rhBMP-2 induces the differentiation and mineralization of MC3T3-E1 cells under hypoxic conditions via activation of PKD and p38 MAPK signaling pathways

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Abstract

Background: Although many studies have examined the effects of rhBMP-2 on osteoblastic differentiation, mineralization and the related signaling pathways, those of rhBMP-2 on osteoblastic cells remain unknown, particularly under hypoxic conditions. Therefore, this study was conducted under a 1% oxygen tension to examine the differentiating effects of rhBMP-2 on osteoblastic cells under hypoxia.
Results: rhBMP-2 increased the ALP activity in a time dependent manner, and expression of ALP, Col-1 and OC mRNA were up-regulated significantly in a time- and concentration-dependent manner. In addition, the area of the mineralized nodules increased gradually in a concentration-dependent manner. Western blot analysis showed that rhBMP-2 significantly promoted the phosphorylation of the p38 mitogen-activated protein kinase (MAPK) in a time-dependent manner. A pretreatment with SB203580, a p38 MAPK inhibitor, and Go6976, a PKD inhibitor inhibited the rhBMP-2-mediated differentiation and mineralization.
Conclusion: These findings suggest that rhBMP-2 induces the differentiation and mineralization of MC3T3-E1 cells under hypoxic conditions via activation of the PKD and p38 MAPK signaling pathways.

Introduction

The effects of hypoxia, a low oxygen tension on the genes involved in osteoblast proliferation and differentiation is controversial but, generally, osteoblasts and their differentiation tends to be inhibited by hypoxia.

rhBMP-2 play an important role in osteoblast differentiation and bone remodeling by inducing different signaling pathways in the cells. The BMP signaling pathways are mediated primarily by Smad proteins. Other non-Smad signaling pathways, such as the mitogen-activated protein(MAP) kinase super family are also involved in osteoblastogenesis. Lemonnier et al. indicated the possible mediators between BMP receptors and MAPKs and suggested the involvement of protein kinase D(PKD) in the activation of c-Jun N-terminal kinases(JNK) and p38 induced by BMP-2. On the other hand, the effects of rhBMP-2 on osteoblastic cells under hypoxic conditions, which clinicians encounter frequently in clinical situations requiring bone regeneration and repair, as well as the precise molecular mechanisms underlying these effects are unknown.

Therefore, this study examined the effects of rhBMP-2 on the differentiation and mineralization of mouse preosteoblastic MC3T3-E1 cells under hypoxic conditions and their underlying pathways.

Materials and Methods

Cell culture
MC3T3-E1 cells, a murine osteoblastic cell line, were cultured in α-MEM with 10% FBS and 1% penicillin-streptomycin, and incubated in a humid incubator at 37°C, 95% O_2 and 5% CO_2.

Differentiation under hypoxic conditions
For osteoblast differentiation, the MC3T3-E1 cells were seeded. At confluence (day 0), the cells were induced with osteogenic medium supplemented with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. The cells were transferred to a hypoxic chamber with 95 % N_2 and 5 % CO_2 at 37°C. The cells were then incubated within the chamber for 1, 3, 7, and 14 days under 1% oxygen tension. The rhBMP-2 and p38 MAPK and PKD inhibitors (SB203580, Go6976) were dissolved in D.W and DMSO, and then diluted 1000-fold. rhBMP-2 was then added to the medium at different concentrations. The final working concentration was 100 ng/ml for rhBMP-2.

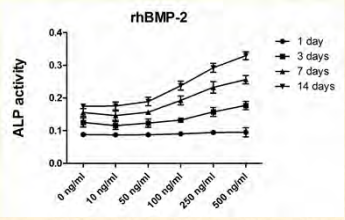
Alkaline phosphatase (ALP) assay
The MC3T3-E1 cells (2×10^5) were plated in 24-well plates and cultured in the osteogenic media for 1 day, and then treated with or without rhBMP-2. The medium and rhBMP-2 were renewed every 3 days. After the treatment with rhBMP-2, the cells were washed twice with PBS. Subsequently, lysis buffer 500 μL was added and the resulting mixture was sonicated on ice to lyse the cells. The final concentration of the protein used in this work was 50 μg. The lysates were incubated with 200 μL ALP reaction buffer for 1 h at 37°C. The ALP activity was measured from the optical absorbance at 405 nm using a microplate reader.

Alizarin Red S Staining for Mineralization analysis
The cells were grown in osteogenic media and treated with rhBMP-2 for 7 - 14 days. The differentiation medium and rhBMP-2 were changed every 3 days. The cells were fixed with 70% ethanol for 1 h, washed 3 times with distilled water, and then stained with 1% Alizarin Red S solution (pH 4.2) for 10 min. The cells were then washed 3 times with distilled water. To quantify the level of calcium deposition, the cells were destained with 10% cetylpyridinium chloride and transferred to a 96-well plate, and the absorbance was measured at 550 nm using a microplate reader.

Western blot assay
The cells (2×10^6) were plated in 100mm culture dishes. After 24 h, the cultured cells were treated with rhBMP-2 and/or a combination treatment of sb203580 and Go6976. The cells were incubated in RIPA buffer at 4℃ for 1 h. The cells were then centrifuged at 14,000 rpm for 30 min at 4 ℃ and the protein extract of the cells was transferred. The protein concentrations were determined using a Bio-Rad Protein Assay Kit. Equal amounts of protein (20 μg) were separated by 10% SDS-PAGE gels and transferred to PVDF membranes. The PVDF membranes were blocked with 5% fat-free dry milk in PBS for 1 h. The membranes were incubated overnight with the primary antibodies and then the membranes were developed using Western blotting detection reagent, and the bands were exposed and analyzed using an Alpha Imager HP.

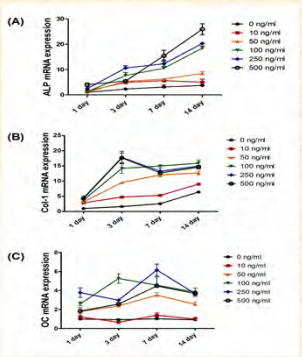
Results

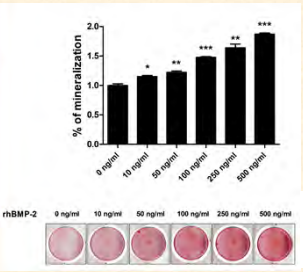
- **Effects of rhBMP-2 on the differentiation and mineralization of hypoxic MC3T3-E1 cells**



Effects of rhBMP-2 on the ALP activity of MC3T3-E1 cells under hypoxic conditions. rhBMP-2 time-dependently promoted the ALP activity($p<.001$). On the other hand, there was no significant correlation between the ALP activity and concentration of rhBMP-2.

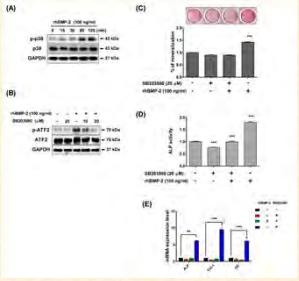
Effect of rhBMP-2 on the expression of the differentiation markers of MC3T3-E1 cells under hypoxic conditions. The total mRNA of ALP, Col-1 and OC was collected on days 1, 3, 7, and 14 from hypoxic cultured cells and the gene expression was determined by a real-time reverse transcription-polymerase chain reaction(A-C). The expression of ALP, Col-1 and OC mRNA were up-regulated significantly in a time- and concentration-dependent manner ($P < 0.001$)





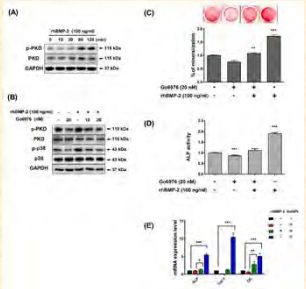
The area of the mineralized nodules, which was positively stained, increased gradually in a concentration-dependent manner after 14 days of culture by Alizarin red S staining.($P < 0.01$)

- **rhBMP-2 induces the differentiation and mineralization of hypoxic MC3T3-E1 cells via the p38 MAPK pathway**



The hypoxic cells were treated with 100 ng/mL rhBMP-2 for 120 min, the phosphorylation of p38 MAPK was promoted in a time-dependent manner. In the presence of SB203580, the phosphorylation of activating transcription factor 2(ATF 2), a specific target protein for p38 MAPK, was blunted significantly. the ALP activity, BMP-2-induced differentiation markers expression (ALP, Col-1 OC) and the formation of mineralized nodules were inhibited when pretreated with SB203580(* $P < 0.01$)

- **rhBMP-2 activates p38 MAPK via the PKD pathway in hypoxic MC3T3-E1 cells**



rhBMP-2 time-dendeepntly stimulated the phosphorylation of PKD and p38, which was inhibited by pretreating the cells with Go6976, a PKD inhibitor In addition, the rhBMP-2-mediated induction of the osteoblast marker genes, ALP activity and the formation of mineralized crystals were all inhibited by Go6976. (* $P < 0.01$)

Discussion & Conclusion

- rhBMP-2 stimulates the maturation and function of osteoblasts by regulating early and late osteoblast differentiation, even under hypoxic conditions.

- rhBMP-2 could phosphorylate p38 MAPK in hypoxic MC3T3-E1 cells in a time-dependent manner

- rhBMP-2 can induce the activation of PKD in osteoblastic cells and that this kinase is involved in the activation of p38 induced by BMP-2.

- In summary, these results suggest that BMP-2 promotes the differentiation and mineralization of osteoblastic MC3T3-E1 cells under hypoxic conditions via the p38 MAPK and PKD signaling pathway.