Original

Bone Morphogenetic Protein-2 Accelerates Osteogenic Differentiation in Spheroid-Derived Mesenchymal Stem Cells

Naoyuki Miyaguchi1,2, Hiroshi Kajiya3, Masahiro Yamaguchi3,4, Ayako Sato2, Madoka Yasunaga5,6, Takuya Toshimitsu2,4, Tsukasa Yanagi2, Ayako Matsumoto2, Hirofumi Kido2, and Jun Ohno3

1 Section of Oral Implantology, Department of Oral Rehabilitation, Fukuoka Dental College, Fukuoka, Japan
2 Research Center for Regenerative Medicine, Fukuoka Dental College, Fukuoka, Japan
3 Section of Cellular Physiology, Department of Physiological Science and Molecular Biology, Fukuoka Dental College, Fukuoka, Japan
4 Section of Oral Implantology, Department of Oral Rehabilitation, Fukuoka Dental College, Fukuoka, Japan
5 Section of Orthodontics, Department of Oral Growth and Development, Fukuoka Dental College, Fukuoka, Japan
6 Dentistry for the Disabled, Department of Oral Growth and Development, Fukuoka Dental College, Fukuoka, Japan

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Abstract: Spheroid culture systems more accurately recreate the in vivo microenvironment and are susceptible to factors that induce differentiation. In this study, we assessed whether bone morphogenetic protein (BMP)-2 induces enhanced osteogenic differentiation in spheroid-derived mesenchymal stem cells (MSCs). MSC spheroids were generated from human adipose tissue-derived MSCs using low-binding plates. Osteogenic differentiation of monolayer and spheroid-derived MSCs was induced by osteogenesis induction medium (OIM) with or without BMP-2. Increased alkaline phosphatase and Alizarin Red staining were observed in spheroid-derived MSCs treated with a mixture of OIM and BMP-2, compared with monolayer MSCs. Spheroid-derived MSCs had increased mRNA and protein expressions of osteogenic runt-related transcription factor 2 (Runx2) and osterix (OSX). The intranuclear expression of OSX was also observed in spheroid-derived MSCs treated with the mixture of OIM and BMP-2. In addition, spheroid-derived MSCS with BMP-2 treatment showed the upregulation of Smad5 mRNA and phosphorylated Smad1/5, suggesting that the Smad-BMP signaling pathway is enhanced in these cells. Our data indicate that the Smad-dependent BMP signaling pathway accelerates osteogenic differentiation in spheroid-derived MSCs, compared with monolayer MSCs.

Key words: Osteogenesis, Mesenchymal stromal cells, Bone morphogenetic protein 2, Spheroid culture, Smad

Introduction

Adult mesenchymal stem cells (MSCs) are found in pulp, cord blood, umbilical cord and others. MSCs possess great potential for tissue engineering and regenerative medicine, because of their capability of self-renewal and multilineage differentiation. MSCs have been utilized as a cell source for osteogenic tissue regeneration because culture in the presence of osteogenic supplements facilitates MSCs to undergo differentiation into the osteogenic phenotypes.

Osteogenic differentiation is regulated by various signaling pathways, including Wnt, bone morphogenetic protein (BMP), Hedgehog, Notch, and fibroblast growth factors. BMPs, members of the transforming growth factor-β (TGF-β) superfamily, regulate proliferation, differentiation, and apoptosis in various types of cells and organs in embryonic development and postnatal physiological function. When BMPs-containing scaffolds are implanted into rodent tissues, ectopic bone formation is induced by BMPs. BMPs, including BMP-2 and -4, accelerate osteoblast differentiation of MSCs via activation of transcription factors, such as Runx2/core binding factor a1 (Cbfa1) and Sp7/osterix. In addition, Smad proteins play an important role in the intracellular signaling of BMPs in mammals. Receptor-activated Smad (R-Smad) proteins, Smad1, Smad2, Smad3, Smad5, and Smad8, are directly phosphorylated by type I TGF-β receptors. Upon activation, R-Smad form complexes with the Co-Smad, Smad4, and translocate to the nucleus to regulate gene expression by binding to regulatory regions of the target genes.

Although relatively easy and traditional, 2D culture is an artificial and less physiologically relevant environment, because in vivo characteristics and traits are lost or compromised. Furthermore, in conventional 2D in vitro monolayer culture techniques, MSCs eventually lose intrinsic properties, such as self-renewal, replication, colony-forming efficiency, and differentiation capacity. Spheroid 3D environments are generally considered more favorable than 2D monolayer culture. Various culture systems have recently been developed to generate 3D multicellular spheroids from MSCs, such as suspension, hanging drop, and micropattern substrates, and non-adherent surfaces. In 3D-cultured spheroids, self-renewal has been reported to be significantly enhanced. Additionally, MSC spheroids formed on micropattern substrates and non-adherent surfaces have higher osteogenic and adipogenic differentiation efficiencies. In this study, we employed a low-attachment culture condition to generate MSC spheroids. The osteogenic properties of spheroid-derived MSCs were investigated. We further examined an effect of BMP-2 on acceleration of osteogenesis in spheroid-derived
MSCs. Possible mechanisms for activation of osteogenesis via the BMP pathway are discussed based on spheroid formation of MSCs.

Materials and Methods

MSC monolayer expansion

The human adipose tissue-derived mesenchymal stem cell line HAdpc-25-Bmi-1-TERT was purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cryopreserved MSCs were thawed and plated onto a tissue culture dish maintained in complete Powerby10 expansion medium (GlycoTechnia Ltd., Yokohama, Japan). Cells were dissociated with 0.25% trypsin/EDTA (Invitrogen, Tokyo, Japan), counted, and either re-plated for monolayer cultures or used for spheroid formation.

Spheroid formation of MSCs

Dissociated MSC monolayers were resuspended in complete Powerby10 expansion medium to obtain a single cell suspension. MSCs (1 x 10^5 cells/ml, corresponding to approximately 10,000 cells/well) were added to Corning®96 well Ultralow Attachment micro plates (Corning, NY, USA) and incubated in Powerby 10 expansion medium at 37°C for 24 h.

Morphology and cell viability of spheroid-derived MSCs

After 24 h of suspension culture, MSC spheroids were washed three times with PBS and incubated with 0.25% trypsin-EDTA solution at 37°C for 15 min with mechanical agitation until a single-cell suspension was obtained. Dissociated cells were plated and cultured as described above for morphology, cell viability, and differentiation assays. Cell morphology of spheroid-derived MSCs was observed using a phase-contrast microscopy. To determine cell viability, cell suspension of monolayer and spheroid-derived MSCs were diluted 1:1 in 0.4% trypan blue solution (Invitrogen) in PBS. Cell viability was calculated using a Countess Automated Cell Counter (Invitrogen) according to the manufacturer’s procedures.

Osteogenic differentiation of monolayer- and spheroid-derived MSCs

For induction of osteogenic differentiation, monolayer- and spheroid-derived cells were seeded and cultured until reaching confluence. The medium was then replaced (Day 0) with osteogenesis-induction medium (OIM) composed of Powerby 10. Ascorbic Acid (TaKaRa, Tokyo, Japan), hydrocortisone (TaKaRa), β-glycerophosphate (TaKaRa) were added to the medium to 1%, 0.2% and 2%, respectively. The medium was changed every three days for 21 days, with or without 20 ng/ml bone morphogenetic proteins (BMP)-2 (Peprotech, Rocky Hill, NJ, USA).

Alkaline phosphatase (ALP) and Alizarin Red (AR) staining

ALP and AR staining were performed in monolayer and spheroid-derived MSCs treated with or without the experimental solution, using an ALP Kit (Sigma-Aldrich) and Calcified Nodule Staining Kit (Cosmo Bio, Tokyo, Japan), respectively, according to the manufacturer’s instructions.

Real-time reverse transcription (RT)-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from monolayer and spheroid-derived cells treated with a mixture of OIM and BMP-2, using ISOGEN reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from total RNA (5 μg) using SuperScript II™ reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. The resulting templates were amplified using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Nucleotides,
TaqDNA polymerase, and a buffer were included in the SYBR Premix Ex Taq II (TaKaRa). β-actin (ACTB) was used as an internal control. Relative mRNA expression was normalized as the ratio of runt-related transcription factor 2 (Runx2), Smad5, or β-catenin mRNA to the level of ACTB expression. All the reactions were run in triplicate. The primers used were ACTB, forward 5'-TGGCACCCAGCACAATGAA-3' and reverse, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'; Runx2, forward 5'-CTGATGCGAGTTAGTCAAGGGTAACGAT-3' and reverse 5'-AAAGACCGTTATGCTAACTGGAAGAAGCAGGAAGTAACGAT-3'; Smad5, forward 5'-GGAGAGTCCAGTCT-3' and reverse 5'-GGCTGTTTGGAGATAAGGGAGCTTAC-3'; β-catenin, forward 5'-GGCTGTTTGGAGATAAGGGAGCTTAC-3' and reverse 5'-GGCTGTTTGGAGATAAGGGAGCTTAC-3'.

**Western blot analysis**

Cells were lysed in Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) containing a 1X Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology). Protein samples were loaded and separated onto 4-20% sodium dodecyl sulfate (SDS) polyacrylamide gel, blotted onto PVDF membrane, and incubated with the primary and secondary antibodies. Blots were developed using SignalFire™ Plus ECL Reagent (Cell Signaling Technology). The band density was quantified using the NIH-Image J software.

**Immunocytochemistry (ICC)**

Monolayer- and spheroid-derived MSCs were fixed with 4% paraformaldehyde for 10 min and washed in 0.1% digitonin in phosphate buffered saline (PBS) for 15 min. The cells were incubated with primary antibodies for 2 h at room temperature. After washing with PBS, the cells were incubated with anti-mouse IgG or anti-rabbit IgG conjugated with Alexa Fluor 488 or 568 (Invitrogen) for 45 min. To visualize the nuclei, the cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

**Statistical analysis**

Two-way analysis of variance and Holm’s multiple comparison test or Student’s t-test were used to determine the statistical differences among the samples (StatView for Windows, version 5; SAS Institute, Inc., Cary, CA, USA). Data are presented as the mean ± SD and P-values < 0.05 were considered to be statistically significant.

**Results**

**Spheroid formation of hMSCs**

First, we examined the generation of spheroids from MSCs in round-bottom, low-binding plates that can generate a single spheroid per well. Spheroid formation was divided into three stages (Fig. 1). Approximately 2 hours after seeding MSC cell into the plates, cells spontaneously and loosely aggregated in the medium. After 4 hours, cellular aggregates began to form a compact accumulation of cells. Finally, cells formed compact multicellular spheroids after 24 hours in suspension culture in low-binding plates.

**Cell viability of spheroid-derived MSCs**

To observe differences in cell morphology and viability between monolayer- and spheroid-derive MSCs, MSC spheroids were dissociated and seeded back into monolayer culture. Both monolayer- and spheroid-derived MSCs showed spindle and triangular shapes (Fig. 2A). As shown in Fig. 2B, there was no difference in cell viability between monolayer- and spheroid-derived MSCs by trypan blue dye exclusion. Taken together, the dissociation procedure for spheroid-derived MSCs did not affect cell morphology or viability.

**A mixture of OIM and BMP-2 accelerates osteogenic differentiation in spheroid-derived MSCs**

To investigate the effect of BMP-2 on spheroid-derived MSCs, we first assessed ALP and AR staining in monolayer- or spheroid-derived cells. Figure 3A shows ALP staining in both monolayer and spheroid-derived MSCs cultured in basic medium with OIM alone or a mixture of OIM and BMP-2. ALP staining was evident in spheroid-derived cells on Day 3, even though monolayer cells remained unstained. ALP staining was much more intense in spheroid-derived cells at any time point, compared with monolayer cells. The intensity of ALP staining was significantly enhanced in spheroid-derived cells treated with the mixture of OIM and BMP-2 on Day 7, compared with OIM alone. Three weeks after osteogenic treatment, AR staining showed increased deposition of calcium in spheroid-derived cells treated with the mixture of OIM and BMP-2, compared with OIM alone.

Figure 3. Alkaline phosphatase (ALP) and Alizarin red (AR) staining in monolayer and spheroid-derived MSCs. A: ALP staining was assessed in monolayer- and spheroid-derived cells cultured in the basic medium with OIM alone or a mixture of OIM and BMP-2 (20 ng/ml) for 3, 5, and 7 days. B: AR staining in monolayer- and spheroid-derived cells treated with OIM alone or a mixture of OIM and BMP-2 (20 ng/ml) for 3 weeks.
mixture of OIM and BMP-2 (Fig. 4A). Increased expression of Runx2 was observed in spheroid-derived MSCs after Day 3. Compared with monolayer-derived MSCs, spheroid-derived cells showed increased expression at all time points, with significant increases on Day 3 and Day 7.

Next, we immunocytochemically examined OSX expression in monolayer- and spheroid-derived MSCs treated with OIM alone or the mixture of OIM and BMP-2 (Fig. 4B). Cytoplasmic expression of OSX was observed in monolayer cells with or without BMP-2 and spheroid-derived cells with OIM alone. In contrast, spheroid-derived cells treated with BMP-2 showed intranuclear expression of OSX in addition to intense cytoplasmic staining.

We then performed western blotting to examine whether expression of osteogenic proteins, such as OSX and Runx2, were upregulated in spheroid-derived MSCs treated with OIM containing BMP-2 (Fig. 4C). Expression of both OSX and Runx2 were upregulated in the spheroid-derived cells treated with OIM containing BMP-2 on Days 3, 5, and 7, compared to monolayer-derived MSCs. These findings indicate that BMP-2 accelerates osteogenic differentiation in spheroid-derived MSCs.

Osteogenesis of spheroid-derived cells is promoted through the BMP signaling pathway

Interactions between the TGF-β/BMP and Wnt/β-catenin signaling pathways play crucial roles in regulation of osteogenesis of MSCs[22]. To elucidate whether both pathways contribute to acceleration of osteogenic induction of spheroid-derived cells, we examined expression of Smads and β-catenin, downstream effectors of BMP and Wnt signaling, respectively, by qRT-PCR, western blotting, and immunocytochemical assays.

Fig. 5A shows the mRNA expression of Smad 5 and β-catenin in monolayer- and spheroid-derived cells treated with the mixture of OIM and BMP-2. Smad 5 expression was significantly increased in spheroid-derived cells on Day 3 and then decreased to levels similar to Day 1. At all observed time points, Smad 5 expression was higher in spheroid-derived cells, compared with monolayer cells. In contrast, β-catenin mRNA expression gradually increased by Day 7 in both monolayer- and spheroid-derived cells. There were no significant differences in β-catenin mRNA expression between monolayer- and spheroid-derived cells at any of the time points.

We next examined whether the expression of phosphorylated Smad 1/5 (p-Smad1/5) and β-catenin proteins were upregulated in monolayer- and spheroid-derived cells treated with the mixture of OIM and BMP-2.
Increased expression of p-Smad 1/5 was observed in spheroid-derived cells, compared with monolayer cells. The relative p-Smad1/5 expression in spheroid-derived cells gradually increased by Day 7. In contrast to p-Smad 1/5 expression, there was no difference in β-catenin expression in either monolayer- or spheroid-derived cells.

Finally, we performed immunocytochemical staining to detect the intranuclear expression of p-Smad 1/5 and β-catenin, because both proteins translocate to the nucleus to initiate downstream gene expression. Intranuclear p-Smad 1/5 expression was significantly upregulated in spheroid-derived cells treated with the mixture of OIM and BMP-2, compared with monolayer cells (Fig. 5C). Increase in the number of intranuclear p-Smad 1/5-positive cells was observed in spheroid-derived cells at any time points. In contrast, β-catenin expression was localized in the cytoplasm of both monolayer- and spheroid-derived cells treated with the mixture of OIM and BMP-2 (Fig. 5D). There was no difference in the number of cytoplasmic β-catenin-positive cells in monolayer- and spheroid-derived MSCs. These findings suggest that acceleration of osteogenic induction in spheroid-derived cells may be regulated in part through the BMP signaling pathway without dependence on Wnt/β-catenin signaling.

**Discussion**

Spheroid culture takes advantage the intrinsic self-assembly tendency of numerous cell types allowing cells to adapt native morphology, promoting greater cell-cell contacts and interaction with the extracellular matrix. Our recent study demonstrated that MSC spheroids possess enhanced osteogenic potential, compared with monolayer MSCs. In this study, we present three lines of evidence to support the conclusion that MSC spheroids exhibit enhanced osteogenic potential: (a) spheroid-derived MSCs exhibited increased calcium deposition, compared with monolayer MSCs; (b) expression of osteogenic proteins was upregulated in spheroid-derived MSCs cultured in the mixture of OIM and BMP-2, compared with those in OIM alone or monolayer-derived MSCs treated with the mixture of OIM and BMP-2; (c) osteogenesis of spheroid-derived MSCs was promoted via the BMP signaling pathway.

The round-bottom, low-binding plates used in this study have desir-
able characteristics such as a single spheroid per well, high reproducibility, and simple harvesting for further analysis. In this study, we dissociated MSC spheroids to seed back into monolayer culture for increased permeability of OIM and BMP-2 to whole spheroid-composed MSCs. There were no differences in cell morphology and viability between monolayer-derived MSCs and spheroid-derived MSCs.

OIM-induced osteogenesis in MSCs is characterized by an increase in ALP at early stages, followed by matrix deposition, matrix maturation, and mineralization at later stages. BMPs are known to be the most important inducers and stimulators of osteogenic differentiation. In this study, increased staining with ALP was observed in spheroid-derived MSCs cultured in the mixture of OIM and BMP-2. Similarly, AR staining showed matrix mineralization when spheroid-derived cells were treated with the mixture of OIM and BMP-2. These findings indicate that compared with monolayer MSCs, spheroid-derived MSCs are more susceptible to BMP-2, resulting in an acceleration of osteogenic differentiation at both early and late stages.

Our data indicate that BMP-2 enhances expression of osteogenic factors in spheroid-derived MSCs, therefore, we investigated the expression of Runx2. Runx2 is a master transcription factor, and both endochondral and intramembranous bone formation are inhibited in Runx2-null mice. BMPs and Runx2 cooperatively interact to stimulate osteoblast gene expression, and Runx2 is essential for BMPs signaling in osteoblast differentiation. In addition, Runx-2 is required for osteoblastic differentiation and bone formation during embryonic development.

In this study, Runx-2 mRNA expression was upregulated in spheroid-derived MSCs treated with the mixture of OIM and BMP-2. Similarly, OSX, a zinc-finger-containing transcription factor, is required for osteoblast differentiation and bone formation. OSX-null preosteoblasts are blocked from differentiating into mature osteoblasts and express chondrogenic markers and can differentiate into both chondrocytes and osteoblasts, indicating that OSX is necessary for mature bone formation. Our results showed increased expression of both Runx-2 and OSX in spheroid-derived MSCs cultured in the mixture of OIM and BMP-2. Furthermore, addition of BMP-2 enhanced intranuclear expression of OSX in spheroid-derived MSCs, indicating promotion of transcriptional activity or osteogenic differentiation.

The BMP and Wnt families are essential for commitment and differentiation of the osteoblast lineage and BMPs play significant roles in bone formation via Smad-dependent pathways. In this study, we demonstrate that spheroid-derived MSCs exhibited upregulation of Smad5 mRNA and increased pSmad 1/5 expression. BMPs induces canonical Smad 1/5/8 phosphorylation and it has been postulated that Smad 1/5/8 are intracellular signaling proteins that transduce signals elicited by members of the BMP signaling pathway in osteoblasts. Following BMP induction, Smad pathways converge at the Runx2 gene to control mesenchymal precursor cell differentiation. The coordinated activity of Smads activated by Runx2 and BMP is critical for bone formation. Additionally, OSX is indispensable for bone formation as it is required for differentiation of preosteoblasts into mature osteoblasts. Increased expression of OIM-induced BMP-2 treatment is mediated by distal-less homebox 5 (Dlx5), an essential regulator of BMP-2-induced osteoblast differentiation and a bone-inducing homeodomain transcription factor expressed in the latter stages of osteoblast differentiation. Therefore, Dlx5 may directly interact with OSX. From these reports, we suggest that increased expression of both Runx2 and OSX is regulated by a Smad-dependent BMP pathway in spheroid-derived MSCs.

β-catenin is a transcription regulator in the canonical Wnt signaling pathway, which has several functions in osteoblastogenesis. BMP-2 and β-catenin synergize to promote osteoblast differentiation by stimulating ALP activity during osteogenic differentiation of multipotent embryonic cell lines which partially relies on Wnt signaling, suggesting that β-catenin may regulate osteoblastic differentiation via multiple mechanisms. However, in our experiments using spheroid-derived MSCs, BMP-2 stimulation did not enhance expression of β-catenin. Therefore, in spheroid-derived MSCs originating from human adipose tissues, enhancement of osteogenic differentiation by BMP-2 is independent of the canonical Wnt signaling pathway, which is supported by previous studies.

There are three possible limitations in our study. First, our study may be limited by the lack of direct evidence to define why spheroid-derived MSCs are susceptible to BMP-2 treatment, compared with MSCs in monolayer culture. Spheroid formation is regulated by integrin-extracellular matrix (ECM) and cadherin-cadherin interactions. Recent studies proposed that integrins are found to enhance BMP-2 receptor activity and induce smad phosphorylation via Cdc42-Src-FAK-ILK pathway. Therefore, we speculate that spheroid-derived MSCs may maintain enhancement of integrin activity. Secondly, although recent studies have suggested that cross-talk between BMP-2 and Wnt signaling pathway promotes osteogenesis in MSCs, BMP-2 treatment did not induce noncanonical Wnt signaling pathway in spheroid-derived MSCs as evidence by no change in β-catenin expression. Induction of the noncanonical Wnt signaling pathway by BMP-2 treatment remains unclear. Therefore, further studies are warranted to examine the mechanisms of cross-talk between BMP-2 and Wnt signaling pathway in spheroid-derived MSCs during the osteogenesis. Finally, this study may be limited by the lack of direct evidence to elucidate whether spheroid-derived MSCs treated with BMP-2 can accelerate bone formation in vivo. However, we expect that an implantation of BMP-2-stimulated spheroid-derived MSCs can promote bone regeneration in an in vivo bone defect model based on evidence of enhanced calcium deposition in vitro.

In conclusion, this study revealed that spheroid-derived MSCs treated with BMP-2 exhibit enhanced osteogenic differentiation via the canonical Smad-dependent BMP signaling pathway, which may be targetted to advance the osteogenesis capacity of MSCs and provide unique strategies for bone regeneration and repair.

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Conflict of Interest

The authors declare that they have no competing interest.

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