Bovine Lactoferrin Stimulates Anchorage-Independent Cell Growth
via Membrane-Associated Chondroitin Sulfate and Heparan Sulfate
Proteoglycans in PC12 Cells

Toshiaki Ishii1,*, Hiroshi Ishimori1, Kaori Mori1, Tomoka Uto1, Kenji Fukuda2, Tadasu Urashima2, and Masakazu Nishimura1

1Department of Pathobiological Science, and 2Graduate School of Food Hygiene, Obihiro University of Agriculture
and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

Received April 27, 2007; Accepted June 21, 2007

Abstract. Bovine lactoferrin (bLf) is an iron-binding secretory protein present in breast milk, mucosal secretions, and the secondary granules of neutrophils. Although bLf has multiple functions, including antimicrobial and immunomodulatory activities, its effect on neuronal cells is not fully understood. We report that bLf prevents cell adhesion of PC12 cells and allows them to be cultivated in suspension. PC12 cells normally adhere well to plastic culture plates and show anchorage-dependent cell growth, but we found that soon after adding bLf, they detach from culture plates and begin to grow in suspension. When bLf was removed from the medium, the cells began to re-adhere to the plates. Thus, bLf inhibits cell adhesion and stimulates anchorage-independent growth in PC12 cells. On the other hand, bLf-induced cell suspension growth was not observed when cells were grown on a laminin matrix, suggesting that bLf does not affect integrin-mediated cell adhesion on a laminin matrix. Treatment of cells with heparin or chondroitin sulfate A or C inhibited bLf-induced growth in cell suspension. Furthermore, pretreatment of cells with heparinase and/or chondroitinase prevented direct binding of bLf to the cell membrane. These results suggest that bLf binds to the membrane of PC12 cells via membrane-associated proteoglycans and leads to anchorage-independent growth.

Keywords: bovine lactoferrin, cell adhesion, PC12 cell, proteoglycan, cell suspension

Introduction

Lactoferrin is an iron-binding glycoprotein present in many different exocrine fluids, including milk, tears, saliva, and bile (1), and is composed of a single 80-kDa polypeptide chain that is folded into two homologous lobes (2). Lactoferrin is also a major constituent of the secondary granules of neutrophilic leukocytes, from which it is released during acute inflammation (3), and it is a potent inhibitor of several enveloped viruses and a wide variety of bacteria, fungi, and parasites (4–6). Thus, lactoferrin is thought to play a role in innate host defense (7). Moreover, pepsin-digested lactoferrin induces apoptotic cell death in oral cancer cells (8). The finding indicates that proteolysis of lactoferrin produces peptides with antitumor activity. In 2004, Cornish et al. (9) demonstrated that lactoferrin potently stimulates the proliferation and differentiation of osteoblasts but inhibits osteoclastogenesis, resulting in the promotion of bone growth. Such a lactoferrin-induced increase in bone formation has also been detected in vivo (9, 10). These findings indicate that lactoferrin acts not only as an antimicrobial agent but also as a growth factor, but the physiological and biological role of lactoferrin in neuronal cells has not been determined. In the present study, we investigated the effect of bovine lactoferrin (bLf) on cell adhesion and growth of neuronal cells.

The PC12 cell line, which is derived from rat phaeochromocytoma cells, is one of the most widely used for investigating the mechanisms of neuronal differentiation, cell adhesion, neuroprotection, and neurodegenerative disease (11–14). We therefore examined the effect...
of bLf on the cell adhesion of PC12 cells, and we characterized the ability of the PC12 membrane to bind bLf. We found that bLf stimulates anchorage-independent growth of PC12 cells via membrane-associated chondroitin sulfate and heparan sulfate proteoglycans.

Materials and Methods

Reagents
PC12 cells were obtained from the Riken Cell Bank (Tsukuba). The Cell Proliferation kit 1 (MTT) and Biotin Labeling Kit were obtained from Roche Diagnostics (Penzberg, Germany). Dulbecco’s Modified Eagle Medium (DMEM) was obtained from GIBCO BRL (Rockville, MD, USA). Sodium chloride and bLf were obtained from Wako Pure Chemical Co. (Osaka). Heparin (HP), heparitin sulfate (HPS), chondroitin sulfate A (CS-A), chondroitin sulfate C (CS-C), heparinase III, and chondroitinase ABC were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade and were obtained from Sigma Chemical Co. or Wako Pure Chemical Co.

Cell culture
PC12 cells were maintained in DMEM containing 5% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 5% horse serum (ICN, Costa Mesa, CA, USA), penicillin G sodium (1 × 10^5 U/L) and streptomycin sulfate (1 × 10^5 μg/L) in 5% CO₂ at 37°C.

Cell proliferation and viability assay
PC12 cells were seeded on 96-well plates and 35-mm culture dishes at a density of 7 × 10^3 cells/well and 7 × 10^4 cells/dish, respectively, and cultivated for 24 h. Cells were then treated with varying concentrations of bLF for 24 h by direct addition to the culture medium. The culture medium containing suspended cells was transferred to new wells or plates, and new culture medium was added. The sum of both optical density (O.D.) (570 nm) values was used as a measure of the total number of cells.

bLF binding assay
The Biotin Labeling Kit was used to label bLf with biotin according to the manufacturer’s instructions. PC12 cells were seeded on 35-mm culture dishes at a density of 7 × 10^4 cells/dish and cultivated for 24 h. Cells were then treated with heparitin III (4 unit/ml) or chondroitinase ABC (4 unit/ml), the treatment of which was determined by considering product information from the manufacturer, in DMEM containing 3% FBS for 30 or 60 min. After washing twice with phosphate-buffered saline, fresh culture medium containing biotin-labeled bLf (7 μM) was added. Cells were cultivated for an additional 6 h and then collected. In experiments examining the effect of heparin sulfate (HS) and chondroitin sulfate (CS) on bLf binding, binding of biotin-labeled bLf to the cell membrane was measured by cultivating PC12 cells for 6 h in medium containing HS or CS and in the presence of biotin-labeled bLf (7 μM). After washing twice with phosphate-buffered saline, the cells were solubilized in 100 μl sample buffer containing 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue, and 62.5 mM Tris-HCl (pH 6.8). The solubilized materials were boiled for 3 min, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5% – 20% gradient polyacrylamide), and electrophoretically transferred to nitrocellulose membranes. After blocking, the blots were probed with streptavidin-conjugated horseradish peroxidase (Vector ABC kit; Vector, Burlingame, CA, USA), and the bound peroxidase was detected using chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

Statistical methods
Data were analyzed by one-way analysis of variance (ANOVA) after a Bartlett test. Either the Tukey-Kramer or Dunnett test was used as a post hoc test. To compare the number of adherent or suspended cells between the cells cultured in the presence and the absence of bLf after removal of bLf, data were analyzed by Welch’s t-test after the F-test. A P value of less than 0.05 was considered to indicate statistical significance.

Results

bLf Inhibits cell adhesion and stimulates anchorage-independent growth in PC12 cells
PC12 cells adhere well to plastic culture plates and show anchorage-dependent cell growth (Fig. 1Aa). To examine the effect of bLf on cell adhesion to plastic plates, we treated the PC12 cells with varying concentrations of bLf for 24 h. We found that bLf dose-dependently inhibited cell adhesion and caused the cells
to be released into suspension (Fig. 1B). The detachment of cells was observed immediately after the addition of bLf, and the cells in suspension began to form clusters (Fig. 1A: b, c, and d). Moreover, the total cell number significantly increased following treatment with 300, 450, and 600 µM bLf (Fig. 1B). Thus, higher concentrations of bLf were required for bLf-induced stimulation of cell growth in comparison with bLf-induced inhibition of cell adhesion. These results indicate that bLf inhibits cell adhesion and stimulates anchorage-independent growth by PC12 cells. On the other hand, when bLf was removed from the medium, the cells began to re-adhere to the plates (Fig. 2: A and B). Thus, bLf-induced inhibition of cell adhesion is a reversible effect, and the cells suspended by bLf are able to re-adhere to plastic plates after removal of bLf from the culture medium.

**Laminin but not fibronectin prevents bLf-induced detachment but enhances bLf-induced cell growth**

Cell interactions with extracellular matrix (ECM) proteins such as laminin and fibronectin are mediated primarily by integrins, transmembrane proteins that are important regulators of cell proliferation and differentiation (15). PC12 cells adhere readily to laminin but poorly to fibronectin (16). We therefore examined whether bLf can inhibit cell adhesion to laminin and/or fibronectin. PC12 cells grown on laminin or fibronectin were treated with 100 µM bLf for 24 h. As shown in Fig. 2C, bLf significantly inhibited cell adhesion to fibronectin but not laminin.

We further examined the effect of varying concentrations of bLf on cell adhesion and growth of PC12 cells grown on laminin. We found that bLf does not prevent cell adhesion to laminin but significantly increased the total number of cells in a dose-dependent manner (Fig. 3). Furthermore, bLf-stimulated cell growth on laminin was observed at lower concentrations than in cells grown on control plates (Figs. 1B and 3), although at concentrations above 450 µM, the effect of bLf gradually decreased (Fig. 3). Thus, laminin prevents bLf-induced cell detachment but enhances bLf-stimu-
lated cell growth. On the other hand, higher concentrations of bLf were required for bLf-induced stimulation of cell growth on plastic plates in comparison with laminin-coated plates. This may arise from the different conditions of the cellular states, that is, the detached or adherent state.

Effect of HS and CS on bLf-induced detachment of PC12 cells
Glycosaminoglycan (GAG) chains are ubiquitous components of cell surfaces. Most GAG chains are covalently attached to core proteins of proteoglycans, and they are involved in cell adhesion and growth (11, 17 – 19). To determine whether GAG is involved in bLf-induced cell detachment and growth, we treated PC12 cells with varying concentrations of HP, HPS, CS-A, or CS-C in the presence of 100 µM bLf. HPS showed the cytotoxic effect on PC12 cells, but HP, CS-A, and CS-C did not affect either cell growth or cell viability (Fig. 4A). We, therefore, could not examine the effect of HPS on bLf-induced cell detachment because of its cytotoxicity. As shown in Fig. 4B, we found that HP, CS-A, and CS-C dose-dependently inhibit bLf-induced cell detachment and increase the number of adherent cells. HP was the most potent of these three GAGs at inhibiting detachment.

Binding of bLF to the cell membrane in the presence or absence of HP or CS and the effect of heparinase III and chondroitinase ABC
To determine whether there is a direct interaction between membrane-bound GSG and bLf, we examined the binding of bLf to the membrane of PC12 cells before and after treatment with heparinase III or chondroitinase ABC. Binding of bLf to the cell membrane was assessed by cultivating PC12 cells for varying time periods in medium containing biotin-labeled bLf (7 µM) (Fig. 5A). It seems that the binding of biotin-labeled bLf to the
cell membrane reaches saturation within 30 min after addition of biotin-labeled bLf. As shown in Fig. 5B, when PC12 cells were cultivated for 6 h in medium containing biotin-labeled bLf (7 \( \mu \)M), specific binding of biotin-labeled bLf to the cell membrane was significantly blocked in the presence of high concentrations (100 and 500 \( \mu \)M) of unlabeled bLf. On the other hand, the specific binding of biotin-labeled bLf was not affected in the presence of 100 \( \mu \)M bovine serum albumin (BSA) but was weakly inhibited in the presence of 500 \( \mu \)M BSA. Because BSA is negatively charged, the weak inhibitory effect of 500 \( \mu \)M BSA may be caused by its negative charge. The binding of biotin-labeled bLf to the cell membrane was significantly reduced in the presence of 100 \( \mu \)M bovine serum albumin (BSA) but was weakly inhibited in the presence of 500 \( \mu \)M BSA. Because BSA is negatively charged, the weak inhibitory effect of 500 \( \mu \)M BSA may be caused by its negative charge. The binding of biotin-labeled bLf to the cell membrane was significantly reduced in the presence of 100 \( \mu \)M bovine serum albumin (BSA) but was weakly inhibited in the presence of 500 \( \mu \)M BSA. Because BSA is negatively charged, the weak inhibitory effect of 500 \( \mu \)M BSA may be caused by its negative charge. The binding of biotin-labeled bLf to the cell membrane was significantly reduced in the presence of 100 \( \mu \)M bovine serum albumin (BSA) but was weakly inhibited in the presence of 500 \( \mu \)M BSA. Because BSA is negatively charged, the weak inhibitory effect of 500 \( \mu \)M BSA may be caused by its negative charge. The binding of biotin-labeled bLf to the cell membrane was significantly reduced in the presence of 100 \( \mu \)M bovine serum albumin (BSA) but was weakly inhibited in the presence of 500 \( \mu \)M BSA. Because BSA is negatively charged, the weak inhibitory effect of 500 \( \mu \)M BSA may be caused by its negative charge. The binding of biotin-labeled bLf to the cell membrane was significantly reduced in the presence of 100 \( \mu \)M bovine serum albumin (BSA) but was weakly inhibited in the presence of 500 \( \mu \)M BSA. Because BSA is negatively charged, the weak inhibitory effect of 500 \( \mu \)M BSA may be caused by its negative charge. The binding of biotin-labeled bLf to the cell membrane was significantly reduced in the presence of 100 \( \mu \)M bovine serum albumin (BSA) but was weakly inhibited in the presence of 500 \( \mu \)M BSA. Because BSA is negatively charged, the weak inhibitory effect of 500 \( \mu \)M BSA may be caused by its negative charge. The binding of biotin-labeled bLf to the cell membrane was significantly reduced in the presence of 100 \( \mu \)M bovine serum albumin (BSA) but was weakly inhibited in the presence of 500 \( \mu \)M BSA. Because BSA is negatively charged, the weak inhibitory effect of 500 \( \mu \)M BSA may be caused by its negative charge.

Discussion

The results of the present study demonstrate that bLf stimulates anchorage-independent growth via membrane-associated CS and HS proteoglycans in PC12 cells. Furthermore, integrin-dependent cell adhesion on a laminin matrix prevents bLf-induced detachment but not bLf-stimulated cell growth.

PC12 cells adhere well to plastic culture plates and show anchorage-dependent cell growth. When bLf was added into the culture medium, the cells immediately detached and began to form clusters in suspension. Also, treatment of PC12 cells with bLf for 24 h stimulated the cell growth. Higher concentrations of bLf, however, were required for bLf-induced stimulation of cell growth in comparison with bLf-induced inhibition of cell adhesion. In contrast to the cell growth on plastic plates, bLf-induced stimulation of cell growth on laminin was observed at lower concentrations of bLf, which were similar to the concentrations at which bLf-induced inhibition of cell adhesion was observed. Therefore, some cellular condition after detachment from the plate may affect the effect of bLf on cell growth and result in the discrepancy in the effective concentrations of bLf between inhibition of cell adhesion and stimulation of cell growth.

Thus, bLf blocked cell adhesion and stimulated anchorage-independent growth in PC12 cells. Moreover, when bLf was removed from the medium, the cells began to re-adhere to the plates. BLf-induced inhibition of cell adhesion is, therefore, a reversible effect. These results suggest that bLf directly binds to the cell membrane and activates intracellular signaling permitting anchorage-independent growth in PC12 cells. In 2005, Lin et al. (20) reported that human lactoferrin reduces the cell viability of PC12 cells at higher concentrations (400 – 700 \( \mu \)g/ml) and also that this unfavorable effect is more obvious for longer treatment. In the present study, however, bLf did not affect cell viability after exposure to bLf (1 – 600 \( \mu \)M) for 24 h. Although this discrepancy in the cell viability between human lactoferrin and bLf may be caused by different properties of lactoferrin derived from different species or by different experimental conditions, for example, serum concentrations, exposure time of bLf, different CO\(_2\) concentrations, and so on, the reason remains unclear.

Cell interactions with ECM proteins are mediated primarily by integrins that act as cell-surface receptors and are heterodimers of \( \alpha \)- and \( \beta \)-subunits. Integrin-ECM interactions are important in the regulation of cell proliferation and differentiation (15). The intracellular pathways activated in various cell types can differ according to the specific integrin-ECM interaction and/or the specific integrin-linked intracellular signaling proteins. In PC12 cells, nerve growth factor-induced neurite outgrowth is strongly affected by cell adhesion...
bf Prevents Cell Adhesion of PC12 Cells 371

and PC12 cells adhere readily to laminin but poorly to fibronectin (16). Both α1β1 and α3β1 integrins mediate PC12 cell adhesion to laminin (23). On the other hand, some of the αβ-heterodimeric integrins such as αvβ3 bind to RGD-containing ECM proteins, fibronectin, and vitronectin, in non-neuronal cells (24). To examine whether bf can block integrin-mediated cell adhesion, we grew PC12 cells on laminin or fibronectin and treated them with 100 µM bf for 24 h. We found that bf significantly inhibited cell adhesion to fibronectin but not laminin. Thus, laminin prevents bf-induced cell detachment. On the other hand, bf-stimulated cell growth was enhanced by laminin. These results suggest that bf blocks integrin-independent cell adhesion but cannot block integrin-ECM binding specific for the cells and that bf-stimulated cell growth is not directly related to bf-induced cell detachment. In 2006, Sakanoto et al. (25) demonstrated that human lactoferrin inhibits cell adhesion to RGD-containing ECM proteins such as fibronectin and vitronectin via interaction with their RGD motif but not to RGD-independent ECM proteins such as laminin and

Fig. 4. Effect of HPS, HP, CS-A, and CS-C on cell viability (A) and on bf-induced cell detachment (B) in PC12 cells. A: Cells were treated with varying concentrations of HPS, HS, CS-A, or CS-C in the presence of 100 µM bf for 24 h. The number of total cells was estimated using an MTT assay. Results represent means ± S.D. of O.D. values from five independent cultures. *P<0.05 vs bf-treated control cells. B: The number of suspended cells and adherent cells was estimated using an MTT assay. Results represent means ± S.D. of O.D. values from five independent cultures. *P<0.05 vs untreated suspended cells; ‡P<0.05 vs untreated adherent cells; *P<0.05 and ‡P<0.05 vs suspended cells and adherent cells, respectively, in bf-treated control cells.
collagen. Therefore, bLf similar to human lactoferrin might block cell adhesion to fibronectin via interaction with its RGD motif.

Recently, Marchetti et al. (6) reported that bLf inhibition of virus infection depends on interference with the binding of virus to GAG chains in cell surface HS and CS. Both CS and HS proteoglycans are present in PC12 cells (11) and modulate the growth and differentiation of PC12 cells (17). We therefore examined whether GAG chains of HS and CS participate in bLf-induced anchorage-independent cell growth. Unexpectedly, HPS but not HP showed cytotoxicity on PC12 cells. Because HPS contains more acetyl groups than HP, the cytotoxic effect of HPS might be caused by an abundance of acetyl groups in its chemical structure. When the cells were treated with varying concentrations of HP, CS-A, or CS-C in the presence of 100 µM bLf, they strongly inhibited bLf-induced cell detachment in a dose-dependent manner, suggesting that these GAGs antagonize the binding between bLf and the cell membrane by directly binding to free bLf. These results indicate that the GAG chains of HS and CS in the cell membrane might be involved in the bLf-induced anchorage-independent cell growth.

To examine whether bLf directly interacts with membrane-associated GAG, we further analyzed the binding of bLf to the cell membrane using biotin-labeled bLfs. We found that HP, CS-A, and CS-C significantly reduced the binding of biotin-labeled bLf to the cell membrane without regard to incubation periods, shorter or longer. Furthermore, the binding of biotin-labeled bLf to the cell membrane was significantly reduced by treating the cells with heparinase III or chondroitinase ABC. These results suggest that bLf stimulates anchorage-independent growth by directly binding to membrane-associated CS and HS proteoglycans in PC12 cells. The internalization of human lactoferrin into the human monocytic cell line THP-1 was previously reported (26). Our bLf-binding assay, however, can not distinguish between the direct binding of bLf to cell membrane and the internalization of bLf into cell. Therefore, further studies are needed to elucidate whether bLf incorporates into the intracellular fraction of PC12 cells via endocytosis.

CS and HS accounts for 70% – 80% and 20% – 30%, respectively, of the [35S]sulfate-labeled proteoglycans present in PC12 cells (11). Although this indicates that CS accounts for a greater proportion of GSGs than HS, we found that heparinase III was a more potent inhibitor of bLf binding to PC12 cells than chondroitinase ABC (Fig. 5C). Moreover, HP was more potent at inhibiting bLf-induced cell detachment than CS-A and CS-C (Fig. 4B). Thus, HS seems to be much more important than CS in binding bLf, even though there is less HS than CS present in the surface proteoglycans of PC12 cells. The binding affinity between HS and bLf, therefore, might be higher than that between CS and bLf.

In conclusion, the results presented in this study demonstrate that the naturally occurring glycoprotein bLf inhibits integrin-independent cell adhesion and stimulates anchorage-independent growth via membrane-associated CS and HS proteoglycans in PC12 cells. BLf
may therefore have a physiological role in neuronal growth. In addition, it might have utility as not only a reagent substitute for trypsin to suspend neuronal cells but also a feasible medicine for neuronal regeneration. Further studies are needed to elucidate the intracellular signaling pathways involved in bLf-induced anchorage-independent cell growth.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (B) (to T.I.) from the Japan Society for the Promotion of Science and by a Grant-in-aid (Research and Development Program for New Bio-industry Initiatives) from the Bio-oriented Technology Research Advancement Institution of the National Agriculture and Food Research Organization of Japan (to T.I.).

References

6 Marchetti M, Trybala E, Superti F, Johansson M, Bergstrom T. Inhibition of herpes simplex virus infection by lactoferrin is dependent on interference with the virus binding to glycosaminoglycans. Virology. 2004;318:405–413.