FULL PAPER *Internal Medicine*

The Antiproliferative Effect of Bovine Lactoferrin on Canine Mammary Gland Tumor Cells

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ABSTRACT. Lactoferrin has several biological activities, including antitumor activities in some human and animal tumor cells. Clinical trials have been carried out in human medicine based on these effects. However, the antitumor effects of lactoferrin in veterinary medicine remain unknown. In this *in vitro* study, we demonstrated that co-incubation of canine mammary gland tumor cells (CIPp and CHMp) and bovine lactoferrin induced growth arrest of tumor cells. This growth arrest was associated with induction of G1 arrest. Furthermore, this effect was stronger in tumor cells than in normal cells. These findings demonstrate that bovine lactoferrin has anti-tumor activity in canine mammary tumors and has the potential for use in tumor-bearing dogs.
KEY WORDS: bovine lactoferrin, canine mammary gland tumor cell, G1 arrest.

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In recent years, advancement of veterinary medicine has led to an increase in the life span of companion animals. This has resulted in increasing incidence of age-related diseases, such as cardiac disorders and tumors. In fact, the likelihood of encountering tumor-bearing animals is increasing in veterinary medicine. However, some of these animals have already progressed to advanced stages by the time they are diagnosed and are poor candidates for aggressive therapies, such as surgery. Mammary gland tumor (MGT) is especially common tumor in dogs. Generally, surgery is the first choice for the treatment of MGT. However, patients with inflammatory MGT or metastatic lesions are not eligible for surgery. MGT also has low responsiveness to chemotherapy. It is therefore imperative that good alternative or palliative therapies be developed.

Lactoferrin (LF) is an approximately 80 kDa iron-binding glycoprotein mainly found in external secretions such as breast milk, tears and the secondary granules of neutrophils [7]. It has several biological activities associated with the immune system, including anti-inflammatory and immunomodulatory activities. Lactoferrin also has antimicrobial properties against bacteria, fungi and several viruses [1, 10]. Additionally, Hayashida *et al.* [3] showed that LF has mu-opioid receptor-mediated antinociceptive activity.

Recently, some studies have also suggested antitumor activities for LF [2, 6, 9, 13, 14]. In previous studies, LF has been shown to inhibit the growth of some tumor cell lines, including head and neck squamous cell carcinoma (SCCVII, O12), human breast cancer epithelial cells (MCF-7, MDA-MB-231) and murine colon adenocarcinoma cells (colon-26) [2, 6, 9, 13, 14]. Furthermore, phase I trials of talactoferrin alfa, a recombinant human lactoferrin, have been completed using human refractory solid tumors [4].

As described above, LF has promising properties as an antitumor agent due to several of its biological activities. Therefore, we considered that LF was worth administering to inoperative tumor-bearing animals.

On the other hand, the factors that are import for control of tumor progression are the cell cycle, angiogenesis, metastasis and tumor immunity. In eukaryotic cells, each phase of the cell cycle is controlled by various cyclin proteins, sequential activation of various cyclin-dependent kinases (Cdks) and inhibition of Cdks activity by cyclin-dependent kinase inhibitors (CdkI). This cell cycle control system breaks down in tumor cells. Lactoferrin has been shown to inhibit G1 cyclin-dependent kinases in human breast carcinoma cells [2], downregulate G1 cyclin-dependent kinases in head and neck cancer cells [13] and control the level and activity of retinoblastoma protein (pRB) [11]. These reports also raise interesting questions about the effects of LF on the canine tumor cell cycle.

Regardless of the extensive studies in human tumors, the efficacy of bLF has not been reported for canine tumors. In this foundational study, we analyzed the effect of bLF on the growth and cell cycle of canine tumor cell lines with the aim of investigating the clinical application of bLF.

MATERIALS AND METHODS

Canine cells: Canine mammary gland adenocarcinoma cells (CIPp, CHMp) were obtained from a previously established cell line at the Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, University of Tokyo [8]. CIPp and CHMp cells were cultured in

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RPMI 1640 supplemented with 10% FBS, 3% L-glutamine, 5% gentamicin and 0.6% amphotericin B and were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Canine fibroblasts (CF) were obtained through skin punch biopsy of a healthy beagle. CF cells were cultured in DMEM supplemented with 10% FBS, 5% gentamicin and 0.6% amphotericin B and were maintained under the same conditions as CIPp cells.

Bovine lactoferrin: Bovine LF was obtained from Morinaga Milk Industry Co., Ltd., Japan. It was reconstituted in ultrapure water (MQ) at 50 mg/m*l* and sterilized by filtration (0.2 μm millipore filter). The concentrations of accretive bLF were decided based on previous reports [13, 14] and our pilot study.

Primary culture: To collect CF cells, the dorsal aspect of the cervical region was clipped and tissue was obtained by skin punch biopsy. The skin tissue was then washed with PBS and minced using scissors. The macerated tissue was put in a culture bottle, allowed to adhere to the bottom and then cultured in DMEM supplemented with 10% FBS, 5% gentamicin and 0.6% amphotericin B.

Cell proliferation: CIPp, CHMp and CF cells (1×10^4 cells/m*l*, 2 m*l*) were cultured with or without bLF (250 or 500 μg/m*l* bLF in medium) for 72 hr. The cell concentrations at passage were decided based on the doubling time of each cells. After 72 hr, the cells were washed twice with PBS and trypsinized for harvesting. The number of viable cells was counted after harvesting using 0.3% trypan blue and a hemocytometer. The cell count was the average of triplicate wells for each sample. Third-passage CF cells were used for the present study.

Flow cytometry for cell cycle analysis: CIPp and CHMp cells were cultured with or without bLF (250 or 500 μg/m*l*) at 1×10^6 cells/well in 12-well flat-bottomed plates for 48 hr. After culture, the cells were washed twice with PBS and trypsinized. The cells were counted and adjusted to 1×10^6 cells/m*l*. After washing twice with PBS, the cells were fixed with 70% ethanol at 4°C for 2 hr. After fixing, the cells were centrifuged at 3,500 rpm for 5 min and washed twice with PBS. The cells were then suspended in PBS and incubated with 0.25 mg/m*l* RNase for 30 min. DNA was stained with 25 mg/m*l* of propidium iodine (PI) for 30 min at 4°C in the dark. The cell cycle distribution was determined by flow cytometry using a FACScan cytofluorimeter (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.). PI fluorescein data was collected using linear amplification. A minimum of 10000 events was collected for each sample. Analysis of the data was performed with the CELLQuest Software program (Becton Dickinson Immunocytometry Systems).

Statistical analysis: The numbers of cells are showed as averages and standard deviations, respectively. The Tukey-Kramer test was performed to evaluate the relationship between cell proliferation and bLF concentration. Statistical analysis was performed for different concentrations of bLF. Differences among the groups were considered significant when P was <0.05.

RESULTS

Cell proliferation: The numbers of CIPp, CHMp and CF cells after incubation with bLF or MQ for 72 hr are shown in Fig. 1–1. The numbers of CIPp and CHMp co-incubated with bLF or MQ for 24, 48 and 72 hr as a pilot study are shown in Fig. 1–2. Furthermore, the cell densities of these cell lines following incubation for 72 hr are shown in Fig. 2.

The number of CIPp cells without bLF at 72 hr was $18.33 \pm 6.03 \times 10^4$ cells. On the other hand, the numbers of CIPp with bLF (250 μg/m*l* and 500 μg/m*l*) at 72 hr were $0.67 \pm 0.58 \times 10^4$ and almost 0 cells, respectively. There was a significant difference between the bLF groups and MQ group. There was no significant difference between the 250 μg/m*l* and 500 μg/m*l* groups, but the number of cells of the high concentration group was tended to decrease compared with the low concentration group. The number of CHMp cells without bLF at 72 hr was $48.33 \pm 17.01 \times 10^4$ cells. On the other hand, the numbers of CHMp with bLF (250 μg/m*l* and 500 μg/m*l*) at 72 hr were $22.67 \pm 2.31 \times 10^4$ and $11.67 \pm 1.53 \times 10^4$ cells, respectively. There was a significant difference between the bLF and MQ groups. There was no significant difference between the 250 μg/m*l* and 500 μg/m*l* groups, but the number of cells of the high concentration group was tended to decrease compared with the low concentration group. In the case of CF cells, the number of cells without bLF at 72 hr was $3.67 \pm 2.08 \times 10^6$ cells. On the other hand, the numbers of cells with bLF (250 μg/m*l* and 500 μg/m*l*) at 72 hr were $3.67 \pm 2.31 \times 10^4$ and 1.67 ± 1.53

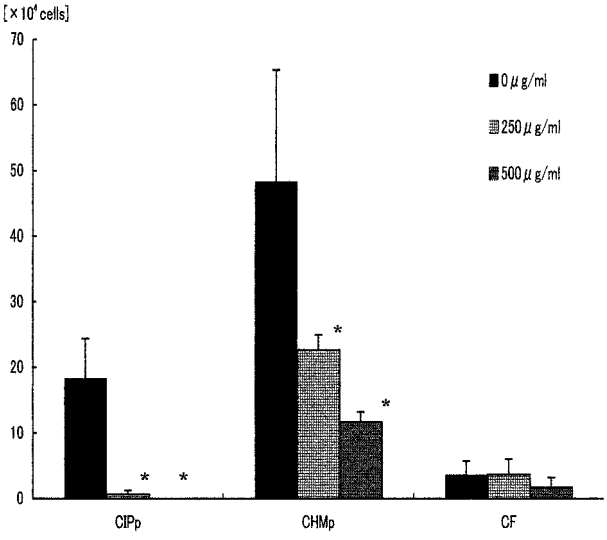

Fig. 1–1. The antiproliferative effect of bLF in canine mammary tumor cell lines and canine fibroblasts at 72 hr. Proliferation of canine mammary tumor cell lines (CIPp and CHMp) was significantly inhibited by bLF (versus without bLF, *: $p<0.05$). The antiproliferative effect of bLF tended to correlate with the bLF concentration. On the other hand, proliferation of the control population (canine fibroblasts), which acted as a control of normal cells, was not inhibited by bLF.

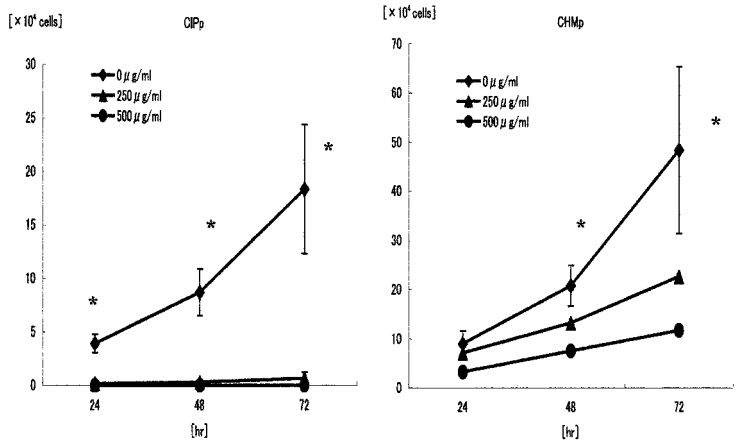

Fig. 1–2. The antiproliferative effect of bLF on CIPp and CHMp from 24 to 72 hr. Proliferation of CIPp and CHMp was inhibited by bLF at 24 and 48 hr, respectively (versus with bLF, *: $p<0.05$).

$\times 10^4$ cells, respectively. There were no significant difference between the bLF and MQ groups. The densities of the CIPp and CHMp cells decreased according to the increase in bLF concentration. On the other hand, the number of CF cells decreased slightly, and a mild morphologic change was observed in the CF cells of the 500 μg/m*l* group. However, there was no significant difference between the bLF and MQ groups in terms of the number of cells.

Cell cycle analysis: Cell cycle analysis was employed to further investigate the inhibitory effects of bLF on mammary tumor cell line proliferation. The results of cell cycle analysis are shown in Fig. 3. The G1 and G2/M peaks in the CIPp cells without bLF were 50.2% and 36.5% of the total. The G1 and G2/M peaks in the CIPp cells with bLF (250 μg/m*l*) were 73.5% and 18.5% of the total. The G1 and G2/M peaks in the CIPp cells with bLF (500 μg/m*l*) were 71.3% and 20.2% of the total. The G1 peak in the CIPp cells with bLF versus without bLF increased to 148.5% (250 μg/m*l*) and 144.0% (500 μg/m*l*), respectively. On the other hand, G2/M peak in the CIPp cells with bLF versus without bLF decreased to 69.5% (250 μg/m*l*) and 51.5% (500 μg/m*l*), respectively. The G1 and G2/M peaks in the CHMp cells without bLF were 81.6% and 11.2% of the total. The G1 and G2/M peaks in the CHMp cells with bLF (250 μg/m*l*) were 86.5% and 6.9% of the total. The G1 and G2/M peaks in the CHMp cells with bLF (500 μg/m*l*) were 92.8% and 4.5% of the total. The G1 peak in the CHMp cells with bLF versus without bLF increased to 106.0% (250 μg/m*l*) and 113.8% (500 μg/m*l*), respectively. On the other hand, the G2/M peak in the CHMp cells with bLF versus without bLF decreased to 61.6% (250 μg/m*l*) and 40.4% (500 μg/m*l*), respectively. In other words, bLF induced G1 arrest in both cell lines of canine mammary gland tumor.

DISCUSSION

In this report, we demonstrated for the first time that bLF inhibited the proliferation of canine mammary gland tumor cell lines. Our results concurred with those found in human cell lines in which LF exhibited antitumor effects on tumor cells [2]. Suzuki *et al.* reported that bLF did not influence the proliferation of human fibroblast cells at the 8th International Conference on Lactoferrin [12]. In this study, bLF (500 μg/m*l*) produced mild inhibition of CF cell proliferation and morphological change of CF cells. However, this antiproliferative effect on CF cells was mild compared with that in canine mammary gland tumor cells. Unlike human fibroblast cells, a high concentration of bLF (>500 μg/m*l*) may influence the proliferation and migration of CF cells. The mechanisms were not known nothing about the difference of response to bLF among CF cells, human fibroblast cells and established canine tumor cell line. The differences in characteristics among the cells may also influence the results. Therefore, these problems require further study.

Furthermore, we investigated the mechanism involved in the inhibitory effect of bLF on the proliferation of canine mammary gland tumor cell line using flow cytometry. We found that the induction of growth arrest at the G1 to S transition was one of factors in the antiproliferative effect of bLF. Our results are in agreement with previous reports that have shown G1 arrest induced by hLF in human mammary cell lines [2].

Hurley *et al.* [5] reported that bLF and hLF had no effect on the growth of MCF-7 human breast tumor cells. On the other hand, Damien *et al.* [2] reported that hLF inhibited the growth of MDA-MB 231 human breast cancer epithelial cells. These results indicate that the inhibitory effect of LF may differ according to the cell line. In the present study, the response of CHMp cells to bLF was slightly lower than that of CIPp cells. This result supports the suggestion that the inhibitory effect of bLF differs according to the cell line in relation to canine tumors. Nakagawa *et al.* [8] reported that CIPp showed higher expression of cyclin D1 than CHMp. A previous study showed that LF reduces cyclin D1

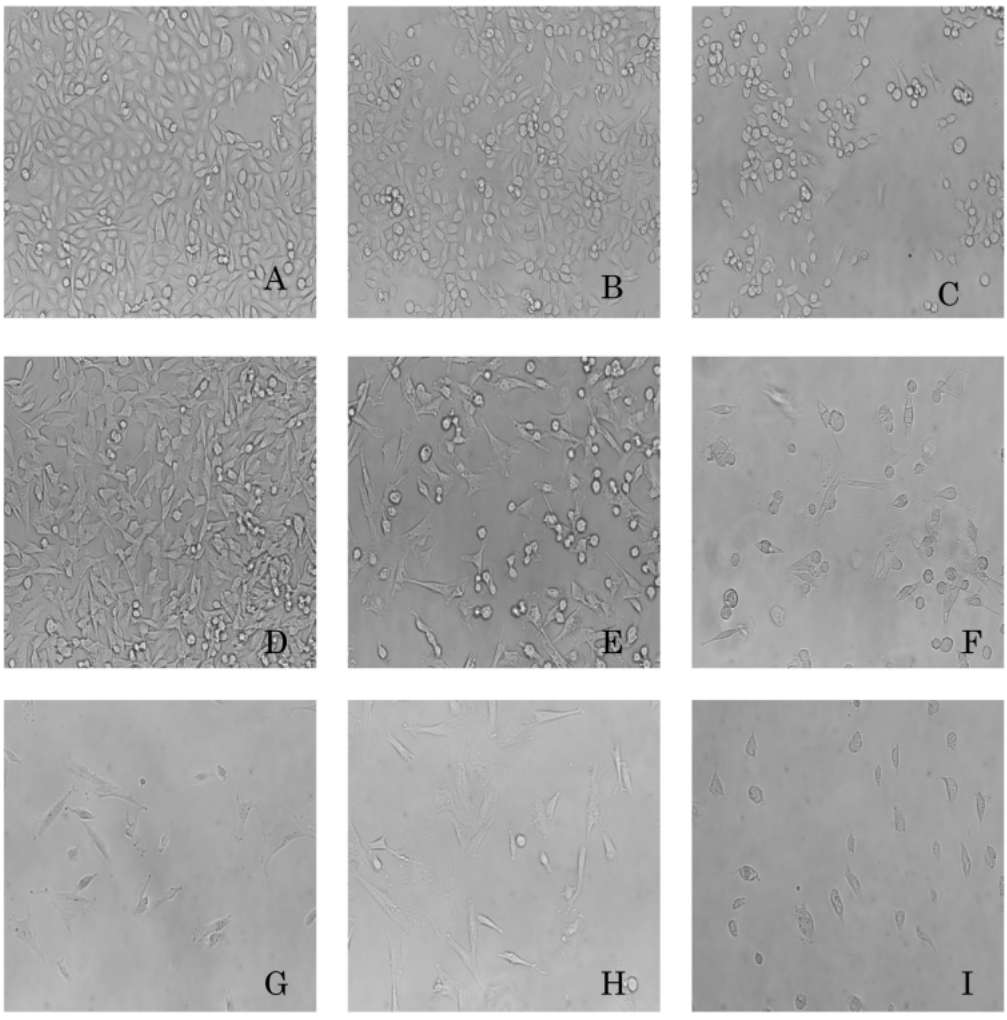

Fig. 2. Cell densities of the cell lines following incubation for 72 hr. The number of CIPp cells without bLF (A: 0 μg/m*l*) increased while the number of cells cultured with bLF (B, 250 μg/m*l*; C, 500 μg/m*l*) decreased. The number of CHMp cells without bLF (D, 0 μg/m*l*) increased, while the number of cells cultured with bLF (E, 250 μg/m*l*; F, 500 μg/m*l*) decreased. However, there were no major changes in the CF cells upon incubation with 0, 250 or 500 μg/m*l*(G, H, I) bLF, respectively.

expression [13]. Therefore, the higher inhibition of bLF in CIPp cells observed in the present study seems to have been caused by the difference in cyclin D1 expression. On the other hand, G1 arrest in CHMp cells as a result of bLF seemed to be clearer than in the CIPp cells. Based on the results of flow cytometric analysis, proliferation of CHMp cells seemed to be more greatly inhibited by bLF than proliferation of CIPp cells. However, there was a larger decrease in the number of CIPp cells compared with the CHMp cells. These results suggest that bLF has a possibility to present the other antitumor mechanisms like the induction of apoptosis except the leading of G1 arrest.

The origin of LF causes different cell responses according to Hurley *et al.* [5]. In our study, bLF had an antiproliferative effect on canine mammary tumor cells. Although we do not make some studies of hLF, bLF at least may be worth applying it for canine mammary gland tumors in clinical setting.

There was no significant difference in bLF antiproliferative activity according to dosage in the present study, but a high concentration of bLF did tend to suppress canine mammary gland tumor cell growth. Previous reports have shown that the LF concentration affects its antiproliferative activity and mechanism [13, 14]. Further study is required to investigate the effect of the bLF concentration on its antiproliferative activity and mechanisms in relation to canine mammary gland tumor cells.

As described above, our study suggests that bLF has antiproliferative activity on canine mammary gland tumor cells through induction of arrest of G1 to S transition. This preliminary work indicates that bLF might have benefits when administered to mammary gland tumor-bearing dogs.

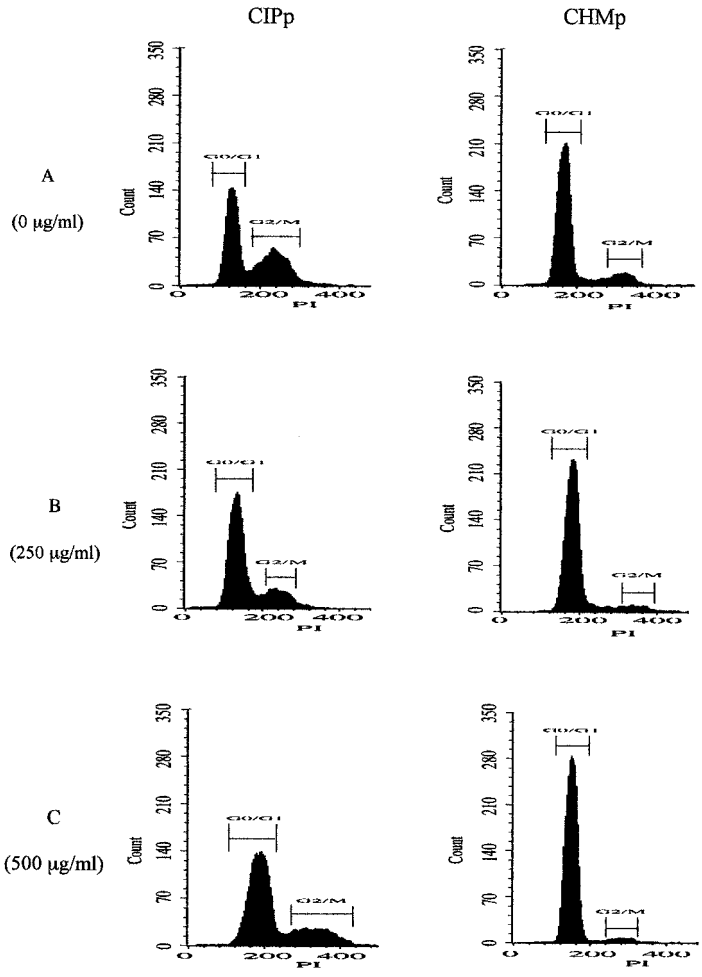

Fig. 3. Cell cycle analysis by flow cytometry showing the effect of culture with and without (A) bLF on the canine mammary tumor cell lines (CIPp and CHMp). Bovine LF induced G1 arrest in both cell lines; the G1 peak increased while the G2/M peak decreased in both cell lines (B and C).

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