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Lupeol acetate ameliorates collagen-induced arthritis and osteoclastogenesis of mice through improvement of microenvironment

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ABSTRACT

Lupeol has been shown with anti-inflammation and antitumor capability, however, the poor bioavailability limiting its applications in living subjects. Lupeol acetate (LA), a derivative of lupeol, shows similar biological activities as lupeol but with better bioavailability. Here RAW 264.7 cells and bone marrow-derived macrophages (BMDMs) stimulated by lipopolysaccharide (LPS) were treated with 0–80 μM of LA, and assayed for TNF-α, IL-1β, COX-2, MCP-1 using Western blotting. Moreover, osteoclatogenesis was examined with reverse transcription PCR (RT-PCR) and tartrate-resistant acid phosphatase (TRAP) staining. For *in vivo* study, collagen-induced arthritis (CIA)-bearing DBA/1J mice were randomly separated into three groups: vehicle, LA-treated (50 mg/kg) and curcumin-treated (100 mg/kg). Therapeutic efficacies were assayed by the clinical score, expression levels of serum cytokines including TNF-α and IL-1β, ^{18}F-fluorodeoxyglucose (^{18}F-FDG) microPET/CT and histopathology. The results showed that LA could inhibit the activation, migration, and formation of osteoclastogenesis of macrophages in a dose-dependent manner. In RA-bearing mice, the expressions of inflammation-related cytokines were suppressed, and clinical symptoms and bone erosion were ameliorated by LA. The accumulation of ^{18}F-FDG in the joints of RA-bearing mice was also significantly decreased by LA. The results indicate that LA significantly improves the symptoms of RA by down-regulating expressions of inflammatory cytokines and osteoclastogenesis.

1. Introduction

Rheumatoid arthritis (RA), a common autoimmune disease, causes the progressive disability, systemic complications and early death [1–3]. Clinical symptoms of RA include swelling, deformity, and limited motion of the affected joints. The cause of RA is a complex interplay among genetic components and environmental factors [1]. At the later stage of RA, abnormal

Abbreviations: ACK, ammonium-chloride-potassium; BCS, bovine calf serum; BMDM, bone marrow derived macrophage; CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; COX-2, cyclooxygenase-2; DC-STAMP, dendritic cell–specific transmembrane protein; DMARD, disease-modifying anti-rheumatoid drugs; ^{18}F-FDG, ^{18}F-fluorodeoxyglucose; GMCSF, granulocyte-macrophage colony-stimulating factor; i.d., intradermally; IL, interleukin; LA, lupeol acetate; RA, rheumatoid arthritis; RANK, receptor activator of NF-κB; RANKL, receptor of activation of NF-κB ligand; ROI, region-of-interest; RT-PCR, reverse transcription-polymerase chain reaction; MCP-1, monocyte chemotactic protein 1; MCSF, macrophage colony-stimulating factor; NFATc1, nuclear factor of activated T cells c1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSAID, nonsteroidal anti-inflammatory drug; SEM, standard errors of mean; SSRB, single-slice rebinning; TGF-β, transforming growth factor beta; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; Treg, regulatory T cell; TRAF6, TNF receptor-associated factor 6; TRAP, tartrate-resistant acid phosphatase; VEGF, vascular endothelial growth factor; VOI, volume of interest.

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expressions of cytokines may trigger the activation of immune system, and cause the damage of synovia and joints. The macrophage has been reported to be critical for the progression of RA. Macrophages and immune cells regulate each other by secreting types of cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and monocyte chemotactic protein 1 (MCP-1). These cytokines further disturb the balance of the immune system, trigger the infiltration of other immune cells and enhance the inflammation severity [4,5]. Inflamed joints, peripheral blood and bone marrow are often found with elevated expressions of these cytokines in RA patients [6], indicating a close correlation between cytokines and the progression of RA.

The macrophage is also one of the precursors of osteoclasts which are responsible for the bone resorption and characterized with multinucleation resulted from cell-cell infusion [7–10]. Osteoclasts will be differentiated from macrophages and monocytes when excessive receptor activator of nuclear factor kappa-B (NF-κB) ligand (RANKL) and MCP-1 are present. Macrophage colony-stimulating factor (M-CSF) is another important cytokine for the differentiation of osteoclasts [11]. M-CSF binds to its receptor expressed on the monocyte, and induces the expression of receptor activator of NF-κB (RANK) which will be bound by RANKL, and initiates the osteoclast differentiation. RANKL, generated by stromal cells and immune cells, induces the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), and increases the expression of dendritic cell–specific transmembrane protein (DC-STAMP) [12] which is essential for cell-cell fusion in osteoclast differentiation [13–15]. RANKL is also correlated with the production of proinflammatory cytokines [16]. The activity and formation of osteoclasts are also modulated by the balance between IL-17 and TGF-β, which are secreted by Th17 and regulatory T cells (Tregs), respectively [17,18]. Over-reactivation of osteoclasts may disrupt the bone homeostasis and cause the damage of cartilage and bone.

As mentioned above, cytokines, chemokines, and progression of RA are tightly correlated and complicated. The COX-2 and MCP-1 are known to be critical for both migration and infiltration of macrophages in various disease models [19–23]. Moreover, COX-2 is also important for induction of osteoclast differentiation as MCP-1 [24,25].

Current treatments for RA are the nonsteroidal anti-inflammatory drug (NSAID), disease-modifying antirheumatic drug (DMARD) and several biologic agents for the relief of inflammatory syndrome or pain [26,27]. However, only parts of patients are benefited from these treatments, but severe complications such as the spine lesion and lung diseases are reported [28–31]. Hence, to elucidate the correlation between the immune system and RA progression for developing alternative medicine is worth to be studied.

Lupeol is one of the triterpenes which are available from types of fruits and vegetables such as mango, green pepper and strawberries. Lupeol has been shown as an immunomodulator with several abilities of anti-inflammation, antioxidant, anti-cancer and anti-arthritis [32–34]. Lupeol acetate (LA), a derivative of lupeol, shows better bioavailability compared with lupeol. Although LA has been reported to alleviate the inflammation in complete Freund's adjuvant (CFA) model [35] and carrageenan-induced paw edema in mice [36], the underlying mechanism of the effects is still ambiguous. Here the therapeutic efficacy of LA on RA using both murine macrophage RAW 264.7 cell line and collagen-induced arthritis (CIA) mouse model were studied.

2. Materials and methods

2.1. Cell line, animal and drugs

The murine macrophage RAW 264.7 cell line was purchased from the Food Industry Research and Development Institute, Hsinchu, Taiwan. Cells were maintained in DMEM (Invitrogen, CA, USA) supplemented with 10% bovine calf serum (Sigma-Aldrich, MO, USA), 2 mM L-glutamine, and 1% penicillin-streptomycin (Mediatech, VA, USA). C57B/6 mice purchased from the National Laboratory Animal Center, Taiwan were used for the separation of bone marrow derived macrophages (BMDMs). DBA/1J mice obtained from the Jackson Laboratory (Bar Harbor, ME, USA) were housed in the Laboratory Animal Center of National Yang-Ming University for establishing the collagen-induced arthritis (CIA)-bearing mouse model. All animal experiments were approved by the Animal Care and Use Committee at National Yang-Ming University (protocol number: 1010414).

Lupeol acetate (LA) was purchased from Extrasynthese (Genay, France). Curcumin purchased from Sigma was used as a positive control. LA and curcumin was dissolved in 0.1% DMSO (Sigma-Aldrich) and 0.025 N NaOH (Sigma-Aldrich), respectively.

2.2. Isolation of bone marrow derived macrophages (BMDMs)

Bone marrows collected from the femurs of 8-week-old C57B/6 mice by flushing with cold PBS using a 25 G syringe. Collected cells were treated with 0.5 M ammonium-chloride-potassium (ACK) buffer to lyse RBC. The derived bone marrow cells were maintained in DMEM supplemented with 30 μg/ml recombinant macrophage colony-stimulating factor (M-CSF, Peprotech, NJ, USA), 10% BCS, 2 mM L-glutamine, and 1% penicillin-streptomycin for 7 days, then stained with CD68-FITC and F4/80-PerCP/Cy5.5 (Biolegend, CA, USA) and analyzed by flow cytometry. More than 95% of cultured cells expressed CD68 and F4/80, the macrophage markers, were recognized as BMDMs.

2.3. Cytotoxicities of lupeol acetate on RAW 264.7 cells and BMDMs with MTT assay

5×10^4/well of RAW 264.7 cells and BMDMs were cultured in 96-well plate for 24 h before treated with lupeol acetate (LA). The cytotoxicity of LA was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and measured by ELISA reader (ELISA plate reader; BIO-TEK instruments, VT, USA).

2.4. Expressions of cell surface markers of RAW 264.7 cells and BMDMs

1×10^6/well of RAW 264.7 cells and BMDMs were cultured in a 6-well plate one day prior to the following experiments. Cells were pretreated with 0–80 μM LA for an hour followed by 24 h stimulation with 1 μg/ml LPS (Sigma-Aldrich). Harvested cells were stained with CD80-APC and CD86-FITC (Biolegend), then analyzed with FACScalibur flow cytometer (BD Biosciences, CA, USA) to determine the activation levels. The data were analyzed by FlowJo (Tree Star, OR, USA) to define the positive region, and the results were presented as the percentage of positive cells.

2.5. Proinflammatory cytokines assay with ELISA

The cultured media collected from both LPS-stimulated RAW 264.7 cells and BMDMs treated with different concentrations of LA to determine the levels of TNF-α and IL-1β. For *ex vivo* study, blood samples of each group were collected on day 20, 32, 39 and 43 from fascial veins and cardiac puncture, respectively, and applied for