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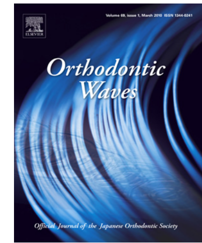

Original article

Gene expression profiles of early chondrogenic markers in dedifferentiated fat cells stimulated by bone morphogenetic protein 4 under monolayer and spheroid culture conditions *in vitro*

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ABSTRACT

Purpose: Human dedifferentiated fat (hDFAT) cells are thought to be a promising cell source for cartilage regeneration therapy. Nevertheless, the responses of hDFAT cells to bone morphogenetic proteins (BMPs) are still unclear. Here, we elucidated the effects of BMP-4 on the mRNA expression of early chondrogenic markers in hDFAT cells under monolayer or pellet cell culture conditions.
Materials and methods: Monolayer and pellet cell cultures of hDFAT cells were grown with control medium or chondrogenic medium (CM) with or without BMP-2, BMP-4, or BMP-7. Real-time polymerase chain reaction was used to analyze the mRNA expression levels of nine genes: chondrogenic markers, *i.e.*, *SOX9*, *SOX5*, *SOX6*, aggrecan, type 2 collagen, type 10 collagen, and matrix metalloproteinase (MMP) 13; type 1 collagen; and *MMP3*. The BMP signaling inhibitor dorsomorphin was used to verify the mechanisms of BMP-4-induced chondrogenesis.
Results: Recombinant BMP-4 (100 ng/mL) increased the expression of *SOX9*, *SOX6*, and aggrecan mRNAs in monolayer cells compared with that in cells treated with BMP-2 or BMP-7 on day 3. Chondrogenically differentiated hDFAT cells induced by CM containing BMP-4 showed higher expression of eight genes (excluding *SOX5*) in monolayer cultures and nine genes (including *SOX5*) in pellet cultures compared with those in control medium on day 14. Dorsomorphin attenuated the effects of BMP-4.
Conclusion: These results showed that BMP-4 had the potential to modulate the early chondrogenesis of hDFAT cells under both monolayer and pellet cell culture conditions.
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1. Introduction

Injuries and congenital morphological anomalies of cartilage, such as cleft lip and palate, cause deleterious effects on facial morphology and decrease the quality of life of patients [1,2]. Currently, autocartilage, bone grafts, and synthetic materials have been used as conventional reconstructive materials for the treatment of such diseases [1,2]. However, these materials have potential disadvantages, such as lack of durability, absorption, calcification, and inflammation [1]. Regenerative therapy using stem cells has attracted much attention as a prospective method to supply chondrocytes [1], which might be beneficial in the management of the above diseases.

Dedifferentiated fat (DFAT) cells exhibit a fibroblastic cell shape and can be obtained from mature adipocytes using ceiling [3] and gel culture techniques [4]. Similar to bone marrow-derived mesenchymal stem cells (BMSCs) [5] and adipocyte-derived stem cells (ADSCs) [3], DFAT cells can differentiate into multiple lineages, such as osteoblasts [3,6–14], adipocytes [3,14], myocytes [13–16], endothelial cells [17], and chondrocytes [3,18,19], under specific cell culture conditions *in vitro*. Although DFAT cells and ADSCs are isolated from the same fat tissues, DFAT cells differentiate into osteoblasts much earlier than BMSCs and ADSCs [8,20]. Moreover, these cells exhibit higher adipogenic efficiency [21]. Despite these advantages of hDFAT cells for regenerative therapies, application of hDFAT cells in cartilage regeneration has not been extensively investigated, and the chondrogenic differentiation process in these cells has not been fully elucidated.

Chondrogenic differentiation of BMSCs and ADSCs *in vitro* has been widely investigated to obtain chondrocyte by application of a variety of growth factors and synthetic glucocorticoid [22–26]. In particular, combinations of transforming growth factor (TGF)-β3, and other growth factors, such as basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF), are potent cocktails that can enhance the chondrogenic differentiation of mesenchymal stem cells (MSCs) [22]. Similar to these growth factors, bone morphogenetic proteins (BMPs) are known to stimulate chondrogenesis in MSCs *in vitro* [22,27,28]. BMP-4 enhances cartilage formation and regulates chondrogenic differentiation in adult stem cells [28]. Nevertheless, little information is available regarding the effects of BMPs on the early chondrogenic differentiation of hDFAT cells *in vitro*.

Given the similarity of DFAT cells to BMSCs and ADSCs in terms of multipotency, we hypothesized that the recombinant BMP-4 could also be an effective stimulant for modulating the chondrogenic differentiation of hDFAT cells, which could be applicable in preparing DFAT-derived chondrocytes for stem cell-based cartilage regeneration. To improve our understating of the mechanisms of chondrogenic differentiation in hDFAT cells *in vitro*, we investigated the mRNA expression profiles of monolayer and pellet cell cultures of hDFAT cells undergoing chondrogenic differentiation using chondrogenic medium (CM) containing BMP-4 *in vitro*. BMP-2 and BMP-7 were also used to compare the early chondrogenic capability of BMP-4. Dorsomorphin (DM), a specific BMP signaling inhibitor, was applied to verify the mechanisms underlying the chondrogenic differentiation of hDFAT cells treated with BMP-4.

2. Materials and methods

2.1. Reagents

Recombinant hTGF-β3, bFGF, hBMP-2, and hBMP-7 were purchased from PeproTech (Rocky Hill, NJ, USA). L-proline, L-ascorbic acid 2-phosphate, dexamethasone, and DM were purchased from Sigma (St. Louis, MO, USA). BMP-4 was obtained from HumanZyme (Chicago, IL, USA). ITS-premix was purchased from Corning Inc. (NY, USA).

2.2. Isolation and culture of hDFAT cells

This study conformed to the tenets of the Declaration of Helsinki, and the protocol was approved by the ethics committees of Osaka Dental University and Amagasaki Chuo Hospital (approval numbers: 110760 and 110790). The hDFAT cells used in the present study were prepared using the ceiling culture technique [3,18]. Briefly, fat tissues were obtained from a healthy 63-year-old man who underwent oral and maxillofacial surgery. Isolated mature adipocytes contained in the fat tissue were seeded into a converted culture flask completely filled with growth medium. The floating adipocytes attached to the inner ceiling of the flask. One week later, the flask was inverted to remove the residual mature adipocytes. Fibroblast-like cells attached to the bottom surface were designated DFAT cells. Surface markers of the original cells have been evaluated using fluorescence-assisted cell sorting (FACS): CD90 and CD105 were regarded as positive, while CD34 and CD45 were regarded as negative [18]. These surface markers showed tendencies similar to those of surface markers on DFAT cells reported in previous studies [3,21]. The isolated DFAT cells were maintained in the medium, consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) with 10% fetal bovine serum (FBS) and antibiotics in an incubator at 37 °C with 5% CO_2. The cells were used at passages 6–9. Images of cell morphology were obtained using an optical microscope (IX70; Olympus, Tokyo, Japan).

2.3. Chondrogenic differentiation

To compare the effects of BMPs on early chondrogenic differentiation of hDFAT cells, we stimulated the cells by using control (CNTL) medium or using CM without or with different concentrations of BMPs (Fig. 1). For monolayer cell cultures, hDFAT cells were seeded at 1.5×10^4 cells/cm^2 in 24-well plates and were cultured in CNTL medium, consisting of DMEM with 1% FBS, antibiotics, and 5 ng/mL bFGF. From one day after cell seeding, the cells were cultured in CNTL medium or CM with or without 10 or 100 ng/mL BMPs for up to 14 days. CM consisted of CNTL medium with 10 ng/mL TGF-β3, 40 μg/mL L-proline, 1 × ITS premix, 15 μM L-ascorbic acid 2-phosphate, and 100 nM dexamethasone. The media were changed at every 3–4 days. For the pellet culture, hDFAT cells were seeded at 100×10^4 cells/tube in cryotubes (Thermo Fisher Scientific Inc., MA, USA) and were cultured in 1 mL CNTL

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