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Establishment of a novel method for enriching osteoblast progenitors from adipose tissues using a difference in cell adhesive properties

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Abstract

In the clinical field, cell-based therapies are used to treat bone defects. Adipose tissues contain many osteoblast progenitors, among other cell types. We separated mouse adipose tissue-derived stromal cells (ATSCs) according to their cell adhesive properties. Cells in a fraction adherent to the culture dishes 0.5 h after inoculation (AF-0.5) had a potent ability to differentiate into both osteoblasts and adipocytes in vitro. Their differentiation pathways depended on the culture conditions. In these cells, the expression of marker genes for osteoblast differentiation was induced in osteogenic medium. Moreover, the AF-0.5 cells, which were induced to differentiate into osteoblasts in vitro, formed abundant bone tissues in vivo. These results suggest that the AF-0.5 cells have been enriched with bi-potential progenitor cells destined for either osteoblasts or adipocytes. This simple and efficient method for preparing osteoblast progenitor cells from ATSCs may be utilized for bone defect treatment clinically.

Keywords: Adipose tissues; Osteoblasts; Adipocytes; Bone formation

Several methods involving scaffolds and growth factors have been studied to treat bone defects in the clinical field, especially for orthopaedic surgery. Cell-based therapies are also used for tissue regeneration in vivo, including bone [1]. Among these therapies, bone marrow cells are the most widely used source of osteoblast progenitors due to the presence of multipotent mesenchymal stem cells (MSCs), which are capable of differentiating into adipocytes, osteoblasts, and chondrocytes [2,3]. However, the preparation of bone marrow is an invasive treatment for patients, and the efficiency of MSCs to differentiate into osteoblasts is not high enough in vivo. Thus, a more simple and non-invasive method is needed to prepare osteoblast progenitors for clinical application.

Recently, it was shown that not only bone marrow but also adipose tissues contain osteoblast progenitors [4,5]. These adipose tissue-derived stromal cells (ATSCs) can be induced to differentiate into osteoblasts under osteogenic conditions in vitro and in vivo [6–8]. The mature adipocytes express tissue-specific markers, such as leptin and lipoprotein lipase (LPL). In contrast, the differentiated osteoblasts show such bone specific markers as alkaline phosphatase (ALP) activity, expression of parathyroid hormone receptor (PTHR), and osteocalcin secretion. The process of differentiation of ATSCs into functionally specialized cells is regulated by the individual tissue-specific transcription factors, such as peroxisome proliferators activated receptor γ (PPARγ) and CCAAT enhancer-binding protein α (C/EBPα) for adipocytes, and runt-related transcription factor 2 (Runx2) and osterix for osteoblasts. Adipose tissues contain a large number of cells and are easily obtained from small samples of subcutaneous tissues. Thus, adipose tissues are useful when preparing autologous progenitor cells for patients who require cell-based therapies to repair damaged tissues.

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Recently, Qu-Petersen et al. separated three different populations of muscle cells using different cell adhesive properties [9,10]. In the present study, we applied a similar technique to mouse ATSCs to enrich the osteoblast progenitors from mixed cell populations. We found that a fraction adhered to the culture dish within 30 min after inoculation had a potent capacity to differentiate into osteoblasts in vitro. Moreover, these cells formed abundant bone tissues in vivo, when they are implanted into mice with a scaffold. Thus, this simple and efficient method of osteoblast progenitor preparation from ATSCs could be used to clinically treat bone defects.

Materials and methods

Preparation of mouse ATSCs. Primary ATSCs were prepared from inguinal adipose tissue of 6-week-old C57BL/6J mice. The adipose tissues were washed extensively with Dulbecco's modified Eagle's medium (DMEM) and digested for 120 min at 37 °C with 0.1% collagenase type I (Wako Pure Chemical Industries, Osaka, Japan). The cells were suspended in DMEM containing 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin) (control medium), and then centrifuged at 3000 rpm for 10 min. The pellet was resuspended in control medium and treated with a 100 μm nylon mesh (Becton–Dickinson, NJ). The cells obtained were used as ATSCs. These ATSCs were inoculated in collagen type I-coated dishes (Becton Dickinson) for 0.5, 2, or 6 h. The non-adherent cells were transferred into new dishes and cultured further until confluence was reached. Cell numbers in the adherent fractions were determined using a hemocytometer after trypsinization. On day 7, the adherent cells in both adherent and non-adherent cultures were ready for experimentation.

Induction of osteoblast and adipocyte differentiation. Osteoblast and adipocyte differentiation were induced by the ATSCs with the osteogenic medium containing 150 ng/ml of bone morphogenetic proteins 2 (BMP-2) (Astellas Pharma, Tokyo, Japan) and adipogenic medium containing 1 μM dexamethasone, 0.01 mg/ml of insulin, 0.5 mM isobutylmethylxanthine, and 0.5 mM indomethacin, respectively. For adipocyte differentiation, the medium was replaced with control medium containing 0.01 mg/ml of insulin on day 2. To induce mineralization, the cells were cultured with osteogenic medium containing 50 μM ascorbate-2-phosphate and 10 mM β-glycerophosphate.

Characterization of cell properties. Cell growth rate was examined using the MTT assay kit (Dojindo Lab., Kumamoto, Japan). The amount of osteocalcin secreted into the culture medium was determined with the mouse osteocalcin EIA kit (Biomedical Technologies, MA). ALP activity and calcification were determined by biochemical techniques, as described previously [4,7]. The adipocytes were stained with Oil red O (Hokudo, Sapporo, Japan).

Reverse transcriptase polymerase chain reactions. Reverse transcriptase polymerase chain reactions (RT-PCR) was performed as described previously [10]. In brief, cDNA was synthesized from total RNA using SuperScript III Reverse Transcriptase (Invitrogen, CA). PCR amplification was performed with Platinum *Pfx* DNA Polymerase (invitrogen) using the Gene Amp PCR System 2400 (PerkinElmer, MA). Primers used are listed in Table 1. The PCR products were semi-quantitatively analyzed with an image analyzer (Densitograph, ATTO, Tokyo, Japan). The relative expression intensity was calculated according to the following

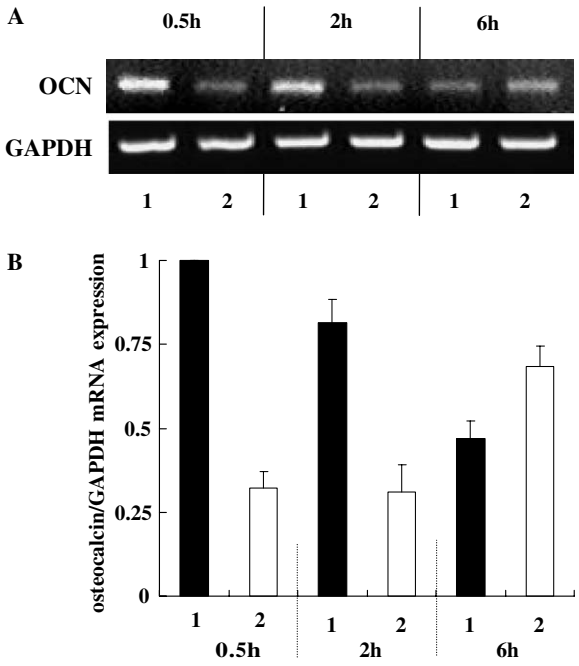

Fig. 1. Comparison of an osteoblast differentiation in fractions prepared from mouse ATSCs. ATSCs were prepared from mouse adipose tissues using collagenase type I. The cells were inoculated into culture dishes for 0.5, 2, and 6 h, and then non-adherent cells were transferred to new dishes. The cells in adherent (lane 1) and non-adherent (2) fractions at each time-point were expanded separately and cultured in the osteogenic medium for 5 days. (A) The osteoblast differentiation was determined by the level of osteocalcin mRNA in RT-PCR. GAPDH was examined as the internal control. Osteocalcin and GAPDH were amplified for 30 and 25 cycles, respectively. (B) Semi-quantitative mRNA analysis for expression ratios of osteocalcin mRNA. The data are presented as the mean mRNA levels (±SD) relative to the cells in adherent fractions at 0.5 h (relative expression = 1.0).

Table 1
Primers sequences used

Primer	Forward	Reverse	Reference
Runx2	GATGATGACACTGCCACCTCTG	CGGGTACCATTGGGAACTGATA	HM
Osterix	GTGAATTCACCTTTCAGCCCCCAAAACC	TGGGATCCCAGCTGTGAATGGGCTTCTT	[15]
ALP	GATCATTCCCACGTTTTCAC	TGCGGGCTTGTGGGACCTGC	HM
PTHR	GTATCTGTGGGGCTTCACCATC	CACCTCACCATTGCAGAAACAG	HM
Osteocalcin	TTCATGTCCAAGCAGGAGGGCAA	ACCGTAGATGCGTTTGTAGGCGGT	[14]
C/EBPα	AAACTCGCTCCTTTTCCTACCG	ACCTGGCCTGTTGTAAGCTGAG	HM
PPARγ	CTGATGCACTGCCTATGAGC	CAGACTCGGCACTCAATGGC	HM
LPL	TCCAGAGTTTGACCGCCTTC	TTGGTCAGACTTCCTGCTACG	[16]
Leptin	TGTCTACTCATGCCAGCACTCA	CACAATCTGGGAACAAGCCATA	HM
GAPDH	TGAAGGTCGGTGTGAACGGATTTGGC	CATGTAGGCCATGAGGTCCACCAC	HM

HM, homemade.