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Inhibitory effect of lactoferrin on hypertrophic differentiation of ATDC5 mouse chondroprogenitor cells

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Abstract The skeleton is formed by two different mechanisms. In intramembranous ossification, osteoblasts form bone directly, whereas in endochondral ossification, chondrocytes develop a cartilage template, prior to osteoblast-mediated skeletogenesis. Lactoferrin is an iron-binding glycoprotein belonging to the transferrin family. It is known to promote the growth and differentiation of osteoblasts. In this study, we investigated the effects of bovine lactoferrin on the chondrogenic differentiation of ATDC5 chondroprogenitor cells. This mouse embryonic carcinoma-derived clonal cell line provides an in vitro model of chondrogenesis. Lactoferrin treatment of differentiating ATDC5 cells promoted cell proliferation in the initial stage of the differentiation process. However, lactoferrin treatment resulted in inhibition of hypertrophic differentiation, characterized by suppression of alkaline phosphatase activity, aggrecan synthesis and N-cadherin expression. This inhibitory effect was accompanied by sustained Sox9 expression, as well as increased Smad2/3 expression and phosphorylation, suggesting that lactoferrin regulates chondrogenic differentiation by up-regulating the Smad2/3-Sox9 signaling pathway.

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Introduction

Ossification is classified into two main processes. In intramembranous ossification, bone is formed directly by osteoblasts derived from mesenchymal cells, and this process is important for formation of flat skull bones, including crania. Endochondral ossification is responsible for development of most other bones. It begins with the differentiation of chondrocytes from mesenchymal cells at growth plates. Growth plate chondrocytes undergo proliferation, condensation and hypertrophic differentiation, providing a scaffold for osteoblasts. As a result of blood vessel invasion and chondrocyte apoptosis, the cartilage template becomes replaced by bone tissue (Cancedda et al. 2000).

Numerous growth factors and cytokines are involved in regulation of this process. Bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β), regulate differentiation and proliferation of chondrocytes (Provot and Schipani 2005; Goldring et al. 2006; Li et al. 2005). Although TGF-β enhances the proliferation of chondrocytes, it inhibits their hypertrophic differentiation (Goldring et al. 2006). On the

other hand, BMP2/4 promotes expression of the hypertrophic phenotype in differentiating chondrocytes (Li et al. 2005). Binding of TGF-β family proteins to their receptor (TGF-β receptor or BMP receptor) induces phosphorylation and nuclear translocation of receptor-regulated Smads (R-Smads), which in turn promote cartilage-specific gene expression; Smad1/5/8 in BMP2/4 signaling and Smad2/3 in TGF-β signaling (Söderberg et al. 2009).

Lactoferrin is an iron-binding glycoprotein that was found initially in milk and colostrum. It is synthesized by glandular epithelial cells and secreted in bodily fluids including tears, saliva, semen, vaginal secretions and milk. Lactoferrin is important in host defense against pathogens, exhibiting a broad spectrum of anti-microbial and anti-viral activities (Brock 2002). Furthermore, lactoferrin is a major component of neutrophil secretory granules, playing important roles in inflammatory- and immune-modulating processes via regulation of lymphocyte activity (Legrand et al. 2008). Recent in vivo and in vitro studies have indicated that lactoferrin promotes development of skeletal tissue by up-regulating the growth and differentiation of osteoblasts, as well as inhibiting osteoblast apoptosis (Cornish et al. 2004; Takayama and Mizumachi 2008; Blais et al. 2009; Guo et al. 2009). In addition, lactoferrin has been shown to inhibit the differentiation and bone resorbing activity of osteoclasts (Cornish et al. 2004; Lorget et al. 2002).

In this study, we examined whether lactoferrin affects chondrogenic differentiation. ATDC5 mouse embryonic carcinoma-derived cells were used in an in vitro model of endochondral bone formation at a growth plate. In response to insulin or ascorbic acid, ATDC5 cells will undergo chondrogenic differentiation, including cell proliferation, condensation (aggregation), cartilage nodule formation and cellular hypertrophy (Shukunami et al. 1996; Altaf et al. 2006). We investigated the effects of lactoferrin treatment on the expression of differentiated cell phenotypes throughout the differentiation process.

Materials and methods

Materials

ATDC5 cells were provided by the RIKEN Bioresource Center, through the National Bio-resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. D-MEM/F-12 (1:1 mixture of Dulbecco's Modified Eagle Medium and Ham's F-12), protease inhibitor cocktail, phosphatase inhibitor cocktail and ascorbic acid, were purchased from Sigma. Fetal bovine serum (FBS), penicillin, streptomycin and horseradish peroxidase-(HRP) conjugated anti-rabbit IgG, were obtained from Invitrogen. Enhanced chemi-luminescence (ECL) Western blotting detection reagent and nitrocellulose membranes were purchased from GE Healthcare. Bovine lactoferrin was obtained from Fonterra. Human lactoferrin was obtained from Cappel. Alcian blue 8GX and crystal violet were from Merck. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was obtained from Promega. Anti-Sox9 and anti-N-cadherin polyclonal antibodies were obtained from Abcam. Anti-type II collagen monoclonal antibody, anti-phospho Smad1 (Ser463/465) polyclonal antibody, anti-Smad2/3 polyclonal antibody and anti-phospho Smad3 (Ser 423/425) polyclonal antibody, were obtained from Millipore. Anti-Smad1 monoclonal antibody was obtained from Cosmo Bio. HRP-conjugated anti-mouse IgG was obtained from Jackson Laboratory. All materials and chemicals not specified above were of the highest grade available.

Chondrocyte culture

ATDC5 cells were cultured in DMEM/F-12 supplemented with 5% fetal bovine serum, 3×10^{-8} M sodium selenite, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37°C in a 5% CO_2 atmosphere and grown in a monolayer. To induce chondrogenic differentiation, ATDC5 cells (4×10^4) were plated into 12-well plates. Upon reaching confluence (culture day 0), the DMEM/F-12 was supplemented with ascorbic acid (20 μg/mL) and these conditions were maintained for 28 days. The culture medium was replaced every other day. At least three independent cultures were examined for each experiment.

Cell proliferation assay

Cell proliferation was evaluated by colorimetrically measuring the reduction of MTS by living cells. ATDC5 cells (100 μL; 10^3 cells) were plated in 96-

well plates. Upon reaching confluence, cells were differentiated into chondrocytes by the addition of 20 μg/mL ascorbic acid. For cell proliferation analysis, MTS solution (20 μL) was added at the times indicated, and then cells were incubated at 37°C for 60 min. The absorbance at 490 nm (A490) was measured using an iMark micro plate reader (Bio-Rad). Results are expressed as means ± standard deviations.

Immunoblotting

ATDC5 cells were washed twice with chilled PBS and homogenized in TNE buffer (20 mM Tris–HCl [pH7.4], 0.15 M NaCl, 1% NP-40, 1 mM EDTA, 5 mM 2-mercaptoethanol) supplemented with 1% protease and phosphatase inhibitor cocktail. Cell homogenates were centrifuged at 15,000 *g* for 15 min and aliquots of supernatant (10 μg protein) were resolved by SDS–PAGE in a Laemmle system. Proteins were then transferred electrophoretically onto nitrocellulose membranes. Subsequently, membranes were treated with a blocking reagent (Tris-buffered saline containing 0.1% Tween-20 and 5% skim milk) for 2 h at room temperature. Blocked membranes were incubated with primary antibody, followed by incubation with HRP-conjugated secondary antibody. Immunoreactivity was detected using ECL Western blotting detection reagents.

Alkaline phosphatase (ALP) activity

ATDC5 cells grown in 12-well plates were lysed in 200 mL 0.2% Triton X-100. After centrifugation of the cell lysate at 15,000 *g* for 15 min, ALP activity was estimated by measuring the release of p-nitrophenol (pNP) from a p-nitrophenyl phosphate (PNPP) solution. Aliquots were incubated with 0.1 M $CaCO_3$ buffer (pH 9.8) containing 6.7 mM pNPP and 2 mM $MgCl_2$, at 37°C for 30 min. The amount of pNP was estimated by measuring absorbance at 405 nm. ALP activity was normalized to the total protein in a well, and total protein was determined by the Bradford method.

Alcian blue staining

Cells were fixed with 100% ethanol for 20 min at −20°C. Alcian blue 8GX solution was prepared by dissolving the solid stain in 0.1 M HCl. Fixed cells were incubated with 0.1% (w/v) Alcian blue 8GX solution for 2 h. Excess stain was removed by washing twice with distilled water.

Statistical analyses

Statistical significance was determined using a one-way analysis of variance (ANOVA), followed by multiple comparisons. Data were considered significant at $P < 0.05$.

Results

MTS cell growth assay

The growth of a skeletal tissue depends upon the precise regulation of chondrocyte proliferation and differentiation. We investigated whether lactoferrin could promote the growth of ATDC5 cells during chondrogenic differentiation. MTS assays revealed that under normal conditions, ATDC5 cell number increased continuously until day 21 (Fig. 1). In the presence of 1 μM of bovine lactoferrin, the increase in cell number reached a plateau at day 7 and then

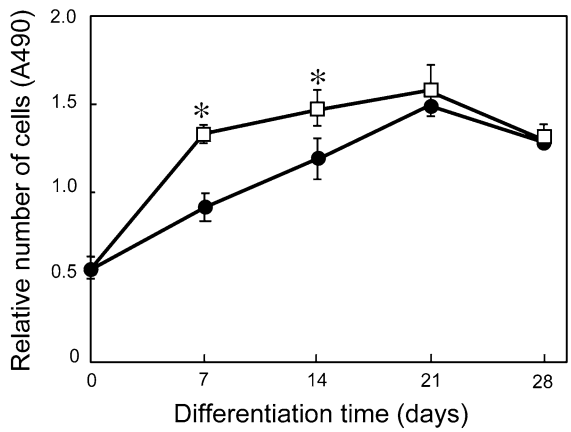

Fig. 1 Effect of lactoferrin on the growth of differentiating ATDC5 cells. ATDC5 cells were differentiated into chondrocytes in the presence (*empty squares*) or absence (*black circles*) of 1.0 μM of bovine lactoferrin. Cells were treated for 28 days. *Error bars* indicate the standard deviations from six measurements. Statistically significant differences ($P < 0.05$) between control and lactoferrin-treated cells at each time point are indicated with an *asterisk*

remained at a similar level until day 21. This observation indicates that lactoferrin promotes the growth of differentiating ATDC5 cells.

Effect of lactoferrin on hypertrophic differentiation

To address whether lactoferrin affects hypertrophic differentiation of ATDC5 cells, we evaluated the effects of lactoferrin on aggrecan synthesis using Alcian blue staining at day 28. Aggrecan is a major component of cartilage proteoglycan and is used commonly as a marker for hypertrophic differentiation of chondrocytes (Kiani et al. 2002). As shown in Fig. 2a, addition of bovine lactoferrin inhibited aggrecan synthesis in a dose-dependent manner.

To confirm that lactoferrin inhibits hypertrophic differentiation, we investigated the effect of lactoferrin on alkaline phosphatase (ALP) activity, another marker of hypertrophic differentiation. ALP activity was reduced significantly at higher concentrations of bovine lactoferrin (1 and 2 μM), but not by 0.5 μM bovine lactoferrin (Fig. 2b). The inhibitory effect of human lactoferrin on ALP activity was comparable to that of bovine lactoferrin (Fig. 2c). This inhibitory effect was not observed when cells were treated with 2 μM bovine transferrin (Fig. 2c).

Effect of lactoferrin treatment period on the expression of hypertrophic phenotypes

Next, we investigated the effect of lactoferrin treatment period on the chondrogenic differentiation of ATDC5 cells. For this purpose, cells were treated with bovine lactoferrin (1 μM) for the first or last 14 days of the differentiation process (initial and hypertrophic stages, respectively). Lactoferrin treatment only inhibited aggrecan synthesis and ALP activity during the hypertrophic stage and the levels observed were comparable to those in cells treated with lactoferrin throughout the entire differentiation process (Fig. 3). In contrast, no significant inhibition of aggrecan synthesis or ALP activity was observed in response to lactoferrin treatment during the initial stage of differentiation. These data demonstrate that it is lactoferrin treatment during the hypertrophic stage that is critical for inhibiting expression of hypertrophic phenotypes in differentiating ATDC5 cells.

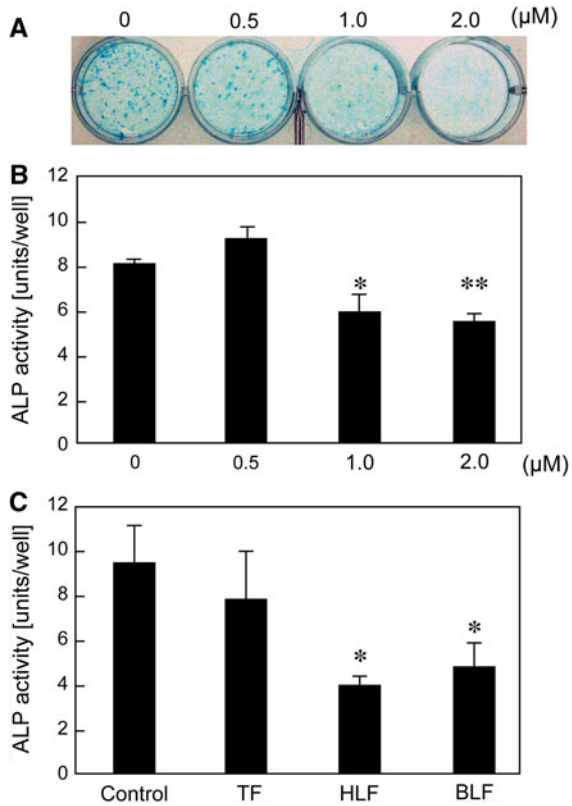

Fig. 2 Effect of lactoferrin on aggrecan synthesis and alkaline phosphatase (ALP) activity during chondrogenic differentiation of ATDC5 cells. ATDC5 cells were differentiated into chondrocytes using ascorbic acid. Cells were incubated in DMEM/F-12 supplemented with various concentrations of bovine lactoferrin (0–2.0 μM). **a** Cell surface aggrecan was detected by Alcian blue staining at day 28. **b** ALP activity was determined from the rate of hydrolysis of 1.0 mM pNPP at day 28. *Error bars* show standard deviations of three measurements. **c** Cells were differentiated in the presence of 2.0 μM of bovine transferrin (*TF*), human lactoferrin (*HLF*), or bovine lactoferrin (*BLF*). ALP activity was measured at day 28. Statistically significant differences between treated and control cells are indicated as follows: *, $P < 0.05$; **, $P < 0.01$

Effect of lactoferrin on the expression of chondrogenic marker proteins

In order to speculate on the molecular mechanisms underlying the effects of lactoferrin on chondrogenic differentiation, we investigated its effects on expression of transcription factors and chondrogenic markers throughout the differentiation process of ATDC5 cells. Sox9 is a master transcription factor that is required for the induction of chondrogenic differentiation (Provot and Schipani 2005; Akiyama 2008).

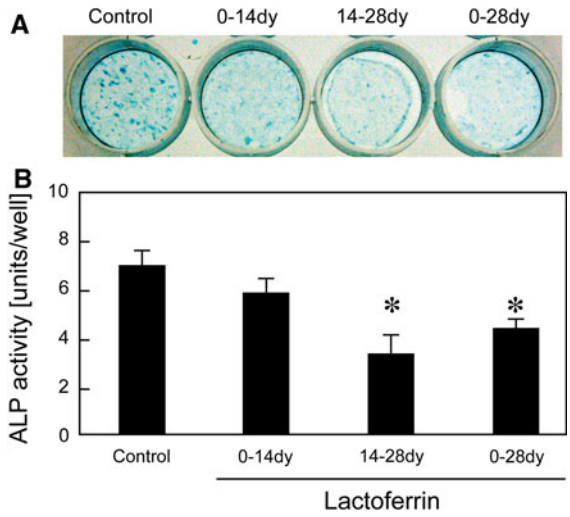

Fig. 3 Effect of lactoferrin treatment period on aggrecan synthesis and alkaline phosphatase (*ALP*) activity. ATDC5 cells were differentiated into chondrocytes using ascorbic acid. Cells were treated with 1 μM bovine lactoferrin for the first 14 days (initial stage), last 14 days (hypertrophic stage), or throughout the entire differentiation process (0–28 days). **a** Cell surface aggrecan was detected using Alcian blue staining at day 28. **b** ALP activity was examined at day 28. *Error bars* show standard deviations of three measurements. Statistically significant differences between treated and control cells are indicated as *, $P < 0.05$

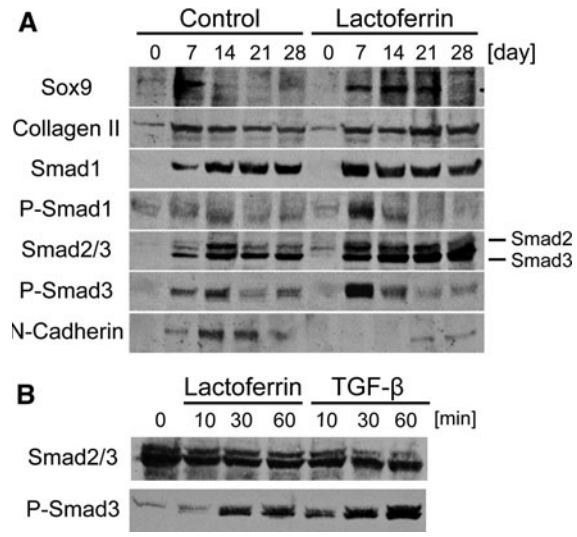

Fig. 4 **a** Effect of lactoferrin on the expression levels of Sox9, type-II collagen, Smad1, Smad2/3 and N-cadherin, as well as the phosphorylation of Smad1 (Ser463/465) and Smad2/3 (Ser 423/425), during chondrogenic differentiation of ATDC5 cells. Cells were maintained under chondrogenic conditions for 28 days in the presence or absence of 1 μM bovine lactoferrin. Cells were harvested in TNE buffer or 6 M urea (type-II collagen), at the times indicated. Cell lysates (10 μg protein) were subjected to separation by SDS–PAGE, followed by immunoblotting using the antibodies against the proteins indicated. **b** ATDC5 cells were maintained under chondrogenic conditions for 7 days and stimulated with bovine lactoferrin (1 μM) or TGF-β (10 ng/mL). The cells were harvested in TNE buffer at the indicated times. Phosphorylation of Smad3 (Ser 423/425) was detected by immunoblotting

On the other hand, Sox9 is known to inhibit the hypertrophic differentiation of chondrocytes (Ikeda et al. 2005; Huang et al. 2001; Yamashiro et al. 2004). Un-differentiated cells exhibited low levels of Sox9 expression, with transient induction observed at day 7, followed by a return to baseline levels at day 14 or later (Fig. 4a). In the presence of 1 μM bovine lactoferrin, Sox9 expression was sustained until day 21 and then decreased to baseline levels by day 28. This observation suggests that lactoferrin-mediated inhibition of the hypertrophic phenotype is dependent upon sustained Sox9 expression.

Type-II collagen is a major component of the cartilage extracellular matrix and it is known to be a direct target of Sox9 (Lefebvre et al. 1997). In control cells, type-II collagen expression peaked at day 7 and then decreased gradually in parallel with the progression of chondrogenic differentiation (Fig. 4a). Lactoferrin treatment resulted in strong type-II collagen expression throughout the differentiation process. This observation suggests that lactoferrin supports cartilage formation by regulating the expression of type II collagen.

Effect of lactoferrin on the expression and phosphorylation of R-Smads

Smad2/3 proteins play a critical role in chondrogenic differentiation by promoting the transcriptional activity of Sox9 (Furumatsu et al. 2005). We investigated the effect of bovine lactoferrin on the expression and phosphorylation of R-Smads. In control cultures, expression of Smad1 and Smad2/3 was induced following the induction of chondrogenic differentiation (Fig. 4a). In the presence of bovine lactoferrin, expression Smad1 and Smad2/3 was enhanced throughout the differentiation process. Increased expression of these proteins was accompanied by a strong up-regulation in phosphorylation of Smad1 (Ser463/465) and Smad3 (Ser 423/425) during the initial stage of chondrogenic differentiation (day 7).

Effect of lactoferrin on N-cadherin expression

During the process of chondrogenesis, cell–cell interactions are important for the regulation of cellular responses to growth factors (Woods et al. 2007). N-cadherin is a well-known cell adhesion molecule that mediates Ca^{2+}-dependent cell–cell adhesion and cellular condensation during chondrogenic differentiation. In control cultures, the highest levels of N-cadherin expression were observed at days 14 and 21 (Fig. 4a). In contrast, bovine lactoferrin strongly antagonized N-cadherin expression in differentiating ATDC5 cells. This result suggests that the inhibitory effect of lactoferrin correlates with abrogation of cellular condensation and cell–cell interactions mediated by N-cadherin.

Promotion of Smad3 phosphorylation by lactoferrin

In differentiating ATDC5 cells (day7), addition of bovine lactoferrin (1 μM) enhanced phosphorylation of Smad3 (Ser 423/425) within 30 min (Fig. 4b). In contrast, the phosphorylation of Smad1 (Ser 463/465) was not affected by bovine lactoferrin (data not shown). This observation suggests that lactoferrin regulates chondrogenic differentiation by directly up-regulating the Smad3 phosphorylation.

Discussion

Lactoferrin is known to be a potent regulator of bone cells. It promotes the differentiation and bone-forming activity of osteoblasts (Cornish et al. 2004). Previously, we have shown that lactoferrin promotes osteogenic differentiation of human osteoblast-like MG63 cells (Takayama and Mizumachi 2008). On the other hand, lactoferrin inhibits the genesis and bone-resorbing activity of osteoclasts (Lorget et al. 2002). In this study, we show that lactoferrin promotes the growth and initial stage of chondrogenic differentiation in ATDC5 cells, whereas it inhibits hypertrophic differentiation of these cells. This inhibitory effect was characterized by the attenuation of aggrecan synthesis, ALP activity (Fig. 2a, b) and N-cadherin expression (Fig. 4a).

Although the intracellular signaling pathways that regulate chondrogenesis remain unknown, our findings suggest that lactoferrin may act as a signaling molecule in this process. We found that lactoferrin treatment of differentiating ATDC5 cells resulted in sustained Sox9 expression (Fig. 4a). Sox9 is a master transcription factor that plays an essential role in establishing pre-cartilaginous condensation and initiation of chondrogenic differentiation (Akiyama 2008). Lactoferrin-induced promotion of Sox9 expression has also been reported in C2C12 mesenchymal cells (Yagi et al. 2009). The suggestion that lactoferrin promotes Sox9 expression was supported by the finding that expression of type-II collagen, a major transcriptional target of Sox9, was also enhanced by lactoferrin in differentiating ATDC5 cells (Fig. 4a). Thus, lactoferrin-mediated inhibition of hypertrophic differentiation in ATDC5 cells may be explained by the sustained Sox9 expression, since Sox9 is also an inhibitor of chondrocyte hypertrophic differentiation (Ikeda et al. 2005; Huang et al. 2001; Yamashiro et al. 2004).

Multiple signaling pathways control chondrogenic differentiation by regulating the transcriptional activity of Sox9. In addition to inhibiting the hypertrophic differentiation of chondrocytes, Smad2/3 signals promote chondrocyte proliferation and are required for induction of chondrogenesis (Yang et al. 2001). In response to TGF-β stimulation, Smad2/3 is translocated to the nucleus, forming a complex with Sox9 to promote the expression of genes involved in chondrogenic differentiation (Furumatsu et al. 2005). In chondrocytes, Smad3 deficiency results in accelerated hypertrophic differentiation (Li et al. 2006). Thus, lactoferrin is likely to increase cell proliferation and inhibit hypertrophic differentiation by promoting the transcriptional activity of Smad2/3 and Sox9.

Chondrogenic differentiation is characterized by drastic changes in cell shape, which results in the conversion of a flattened shape to a round or polygonal morphology (Woods et al. 2007). Rho GTPase acts as a cell shape regulator by controlling organization, polymerization and de-polymerization of actin stress fibers in chondrocytes, as well as other types of cells (Burridge and Wennerberg 2004). Activated (GTP-bound) RhoA stabilizes actin stress fibers by up-regulating both RhoA kinase (ROCK) and LIM kinase. It has been shown that overexpression of RhoA enhances cell proliferation and inhibits hypertrophic differentiation of ATDC5 cells (Wang et al. 2004), while inhibition of ROCK activity

results in promotion of hypertrophic differentiation (Woods et al. 2005). Thus, RhoA/ROCK signaling inhibits the hypertrophic differentiation of chondrocytes, while promoting the proliferation and initial stage of chondrogenic differentiation. Data from our laboratory demonstrate that lactoferrin enhances collagen gel contractile activity in human fibroblasts by activating Rho and MLCK, which suggests that RhoA/ROCK may be involved in lactoferrin-mediated inhibition of hypertrophic differentiation in ATDC5 cells (Takayama and Mizumachi 2001).

Mesenchymal cells undergo chondrogenic differentiation through a cellular condensation stage. N-cadherin is a calcium-dependent cell adhesion molecule that is required for both pre-cartilage condensation and cartilage nodule formation during chondrogenic differentiation. Inhibition of N-cadherin-mediated cell adhesion results in abrogation of cell condensation and subsequent chondrogenesis (Oberlender and Tuan 1994). Here, we observed that lactoferrin antagonized N-cadherin expression in differentiating ATDC5 cells. Although Sox9 was reported to bind specifically to an N-cadherin gene promoter region and to increase the transcription and translation of N-cadherin (Panda et al. 2001), Sox9 is not essential for N-cadherin expression in vivo (Akiyama et al. 2002). This finding suggests that in addition to Sox9, it is possible that lactoferrin controls the expression of other genes required for N-cadherin expression.

This is first study describing the effects of lactoferrin on chondrogenic differentiation. These experiments were performed in vitro using an ATDC5 cell line. To confirm the effect of lactoferrin on endochondral bone formation, further studies using primary chondrocytes and mesenchymal stem cells will be required.

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