Effect of lactoferrin on osteogenic differentiation of human adipose stem cells

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Abstract

Purpose Many in vitro studies of the analysis of the lactoferrin (LF) effect on cells have been reported. However, no study has yet investigated the effect of LF on osteogenic differentiation of human adipose-derived stem cells (hADSCs). The aim of this study was to evaluate the effect of LF on osteogenic differentiation of human adipose stem cells.

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Methods The hADSCs were cultured in an osteogenic medium with 0, 10, 50 and 100 µg/ml LF, respectively. hADSC proliferation was analysed by Cell Counting Kit-8 (CCK-8) assay, and cell osteogenic differentiation was evaluated by alkaline phosphatase (ALP) activity assay, von Kossa staining and real-time polymerase chain reaction (RT-PCR).

Results Cell proliferation was significantly increased by LF in a dose-dependent manner from days 4 to 14. Cells cultured with 100 µg/ml LF presented a higher activity compared with the control. The deposition of calcium was increased after the addition of LF. The mRNA expression of type I collagen (COL-I), ALP, osteocalcin (OCN) and RUNX2 increased markedly as a result of LF treatment.

Conclusions We have shown for the first time that LF could promote the proliferation and osteogenic differentiation of hADSCs, which could be a promising approach for enhancing osteogenic capacity of cell-based construction in bone tissue engineering.

Introduction

Some bone pathologies such as bone defects, nonunion fractures, osteonecrosis and osteoporosis still lack efficient treatments. The use of stem/progenitor cells as osteo-regenerative cells to give rise to new functional bone or as tools for studying both normal and pathological osteogenesis could be of interest for medicine. Among osteo-regenerative cells, mesenchymal stem cells (also named mesenchymal stromal cells, MSCs) are of primary interest. It is currently well accepted that the ability of MSCs to differentiate into various cell phenotypes may be critical in the progression of bone disease. Adipose-derived stem cells (ADSCs), which have emerged as an attractive source of multipotent cells, have shown the ability to differentiate into osteogenic lineages in vitro [1] and have

also been used to treat bone defects in clinical cases [2, 3]. Additionally, it is easy to procure ADSCs: their expansion *in vitro* is rapid and their harvest yield is approximately 40-fold higher compared to bone marrow MSCs [4].

Lactoferrin (LF), an iron-binding glycoprotein, belongs to the transferrin family [5]. It was initially identified as an abundant protein in milk and colostrum. It is produced by many exocrine glands and consequently is widely distributed in body fluids, including milk, saliva, tears, bile and pancreatic fluid [6]. The serum level of LF in healthy subjects ranges from 2 to 7 µg/ml and is predominantly neutrophil derived, but in the presence of inflammation and sepsis, local concentrations may be much higher (1–100 µg/ml) [7]. LF participates in host defences due to its antibacterial and antiviral activities [8, 9].

Recently, LF has been demonstrated to be an anabolic factor for bone both *in vivo* and *in vitro*. Local administration of LF over the calvariae of adult male mice increased bone formation [10], and oral administration of LF to ovariectomised rodents improved bone density [11]. Many bone metabolic diseases develop when there is an imbalance between the formation and resorption of bone, which depend on the interactions between osteoblasts and osteoclasts [12]. Interestingly, LF has been reported to stimulate proliferation and differentiation of osteoblasts [13–15] and to inhibit the differentiation of osteoclasts [13, 16]. Thus, LF may be a promising therapeutic agent for treatment of bone diseases.

Many *in vitro* studies of the analysis of the LF effect on cells, from rat to human, and from osteoblasts to osteoclasts, have been reported. However, no study has yet investigated the effect of LF on osteogenic differentiation of human ADSCs (hADSCs). The aim of this study was to evaluate the effect of LF on osteogenic differentiation of hADSCs by examining the mRNA expression of genes related to this process.

Materials and methods

Isolation and culture of hADSCs

hADSCs were isolated as previously described and used at passage 3 [17, 18]. In brief, hADSCs were isolated from adipose tissue from three healthy volunteers, aged 33–62 years (two men and one woman), by enzymatic digestion with 0.0075% type I collagenase (Sigma) supplemented with 100 IU penicillin or 100 µg/ml streptomycin (Gibco) in DMEM basal media (Gibco). Full ethical consent was obtained from all patients and the study was granted ethical approval by the Medical Ethics Committee of the Second Affiliated Hospital, Wenzhou Medical College. After digestion, the tissue was centrifuged for ten

minutes at 10,000 g and the supernatant was discarded. The remaining cell pellet was resuspended in DMEM basal media supplemented with 10% foetal bovine serum (FBS, Gibco), 1% penicillin-streptomycin (Gibco) and 0.1% amphotericin B (Invitrogen) in a standard humidified incubator (37°C/5% CO₂/21% O₂). Cultures were grown to 80% confluence and passaged. To examine the effects of LF on terminal differentiation, the hADSCs were cultured in an osteogenic medium [growth culture medium supplemented with 10⁻⁸ M dexamethasone (Sigma), 50 µg/ml ascorbic acid (Sigma) and 5 mM β-glycerol phosphate (Sigma)] at an initial density of 1×10⁴ cells/cm² with 0, 10, 50 or 100 µg/ml LF (Fonterra), based on a report that the local concentration of LF is 1–100 µg/ml during inflammation [7]. The medium was changed every four days during osteogenic differentiation.

Cell proliferation by Cell Counting Kit-8 (CCK-8) assay

For the cell proliferation assays, hADSCs were cultured in 96-well plates at 1×10⁴ cells per well with growth culture medium. Twenty-four hours later, cells were switched to LF-containing media for up to 14 days. The proliferation of hADSCs was determined by the CCK-8 (Kumamoto, Japan) and measured by microplate reader scanning (ELx800, BioTek) at 450 nm as previously described elsewhere [19].

Alkaline phosphatase (ALP) assay

ALP staining was performed at day seven after cells were cultured in 24-well tissue culture plates at a cell density of 1×10⁴. Cells which were cultured in osteogenic medium were rinsed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde. ALP substrate mixture (ALP staining kit, Sigma) was then added and incubated for ten minutes. The ALP activity was determined at 405 nm using *p*-nitrophenyl phosphate (pNPP) (Sigma) as the substrate, and the total protein contents were determined with the bicinchoninic acid (BCA) method, which was previously described in the literature [20, 21].

Quantitation of the deposition of calcium

To qualitatively assess cell-mediated calcium deposition, hADSCs which were cultured in osteogenic medium were visualised by von Kossa staining, which was performed at day 21 after cells were cultured in 24-well tissue culture plates at a cell density of 1×10⁴. Cells in the well plates were fixed in 4% paraformaldehyde, then stained with 1% silver nitrate, placed under a UV lamp for 20 min and rinsed with distilled water before treatment with 5% sodium thiosulfate for two minutes. von Kossa-positive (black) deposits was observed after alcohol washing.

Table 1 Primers for real-time PCR

Gene	Primer sequences (5'–3')	
COL-I	Forward	GAG AGC ATG ACC GAT GGA T
	Reverse	ATG TTT TGG TGG TTC AGG AGG
ALP	Forward	CGC TGT GTC AAC TCC ACC T
	Reverse	CCA GAA GGT TCT GTT AAC TTG
OCN	Forward	GTG CAG CCT TTG TGT CCA AG
	Reverse	GTC AGC CAA CTC GTC ACA GT
RUNX2	Forward	GCG TCA ACA CCA TCA TTC TG
	Reverse	CAG ACC AGC AGC ACT CCA TC
GAPDH	Forward	CCT CAA GAT CAT CAG CAA T
	Reverse	CCA TCC ACA GTC TTC TGG GT

Real-time polymerase chain reaction (PCR) assay

Real-time PCR was used to detect the expression of several osteogenic differentiation-related marker genes [type I collagen (COL-I), ALP, osteocalcin (OCN), RUNX2] at days four and seven, respectively. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions and quantified. Its concentration was determined spectrophotometrically at 260 nm (HP 8452A Diode Array Spectrophotometer). First strand complementary DNAs (cDNAs) were synthesised from 0.3 mg of the isolated RNA by oligo(deoxythymidine) using the DyNamo™ cDNA Synthesis Kit (Fermentas) and used as templates for real-time PCR. The expression of mRNAs was determined quantitatively using DyNamo SYBR1 Green qPCR kit (Takara, Japan). The PCR was performed on a final volume of 25 ml containing 2 ml cDNA, 7.5 pmol of each primer, 1 ml ROX reference dye and 12.5 ml of SYBR Green Master mix (Tiangen), with ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA). The samples underwent 40 cycles consisting of the following steps: initial denaturation at 95°C for five minutes, followed by a set cycle of denaturation at 94°C for ten seconds, different annealing temperatures for each pair of primers (ranging between 53 and 62°C) for 10 s, extension at 72°C for 27 seconds and a final elongation at 72°C for five minutes. Fold increment of any assayed gene was calculated by normalising its expression level to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, which was used as an internal control. Each gene analysis was performed in triplicate. Primer's sequences of the targeted genes are listed in Table 1.

Statistical analysis

All experiments reported in this study were performed independently at least three times. Results are expressed as

mean±standard deviation. Student's *t* test was used to determine statistical significance; values of $P<0.05$ were considered significant.

Results

Cell proliferation in vitro

The proliferation of hADSCs was analysed at days one, four, seven and 14, respectively. By the CCK-8 assay (Fig. 1), no stimulatory effect of LF on cell proliferation was noted at day one ($P>0.05$). However, cell proliferation was significantly increased by LF in a dose-dependent manner from days four to 14 ($P<0.05$).

ALP activity

The ALP activity of hADSCs was measured in the presence of 0, 10, 50 and 100 µg/ml LF by pNPP assay at day seven. Cells treated with 100 µg/ml LF presented a higher activity compared with the control ($P<0.05$), and no significant difference was found in the ALP activity of hADSCs by the addition of 10 or 50 µg/ml LF compared to the control ($P>0.05$) (Fig. 2).

Quantitation of the deposition of calcium

Compared to the control group, the calcium depositions were no different from the control group when 10 µg/ml LF ($P>0.05$) was added; however, increased calcium depositions were observed in the 50 and 100 µg/ml LF treatment groups ($P<0.05$) (Fig. 3).

Fig. 1 The proliferation of hADSCs was analysed at days 1, 4, 7 and 14, respectively. The data are presented as mean±standard deviation. * $P<0.05$

Fig. 2 ALP staining was performed at day 7 after cells were cultured in 24-well tissue culture plates at a cell density of 1×10^4 . The ALP activity of hADSCs was measured with the pNPP assay. The data are presented as mean $\pm$ standard deviation. * $P < 0.05$

The mRNA expression of several osteogenic differentiation-related marker genes

Real-time PCR (Fig. 4) was used to detect the expression of several osteogenic differentiation-related marker genes when the hADSCs were cultured in osteogenic medium supplemented with LF for four and seven days. The mRNA expression of ALP was increased in hADSCs cultured with

Fig. 3 von Kossa staining was performed at day 21 after cells were cultured in 24-well tissue culture plates at a cell density of 1×10^4 . The data are presented as mean $\pm$ standard deviation. * $P < 0.05$

100 µg/ml LF at days four and seven ($P < 0.05$) (Fig. 4a). OCN was significantly increased in hADSCs cultured with 100 µg/ml LF at day seven ($P < 0.05$) (Fig. 4b). COL-I is an early osteoblastic marker, and as shown in Fig. 4c, upon incubation of cells with LF from 10 to 100 µg/ml, the expression of COL-I was increased significantly compared to the control at day seven ($P < 0.05$). At day four, it was only increased in the 100 µg/ml LF treatment group ($P < 0.05$), and there was no difference at the concentrations 10 or 50 µg/ml LF compared to the control, respectively. The level of RUNX2 mRNA expression was increased about 1.2- and 1.5-fold in the 50 and 100 µg/ml LF treatment groups at day four. There was no difference at the concentrations 10, 50 and 100 µg/ml LF compared to the control at day seven, respectively (Fig. 4d).

Discussion

To our knowledge, we have shown for the first time that LF can promote the proliferation and osteogenic differentiation of hADSCs.

The proliferation of hADSCs was increased as a result of the addition of LF in a dose-dependent manner from days four to 14. The result is similar to the data of Cornish et al. [10] that the proliferation of primary human osteoblasts in the two cell lines, Saos-2 and ST2, increased in the presence of a physiological concentration (1–100 µg/ml) of LF. Yagi et al. [22] also proved that LF could promote the proliferation of a typical pluripotent mesenchymal cell line, C2C12. Both of their results were consistent with ours.

ALP, a cell membrane-associated enzyme, appeared early during osteoblast differentiation and was the most widely recognised marker of osteoblastic differentiation [23–25]. ALP activity correlates with matrix formation in osteoblasts prior to the initiation of mineralisation [26]. In our study, by ALP staining, we discovered that cells treated with 100 µg/ml LF presented a higher activity when compared with the control. By real-time PCR, the mRNA expression of ALP was increased in hADSCs cultured with 100 µg/ml LF at days four and seven. Our results were similar to Yagi et al. [22]; they found ALP activity was increased significantly in the presence of LF after day three of culture in comparison to controls in C2C12 cells.

OCN was involved in controlling the mineralisation process, appeared at a late stage of osteogenic differentiation and was characterised by mature cells of the osteoblastic lineage [27, 28]. COL-I is the most abundant protein in bone matrix; it constitutes about 90% of the organic bone matrix and high levels of COL-I mRNA expression would be observed during proliferation [29]. To confirm that LF enhances osteoblastic differentiation, we examined the mRNA expression of important osteogenic markers such

Fig. 4 Real-time PCR reactions were performed using primers for osteoblastic genes, including ALP (a), OCN (b), COL-I(c) and RUNX2 (d) for 4 and 7 days. Each bar represents the mean $\pm$ standard deviation. * $P < 0.05$

as OCN and COL-I. By real-time PCR, the mRNA expression of OCN was significantly increased in hADSCs cultured with 100 $\mu\text{g/ml}$ LF at day seven. Also the expression of COL-I was increased significantly compared to the control at day seven. Our results suggested that LF stimulated osteogenic differentiation of hADSCs not only at the early stage, but also at the maturation stage.

Several signalling pathways have been shown to regulate the lineage commitment and terminal differentiation of hADSCs. During osteogenic differentiation, RUNX2, bone morphogenetic proteins (BMPs), transforming growth factor- β (TGF- β), osterix, β -catenin and Wnt signalling play essential roles in the commitment of mesenchymal cells to the osteoblastic lineage [30, 31]. RUNX2 is a transcription factor essential for osteoblast differentiation [32, 33]. It was reported that RUNX2 null mice had no bone tissue, osteoblasts or osteoclasts. RUNX2 is also active in mature osteoblasts, and when active RUNX2 levels have been reduced, decreased expression of the genes encoding the main bone matrix proteins including bone sialoprotein (BSP), OCN, osteopontin (OPN) and COL-I were also observed [34, 35]. In

our study, the level of RUNX2 mRNA expression was increased about 1.2- and 1.5-fold in the 50 and 100 $\mu\text{g/ml}$ LF treatment groups at four days. The results of our study suggested that RUNX2 acted as a critical transcription factor in hADSCs after LF treatment. This may be one of the mechanisms applied in osteogenic differentiation of hADSCs. Further studies are required to realise other signalling pathways of the regulation of osteogenic differentiation of hADSCs.

In our study, the proliferation of hADSCs was increased as a result of the addition of LF in a dose-dependent manner from days four to 14, and the osteogenic differentiation of hADSCs was observed by detecting the expression of several osteogenic differentiation-related marker genes at days four and seven, respectively. Although COL-I, ALP and RUNX2 expressed mainly at the early stage of osteogenic differentiation, we thought a longer time study was needed in order to better explain the complementary processes between proliferation and differentiation. Also there are some limitations of these kinds of in vitro studies (they cannot always mimic in vivo conditions), so the results still need further validation in vivo.

Conclusions

We report for the first time that LF can increase the osteogenic effect by stimulating synthesis of osteogenic differentiation-related marker genes during the proliferation and differentiation phase in hADSCs and could be a promising approach for enhancing osteogenic capacity of cell-based construction in bone tissue engineering.

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