International Immunopharmacology 24 (2015) 239–246

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Celastrol attenuates bone erosion in collagen-Induced arthritis mice and inhibits osteoclast differentiation and function in RANKL-induced RAW264.7

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ARTICLE INFO

Article history:
Received 30 September 2014
Received in revised form 18 November 2014
Accepted 8 December 2014
Available online 19 December 2014

Keywords:
Celastrol
Osteoclast
Collagen-Induced arthritis

ABSTRACT

Recently, the traditional Chinese medicine *Tripterygium wilfordii* Hook f (TwHF) of the Celastraceae family has attracted increasing attention for its potential therapeutic application in patients with rheumatoid arthritis (RA). It is well accepted that TwHF exerts the antirheumatic activity and mainly depends on its potent anti-inflammatory property. To further explore the therapeutic potential of the well-defined TwHF-derived single compound — celastrol in RA, we study the therapeutic efficacy of celastrol on bone erosion in collagen-induced arthritis (CIA) mice and delineate its effects on osteoclast differentiation and functions in RANKL-induced osteoclast precursors RAW264.7 cell line. In CIA mice, daily injection of celastrol (beginning on day 28 after arthritis induction) markedly suppressed arthritis, and reduced bone damage in the joints as demonstrated by histology and bone micro-computed tomography (CT). The effects were accompanied by reductions of osteoclast cells in joints, serum tartrate-resistant acid phosphatase (TRAP) 5b, and expression of osteoclastic genes (Trap, Ctsk, Ctr, Mmp-9) and transcriptional factors (c-Fos, c-Jun and NFATc1). When RAW264.7 cells were treated with RANKL, celastrol inhibited the formation of TRAP + multinucleated cells and the bone-resorbing activity in dose-dependent manners. Furthermore, celastrol reduced the RANKL-induced expression of osteoclastic genes and transcriptional factors, as well as phosphorylation of NF-kB and mitogen-activated protein kinases (MAPK). These findings show that celastrol could directly inhibit osteoclast formation and function, suggesting a novel therapeutic strategy of celastrol for managing RA, especially in preventing bone destruction.

1. Introduction

Recently, the traditional Chinese medicine *Tripterygium wilfordii* Hook f (TwHF, or léi gōng téng/thunder god vine in Chinese) of the Celastraceae family has attracted increasing attentions for its potential therapeutic application in patients with rheumatoid arthritis (RA) [1–4]. Significantly, Lipsky led a multiple-center, randomized study of an ethanol and acetate extract of TwHF versus sulfasalazine in active RA patients [4]. The results showed that, compared with sulfasalazine (2 g/day, an approved standard therapy for RA at that time) during the 24-week study, the TwHF extract led to statistically significantly greater improvement in terms of patients achieving ACR 20, ACR 50 and ACR 70 responses [4]. Moreover, the TwHF group trended to slower progression in radiographic joint damage than the sulfasalazine group [4]. Considering that sulfasalazine had been shown to limit radiographic progression, the results indicated that TwHF was at least as effective as sulfasalazine in controlling radiographic progression in RA patients. Celastrol is an active compound in the TwHF extract [5,6]. It was shown to suppress adjuvant-induced arthritis and protect against bone damage in arthritic rats, in part via modulating pro-inflammatory cytokines and chemokines [7,8].

The persistent synovitis and thereby bone erosion are the hallmark of RA [9–11]. Bone erosion in RA represents a combination of inflammatory immune reaction and accelerated osteoclastogenesis in affected joints in RA [11]. Of note, osteoclastogenesis occurs in the synovial tissue when osteoclast precursors are exposed to macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). The latter induces a signaling pathway leading to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), three mitogen-activated protein kinases (MAPKs; i.e, ERK, JNK and p38 MAPK), and c-Fos, a component of activator protein-1 (AP-1) transcription factor complex [12–14]. A common target gene of NF-κB and AP-1/c-Fos is nuclear factor of activated T-cells, cytoplasmic 1

Abbreviations: ALT, aminotransferase; CIA, collagen-Induced arthritis; Ctr, calcitonin receptor; Ctsk, cathepsin K; CM, culture medium; MAPK, mitogen-activated protein kinases; Mmp-9, matrix metallopeptidase 9; M-CSF, macrophage colony-stimulating factor; IP, intraperitoneally; PBS, phosphate buffered saline; RA, rheumatoid arthritis; RANKL, nuclear factor κB ligand; TRAP, tartrate-resistant acid phosphatase

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http://dx.doi.org/10.1016/j.intimp.2014.12.012
1567-5769/

(NFATc1), the master regulator of osteoclastogenesis. It regulates expression of several osteoclast-specific genes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (Ctsk), calcitonin receptor (Ctr) and matrix metallopeptidase 9 (Mmp-9). Of note, TRAP, Ctsk, and Mmp-9 are matrix enzymes that degrade the bone matrix.

Bone erosion results in severe structural damage, constituting a critical factor in poor functional outcome in RA [11]. Though the precise etiology of RA remains elusive, osteoclast is the cell ultimately responsible for bone destruction in RA [9,15]. Substantial evidence demonstrates that mature osteoclasts are abundant at sites of arthritic bone erosions in RA patients, making osteoclastogenesis and osteoclasts attractive therapeutic targets for RA [11].

To further explore the therapeutic potential the well-defined TwHF-derived single compound – celastrol in RA – we study celastrol for its effects on osteoclast differentiation and functions in murine monocytic cells. These cells can be induced to differentiate into giant osteoclast-like cells upon stimulation with RANKL and thereby is considered as a cell line with osteoclast precursor-like capacity. In addition, we delineate the therapeutic efficacy of celastrol on preventing bone erosion in collagen-induced arthritis (CIA) mice. It is well accepted that TwHF exerts the antirheumatic activity and mainly depends on its potent anti-inflammatory property. Our results show that celastrol inhibits osteoclast differentiation and functions and suppresses arthritis in CIA mice, suggesting a novel therapeutic strategy of celastrol for preventing bone destruction in RA.

2. Materials and methods

2.1. Reagents

Celastrol was purchased from Sigma-Aldrich Co Ltd, and dissolved in dimethyl sulfoxide to a concentration of 0.01 M as a stock solution, which was kept at −20 °C. Thereafter, it was diluted in the culture medium (CM) to the final concentrations as indicated in every experiment. Recombinant RANKL and M-CSF proteins were purchased from PeproTech and R&D Systems China Co. Ltd., respectively. A rabbit polyclonal antibody (Ab) against β-actin was purchased from Abcam. Rabbit polyclonal Ab against phospho-NF-kappaB p65,phospho-c-Jun N-terminal kinases (JNK), phospho-extracellular signal-regulated kinases (ERK), and phospho-p38 MAPK was purchased from Cell Signaling Technology. Rabbit polyclonal Abs against TRAP, c-Fos, c-Jun and NFATc1 were purchased from Santa Cruz Biotechnology.

2.2. Induction of collagen-induced arthritis in DBA/1J mice

Six-week-old male DBA/1J mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Science. All procedures involving animals and their care have been conducted following the institutional guidelines and in accordance with international laws and policies (EEC Council Directives 86/609, OJ L 358, 1 DEC.12, 1987; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). The mice were maintained under specific pathogen-free conditions and were fed standard mouse chow and water *ad libitum*. The arthritis was induced as described previously [16]. Briefly, mice were injected intradermally with 0.1 ml of an emulsion containing 100 μg of bovine type II collagen (CII; Chon-drex) and Freund's complete adjuvant (Sigma), into the base of the tail as a primary immunization. On day 21 after the initial immunization, mice were boosted with100 μg CII emulsified 1:1 with Freund's incomplete adjuvant (Sigma). From days 28 to 42, mice were treated intraperitoneally (IP) with celastrol in phosphate buffered saline (PBS; 3 mg/kg) or PBS alone. All procedures performed were in compliance with the Animal Welfare Act and US Department of Agriculture regulations and were approved by Nanjing Medical University Animal Care and Use Committee (reference number 12H-0378).

Mice were monitored daily in a blinded manner for signs of arthritis. The symptoms were graded and scored as previously described [16]. Briefly, all four limbs of the mice were evaluated and scored from 0 to 4 according to the following scale: 0 = no swelling; 1 = slight swelling and erythema confined to either ankle or mid foot; 2 = slight swelling extending from ankle to mid foot; 3 = moderate welling from ankle to metatarsal joints; 4 = severe swelling in the ankle, foot, and digits.

2.3. Ex vivo experiments

2.3.1. Histologic and immunohistochemical analyses

For standard histologic assessment, when mice were euthanized on day 42, their ankles or paws were isolated, fixed in 4% paraformaldehyde, decalcified in ethylenediaminetetraacetic acid (EDTA) bone decalcifier, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (H&E) to examine joint changes, and were evaluated blindly, as described previously [17]. The histological arthritis score was determined in a blinded fashion for changes in synovial lining, cellular infiltrate, cartilage damage, and pannus as previously described [18]. Briefly, for synovial lining, grade 1 indicated localized monolayer cubical transformation; 2, localized multilayer cubical transformation; and 3, multilayer synovial lining with extensive necrosis. For cellular infiltrate, grade 1 indicated few focal infiltrates; 2, extensive focal infiltrates; and 3, extensive infiltrates invading the capsule with aggregate formation. For cartilage damage, grade 1 indicated superficial, localized cartilage degradation in more than one region; 2, localized deep cartilage degradation; and 3, extensive deep cartilage degradation at several locations. For pannus, grade 1 indicated pannus formation at up to two sites; 2, pannus formation at up to four sites, with infiltration or flat overgrowth of joint surface; and 3, pannus formation at more than four sites or extensive pannus formation at two sites. For each category, the maximum score of the four limbs analyzed was used for the mouse, resulting in a minimum score of 0 (no changes at all) and a maximum score of 12 for a mouse.

To analyze osteoclast in joint tissues, each joint section was treated sequentially with anti-mouse TRAP antibody (Santa Cruz Biotechnology, Inc.) and the peroxidase conjugated goat anti-mouse IgG (H + L; Millipore). Sections were counter stained with hematoxylin (Sigma).

2.3.2. Micro-computed tomography (CT) analyses

Computed tomographic images of the knee joints and paws of the mice in all three groups ($n = 3$) were acquired on day 42, using a micro-CT Scan SkyScan1176S scanner at a resolution of 9 μm. For verification of bone destruction, 3-dimensional models of the knee joints and paws were reconstructed using SkyScan CT Analyzer version 1.8.

2.3.3. The relative RNA expression of osteoclastogenesis-related genes and transcription factors by real time reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from joint tissue of CIA mice and RAW264.7 cells with TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA) following the manufacturer's instructions. A SuperScript TM III Platinum® SYBR® Green one-step quantitative RT-PCR (qRT-PCR) kit (Invitrogen) was used. Relative expressions of osteoclastic markers (including *Trap*, *Ctsk*, *Ctr* and *Mmp-9*), and transcriptional factors (including *c-Fos*, *c-Jun* and *NFATc1*) were determined, normalized (using the expression levels of *glyceraldehyde-3-phosphate dehydrogenase — GAPDH*) and calculated by the $2^{-\Delta\Delta Ct}$ method. The following primers were used: *Trap*, 5′-CCAA TGCCAAAGAGATCGCC-3′ (sense) and 5′-TCTGTGCAGAGACGTTGCCAAG-3′ (anti-sense); *Ctsk*, 5′-GACGCAGCGATGCTAACTAA-3′ (sense) and 5′-CCAGCACAGAGT-CCACAACT-3′ (anti-sense); *Ctr* 5′-TCAGGAACCACGGA ATCCTC-3′ (sense) and 5′-ACATTC-AAGCGGATGCGTCT-3′ (anti-sense); *Mmp-9*, 5′-CTGGACAGCCAGACACTAAAG-3′ (sense) and 3′-CTCGCGGC AAGTCTTCAGAG-3′ (anti-sense); *c-Fos*, 5′-ATGATGTTCTCGGGTTTCAA-CG-3′ (sense) and 5′-CAGTCTGCTGCATAGAAGGAACCG-3′ (anti-sense); *c-Jun*, 5′-ACTCGGA-CCTTCTCACGTCG-3′ (sense) and 5′-TAGACCGGAG