学 位 論 文 の 要 旨

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学 位 論 文 名	BMP/Wnt Antagonists are Upregulated by Dexamethasone in Osteoblasts and Reversed by Alendronate and PTH: Potential Therapeutic Targets for Glucocorticoid-induced Osteoporosis
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論 文 内 容 の 要 旨

Introduction

Glucocorticoid is known to induce osteoporosis mainly by suppressing osteoblast-mediated osteogenesis. Both the bone morphogenetic protein (BMP)-Runx2 and the Wnt signal pathways are known to play crucial roles in osteogenesis and to stimulate osteoblast differentiation. Glucocorticoid may affect these signal pathways, but the precise mechanisms are still unclear.

The BMP-Runx2 and the Wnt pathways are regulated by several processes. One of known mechanisms is the regulation by a variety of secreted proteins that act as extracellular antagonists. Noggin, Chordin, Twisted gastrulation (Tsg), Gremlin, Sclerostin, follistatin, and Dan are BMP antagonists, while secreted frizzled-related protein (sFRP), Dickkopf (Dkk) and Cerberus are Wnt antagonists. Follistatin binds to the complex of BMP and its receptor, leads to suppression of BMP activity. Also, Dan binds to BMP, acts as an antagonist, and leads to the BMP signal suppression. On the other hand, sFRPs have been found to act as competitive inhibitors of Wnt signaling by competing with membrane-bound frizzled proteins for Wnt binding. Axin is an intracellular inhibitor of canonical Wnt signaling, by promoting the phosphorylation and consequent degradation of β-catenin , and deletion of Axin-2 led to an enhancement in the proliferation and differentiation of osteoblasts.

It has been suggested that glucocorticoid induces impaired osteoblastic function through increased Wnt antagonists and subsequent suppression of the Wnt pathway. In contrast, little is known about whether or not the BMP pathway would be involved in glucocorticoid-induced osteoblastic dysfunction through modulation by BMP antagonists. In the present study, to settle this issue, we examined whether or not Dex suppresses the BMP pathway in addition to the Wnt pathway by increasing their antagonists, follistatin/Dan and sFRP-1/axin-2, respectively. Moreover, we investigated whether or not alendronate or parathyroid hormone (PTH), which is known to be effective in treating glucocorticoid-induced

osteoporosis (GIO), would have the abilities to modulate these processes.

Materials and methods

Cell culture MC3T3-E1 cells were cultured in α-Modified Minimal Essential Medium (α-MEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in 5% CO_2 at 37°C. To support differentiation, 10 mM β-glycerophosphate were added to confluent cultures and the medium was then changed twice a week.

Mineralization assay The cultured cells were fixed with 95 % ethanol and stained with $AgNO_3$ by the von Kossa method to detect phosphate deposits in bone nodules. At the same time, the order plates were fixed with ice-cold 70% ethanol and stained with Alizarin red to detect calcification. For quantification, cells stained with Alizarin red ($n \geq 6$) were destained with ethylpyridium chloride, then the extracted stain was transferred to a 96-well plate, and the absorbance at 550 nm was measured using a microplate reader.

Real-time PCR MC3T3-E1 cells were cultured and treated with Dex 10^{-7}M on days 3, 5, and 7. RNA was extracted after 2.5 days of the treatment. Total RNA was reverse-transcribed with the Omniscript Reverse Transcription Kit. The sense and antisense primers were designed using the Primer Express Version 2.0.0 based on published cDNA sequences. PCR was performed using 1 μl of cDNA in a 25 μl reaction volume with ABI PRISM7000. Expression of mRNA were corrected by house keeping gene, 36B4. Each experiment was repeated at least 5 times.

Immunoblotting Cells were rinsed with ice-cold PBS and scraped on ice into lysis buffer that contained 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EGTA, 1 mM Na_2EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin. The cell lysates were then sonicated for 30 sec. Nuclei and cell debris were removed by centrifugation (12,000 × g for 10 min). The cell lysates were electrophoresed by 10% SDS-PAGE and transferred to nitrocellulose membrane. The blots were incubated overnight at 4°C with gentle shaking with a β-catenin antibody at a 1:200 dilution. The blots were extensively washed with PBS containing 1% Tween 20 (BIO-RAD) and 0.15% dry milk at room temperature and were further incubated with a 1:2000 dilution of horseradish peroxidase-coupled secondary antibody in PBS containing 1% Tween 20 for 1 hr at room temperature. The blots were then washed, and the signal was visualized by chemiluminescence.

Statistics Results are expressed as a mean ± SEM. Statistical evaluation for differences between groups was carried out using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD). For all statistical tests, a value of $P < 0.05$ was considered to be a statistically significant difference.

Results

Dex (10^{-9}–10^{-7} M) strongly suppressed mineralization of the cells by both von Kossa and Alizarin red stainings on day 21. Quantification of the Alizalin red staining showed that this inhibition was significant ($p<0.01$) and dose-dependent. On the other hand, neither alkaline phosphatase staining nor activity were significantly affected by Dex. Dex (10^{-7} M) increased mRNA expression of BMP antagonists, follistatin and Dan as well as mRNA expression of a Wnt antagonist, sFRP-1 and a Wnt signal inhibitor, axin-2. In contrast, the mRNA expression of Runx2, downstream of the BMP signal pathway and the protein

expression of β-catenin were decreased by Dex (10^{-7}M). Moreover, pretreatments with alendronate (10^{-8} M) or human PTH (1-34) (10^{-8} M) totally or partially antagonized not only the Dex-induced enhancement in mRNA expression of follistatin/Dan and sFRP-1/axin-2 but also the Dex-induced reduction in mineralization and Runx2 mRNA expression.

Discussion

Both the BMP and the Wnt pathways are known to play crucial roles in the osteogenesis and in stimulating osteoblast differentiation. Leclerc et al. have recently reported that Dex stimulated mRNA expression of follistatin and Dan by cDNA microarrays. Thus, we focused on these two BMP antagonists and additionally showed that Dex-induced increase in follistatin and Dan may play a physiological role in the inhibition of osteoblastic mineralization. On the other hand, several studies have shown that glucocorticoid could inhibit the Wnt pathway through enhanced expression of its antagonists. The present findings of Dex-induced increase in sFRP-1 expression in osteoblastic MC3T3-E1 cells seem to accord with these previous findings, and we for the first time showed that Dex also increased another Wnt signal inhibitor, axin-2, which acts intracellularly.

Bisphosphonates are known to be effective in treating osteoporosis by increasing bone mineral density and suppressing bone fractures. Although the primary action of bisphosphonates is the inhibition of osteoclastic bone resorption, there is increasing evidence that bisphosphonates also interact with osteoblasts. Bisphosphonates have been reported to enhance proliferation of human bone marrow stromal cells and to initiate osteoblastic differentiation. Moreover, it has been reported that alendronate increased proliferation and ALP activity, and enhanced mRNA expression of BMP-2, type I collagen, and osteocalcin in human trabecular bone cell culture and MG63 osteoblast-like cells. Our findings also suggest that alendronate interacts with osteoblasts and alleviates GIO through totally or partially reversing Dex-induced enhancement in BMP and Wnt antagonists in the cells.

Bone anabolic effects of intermittent PTH treatment are well documented, but the mechanisms by which this hormone stimulates bone formation are still not clear. PTH signaling has been observed to modulate the Wnt pathway in osteoblasts and bone. The present findings suggest that the effectiveness of PTH in treating GIO may also be related to the modulation of the BMP pathway via totally or partially suppressing Dex-induced enhancement in their antagonists, although we provided no data on the precise mechanism by which PTH affects the BMP pathway.

Conclusion

The present study shows that Dex suppresses both of the BMP and Wnt pathways by enhancing the expression of their antagonists, suggesting that glucocorticoids can suppress osteoblastic differentiation and activity through the dual signaling modulation. Moreover, we showed that the effectiveness of bisphosphonate and PTH in treating GIO may be partly explained by reversion of these processes. Thus, diminishing the activities of Wnt and BMP antagonists seems to be one of potential therapeutic targets for GIO.