Effects of lactoferrin on collagen gel contractile activity and myosin light chain phosphorylation in human fibroblasts

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Abstract When fibroblasts are plated on a type I collagen gel they reduce the size of the gel and the extent of collagen gel contraction reflects the motile activity of the fibroblasts. We found that both bovine and human lactoferrin (Lf) enhanced the collagen gel contractile activity of WI-38 human fibroblasts. Rho inhibitor (exoenzyme C3), Rho kinase inhibitor (Y-27632), myosin light chain kinase inhibitor (ML-7), MEK inhibitor (PD98059) and Src family tyrosine kinase inhibitor inhibited the Lf-enhanced collagen gel contraction. Treatment of fibroblasts with Lf induced the phosphorylation of myosin light chain (MLC) within 30 min. Lf-enhanced MLC phosphorylation was inhibited by Y-27632 and ML-7. These results suggest that Lf promotes the motility of fibroblasts by regulating MLC phosphorylation. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Lactoferrin; Cell migration; Collagen gel contraction; Myosin light chain phosphorylation

1. Introduction

A cell culture system utilizing collagen gel provides an excellent model for evaluating the effects of the three-dimensional collagen matrix on cellular physiological properties (e.g. organization of the cytoskeleton, regulation of signal transduction pathways). When fibroblasts are embedded or plated on a collagen gel, they show an ability to reduce the collagen gel surface area, and eventually they form a tissue-like structure. This phenomenon is called collagen gel contraction, and it is considered to mimic the reorganization of the collagen matrix that accompanies wound healing in skin [1,2]. In addition, the extent of collagen gel contraction reflects the motility of the fibroblasts [1]. Both growth factors and cytokines, such as transforming growth factor (TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor and lysophosphatidic acid (LPA) are known to promote collagen gel contraction by fibroblasts [3–6].

Bovine milk and colostrum are known to be a rich source of growth factors [7]. Reyners and coworkers reported that bovine milk whey stimulated the wound repair activity of myofibroblasts [8]. In addition, Belford and coworkers reported that the cell growth activity found in bovine milk was not completely blocked by mixtures of antibodies against known growth factors, suggesting the existence of additional cell growth factor(s) in bovine milk and colostrum [9].

Lactoferrin (Lf) is a member of the transferrin family of iron binding glycoproteins. Lf was first identified as an abundant protein in bovine milk and colostrum. It has subsequently been found to be expressed in many mammalian tissues and secretions [10]. Lf has a broad spectrum of antimicrobial activity, and is thought to have a role in host defense against microbes [10]. In addition, several studies indicate that Lf acts directly on mammalian cells, resulting in the activation of natural killer cells and neutrophils [11,12], promotion or inhibition of epithelial cell and fibroblast proliferation [13,14], and stimulation of nerve growth factor synthesis and secretion from L-M cells [15]. Interestingly, some activities of Lf in mammalian cells are independent of its iron binding properties [13,15]. However, the signal transduction pathway that mediates the intracellular responses of Lf in mammalian cells is still poorly understood.

We report here that both human Lf (hLf) and bovine Lf (bLf) are able to enhance collagen gel contraction induced by human fibroblasts. To elucidate the mechanisms of Lf-induced collagen gel contraction, we focused our study on the relationship between myosin light chain (MLC) phosphorylation and the gel contraction promoting activity of Lf.

2. Materials and methods

2.1. Cell culture

WI-38 human fetal fibroblasts were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere.

2.2. Materials and antibodies

Materials and chemicals were obtained as follows: purified bLf from Nippon Protein (Tokyo, Japan); purified hLf from ICN (Aurora, OH, USA); type I collagen solution from Nitta Gelatin (Osaka, Japan); Rho kinase inhibitor (Y-27632) from Welfide Corporation (Osaka, Japan); Rho inhibitor (exoenzyme C3), myosin light chain kinase (MLCK) inhibitor (ML-7), Src family tyrosine kinase inhibitor (PP1) and pertussis toxin from Biomol (Plymouth Meeting, PA, USA); MEK inhibitor (PD98059) from Wako (Osaka, Japan); okadaic acid from Calbiochem (San Diego, CA, USA); anti-MLC monoclonal antibody, LPA and SB203580 from Sigma (St. Louis, MO, USA). A monoclonal antibody against phosphorylated serine 19 of MLC was a gift from Dr. Minoru Seto (Asahi Chemical Indus-

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Abbreviations: bLf, bovine lactoferrin; hLf, human lactoferrin; FBS, fetal bovine serum; DMEM, Dulbecco's minimal essential medium; Rock, Rho kinase; MLC, myosin light chain; MLCK, myosin light chain kinase; LPA, lysophosphatidic acid; TGF-β, transforming growth factor; PDGF, platelet-derived growth factor; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; PTK, protein tyrosine kinase

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try, Shizuoka, Japan). Iron-free bLf (apo-Lf) and iron-saturated bLf (holo-bLf) were prepared according to procedures described by Masson and Heremans [16] and Suzuki et al. [17], respectively.

2.3. Biotinylation of lactoferrin and cell surface binding assay

Biotinylation of bLf and the detection of Lf binding to WI-38 cells was performed according to the method described by Rejman et al. [18]. Briefly, WI-38 cells were plated on a collagen-coated 96-well tissue culture plate (2×10^4 cells/well). Cells were incubated with biotinylated Lf for 4 h at 4°C in the presence or absence of a 100-fold molar excess of unlabeled Lf and transferrin, and with horseradish peroxidase (HRP)-conjugated streptavidin for 1 h at room temperature. The biotin–avidin complex was detected with tetramethylbenzidine substrate reagent (BD PharMingen). The reaction was terminated with 0.18 N H_2SO_4. Absorbance at 450 nm was read using an automated plate reader.

2.4. Collagen gel contraction assay

For a 24-well culture plate, 7 ml of cold collagen solution (3 mg/ml) was mixed gently with 2 ml of five-fold concentrated cold DMEM. The pH of the mixture was adjusted to 7.4 with 200 mM HEPES containing 2.2% Na_2HCO_3 and then the total volume was adjusted to 10 ml using distilled water. A 400-μl aliquot of this collagen mixture was poured into each well of the plate. Plates were allowed to settle at 37°C for 2 h to allow for collagen gelation. A cell suspension containing 3.2×10^4 WI-38 fibroblasts per ml was plated onto each polymerized gel. Gel contraction was initiated by detaching the edges of the collagen gels from the side of the wells. To document collagen gel contraction, the gels were photographed using a charge-coupled device camera at the indicated intervals. The collagen gel area was quantified by NIH image software.

2.5. Wound healing assay

WI-38 cells were plated on a collagen-coated 35-mm tissue culture dish at a density of 3.5×10^4 cells/cm^2. Confluent cells maintained in serum-free DMEM for 2 days were wounded with a silicone cell scraper with a 17-mm blade (Sumitomo Bakelite). The wounded monolayer was rinsed with serum-free DMEM two times to remove scratched cells and then cultured for 24 h in serum-free DMEM with or without 1 μM Lf. The cells were fixed with phosphate-buffered saline (PBS) containing 3.7% formamide. Migration of the monolayer cells into the wounded area was observed using an inverted phase-contrast microscope (Nikon).

2.6. Western blotting

WI-38 cells were rinsed with cold PBS, and then harvested in TNE buffer (20 mM Tris–HCl pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 1 mM EDTA, 5 μM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin). The cell lysates were then clarified by centrifugation (15 000*g*, 15 min) and the supernatants (containing 10 μg of protein) were resolved by SDS–PAGE (15% gel), and then electrically transferred onto a nitrocellulose membrane (Schleicher and Schuell). The membranes were then probed with primary antibodies and further incubated with a secondary antibody conjugated with HRP. Immunoreactivity was visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

3. Results

3.1. Specific binding of bLf to human fibroblasts

We examined the binding of the iron-saturated form of bLf to WI-38 human fibroblasts. As shown in Fig. 1A, biotinylated bLf interacted with WI-38 fibroblasts in a saturating manner. In the presence of a 100-fold molar excess of unlabeled bLf, bLf binding was reduced to the background level. bLf binding was not blocked by a 100-fold molar excess of bovine transferrin. These observations indicate that bovine bLf specifically binds WI-38 fibroblasts. Scatchard analysis of the binding data resulted in a linear relationship. The estimated association constant was 6.37×10^7 M^{-1} (Fig. 1B).

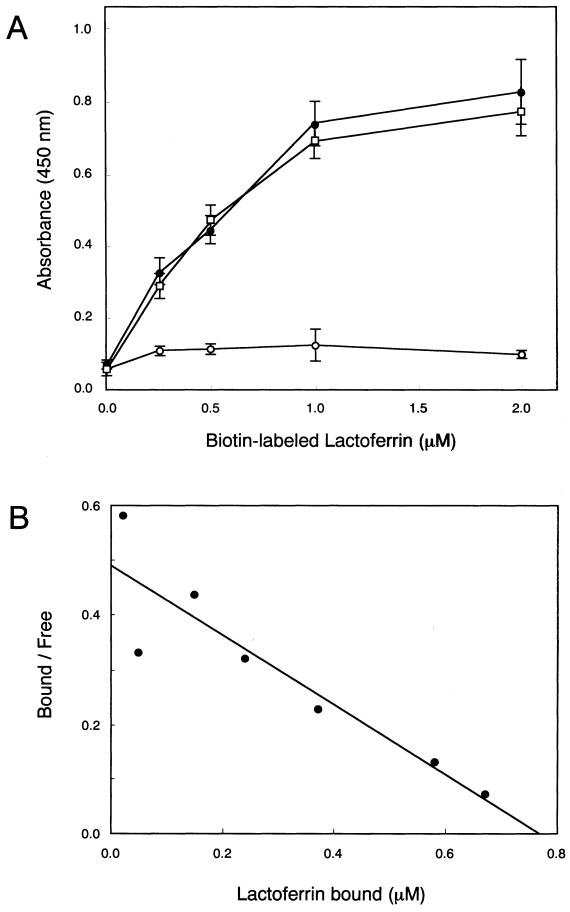

Fig. 1. Specific binding of bLf to WI-38 fibroblasts. A: WI-38 fibroblasts were incubated with increasing concentrations (0–2 μM) of bLf in the absence (closed circle) or presence of a 100-fold molar excess of unlabeled bLf (open circle), or bovine transferrin (open square). The binding of biotin-labeled Lf to the WI-38 cells was detected by incubation with HRP-conjugated avidin. Mean values of three parallel measurements ± S.D. are shown. B: To determine binding affinity of bLf to WI-38 fibroblasts, a constant concentration of biotinylated bLf (2 μM) was incubated with the cells in the presence of increasing concentrations (0–200 μM) of unlabelled bLf. The association constant was calculated following the procedure of Scatchard.

3.2. Lf facilitates the collagen gel contractile activity of human fibroblasts

To examine the effect of Lf on type I collagen gel contraction mediated by fibroblasts, WI-38 cells were plated on type I collagen gels as described in Section 2. bLf and hLf were added to the culture media at a final concentration of 1 μM at the start of collagen gel contraction. Fig. 2A shows the time course for collagen gel contraction in the presence or absence of hLf and bLf. Addition of both bLf and hLf enhanced the rate of collagen gel contraction. The area of the collagen gel was decreased by about 50% within 6 h after the initiation of gel contraction in the presence of both bLf and hLf. On the other hand, addition of holo-bovine transferrin (final 1 μM) did not enhance collagen gel contraction.

Lf has been demonstrated to have cell growth promoting activity for several types of mammalian cells [13,14], and therefore it is possible that enhanced gel contraction is caused by an increase in the number of plated cells. We examined the

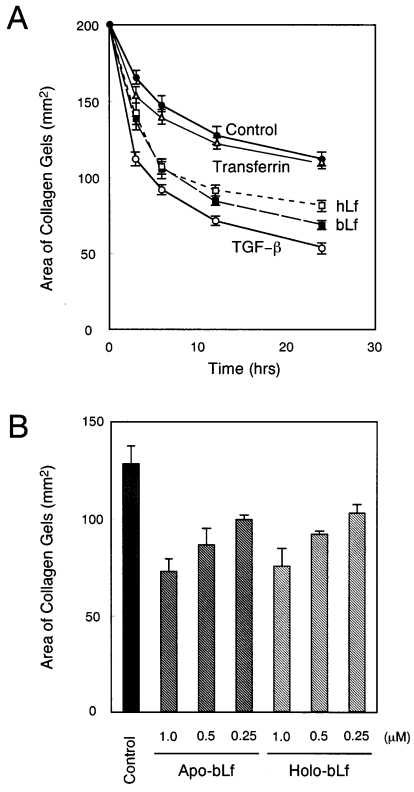

Fig. 2. The effects of Lf on collagen gel contractile activity of WI-38 human fibroblasts. A: WI-38 fibroblast suspensions (containing 3.2×10^5 cells) were plated on polymerized collagen gels in 24-well plates (growth area: 200 mm^2). Two hours after plating, bLf, hLf, bovine transferrin (each 1 μM), and TGF-β (10 ng/ml) were supplemented into the culture media, respectively. Collagen gel contraction was initiated by detaching the gels from the side of the wells. The extent of gel contraction was evaluated by measuring the collagen gel areas at 3, 6, 12 and 24 h after the initiation of collagen gel contraction. Mean values of four parallel measurements are shown. B: Apo-Lf or holo-Lf were supplemented into the culture media. The concentrations of both types of Lf were increased from 0.25 μM to 1.0 μM. The collagen gel area was measured 24 h after the initiation of collagen gel contraction. Mean values of four parallel measurements ± S.D. are shown.

effect of Lf on the growth of WI-38 fibroblasts by quantifying the amount of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium incorporated into the cells. Addition of bLf and hLf (final concentration 1 μM) did not increase the number of WI-38 fibroblasts within 48 h (data not shown). This result indicates that the gel contraction promoting activity of Lf is not due to an increase in cell number in the collagen gels.

3.3. Effects of apo- or holo-Lf on collagen gel contraction

Lf is known to be an iron binding protein, and some of its functions, including its antimicrobial activity, depend upon the iron ion [10]. We investigated the involvement of the iron ion in the gel contraction promoting activity of Lf by comparing the effects of the iron-saturated form of bLf (holo-Lf) and the iron-deprived form of bLf (apo-Lf) on collagen gel contraction. We varied the concentration of these two forms of Lf from 0.25 μM to 1.0 μM. As shown in Fig. 2B, the effect of apo-Lf on gel contraction was almost the same as that of holo-Lf at each concentration. Furthermore, the effect of both types of Lf was dose-dependent. We conclude that the gel contraction promoting activity of Lf is independent of the iron ion.

3.4. Lf facilitates the motility of WI-38 fibroblasts on collagen

It has been previously demonstrated that the extent of collagen gel contraction reflects the motile activity of the cells in the collagen gels [1,2]. We confirmed the fibroblast migration promoting activity of Lf by using an in vitro wound healing assay. Addition of bLf (final concentration 1 μM) into serum-free DMEM enhanced the infiltration of WI-38 fibroblasts into the wounded area within 24 h (Fig. 3C). This observation supports the results of the collagen gel contraction assay.

3.5. Inhibition of Lf-enhanced collagen gel contraction by the inhibitors Rho, Rock and MLCK

The small G protein Rho has been implicated in fibroblast-mediated collagen gel contraction and migration of fibroblasts [6,19]. To determine whether Rho is involved in Lf-enhanced collagen gel contraction, we investigated the effects of exoenzyme C3, which ADP ribosylates and inactivates Rho, on Lf-enhanced collagen gel contraction. WI-38 cells plated on collagen gels were incubated with 25 μg/ml of exoenzyme C3 for 12 h prior to the initiation of collagen gel contraction. As shown in Fig. 4A, exoenzyme C3 markedly blocked Lf-enhanced collagen gel contraction.

Rho kinase (Rock/Rok) is one of the targets of Rho and has been implicated in the regulation of phosphorylation of myosin light chain (MLC) and the contractile activity of fibroblasts by phosphorylating, and thereby inactivating, myosin light chain phosphatase [20]. To investigate whether Rock participates in Lf-enhanced collagen gel contraction, we added the Rock-specific inhibitor Y-27632 to fibroblast cultures. Incubation of fibroblasts with Y-27632 (final concentration 10 μM) attenuated Lf-enhanced collagen gel contraction (Fig. 4B). These observations indicate that Rho- and Rock-dependent mechanisms are involved in Lf-enhanced collagen gel contraction.

Recently, it was shown that the phosphorylation level of MLC is also regulated by MLCK in fibroblasts as well as smooth muscle cells [20]. We investigated the effects of a MLCK inhibitor (ML-7, final concentration 3 μM) on Lf-enhanced collagen gel contraction (Fig. 4B). Gel contraction

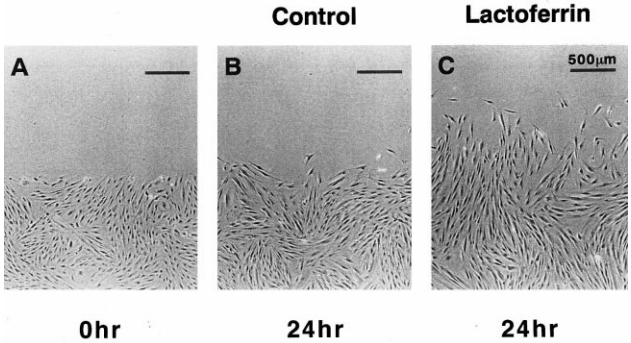

Fig. 3. Migration of fibroblasts on collagen-coated dishes in the presence or absence of bLf. Confluent monolayers of WI-38 fibroblasts in collagen-coated dishes were scratched with a silicone cell scraper as described in Section 2 (A). The cells were further maintained for 24 h with serum-free DMEM (B), or DMEM containing 1 μM of bLf (C). Cell migration into the wounded area was documented by photography. Each calibration bar indicates 500 μm.

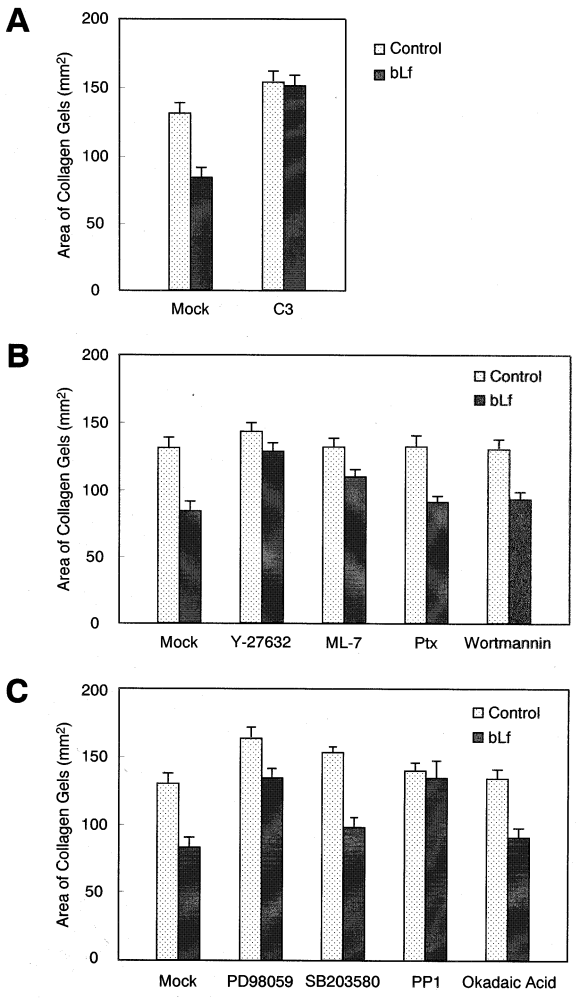

Fig. 4. Effects of Rho, Rock and MLCK inhibitors and MEK inhibitor on Lf-enhanced collagen gel contractile activity of WI-38 fibroblasts. A: WI-38 fibroblasts plated onto collagen gels in a 24-well plate were incubated with 25 μg/ml of exoenzyme C3 for 12 h. The collagen gel contraction was initiated in the presence or absence of 1 μM of bLf. Twelve hours later, the extent of gel contraction was evaluated by measuring the area of collagen gels. Mean values of four parallel measurements ± S.D. are shown. B,C: The effects of Y-27632 (10 μM), ML-7 (3 μM), pertussis toxin (Ptx: 25 ng/ml), wortmannin (1 μM), PD98059 (50 μM), SB203580 (20 μM), PP1 (1 μM) and okadaic acid (1 μM) on Lf-enhanced collagen gel contraction. The experimental procedures were the same as in A, except cells were incubated with the reagents for 1 h.

was inhibited by ML-7; however, its effect was milder than that of the Rho/Rock inhibitors. On the other hand, pertussis toxin (an inhibitor of the Gi class of heterotrimeric G proteins) and wortmannin (a phosphatidylinositol 3-kinase inhibitor) had no effect on Lf-enhanced collagen gel contraction (Fig. 4B).

3.6. *Effects of MEK/ERK cascade inhibitor and protein tyrosine kinase inhibitor on Lf-enhanced collagen gel contraction*

Mitogen-activated protein kinase (MAPK) cascades participate in various cellular responses to stimuli, including cell to extracellular matrix interaction. It has been suggested that extracellular-regulated kinase (ERK) participates in cell migration and the wound healing process [21,22]. To determine whether MAPK cascades are involved in Lf-enhanced collagen gel contraction, we examined the effects of PD98059 (MEK inhibitor) and SB203580 (p38 MAPK inhibitor) on Lf-enhanced collagen gel contractile activity of WI-38 fibroblasts. MEK is a member of the MAPK kinases and specifically activates ERK. As shown in Fig. 4C, PD98059 (50 μM) inhibited gel contractile activity of WI-38 fibroblasts in the presence or absence of bLf, suggesting that MEK/ERK cascades are involved in the collagen gel contractile process. The effects of SB203580 (20 μM) were not so obvious as those of PD98059.

The Src family protein of tyrosine kinases (PTKs) has been implicated in activation of MAP kinase, which is induced by cell–extracellular matrix interaction [23,24]. We next investigated the effects of PP1 (specific inhibitor of Src family PTKs) on Lf-enhanced collagen gel contraction. As shown in Fig. 4C, 1 μM of PP1 abrogated the gel contraction promoting activity of bLf, suggesting that Src family PTKs are involved in the process of Lf-enhanced collagen gel contraction. On the other hand, okadaic acid (1 μM) did not affected collagen gel contractile activity of WI-38 cells in the presence or absence of bLf (Fig. 4C).

3.7. *Lf-induced MLC phosphorylation*

To study whether the contractile activity of fibroblasts is associated with the phosphorylation level of MLC, we investigated the effects of bLf on the phosphorylation of MLC in WI-38 fibroblasts using Western blotting and an antibody against the phosphorylated serine 19 residue of MLC. As shown in Fig. 5A, bLf (final concentration 1 μM) induced serine 19 phosphorylation of MLC within 30 min. The phosphorylation of MLC was sustained for 60 min following stimulation. The effect of hLf on MLC phosphorylation was com-

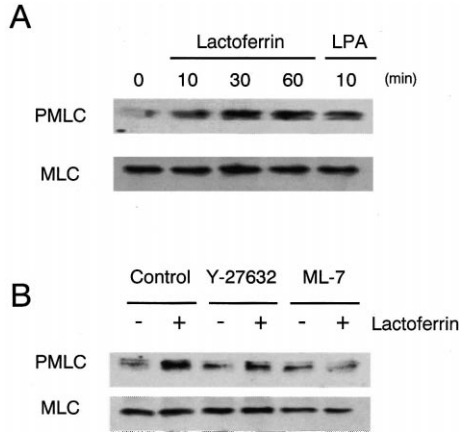

Fig. 5. Effects of Rho and MLCK inhibitors on Lf-induced MLC phosphorylation. A: WI-38 fibroblasts plated in collagen-coated dishes were maintained in DMEM containing 10% FBS, and in serum-free DMEM for 12 h. Subsequently, 1 μM of bLf or 10 nM of LPA were added to the culture media. The cells were harvested at the indicated times. The phosphorylation of MLC was detected by Western blotting using monoclonal antibodies against phosphorylated serine 19 residue of MLC (upper panel) or total MLC (lower panel). B: WI-38 fibroblasts were stimulated with 1 μM of bLf in the presence of Rho kinase inhibitor (Y-27632, 10 μM) or MLCK inhibitor (ML-7, 3 μM). The cells were harvested 30 min following stimulation. The phosphorylation of MLC was investigated as described in A.

parable to that of bLf (data not shown). To investigate the signaling pathway responsible for Lf-enhanced MLC phosphorylation, we examined the effect of Y-27632 and ML-7 on Lf-enhanced MLC phosphorylation. MLC phosphorylation was blocked by both Y-27632 and ML-7 (Fig. 5B). These observations suggest that the regulation of MLC phosphorylation is a critical step in Lf-enhanced collagen gel contraction, and that the regulation of MLC phosphorylation is achieved through two distinct pathways: a Rho/Rock-dependent pathway and an MLCK-dependent pathway.

4. Discussion

We have shown here that both bLf and hLf have novel activity to enhance the collagen gel contractile activity of fibroblasts. Despite differences in their amino acid sequence and their patterns of glycosylation, the three-dimensional conformation of Lf is conserved among different mammalian species and cells [10]. In addition, it has been shown that Lf has the ability to interact with Lf receptors from different species [10]. bLf was also found to specifically interact with WI-38 fibroblasts. The interaction could not be inhibited by bovine transferrin, suggesting that the effects of Lf on WI-38 cells are achieved through a receptor-mediated pathway. The Lf receptor has been characterized in T-cells [25], intestinal cells [26] and platelets [27]. In addition, the low-density lipoprotein receptor-related protein also has a high affinity for Lf [28,29]. Based on these previous findings, identification of the cell surface molecule responsible for Lf-enhanced collagen gel contraction is important for further investigations into the pathways mediating fibroblast gel contraction.

It has been shown that the gel contractile activity of fibroblasts reflects the motility of the cells in the gels [1,2]. We confirmed the fibroblast migration promoting activity of Lf by using an in vitro wound healing assay (Fig. 3). It has been shown that Lf has the ability to modulate the migration of gastrointestinal cells and Langerhans cells [30,31]. The process of cell migration consists of many steps, including the modulation of cell adhesion and reorganization of the cytoskeleton. It has been shown that Lf inhibits adhesion of fibroblasts and intestinal epithelial cells [32,33]. These observations suggest that Lf affects the migration of these cells by regulating cell adhesion. On the other hand, we report here that MLC phosphorylation plays an important role in Lf-enhanced collagen gel contraction. MLC phosphorylation is a critical step in the formation of actin stress fibers, the induction of cell migration [6,19,20], platelet aggregation [34], leukocyte phagocytosis [35], and neurite retraction [36]. We report here that both a Rho/Rock-dependent pathway and an MLCK-dependent pathway contribute to Lf-enhanced MLC phosphorylation and the collagen gel contractile activity of fibroblasts. However, the exact mechanism through which collagen gel contraction is achieved is still controversial. LPA has the ability to phosphorylate MLC, and it promotes the gel contractile force of fibroblasts [6]. On the other hand, LPA-induced collagen gel contraction was inhibited by pertussis toxin [37]. PDGF-induced collagen gel contraction requires phosphatidylinositol 3-kinase activity, but this does not appear to involve MLC phosphorylation [37,38]. In any case, Rho is implicated in the gel contractile activity of fibroblasts [39]. These lines of evidence suggest that the mechanism of Lf-enhanced collagen gel contraction is different from that of LPA and PDGF. The mechanisms of Lf-enhanced MLC phosphorylation have yet to be elucidated.

Our experiments using the MEK inhibitor suggest that the MEK/ERK pathway correlates with collagen gel contraction. It was also shown that ERK was activated during collagen gel contraction by rat mesangial cells and human fibroblasts, and PD98059 inhibited collagen gel contraction by mesangial cells in serum- and agonist-free conditions [38,40,41]. On the other hand, it was reported that PD98059 did not affect gel contractile activity in the presence of FBS [38], suggesting that the MEK/ERK pathway is not involved in agonist-induced collagen gel contraction. Klemke et al. reported that activated ERK phosphorylated MLCK, and eventually enhanced MLC phosphorylation in COS cells [21]. However, neither PD98059 nor SB203580 inhibited Lf-enhanced MLC phosphorylation in WI-38 fibroblasts (unpublished observations), suggesting that the MEK/ERK pathway is not responsible for Lf-enhanced MLC phosphorylation in WI-38 fibroblasts. Further studies are required to elucidate the role of the MEK/ERK pathway on the process of collagen gel contraction.

It has been shown that bovine milk whey has the ability to facilitate wound healing and gel contractile activity of dermal fibroblasts [7]. Bovine milk and colostrum is known to be an abundant source of cytokines and growth factors [8]. The results of this study suggest that Lf is a major contributor to the gel contraction promoting activity found in colostrum, along with the other previously identified milk-derived growth factors. Because Lf has anti-inflammatory and antimicrobial activity, it may be suitable for the treatment of cutaneous wounds. However, further in vivo experiments are required to confirm this.

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