Biochemical and Biophysical Research Communications xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Co-stimulation with bone morphogenetic protein-9 and FK506 induces remarkable osteoblastic differentiation in rat dedifferentiated fat cells

Toshiaki Nakamura [1], Yukiya Shinohara [1], Sawako Momozaki, Takehiko Yoshimoto, Kazuyuki Noguchi *

Department of Periodontology, Kagoshima University, Graduate School of Medical and Dental Sciences, Kagoshima, Japan

ARTICLE INFO

Article history:
Received 9 September 2013
Available online xxxx

Keywords:
Dedifferentiated fat cells
BMP-9
FK506
Smad
Osteoblastic differentiation

ABSTRACT

Dedifferentiated fat (DFAT) cells, which are isolated from mature adipocytes using the ceiling culture method, exhibit similar characteristics to mesenchymal stem cells, and possess adipogenic, osteogenic, chondrogenic, and myogenic potentials. Bone morphogenetic protein (BMP)-2 and -9, members of the transforming growth factor-β superfamily, exhibit the most potent osteogenic activity of this growth factor family. However, the effects of BMP-2 and BMP-9 on the osteogenic differentiation of DFAT remain unknown. Here, we examined the effects of BMP-2 and BMP-9 on osteoblastic differentiation of rat DFAT (rDFAT) cells in the presence or absence of FK506, an immunosuppressive agent. Co-stimulation with BMP-9 and FK506 induced gene expression of *runx2*, *osterix*, and *bone sialoprotein*, and ALP activity compared with BMP-9 alone, BMP-2 alone and BMP-2 + FK506 in rDFAT cells. Furthermore, it caused mineralization of cultures and phosphorylation of smad1/5/8, compared with BMP-9 alone. The ALP activity induced by BMP-9 + FK506 was not influenced by addition of noggin, a BMP antagonist. Our data suggest that the combination of BMP-9 and FK506 potently induces osteoblastic differentiation of rDFAT cells.

© 2013 Published by Elsevier Inc.

1. Introduction

Transplant cells are considered to be a crucial component in the research field of tissue regenerative therapy. Several types of adult mesenchymal stem/stromal cells (MSCs) including bone marrow-derived stromal cells (BMSCs), adipose tissue-derived stem cells (ADSCs), and dental pulp-derived stem cells have been considered to be attractive cell sources in stem cell-based tissue engineering [1]. Recently, dedifferentiated fat (DFAT) cells, which are isolated from mature adipocytes using the ceiling culture method, have become the focus of potential sources of MSCs. It has been reported that DFAT cells have a multilineage differentiation capacity, and that they possess similar properties to BMSCs [2] with a higher purity of stem cell population than ADSCs [3]. Comparative transcriptome analyses have revealed the reduction of genes for lipid metabolism and the increase of genes involved in cell proliferation, altered cell morphology, and regulation of differentiation during the dedifferentiation process of DFAT cells [4]. It has been reported that when DFAT cells are transplanted subcutaneously in mice, osteogenically differentiated DFAT cells can form ectopic osteoid tissue, although non-differentiated DFAT cells cannot [5]. This finding may indicate that the effective stimulation of osteogenic differentiation is critical to the DFAT cells-based bone regeneration.

Bone morphogenetic proteins (BMPs) belong to the major subgroup of the transforming growth factor (TGF)-β superfamily. At least 15 types of BMPs have been identified in humans, and BMPs are well known to function in embryogenesis, hematopoiesis, neurogenesis, and skeletal formation [6]. BMPs exert their biologic effects to bind mainly to type I and II serine/threonine kinase receptors and activate intracellular signaling including smad and/or non-smad pathways. In particular, BMP-2, a member of the BMP-2/-4 subfamily, has been well studied, and possesses potent osteoinductive activity [7]. BMP-2 mainly interacts with activin receptor-like kinase (ALK)-3 (BMPR-IA) and ALK6 (BMPR IB), type I receptors, and activates smad1/5/8. The phosphorylated smads, which complex with smad4, translocate into the nucleus, activating the transcription of target genes including *runx2* and *osterix*, and regulate transcription of osteogenic genes such as *alkaline phosphatase* (*ALP*) and *bone sialoprotein* (*BSP*) [8–10]. The USA Food and Drug Administration has already approved the use of recombinant human BMP-2 with resorbable collagen sponges to treat tibial bone fractures and for spinal fusion [11]. It is reported that FK506, known as an immunosuppressive drug, modulates smad signaling pathway [12] and promotes the differentiation of osteoblastic cells [13]. Kaihara et al. have demonstrated that FK506 promotes the early stage of BMP-2-induced osteoinduction after short-term administration [14].

* Corresponding author. Address: Department of Periodontology, Kagoshima University, Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan.
E-mail address: kazuperi@dent.kagoshima-u.ac.jp (K. Noguchi).
[1] These authors equally contributed to this work.

0006-291X/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.09.073

BMP-9 (also known as growth differentiation factor-2 [GDF-2]), which was originally identified from fetal mouse liver [15], has been shown to be possess osteogenic activity [16], and to induce osteogenic differentiation and bone formation of MSCs *in vitro* and *in vivo* [17]. Recently, it has been reported that BMP-9 is more osteogenic than BMP-2, and is not antagonized by noggin, which is a BMP antagonist [18,19]. However, it is unknown whether BMP-2 and BMP-9 can induce osteogenic differentiation in rDFAT cells. The aim of present study is to examine the effects of BMP-2 and BMP-9 in the presence or absence of FK506 on osteoblastic differentiation in rat DFAT (rDFAT) cells.

2. Materials and methods

2.1. Animals

9–10-week-old male Wistar rats were purchased from Kyudo Co. Ltd (Tosu, Japan). All animal experiments were approved by the Ethical Committee of the Animal Research Center of Kagoshima University.

2.2. Reagents

Recombinant human BMP-2, BMP-9, and Noggin were purchased from R&D Systems Inc (Minneapolis, MN, USA), FK506 from Sigma–Aldrich (St. Louis, MO, USA), and anti-phospho-Smad1/5/8 and anti-Smad1 polyclonal antibody from Cell Signaling (Beverly, MA, USA).

2.3. Cell isolation and culture

The preparation of rDFAT cells from adipose tissue was performed according to the method of Jumabay et al. [20], with a minor modification. The adipose tissue (1–2 g) was minced and digested using 0.2% collagenase I solution (Invitrogen, Carlsbad, CA, USA) at 37 °C for 45 min with gentle shaking. After filtration through 140 μm mesh (Sigma–Aldrich) and centrifugation at 135 g for 3 min, the top layer of unilocular adipocytes was collected. The layer containing adipocytes was washed with phosphate-buffered saline (PBS) and centrifuged three times. The cells were placed in a 25 cm^2 culture flask filled completely with Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich), supplemented with 20% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin), and cultured in this floating condition at 37 °C in 5% CO_2. After 1 week, cells adhered to the top inner ceiling surface. The medium was removed, and the flasks were inverted. Then the cells were cultured in culture medium (CM: DMEM supplemented with 10% FBS and antibiotics). rDFAT cells were subcultured and used for experiments at passages 4–7. For osteogenic differentiation, the cells were cultured in osteogenic differentiation medium (ODM), which consisted of DMEM supplemented with 10% FBS, antibiotics, 10 mM β-glycerophosphate, 10 μg/ml ascorbic acid, with 10 μM all-trans retinoic acid for the initial 3 days, and without retinoic acid following the first 3 days [5].

2.4. ALP activity assay and mineralization assay

Cells were cultured for 6 days, and then lysed for the purpose of an ALP activity assay. ALP activity was measured as described previously [21], and expressed relative to the protein content of the samples as determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For the mineralization assay, rDFAT cells were cultured for 21 days. The cells were fixed in 3.7% formaldehyde neutral buffer solution and then stained with alizarin red S.

2.5. Reverse-transcriptase PCR and real-time PCR analysis

Total RNA was extracted from cells using Isogen (Nippon Gene, Tokyo, Japan). RT-PCR was conducted as described previously [22]. Total RNA was converted to cDNA using SuperScript III First-strand Synthesis SuperMix (Invitrogen). The PCR amplifications were carried out using a Taq PCR Core Kit (Qiagen, Hilden, Germany) under the following conditions: 94 °C for 3 min, followed by specified numbers of cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The PCR products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide, and visualized with an ultraviolet light transilluminator. Real-time PCR amplifications were performed using a QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer's instruction. The amplification conditions were 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. We used the comparative Ct method to calculate the relative mRNA expression. All quantitation was normalized by the corresponding GAPDH expression and presented relative to the control levels. Three measurements were performed for each sample. The primer sequences, GenBank accession numbers, and predicted sizes of the PCR product are shown in Supplementary Tables 1 and 2.

2.6. Western blotting

rDFAT cells were treated with either vehicle, BMP-9 or BMP-9 + FK506, for the indicated times, and the cells were then lysed with RIPA buffer (Nacalai tesque, Kyoto Japan). Extracts containing the same amounts of protein (10 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane using the Trans-blot Turbo Transfer system (Bio-Rad Laboratories). Membranes were blocked in blocking buffer (Block Ace Powder; Dainippon, Osaka, Japan), and then probed with anti-phospho-Smad1/5/8 (1:1000) or anti-Smad1 polyclonal antibody (1:1000), followed by incubation with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling). The immunoreactive bands were visualized using an ECL plus detection system (GE Healthcare Biosciences, Piscataway, NJ, USA). Band densities were measured with a ChemiDoc XRS Plus system (Bio-Rad Laboratories), and normalized for the optical density of smad1 bands.

2.7. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). The statistical significance of differences between groups was analyzed by one-way analysis of variance (ANOVA) and the Bonferroni–Dunn test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. mRNA expression of BMP receptors in rDFAT cells, and expression levels of bone-related genes in rDFAT cells stimulated with BMP-2, BMP-9, and FK506

We examined the gene expression of BMP receptors in rDFAT cells. RT-PCR analysis revealed the expression of mRNA of BMP receptors, including type I receptors (ALK-1, -2, -3) and type II receptors (BMPRII, ActRIIA, and ActRIIB), in rDFAT cells (Fig. 1A). The level of bone-related gene expression, including *runx2*, *osterix*, and *bone sialoprotein* (*BSP*), was significantly increased in rDFAT

UNCORRECTED PROOF

Please cite this article in press as: T. Nakamura et al., Co-stimulation with bone morphogenetic protein-9 and FK506 induces remarkable osteoblastic differentiation in rat dedifferentiated fat cells, Biochem. Biophys. Res. Commun. (2013), http://dx.doi.org/10.1016/j.bbrc.2013.09.073

Download English Version:

https://daneshyari.com/en/article/10757755

Download Persian Version:

https://daneshyari.com/article/10757755

Daneshyari.com