INTRODUCTION

Lactoferrin (LF) is an iron-binding glycoprotein that is involved in the host innate immune defense and that is found in biological fluids such as saliva, tears, blood, and milk. LF possesses many biological functions, including anti-inflammatory, anti-oxidative, anti-viral, anti-microbial, anti-tumor, and immunomodulatory effects. Even though LF has a molecular mass of 80 kDa, it is still absorbed intact through the small intestine and transported to the blood circulation via the lymphatic pathway. Due to its absorption and efficacy profile, this protein is an attractive oral drug candidate.

The Caco-2 cell line is derived from human colon adenocarcinoma cells and is the most commonly used model for human enterocytes. Previous studies have reported that LF can be taken up into Caco-2 cells via a clathrin-mediated endocytic process mediated by the human intestinal lactoferrin receptor (also called human intelectin-1). Moreover, bovine LF (bLF) has been shown to be taken up into 10 d-cultured Caco-2 cells, after which degraded bLF is released back into the culture medium. Another study used spontaneously differentiated cell monolayers of 21 d-cultured Caco-2 as an in vitro model of the intestinal barrier and showed, using a permeability assay, that LF transported across Caco-2 monolayers was more likely to be degraded, which was inconsistent with in vivo events. In this study, to confirm the earlier published results, we have reevaluated the cellular uptake and release of LF using an enterocyte model of human small intestinal cells derived from the Caco-2 cell line. In contrast to a previous report, we observed that intact bovine LF was taken up into seven and 21 d-cultured Caco-2 cells and successfully released back into the culture medium, even though the human intestinal LF receptor, intelectin-1, was not immunodetectable. Similar observations were made for human LF and its derivatives (the N-terminal half of LF designated N-lobe and Fc fusions). These observations regarding the uptake and release of intact LF in Caco-2 cells were consistent with in vivo observations. Therefore, we propose that the uptake and release of intact LF by Caco-2 cells should be assessed as a potential in vitro model of in vivo LF absorption in human intestines.

Key words lactoferrin; Caco-2 cell; cellular uptake; release
A PCR fragment cleaved by EcoRI and NotI enzymes was inserted into the corresponding sites of the expression vector pOptiVecMCS. The resulting plasmid was named pOptiVec/N-lobe. dhfr-CHO DG44 cells were then transfected with pOptiVec/N-lobe and pools of stably transfected cells were selected by a stepwise increase of methotrexate (MTX) at concentrations of 0, 50, 100 and 250 nM in dihydrofolate reductase (DFHR)-selective medium (MEM-α without nucleosides and deoxynucleosides). A single stable transfectant expressing high levels of N-lobe was isolated from a pool of transfectants using a limited dilution technique in MEM-α with 250 nM MTX. This clone was then selected in MEM-α containing up to 4 µg M MTX (250, 500 nM, 1, 2, and 4 µg M). CHO DG44 cells producing N-lobe or hLF-Fcs (conventional hLF-hinge-CH2-CH3) and hinge-deficient hLF-CH2-CH3 were grown in Hybridoma SFM (Invitrogen). Condition media collected from the transfectants were directly subjected to purification via cation exchange chromatography using Macropac SP (GE Healthcare Bio-Sciences KK, Tokyo, Japan), as described previously.

### Uptake and Release of LF by Caco-2 Cells
Caco-2 cells (5 × 10^4 cells) were seeded in a 12-well culture plate and grown in 5% CO₂ at 37°C for 7 or 21 d, as indicated. After washing the cells with phosphate buffered saline (PBS containing 137 mM NaCl, 2.7 mM KCl, 9.6 mM Na₂HPO₄ and 1.5 mM KH₂PO₄) three times, 0.38 µM of various LFs in PBS were added and incubated for 1 h at 4°C or 37°C for their uptake. Cells incubated with PBS only were used as a negative control. All cells were washed with PBS twice and then exposed to trypsin–ethylenediaminetetraacetic acid (EDTA) treatment for 3 min to detach them from the plate and to remove any LF attached to the cell surface. Detached cells were collected into a new tube and washed with 1 mL PBS three times. The presence of LFs in the third wash fraction (12 µL) was determined by immunoblotting. The uptake or release of LF by Caco-2 cells was evaluated as follows:

For the analysis of LF uptake, 100 µL cell lysis buffer (PBS containing 1% Triton X-100 and a mixture of protease inhibitors [complete™, Roche Diagnostics K.K., Tokyo, Japan]) was added to the cells, which were then incubated at 4°C for 1 h. The cell suspension was centrifuged at 21500×g for 15 min at 4°C. The protein concentration was measured using a Bradford assay. All cells were washed with PBS twice and then exposed to trypsin–ethylenediaminetetraacetic acid (EDTA) treatment for 3 min to detach them from the plate and to remove any LF attached to the cell surface. Detached cells were collected into a new tube and washed with 1 mL PBS three times. The presence of LFs in the third wash fraction (12 µL) was determined by immunoblotting. The uptake or release of LF by Caco-2 cells was evaluated as follows:

For the analysis of released LFs taken up into Caco-2 cells, 100 µL DMEM (high glucose) was added to the cells, which were then incubated at 37°C for the indicated time. An aliquot (12 µL) of the medium was then used for the immunoblotting analysis. To examine the effect of heparin on the uptake of hLF into Caco-2 cells, heparin with the same concentration as hITLN-1 was added to the cell suspension. After centrifugation at 21500×g for 15 min at 4°C, the protein concentration was measured using a Bradford assay. All cells were washed with PBS twice and then exposed to trypsin–ethylenediaminetetraacetic acid (EDTA) treatment for 3 min to detach them from the plate and to remove any LF attached to the cell surface. Detached cells were collected into a new tube and washed with 1 mL PBS three times. The presence of LFs in the third wash fraction (12 µL) was determined by immunoblotting. The uptake or release of LF by Caco-2 cells was evaluated as follows:

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**Immunoblotting** A 10% polyacrylamide gel was used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The separated proteins were then electrotransferred onto a nitrocellulose membrane (Amersham Protran supported 0.45 µm NC [GE Healthcare]) that was blocked for 1 h at 25°C in blocking buffer. Thereafter, each primary antibody at the indicated concentration was incubated at 4°C overnight, except for horseradish peroxidase (HRP)-conjugated anti-human immunoglobulin G (IgG) (H + L) (1 : 10000, Promega Corporation, Madison, WI, U.S.A.), which was incubated for 1 h at 25°C. After washing with tris-buffered saline containing 0.05% Tween 20 (TBS-T) three times, the bound antibody was detected using an HRP-conjugated second antibody by incubating for 1 h at 25°C, if necessary. The membrane was then washed with TBS-T three times. Bound antibody was detected using a sensitive luminescent reagent (Immunostar Zeta, FUJIFILM Wako Pure Chemical Corporation). Band intensities were quantified by ATTO CS analyzer 3 (ATTO, Tokyo, Japan). Conditions for immunoblotting with each antibody are summarized below.

Human LF receptor (hITLN-1): Caco-2 or CHO DG44 transfectants expressing human LF receptor were lyzed in cell lysis buffer, as described above. Protein concentration was determined using the Bradford assay. Fifty micrograms of Caco-2 lysate or 10 µg of CHO DG44 transfectant lysate was run on an SDS-PAGE gel under non-reducing or reducing conditions. Samples were not boiled prior to electrophoresis. TBS-T containing 2% skim milk was used as a blocking buffer. Anti-human omentin 1 antibody (Omentin 1 [human]), mAb [Saly-1]: 1 : 3000, Enzo life sciences, Farmingdale, NY, U.S.A.) and HRP-conjugated anti-mouse IgG (H + L) (1 : 10000, Promega Corporation, Madison, WI, U.S.A.) were used as primary and secondary antibodies, respectively.

blF: Samples were not boiled prior to electrophoresis and were run in a 10% gel under non-reducing conditions. TBS-T containing 2% skim milk was used as the blocking buffer. HR conjugated Goats anti-bovine LF antibody (1 : 5000, E10-126P, Bethyl laboratories Inc., Montgomery, TX, U.S.A.) was used.

hLF: Samples were not boiled prior to electrophoresis and were run in a 10% gel under non-reducing conditions. TBS-T containing 1% bovine serum albumin (BSA) was used as the blocking buffer. Human LF antibody (1 : 10000, A80-144 A, Bethyl Laboratories, Inc.) and HRP-conjugated anti-rabbit IgG (H + L) (1 : 10000, Promega Corporation) were used as primary and secondary antibodies, respectively.

N-lobe: Samples were boiled prior to electrophoresis and were run in a 10% gel under reducing conditions. Reactions of primary and secondary antibodies were conducted under the
same conditions as described for hLF.

Human IgG Fc: Samples were not boiled prior to electrophoresis and were run in a 10% gel under non-reducing conditions. TBS-T containing 2% skim milk was used as the blocking buffer. HRP-conjugated Anti-Human IgG (H+L) (1:10000, Promega Corporation) was added prior to incubation for 1h at 25°C.

Human γ1 actin: The amount of γ1 actin in cell lysates was used to show the equivalence of protein loading. After immunoblotting, the antibodies on blot membranes were removed using stripping buffer (FUJIFILM Wako Pure Chemical Corporation) followed by incubation for 10min at 25°C. Membranes were washed with TBS-T three times and blocked with TBS-T containing 1% BSA. Human anti-γ1-actin antibody (1:5000, FUJIFILM Wako Pure Chemical Corporation) and HRP-conjugated anti-mouse IgG (H+L) (1:10000, Promega Corporation) were used as primary and secondary antibodies, respectively.

RESULTS AND DISCUSSION

Loss of Human LF Receptor Expression in 7 and 21d-Cultured Caco-2 Cells

First, we attempted to confirm the expression of human intestinal LF receptor (referred to as hITLN-1) in Caco-2 cells cultured for 7d (D7) and 21d (D21) via Western blotting, since previous reports have shown that 16d-cultured cells express hITLN-1 and take up LF via

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**Fig. 2. Evaluation of LF Uptake and Release by Caco-2 Cells**

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**Fig. 3. Intact Bovine Lactoferrin (bLF) Taken Up into Caco-2 Cells Was Later Successfully Released Back into the Culture Medium**

(A) bLF as an intact molecule was taken up from PBS by Caco-2 cells cultured for both 7 and 21d, and was subsequently released into the culture medium. (B) Intact bLF was also taken up from the medium (DMEM) by D7 cells with an incubation time of 1 and 3h, and was later successfully released into the culture supernatant during the release period for 120min. (C) The temperature-dependent uptake of bLF into Caco-2 cells. Cells incubated with PBS-only at 37°C did not show any signal (—, left). (D) Release of intact bLF into the culture medium after being taken up by Caco-2 cells. Cells incubated with PBS-only for 120min (--) did not show any signal in the release step. No bLF was detected in the wash fraction. (B)–(D) Control bLF (10ng) was detected via immunoblotting (Ctrl, left panel). Relative band intensities are represented graphically in A, C and D (right).
hITLN-1-mediated endocytosis. As a positive control, CHO DG44 transfectants expressing hITLN-1 were analyzed, and showed a monomeric hITLN-1 (molecular mass of 40 kDa) under reducing conditions and a trimeric hITLN-1 (molecular mass of 120 kDa) under non-reducing conditions (Fig. 1). Unfortunately, hITLN-1 could not be immunochemically detected either in D7 or D21 Caco-2 cells (Fig. 1). This phenomenon was verified using cells obtained from a different source (Riken BRC, data not shown). The discrepant results seen here might be ascribed to cell culture conditions which affect Caco-2 cells differentiation.

After bLF Endocytosis, D7 and D21 Caco-2 Cells Released Intact bLF into the Culture Medium. It has been reported that 1.25 µM bLF in the medium (DMEM) was taken up by Caco-2 cells cultured on a flat-bottom plate for 10 d, after which degraded bLF was released back into the culture medium. We next examined the uptake of bLF (suspended in PBS, pH 7.4) by Caco-2 cells, according to the protocol shown in Fig. 2. Unlike the previously reported results, bLF as an intact molecule was taken up from PBS by both D7 and D21 cells and subsequently released from these cells into the culture medium (Fig. 3A), even though the hITLN-1 receptor was not immunochemically detectable. Intact bLF was also taken up from the medium (DMEM) by D7 cells with an incubation time of 1 and 3 h, and was successfully released into the culture supernatant (Fig. 3B). For further analysis, we selected PBS for the cellular uptake of LF because similar results were obtained with both PBS and the medium (DMEM).

Temperature dependence of bLF uptake into D7 cells was observed (Fig. 3C). After its endocytosis into the D7 cells, bLF was released into the culture medium as an intact molecule, as was seen from the fact that neither the wash fraction nor the culture medium of D7 cells itself contained bLF (Fig. 3D). This phenomenon was also verified with cells provided...
by Riken BRC (data not shown). Thus, D7 Caco-2 cells were used for further experiments due to the facile preparation of the cells.

**Intact hLF and Its N-Lobe Were Taken up into Caco-2 Cells and Then Released into the Culture Medium**

Like bLF, intact hLF was also taken up into Caco-2 cells in a temperature-dependent fashion (Fig. 4A). Further, after its internalization, hLF was released intact into the culture medium (Fig. 4B). We have already reported that fluorescent-labeled hLF is taken up into Caco-2 cells in an ATP-dependent manner due to the inhibitory effects on its uptake by an ATP-synthesis inhibitor, sodium azide.\(^{10,11}\) It is well known that ATP is required for receptor-mediated endocytosis.\(^{12}\) Thus, a putative receptor or receptor-like molecule other than hITLN-1 might be involved in hLF internalization into Caco-2 cells.

Next, the uptake and release of the N-terminal half of hLF (N-lobe) by Caco-2 cells was examined, since N-lobe has been reported as the structural unit responsible for the internalization of hLF into Caco-2 cells.\(^{13}\) The results showed that hLF N-lobe (molecular mass of 40 kDa) taken up by Caco-2 cells was released intact back to the culture medium (Figs. 5A, B).

**hLF-Fc Fusion Endocytosis and Subsequent Release from Caco-2 Cells**

Recently, we developed two long-acting hLF-Fc fusions, a conventional hLF-hinge-CH2-CH3\(^{10}\) and a hinge-deficient hLF-CH2-CH3.\(^{11}\) Here, we examined their endocytosis and subsequent release from Caco-2 cells for their potential use as oral drugs. Intact hLF-hinge-CH2-CH3 was taken up into Caco-2 cells and successfully released into the culture supernatant (Figs. 6A, B). Although some degraded hLF-CH2-CH3 was found in the release fraction, intact hLF-CH2-CH3 internalized into Caco-2 cells was also found to be released into the culture medium (Figs. 7A, B). Degraded fragments with molecular masses of approx. 80 kDa (similar to hLF) were detectable with anti-hLF antibodies but not with anti-human IgG antibodies. It is possible that the spacer sequence flanked by hLF and CH2-CH3 may have been partially degraded (Fig. 7B). The uptake of conventional and hinge-deficient hLF-Fc fusions by Caco-2 cells in PBS (pH 7.4) was mainly mediated by hLF due to relatively poor human IgG uptake into Caco-2 cells at pH 7.4.\(^{10}\) Further, the uptake of fluorescent-labeled hLF-Fc fusions into Caco-2 cells was completely abolished by the addition of excess amounts of unlabeled bLF,\(^{10,11}\) which also indicated that hLF was mainly involved in the endocytosis of hLF-Fc fusions.
Interaction between LF and Heparin Blocks the Uptake of hLF

It has been reported that LF interacts with heparan sulfate present on the surface of most cells, since heparinase treatment\(^{15}\) or the addition of heparin has led to the attenuated binding of LF to some cells.\(^{16}\) Here, we examined the effects of heparin on the uptake of hLF into Caco-2 cells. As reported previously,\(^{17}\) the addition of heparin at an equimolar concentration of hLF prevented the uptake of hLF into Caco-2 cells (Fig. 8), suggesting that the heparan sulfate-recognizing sequence on hLF is required for its internalization, or alternatively, that heparan sulfate proteoglycans on Caco-2 cells might act as endocytosis receptors.\(^{18}\)

Taken together, unlike the previous report,\(^{6}\) our study shows that intact LF and its derivatives taken up by Caco-2 cells were released extracellularly, consistent with in vivo observations.\(^{3}\) So far, the possible causes for the discrepant results are not certain. Due to the heterogeneous features of the Caco-2 cell line,\(^{19}\) different culture-related conditions or sources of cell clones might yield inconsistent results. However, Caco-2 cells from the two different sources (DS Pharma Biomedical and Riken BRC) both exhibited reproducible results regarding the uptake and release of bLF and hLF.

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Fig. 7. Intact hLF-CH2-CH3 Taken up by Caco-2 Cells Was Then Released into the Culture Medium with Slight Degradation

(A) The temperature-dependent uptake of hLF-CH2-CH3 into Caco-2 cells. Cells incubated with hLF-CH2-CH3 showed uptake of intact hLF-CH2-CH3 in a temperature-dependent manner (immunoblotting with polyclonal anti-LF antibody [left] and anti-human IgG (H + L) antibody [right]). Cells incubated with PBS-only at 37°C did not show any signal (—, left). (B) hLF-CH2-CH3 taken up into Caco-2 cells was released into the culture medium along with its degradate (immunoblotting with polyclonal anti-LF antibody [left] and anti-human IgG (H + L) antibody [right]). Cells incubated with PBS-only for 120 min (—) did not show any signal in the release step. No hLF-CH2-CH3 was detected in the wash fraction. (A) and (B) Control hLF-CH2-CH3 (26 ng) was detected via immunoblotting (Ctrl, left panel).

Fig. 8. Heparin Inhibits Human Lactoferrin (hLF) Endocytosis into Caco-2 Cells Due to Its Binding

HP: Heparin added at an equimolar concentration of hLF (0.38 µM). Control hLF levels (10 ng) detected via immunoblotting (Ctrl, left panel).
The Caco-2 monolayers have been widely used as an in vitro model of the intestinal barrier to study the intestinal absorption of drugs. This cell culture system could be a valuable approach to examine the intestinal absorption of LF. However, this assay can often be laborious and time-consuming. Thus, the simple assay to assess its uptake and release by Caco-2 cells could be a potential alternative approach to evaluate the intestinal absorption of LF.

In conclusion, the simple uptake and release of intact LF by Caco-2 cells shows its potential for use as an in vitro model for the intestinal absorption of LF.

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Conflict of Interest The authors declare no conflict of interest.

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