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Short communication

Inhibition of osteoblast differentiation by aluminum trichloride exposure is associated with inhibition of BMP-2/Smad pathway component expression

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ABSTRACT

Bone morphogenetic protein-2 (BMP-2)/Smad signaling pathway plays an important role in regulating osteoblast (OB) differentiation. OB differentiation is a key process of bone formation. Aluminum (Al) exposure inhibits bone formation and causes Al-induced bone disease. However, the mechanism is not fully understood. To investigate whether BMP-2/Smad signaling pathway is associated with OB differentiation in aluminum trichloride ($AlCl_3$)-treated OBs, the primary rat OBs were cultured and exposed to 0 (control group, CG), 1/40 IC_{50} (low-dose group, LG), 1/20 IC_{50} (mid-dose group, MG), and 1/10 IC_{50} (high-dose group, HG) of $AlCl_3$ for 24 h, respectively. We found that the expressions of OB differentiation markers (Runx-2, Osterix and ALP) and BMP-2/Smad signaling pathway components (BMP-2, BMPR-IA, p-BMPR-IA, BMPR-II, p-Smad1/5/8 and p-Smad1/5/8/4) were all decreased in $AlCl_3$-treated OBs compared with the CG. These results indicated that inhibition of OB differentiation by $AlCl_3$ was associated with inhibition of BMP-2/Smad pathway component expression. Our findings provide a novel insight into the mechanism of $AlCl_3$-induced bone disease.

1. Introduction

Aluminum (Al) is a ubiquitous environmental metal toxicant (Sun et al., 2016). Bone is mainly target tissue for Al, approximately 70% of the Al^{3+} accumulates in bone of the body (VanDuyn et al., 2013). Excessive Al accumulation disrupts bone formation and causes bone disease which defined as “Al-induced bone disease” (AIBD), ending with spontaneous fractures, including osteomalacia and osteoporosis (Kasai et al., 1991; Boyce et al., 1992; Crisponi et al., 2011; Cao et al., 2016a). Al has been implicated in all forms of renal osteodystrophy; about 90% of low turnover osteomalacia and adynamic bone disease and 50% mixed uraemic osteodystrophy at one point involved Al (Malluche, 2002). Osteomalacia induced by Al manifests as the bone formation impairment, low bone mineral density (BMD), proximal muscle weakness, diffuse bone pain and tenderness (Priest, 2004; Hellström et al., 2005; Maricic, 2008). Osteoporosis induced by Al manifests as low bone mineral mass and changed bone microarchitecture (Leung and Siu, 2013; Cooper and Martyn, 1991). In our previous research, we found that aluminum trichloride ($AlCl_3$) disturbed the metabolism of calcium and phosphorus, disrupted the microstructure of the bone, decreased the BMD, and inhibited bone formation (Li et al., 2011; Sun et al., 2015). Inhibited bone formation is the key in AIBD. Osteoblast (OB) differentiation is a central event in bone formation (Gunton et al., 2015). Basic research evidence showed that $AlCl_3$ could suppress bone formation by inhibiting OB differentiation (Cao et al., 2016b). However, the inhibition mechanism of OB differentiation caused by $AlCl_3$ remains elusive.

Bone morphogenetic protein-2 (BMP-2)/Smad signaling pathway plays an important role in regulating OB differentiation and bone formation (Katagiri and Takahashi, 2002). Activating

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BMP-2/Smad pathway promotes OB differentiation, conversely, inhibiting BMP-2/Smad pathway suppresses OB differentiation (Huang et al., 2014; Yonezawa et al., 2011). Accordingly, we deduce that BMP-2/Smad pathway is associated with the inhibition of OB differentiation induced by $AlCl_3$. In this pathway, BMP-2 ligand exerts its biological functions via activating the transmembrane receptor I (BMPR-IA) and II (BMPR-II) (Cárcamo et al., 1995; Wieser et al., 1995). Subsequently, the activated BMPR-IA (p-BMPR-IA) transmits the signal by recruiting and phosphorylating Smad1/5/8 (p-Smad1/5/8) (Hoodless et al., 1996; Chen et al., 2004; Shen et al., 2016). Then, p-Smad1/5/8 and Smad4 form p-Smad1/5/8/4, which translocates into the nucleus to regulate the expressions of osteogenic transcription factors Runt-related transcription factor-2 (Runx-2) and Osterix, and their downstream gene target, alkaline phosphatase (ALP) (Zhang et al., 2000; Nakashima et al., 2002; Afzal et al., 2005; Vimalraj et al., 2015). Runx-2, Osterix and ALP are positive regulators and markers of OB differentiation (Nishide et al., 2015; Vimalraj et al., 2015).

Therefore, we examined the expressions of OB differentiation markers and BMP-2/Smad signaling pathway components in $AlCl_3$-treated OBs to verify the relationship between the inhibition of OB differentiation and BMP-2/Smad signaling pathway.

2. Materials and methods

2.1. Cell culture and treatment

Experimental design and procedures were approved by the Animal Ethics Committee of the Northeast Agricultural University (Harbin, China). The procedure of isolation and cell culture was consistent with Cao et al. (Cao et al., 2016b). Briefly, primary OBs were isolated from the calvaria of 1-day-old Sprague-Dawley rats, then cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gibco, USA), 2 mM glutamine (Gibco, USA), 1% penicillin/streptomycin (Gibco, USA) and incubated overnight at 37 °C and 5% CO_2. Cells between 2nd and 3rd passage were harvested after reaching 90% confluence, and then were plated in the medium supplemented with 50 μg/mL Ascorbic Acid (Sigma, USA) and 10 mM β-glycerol phosphate (Sigma, USA) for 12 days, with medium changed every 2 days. OBs incubated with 0 (control group, CG), 1/40 IC_{50} (low-dose group, LG), 1/20 IC_{50} (mid-dose group, MG), and 1/10 IC_{50} (high-dose group, HG) of $AlCl_3$ for 24 h or with $AlCl_3$ for 3, 6, 12 and 24 h in the MG. Our previous work had demonstrated that the IC_{50} of $AlCl_3$ for OBs was 8.16 mmol/L (Cao et al., 2016b). After $AlCl_3$ treatment, OBs were harvested and used in the following studies.

2.2. ALP activity

OBs were incubated with $AlCl_3$ at 3, 6, 12, 24 and 48 h to determinate intracellular ALP activity by commercially available kits (Beyotime, CHN), the procedure was according to Cao et al. (2016b).

2.3. Quantitative RT-PCR

ALP, Runx-2, Osterix, BMP-2, BMPR-II and BMPR-IA mRNA expression were analyzed by qRT-PCR. Total OB mRNA was extracted by Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. cDNA was synthesized using First Strand cDNA Synthesis kit (TransScript First-Strand cDNA Synthesis SuperMix, TransGen Biotech, China). qRT-PCR was performed using SYBR Green/Fluorescein qPCR Master Mix on 7000 real-time PCR detection system (ABI, USA). Each sample was analyzed in triplicate, and the mean value was calculated. Relative mRNA expression was normalized to β-actin levels. Gene-specific primer pairs were shown in Table 1.

2.4. Western blotting

Runx-2, Osterix, BMP-2, p-BMPR-IA, p-Smad1/5/8 and p-Smad1/5/8/4 protein expression were examined in all $AlCl_3$-treated groups at 24 h and in the MG and CG at 3, 6, 12 and 24 h. OB cytoplasmic and nuclear proteins were extracted by protein extraction kits (Sangon Biotech Co., Ltd, China; BestBio Corp., Shanghai, China). Protein content was quantified by BCA protein assay reagent (Beyotime Institute of Biotechnology, China). Protein lysates were separated via SDS-PAGE and electro-transferred onto PVDF membrane. Specific protein detection was carried out by membrane incubation with anti-BMP-2, -p-Smad1/5/8, -Runx-2, -p-BMPR-IA and -Osterix antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. An appropriate secondary antibody was used subsequently (ZSGB-BIO, China). Finally, protein bands were visualized via the enhanced chemiluminescent reagent (Beyotime Institute of Biotechnology, China). The relative expression levels of membrane and cytoplasmic proteins were normalized to β-actin levels. Nuclear proteins were normalized to Histone H3. All assays were performed in triplicate.

2.5. Co-immunoprecipitation

The procedures were consistent with the research of Schenk et al. (Schenk and Horowitz, 2006). Immunoprecipitation was performed using an immunoprecipitation kit (Roche, China).

Table 1
Primer sequences and amplification lengths of destination fragments.

Gene	Primer sequence	Primer length (bp)	Product length (bp)
BMP-2	UP 5′ ACAAACGAGAAAAGCGTCAA 3′	22	291
	LOW 5′ CCAGTCATTCCACCCCACAT 3′	22	
BMPR-IA	UP 5′ TGCTCATCTCTATGGCTGTC 3′	19	325
	LOW 5′ AATGCTTCATCCTGTTCCA 3′	21	
BMPR-II	UP 5′ AGTCCGCCTCATTCATCTA 3′	19	321
	LOW 5′ CTGTTTCCTGTCTCCTGTC 3′	22	
Runx-2	UP 5′ GCACTATCCAGCCACCTTCA 3′	21	321
	LOW 5′ CTTCCATCAGCGTCAACA 3′	21	
Osterix	UP 5′ GAGACTCAACAGCCCTGGG 3′	22	325
	LOW 5′ GGGTGGGTAGTCATTGGCAT 3′	22	
ALP	UP 5′ ACCCTGCCTTACCAACTCA 3′	21	233
	LOW 5′ TCTCCAGCCGTGTCTCCTC 3′	19	
β-actin	UP 5′GCCAACACAGTGCTGTCT3′	18	114
	LOW 5′AGGAGCAATGATCTTGATCTT3′	21	