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Aryl hydrocarbon receptor suppresses the osteogenesis of mesenchymal stem cells in collagen-induced arthritic mice through the inhibition of β-catenin

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ABSTRACT

The contributions of aryl hydrocarbon receptor (Ahr) to the pathogenesis of rheumatoid arthritis (RA), particularly bone loss, have not been clearly explored. The imbalance between osteoblasts and osteoclasts is a major reason for bone loss. The dysfunction of osteoblasts, which are derived from mesenchymal stem cells (MSCs), induced bone erosion occurs earlier and is characterized as more insidious. Here, we showed that the nuclear expression and translocation of Ahr were both significantly increased in MSCs from collagen-induced arthritis (CIA) mice. The enhanced Ahr suppressed the mRNA levels of osteoblastic markers including Alkaline phosphatase (Alp) and Runt-related transcription factor 2 (Runx2) in the differentiation of MSCs to osteoblasts in CIA. The 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD)-mediated activation of Ahr dose-dependently suppressed the expression of osteoblastic markers. In addition, the expression of β-catenin was reduced in CIA MSCs compared with control, and the TCDD-mediated activation of the Ahr significantly inhibited β-catenin expression. The Wnt3a-induced the activation of Wnt/β-catenin pathway partly rescued the osteogenesis decline induced by TCDD. Taken together, these results indicate that activated Ahr plays a negative role in CIA MSCs osteogenesis, possibly by suppressing the expression of β-catenin.

1. Introduction

Bone remodeling depends on a functional balance among bone-resorbing cells, osteoclasts, and bone-forming cells, osteoblasts [1]. Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that primarily involves joints [2]. Synovitis exacerbates articular erosion, periarticular bone loss and osteoporosis, ultimately leading to disability [3,4]. Once bone erosions is established, limited signs of repair are observed, even with potent anti-inflammatory therapeutic strategies [5]. Indeed, synovitis fosters a fundamental imbalance in bone homeostasis, resulting in focal articular bone loss through the blockage of local bone formation [6,7]. Typically, osteoclasts, which are derived from the monocyte/macrophage lineage, are the principal cells of bone erosions. However, recent reports have demonstrated that the dysfunction of osteoblast-mediated bone erosion occurs earlier and is

characterized as more insidious [8,9]. Therefore, in the present study, we focused on the crucial roles of osteoblast differentiation factors in the development of early RA.

Mesenchymal stem cells (MSCs) isolated from bone marrow or adipose tissue, are multipotent progenitor cells, capable of differentiating into tissue-forming cells, such as bone and cartilage [10]. Many studies have confirmed that MSCs can differentiate into osteoblasts after being isolated and cultured *in vitro* [11]. They are increasingly being used for many clinical applications, such as orthopedic and reconstructive surgery. Recently, researchers have realized the important role of MSCs in anabolic processes in bone and have begun to fundamentally study the mechanisms of abnormal MSCs leading to bone loss and osteoporosis [12].

Aryl hydrocarbon receptor (Ahr) is a ligand-activated transcription factor that belongs to the basic helix-loop-helix/PER-ARNT-SIM

family. Upon binding with its ligand, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), Ahr undergoes conformational changes, translocates to the nucleus and interacts with the Ahr nuclear translocator (Arnt) to activate target gene transcription [13,14]. Ahr was initially identified as a receptor for environmental contaminants, which mediates the toxic and biochemical effects of dioxins. Recent studies have demonstrated that Ahr is closely correlated with the development of RA and bone metabolism. The high expression of Ahr was observed in synovial tissue, and increased Ahr exacerbated inflammatory responses and disease progression in RA [15]. However, Ahr deficiency in T cells alleviates collagen-induced arthritis (CIA) by suppressing both the differentiation of Th17 cells and the production of pro-inflammatory cytokines, such as IL-6 and IL-1β [16]. In addition, activated Ahr interferes with the balance of osteoblasts and osteoclasts and aggravates bone resorption [17]. In our previous study, we showed that the high expression of Ahr suppressed the differentiation and proliferation of osteoblasts in CIA and eventually induced bone destruction [18]. But whether Ahr influences the differentiation from MSCs to osteoblasts in CIA has not been completed elucidated.

The Wnt/β-catenin pathway plays an important role in the bone cell biology, which includes skeletal development, bone mass maintenance and osteoblast differentiation, through β-catenin-dependent and β-catenin-independent signaling [19,20]. Ahr activation leads to the downregulation of Wnt/β-catenin signaling and inhibits the differentiation and proliferation of liver progenitor cells and endometrial epithelial cells [21]. Conversely, other studies have also demonstrated that TCDD increased the expression of β-catenin to promote cell differentiation [22–24]. Thus, more studies are needed to explore the relationships between bone metabolism and Wnt/β-catenin pathway.

In the present study, we isolated the primary MSCs from CIA or control mice and examined the expression of Ahr pathway and osteoblastic markers and further explored the potential mechanisms on Wnt/β-catenin pathway.

2. Materials and methods

2.1. Animals

Six-week-old male DBA/1 mice were purchased from Beijing Vital River Laboratories (VRL) Co., Ltd. The mice were maintained under specific pathogen-free conditions. All experiments were conducted in accordance with the requirements of the Animal Care and Use Committee of Capital Medical University (Permit ID: SCXK-2014-0004).

2.2. CIA model and assessment of arthritis

The CIA model was established as previously described [25]. The incidence of arthritis was recorded every two days after the booster immunization, and severity of arthritis was assessed using clinical scores: 0=no signs of arthritis; 1=swelling and/or redness of the paw or one digit; 2=two joints involved; or 3=more than two joints involved and 4=severe arthritis of the entire paw and digits, as previously described [26]. The maximum clinical score was 16 per mouse, and each limb was graded independently. Animal clinical scores equal to or greater than 6 were used for the following cell experiments.

2.3. MSC isolation, culture and treatment

MSCs were isolated from the bone marrow of femurs and tibias of CIA or control mice as previously described [17]. The MSCs for one experiment were collected from 3 to 4 animals. The bones were aseptically excised, cleaned of adhering connective tissues, and soaked in ethanol, followed by PBS. The ends of the bones were removed, and the marrow cavity was flushed using 10 ml of culture medium containing α-MEM (Hyclone, Logan, UT, USA) medium, 10% fetal bovine

serum (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Hyclone) expelled from a 10 ml syringe through a 1 ml syringe needle. After flushing up and down, the suspension was filtered through a 40-µm cell strainer (BD Bioscience, San Jose, CA, USA). The released cells were collected in a 100-mm dish and maintained in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) with a humid atmosphere at 37 ºC and 5% CO_2.

The nonadherent cells were removed after three days, and fresh culture medium was added. After 3–4 days in primary culture, the cells were digested and resuspended with osteogenic medium comprising cultured medium supplemented with 10 nM dexamethasone (Sigma, St. Louis, MO, USA), 10 mM β-glycerol phosphate (Sigma) and 50 µg/ml ascorbic acid (Wako, Tokyo, Japan). Subsequently, the cells were counted and seeded onto 60-mm dishes (5×10^5 cells/well) for mRNA, protein and supernatant detection and onto a 96-well plate (1×10^4 cells/well) for Alp staining and cell proliferation detection. The cells were treated with variable concentrations (0, 0.1, 1, 10, 100 nM) of TCDD (Cambridge, MA, USA) in the presence or absence of 10 µM 2-Methyl-2H-pyrazole-3-carboxylic acid (CH223191, Sigma) or 1 µM Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132, Sigma) or 20 ng/ml Wnt3a (R & D Systems, Minneapolis, MN, USA). TCDD, CH223191 and MG132 were dissolved in 0.1% DMSO and Wnt3a was dissolved in sterile PBS.

2.4. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from MSCs was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. The cDNA was synthesized using the QuaniTect Reverse Transcription Kit (Qiagen, Hilden, Germany). qRT-PCR was performed in duplicate using a Rotor-Gene Q Detection Instrument (Qiagen). The mRNA expression levels of *Runx2*, *Alp* and *Ocn* were analyzed using the QuantiFast SYBR Green PCR Kit and the PCR primers are listed in Table 1. A melting curve was used to confirm the specific amplification. Taqman probes specifically designed for *Ahr* (Mm00478932_m1, Applied Biosystems, Foster City, CA, USA), *Cyp1a1* (Mm03646707_g1, Applied Biosystems), *Gapdh* (Mm9999915_g1, Applied Biosystems) were used to detect the expression of *Ahr* and *Cyp1a1*. The mRNA expression levels were normalized to *Gapdh* using the comparative CT method.

2.5. Western blotting

Primary MSCs were lysed in RIPA buffer (Beyotime, Shanghai, China) that contained a protease inhibitor cocktail (Roche, Mannheim, Germany). Nuclear and Cytoplasmic Protein Extraction Reagents (Thermo) were used to extract nuclear and cytoplasmic protein according to the manufacturer's instructions. The cell concentrations were measured using the BCA Protein Assay Kit (Thermo), and the cell lysates were subjected to 10% SDS-PAGE and transferred onto PVDF

Table 1
Primer sequences for qRT-PCR.

Gene	ID	Primer sequence
Alp	11647	F: TCCGTGGGCATTGTGACTAC R: CCTCTGGTGGCATCTCGTTA
Runx2	12393	F: TAGCCCTCGGAGAGGTACCA R: TTTCATAACAGCGGAGGCATT
Ocn	12097	F: CCCTGAGTCTGACAAAGCCT R: GCGGTCTTCAAGCCATACTG
Gapdh	14433	F: GTATGACTCCACTCACGGCAAA R: GGTCTCGCTCCTGGAAGATG