Effect of lactoferrin on rat osteoblast proliferation

Jun Wang, Bi-Yu Zhang, Chuang-Yue Zhong

Department of anesthesia, Huai’an Second People’s Hospital, Huaian, Jiangsu 223002, China

Abstract

Objective: To investigate the effect of lactoferrin on osteoblastic proliferation of rats and to explore the possible mechanisms. Methods: Isolation and purification of rat osteoblasts were performed, the second generation of osteoblasts in the logarithmic growth phase were tested. Osteoblast cells were seeded into the porous plate, adding lactoferrin solution to the final concentration 0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL, respectively, set up the control group (lactoferrin concentration was 0 μg/mL), rat osteoblast cells proliferation was detected by MTT method, insulin-like growth factor-1 (IGF-1) mRNA expression was detected by fluorescence quantitative PCR (real-time PCR). Results: MTT results showed that compared with the 0 μg/mL group, the number of rat osteoblast cells in the other concentration groups increased significantly (P<0.05) except in the 0.1 μg/mL group; The number of 3 d and 5 d rat osteoblasts in 1 μg/mL group was significantly higher than that in 0 μg/mL group, the number of 1 d, 3 d, 5 d and 7 d rat osteoblasts in 10 μg/mL and 100 μg/mL group was significantly higher than that in 0 μg/mL group (P<0.05); With the extension of time, the number of rat osteoblasts in each concentration group was significantly increased with the increase of the concentration, the number of 7 d rat osteoblasts in the 100 μg/mL group was the highest. Real-time PCR results showed that different concentrations of lactoferrin could promote rat osteoblast cells IGF-1 mRNA expression and was dose dependent. The 7 d IGF-1 mRNA expression in 0.1 μg/mL group was significantly higher than that in 0 μg/mL group, the 3 d, 5 d and 7 d IGF-1 mRNA expression in 1 μg/mL and 10 μg/mL were significantly higher than that in 0 μg/mL group, the 1 d, 3 d, 5 d and 7 d IGF-1 mRNA expression in 100 μg/mL were significantly higher than that in 0 μg/mL group (P<0.05); With the extension of time, the expression of rat osteoblasts IGF-1 mRNA in each concentration group was significantly increased with the increase of the concentration, the expression level of 7 d IGF-1 mRNA in the 100 μg/mL group was the highest. Conclusions: Lactoferrin may promote the proliferation of rat osteoblast cells by increasing the IGF-1 mRNA expression, and the effect was dose dependent.

1. Introduction

Osteoporosis is mainly due to the amount of bone formation less than the amount of bone resorption, resulting in bone loss, bone microstructure destruction and the increased bone fragility[1-3]. The amount of bone formation and bone resorption were mainly related to the balance of osteoblasts and osteoclasts[4]. Bisphosphonates and estrogen are clinical commonly used anti-absorption drugs for postmenopausal osteoporosis treatment, but the ability of these drugs on bone mass recovery was limited and had different degrees of side effects. Lactoferrin is an iron binding glycoprotein, mainly exists in epithelial secretions and breast milk[5]. Studies found that[6,7] lactoferrin had multiple biological effects like immune regulation and antibacterial. In recent years, studies suggested that lactoferrin can promote bone growth[8]. Therefore, our study investigate the osteoblastic proliferation promoting effect of lactoferrin on rats and to explore the possible mechanisms.
2. Materials and methods

2.1. Materials and reagents

Lactoferrin was purchased from Suzhou Yong Wei Biotechnology Co., Ltd. The reverse transcription kit, real-time PCR kit was purchased from American BD Company. The 2720 thermal cycler PCR instrument was purchased from the German Eppendorf Company. The DNM-9602 enzyme standard instrument was purchased from Shanghai Jinggong Industrial Co., Ltd. The DU640 ultraviolet spectrophotometer was purchased from Beckman Company in the United States.

2.2. Cell collection and culture

The rat osteoblasts were isolated and purified, and the primary cells were cultured and passaged. Further purified cell and osteoblasts from the second generation were used for subsequent experiments.

2.3. Lactoferrin solution configuration

Dimethyl sulfoxide (DMSO) was used to dissolve lactoferrin and the mother liquor of lactoferrin concentration was 1 000 μg/mL, diluted into 4 kinds of concentration: 0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL.

2.4. Detection of rat osteoblast cells proliferation by MTT method

Rat osteoblast cells were digested and suspended by Trypsin, then inoculated into the porous plate (Each group consisted of 4 plates) and added with 2 mL cell suspension, using low sugar DMEM medium. After cell growth and fusion up to 90% then lactoferrin solution was added to the final concentration 0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL, respectively; the control group was added with equal amount of drug dissolution media, 1 porous plate was taken on the 1 d, 3 d, 5 d and 7 d after drugs administration respectively. The culture and collection of cells was terminated. Each group had 2 holes to carry out the experiment. The experiment was repeated 3 times. Total RNA was extracted from the cells according to the kit instructions. Primers was designed, synthesized and packaged, two step method was used for real-time PCR amplification. It was pre denatured at 95 °C for 30 s, denatured at 95 °C for 30 s, annealed at 60 °C for 30 s, total 40 cycles of amplification. Melting curve analysis was used with β actin as the housekeeping gene. The expression of IGF–1 mRNA was measured by double standard curve method.

2.5. Detection of IGF–1 mRNA by fluorescence quantitative PCR (real–time PCR)

Rat osteoblast cells were digested by Trypsin, then inoculated into the porous plate (Each group consisted of 4 plates) and added with 2 mL cell suspension, using low sugar DMEM medium. After cell growth and fusion up to 90% then lactoferrin solution was added to the final concentration 0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL, respectively; the control group was added with equal amount of drug dissolution media, 1 porous plate was taken on the 1 d, 3 d, 5 d and 7 d after drugs administration respectively. The culture and collection of cells was terminated. Each group had 2 holes to carry out the experiment. The experiment was repeated 3 times. Total RNA was extracted from the cells according to the kit instructions. Primers was designed, synthesized and packaged, two step method was used for real-time PCR amplification. It was pre denatured at 95 °C for 30 s, denatured at 95 °C for 30 s, annealed at 60 °C for 30 s, total 40 cycles of amplification. Melting curve analysis was used with β actin as the housekeeping gene. The expression of IGF–1 mRNA was measured by double standard curve method.

2.6. Statistical treatment

Statistical software SPSS19.0 was used for data analysis, the measurement data were expressed by mean±standard deviation, variance analysis was used for comparison between groups, SNK-q tests for comparison between the two, \( P<0.05 \) indicated difference was statistically significant.

3. Results

3.1. Lactoferrin promotes the proliferation of rat osteoblast cells

MTT results showed that compared with the 0 μg/mL group, the number of rat osteoblast cells in the other concentration groups increased significantly \( (P<0.05) \) except in the 0.1 μg/mL group; The number of 3 d and 5 d rat osteoblasts in 1 μg/mL group was significantly higher than that in 0 μg/mL group, the number of 1 d, 3 d, 5 d and 7 d rat osteoblasts in 10 μg/mL and 100 μg/mL group was significantly higher than that in 0 μg/mL group \( (P<0.05) \); With the extension of time, the number of rat osteoblasts in each concentration group was significantly increased with the increase of

<table>
<thead>
<tr>
<th>Time</th>
<th>0 μg/mL</th>
<th>0.1 μg/mL</th>
<th>1 μg/mL</th>
<th>10 μg/mL</th>
<th>100 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 d</td>
<td>0.213±0.008</td>
<td>0.211±0.009</td>
<td>0.228±0.006</td>
<td>0.301±0.008</td>
<td>0.332±0.011</td>
</tr>
<tr>
<td>3 d</td>
<td>0.417±0.10</td>
<td>0.420±0.011</td>
<td>0.593±0.010</td>
<td>0.687±0.009</td>
<td>0.773±0.013</td>
</tr>
<tr>
<td>5 d</td>
<td>0.709±0.009</td>
<td>0.723±0.007</td>
<td>0.739±0.012</td>
<td>0.843±0.006</td>
<td>0.910±0.015</td>
</tr>
<tr>
<td>7 d</td>
<td>0.978±0.008</td>
<td>0.980±0.009</td>
<td>1.019±0.009</td>
<td>1.208±0.013</td>
<td>1.226±0.011</td>
</tr>
</tbody>
</table>

Ps: Compared with 0 μg/mL group, \( P<0.05 \).
the concentration, the number of 7 d rat osteoblasts in the 100 μg/mL group was the highest (Table 1).

3.3. Lactoferrin promotes IGF–1 mRNA expression of rat osteoblast cells

Real-time PCR results showed that different concentrations of lactoferrin could promote rat osteoblast cells IGF–1 mRNA expression and had dose dependent. The 7 d IGF–1 mRNA expression in 0.1 μg/mL group was significantly higher than that in 0 μg/mL group, the 3 d, 5 d and 7 d IGF–1 mRNA expression in 1 μg/mL and 10 μg/mL were significantly higher than that in 0 μg/mL group, the 1 d, 3 d, 5 d and 7 d IGF–1 mRNA expression in 100 μg/mL were significantly higher than that in 0 μg/mL group (P < 0.05); With the extension of time, the expression of rat osteoblasts IGF–1 mRNA in each concentration group was significantly increased with the increase of the concentration, the expression level of 7 d IGF–1 mRNA in the 100 μg/mL group was the highest (Table 2).

Table 2
<table>
<thead>
<tr>
<th>Time</th>
<th>0 μg/mL</th>
<th>0.1 μg/mL</th>
<th>1 μg/mL</th>
<th>10 μg/mL</th>
<th>100 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 d</td>
<td>1.009±0.004</td>
<td>1.132±0.078</td>
<td>1.174±0.036</td>
<td>1.529±0.044</td>
<td></td>
</tr>
<tr>
<td>3 d</td>
<td>1.082±0.049</td>
<td>1.563±0.072</td>
<td>1.669±0.068</td>
<td>1.893±0.059</td>
<td></td>
</tr>
<tr>
<td>5 d</td>
<td>1.143±0.075</td>
<td>1.842±0.066</td>
<td>1.869±0.064</td>
<td>2.138±0.079</td>
<td></td>
</tr>
<tr>
<td>7 d</td>
<td>1.332±0.067</td>
<td>1.877±0.064</td>
<td>2.014±0.069</td>
<td>2.315±0.060</td>
<td></td>
</tr>
</tbody>
</table>

Ps: Compared with 0 μg/mL group, P<0.05.

4. Discussion

Lactoferrin belongs to the transferrin family, it’s an iron binding glycoprotein, and the molecular weight is 80 kDa. Zong et al[9] found that serum lactoferrin mostly derived from neutrophil, so the concentration of local serum lactoferrin increased during inflammation. Hou et al[10] found that the proximal femur bone mineral density of the lactoferrin feeding rats was significantly increased and bone morphology has been significantly improved. Subsequent studies of Montesi[11] also obtained similar results. Careba et al[12] found that postmenopausal women’s bone formation increased and bone resorption decreased significantly after adding lactoferrin. Therefore, we hypothesize that as an anabolic factor that lactoferrin may have a physiological role on bone healing and bone growth.

In this study, the primary culture was used to obtain osteoblasts, and the biological characteristics of the cells did not change significantly. We used MTT assay to detect osteoblast cells proliferation, the method with objective, quantitative and accurate advantages, mainly through the detection of mitochondria activity in living cells, sensitively reflect the proliferation of attached cells[13,14]. We found that compared with the 0 μg/mL group, the number of rat osteoblast cells in the other concentration groups increased significantly (P<0.05) except in the 0.1 μg/mL group; The number of 3 d and 5 d rat osteoblasts in 1 μg/mL group was significantly higher than that in 0μg/ml group, the number of 1 d, 3 d, 5 d and 7 d rat osteoblasts in 10 μg/mL and 100 μg/mL group was significantly higher than that in 0 μg/mL group (P<0.05); With the extension of time, the number of rat osteoblasts in each concentration group was significantly increased with the increase of the concentration, the number of 7 d rat osteoblasts in the 100 μg/mL group was the highest. The results indicated that lactoferrin could promote the proliferation of rat osteoblast cells, and promote effect was dose and time dependent. The higher drug concentration, the longer action time, the larger number of rat osteoblasts. The postmenopausal osteoporosis often use bisphosphonates and estrogen therapy, compared with this, through physiological supplement, with less side effect and low price, lactoferrin has a potential therapeutic role on osteoporosis and has an irreplaceable advantage[15,16].

Bone mass decrease is mainly due to the excessive activation of osteoclast cells, resulting in a significant reduction in the rate of value added ratio of osteoblast cells, the decreased bone resorption and bone formation[17]. In the process of bone formation, many cytokines and hormones are involved in the balance between osteoclast and osteoblast cell[18,19]. IGF–1 is a protein composed of 70 amino acid residues, mainly secreted by osteoblast cells, it’s one of the main cellular factors of bone metabolism[20]. IGF–1 can combine with insulin like growth factor 1 receptor (IGF–1R), causing IRS–1 phosphorylation and serine/threonine protein kinase (Akt) activation, resulting in bone cell survival and proliferation[21]. Some signal pathways of lactoferrin activated bone cells have been identified, however, details about the downstream paths cross series have yet to be explored. We used real-time PCR to detect different concentrations of lactoferrin treated rat osteoblasts IGF–1 mRNA expression, we found that different concentrations of lactoferrin could promote rat osteoblast cells IGF–1 mRNA expression, the 7 d IGF–1 mRNA expression in 0.1 μg/mL group was significantly higher than that in 0 μg/mL group, the 3 d, 5 d and 7 d IGF–1 mRNA expression in 1 μg/mL and 10 μg/mL were significantly higher than that in 0 μg/mL group, the 1 d, 3 d, 5 d and 7 d IGF–1 mRNA expression in 100 μg/mL were significantly higher than that in 0 μg/mL group (P<0.05); With the extension of time, the expression of rat osteoblasts IGF–1 mRNA in each concentration group was significantly increased with the increase of the concentration, the expression level of 7 d IGF–1 mRNA in the 100 μg/mL group was the highest. The results showed that lactoferrin may promote the rat osteoblast cells proliferation by promoting IGF–1 mRNA expression, the increased IGF–1 mRNA expression dependent on dose and time. The higher drug concentration, the longer action time, the higher rat osteoblasts IGF–1 mRNA expression.

In summary, lactoferrin can promote primary cultured rat osteoblast cells proliferation and increase the expression of IGF-1. Our results shows that lactoferrin may have potential therapeutic effect on osteoporosis and its mechanism may be related to the increase of
IGF-1 expression.

References


