Modified Recombinant Human Bone Morphogenic Protein 2 with Enhanced Osteoinductivity

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Introduction: Bone morphogenic proteins (BMPs) play important roles in chemotaxis, mitogenesis, and differentiation of mesenchymal stem cells or progenitor cells [1]. Recombinant human BMP-2 (rhBMP-2) and rhBMP-7 have been used clinically to treat musculoskeletal disorders. However, recent clinical studies have shown various side effects due to high doses of rhBMP used in the local environment [2-4]. Recently, rhBMP-2 was modified in our laboratory to enhance its biological function so that lower doses can potentially be used. The objective of the current study was to compare the osteoinductivity and receptor binding potential of modified rhBMP-2 with unmodified mature rhBMP-2 using in vitro bioassays.

Methods: Preparation of Modified rhBMP-2: Modified rhBMP-2 was produced by truncation of the amino-terminus, purified with HPLC, and quantified by amino acid analysis. The new amino-terminus of modified rhBMP-2 was confirmed by N-terminal sequencing. In vitro bioassay: C2C12 cells were seeded at 25,000 cells/cm² in 24-well plates and cultured in osteogenic (OG) media (DMEM, 1% FBS, 10 mM β-glycerolphosphate, 50 mcg/mL L-ascorbic acid) with or without rhBMP-2 or modified rhBMP-2 (50 ng/ml). Media was changed every 3 days for up to 12 days. Alkaline Phosphatase (AP) Activity: Cell lysates were analyzed by AP assay and BCA protein assay on day 6. AP activity for each sample was quantified using p-NP standard and normalized to the protein quantity. Mineralization: Cells were fixed in 10% formalin and stained with 2% Alizarin Red S after 12 days of culture.

Gene Expression: Taqman primers and probes (Life Technologies) of AP, osteocalcin (BGLAP), collagen type I, and Runx2 were used for qRT-PCR analysis to study gene expression over time. Phosphorylated SMAD1/5/8 Expression: Cell lysates were collected after C2C12 cells treated with rhBMP-2 or modified rhBMP-2 for 2, 24, 48, and 72 hours. Five micrograms of protein from each group were separated by electrophoresis on SDS-PAGE gel and blotted to PVDF membrane. The phosphorylated SMAD-1-5-8 and non-phosphorylated SMAD-5 control were detected using antibodies (Cell Signaling, Inc) and colorimetric development kit (Bio-Rad, Inc.). Soluble Receptor Binding Assay: C2C12 cells were cultured in media containing rhBMP-2 or modified rhBMP-2 (50ng/ml) with the addition of soluble BMP receptors RhBMPR1A or RhBMPR1B at concentrations of 0, 40, 80, 160, 320, 640, or 1280 ng/mL (R&D Systems, Inc.). After 3 days, AP activity in cells was quantified. The percentages of AP activity inhibition were calculated by comparing the AP activity in cells at different BMP receptor concentrations to that in cells with no BMP receptor control.

Results: After 6 days of culture, the AP activity in C2C12 cells treated with 50 ng/ml modified rhBMP-2 was about 70-times higher (p<0.01) than that in cells treated with 50 ng/ml unmodified rhBMP-2. In addition, 50 ng/ml modified rhBMP-2 also induced about 50% higher (p<0.01) AP activity than 200 ng/ml unmodified rhBMP-2 (Fig 1A). Alizarin Red S staining showed significantly more calcium deposition in C2C12 cells cultured with modified rhBMP-2 than in cells cultured with unmodified rhBMP-2 after 12 days (Fig 1B). The gene expression analysis over time revealed significantly higher expression of the AP, osteocalcin, collagen type I, and Runx2 genes in cells cultured with modified rhBMP-2 on day 2 and day 3 compared to those in cells cultured with unmodified rhBMP-2 (Fig 2).

Higher expression of the phosphorylated SMAD 1/5/8 protein was found in cells cultured with modified rhBMP-2 than that in cells cultured with unmodified rhBMP-2 on day 1 through day 3 (Fig 3). Phosphorylated SMAD 1/5/8 was undetectable on day 3 in cells cultured with unmodified rhBMP-2. The control non-phosphorylated SMAD5 expression was consistent for all the time points.

The AP activity induced by rhBMP-2 or modified rhBMP-2 was inhibited at different levels by various concentrations of soluble BMP receptors. RhBMPR1A at 40ng/mL inhibited 78% of modified rhBMP-2-induced AP activity while RhBMPR1A inhibited 32% of unmodified rhBMP-2-induced AP activity (Fig 4A). RhBMPR1B at 320 ng/mL inhibited 82% and 54.5% of modified rhBMP-2 and unmodified rhBMP-2-induced AP activity, respectively (Fig 4B).

Discussion: Results from the present study demonstrate that modified rhBMP-2 has significantly higher osteoinductivity and binds to both soluble BMPR1A and BMPR1B more efficiently than unmodified rhBMP-2. Modification of rhBMP-2 provides the potential to significantly reduce rhBMP-2 therapeutic doses. Further investigations will be conducted to support clinical applications with less adverse effects.

Significance: Modification of rhBMP-2 provides the potential to significantly reduce rhBMP-2 therapeutic doses.

Acknowledgments:

Figure 1: (A) AP activity in C2C12 at day 6. (B) Alizarin Red S staining of C2C12 in OG media, rhBMP-2, or modified rhBMP-2.

*p<0.01. (B) Alizarin Red S staining of C2C12 in OG media, rhBMP-2, or modified rhBMP-2.

Figure 2: Relative gene expressions in C2C12 cells cultured with rhBMP-2 (Black) or modified rhBMP-2 (Gray): (A) AP, (B) osteocalcin, (C) collagen type 1, (D) Runx2. * p < 0.01, # p < 0.05.

Figure 3: Time course study of phosphorylated SMAD1/5/8 expression in C2C12 cells treated with rhBMP-2 or modified rhBMP-2 (top panel). Control non-phosphorylated SMAD-5 expression in C2C12 cells treated with rhBMP-2 or modified rhBMP-2 (bottom panel).

Figure 4: AP activity of C2C12 cells cultured with rhBMP-2 (circle) or modified rhBMP-2 (square) inhibited by soluble BMP receptors (A) rhBMPR1A, (B) rhBMPR1B at different concentrations.