Original

Effects of Leptin on Differentiation and Proliferation of Chondrocytes

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Abstract: Osteoarthritis (OA) is a degenerative joint disease which is one of the most frequent and disabling diseases encountered in elderly individuals. Leptin was unregulated significantly in OA cartilage and was regarded as a metabolic link between obesity and OA. In this study, we evaluated the effect of 10 or 100 ng/ml leptin on the ATDC5 cells. Results revealed that 10 or 100 ng/ml of leptin significantly decrease the expression of aggrecan and type II collagen mRNA and protein levels compare to control (0 ng/ml). Interestingly, sex determining region Y-box 9 mRNA and protein was down-regulated by leptin. Collagen X is a specific marker of hypertrophic and calcified chondrocyte. It has been noted that both mRNA and protein of Collagen X was significantly upregulated with leptin treatment. Meanwhile, Alcian Blue staining indicated that leptin reduces the synthesis of proteoglycan. Alizarin red S staining and alkaline phosphatase activity detection demonstrated that leptin induced cartilage nodules formation and mineralization in a dose dependent manner. We conclude that leptin slightly inhibits the proliferation of ATDC5 cells and affects cartilage-specifically marked proteins and genes. In summary, it has been suggested that leptin stimulates hypertrophic differentiation of ATDC5 cells.

Key words: Leptin, ATDC5 cells, Differentiation, Proliferation

Introduction

Osteoarthritis is one of the most common degenerative joint diseases which results from the loss of balance between degradation and repair in cartilage, and characterized by increased activity of matrix metalloproteinases (MMP-1, -3 and -13) and decreased expression of COL2 (type II collagen) and aggrecan. Previous studies have reported that obesity may result in cartilage degeneration not only in weight-bearing but also non-weight-bearing joints. In terms of mechanism, there were evidence suggested that leptin, secreted by white adipose and correlated with obesity and OA. Overexpression of leptin and its receptor involvement in chondrocytes derived from osteoarthritis. Accordingly, it is reasonable for us to conjecture that leptin might be the underlying link between obesity and OA.

The clonal mouse embryonic cell line ATDC5 cells, which are capable of differentiating into chondrocytes in vitro in a multistep process, from chondrogenitor mesenchymal cells to calcified chondrocytes were used as a model of endochondral cartilage.

In the present study, we analyzed the effects of leptin on chondrocytic proliferation and differentiation using murine ATDC5 chondrocytic cell line. The present results suggest that leptin plays an important role in the regulation of differentiation of ATDC5 cells. It stimulates the hypertrophic differentiation and inhibits proliferation of chondrocytes.

Materials and methods

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Cellculture

The murine chondrocytic cell line ATDC5, derived from the rib growth plate cartilage of a p53-null mouse, was chosen for these studies because it has been shown to be a useful in vitro model for examining the whole process of chondrocyte maturation and terminal differentiation toward hypertrophic as characterized by their morphology and expression of genes including type II and X collagens and aggrecan. Undifferentiated ATDC5 cells proliferate rapidly until they reach confluence, at which point they undergo growth arrest. Insulin treatment causes confluent ATDC5 cells to re-enter a proliferative phase and form cartilaginous matrix nodules.

ATDC5 cells were purchased from Shanghai Mito Biological Technology co., LTD. ATDC5 cells were plated on dishes at an initial density of 2×10⁴ cells per well in 6-multiwell plate at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Once the cells became confluent (on day 4 after seeding), ATDC5 cells were cultured in a 1:1 mixture of Dulbecco’s Modified Eagle Medium (DMEM)/F12, supplemented with 5% fetal bovine serum (FBS), 10 μg/ml Insulin (sigma, USA), 10 μg/ml human transferrin (sigma, USA), 3×10⁻⁸ mol/l sodium selenite (sigma, USA), 2 mM glutamine and antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin).

To examine the effect of leptin on proliferation and differentiation of ATDC5 cells, the culture medium was replaced every other day from Day 4 to Day 14 supplemented with recombinant human leptin (ProSpec-Tany TechnoGene Ltd., USA) at various concentrations (0, 10, 100 ng/ml respectively).

RNA isolation

Total RNA was isolated from ATDC5 cells using trizol reagent ac-
According to the manufacturer's protocol. The concentration was determined by measurement of the optical density at 260 nm using a NanoDrop ND-1000 Spectrophotometer (Labtech, Palaiseau, France). Reverse transcription reactions were performed from 0.2 μg of total RNA using PrimeScript™ RT Master Mix kits (Takara Biotechnology (Dalian) Co., Ltd.) following the manufacturer's protocol. Three different samples were used (n=3). Relative quantification real-time PCR reactions was carried out in S1000™ Thermal Cycler (BIO-RAD) system using SYBR® Green I PCR kit (THUNDERBIRD™ SYBR® qPCR Mix, TOYOBO CO., LTD.).

Reaction conditions were as follows: denaturation at 94°C for 30 seconds; annealing for 34s at 50.0°C for GAPDH and COL2A1, 50°C for COL2A1, 48°C for SOX9 (sex determining region Y-box 9) and COL10a1; and extension at 72°C for 20s (all genes 45 cycles). Primers were designed in-house and synthesized by Generay Biotech Co., Ltd, Shanghai, China.

Reactions were run in triplicate and relative expression level of each target gene was normalized to GAPDH using the comparative CT method. The results are presented as mean ± SD of three duplicate runs from a representative experiment.

Western Blot
ATDC5 Cell were rinsed with ice-cold phosphate buffered saline and harvested in lysis buffer for protein extraction (1M Tris/HCl, pH 6.8, 5 mM EDTA, 15 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM PMSF and protease inhibitor cocktail from Thermo Scientific). Protein concentrations of the cell lysates were quantified using the Coomassie Plus Protein Assay Kit (Bio-Rad). Identical amounts (30 μg of protein) of total cellular lysates from ATDC5 cells were separated by electrophoresis on Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (30% acrylamide) and then transferred onto a nitrocellulose filter membrane (NC) (Pall Corporation, 8780 Ely Rd, Pensacola, FL). Loading amounts of the protein was adjusted according to the protein concentration of each specimen. The membranes were blocked in PBS containing 20% tween-20 (PBST) plus 5% nonfat dry milk for 1 h at room temperature, and then incubated overnight at 4°C with primary antibody. Specific primary antibodies including rabbit anti GAPDH at a dilution of 1:10,000 (Abcam), rabbit anti-COL2 at a dilution of 1:3000 (Abcam), rabbit anti-COX 10 at a dilution of 1:300 (bioso, Beijing, China), rabbit anti-SOX9 at a dilution of 1:1000 (Abcam), rabbit anti-AGG at a dilution of 1:600 (Abcam) were used for the assays. Incubation with GAPDH (1:10,000, abcam) was used as the loading internal control. After 3 times washing with TBST, the membranes were incubated for 1 h at room temperature with goat anti-rabbit-IgG (ZSGB-BIO, Beijing, China) diluted 1:2000 in 5% nonfat dry milk. The detection of bound antibodies was performed using the Amersham Imager 600 (GE Healthcare Life Science, USA). Densitometry was calculated using Scion Image software, and values are expressed as mean ± 95% confidence interval with statistical significance determined by Student’s t-test.
Quantitative densitometric values of each protein were normalized to GAPDH.

**Alcian blue staining and Alizarin red staining**

ATDC5 cells were rinsed twice with phosphate buffered saline (PBS), and then fixed in 4% (v/v) formalin for 10 min at room temperature and stained with 0.1% Alcian blue solution (SIGMA-ALDRICH, Co., 3050 Spruce Street, St. Louis, MO, USA) for 30 minutes or with 1% (w/v) Alizarin red (Beijing Solarbio Science & Technology Co., Ltd, China) for 10 min. Stained cells were washed three times with PBS and photographed with a scanning camera (Epson Perfection 4490 Photo).

**Alkaline phosphatase activity**

Cell layers were homogenized in lysis buffer (20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 protease inhibitor cocktail) at 0°C and centrifuged for 15 minutes at 12,000 g. The supernatant collected as the cell extract, which contained 95% of the total activity, was assayed assessed in a reaction mixture of 0.5 mM pNPP (Aladdin) as the substrate and 0.5 mM MgCl$_2$ for 15 min at 37°C. The reaction was terminated by the addition of 0.25 volume of 1 M NaOH, and p-Nitrophenol concentration was determined by spectrophotometry at 410 nm. p-nitrophenol was used as a standard. The enzyme activity was expressed in nanomoles of pNPP cleaved per minute per microgram of DNA. DNA content was determined by the method of Johnson-Wint.

**Results**

**Effects of leptin on production of proteoglycan and formation of mineralization nodules in ATDC5 cells**

Aggregated proteoglycans is one of the major structural components of the matrix, which was stimulated by the addition of leptin to ATDC5 cells as compared to the control group. The results showed that the expression of proteoglycan decreased with the increase of leptin concentration (Fig. 1A).

Alizarin red densely stained the nodules was detectable in Fig. 1B. Indicating enhanced mineralization of ATDC5 cells following leptin treatment as compared to the controls. The cultures were relatively less mineralized in the absence of leptin, and few Alizarin red S positive nodules were seen in the control. The cartilage nodules were increased in size and conjoin over the ECM with the increase of leptin concentration.

**Effects of leptin on Alkaline phosphatase activity of ATDC5 cells.**

Alkaline phosphatase (ALP) activity can be used as a marker of hypertrophic chondrocytes involved in the mineralization process. To understand the role of leptin on mineralization in ATDC5 cells, enzymatic activity of ALP in ATDC5 cells were measured (Fig. 2). The results showed that ALP activity was significantly increased in cells treated with leptin compared to control cells in a dose dependent manner.

**Effect of leptin on the expression of ATDC5 cell proliferation and differentiation marked gene**

To determine the effect of different amounts of leptin on proliferation of ATDC5 cells, we treated ATDC5 cells with leptin (0, 10 and 100 ng/ml) for 24 h and monitored expression levels of type II collagen, SOX9, aggrecan as early markers for the onset of chondrogenesis by real-time PCR and western blot analysis (Fig. 3).

We found that either 10 ng/ml or 100 ng/ml of leptin were sufficient to inhibit early chondrogenesis in ATDC5 cells with a significantly decrease expression of aggrecan in mRNA and protein levels (Fig. 3A and B). The mRNA expression levels of aggrecan in ATDC5 cell stimulated with either 10 ng/ml or 100 ng/ml of leptin were significantly lower than those without leptin treatment. Protein level of aggrecan was slightly down-regulated by 10 ng/ml of leptin, but the difference with untreated cells reached statistical significance only by 100 ng/ml of leptin. Leptin suppress the expression of aggrecan in a concentration-dependent man-

**Table 1. List of primer sequences**

| Name     | Sequence                | Product   | size/bp |
|----------|-------------------------|-----------|---------|
| GAPDH-F  | GATGCCCATGTTTGATG       | GU214026.1| 162     |
| GAPDH-R  | GGCATGGACTGTTGCAATG     | NM_009925 | 150     |
| COL10A1-F| CCGCTTGTCAGTGCTAACCA    | NM_031163.3| 150 |
| COL10A1-R| ACCAGATCTTGGTGTAGTCATG  | NM_031163.3| 150 |
| COL 2A1-F| CGAGTGGAGAGCCGAGACT     | NM_031163.3| 150 |
| COL 2A1-R| CTCTTTGCTGCCACACCAGTT   | NM_031163.3| 150 |
| Aggrecan-F| AATTGGAGAATGGCGGTCCAA   | L07049.1  | 150     |
| Aggrecan-R| AGGCCACTGTGCCCCTTTTTA   | NM_080403.1| 153    |
| SOX9-F   | CTCCCAAAACAGACGTGCA     | NM_080403.1| 153    |
| SOX9-R   | CGAAGGTCCTGATGTTGGAGAT  | NM_080403.1| 153   |
Figure 3. Gene and protein expression profiles of proliferation markers of ATDC5 cells: Aggrecan mRNA (A), Aggrecan protein (B), COL2A1 mRNA (C), COL2 protein (D), SOX9 mRNA (E), SOX9 protein (F), COL10A1 mRNA (G), COL10 protein (H). ATDC5 cells were treated with leptin (0, 10 and 100 ng/ml) for 24 h after cultured for 14 days. The expression of ATDC5 differentiation mark gene and protein were measured by real-time PCR and/or Western blot analysis. For every group, experiments were carried out in triplicate and results were expressed as means ± standard error of the mean over control values. mRNA expression levels in ATDC5 cells treated with leptin are relative to those of control (0 ng/ml). GAPDH was used as a protein loading control in western blot. *, # indicate P < 0.05 between two groups as indicated. ** or ## indicate P < 0.01 between two groups as indicated.
ner. Gene and protein of COL2 was significantly downregulated by leptin (100 ng/ml) treatment. Additionally, depressed level of COL2 gene and protein was also found at 10 ng/ml of leptin, but the difference with unstimulated cells did not reach statistical significance. It has been reported that SOX9 plays an essential role in the chondrogenic differentiation by regulating the transcription of collagen type II and aggrecan. So, we examined the expression of SOX9 in ATDC5 cells treated with different doses of leptin. Protein and mRNA expression level of SOX9 was decreased upon leptin treatment in a concentration-dependent manner (Fig. 4E and F).

To determine whether expression level of COL10 (type X collagen), which is hypertrophic and calcified chondrocyte-specific and altered by exogenous leptin stimulation, the ATDC5 cells were analyzed by real-time PCR and western blot. Real-time PCR and Western blot analysis of COL10 expression in ATDC5 cells treated with the indicated concentrations of leptin for 24 h. The experimental set-up was paralleled and identical to the methods used to measure COL2, aggrecan and SOX9.

Quantitative real-time PCR analysis showed that Leptin was able to significantly up-regulate COL10 gene expression in ATDC5, Leptin at 10 ng/ml and 100 ng/ml upregulated the COL10 mRNA expression by approximately 12.57 and 113.7-fold respectively compared with control (0 ng/ml) (Fig. 3G). Western blot analysis showed that protein of COL10 was 2 fold and 4 fold increased respectively, after treated with 10 ng/ml and 100 ng/ml of leptin compared to control group (Fig. 3H). The difference of expression level of COL10 mRNA between control and cells upon treatment with 100 ng/ml of leptin was significantly higher.

**Discussion**

It is known that leptin is a very important adipocyte-derived factor that involved and plays key roles in many processes including immune, inflammatory response, neuroendocrine7. Leptin is associated with many inflammatory conditions of articular cartilage mainly, infectious arthritis, rheumatoid arthritis and Osteoarthritis8,9. One study has demonstrated that Leptin-deficient ob/ob mice develop resistance to experimental antigen-induced arthritis in comparison with wild-type mice9. Osteoarthritis is a common chronic degenerative joint disease characterized by progressive articular cartilage destruction. It can be confirm by a serious of observations that leptin is involved in OA development and related to the grade of cartilage destruction10. Although experiments and clinical evidences have confirmed that leptin involve in the homeostasis of cartilage and development of OA, but the effects it exerted on these pathophysiological processes seemed to be contradictory and still remain unclear. It was reported that leptin enhanced the proliferation of cultured normal chondrocytes, but reduced the proliferation of OA chondrocytes with the same culture medium11. Another study showed that leptin affected proliferation of chondrocytes in a biphasic manner, which stimulated proliferation in the range between 10 ng/ml and 100 ng/ml12. Thus, taken together, it is plausible that leptin involving in the proliferation and differentiation processes of chondrocyte is correlated with the local microenvironment and itself. Therefore, further studies are clearly needed to explore the exact effects of leptin on chondrocyte by cultivating chondrocyte in vitro and illustrate the possible mechanisms. To exclude all kinds of extracellular interferences, ATDC5 cell treated with different concentration of leptin were used in this experiment and three representative factors of chondrocyte differentiation including aggrecan, type II collagen, SOX9 which are highly expressed in early chondrogenesis were detected13. The expression of Type II collagen, which is known as the cartilage-characteristic extracellular matrix, becomes higher in the differentiated cells14. One previous research indicated that leptin promotes the expression of collagen type 2B through different downstream signaling pathway15. However, the effects of leptin on the expression of collagen type 2A in ATDC5 cells still remains unknown. Real-time PCR results in this experiment showed that leptin decreased the expression of COL2A1 mRNA in a dose-dependent manner. The effect of leptin on the expression of COL2 is consistent with the results of the gene. To further verify the effects of leptin on COL2A1 expression, the expression of SOX9 representing one important transcription factor of COL2A1 was measured by real-time PCR and Western blot. Leptin promote the expression of SOX9 mRNA and stimulate the expression of its protein in a dose-dependent manner, respectively. It is well known that the transcription factor SOX9 plays a key role in the chondrogenic differentiation by regulating collagen type II and aggrecan. In order to evaluate the influence of leptin on the differentiation of ATDC5 cells, the expression of aggrecan was investigated using real-time PCR and Western blot respectively. The results of these two arrays indicated leptin significantly inhibited the expression of aggrecan both at mRNA and protein levels. Taken together, the data highlighted the fact that leptin suppresses the chondrogenesis by affecting the expression of chondrocyte specialized factors such as aggrecan, type II collagen and SOX9. Previous studies have shown that leptin treatment promotes cartilage formation and proteoglycan and collagen synthesis in chondrocytes16. The results shown by others that leptin stimulate anabolic functions on chondrocytes may due to the induction of growth factors synthesis by leptin indirectly but not by leptin directly17. In addition to the effects of leptin on chondrogenic, it has been demonstrated that leptin also has effects on differentiation of chondrocyte. In an attempt to determine the concrete effects of leptin on chondrocyte, expression of type X collagen was detect in ATDC5 cell stimulated with a series of leptin concentrations. Both real-time PCR and Western blot analysis showed that leptin significantly increase the expression of type X collagen, a unique marker of hypertrophy in ATDC5 chondrocytes, Leptin upregulated the type X collagen mRNA expression by approximately 12.57 and 113.7-fold respectively when used used at either 10 ng/ml and 100 ng/ml. These results are in line with many earlier studies which showed that type X collagen in chondrocyte stimulated with leptin. Especially, Kishida et al have proven that type X collagen expression was decreased significantly in growth plates from ob/ob mice compared with those from wild-type mice. Type X collagen is expressed exclusively by mature growth-plate chondrocytes18. Therefore, the current study suggests that leptin significantly promote endochondral ossification process in a concentration-dependent manner, which may be contributed to the formation of osteophyte in the late stage of OA. In addition to the effects on the proliferation and differentiation of chondrocyte, a lot of studies have confirmed that leptin have an impact on catabolism of chondrocyte through production of many pro-inflammatory cytokines such as IFN-g, IL-1 and nitric oxide, ultimately cause the degradation of cartilage. In summary, our results indicate that leptin slightly inhibit the proliferation of chondrocytes by down-regulating the expression of proliferation-related marks such as aggrecan and type II collagen. Meanwhile, it stimulates the hypertrophic differentiation of chondrocytes and results in the calcification of cartilage. It has been suggested that leptin induced chondrogenic differentiation to form cartilage nodules in a dose dependent manner.

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Competing Interests
The authors declare there are no COI regarding the publication of this paper.

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