Journal of Oral Science, Vol. 50, No. 4, 419-425, 2008

Original

Lactoferrin suppress the adipogenic differentiation of MC3T3-G2/PA6 cells

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(Received 1 October and accepted 15 October 2008)

Abstract: Lactoferrin accelerates the differentiation of osteogenic and chondrogenic lineage cells, whereas it inhibits the myogenic and adipogenic differentiation of pluripotent mesenchymal cells; however, the effect of lactoferrin on the differentiation of preadipocytes is unknown. In this study, we examined the effect of lactoferrin on adipogenic differentiation using a mouse preadipocyte cell line, MC3T3-G2/PA6. The cells were cultured in differentiation medium with or without lactoferrin to induce cellular differentiation. The cell lineage was then determined by Oil Red O staining, real-time PCR screening for the mRNA expression of phenotype-specific markers, and Western blot analysis. The number of Oil Red O-positive lipid droplets decreased following treatment with lactoferrin, as did the mRNA expression of C/EBPα, PPARγ, aP2, and adiponectin. Furthermore, our Western blot data revealed a decrease in PPARγ expression attributable to lactoferrin exposure. These results suggest that lactoferrin suppresses the adipogenic differentiation of MC3T3-G2/PA6 cells. (J. Oral Sci. 50, 419-425, 2008)

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Keywords: MC3T3-G2/PA6 cells; lactoferrin; adipogenic differentiation; PPARγ

Introduction

Lactoferrin is an 80-kDa iron-binding glycoprotein that belongs to the transferrin family. It is present in breast milk, epithelial secretions, and the secondary granules of neutrophils. In healthy subjects, lactoferrin circulates at concentrations of $2\text{-}7 \times 10^{-6}$ g/ml. Lactoferrin is a pleiotropic factor with potent antimicrobial and immunomodulatory activities (1). Given its presence in exocrine glands and the secondary granules of neutrophilic leukocytes, lactoferrin is found in many body fluids, including milk, saliva, tears, bile, and pancreatic fluid (2). To date, lactoferrin has been shown to be involved in a variety of biological functions such as cell growth and differentiation (3-5), modulation of the inflammatory response (6), anti-viral effects (7), and the regulation of myelopoiesis (8,9).

Recently, we demonstrated that lactoferrin significantly increased ALPase activity; the mRNA expression of Runx2, osteocalcin, and Sox9; and the protein expression of Runx2 and Sox9 in pluripotent mesenchymal cells, whereas it decreased the mRNA expression of MyoD, desmin, and PPARγ. These results suggested that lactoferrin diverts the differentiation of C2C12 cells from myogenic and adipogenic lineage cells to osteogenic and chondrogenic lineage cells (10).

Fat-storing adipocytes and bone-forming osteoblasts are derived from the same mesenchymal stem cells. During differentiation, gene expression programs establish the fate of these cells. Recently, the transcription factor PPARγ was found to determine whether mesenchymal stem cells differentiate into adipocytes or osteoblasts (11).

Little is known, however, about the effect of lactoferrin on the differentiation of preadipocytes. Thus, we studied the effect of lactoferrin on the adipogenic differentiation of MC3T3-G2/PA6 cells by examining the mRNA and protein expression of genes related to this process.

Materials and Methods

Cell culture

MC3T3-G2/PA6 cells were obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan). MC3T3-G2/PA6 is a preadipocyte cell line that was originally isolated from the calvarium of a mouse (12). The cells were maintained in α-minimum essential medium (α-MEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Logan, UT, USA) and 1% (v/v) penicillin-streptomycin (50 U/ml penicillin and 50 µg/ml streptomycin; Sigma, St. Louis, MO, USA) in an atmosphere of 5% CO_2 at 37°C. To examine the effects of lactoferrin on the adipogenic differentiation of the cells, cells were inoculated onto tissue culture plates at a density of 2×10^4 cells/cm^2 and incubated overnight. The growth medium was then replaced with α-MEM containing with 10% FBS; 0.5 mM 3-isobutyl-1-methylxanthine (IBMX); 0.25 mM dexamethasone (Dex); and 0, 10, 50, or 100 µg/ml lactoferrin, based on data showing that the local concentration of lactoferrin is 1-100 µg/ml during inflammation (13). In a previous report, Dex is an essential component to differentiate into adipocyte in MC3T3-G2/PA6 cells (14).

Determination of cellular proliferation

MC3T3-G2/PA6 cells were dispensed into 96-well microplates at a density of 2×10^4 cells/cm^2 and cultured with 0, 10, 50, or 100 µg/ml lactoferrin for up to 7 days. At the times indicated, the medium was replaced with fresh medium containing 10% (v/v) cell counting kit reagent (Wako Fine Chemicals, Osaka, Japan), and the cells were incubated for 1 h. The intensity of the reaction products was then measured at 450 nm using a microtiter plate reader (Titertek Multiskan Plus, Flow Laboratories, McLean, VA, USA). The relative cell numbers were calculated from the relative absorbance values on the basis of a standard curve.

Real-time PCR

Total RNA was isolated from cultured MC3T3-G2/PA6 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Aliquots containing equal amounts of mRNA were then subjected to a PCR assay. First-strand cDNA synthesis was performed at 42°C for 60 min using 1 µg of DNase-treated total RNA in a 20 µl reaction mix containing first-strand buffer, 50 ng of random primers, 10 mM dNTPs, 1 mM DTT, and 0.5 U of reverse transcriptase. The cDNA mixtures were diluted five-fold with sterile distilled water, and 2 µl aliquots were used for PCR. Real-time PCR was performed in a 25 µl reaction mix containing 1× SYBR Premix Ex Taq (Takara, Tokyo, Japan) and 0.2 µM specific primers (Table 1). PCR was carried out using a Smart Cycler II system (Cepheid, Sunnyvale, CA, USA), and the data were analyzed using Smart Cycler software Version 2.0d. The PCR conditions were: 35 cycles of 95°C for 3 s and 60°C for 20 s, with measurements taken at the end of the annealing period in each cycle. The specificity of each product was verified by melting curve analysis between 60 and 95°C. The reactions were performed in triplicate, and the levels of mRNA were calculated and normalized to the level of GAPDH mRNA at each time point.

Extraction of proteins from the cultured cells

To obtain whole-cell extracts, MC3T3-G2/PA6 cells that had been cultured with or without lactoferrin were rinsed with phosphate-buffered saline (PBS) and exposed to a lysis buffer consisting of 50 mM Tris-HCl, 0.1% Triton X-100, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The cells were then sonicated three times for 10 s each. Aliquots containing equal amounts of protein were subjected to SDS-PAGE.

SDS-PAGE and Western blotting

SDS-PAGE was conducted at 150 V for 60 min in 5-20% gradient cross-linked polyacrylamide gels using a discontinuous Tris-glycine buffer system as described by Laemmli (15). Immunotransfer was performed using a semi-dry transfer unit with a continuous buffer system at a constant amperage of 0.8 mA/cm^2 for 90 min. The transfer membrane was blocked at 4°C for 16 h and then washed with Tris-buffered saline containing Tween 20 (TBS-T). For immunodetection, the blocked membrane was incubated at room temperature for 60 min with anti-PPARγ (sc-7273) and anti-β-tubulin (sc-5274) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then washed with TBS-T. Liu and Xu (16) suggested that β-tubulin is more suitable than β-actin as an internal control for Western blotting; therefore, we selected β-tubulin as

our internal control. The membrane was then incubated at room temperature for 60 min with the appropriate biotin-conjugated secondary antibody, washed with TBS-T, and incubated for 20 min at room temperature with horseradish peroxidase-conjugated streptavidin. The immunoreactive proteins were visualized by chemiluminescence using an ECL kit (Amersham Life Sciences, Buckinghamshire, UK) with exposure of the transfer membrane to X-ray film (Eastman Kodak, Rochester, MN, USA).

Oil Red O staining

MC3T3-G2/PA6 cells were placed into 24-well microplates at a density of 2×10^4 cells/cm^2 and cultured with 0, 10, 50, or 100 μg/ml lactoferrin for 10 days, after which the cells were rinsed with PBS and fixed with 10% formalin. The cells were then rinsed again with PBS and incubated with Oil Red O (Sigma, St. Louis, MO, USA) for 20 min.

Statistical analysis

Each value represents the mean ± standard deviation. Significant differences were detected using Bonferroni's *t*-test. A *P*-value < 0.05 was considered significant.

Results

Cellular proliferation

Cellular proliferation was analyzed in the presence of 0, 10, 50, and 100 μg/ml lactoferrin over 7 days of culture. Proliferation was unaffected by the addition of lactoferrin on day 1 of culture; however, it was increased by lactoferrin in a dose-dependent manner from days 3 to 7 (Fig. 1).

The effect of lactoferrin on lipid accumulation

Cells that had been cultured with 0, 10, 50, or 100 μg/ml lactoferrin for 10 days were incubated with Oil Red O for 20 min. Large numbers of Oil Red O-positive lipid droplets were observed in the cytoplasm of the cells cultured without lactoferrin. In contrast, the number of stained cells in the lactoferrin-treated group was reduced dose-dependently with 10, 50, and 100 μg/ml lactoferrin (Fig. 2).

Effects of lactoferrin on the mRNA expression of adipogenic differentiation markers

The expression of C/EBPα, PPARγ, adiponectin, and aP2 in MC3T3-G2/PA6 cells was monitored by real-time PCR using cells that had been cultured for up to 7 days with or without lactoferrin. C/EBPα expression was significantly reduced on days 3, 5, and 7 of culture with 10, 50, or 100 μg/ml lactoferrin (Fig. 3a). The mRNA expression of PPARγ was also significantly reduced on day 7 of culture with 10, 50, or 100 μg/ml lactoferrin (Fig. 3b),

Table 1 Specific primers used in real-time PCR

Target	Sequence (5' → 3')	Genbank accession No.
C/EBP a-f	TGGACAAGAACAGCAACGAGTA	BC058161
C/EBP a-r	CGAGTTGCCATGGGCCTTGA	
PPAR γ-f	ATGGAGCCTAAGTTTGAGTTTGCTG	NM_011146
PPAR γ-r	GATGTCCTCGATGGGCTTCAC	
aP2-f	GCTACCATCCGGTCAGAGAGTAC	NM_011547
aP2-r	TCGTCTGCGGTGATTTCATC	
adiponectin-f	GCAGAGATGGCACTCCTGGA	NM_004794
adiponectin-r	CCCTTCAGCTCCTGTCATTCC	

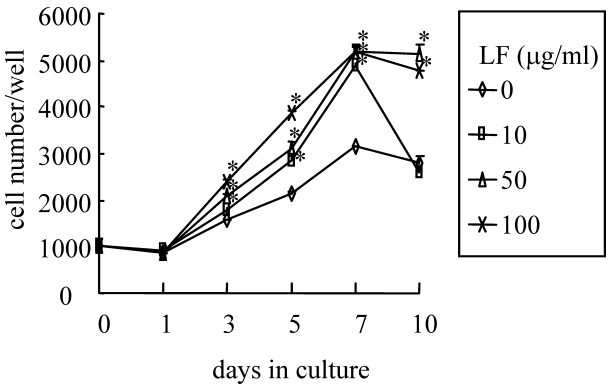

Fig. 1 Effect of lactoferrin on cellular proliferation. Cells were cultured with 0, 10, 50, or 100 μg/ml lactoferrin for up to 7 days. The number of cells was determined on days 3, 5, and 7. Each bar indicates the mean ± standard deviation from three experiments. *$P < 0.05$, lactoferrin treatment versus control.

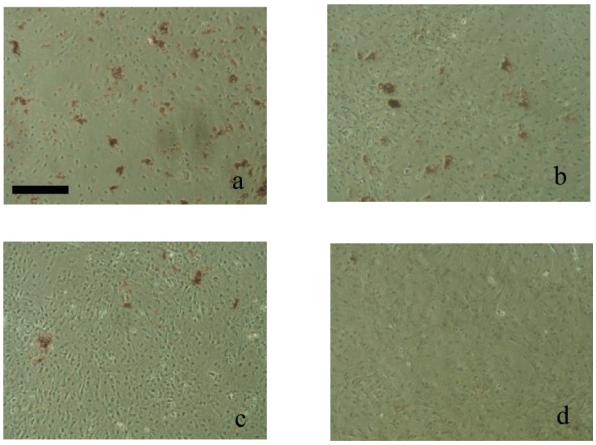

Fig. 2 Effect of lactoferrin as determined by Oil Red O staining. Cells were cultured with 0, 10, 50, or 100 μg/ml lactoferrin for 10 days (a: 0, b: 10, c: 50, d: 100 μg/ml). Oil Red O-positive lipid droplets are shown in red. Scale bar = 100 μm. (original magnification ×100)

as was the expression of adiponectin by the addition of 50 or 100 μg/ml lactoferrin (Fig. 3c). The expression of aP2 was also significantly decreased on days 5 and 7 of culture with 10, 50, or 100 μg/ml lactoferrin (Fig. 3d).

Effects of lactoferrin on the protein expression of adipogenic differentiation markers

The expression of PPARγ was examined by Western blotting using cells that had been cultured for up to 7 days with or without lactoferrin (Fig. 4). Immunoreactive bands corresponding to PPARγ were observed; however, the intensity was decreased in a dose-dependent manner by the addition of lactoferrin.

Discussion

Here, we showed that lactoferrin suppresses the adipogenic differentiation of MC3T3-G2/PA6 cells, as evidenced by the reduced mRNA and protein expression of genes related to that process. We also demonstrated that cellular proliferation increased as a result of the addition of lactoferrin, which is similar to data from Cornish et al. (17) showing that the proliferation of primary human

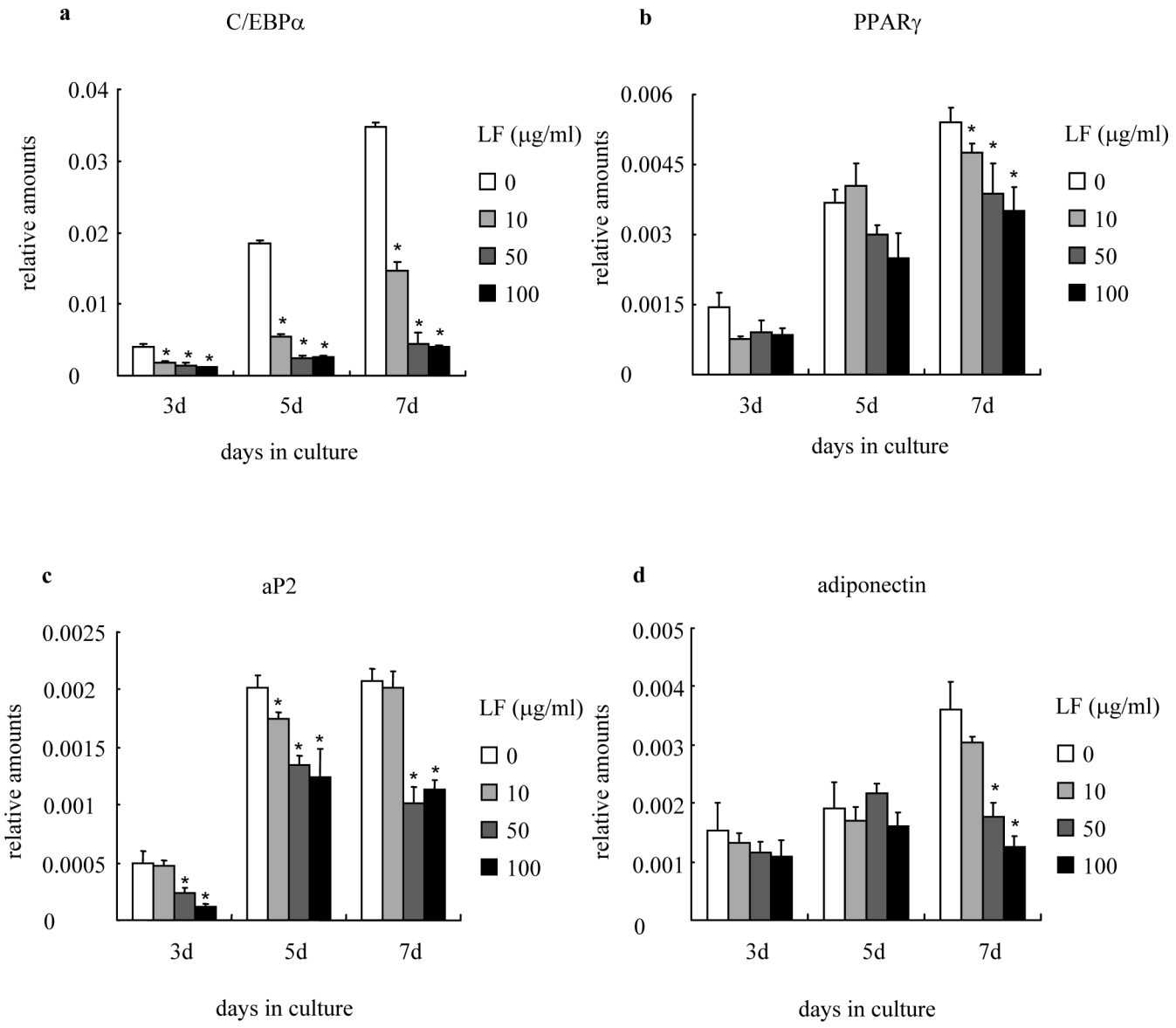

Fig. 3 Effect of lactoferrin on C/EBPα, PPARγ, aP2, and adiponectin mRNA expression. Cells were cultured with 0, 10, 50, or 100 μg/ml lactoferrin for up to 7 days. At the times indicated, the mRNA expression of (a) C/EBPα, (b) PPARγ, (c) aP2, and (d) adiponectin was analyzed by real-time PCR. Each bar indicates the mean ± standard deviation from three experiments. *$P < 0.05$, lactoferrin treatment versus control.

osteoblasts from the Saos-2 and ST2 cell lines was increased in the presence of a physiological concentration (1-100 μg/ml) of lactoferrin.

To investigate the effect of lactoferrin on the adipogenic differentiation of MC3T3-G2/PA6 cells, we first used Oil Red O staining with 0.5 mM IBMX and 0.25 mM Dex to observe the lipids in the cells. Large numbers of Oil Red O-positive lipid droplets were detected in the cells cultured without lactoferrin; however, the number of Oil Red O-positive droplets was decreased in a dose-dependent manner by 10, 50, and 100 μg/ml lactoferrin. These data suggest that lactoferrin suppresses the adipogenic differentiation of MC3T3-G2/PA6 cells.

To investigate how lactoferrin affects adipogenic differentiation, we analyzed the mRNA expression of C/EBPα PPARγ, and aP2, which are transcription factors that have been shown by real-time PCR to be related to adipocyte differentiation (18-20).

C/EBPα plays an important role in the transcriptional activation of adipose differentiation (21). It is expressed just before the transcription of most adipocyte-specific genes is initiated. Wang et al. (22) reported that the disruption of C/EBPα resulted in mice that failed to develop white adipose tissue. These findings indicate that C/EBPα is necessary and sufficient to induce adipocyte differentiation. PPARs have also been implicated in the transcriptional activation of adipocyte differentiation, and PPARγ is the most adipose-specific PPAR. It is expressed at its highest level in adipose tissue and adipocyte cell lines, whereas it is expressed at low levels or not at all in other tissues and cell lines (23-25). C/EBPα and PPARγ appear to act synergistically by triggering an adipocyte differentiation program and reciprocally activating each other's transcription. This is supported by the finding that the ectopic expression of C/EBPα or PPARγ in myoblasts and NIH 3T3 fibroblasts promoted only partial differentiation, while the co-expression of both factors provoked full differentiation (21). Furthermore, it has been reported that the aP2 promoter possesses C/EBP-and PPAR-binding sites, which mediate transactivation by the corresponding transacting factors (26). Our results revealed that lactoferrin decreases the mRNA expression of C/EBPα, PPARγ and aP2; in other words, lactoferrin suppresses the adipogenic differentiation of MC3T3-G2/PA6 cells at the level of transcription. In addition, the protein expression of PPARγ was dose-dependently reduced by the addition of lactoferrin. We also investigated the mRNA expression of adiponectin, a marker of adipocyte cell differentiation, by real-time PCR. Adiponectin is a protein hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism. The level of adiponectin is inversely correlated with the percentage of body fat in adults, although the association in infants and young children is unclear. The hormone also plays a role in the suppression of metabolic derangements that may result in type 2 diabetes, obesity, atherosclerosis, and non-alcoholic fatty liver disease (27-29). The mRNA expression of adiponectin was dose-dependently suppressed on day 7 of culture in the presence of lactoferrin.

These data suggest that lactoferrin affects adipogenic differentiation at not only the transcriptional stage but also the maturation stage in MC3T3-G2/PA6 cells. Based on these results, we propose that lactoferrin, at least in the presence of Dex, suppresses adipogenic differentiation by altering the levels of the transcription factors C/EBPα, PPARγ and aP2 in MC3T3-G2/PA6 cells.

To our knowledge, this is the first study to show how lactoferrin suppresses the adipogenic differentiation of MC3T3-G2/PA6 cells.

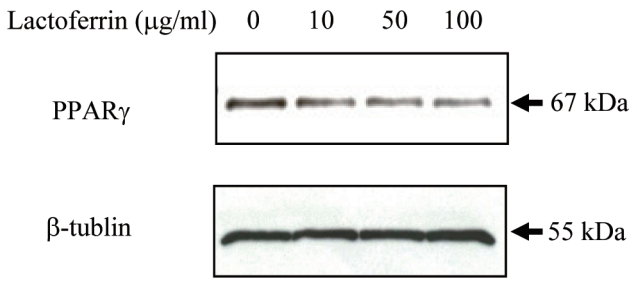

Fig. 4 Effect of lactoferrin on PPARγ expression. Whole-cell extracts were prepared from MC3T3-G2/PA6 cells that had been incubated with various concentrations of lactoferrin for up to 7 days. PPARγ (67 kDa) and β-tubulin (55 kDa) expression was analyzed by Western blotting.

Acknowledgments

This study was supported by the Uemura Fund from the Nihon University School of Dentistry, Japan. Support was also provided by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to promote multidisciplinary research projects.

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