BMP2 Increases Adipogenic Differentiation in the Presence of Dexamethasone, which is Inhibited by the Treatment of TNF-α in Human Adipose Tissue-Derived Stromal Cells

Sun Young Lee, Jung Hee Lee, Jee young Kim, Yong Chan Bae, Kuen Tak Suh, Jin Sup Jung

Department of Physiology, School of Medicine, Pusan National University, Yangsan, Korea
Department of Plastic Surgery, School of Medicine, Pusan National University, Pusan, Korea
Department of orthopedic surgery, School of Medicine, Pusan National University, Pusan, Korea

Key Words
BMP2 • Adipogenic differentiation • Osteogenic differentiation• hADSCs • TNF-α

Abstract
Background/Aims: The aim of this study was to analyze the effect of BMP2 on osteogenic differentiation of human adipose tissue-derived stromal cells (hADSCs). Methods: Cultured cells were differentiated into osteogenic lineage in the presence of BMP2. Gene expressions were determined by real time PCR. Results: BMP2 increased (2/8) or inhibited (6/8) osteogenic differentiation according to hADSCs batches. Regardless of the BMP2 action on osteogenic differentiation, BMP2 induced lipid droplet formation under an osteogenic differentiation condition in all batches of hADSCs, not hBMSCs, to be tested, which was confirmed by analysis of adipogenesis related genes expression. hADSCs expressed various BMP receptors. BMP2 increased expression of BMP2-responsive genes such as DLX3 and ID2, and induced SMAD1 phosphorylation in hADSCs and hBMSCs. BMP2 increased osteogenic differentiation of hADSCs in osteogenic medium in which dexamethasone was omitted. The addition of BMP2 in the control culture media containing dexamethasone alone lead to formation of lipid droplets and increased C/EBP-α expression in hADSCs. In the presence of TNF-α, BMP2 stimulated osteogenic differentiation of hADSCs even in hADSCs batches in which treatment of BMP2 alone inhibited osteogenic differentiation. Conclusion: These data indicate that the control of osteogenesis and adipogenesis in hADSCs is closely related, and that hADSCs have preferential commitment to adipogenic lineages.

Jin Sup Jung, MD, PhD.
Department of Physiology, School of Medicine, Pusan National University, Beomeo-ri, Mulgeum-eup, Yangsan-si, Gyeongsangnam-do 626-870 (South Korea)
Tel. +8251-510-8071, Fax +8251-510-8076, E-Mail jjsjung@pusan.ac.kr
Introduction

Mesenchymal stem cells (MSCs) were first identified in bone marrow [1]. They can be expanded in culture while retaining their multipotency and can differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes, and adipocytes [2, 3]. Therefore, the development of MSC-based techniques has become a major research objective in the field of tissue engineering, and provides a unique model for a better understanding of early differentiation events, as these cells are able to differentiate into multiple mesenchymal lineages.

Recent studies show that several other tissues also contain MSCs. In 2001, Zuk et al. demonstrated that human adipose tissue contains multipotent stem cells that can differentiate along different lineages such as bone, cartilage, fat, and muscle [4]. Adipose tissue-derived stromal cells (ADSCs) share many of the characteristics of their counterparts in bone marrow, including extensive proliferative potential and the ability to differentiate into cells with adipogenic, osteogenic, chondrogenic, and myogenic lineages [5, 6]. There is strong evidence that ADSCs undergo rapid osteogenesis both in vitro and in vivo [7, 8].

The BMP family belongs to the transforming growth factor-beta (TGF-β) superfamily. The classical role for BMPs was considered to be induction of (ectopic) cartilage and bone formation [9], BMP2, BMP4, BMP6, and BMP7 are the most important cytokines in bone tissue engineering [10], as they play paramount roles in osteogenic differentiation of various MSCs [11, 12]. In contrast to bone marrow mesenchymal stem cells (BMSCs), the effect of BMP2 on osteogenic differentiation in hADSCs is not clearly defined. Zuk et al. reported that hADSCs have a defective BMP2 signaling pathway [13] and Cruz et al. showed that BMP2 fails to enhance osteogenic differentiation of hADSCs [14]. However, Panetta et al. showed that BMP2 increases osteogenic differentiation in hADSCs [15]. BMP2 has been reported to increase in vivo bone formation by transplantation of hADSCs [16-18]. To develop optimal in vitro manipulation technique for enhancing in vivo bone regeneration of by transplantation of hADSCs, clear understanding about BMP2 actions on osteogenic differentiation of hADSCs is necessary.

The present study was designed to clarify the effect of BMP2 on osteogenic differentiation of hADSCs and to determine the extent to which BMP2 and TNF-α alone and in combination alter hADSCs differentiation. The data showed that BMP2 induces lipid droplet formation even at osteogenic condition in the presence of dexamethasone in contrast with hBMSCs and that inhibition of this process enhances osteogenic differentiation of hADSCs.

Materials and Methods

Cell culture

All protocols involving human subjects were approved by the Institutional Review Board of Pusan National University. After informed consent, superfluous materials were obtained from eight individuals undergoing total hip arthroplasty and elective abdominoplasty. hBMSCs and hADSCs were isolated and characterized according to the methods described in the previous studies [19, 20]. The isolated cells were cultured in Low-glucose DMEM with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 mg/ml penicillin (CCM) in 5% CO2 environment at 37°C. Cells between third and fifth passages were used for experiments.

Osteogenic and adipogenic differentiation

After plating the cells on a 12-well plate (100,000 cells/well), they were grown to confluency. Then, osteogenic differentiation was induced by culturing the cells for 2–3 weeks in osteogenic induction medium (OIM; 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 250 μM ascorbic acid in L-DMEM) and the level of extracellular matrix calcification was examined by Alizarin Red S staining. In order to obtain quantitative data, 300 μl of 10% cetylpyridiniumchloride in 10 mM sodium phosphate (pH 7.0) was added to the stained culture dish. After 15 min, the extract was diluted 10 fold with 10% cetylpyridiniumchloride...
in 10 mM sodium phosphate (pH 7.0), then the absorbance of the diluted extract was assayed by a spectrophotometer at 562 nm.

Adipogenic differentiation was induced by culturing MSCs for 10–14 days in adipogenic medium (AIM; 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 200 μM indomethacin in L-DMEM) and lipid droplets formation was visualized using Oil Red O stain as an indicator of intracellular lipid accumulation.

Quantitation of mineral formation

For the calcium deposition assay, cultures were washed twice with PBS. Mineral was then collected after dissolution with 30 ml of 0.5 N hydrochloric acid at room temperature overnight and the samples assayed the following day. Incorporation of calcium in the extracellular matrix was quantified using a commercial diagnostic kit (QuantiChrom Calcium Assay Kit, DICA-500; Bio Assay Systems, Hayward, CA, USA), in accordance with the manufacturer’s instructions. Absorbance was compared with curves prepared using standard solutions of calcium. Calcium deposition was expressed as mM per well of tissue culture 12-well plates.

Real-Time polymerase chain reaction (PCR)

Total RNA was extracted using Trizol (Invitrogen), according to the manufacturer’s instructions and reverse-transcribed into cDNA with the Reverse Transcriptase M-MLV (Promega, Madison, WI, USA). Real time PCR was performed using a Power SYBR Green PCR Master Mix on the ABI 7500 Instrument (Applied Biosystems, Warrington, UK). The primer sequences used in the experiment are listed in the Table 1. For each primer pair, the linearity of detection was confirmed by measuring a dilution curve with cDNA isolated from hBMSCs or hADSCs. GUSB (Beta-D-glucuronidase) and β-actin were determined to be appropriate internal controls for hADSCs and hBMSCs, respectively. Because β-actin mRNA levels were changed during differentiation of hADSCs (unpublished data), GUSB mRNA, of which expression was maintained at a constant levels during differentiation, was used for an internal control in differentiated hADSCs. The data were presented as the relative abundance of the gene of interest to GUSB or β-actin.

Western blot analysis

Confluent hBMSCs and hADSC were treated under the appropriate conditions and lysed, after which the protein content of the lysate was determined using a protein assay kit. The proteins were loaded on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA), and probed with monoclonal or polyclonal antibodies (anti-SMAD1, antiphospho (Ser463/465)-SMAD1 and anti β-actin; Cell Signaling Technology, Danvers, MA,

<table>
<thead>
<tr>
<th>gene</th>
<th>Sequences of real time PCR primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GUSB</td>
<td>F: 5'-GGGTGACCTAGAACTTCCTG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CATACGGGACCCTTCTTG-3'</td>
</tr>
<tr>
<td>BMPR1A</td>
<td>F: 5'-GAAGAATGGCAACCATCTGCTG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GCCATAGGAATGAGCAGAGAAC-3'</td>
</tr>
<tr>
<td>ALK2</td>
<td>F: 5'-AGTAAAGTCATGGTGCCAGG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CGAAGCCGCAGTAACTCTTGAT-3'</td>
</tr>
<tr>
<td>ACVR2A</td>
<td>F: 5'-TACAGGGCCAAACAGATGTCG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GAGCACCTTCTAAGAGAGAC-3'</td>
</tr>
<tr>
<td>ACVR2B</td>
<td>F: 5'-TTCGGTCCAAGTGATGATG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTGTAAGCTCTTCTCCACTC-3'</td>
</tr>
<tr>
<td>PPAR-γ isofom2</td>
<td>F: 5'-GAGACATCTATCTCAAGG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTTGTAAGCTCTTCTCCACTC-3'</td>
</tr>
<tr>
<td>C/EBP-α</td>
<td>F: 5'-GAAGCCAAAAGTGGTGGGAG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TCGGTGAAGCCAAGAGAGGAG-3'</td>
</tr>
<tr>
<td>Dlx3</td>
<td>F: 5'-GCAGAGCGACAGACAGAGAGGAG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGGAGGTGCTTCTTCCATT-3'</td>
</tr>
<tr>
<td>ID2</td>
<td>F: 5'-AATCCGCTTCTTCCATT-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GAGACATCTATCTCAAGG-3'</td>
</tr>
</tbody>
</table>
USA). Immunoreactive bands were detected with anti-rabbit peroxidase conjugated secondary antibodies and visualized via enhanced chemiluminescence.

**Statistical analysis**

All results are presented as mean ± SEM. The data were analyzed using Student’s t-test when two groups were compare or by ANOVA for more than two groups. Post hoc range tests and pairwise multiple comparisons were conducted with Tukey adjustment to determine which means differ. Graphpad Prism 6.0 (GraphPad Software San Diego, CA, USA) was used for statistical analysis. Probability values of P < 0.05 were considered statistically significant.

**Results**

**Effect of BMP2 on osteogenic differentiation of hADSCs**

We first determined the effect of BMP2 on osteogenic differentiation of hADSCs and hBMSCs. Adding BMP2 increased osteogenic differentiation of hBMSCs in a dose-dependent manner (Fig. 1A and B). In contrast, the response to BMP2 in hADSCs vary according to cell donors. Although the addition of BMP2 increased osteogenic differentiation in hADSCs isolated from 2 donors among 8 donors to be examined in this experiment donors, in hADSCs isolated from 6 donors (6/8) the addition of BMP2 inhibited osteogenic differentiation (Fig. 2A). Oil Red O staining of hADSCs treated with BMP2 revealed that adding BMP2 increased the formation of lipid droplets in hADSCs in all batches to be tested in this experiment regardless of the BMP2 response in osteogenic differentiation (Fig. 2B). In the following experiments, hADSCs in which BMP2 inhibited osteogenic differentiation were used.
Lee et al.: BMP2 and TNF-α on Differentiation of Human Adipose Tissue-Derived Stromal Cells

We measured changes in the expression levels of adipogenesis-related genes by real time PCR to confirm the effect of BMP2 on hADSCs. The induction of osteogenic differentiation in the presence of BMP2 increased expression of adipogenesis-related genes such as PPAR-γ isoform2 and CCAAT/enhancer binding protein-alpha (C/EBP-α) (Fig. 2C).

To determine whether BMP2 adequately conveyed the hADSCs downstream signal, we first determined expression of BMP2 receptors and BMP2 downstream genes in hADSCs. Real time PCR analyses showed that hADSCs expressed various BMP receptor isoforms and their expression level is similar with that of hBMSCs (Fig. 3A), and that a 2 day BMP2 treatment of hADSCs increased expression of distal-less homeobox 3 (DLX3) and inhibitor of DNA binding 2 (ID2), well-known genes that are altered in response to BMP2 signaling (Fig. 3B). We next determined if BMP2 induces SMAD1 phosphorylation in hADSCs and hBMSCs. Western blot analysis showed that the 100 ng/ml BMP2 treatment induced SMAD1 phosphorylation in hADSCs as well as hBMSCs (Fig. 3C).
Role of dexamethasone on BMP2-induced lipid droplet formation

Then, we determined whether dexamethasone was involved in BMP2-induced adipogenic differentiation. Omitting dexamethasone in OIM decreased basal osteogenic differentiation of hADSCs compared with OIM containing dexamethasone. BMP2 increased osteogenic differentiation in the absence of dexamethasone without inducing lipid droplet formation.

Fig. 3. Analysis of BMP2 downstream signal in hADSCs and hBMSCs. (A) Expression of BMP receptors in hADSCs and hBMSCs. Total RNA were isolated from hADSCs and hBMSCs. The expression of BMP receptors was analyzed by real time PCR. Data represent mean ± SEM of the relative ratio to β-actin levels of the corresponding samples (n=8). (B) Effect of BMP2 on DLX3 and ID2 expression in hADSCs. Total RNA were isolated from hADSCs in CCM (Cont) or from hADSCs at 2 days after treatment of 100 ng/ml BMP2 in CCM. Gene expression was analyzed by real time PCR. Experimental data were converted as the relative ratio to GUSB levels of the corresponding samples. Values were presented as fold change relative to the value of hADSCs in CCM (mean ± SEM, n=3). *p < 0.05 compared with hADSCs in CCM. (C) Western blot analysis of SMAD1 phosphorylation in hADSCs and hBMSCs. hADSCs or hBMSCs were treated for 0, 15 or 30 mins with 100 ng/ml BMP2 in FBS-free CCM. Lysates were prepared at the indicated times and were subjected to immunoblot analysis using p-SMAD1, and total SMAD1 antibodies. To confirm equal loading of protein, the quantities of β-actin were determined by anti-β-actin antibody.
in hADSCs. However, the level of osteogenic differentiation was similar with differentiation in control hADSCs in OIM containing dexamethasone (Fig. 4A and B). We observed that increased cell number during osteogenic differentiation in the absence of dexamethasone resulted in curling up of hADSCs. Therefore, we determined the effect of dexamethasone on hADSCs proliferation. Adding 0.1 μM dexamethasone to the control culture medium (CCM) at a concentration that induced osteogenesis inhibited cell proliferation. OIM without dexamethasone significantly increased cell number compared with that in CCM and in the OIM containing dexamethasone (Fig. 4C).

To further confirm whether dexamethasone is necessary for BMP2-induced adipogenic differentiation, we determined the effect of BMP2 in the presence of dexamethasone in CCM. Dexamethasone alone did not induce the formation of lipid droplets. Adding 100 ng/ml BMP2 with dexamethasone increased the formation of lipid droplets of hADSCs in CCM (Fig. 5A). Real time PCR analysis showed that adding dexamethasone increased C/EBP-α and PPAR-γ isoform2 expression in hADSCs. Adding BMP2 further increased C/EBP-α expression but not PPAR-γ isoform2 expression in hADSCs (Fig. 5B).
To determine whether inhibiting BMP2-induced adipogenesis in hADSCs under osteogenic conditions increased osteogenic differentiation of hADSCs in the presence of BMP2, we determined the effect of TNF-α treatment. Previous studies have shown that TNF-α increases osteogenic differentiation [21] and inhibits adipogenic differentiation of hADSCs [22]. Treatment with 10 ng/ml TNF-α in OIM increased osteogenic differentiation in hADSCs, and BMP2 inhibited osteogenic differentiation and induced lipid droplet formation. The combined treatment of TNF-α and BMP2 further increased osteogenic differentiation compared with TNF-α alone (which was determined by measurement in the optical density of Alizarin Red S staining and calcium deposition amount), and inhibited BMP2-induced adipogenic differentiation (Fig. 6A-D). Real time PCR analysis showed that TNF-α treatment inhibited BMP2-induced C/EBP-α expression and dexamethasone-induced PPAR-γ isoform2 expression (Fig. 6E).

Discussion

In this study we found that BMP2 inhibits osteogenic differentiation in hADSCs isolated from most donors (6/8). To elucidate underlying mechanisms of donor variation, further
investigations including variation of epigenetic status and expression profile of genes that are involved in BMP2 signaling and in osteogenic and adipogenic differentiation are required.
The in vitro differentiation study and real time PCR analysis of lineage-related genes showed that BMP2 induced adipogenic differentiation in the presence of dexamethasone even under osteogenic conditions in all hADSCs to be examined in this study, although there is a donor variation in the BMP2 action on osteogenic differentiation. Zuk et al. reported a defect of BMP2 signaling in hADSCs [13]. However, we observed expression of various isoforms of BMP receptors and increase in expression of BMP2 downstream target genes such as DLX3 and ID2 [23] as like in hBMSCs. BMPs binding induces formation of heteromultimers of both type I and II receptors [24]. Activated BMP type I receptors phosphorylate their downstream targets SMAD1, SMAD5, and SMAD8 proteins, which interact with SMAD4 and translocate to the nucleus [25]. We observed BMP2-induced SMAD1 phosphorylation in hADSCs as well as hBMSCs. All of these findings indicate preservation of intact BMP2 signaling pathways in hADSCs.

PPAR-γ and the CCAAT/enhancer-binding protein (C/EBP) family regulate adipocyte differentiation. C/EBP-β and C/EBP-δ are expressed early during adipogenesis [26, 27] and are involved in the induction of PPAR expression [28, 29]. The overlap between PPAR-γ and C/EBP-α binding indicates cooperativity between the two transcription factors [30, 31]. In this study, the treatment of dexamethasone alone did not induce the formation of lipid droplets in hADSCs in spite of the increase of PPAR-γ isoform2 and C/EBP-α expression. The addition of BMP2 induced further increase of C/EBP-α expression and formed lipid droplets. These data suggest that the later stage of adipose differentiation may require an adequate level of C/EBP-α expression in hADSCs.

We showed that dexamethasone is an essential component of the BMP2-induced increase in adipogenic differentiation in OIM, demonstrating that adding dexamethasone alone in CCM is sufficient for BMP2-induced adipogenesis. Although the presence of dexamethasone inhibited BMP2-induced osteogenic action in most hADSCs, the removal of dexamethasone inhibited osteogenic differentiation in hADSCs, indicating that dexamethasone is necessary to support osteogenic differentiation of hADSCs in the absence of BMP2 (100 ng/ml). Dexamethasone (0.1 µM) was sufficient at inhibiting hADSCs proliferation in control and OIM, and the presence of dexamethasone in OIM showed better osteogenic differentiation in hADSCs than that in OIM in which dexamethasone was omitted. Therefore, the inhibited cell proliferation and the increase of mineralization by glucocorticoids [32, 33] could be related to the dexamethasone-induced increase in osteogenic differentiation of hADSCs.

In this study, treatment of TNF-α inhibited BMP2-induced formation of lipid droplets, and PPAR-γ and C/EBP-α expression. These results are consistent with the previous findings that TNF-α inhibits adipogenic differentiation through the inhibition of PPAR-γ and C/EBP-α expression in murine cells [34, 35]. PPAR-γ plays a critical role in commitment switch of progenitor cells between adipogenesis and osteogenesis [36, 37]. It has been reported that PPAR-γ silencing increases osteogenic differentiation of hADSCs [38]. We observed that the treatment of TNF-α restored the BMP2-induced increase in osteogenic differentiation in hADSCs in which the addition of BMP2 alone inhibited osteogenic differentiation, which results from TNF-α-induced inhibition of PPAR-γ and C/EBP-α expression. ADSCs have been shown to be inferior to BMSCs in osteogenic potential [7, 39]. Osteogenically predifferentiated BMSCs showed better in vivo bone regeneration than undifferentiated BMSCs [40] and chondrogenic preinduction enhanced bone regeneration of ADSCs transplantation [39]. Therefore, the findings in this study indicated that the combined pretreatment of TNF-α and BMP2 may enhance the efficacy of in vivo bone regeneration by hADSCs transplantation.

In this study, we elucidated the BMP2 action on osteogenic differentiation of hADSCs. The results of our current study show that treatment of TNF-α inhibited of BMP2-induced lipid droplet formation at the osteogenic condition and restored BMP2-induced increase of osteogenic differentiation in hADSCs in which the addition of BMP2 alone inhibited osteogenic differentiation, indicating that osteogenesis and adipogenesis in hADSCs is closely regulated. These findings contribute to develop efficient methods of hADSCs manipulation for in vivo bone regeneration using BMP2.
Acknowledgments

This research was supported by the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2012M3A9B4028558).

References

Lee et al.: BMP2 and TNF-α on Differentiation of Human Adipose Tissue-Derived Stromal Cells


