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Osteoactivin inhibits dexamethasone-induced osteoporosis through up-regulating integrin β1 and activate ERK pathway

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ABSTRACT

Backgrounds: Dexamethasone (Dex) is widely used in autoimmune diseases and inflammation treatment. A sever side effect of prolonged exposure to Dex is increased risk of osteoporosis (OP) or even femoral head necrosis, which would cause much suffer to patients. To reveal the mechanism behind this phenomenon, provide therapeutic guidance and potential target, we analyzed the inhibitory mechanism of Dex on osteogenesis of rat-BMSC.

Methods: Rat BMSC were obtained and characterized with FACS analysis. Osteogenesis and adipogenesis abilities were detected with Oil-O-Red staining, Alizarin Red staining and ALP activity analysis. These BMSC were then treated with Dex in combination with recombinant OA or not and detected for osteogenesis related gene expression with qRT-PCR. Protein interaction and expression were detected by Co-Immunoprecipitation and western blot.

Results: Osteoactivin (OA) could promote integrin β 1 expression and interact with this protein physically, leading to ERK activation and promoting osteogenesis related genes' expression including Runx2, Col1a and OCN in BMSC. Dex, however, could block expression of several upstream genes of OA and decrease OA mRNA and protein level, and eventually suppress integrin β1-ERK activation and lead to decreased osteogenesis, which could finally develop into OP.

Conclusion: Recombinant OA treated BMSC exerted better osteogenesis potency even with Dex administration. This is because additional OA in medium counter-acts with Dex's influence and rescued osteoblast differentiation via up-regulating integrin β1 and activate ERK/MAPK pathway which promotes osteogenesis. Hence, OA/integrin β1 could serve as potential therapeutic target for OP.

1. Introduction

Osteoporosis could lead to increased bone fractures, low bone mass and bone microarchitecture damage. It is a world wild disease and effefts around 28 million people [1]. It falls into two major categories, primary OP and secondary OP. Primary OP is closely related to gender and age, while secondary OP is primarily a consequence of prolonged medical administration [2]. Dexamethasone (Dex) is an immune inhibitor widely used in clinical treatment for autoimmune diseases, immunological rejection and inflammations [3]. Dex inhibited osteogenesis has been described as the most severe side effect of such therapies [4]. Prolonged administration of such compounds would lead to osteoporosis or even femoral head necrosis, a serious disease with no effective therapy so far [3,4]. Molecular mechanism underlying this event is not clear yet.

Bone marrow stromal cell (BMSC) is a fibroblast-like adult stem cells with multipotency. BMSC could differentiate into adipocytes, osteoblasts and chondroblasts. It therefore bears the potential to maintain and repair bone tissue. It is also investigated as the most suitable cell source for bone regeneration [5,6]. Differentiation of BMSC into osteoblasts is strictly regulated and involves several pathways and stimulus, including BMP2, Notch/Hedgehog pathway, ERK pathway and

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ant-beta catenin pathway [7]. Hence, decreased osteogenesis of BMSC could increase risk of OP due to reduced bone tissue reiteration and remodeling. It is also reported that dexamethasone treated BMSC showed increased adipogenesis and decreased osteogenesis [4,8]. This is because Dex manipulates BMSC into adipocyte favoring differentiating fashion over osteogenesis. Dexamethasone shifts bone marrow stromal cells from osteoblasts to adipocytes by C/EBP alpha promoter methylation [4]. So we reasoned that dexamethasone induces OP via affecting differentiating preference of BMSC and inhibiting osteogenesis. It would bare great therapeutic potential for hormone induced OP via re-modulating BMSC differentiation.

One essential factor involved in bone marrow stromal cell differentiation is osteoactivin (OA) [9]. OA is also known as GPNMB or DC-HIL [10]. Osteoactivin is a novel osteoclastic protein and plays a key role in osteoclast differentiation and activity. This protein is wildly expressed in organs including bone, liver and kidney with different expression levels, and is especially highly expressed during osteoblast differentiation [11,12]. OA participates in multiple biological processes such as proliferation, cell adhesion, differentiation and ECM synthesis [10]. One of its most important roles is that it modulates bone marrow stromal cell osteogenesis [11]. Noteworthy, there's little study about whether OA is involved in dexamethasone induced BMSC osteogenesis so far.

In this study, we found that dexamethasone could decreased OA expression by blocking BMP2 pathway, which further lead to integrin β1 and ERK inhibition. Decreased integrin β1 signal pathway would then affect expression of osteogenesis related genes such as Runx2, OPN, Osx, OCN, and osteogenesis associated ERK pathway, and finally lead to osteogenesis inhibition. This results could provide clues for further studies.

2. Materials and methods

Alizarin Red staining. Cells were rinsed with PBS and stained with 40 mM Alizarin Red (pH 4.2) for 30 min at 37 °C. After removing medium, cells were then washed with PBS twice and imaged.

Oil Red O staining. Cells were washed twice with PBS and fixed with 4% PFA for 2 h at 4 °C. After two washes in PBS, cells were stained for 2 h in freshly diluted Oil Red O solution consisting of six parts Oil Red O stock solution (0.5% in isopropanol) and four parts water at 4 °C. Solution was removed from the cells with two PBS washes and cells were examined with an inverted microscope.

qRT-PCR. Total RNA were extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and real-time PCR reaction were conveyed using SYBR Green PCR Master Mix (Takara Bio Inc., Otsu, Japan). comparative $2-\Delta\Delta Ct$ method was used to calculate the relative expression of each target gene; the expression levels of all genes were normalized to GAPDH. Primers used in this study were listed in Table 1.

Western Blot. Cells were collected and extracted for proteins with RIPA. Proteins were then separated on SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with 5% milk in TBST for 1 h at room temperature and incubated with antibodies at 4℃ overnight. Membranes were washed with PBS 3 times and incubated with 2nd antibodies for 1 h at room temperature. Wash membranes with PBS 3 times in dark chamber, and subject to ECL detection. Actin, integrin β1, GAPDH antibodies were obtained from Santa Cruz; OA, ERK and pERK antibodies were obtained from CST.

Alkaline phosphatase (ALP) activity. Cells were lysed and extracted cellular components. Supernatants were detected for ALP activity using Alkaline Phosphatase Assay Kit (Beyotime, China) following manufactures' instructions. Briefly, extracts were mixed with working solution and incubated for 10 min at 37℃, and immediately mixed with stop buffer. OD405 were detected and analyzed ALP activity.

Co-Immunoprecipitation (Co-IP). Cells were washed by PBS and collected at 3000 rpm for 5 min at 4℃. For co-IP, cells were lysed with Co-IP buffer (50 mM Tris pH 8.0, 0.5% NP-40, 1 mM EDTA, 150 mM NaCl). Lysate was incubated with primary antibody for 2–4 h at 4 °C, followed by addition of 50% slurry of protein G beads and overnight incubation at 4 °C.

Table 1
The primer sequence used for qRT-PCR.

Gene name	Primer Sequence
OA-F	5′-CCAATTACGTGGCTGGTCTT-3′
OA-R	5′-GATATTGCCGTTGGCATCTT-3′
Runx2 -F	5′-ACCTCCAGGAAGCCTTTGAT-3′
Runx2 -R	5′-CCTGGTGGTGTCACTGAATG-3′
BMP2 -F	5′-CGGAAGCGTCTTAAGTCCAG-3′
BMP2 -R	5′-CGCTAAGCTCAGTGGGGACAC-3′
Dlx5 -F	5′-GTAACTCGCCACAGTCACCA-3′
Dlx5 -R	5′- CAGGAAGCCGAGTTCTCCAG -3′
Dlx3-F	5′- ACCTAGGACAACAATGGGCA -3′
Dlx3-R	5′- GCGGGTATTTGGGGTTTTGT -3′
Smad1-F	5′- ATCAATAGAGGAGATGTTCAAGCAG -3′
Smad1-R	5′- ACACCTCTCCTCCGACGTAA -3′
Glut4-F	5′- GCCGGACATTTGACCAGATC -3′
Glut4-R	5′- GAGGTAAGGGAAGAGAGGGC -3′
aP2-F	5′- ATTCGGCACGAGTCCTTGAA -3′
aP2-R	5′- GACCAAGTCCCCTTCTACGC -3′
Ocn-F	5′- CAAGTCCCACACAGCAACTC -3′
Ocn-R	5′- GTCCATTGTTGAGGTAGCGC -3′
Osx -F	5′-CAATTGGTTAGGTGGTGGGC-3′
Osx -R	5′-TCTTGGGGTAGGACATGCTG-3′
Col1a1-F	5′- AACAAGGGAGGAGAGAGTGC-3′
Col1a1-R	5′- AGTCTCTTGCTTCCTCCCAC-3′
Integrin β1-F	5′-GGACGCTGCGAAAAGATGAA-3′
Integrin β1-R	5′-CACATCGTGCAGAAGTAGGC-3′
GAPDH-F	5′-AGCCCAAGATGCCCTTCAGT-3′
GAPDH -R	5′-CCGTGTTCCTACCCCCAATG-3′

Statistical analysis. All data values were shown as mean ± standard deviation (SD). Comparison of all other results was performed by one-way analysis of variance (ANOVA) with Tukey's comparison analysis and the statistical significance was analyzed using Student *t*-test and analysis of variance. Data was considered significant when $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The Graphpad Prism 5 (Graphpad Software Inc,) was used to compare groups.

3. Results

3.1. Dex inhibited OA and its upstream protein expression in BMSC

To gain insight into Dex induced osteogenesis inhibition, we turned our sight to OA. Firstly, we successfully isolated and acquired rat BMSC with fine osteogenesis and adipogenesis ability, which would fit for our following experiments (Supplement Fig. 1). We therefore cultured BMSC in osteogenesis inducing medium, and detected the mRNA level of OA during osteogenesis. In BMSC undergoing osteogenesis induction at day 3, day 7, day 14 and day 21, mRNA was isolated and subjected to qRT-PCR analysis. Results indicated that mRNA level of OA was dramatically decreased by Dex administration. In cells cultured in inducing medium (IM) and additional Dex, OA mRNA level decreased by 39% compared to IM. This was firstly observed at day 7, and the degree of decline increased to about 82% at day 21 (Fig. 1A). Similar result was observed when we detected OA protein level by western blot (Fig. 1B). Protein level of OA in Dex treated BMSC was much lower than that in cells without Dex administration.

Because no direct interaction between OA and Dex was ever reported, we reasoned that Dex might affected other proteins upstream of OA. As a consequence, disturbance on upstream signal finally inhibited OA expression. So we conveyed another qRT-PCR analysis and detected mRNA level of BMP2, SMAD1, Dlx5 and Dlx3, all of which were genes upstream of OA [4,13,14]. These genes were also closely associated with osteogenesis. Cells were treated as in Fig. 1C. mRNA level of these genes also decreased with Dex administration, among which Dlx5 and