after C3-G reacts with biological radicals, a second radical scavenger is produced. C3-G also gives rise to PC *in vivo*: after oral administration of C3-G, both C3-G itself and PC are found in the plasma.[11] The plasma C3-G concentration reaches a maximum at 30 min after single oral administration. The half life of plasma C3-G is about 2 h.[11] Therefore, C3-G in the diet is expected to improve the body's antioxidant capability and inhibit carcinogenesis *in vivo*.

We have established a rat line carrying copies of the human c-Ha-*ras* proto-oncogene under the regulation of its own promoter region (Hras128). This line is highly susceptible to *N*-methyl-*N*-nitrosourea (MNU)- and DMBA-induced mammary carcinogenesis.[12–14] Tumors develop in almost all females within as short a period as 8–12 weeks after a single MNU or DMBA treatment. The animals have also been found to be susceptible to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder,[15] DMBA-induced skin,[16] and 4-nitroquinoline 1-oxide-induced tongue[17] carcinogenesis. This model can be used for the short-term assay of test compounds including chemopreventive compounds,[18] genotoxic compounds[19] and non-genotoxic promoting agents.[20] In the present study, this short-term tumor model was employed to investigate the effects of PCC on DMBA mammary carcinogenesis. Non-transgenic rats were then used to confirm the effects that PCC had on transgenic rats. PCC inhibited mammary carcinogenesis in both transgenic and non-transgenic rats.

Recently, we established cell lines from mammary carcinomas induced by DMBA in Hras128 rats.[21] These cells can be utilized for mechanistic analysis of compounds showing a modifying influence on mammary carcinogenesis in Hras128 rats. Accordingly, we used the cell lines for the analysis of the mechanism by which PCC inhibited carcinogenesis.

Materials and Methods

Animals. Female c-Ha-*ras* transgenic (Hras128, Tg) and non-transgenic (non-Tg) rats were bred by CLEA Japan, Tokyo, Japan. They were maintained in plastic cages in an air-conditioned room with a 12-h light/12-h dark cycle. In total, 62 Tg and non-Tg rats received a single dose of DMBA (Tokyo Chemical Industry, Tokyo, Japan) (25 mg/kg body weight) by gavage at 7 weeks of age. One day thereafter, they were placed on powdered basal diet MF (Oriental Yeast, Tokyo, Japan) containing either purple corn color (PCC) (San-Ei Gen F.F.I., Osaka, Japan) or no supplement. PCC was prepared as described previously.[4] The specifications of PCC used in this study were as follows: trade name San RED No.5, lot No. 040421, purity 33.7% as anthocyanin concentration. Gross observation and palpation of the mammary gland were regularly performed to monitor the development of mammary tumors after DMBA

[4]To whom correspondence should be addressed. E-mail: htsuda@med.nagoya-cu.ac.jp
Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; PCC, purple corn color; C3-G, cyanidin 3-*O*-β-D-glucoside; PC, protocatechuic acid.

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treatment. The surviving animals were killed by exsanguination under deep ether anesthesia at the end of week 8 for Tg and week 22 for non-Tg rats. The numbers of visible tumors were recorded before they were measured and sampled for histological examination. Values are expressed as average tumor weight of the total tumors for each rat. Body and liver and kidney weights were also recorded. The experiments were conducted according to the 'Guidelines for Animal Experiments of the Nagoya City University Graduate School of Medical Sciences'.

Cell culture. Rat mammary carcinoma cells (C3, C11 and C17),[21] which were established from DMBA-induced tumors in Hras128 rats, were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). Cells were seeded in 96-well plates, and then incubated with PCC, C3-G (Extrasynthese, Genay Cedex, France) or PC (Wako Pure Chemicals, Osaka, Japan) for 1 day. Cell viability was measured using a CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

In some experiments, the cells were grown to confluency, then rendered quiescent by incubation in DMEM containing 0.5% FCS. After serum starvation for 48 h, the cells were treated with 10% FCS to initiate signaling cascades.

Western blot. Cells or tissues were lyzed in a minimal volume of lysis buffer (50 mM Tris-HCl [pH 7.4], 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 1 mM NaF and protease inhibitors [protease inhibitor cocktail, Sigma, Saint Louis, MO, USA]). Total proteins were resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA). The blots were incubated with primary antibodies after blocking with 3% non-fat milk. The blots were then washed and incubated with horseradish peroxidase-conjugated antirabbit and antimouse immunoglobulin G (IgG) antibodies (Southern Biotechnology Associates, Birmingham, AL, USA). The bound antibodies were detected using enhanced chemiluminescence (ECL) plus Western blotting detection system (GE Healthcare Bio-sciences, Piscataway, NJ, USA). Detection of activated Ras protein was performed using a Ras activation assay kit (Upstate, Lake Placid, NY, USA) as described previously.[22,23]

The following antibodies were used: pan-Ras (1/4000, clone RAS10; Upstate), extracellularsignal- regulated kinase (Erk) 1/2 (1/50 000, 06–182; Upstate), phospho-ERK 1/2 (1/1000, #9106; Cell Signaling Technology, Danvers, MA, USA), Akt (1/1000, #9272; Cell Signaling Technology), phospho-Akt (Ser473) (1/1000, #4051; Cell Signaling Technology), caspase-3 (1/1000, #9662; Cell Signaling Technology), cleaved caspase-3 (1/1000, #9661; Cell Signaling Technology), caspase-8 (1/1000, H-134; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (1/10 000, A5441; Sigma, Saint Louis, MO, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of apoptotic cells. Apoptotic cells were detected by TUNEL assay using an *In situ* Apoptosis Detection Kit (Takara Bio, Otsu, Japan) according to the manufacture's instructions. Fluorescent nuclear staining was performed with TO-PRO-3 iodide (Molecular Probes, Eugene, OR) at a dilution of 1/1000. A confocal microscope FLUOVIEW FV300 (Olympus, Tokyo) was utilized for imaging.

Real-time polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), and the RNA was reverse-transcribed using Superscript III Reverse Transcriptase with Random primers (Invitrogen) according to the manufacturer's instructions. PCR amplification was carried out using SYBR *Premix Ex Taq* and the Smart Cycler II System (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. All PCR amplifications were done for 40 cycles and a melt curve analysis was used to examine the specificity of the amplified products. The following primers were used: H*ras*, 5′-CAGTACAGGGAGCAGATCAA-3′ and 5′-AGCACACACTTGCAGCTCAT-3′; K*ras*, 5′-GCGTAGGCAAG AGTGCCTTGA-3′ and 5′-GACCTGCTGTGTCGAGAATATCCA-3′; N*ras*, 5′-AGCAGTGAGGATGGCACTCAAG-3′ and 5′-GATGTC AGAACCAGGGCATCAG-3′; β-*actin*, 5′-CCGTAAAGACCT CTATGCCAACA-3′ and 5′-CGGACTCATCGTACTCCTGCTT-3′.

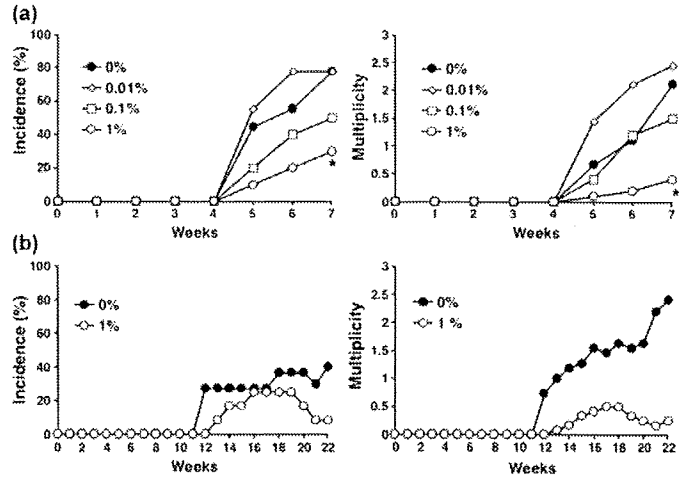

Fig. 1. Periodic observation of palpable mammary tumors after 7,12-dimethylbenz[a]anthracene (DMBA) treatment in female (a) transgenic (Tg) and (b) non-Tg rats fed purple corn color. The *x*-axis indicates weeks after DMBA treatment. $^*P < 0.05$ as compared with the 0% group.

Statistics. All the average values are expressed as the means ± SD. Analysis was performed using the JMP software package (SAS Institute, Cary, NC, USA). Fisher's exact test was conducted for tumor incidence data. Dunnett's *t*-test was conducted for body and organ weight. Mann–Whitney tests were conducted for tumor weight data and multiplicity data.

Results

General observations in the animal experiments. Modifying effects of PCC on DMBA-induced mammary carcinogenesis in female Tg and non-Tg rats were examined. Two rats died in the non-Tg 0% group before the experiment was terminated. There were no consistent significant differences noted in the intake of food or the weights of body, liver or kidneys (data not shown). Coloring of black feces, considered to be related to PCC treatment, was noted in rats fed 1% PCC.

Effects of PCC on mammary tumor induction. In the Tg rats, palpable mammary tumors were first observed at 5 weeks after administration of DMBA. After 5 weeks, the incidence increased rapidly. Tumor incidence and multiplicity increased with time, but was suppressed in a dose-dependent manner in animals fed PCC (Fig. 1a). Final mammary tumor incidences and multiplicity data determined by histological examination are summarized in Table 1. Most of the mammary tumors were diagnosed as adenocarcinomas. The weight of the mammary tumors was significantly lower in the 1% group compared with the 0% group ($P < 0.05$). PCC significantly decreased the incidence of middle-sized (0.5–2.0 g) mammary tumors in Tg rats ($P < 0.05$). It was not statistically significant, but the number of large-sized (>2.0 g) mammary tumors was also decreased by PCC. On the other hand, the number and incidence of smaller-sized (<0.5 g) mammary tumors was not suppressed by PCC, indicating that PCC is not able to inhibit the emergence of mammary tumors in Tg rats.

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Table 1. Inhibitory effects of purple corn color on mammary tumor induction

	Dose (%)	No. of rats	Tumor incidence and multiplicity							Weight of tumor (g)
			~0.5 g†		0.5 g ~2 g†		2 g~†		Total	
			Incidence (%)	No./rat	Incidence (%)	No./rat	Incidence (%)	No./rat	Incidence (%)	No./rat	
Tg	0	9	8 (88.9)	3.4 ± 2.1	8 (88.9)	1.8 ± 1.7	4 (44.4)	1.0 ± 1.4	8 (88.9)	6.2 ± 4.2	1.01 ± 1.34
	0.01	9	9 (100)	5.3 ± 4.4	8 (88.9)	2.9 ± 2.4	6 (66.7)	1.2 ± 1.1	9 (100)	9.4 ± 5.5	0.99 ± 1.52
	0.1	10	6 (60.0)	3.1 ± 4.0	6 (60.0)	1.9 ± 3.1	4 (40.0)	0.8 ± 1.1	8 (80.0)	5.4 ± 5.4	1.06 ± 1.68
	1	10	10 (100)	3.9 ± 3.5	2 (20.0)**	0.6 ± 1.3	3 (30.0)	0.4 ± 0.7	10 (100)	4.9 ± 4.6	0.69 ± 1.64*
non-Tg	0	10	6 (60.0)	4.1 ± 8.6	4 (40.0)	2.6 ± 5.1	3 (30.0)	0.9 ± 1.9	7 (70.0)	7.6 ± 15.3	0.90 ± 1.17
	1	12	2 (16.7)	1.0 ± 2.7	1 (8.3)	0.16 ± 0.58	0 (0)	0	2 (16.7)*	1.2 ± 3.2*	0.24 ± 0.31***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, as compared with 0% group; †tumor weight. Tg, transgenic; non-Tg, non-transgenic.

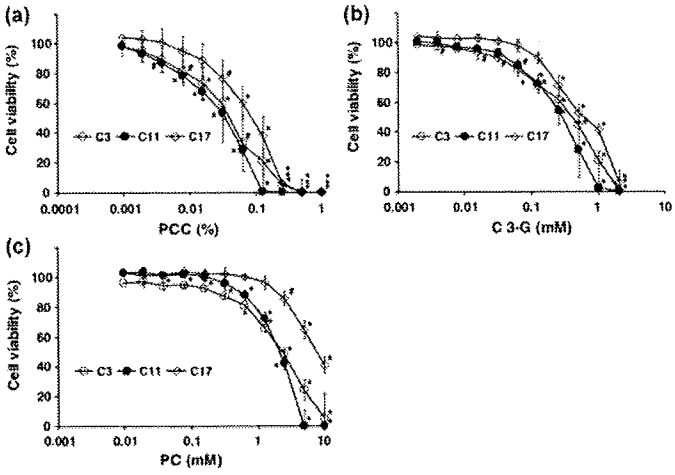

Fig. 2. Cytotoxicity induced by purple corn color (PCC) in mammary tumor cells. Rat mammary tumor cells (C3, C11 and C17) were established from 7,12-dimethylbenz[a]anthracene (DMBA)-induced tumors in a transgenic (Tg) rat. These three cell lines have mutated human Ha-*ras* but not rat Ha-*ras*. Cells were incubated with each compound for 2 days. Dose dependent inhibition of cell proliferation by (a) PCC, (b) cyanidin 3-*O*-β-D-glucoside (C3-G) and (c) protocatechuic acid (PC). #$P < 0.05$, * $P < 0.01$ as compared with no treatment cells.

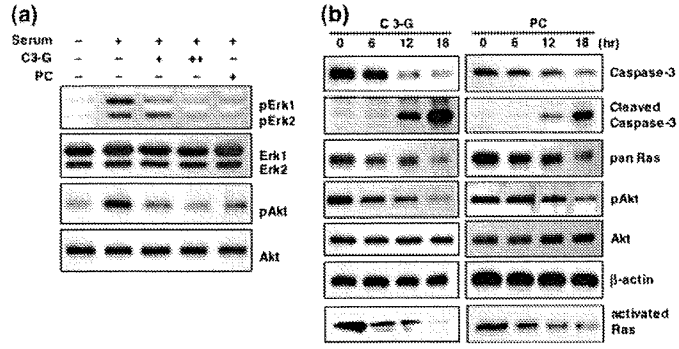

Fig. 3. Cyanidin 3-*O*-β-D-glucoside (C3-G) and protocatechuic acid (PC) inhibit Ras signaling and induce caspase-3 activation. (a) Effect of C3-G and PC on the activation of Erk1/2 and Akt in mammary tumor cells. C11 cells were serum starved for 48 h (0.5% FCS). The cells were incubated in the absence or presence of C3-G (+; 0.25 mM, ++; 0.5 mM) or PC (5 mM) in 0.5% FCS for 24 h prior to serum stimulation. After incubation in media with 10% FCS for 30 min, proteins were extracted. (b) C3-G and PC induced caspase-3 activation by degradation of Ras protein in mammary tumor cells. C11 cells were treated by C3-G (0.5 mM) or PC (5.0 mM) for the indicated time. The protein levels were assessed by Western blot. Activated Ras was precipitated by Raf-1 agarose and detected by antipan-Ras antibody.

Since PCC inhibited mammary carcinogenesis in Tg rats, we tested the inhibitory effect of 1% PCC on mammary carcinogenesis in non-Tg female rats. In non-Tg rats, palpable mammary tumors were first observed at week 12 and the incidence increased with time. PCC reduced mammary tumor incidence and multiplicity (Fig. 1b). Surprisingly, some palpable tumors regressed and became undetectable in rats fed 1% PCC. This suggests that PCC exhibits chemopreventive and chemotherapeutic activity. In non-Tg rats, the incidence and weight of total mammary tumors (Table 1) was significantly lower in the 1% group compared with the 0% group ($P < 0.05$ and $P < 0.005$ respectively). Importantly, not only was the overall number of mammary tumors decreased by PCC in non-Tg rats, but there was complete suppression of the formation of large tumors (2.0 g <) in the 1% PCC group. Most of the mammary tumors in the control group and all of the mammary tumors in the 1% PCC group were diagnosed as adenocarcinomas in non-Tg rats. There was no histological difference between the 0% and the 1% PCC group.

C3-G, and PC inhibit Ras signaling and reduce cell viability in mammary cancer cells. We examined the effects of PCC on the viability of rat mammary cancer cells. Data for the effects of PCC, C3-G, and PC on the viability of mammary cancer cell lines C3, C11, and C17 are summarized in Fig. 2. PCC, C3-G and PC decreased the viability of the cells dose dependently. PCC, C3-G and PC caused a 50% reduction in cell viability at concentrations of 0.051%, 0.395 mM, and 4.37 mM respectively. On the basis of these results, a concentration of 0.5 mM for C3-G and 5 mM for PC were selected for further analysis.

To examine possible effects of C3-G and PC on the Ras signaling pathway, Ras protein levels and phosphorylation of Erk and Akt were examined. Total Ras and activated Ras protein levels were decreased by treatment with C3-G or PC (Fig. 3b). Serum stimulation of serum-starved C11 cells led to activation of Erk and Akt after 30 min. When the cells were pretreated with C3-G or PC for 1 day before serum stimulation, activation of Erk and Akt by growth stimuli was suppressed (Fig. 3a). Although C3-G and PC reduce Ras protein levels and suppress Ras signaling, the gene expression level of *ras* was not changed by treatment with C3-G or PC (data not shown). This suggests that C3-G and PC reduce Ras protein levels by a post-transcriptional mechanism.

To determine whether C3-G and PC induce apoptosis, C11 cells were treated with C3-G or PC for 6, 12 and 18 h, and apoptotic cells were detected using TUNEL. TUNEL staining showed that C3-G and PC induced apoptosis in these mammary tumor cells (Fig. 4).

Caspase-3 is a key protease associated with DNA fragmentation and apoptosis. C3-G and PC induced activation of caspase-3 after

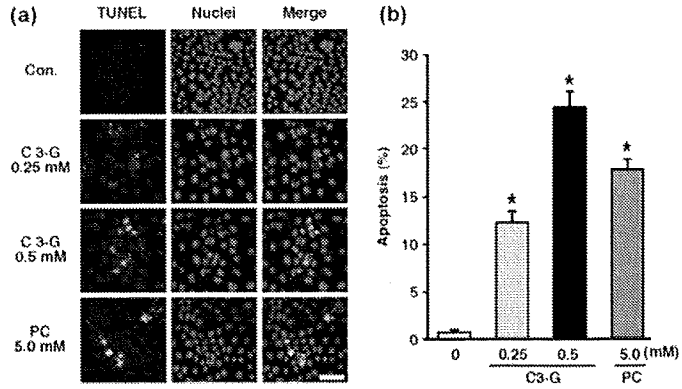

Fig. 4. Cyanidin 3-*O*-β-D-glucoside (C3-G) or protocatechuic acid (PC) induced apoptosis in mammary tumor cells. (a) Terminal deoxynucleotidyl transferase dUTP nick end (TUNEL) staining of mammary tumor cells treated with C3-G or PC. Bar = 50 μm. (b) The percentage of apoptotic cells was determined by counting TUNEL-positive cells from at least 3 fields. $*P < 0.01$ as compared with control.

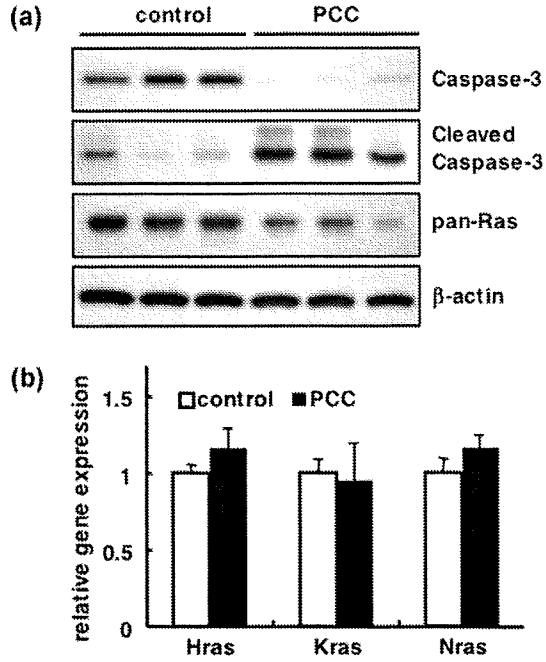

Fig. 5. Purple corn color (PCC) induced degradation of Ras protein in mammary tumors *in vivo*. Non-transgenic (Tg) rats were fed 0 or 1% PCC for 22 week after receiving 7,12-dimethylbenz[a]anthracene (DMBA). The tumors were removed, and proteins and RNA were extracted. (a) Western blot analysis: PCC reduced Ras protein level and activated caspase-3 in mammary tumors *in vivo*. (b) Quantitative real-time PCR analysis: The expression level of *ras* genes, H*ras* (the endogenous gene and the transgene), K*ras* and N*ras*, was not significantly changed in mammary tumors by PCC.

12 h (Fig. 3b). The level of cleaved caspase-3 correlated well with suppression of Ras protein expression and suppression of phosphorylation of Akt. C3-G and PC did not induce activation of caspase 8 (data not shown). This suggests that the TNFα/Fas signaling pathway is dispensable for C3-G and PC apoptotic activity.

PCC inhibits Ras and activates caspase-3 *in vivo*. In determining the true biological significance of a novel pathway, it is always important to confirm *in vitro* results in an *in vivo* context. We evaluated the effect of PCC on the stability of the Ras protein and activation of caspase-3 *in vivo*. As shown in Fig. 5a, PCC suppressed the level of Ras protein and induced the activation of caspase-3 in non-Tg rats. In line with *in vitro* data, total H*ras* (the endogenous gene and the transgene), K*ras* and N*ras* mRNA level was not changed by PCC (Fig. 5b).

Discussion

In the present study, we showed that PCC inhibits mammary tumor development in both cancer prone transgenic rats and their non-transgenic counter parts, and that this inhibition was clearly in line with induction of apoptosis in mammary cancer cell lines derived from the transgenic rats. A previous study reported that 5.0% PCC in the diet did not show any evidence of adverse effects.[4,24] Cyanidin 3-*O*-β-D-glucoside (C3-G) is the major anthocyanin in PCC. C3-G treatment decreased the number of skin tumors induced by 12-0-tetradecanoylphorbol 13-acetate (TPA) in DMBA-initiated mouse skin.[25] C3-G also reduced the size of A549 tumor xenograft growth and significantly inhibited metastasis in nude mice.[25] C3-G is converted to protocatechuic acid (PC) *in vivo*,[11] and PC possesses chemopreventive activities on liver, colon, oral and urinary bladder carcinogenesis.[26–30] Consistent with these observations, PCC inhibited development of mammary tumors in Tg and non-Tg rats.

We also demonstrated that Ras protein levels were decreased by treatment with PCC, C3-G and PC. One mechanism by which this could occur is via reduction of reactive oxygen species (ROS) levels. High ROS levels in conjunction with activation of ERK1/2 stabilize Ha-Ras protein by inhibiting proteasome degradation,[31] and anthocyanins show strong free radical scavenging and antioxidant activities.[32–34]

Down-regulation of Ras levels protects primary cells from inappropriate growth factor signaling, which may result in DNA damage, oxidative stress, and ultimately in apoptosis. However, Ras can activate seemingly contradictory intracellular pathways and in certain conditions Ras has antiapoptotic effects.[35–38] ROS generation is common in cancer cells experiencing oncogenic stimulation by factors such as Ras and Myc,[39,40] and, as noted above, high ROS levels stabilize the Ha-Ras protein. Therefore, PCC, C3-G, and PC might induce apoptosis in cancerous cells by decreasing Ras via reducing ROS levels.

Interestingly, it has been shown that Ha-Ras proteins are modified by mono- and diubiquitination which targets them to endosomes,[41] however, there are no reports of Ras polyubiquitination, which would target Ras proteins for proteasomal degradation. Therefore, it is likely that the effect of PCC on Ras protein expression is mediated through the proteasomal degradation of a factor that affects the stability of Ras.

Ras is found mutated in 30% of all tumors. Pancreatic cancer is the tumor type with the highest incidence of Ras mutations (90%), followed by colon (40%), thyroid (50%), and lung adenocarcinomas (30%).[42] Ras signaling to the PI3 kinase–Akt pathway is an important contributor to tumor cell survival. Aberrant Ras activation can occur as the result of several different cellular abnormalities, not only from mutation of the *ras* gene itself, but, for example, overexpression of the epidermal growth factor receptor. Amplification and/or overexpression of proto-oncogenes such as H-, K-, and N-ras, and neu/erbB-2/HER-2 are frequent events in mammary malignancies of humans,[43,44] and mice.[45] The level of active Erk is clearly elevated in the terminal end buds in the mammary glands of Hras128 transgenic rats.[46] Altogether, it is believed that 80% of all tumors have aberrant Ras pathway activation.[47]

Previous studies have shown that anthocyanidins inhibit TPA-induced Erk, AP-1 activation, and cell transformation.[48] Another flavonoid, silymarin, inhibited both ligand-induced and constitutive activation of erythroblastosis B1/epidermal growth factor receptor(erbB1) [Correction added 1 August 2008: in the preceding sentence erB1 has been corrected to erbB1] and its downstream signaling events.[49] These studies suggest that flavonids including anthocyanin have inhibitory effects on the Ras signaling

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cascade. Previous studies showed that treatment with limonene, an inhibitor of Ras protein isoprenylation, inhibited mammary tumor development,[50,51] and limonene is effective in preventing Ras-initiated mammary carcinomas. Therefore, inhibitors of the Ras signaling cascade might be good candidates for cancer preventive/therapeutic agents.

In the present study, we showed that the activation of Erk was inhibited by C3-G and PC. Our finding suggests that PCC induces apoptosis in mammary tumors by decreasing Ras protein levels. Withdrawal of oncogenic Ras results in regression of tumors.[52] The initial stages of regression involved marked apoptosis in the tumor cells and the surrounding endothelial cells. Apoptosis has been reported to play an important role in elimination of seriously damaged cells and tumor cells by chemopreventive agents.[53] Therefore, apoptosis-inducing agents are expected to be ideal anticancer drugs. Consequently, PCC and it constituent anthocyanidin C3-G are promising candidate chemopreventive and chemotherapeutic agents for cancers that have abnormally high Ras activity.

In summary, dietary administration of PCC significantly suppressed the development of DMBA-induced rat mammary adenocarcinomas. Such cancer protective effects mediated by PCC most likely relate to the modulation of cell proliferation and apoptosis in the mammary neoplastic lesions by reducing Ras protein levels. Furthermore, our *in vivo–in vitro* system, which utilizes Hras128 transgenic rats, non-transgenic rats and mutant Ras-expressing mammary cancer cell lines, can be used for screening for chemopreventive agents that act via suppressing the Ras signaling pathway.

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RESEARCH COMMUNICATION

Lack of Chronic Oral Toxicity of Chemopreventive Bovine Lactoferrin in F344/DuCrj Rats

Seiko Tamano[1], Kazunori Sekine[2], Mitsunori Takase[2], Koji Yamauchi[3], Masaaki Iigo[4], Hiroyuki Tsuda[5]*

Abstract

Studies were undertaken to determine whether bovine lactoferrin (bLF) and related compounds, shown to prevent carcinogenesis in the colon and other organs in rats, have any toxic effects in long-term feeding studies. In experiment I, male F344/DuCrj rats received a basal diet containing 0.2% bLF for 40 weeks. No adverse findings were noted, furthermore, serum triglyceride level was significantly decreased to 72% of the control level, suggesting preventive effects against the metabolic syndrome. In experiment II, male and female F344/DuCrj rats were fed a basal diet containing 0.02, 0.2, 2.0 and 5.0% bLF, 2.0% bLF hydrolysate (bLF-H) or 0.1% lactoferricin (LFcin), a peptide derived from bLF, for 60 weeks in males and 65 weeks in females. No toxicological effects, including carcinogenicity, were evident in either sex. The results of the studies provide subjective support for safety of clinical studies of bLF for supplement use.

Key Words: Chemopreventive agent - bovine lactoferrin - *in vivo* toxicity - F344/DuCrj rat

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Introduction

Lactoferrin is a multi-functional iron-binding glycoprotein, which is present at high concentration in mammalian colostrums, as well as neutrophilic leukocytes (Masson et al., 1969), which release the compound in response to inflammatory stimuli (Lash et al., 1983). The primary biological function of lactoferrin is related to its anti-bacterial, anti-viral, anti-fungal and immune-modulating effects (Bullen, 1975; Arnold et al., 1980; Brock, 1995; Lönnerdal and Iyer, 1995). Furthermore, lactoferricin (LFcin), a pepsin digested peptide of lactoferrin, has been shown to possess potent and wide-spectrum anti-microbial effects (Tomita et al., 1991; Bellamy et al., 1992), as confirmed by extensive analyses of the mechanisms (Wakabayashi et al., 2003). Recently, bovine lactoferrin (bLF) was shown to exert cancer preventive effects in various organs of rodents including the colon, lung and esophagus (Sekine et al., 1997; Tsuda, et al., 2000b, 2004). Furthermore, bLF was also demonstrated to have anti-metastatic effects in mice (Iigo et al., 1999; Tsuda et al., 2000a).

However, for application of bLF in human trials of its ability to prevent carcinogenesis and tumor metastasis with long-term ingestion, it is obviously necessary to conduct chronic feeding studies to detect any adverse effects. Prior to the current studies, acute and subchronic exposure indicated no obvious toxicological effects after 4-weeks and 13-weeks feeding with doses up to 2,000 mg/kg/day (Yamauchi et al., 2000a). Lack of mutagenicity was also reported (Yamauchi et al., 2000b). However, chronic toxicology studies have hitherto not been performed, promoting the present studies of long-term toxic effects of dietary bLF in F344 rats of both sexes.

Materials and Methods

Test Chemical

Bovine lactoferrin (bLF), lactofferin hydrolysate (bLF-H), generated by acid-pepsin hydrolysis (Tomita et al., 1991), and lactoferricin (LFcin), identified as an antimicrobial peptide derived by pepsin digestion of lactoferrin (Bellamy et al., 1992)(Morinaga Milk Industry Co., Ltd., Zama, Japan) were used.

Diet preparation and analysis

The compounds were mixed at the designated levels into powdered basal diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan) after being previously confirmed to be stable in diet for 3 months when stored in a cold room controlled at the Food Science and Technology Institute, Morinaga Milk Industry Co., Ltd.. Therefore, the diets were prepared at intervals of 3 months and stored in a cold room. Amounts of bLF in the diet preparations were

*[1]DIMS Institute of Medical Science, Inc., 64 Goura, Nishiazai, Azai-cho, Ichinomiya Aichi Pref. 491-0113, [2]Nutritional Science Institute, [3]Food Science and Technology Institute, Morinaga Milk Industry Co., Ltd. 1-83, 5-Chome, Higashihara, Zama, Kanagawa Pref. 228-8583, [4]Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, [5]Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, 1, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan *For correspondence, email: htsuda@med.nagoya-cu.ac.jp*

within ± 5% of the target concentrations. Homogeneity was confirmed as satisfactory.

Animals and their maintenance

Male and female F344/DuCrj (F344) rats, 5 weeks of age, were purchased from Charles River Japan (Atsugi, Japan) and allowed a 7 days quarantine and acclimation period. After confirmation of normal health status, they were used for the studies.

Animals of the same sex were housed two or three to a polycarbonate cage, with wood chip (Oriental Yeast Co., Ltd., Tokyo, Japan) for bedding. They were placed on powdered MF basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) with or without test compounds and tap water ad libitum. The room temperature and relative humidity were controlled at 21~25°C and 50~60%, respectively, and the room air was changed 15 or more times per hour. Fluorescent tube lighting was employed to provide a 12-hr light/dark cycle.

Experimental procedures

Experiment I: Starting at 6 weeks of age, groups of 15 male rats were given diet containing 0% (control) or 0.2% of bLF for 40 weeks. Diet and drinking water were available ad libitum. The animals were observed for general conditions every day and weighed once weekly for the initial 4 weeks and once every 4 weeks thereafter. Determination of food consumption by cage was performed at the same time as body weight measurement. Test material intake (mg/kg body weight/day) was calculated for each group from mean food consumption and body weight data and the nominal dietary levels.

At the end of the treatment, all animals were fasted overnight and then killed in the next morning under deep ether anesthesia. Whole blood samples were collected from the all rats via the inferior vena cava and blood biochemistry determinations were performed with an Automatic Analyzer Model 7070 (Hitachi Co., Ltd., Tokyo, Japan). Parameters were aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (É¡-GTP), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), total cholesterol (T-CHO), trigryceride (TG), total protein (TP), albumin (ALB), serum iron. Gross inspections for any lesions were made at autopsy and the findings recorded. The liver, kidneys and spleen were weighed and the organ-to body weight ratios were determined.

Experiment II: A total of 100 F344 rats each sexes were used. Starting at 17 (in males) or 11 (in females) weeks of age, groups of 25 rats (groups 1 and 5) and 10 rats (groups from 2 to 4, and 6 and 7) of each sex were given powdered diet as in Experiment Öü containing 0% (control), 0.02, 0.2, 2.0 and 5.0% bLF, 2.0% bLF-H or 0.1% bLFcin for 60 weeks in males and 65 weeks in females. The animals were observed for general conditions every day and weighed 8 times during the experiment. Measurement of food consumption and water intake by cage were performed once every 2 weeks for the first 16 weeks and once every 4 weeks thereafter. Test material intake (mg/kg body weight/day) was calculated for each group from mean food consumption and the nominal dietary levels. Careful gross examinations were made at autopsy. The following organs, the liver, kidney, spleen, adrenal and pituitary were weighed for each animal. Samples of these organs, thymus, lungs (including trachea), salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, pancreas, urinary bladder testes, prostate, seminal vesicle, ovaries (including oviducts), uterus, vagina, spinal cord and grossly visible lesions were fixed in 10% buffered formalin solution. Tissues were routinely processed for histopathological examination.

Statistical analyses

For body weight, blood biochemistry and organ weight data, the significance of inter group differences was assessed using the Bartlett's test (Bartlett, 1937). If homogeneous, the data were analyzed with the Dunnett's multiple comparison test (Dunnett, 1955) and if not then with the Steel's test (Steel, 1959). For the incidences of histopathological lesions, the significance of differences observed between the control and treated groups was evaluated with the Fisher's exact probability test (Fisher, 1955). The Mann-Whitney U test was employed for comparison of degrees of change (Gad and Weil, 1989). The levels of significance were set at $P<0.05$ and $P<0.01$.

Results

Experiment I

Neither clinical signs related to bLF treatment nor deaths were observed throughout the 40 weeks of the study. No alteration in body weights related to bLF treatment was noted (data not shown). However slight, but significantly decreased relative liver weights (but not absolute weights) were noted in the 0.2% bLF treated animals (data not shown). No treatment-related macroscopic changes were observed in the bLF fed animals (data not shown). Selected blood biochemistry data are given in Table 1. AST, ALT, ALP, BUN and TG were significantly lowered in the 0.2% bLF group. No treatment-related histopathological lesions were observed.

Experiment II

Neither clinical signs related to bLF treatment nor deaths were observed throughout the study period. No significant alteration in body weights related to bLF treatment was noted. Average food consumption values were comparable to the targeted doses of test compound in both sexes, exhibiting dose-dependent increase in total bLF intake. Average water intake values in bLF-treated groups were not different from controls in either sex. There were no significant differences in final body, liver, kidneys, spleen, adrenal and pituitary weights between treated groups and control groups in either sex. No treatment-related macroscopic changes were found (data not shown).

No treatment-related histopathological changes were observed in either sex of the treated and control groups. All incidences of histopathological alterations observed in the present study were within the ranges for spontaneously occurring lesions in F344 rats (Goodman et al., 1979; Haseman et al., 1990).

Table 1. Blood Biochemistry Data for F344 Rats Fed a Diet Containing 0.2% bLF for 40 weeks

	Control	0.2% bLF
GOT (IU/L)	135.1 ± 30.1	100.0 ± 24.0[c]
GPT (IU/L)	85.3 ± 11.7	68.3 ± 19.0[b]
γ-GTP (IU/L)	N.D.	N.D.
ALP (IU/L)	714.6 ± 108.5	585.3 ± 93.7c
Creatinine (mg/dL)	0.3 ± 0.028	0.28 ± 0.025
BUN (mg/dL)	18.5 ± 2.00	16.0 ± 1.33c
Total Protein (g/dL)	6.7 ± 0.38	6.6 ± 0.22
Albumin (g/dL)	4.3 ± 0.26	4.4 ± 0.13
Cholesterol (mg/dL)	77.1 ± 10.9	78.3 ± 8.26
Triglyceride (mg/dL)	117.3 ± 44.9	83.9 ± 21.8[a]
Blood Glucose (g/dL)	174.4 ± 24.1	168.5 ± 21.3
Serum Iron (μg/dL)	152.7 ± 19.5	155.9 ± 25.6

Values are means±S.D. [a,b,c] P<0.05, P<0.01, P<0.005 compared to the control group. N.D., Not detected

Discussion

The present 40 week-chronic oral administration of dietary 0.2% bLF (Experiment I) demonstrated no cause of any toxicological lesions in male F344 rats. Decrease in serum GOT, GPT and ALT may be related to improvement of the impaired liver function possibly due to aging. Similarly, the lower level of BUN may be at least partly associated with protection of kidney function.

The findings, however, could not be confirmed in Experiment II in which animals were fed longer duration because serum was not taken. However, the serum triglyceride level was clearly decreased to 72 % of the control level. Although serum glucose level did not decrease, the result may be beneficial for protecting against the metabolic syndrome associated with hyperlipidemi. Administration of bLF has been reported to induce the production of cytokine IL-18 in the mouse small intestine (Kuhara et al., 2000). Recently, Netea et al. reported that deficiency of interleukin-18 leads to hyperphagia, obesity and hyperglycemia resulting from insulin resistance in Il-18 knockout mice (Netea et al., 2006). Furthermore, the molecular mechanisms responsible for the hepatic insulin resistance in Il-18 knockout mice involve an enhanced expression of genes associated with gluconeogenesis in the liver, resulting from defective phosphorylation of STAT3 (Netea et al., 2006). Decreased triglyceride level might therefore imply induction of cytokine IL-18 by lactoferrin in experiment I. Practically, serum IL-18 levels are significantly increased in patients with HVC associated chronic hepatitis C (Ishii, 2004). In this context it should be noted that blood biochemistry revealed significant lowering of AST and ALT in the 0.2% bLF treatment group, which might be related to gluconeogenesis in the liver.

In experiment II, there was no evidence of long term toxicity or carcinogenicity in either sex of rat fed 5.0% bLF for 60 or 65 weeks. Total and daily intake in the 5.0% bLF group were 46,201 g or 54,175 g, and 109 g or 119 g, when extrapolated to male or female human beings, respectively. Thus, we conclude that these ranges of dosage levels can be administered to humans safely in clinical trials. Our results are in line with the lack of any adverse effects reported in lactoferrin-treated patients with chronic hepatitis C, healthy subjects positive for helicobacter pylori infection and high risk subjects of colon and lung cancers (Tanaka et al., 1999; Iwasa et al., 2002; Okuda et al., 2005).

Accordingly, the results indicated that the NOAEL for bLF with 60 or 65 weeks dietary treatment is at least 5.0% for both sexes. Therefore, the results of the current study provide strong support for safety in accepted dose ranges of lactoferrin and related compounds for further clinical and intervention studies.

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Research paper

Anticarcinogenesis pathways activated by bovine lactoferrin in the murine small intestine

Masaaki Iigo [a,b], David B. Alexander [b], Ne Long [b], Jiegou Xu [b], Katsumi Fukamachi [b], Mitsuru Futakuchi [b], Mitsunori Takase [c], Hiroyuki Tsuda [b,*]

[a] *Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo*
[b] *Department of Molecular Toxicology, Nagoya City University, Graduate School of Medical Sciences, Nagoya-shi, Aichi-ken, Japan*
[c] *Food Science & Technology and Nutritional Science Institutes, Morinaga Milk Industry Co., Ltd, Zama, Japan*

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Abstract

Oral administration of bovine lactoferrin (bLF) inhibits carcinogenesis in the colon and other organs in rats, and lung metastasis in mice. A likely mechanism by which bLF mediates its anticarcinogenesis effects is by enhanced expression of cytokines and subsequent activation of immune cells. Oral administration of bLF enhances expression of interleukin-18 (IL-18) mRNA in the mucosa of the small intestine of mice. Importantly, the pepsin hydrolysate of bLF (bLFH) also induced expression of IL-18 mRNA in the mouse small intestine and a peptide produced by pepsin digestion of bLF, bovine lactoferricin (bLFcin), induced expression of mature IL-18 in organ culture. In addition to IL-18, bLF and bLFcin both induced significant increases in caspase-1 activity in peritoneal macrophages and in organ cultures. The increase of mature IL-18 by macrophages was inhibited by caspase-1 inhibitor: caspase-1 is known to cleave the proform of IL-18 to produce active mature IL-18. Finally, bLF also induced expression of IFNγ by peritoneal macrophages. Importantly, in IFNγ knockout (GKO) mice, bLF administration resulted in increased expression of caspase-1 protein, but induction of IL-18 mRNA, caspase-1 activity, and mature IL-18 was not observed. These results indicate that orally administered bLF can induce expression of IFNγ and caspase-1 in the small intestine. IFNγ in turn increases expression of target genes, including IL-18. Active caspase-1 then cleaves pro-IL-18 to generate mature IL-18. Thus, bLF activates an effector pathway mediated by IFNγ, caspase-1, and IL-18. We also show that ingested bLF is able to activate more than a single effector pathway. For example, in GKO mice while bLF administration could not activate the IFNγ/caspase-1/IL-18 effector pathway, it was able to inhibit tumor growth and metastasis by activation of an IFNα/IL-7 effector pathway.

Keywords: Lactoferrin; Innate immune system; Cytokine; Chemoprevention; Carcinogenesis

1. Introduction

Lactoferrin (LF), an approximately 80 kDa iron binding glycoprotein initially purified from milk, is an important component of the innate immune system. A potent antimicrobial and antiviral agent [1–5], LF is a major component of antimicrobial host defense and is found in a variety of exocrine secretions, e.g., tears, nasal exudate, saliva, bronchial mucus, gastrointestinal fluids, cervicovaginal mucus, and seminal fluid. In addition to its presence in mucosal secretions, LF is released by activated neutrophils at septic sites.

The concentration of LF in human colostrum is particularly high (7–10 mg/ml) [6] resulting in ingestion of 1–2 g/day for several months by a suckling infant [7]. It is thought that orally ingested LF may be able to interact with epithelial cells and immune cells in the mucosa of the intestine: ingested LF is reported to stimulate cytokine

* Corresponding author at: Department of Molecular Toxicology, Nagoya City University, Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-ch-, Mizuho-ku, Nagoya-shi, Aichi-ken 467-8601, Japan. Tel.: +81 52 853 8991; fax: +81 52 853 8996.
E-mail address: htsuda@med.nagoya-cu.ac.jp (H. Tsuda).

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production [8] and enhance mucosal immunity [9], NK cell activity [10,11], LAK activity [11], neutrophil activity [12,13], and macrophage cytotoxicity [14,15]. Previously we found that the pro-inflammatory cytokine interleukin-18 (IL-18) was produced by epithelial cells of the small intestine following administration of bLF [9,16]. IL-18 enhances Th1 type T and NK cell responses and generates $CD8^+$T cells [17].

Bezault et al. [18] report a protective effect of human-LF against the growth of solid tumors and the development of metastases in mice. bLF also inhibits lung metastasis of B16 melanoma and colon 26 tumor cells in mice [19,20] and is protective against tongue, esophagus, intestinal, lung, and bladder carcinogenesis in rats [21–24]. bLF administration results in a significant increase of $CD4^+$ and $CD8^+$T cells and asialo GM_1^+ (NK) cells in the blood [16] and in the lymphoid tissues and lamina propria of the small intestine [9]: $CD8^+$T and asialo $GM1^+$ cells exhibit marked cytotoxicity against Co26Lu cells *in vitro* [16]. The observed increase in T and NK cells may be due to the increased levels of mature IL-18 produced by epithelial cells of the small intestine after treatment with bLF [16].

In Co26Lu bearing-SCID mice, which are deficient in T and B cells, bLF also showed significant inhibition of tumor growth (Fig. 1) and lung metastatic colony formation [7]. However, the number of lung metastatic colonies was markedly increased when these mice were treated with anti-asialo GM_1 antibodies to weaken their NK cell activity. Bezault et al. [18] also report that LF-mediated antitumor effects were lost upon elimination of NK cell activity by pretreatment of mice with anti-asialo GM_1 antibodies. These results demonstrate the importance of activated NK cells for LF-mediated inhibition of tumor growth and metastasis.

The immunomodulatory and antitumor activities of bLF suggest that it may be a promising chemopreventer of carcinogenesis [25,26]. In this study we investigated pathways by which ingested bLF can exert its effects.

2. Materials and methods

2.1. bLF, bLFH, bLFcin and bTF

Bovine lactoferrin (bLF) (purity: greater than 96% of total protein by HPLC; endotoxin: less than 5.0 pg/mg of bLF; iron content: 143 ± 19 ng/mg of bLF, $n = 3$), a pepsin-generated hydrolysate of bLF (bLFH: endotoxin, 101.3 ± 25.7 pg/mg of bLFH; iron content, 100 ± 0 ng/mg of bLFH, $n = 3$), and lactoferricin (bLFcin: endotoxin, 101.3 ± 25.7 pg/mg of bLFcin, $n = 3$) were obtained from Morinaga Milk Industry Co., Ltd. (Zama, Japan). Bovine transferrin (bTF) (purity: greater than 98% of total protein by agarose electrophoresis; endotoxin, 16.4 ± 2.8 pg/mg of bTF; iron content, 13 ± 3 ng/mg of bTF, $n = 3$) was purchased from Sigma Co. Ltd. (St. Louis, MO). Endotoxin (by the modified limulus test) and iron (by the 2-nitroso-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)amino]phenol test) content were determined by SRL Inc., Hachioji, Japan.

2.2. Cytokines and antibodies

Anti-asialo GM_1 Ab was purchased from Wako Pure Chemical Industries (Osaka, Japan; caspase-1/ICE inhibitor (Acyl-Tyr-Val-Ala-Asp-chloromethylketone) was purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan); recombinant murine IFNγ was purchased from Intergen Company (Purchase, NY); recombinant murine IL-7 was purchased from PeproTech House (London, England); recombinant murine IL-18 was purchased from Medical & Biological Laboratories Co., Ltd.; recombinant IFNαA/D (2.04×10^7 IU/ml) was obtained from Nippon Roche Research Center, (Kamakura, Japan); anti-mouse IFNα antibody (clone F18, rat IgG_1) was purchased from HyCult Biotechnology b.v.; goat anti-murine IL-1β (M-20, goat IgG) was purchased from Santa Cruz Biotechnology, Inc.; anti-murine IL-7 polyclonal antibody was purchased from Pepro Tech EC Ltd.; anti-mouse IL-12 antibody (p40/p70) was purchased from PharMingen, BD Biosciences; rabbit anti-mouse IL-15 antibody was purchased from Torrey Pines Biolabs, Inc.

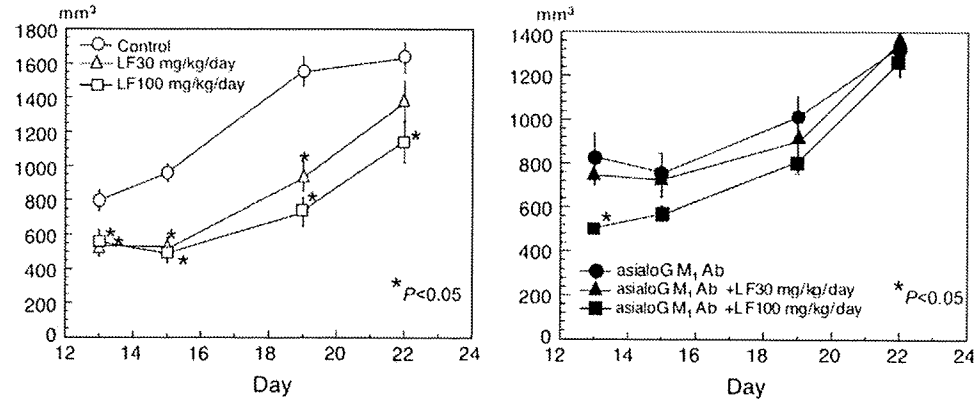

Fig. 1. Importance of activated NK cells for bLF-mediated inhibition of tumor growth (Co26Lu tumor-bearing SCID mice). 1×10^5 cells/mouse were subcutaneously implanted into the right thigh of SCID mice (Charles River Japan, female). bLF was administered at 30 or 300 mg/kg per day on days 5–9 and 12–16. Anti-asialo GM_1 Ab was injected (dilute 1/40, 0.2 ml/mouse i.v.) on days 5, 7, 12, and 14. The longest (a) and shortest (b) diameters of tumors at the Co26Lu cell injection site were measured twice a week using calipers, and the volume was calculated using the formula: $ab^2/2$ (mm^3). The experiment was terminated 22 days after implantation. The symbols represent the mean and standard error of tumor sizes ($n = 5–7$). *$p < 0.05$, Dunnett's multiple comparison *t*-test.

(Houston, TX); anti-rat IFNγ antibody (clone DB-1, mouse IgG_1) was purchased from BioLegend (San Diego, CA); and anti-TNFα (L-19) polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-human lactoferrin, specific for human lactoferrin, and rabbit anti-bovine lactoferrin antibodies, specific for bovine lactoferrin, were obtained from Morinaga Milk Industry Co. Ltd. (Zama, Japan). Anti-human IL-12/IL-23 p40 (clone: C8.3, mouse IgG_1) was purchased from BioLegend; anti-human IL-18 antibody (clone: 159-12B, rat IgG_{2a}:detects both pro-IL-18 and mature IL-18) and anti-human IFNα antibody (clone: #14, mouse IgG_1) were purchased from Medical & Biological Laboratories Co., Ltd.; anti-human IFNγ antibody (clone: MD-1, mouse IgG_1) was purchased from BioLegend (San Diego, CA).

2.3. Mice

BALB/c mice (wild type mice), 5-week-old male and female, were obtained from Charles River Japan (Atsugi, Japan). IFNγ knockout mice (mice with a targeted disruption of the IFNγ gene, BALB/c-Ifng^{tm1Ts}, 4 ~ 5-weeks-old female, GKO mice) [27] were purchased from the Jackson Laboratory, Bar Harbor, ME. The animals were allowed free access to CE-2 pellet diet (CLEA Japan, Tokyo, Japan) and water, and were maintained in plastic cages on woodchip bedding under specific pathogen-free conditions in our animal facility with a controlled temperature of 24 ± 2 °C, humidity 60 ± 10%, and a 12 h light–dark cycle. All experiments were initiated when the mice became 6 weeks-old, and performed according to "Guide for the Care and Use of Laboratory Animals" of the Animal Study Committee of National Cancer Center Research Institute, Tokyo, Japan.

2.4. Organ culture of the small intestine

BALB/c mice were anesthetized with diethyl ether and then killed. The jejunum was excised (2 cm) and then washed with physiological saline. The jejunum was turned inside out and cultured in RPMI-1640 culture medium at 37 °C for 30 min with bLF, bTF, or bLFcin as shown in Fig. 2. After the 30 min culture period, the tissue specimens were cut perpendicularly with scissors and opened onto glass plates. The entire surface (epithelium, lamina propria, submucosa) of the jejunum was harvested by scraping with a slide glass. The sample was then placed on an aluminum plate cooled by acetone-dry ice and pressed flat using another plate. These samples were stored at −80 °C until use.

2.5. Peritoneal macrophage culture

Mice were injected intraperitoneally with 4 ml of thioglycollate broth (3 g/100 ml, Eiken Chemical Co. Ltd., Tokyo). Four days after injection, peritoneal exudate macrophages were obtained: using a plastic 10 ml syringe with a 22-gauge needle, mice were injected intraperitoneally with saline and massaged and the saline solution containing peritoneal exudate macrophages was recovered. Macrophages were washed once with Hanks' balanced salt solution. The harvested cells were centrifuged at 200 × *g* for 10 min, resuspended in culture medium (RPMI-1640, 10% FBS), and plated into a 24-well tissue culture plate (2×10^6 cells/2 ml/well). After incubation for 2 h, non-adherent cells were removed by vigorous washing, and fresh culture medium (RPMI-1640, 5% FBS) was added to the monolayer.

2.6. Spleen cell culture

Mice were anaesthetized and killed and the spleen harvested. Spleens were minced in petri dishes containing 5 ml media and pressed through a 120-stainless-steel mesh. Cells obtained were washed with Hanks' balanced salt solution, centrifuged at 200 × *g* for 10 min, resuspended in culture medium (RPMI-1640, 10% FBS), and plated into a 24-well tissue culture plate (2×10^6 cells/2 ml/well).

2.7. Enzyme-linked immunosorbent assay (ELISA) – frozen organ culture samples

ELISA was used to determine the levels of various cytokines. Frozen tissues were homogenized with a Sonifier 450 (Branson Ultrasonics Corp., Daburg, CT) in lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), homogenates were clarified by centrifugation at 15,000 rpm for 10 min at 4 °C, and the supernatants were collected and stored at −80 °C until testing. ELISA kits for murine IL-1β were purchased from Genzyme Techne (Minneapolis, MN). Mature IL-18 levels were determined using a mouse IL-18 ELISA kit (minimum detection limit: 25.0 pg/ml) purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan).

2.8. Enzyme-linked immunosorbent assay (ELISA) – peritoneal macrophage, RAW 264.7 cells, and spleen cell cultures

Peritoneal macrophages and spleen cells were harvested as described above. RAW 264.7 mouse macrophages were obtained from Riken (Tsukuba, Japan). Cells were plated into 24-well tissue culture plates (2×10^6 cells/2 ml/well). After incubation for 24 h in 300 μg/ml bLF, cells were harvested and lysed in 100 μl of chilled lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), and then clarified by centrifugation at 15,000 rpm for 10 min. The supernatants were collected and stored at −80 °C until testing. An ELISA kit for mature mIL-18 (minimum detection limit: 25.0 pg/ml) (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was used to measure IL-18 levels.

2.9. Enzyme-linked immunosorbent assay (ELISA) – peritoneal macrophage cultures

Peritoneal macrophages were harvested as described above. Cells were plated into 24-well tissue culture plates

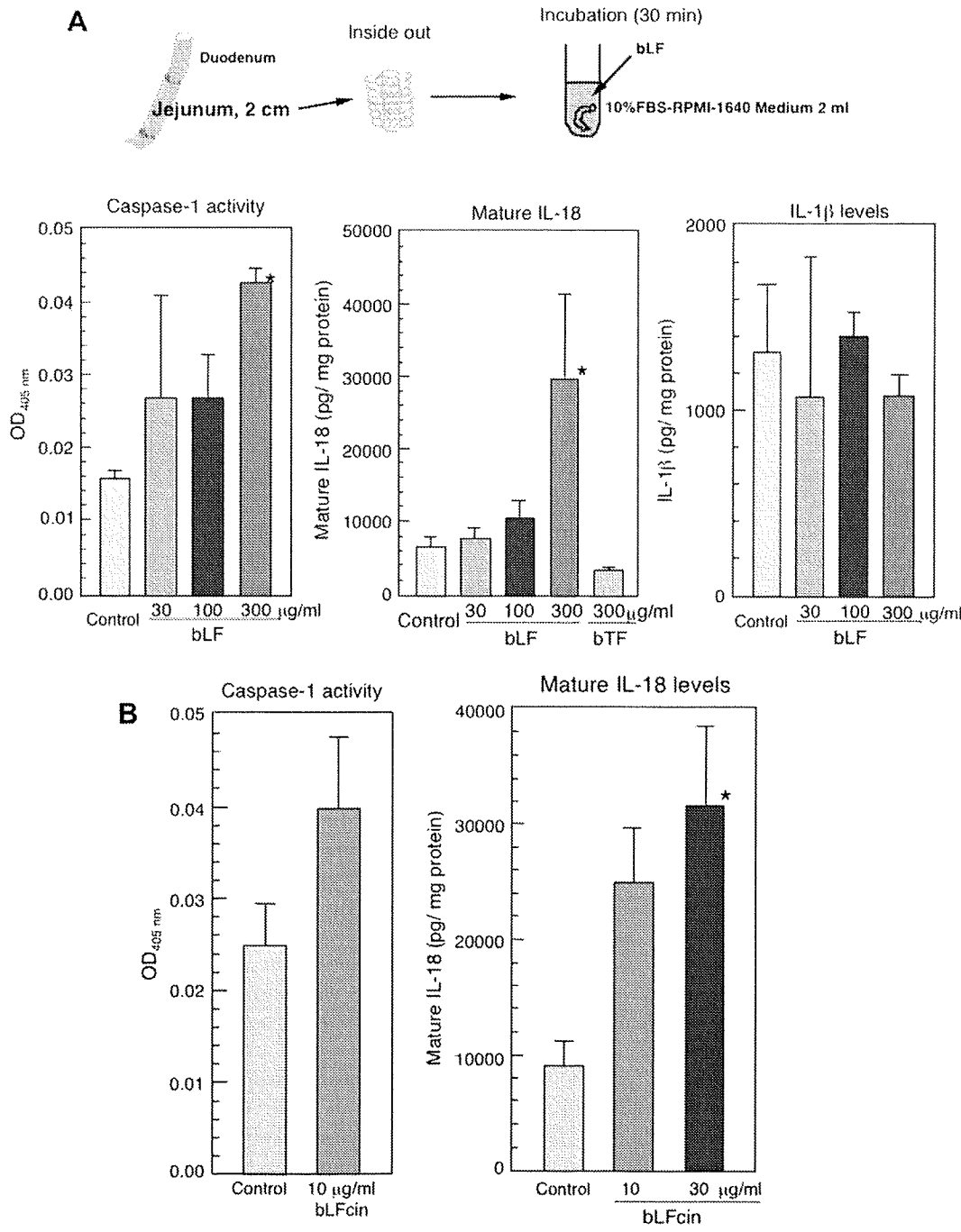

Fig. 2. Effects of bLF and bLFcin on caspase-1 activity and the level of mature IL-18 in the mucosa of organ cultured small intestine. Mouse jejunum in organ culture was incubated with lactoferrin (A) ($n = 7$) or lactoferricin (B) ($n = 3$) for 30 min, and caspase-1 activity and mature IL-18 or IL-1β levels were measured by ELISA. Error bars represent the standard error. *$p < 0.05$, Dunnett's multiple comparison t-test.

(2×10^6 cells/2 ml/well). After incubation for 24 h in bLF, rmIFNγ, anti-mIFNγ antibody, or caspase-1 inhibitor, cells were harvested and lysed in 100 μl of chilled lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), and then clarified by centrifugation at 15,000 rpm for 10 min. The supernatants were collected and stored at −80 °C until testing. An ELISA kit for mature mIL-18 (minimum detection limit: 25.0 pg/ml) (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was used to measure IL-18 levels.

2.10. Caspase-1 activity

For assaying the activity of caspase-1, colorimetric protease assay kits were used (BioVision Research Products, Mountain View, CA). The samples were diluted to 200 μg protein in 50 μl Cell Lysis buffer and then 50 μl of Reaction buffer containing 10 mM DTT and 5 μl of 4 mM Tyr-Val-Ala-Asp-pNA

(chromophore *p*-nitroanilide) was added. The samples were incubated at 37 °C for 2 h. Caspase-1 activity levels were determined at 405 nm in a microtiter plate reader. In some experiments, Acyl-Tyr-Val-Ala-Asp-chloromethylketone (Bachem Biochemica GmbH, Heidelberg, Germany) at a concentration of 50 μM was added to inhibit caspase-1 activity; the inhibitor was applied 30 min before bLF addition.

2.11. Western blot analysis of cell cultures

Peritoneal macrophages were harvested as described above; Lewis Lung carcinoma cells and MCF7 human breast cancer epithelial cells were obtained from the National Cancer Center Research Institute (Tokyo, Japan); SW620 colon carcinoma cells were obtained from the American Type Culture Center (Rockville, MD); and SK-MEL 19 melanoma cells [28] were obtained from the Medical Research and Development Center, the Tokyo Metropolitan Institute of Medical Science (Tokyo, Japan). Cells were seeded in 24 well plates in RPMI-1640 medium with 10% FBS at a density of 1×10^6 cells/well and incubated for 24 h. The medium was then changed to serum-free medium containing various concentration of bLF. After a 24 h incubation, cells were washed twice with serum-free medium and lysed in 75 μl chilled lysis buffer containing 125 mM Tris–HCl (pH 6.8), 4.3% SDS, 30% glycerol, 10% 2-mercaptoethanol, 0.01% BPB, and protease inhibitor cocktail "Complete Mini" (Roche, Mannheim, Germany). Lysates were sonicated and clarified by centrifugation at 15,000 rpm for 45 min at 4 °C. Ten μg of each sample was separated by SDS-PAGE on 15/25% polyacrylamide gradient gels (Multigel II Mini, Daiichi Pure Chemicals Co. Ltd., Tokyo). Proteins were transferred to PVDF membrane (Immobilon-P, Millipore Corp., Bedford, MA) and detected by Western blot with appropriate antibodies. Immunoreactive bands were visualized with Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Densitometric analysis of digitized blot images was carried out using NIH Image software.

2.12. Protein assay

Total protein content of the samples was assayed using Coomassie Dry Protein Assay Plates (Pierce, Rockford, IL) with bovine serum albumin as the protein standard (Fraction V, Sigma Chemical Co. St. Louis, MO).

2.13. Lung metastasis assay

Colon carcinoma 26 (obtained from the Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan), are of BALB/c origin and have low metastatic potential. Metastatic tumor cells (Co26Lu) were obtained by sequential selection of tumor colonies in the lung: colon carcinoma 26 cells were subcutaneously implanted into the back of synergistic BALB/c mice and after tumor formation, colonies in the lung were recovered and cells from these lung metastases were subcutaneously implanted into the back of another mouse. After several rounds of sequential selection, highly metastatic Co26Lu cells were obtained.

Freshly excised Co26Lu cells from tumor-bearing mice were minced in Hanks' balanced salt solution (Life Technologies, Grand Island, NY) and strained through a 120-stainless-steel mesh. Viability was determined by trypan blue dye exclusion. Cells were diluted to the desired concentration and 1×10^5 cells/mouse were subcutaneously implanted into the right thigh of GKO and BALB/c mice ($n = 15$). bLF was administered at 30 or 300 mg/kg per day on days 3–7, 10–14, and 17–21 and the mice were killed on day 22. The lungs were excised and fixed in acetone and lung metastases were counted.

2.14. Treatment with bLF, bLFcin, and bTF and preparation of tissues

Normal BALB/c (WT) and GKO mice were used (five mice/group). bLF, bLFH, and bTF were dissolved in physiological saline (3 or 30 mg/ml) and administered at 30 mg/kg per day in consecutive treatments for 7 days or a single administration at 300 mg/kg. Three hours after the last oral administration of bLF, bLFH or bTF the mice were anesthetized with diethyl ether and killed. The small intestine, jejunum and ileum, was excised and washed with physiological saline. It was then cut perpendicularly with scissors and opened onto a glass plate. The entire surface (epithelium, lamina propria, submucosa) of the ileum was harvested by scraping with a slide glass. A portion of the harvested mucosa was used for RNA extraction; the remaining mucosa was placed on an aluminum plate cooled by acetone-dry ice and pressed flat using another plate. These samples were stored at −80 °C until use. The jejunum was fixed in acetone and used for immunohisochemistry.

2.15. Enzyme-linked immunosorbent assay (ELISA) – frozen tissue samples

ELISA was used to determine the levels of various cytokines. Frozen tissues were homogenized with a Sonifier 450 (Branson Ultrasonics Corp., Daburg, CT) in lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), homogenates were clarified by centrifugation at 15,000 rpm for 10 min at 4 °C, and the supernatants were collected and stored at −80 °C until testing. ELISA kits for murine IL-1β, IL-4, IL-6, IL-12, GM-CSF, and TNFα were purchased from Genzyme Techne (Minneapolis, MN). Mature IL-18 levels were determined using a mouse IL-18 ELISA kit (minimum detection limit: 25.0 pg/ml) purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan).

2.16. PCR detection of IL-7, IL-15, IL-18, and caspase-1 mRNA expression

For investigation of IL-7, IL-15 IL-18, and caspase-1 mRNA levels, in the intestinal epithelium, bLF was administered orally to tumor-bearing mice on day 7 or days 1–7,

and the mice were killed under deep anesthesia with diethyl ether 3 h after the last oral administration and the small intestine was immediately removed. Total RNAs from mucosal tissues were isolated using ISOGEN (Nippon Gene, Toyama, Japan), followed by ethanol precipitation. Aliquots (5 μg) of total RNA were subjected to a DNase I digestion before reverse transcription (RT) to eliminate any remaining genomic DNA. The RT reaction was carried out for 1 h at 42 °C in a reaction mixture (20 μl) containing 50 ng of oligo(dT)12–18 primer, dNTP (0.5 mM each of dATP, dGTP, dCTP and dTTP), 200 units of Superscript™II (Life Technologies Inc., Gaithersburg, MD) and the first strand buffer, and terminated by incubation at 95 °C for 5 min.

PCR was performed in a reaction mixture (30 μl) containing cDNA, primers (10 pmol each), 0.2 mM dNTP and 1.5 units of Takara Ex Taq™ DNA polymerase (Takara Shuzo, Kyoto, Japan) in Ex Taq buffer. Primers (5′–3′) specific for murine IL-7, IL-15, IL-18, caspase-1 and β-actin sequences [29–32] were as follows:

IL-7, GCGGATCCATGTTCCATGTTTCTTTTAG and GCAAGCTTGTTTATATACTGCCCTTC, 483 bp;
IL-15, GCGGATCCAATGAAAATTTTGAAACC and GCGAATTCAGTCAGGACGTGTTGATG, 508 bp;
IL-18, GCGAATTCCAATGGCTGCCATGTCAG and GCAAGCTTACCTAACTTTGATGTAAG, 599 bp;
Caspase-1, GCCTGCAGATGGCTGACAAGATCCTGAGG and GCCTCGAGTTAATGTCCCGGGAAGAGGTAG, 1225 bp
β-actin, GCGGATCCATGGATGATGATATCGCCGC and GCAAGCTTCCTAGAAGCATTTGCGGTGG, 1144 bp.

Before the first cycle, a denaturation step was performed at 95 °C for 7 min, PCR was carried out using a PCR thermal cycler sp (Takara Shuzo, Kyoto, Japan) with a regimen of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min (30 cycles), and a final extension period of 7 min at 72 °C. The products were analyzed by electrophoresis through 1.5% agarose gels and stained with ethidium bromide, and densitometric analysis of digitized gel images was carried out using NIH Image software.

2.17. Immunohistochemical studies

For investigation of the effect of bLF on immune cells, 3 h after the final oral administration of bLF (30 mg/kg per day for 7 days; four mice/group) mice were anesthetized and killed, and the jejunum was removed. Jejuna obtained from mice were fixed in acetone at 4 °C and embedded in paraffin. After cutting and deparaffination, sections were incubated in 1% skim milk (Yukijirushi, Sapporo, Japan) − PBS at room temperature for 1 h to block non-specific antigens.

$CD4^+$ and $CD8a^+$ cells were detected with rat-anti-mouse CD4 mAb and CD8a mAb (Japan BD Biosciences Pharmingen, Tokyo, Japan). After washing blocked sections three times, sections were incubated in PBS containing 10% hydrogen peroxide at room temperature for 30 min to remove endogenous peroxidase activity before antibody was added. Sections were then incubated with antibody in a humidified chamber at room temperature for 2 h. After washing, the sections were incubated with biotin-conjugated anti-rat IgG antibody (Vectastain Elite ABC kit, Vector Laboratories, Burlingme, CA) for 3 h. Labeled cells were visualized with 0.03% 3,3-diaminobezidine tetrahydrochroride solution containing 0.1% hydrogen peroxide for 5 min.

IgM^+ and IgA^+ cells were detected with goat anti-mouse IgM Ab-FITC and goat anti-mouse IgA Ab-FITC (Sigma). After washing blocked sections three times, sections were incubated with antibody in a humidified chamber at 4 °C for 2 h. Labeled cells were visualized by fluorescent microscopy.

IL-7, IFNα, and caspase-1 (p-20) expressing cells were detected with rat anti-mouse IL-7 Ab (PeproTech EC. Ltd. London, UK), rat anti-mouse IFNα mAb (HyCult Biotechnology b.v., Uden, The Netherlands), and rabbit anti-mouse caspase-1 (p-20) antibody (Santa Cruz Biotechnology), respectively. After washing blocked sections three times, sections were incubated in PBS containing 10% hydrogen peroxide at room temperature for 30 min to remove endogenous peroxidase activity before antibody was added. Sections were then incubated with antibody in a humidified chamber at room temperature for 2 h. After washing, the sections were incubated with biotin-conjugated anti-rat IgG antibody or biotin-conjugated anti-rabbit IgG antibody (Vectastain Elite ABC kit, Vector Laboratories, Burlingme, CA) for 3 h. Labeled cells were visualized with 0.03% 3,3-diaminobezidine tetrahydrochroride solution containing 0.1% hydrogen peroxide for 5 min.

Results were expressed as the mean of the number of positive cells in the lamina propria per 10 light microscope fields (×200).

2.18. Western blot analysis of frozen tissue samples

Frozen tissue samples (100 mg) were homogenized in 1 ml chilled lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail "Complete Mini™" (Roche, Mannheim, Germany). Lysates were centrifuged at 600 rpm for 20 min at 4 °C to remove nuclei and cell debris, and then clarified by centrifuged at 15,000 rpm for 45 min at 4 °C. Twenty μg of protein from each sample were separated by SDS-PAGE on 10% acrylamide, 0.2% bis gels. Proteins were transferred to Immobilon™ transfer membranes (Millipore Corp., Bedford, MA) and detected by Western blot with appropriate antibodies. Immunoreactive bands were visualized with ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Densitometric analysis of digitized blot images was carried out using NIH Image software.

2.19. Treatment of GKO mice with rmIL-7, rmIL-18 or rIFNαA/D and metastasis assay

Co26Lu cells (1×10^5 cells/mouse) were subcutaneously implanted into the right thigh of GKO mice. Recombinant

murine IL-7 (*Escherichia coli*, PeproTech House, London, England), recombinant murine IL-18 (Medical & Biological Laboratories Co., Ltd.), and recombinant IFNαA/D (2.04×10^7 IU/ml, Nippon Roche Research Center, Kamakura, Japan) were administered intraperitoneally (i.p.) to GKO mice at 1 and 0.1 μg/mouse, 0.02 μg/mouse and 1×10^5 IU/mouse, respectively, on days 10, 13, 17, and 20. Endotoxin levels of rmIL-7 and IL-18 were less than 0.1 ng/μg protein by the LAL method. The longest (a) and shortest (b) diameters of tumors at the Co26Lu cell injection site were measured twice a week using calipers, and the volume was calculated using the formula: $ab^2/2$ (mm^3). The experiment was terminated 22 days after implantation, and the mice were killed. The lungs were removed, rinsed with saline, and fixed in acetone, and the lung metastatic colonies counted.

3. Results

3.1. Caspase-1 activity and generation of mature interleukin-18 levels in the mucosa of the small intestine in organ culture by bLF

In C57BL/6 or BALB/c mice, oral treatment with bLF or bLFcin increases mature IL-18 levels in the mucosa of the small and large intestines [7]. However, bLF is digested into numerous peptides by pepsin in the stomach [33]. We employed an organ culture system using mouse small intestine to investigate the ability of intact bLF and an N-terminal peptide fragment of bLF (bLFcin) to affect the activity of caspase-1 and the generation of mature IL-18: caspase-1 cleaves the proform of IL-18 to generate the 18 kDa bioactive molecule [34–36].

In organ culture experiments, caspase-1 activity and mature IL-18 levels in the mucosa of the small intestine were significantly increased following addition of bLF (Fig. 2A). Exposure to bLFcin also resulted in increases in caspase-1 activity and mature IL-18 levels (Fig. 2B). These results were specific since exposure to bTF did not result in elevated levels of mature IL-18, and exposure to bLF did not affect IL-1β levels. These results are similar to those obtained from previous *in vivo* experiments in which treatment with bLF or bLFcin caused an increase in IL-18 levels and caspase-1 activity in the mucosa of the small intestine [9]. Therefore, both intact bLF and an N-terminal peptide fragment of bLF, bLFcin, stimulate caspase-1 activity and the generation of mature IL-18.

3.2. bLF or bLFcin induction of cytokine production by peritoneal macrophages

Next, we investigated caspase-1 and cytokine production by peritoneal macrophages treated with bLF or bLFcin *in vitro*. Peritoneal macrophages, the macrophage cell line RAW264.7, and spleen cells were treated with bLF, and caspase-1 activity and mature IL-18 levels were measured. Treatment with 300 μg/ml bLF enhanced caspase-1 activity in peritoneal macrophages and possibly to some extent in spleen cells, but not in RAW264.7 cells (Fig. 3 left panel). bLF markedly enhanced production of mature IL-18 in peritoneal macrophages but not in Raw264.7 or spleen cells (Fig. 3 right panel). bLF-mediated induction of mature IL-18 by peritoneal macrophages was dose dependent and bovine transferrin (bTF) had no effect (Fig. 4). Recombinant murine interferon-gamma (rmIFNγ) also induced mature IL-18 production by peritoneal macrophages (Fig. 4). Importantly, bLF-mediated induction of mature IL-18 was abrogated by co-incubation of the cells with caspase-1 inhibitor (Acyl-Tyr-Val-Ala-Asp-chloromethylketone, Ac-YVAD-CMK) (Fig. 5). bLF-mediated induction of mature IL-18 was also inhibited by co-incubation with anti-mIFNγ antibody (Fig. 5). Finally, IFNγ induced production of mature IL-18 (Fig. 5), and this induction was inhibited by co-incubation of the cells with anti-mIFNγ antibody (data not shown), confirming the validity of this assay.

In Western blot analysis of macrophage lysates after addition of bLF or bLFcin at 1000 or 30 μg/ml, respectively, bLF induced expression of IFNγ and IL-15, and bLFcin induced expression of IL-15 (Fig. 6). Treatment with bLF decreased expression of IFNα while treatment with bLFcin decreased expression of TNFα (Fig. 6).

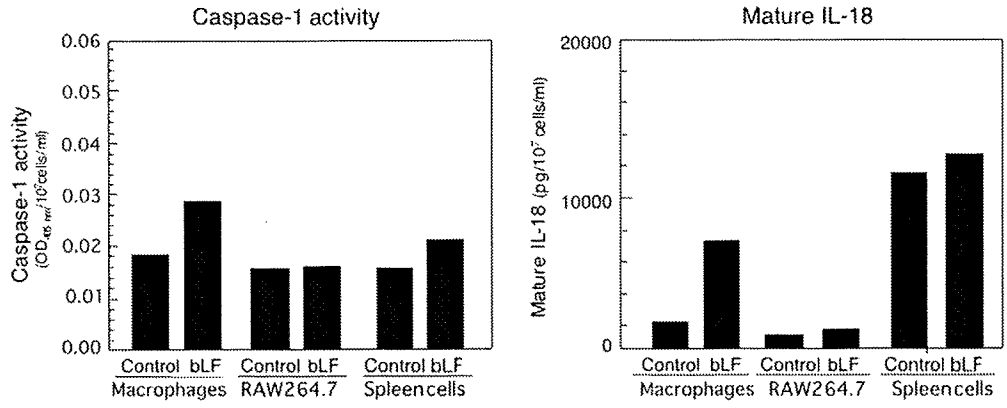

Fig. 3. Effects of bLF on caspase-1 activity and mature IL-18 production in peritoneal macrophages, RAW264.7 cells, and spleen (BALB/c mouse) cells. Cells were incubated for 24 h in bLF (300 μg/ml). Caspase-1 activity and mature IL-18 levels were determined by ELISA. Data are representative of two independent experiments.

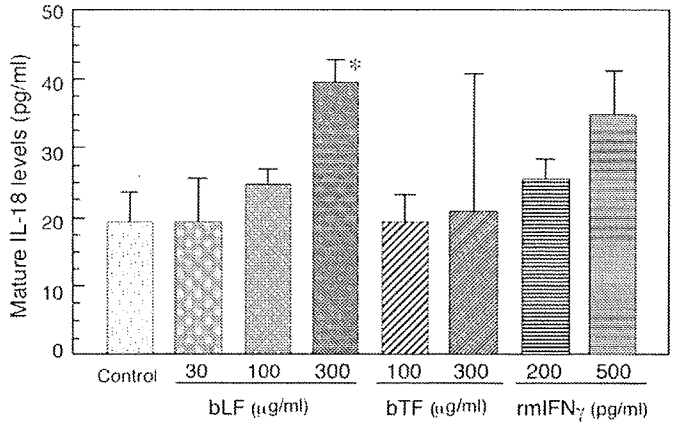

Fig. 4. Effect of bLF, bTF, and rmIFNγ on the production of mature IL-18 by macrophages. Macrophages were incubated for 24 h in bLF, bTF or rmIFNγ and mature IL-18 levels were measured by ELISA. Error bars represent the standard error ($n = 3$). *$p < 0.05$, Dunnett's multiple comparison t-test.

3.3. Cytokine induction in various tumor cell lines by bLF or bLFcin

bLF can induce caspase-1 activity and the generation of mature IL-18 in mouse intestinal tissue and macrophages and IFNγ and IL-15 expression in macrophages; bLFcin can induce caspase-1 activity and the generation of mature IL-18 in mouse intestinal tissue and IL-15 expression in macrophages. We next investigated the effect bLF or bLFcin had on cytokine expression in mouse and human tumor cell lines: Lewis lung carcinoma was the mouse tumor cell line used, and SW620 (colon), MCF-7 (breast), and SK-MEL-19 (melanoma) were the human cell lines used.

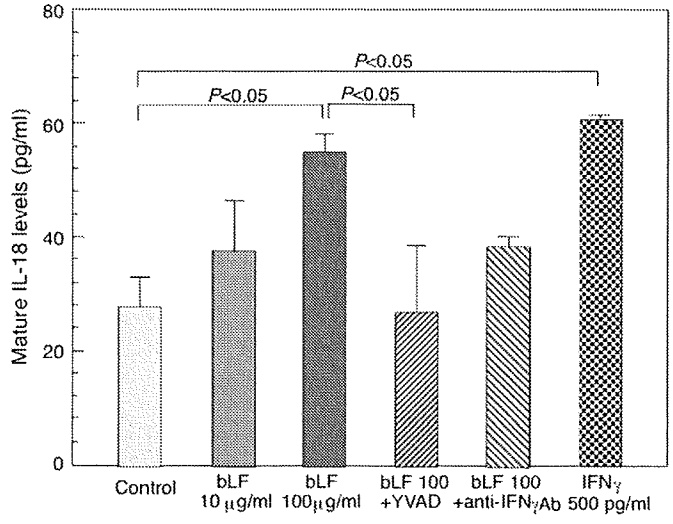

Fig. 5. Caspase-1 inhibitor (YVAD) and IFNγ antibodies block mature IL-18 production by macrophages treated with bLF. Peritoneal macrophages were treated with bLF, bLF + the caspase-1 inhibitor YVAD or bLF + IFNγ antibodies, and mature IL-18 levels were measured by ELISA. The error bars represent the standard error ($n = 3$), p values were calculated by Dunnett's multiple comparison t-test. YVAD = Acyl-Tyr-Val-Ala-Asp-chloromethylketone.

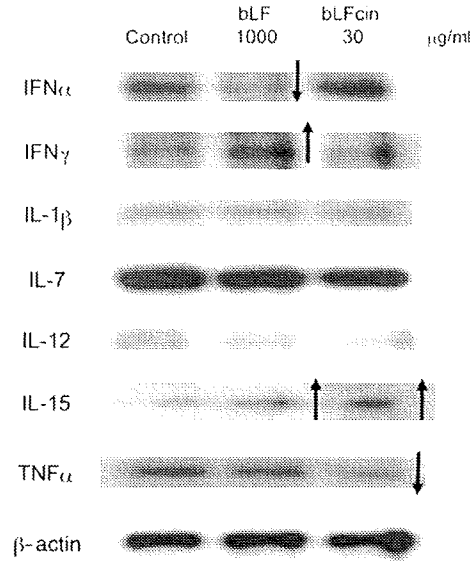

Fig. 6. Western blot analysis of cytokine production in peritoneal macrophages following addition of bLF or bLFcin (BALB/c mice). Macrophages were incubated for 24 h in bLF (1000 μg/ml) or bLFcin (30 μg/ml). Arrows indicate increases or decreases in expression compared to the control. Data are representative of two independent experiments.

Incubation of Lewis Lung carcinoma cells in bLFcin at 100 or 300 μg/ml for 48 h significantly decreased cell number, however, bLF at 1000 μg/ml or bLFcin at 30 μg/ml had no effect on cell number (Table 1). In Western blot analysis of Lewis lung carcinoma cells treated with bLF or bLFcin for 24 h, bLF induced increased expression of IFNγ (2-fold) and IL-1β (3-fold), and bLFcin (lower doses) induced increased expression of IL-1β (4-fold) (Fig. 7).

In Western blot analysis of human cells treated with bLF or bLFcin for 24 h, both bLF and bLFcin induced expression of human lactoferrin (hLF) (2-fold) and mature IL-18 (2 ~ 4-fold) by colon cancer SW620 cells (Fig. 8A); both bLF and bLFcin induced expression of hLF (7 ~ 29-fold), IFNα (4-fold), IL-12 (2 ~ 4-fold), and mature IL-18 (2 ~ 3-fold) by mammary cancer MCF-7 cells (Fig. 8B); and both bLF and bLFcin induced expression of IFNα (4 ~ 11-fold), IL-12 (2 ~ 5-fold) and mature IL-18 (3 ~ 4-fold) by melanoma SK-MEL19 cells (Fig. 8C). SK-MEL19 cells did not express hLF. In HT29 human colon cancer cells, hLF levels were unaffected by incubation in 1000 μg/ml bLF (87% of basal line), but were significantly decreased by incubation in 30 μg/ml bLFcin (46% of basal line) (data not shown).

Table 1
Effects of lactoferrin and lactoferricin on the growth of Lewis lung carcinoma cells 5% FCS in RPMI-1640 medium, 48 h incubation

	Concentration (μg/ml)	Growth (% of Control)
bLF	1000	96.2
bLFcin	30	100.0
	100	46.5
	300	13.2

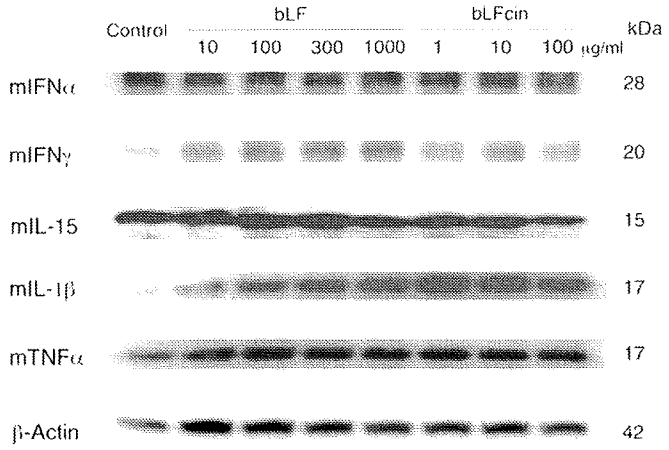

Fig. 7. Western blot analysis of cytokine production in Lewis Lung carcinoma cells following incubation with bLF or bLFcin for 24 h. Data are representative of two independent experiments.

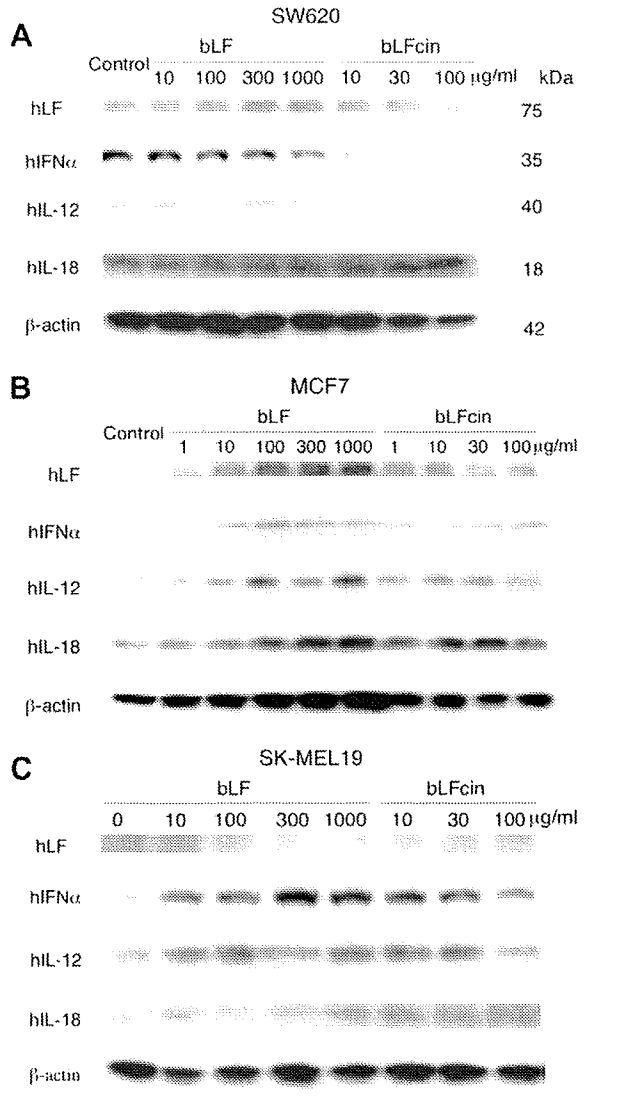

Fig. 8. Western blot analysis of cytokine production in SW620 (A) colon cancer cells, MCF7 (B) mammary carcinoma cells, and SK-MEL19 (C) melanoma cells following incubation with bLF or bLFcin for 24 h. Data are representative of three independent experiments.

In summary, exposure of cells to bLF or bLFcin tends to cause induction of a variety of cytokines. In addition, LF may activate an autocrine loop in some cells; notice the extremely high induction of hLF in the breast cancer cell line, MCF7. The specific effects of bLF and bLFcin, however, depend on the cell type.

3.4. Anti-metastatic activity following oral administration of bLF in GKO and BALB/c mice

We have shown that bLF and bLFcin can induce mouse small intestine to increase its production of mature IL-18. An important function of IL-18 is activation of T-cells and NK cells involved in cell mediated immune responses. Through its stimulation of immune cells, IL-18 has been shown to protect experimental animals against repeated challenges with tumor cells [37,38]. Therefore, we investigated the ability of bLF to inhibit lung metastasis by Co26Lu tumor cells. In addition, we used IFNγ KO (GKO) mice to assess the importance of IFNγ on the ability of bLF to inhibit lung metastasis of Co26Lu tumor cells.

The number of lung metastatic colonies in tumor-bearing GKO mice were markedly higher than in tumor-bearing BALB/c mice (Fig. 9), indicating that IFNγ inhibits the metastatic ability of Co26Lu cells. Oral treatment with bLF significantly inhibited lung metastatic colony formation in both GKO mice and BALB/c mice (Fig. 9), indicating that bLF can inhibit the metastatic ability of Co26Lu cells in the absence of IFNγ.

3.5. Cytokine levels in the small intestines in GKO and BALB/c mice following treatment with bLF

Since IFNγ is not required for bLF-mediated inhibition of Co26Lu metastasis to the lung, we compared bLF-mediated induction of cytokines in the small intestines of GKO and BALB/c mice. IL-1β, IL-6, and TNFα levels in the mucosa of the small intestine in both GKO and BALB/c mice were not markedly changed after treatment with bLF (Fig. 10). IL-12 levels showed a tendency to decrease in BALB/c mice. IL-4 and GM-CSF showed a tendency to increase in both GKO and BALB/c mice. Mature IL-18 levels in the small intestine of GKO mice were not affected by treatment with bLF, however, bLF did cause a significant increase in mature IL-18 in BALB/c mice (Fig. 11).

Expression of IL-7, IL-15, and IL-18 mRNA was examined in freshly isolated mucosal tissues of the small intestine of GKO and BALB/c mice following treatment with bLF at 30 or 300 mg/kg per day. IL-7 mRNA levels were significantly enhanced in GKO mice by administration of bLF (1.4- and 1.5-fold increase, $p < 0.05$ and $p < 0.01$, respectively), but bLF had no effect on IL-7 mRNA levels in BALB/c mice (0.9 ~ 1.3-fold increase $p > 0.05$); bLF had no significant effect on IL-15 mRNA levels in either GKO or BALB/c mice (Fig. 12); bLF had no significant effect on IL-18 mRNA levels

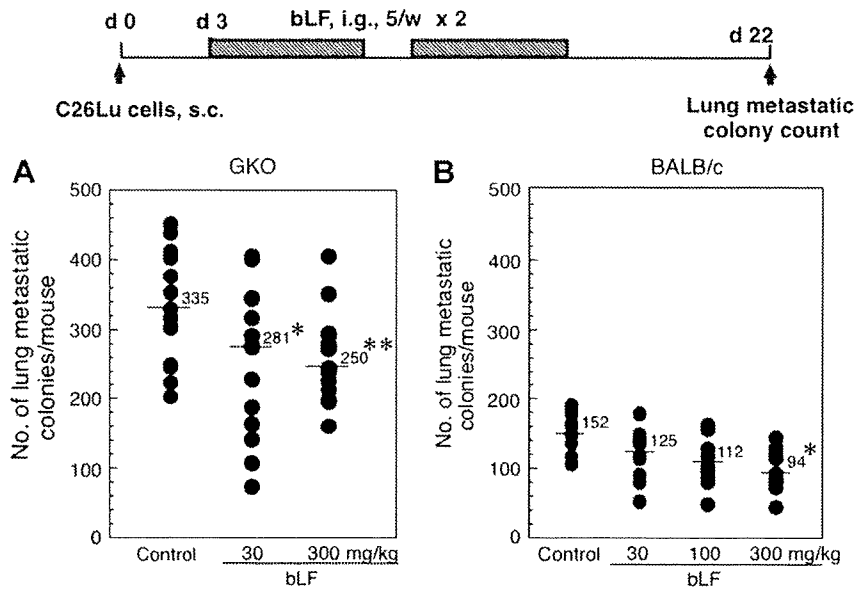

Fig. 9. Effects of oral administration of bLF on the number of lung metastatic colonies in Co26Lu-bearing GKO (A) and Co26Lu-bearing BALB/c (B) female mice. The numerical values represent the median of the number of lung metastatic colonies in 15 animals. *$p < 0.05$, **$p < 0.01$ vs Control, Mann–Whitney U-test.

in GKO mice (about 1.3-fold increase, $p > 0.05$), but bLF markedly elevated IL-18 mRNA levels in BALB/c mice (1.3 and 1.7-fold increase, $p > 0.05$ and $p < 0.01$, respectively) (Fig. 12).

Thus, bLF induced expression of IL-18 mRNA and generation of mature IL-18 in BALB/c mice. Since bLF had no effect on IL-18 expression in GKO mice, bLF induction of IL-18 is IFNγ dependent. In GKO mice, bLF induced expression of IL-7.

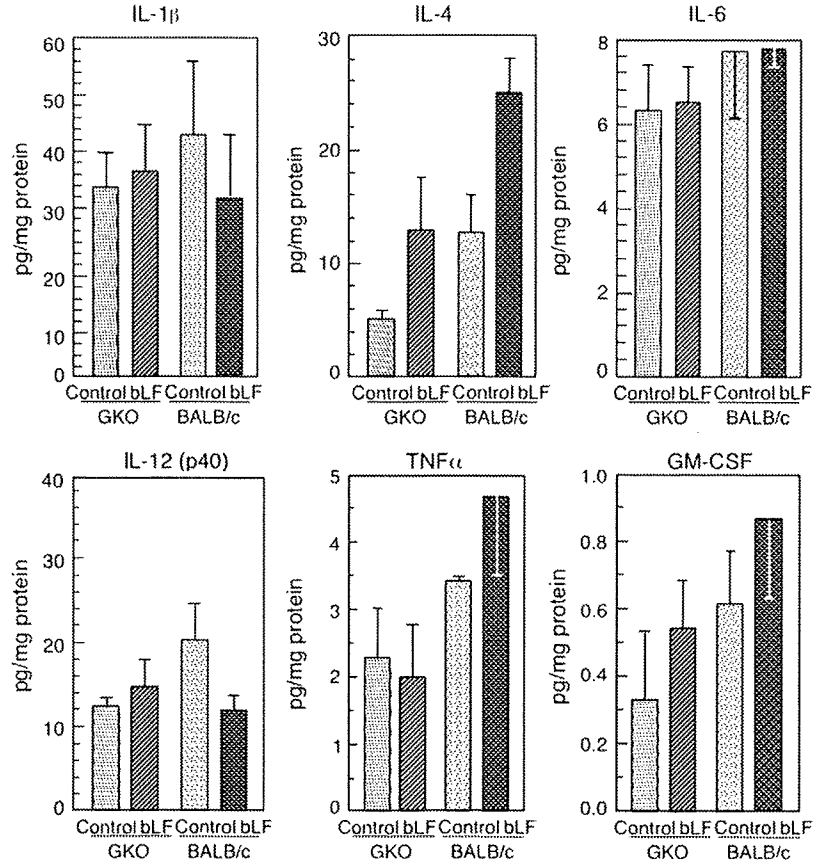

Fig. 10. Effect of bLF on various cytokine levels in the mucosa of the small intestines of GKO and BALB/c female mice. bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration the animals were anesthetized and killed, the jejunum removed, and cytokine levels measured. Error bars represent the standard error ($n = 5$).