Effect of Pioglitazone on Transdifferentiation of Preosteoblasts from Rat Bone Mesenchymal Stem Cells into Adipocytes

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Rat Bone Mesenchymal Stem Cells into Adipocytes

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Summary: We aimed to examine the effect of pioglitazone on transdifferentiation of preosteoblasts from rat bone marrow mesenchymal stem cells (BMSCs) into adipocytes and investigate its effect on bone metabolism. BMSCs were harvested from the femurs and tibias of a rat, then separated, purified, proliferated for 3 generations and differentiated into preosteoblasts for 5 days and 14 days respectively in the presence of osteogenic medium. Thereafter, the preosteoblasts were cultured for 21 days in the presence of adipogenic medium with and without pioglitazone (1 μg/mL). Partially-differentiated osteoblasts were identified by mineralized nodules with Alizarin red S staining. Transdifferentiated adipocytes were identified by Oil Red O staining. Reverse transcription PCR (RT-PCR) was performed to assay the expression levels of osteogenic markers Runx2 and ALP, and an adipogenic marker PPARγ. Those cells cultured for 5 days showed much more adipocytes separately as detected by Oil Red O staining. Whatever the time was 5 days or 14 days of BMSCs osteogenic differentiation, the cells cultured with pioglitazone showed much more adipocytes than those without pioglitazone. Our experiment showed that the less time it took for BMSCs osteogenic differentiation, a stronger ability remained for BMSCs to transdifferentiate into adipocytes. The mRNA expression levels of Runx2 and ALP were decreased by 1.79 and 1.90 times respectively in 5EG (P<0.05) as compared with 5CG, and that of PPARγ was increased by 1.31 times in 5EG (P<0.05) as compared with 5CG. The mRNA expression levels of Runx2 and ALP were decreased by 1.45 and 1.54 times respectively in 14EG (P<0.05) as compared with 14CG, and that of PPARγ was increased by 1.39 times in 14EG (P<0.05) as compared with 14CG. It was concluded that pioglitazone stimulated the transdifferentiation of BMSCs into adipocytes. These observations provided a potential mechanism of imbalance in thiazolidinedione induced bone metabolism.

Key words: osteogenesis; adipogenesis; pioglitazone; bone metabolism

Thiazolidinediones (TZDs) are a group of effective and frequently prescribed medications for diabetes and may have the potential to be used as a prevention treatment in adults and adolescents at high risk of diabetes[1-3]. TZDs increase insulin sensitivity via activation of peroxisome proliferator-activated receptor (PPAR)-γ receptors. However, TZDs treatment increases the loss of bone mass and bone marrow fat content, decreases bone mineral density, and at last, increases the risk of bone fracture in type 2 diabetic patients[1-3]. Until now the mechanism of its effects on bone metabolism has not yet been elucidated. Schilling et al found that the plasticity between osteogenesis and adipogenesis extends into the differentiation pathways of both cell lineages and may contribute to the age-related expansion of adipose tissue in human bone marrow[4]. Ali et al reported that the secretion of calcium deposition and osteocalcin in femur bone marrow mesenchymal stem cells (BMSCs) differentiating to osteoblasts in roglitazone (which belongs to TZDs) intervention group is decreased by 90% or more at the 3rd and 7th day, whereas only a modest inhibitory effect was observed when the roglitazone was added on the 11th or 15th day[5]. It shows that long-term osteoblast differentiation culture inhibits the ability of osteoblast precursor cells to differentiate to fat cells. Concluding with these findings, we consider that BMSCs are common precursors of osteoblasts and adipocytes. The differentiation of them can be influenced by TZDs. In the present study we established a cell culture system of BMSCs that allows for osteogenic and adipogenic differentiation. The effect of pioglitazone, a representative of TZDs, on transdifferentiation of preosteoblasts into adipocytes was investigated.
1 MATERIALS AND METHODS

1.1 Materials and Reagents

Healthy Sprague-Dawley (SD) male and female rats, weighing 120–180 g, were obtained from the Experimental Animal Center, Tongji Medical College, HUST (China). Cell culture regents L-DMEM, Taq enzyme and premium grade fetal bovine serum (FBS) were purchased from Gibco (USA). Penicillin, streptomycin and recombinant human insulin were purchased from Novo Nordisk (Denmark). β-glycerophosphate (β-GP), VitC powder, dexamethasone, 3-isopropyl-1-methyl xanthine (IBMX), oil red-O, alizarin red powder and trypsin were purchased from SIGMA (Germany). Original pioglitazone drug and reverse transcriptase kit were purchased from Promega Corporation (USA). PCR primers were purchased from Invitrogen Biotechnology Co. (China).

1.2 Cell Culture

BMSCs were isolated from rat tibia and femoral bones and resuspended in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. The culture medium was replaced every other day. Homogenous BMSCs were obtained through 3 generations. Differentiation of osteoblasts was induced by the addition of osteogenic medium containing 2% FBS, 50 µg/mL VitC, 10⁻⁶ mol/L dexamethasone and 10 mmol/L β-glycerophosphate (β-GP). Differentiation of adipocytes was induced by the addition of adipogenic medium containing 2% FBS, 10 mg/mL insulin, 10⁻⁶ mol/L dexamethasone and 0.5 mmol/L IBMX.

1.3 Experimental Groups

BMSCs were cultured in osteogenic medium for 5 days then in adiogenic medium for another 21 days without pioglitazone (5-day control groups, 5CG); BMSCs were cultured in osteogenic medium for 5 days then in adiogenic medium for another 21 days with 1 µg/mL pioglitazone (5-day experimental groups, 5EG); BMSCs were cultured in osteogenic medium for 14 days then in adiogenic medium for another 21 days without pioglitazone (14-days control groups, 14CG); BMSCs were cultured in osteogenic medium for 14 days then in adiogenic medium for another 21 days with 1 µg/mL pioglitazone (14-day experimental groups, 14EG).

1.4 Osteogenic Differentiation

Confirmation of osteogenesis was done by means of alizarin red S staining, and the assessment of ALP activity. In brief, BMSCs cultures were fixed as described for ALP staining and then treated with a 40 mmol/L alizarin red S solution (pH 4.2) for 10 min. Stained cultures were imaged and then quantified as described for ALP staining.

1.5 Adipogenic Differentiation

Adipogenic differentiation was evaluated using Oil Red O staining, which shows the presence of triglyceride deposits. In brief, BMSCs cultures were washed with PBS, fixed in 4% paraformaldehyde in 0.12 mol/L phosphate buffer for 10 min and stained with three volumes of Oil Red O (0.3% in isopropanol) and two volumes of H₂O for 15 min at room temperature. Adipogenic-differentiated cells were recognized by their characteristics being round shape and containing a single large lipid droplet. For counts randomly selected, microscopic fields were photographed at a magnification of 10×times.

1.6 RNA Isolation

Total cellular RNA was isolated using TRIZOL (Invitrogen, Germany). One microgram of extracted total RNA was digested with DNase I (Invitrogen Life Technologies, Germany) according to the manufacturer’s protocol.

1.7 Reverse Transcription PCR (RT-PCR)

RNA was reverse-transcribed with MMLV reverse transcriptase (Promega GmbH, Germany) and cDNA was amplified with Taq DNA polymerase from Peqlab GmbH (Erlangen, Germany). The primer sequences were as follows: PPARγ, 5′-CCCTTTACCAGGGTGATTTCTC-3′ (sense primer) and 5′-GCAAGGCTCTACTTTGATCGCAC-3′ (antisense primer); Runx2, 5′-CCATAACGGTCTTCACAAATCC-3′ (sense primer) and 5′-ACTTGGTGCTGATTCAGGGAG-3′ (antisense primer); ALP, 5′-TATGGGCTACCTGTCTTCA-3′ (sense primer) and 5′-GCTGTTCCATTTGGCTC-3′ (antisense primer). GAPDH was used as a reference gene (sense primer: 5′-GGAGTCTACTGGCGTCTTCA-3′, antisense primer: 5′-ATGGACTGTGGTGATGCC-3′). The PCR products were separated using electrophoresis on 6% polyacrylamide gels and then dried, and autoradiography and image analysis was carried out using MacBAS (Fuji Film, Japan).

1.8 Statistical Analysis

The results were expressed as X±s. The statistical difference was analyzed by one-way ANOVA followed by Dennett’s test. *P<0.05 was considered to be significant. All assays were performed in triplicate.

2 RESULTS

2.1 Alizarin Red Staining

The cells were stained positively for extracellular mineralization after 14 days of osteogenic induction, as confirmed by alizarin red staining. The cells were stained negatively for extracellular mineralization after 5 days of osteogenic induction although cells were confluent. At 14th day, brown mineralized nodes were found. These findings may be related to the short time of osteogenic differentiation (fig. 1).

![Fig. 1 Alizarin red staining after osteogenic differentiation](image)

A: Cells were confluent but no Alizarin red staining was noted after 5 days; B: Brown mineralized nodes were found after 14 days (×100).

2.2 Oil Red O Staining

The Oil Red O staining was performed at the indicated time points in four groups. The positive fat cells were orange red under a light microscope. As compared with control groups, the number of fat cells, the lipid droplets and the volume of fat cells were increased in