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IL-15 promotes osteoclastogenesis via the PLD pathway in rheumatoid arthritis

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ABSTRACT

Osteoclastogenesis plays an important role in joint destruction in rheumatoid arthritis (RA). IL-15 is a pleiotropic proinflammatory cytokine that appears to help mediate the pathological bone loss. This study was undertaken to explore the signaling molecules essential for osteoclastogenesis mediated by IL-15 in rheumatoid synovial fibroblasts. Expression of phospholipase D1 (PLD1) and osteoclast-related gene expression in synovial tissues and their modulation by treatment with IL-15 and different inhibitors in synovial fibroblasts of RA patients were evaluated using immunohistochemistry and quantitative polymerase chain reaction. The levels of IL-15 in serum and synovial fluid were measured by ELISA. The effects of IL-15 and phosphatidic acid (PA) on osteoclast formation were evaluated in cocultures of rheumatoid synovial fibroblasts and peripheral blood monocytes or monocytes alone in the presence of M-CSF and RANKL. The levels of RANKL and PLD1 but not PLD2 were upregulated significantly by IL-15, and the RANKL level was significantly upregulated by PA in rheumatoid synovial fibroblasts. Blocking PA production with 1-butanol and siRNA against PLD1 significantly inhibited the IL-15-stimulated expression of RANKL and PLD1. IL-15 levels were significantly higher in serum and synovial fluid from patients with RA than in osteoarthritis patients and healthy controls. IL-15 and PA induced osteoclast formation through the mitogen-activated protein kinases (MAPKs) and NF-κB signaling pathways. Activation of PLD1 contributes to IL-15-mediated osteoclastogenesis via the MAPKs and NF-κB signaling pathways in rheumatoid synovial fibroblasts. Our data suggest that PLD1 might be an efficient therapeutic strategy for preventing bone destruction in rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the progressive destruction of articular cartilage and bone in the chronic phase. Invasion of a hypertrophic synovial membrane is the typical histopathological feature of bone erosion in RA [1], and the infiltration of synovial inflammatory tissue into bone is considered the pathogenic mechanism responsible for bone erosion. Activated fibroblasts within the synovial membrane participate directly in the invasion [2] and resorption of bone in RA. Bone-resorbing osteoclasts formed in the synovium are implicated as important mediators of bone damage in RA [3].

RA synovial tissue can be considered a suitable microenvironment for osteoclastogenesis because activated synovial T cells and fibroblasts express receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) in situ [4,5]. These cells may act as nursing cells [6], and a large number of cells of the monocyte/macrophage lineage may act as osteoclast progenitors to accumulate in the inflammatory lesions of RA [7]. The role of rheumatoid synovial fibroblasts in osteoclastogenesis and bone destruction is supported by the finding of the formation of osteoclasts in cocultures of rheumatoid synovial fibroblasts and peripheral blood mononuclear cells (PBMCs) [3,8]. In addition, the proinflammatory cytokines including interleukin-17 (IL-17), IL-1, and tumor necrosis factor-α (TNF-α) accumulate in the RA synovial joint, where they induce the multinucleation of osteoclast precursors and/or commitment to the osteoclast phenotype [9–11] and may act synergistically with RANKL.

IL-15 is a pleiotropic proinflammatory cytokine that appears to help regulate the pathological bone loss that occurs in diseases such as RA. IL-15 may be an important cytokine for modulating osteoclast differentiation. Supporting this notion is the observation that IL-15 stimulates the formation of multinucleated osteoclast-like cells in rat bone marrow cultures [12] and that blocking IL-15 reduces the destruction of cartilage and bone [13]. IL-15 produced

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by RA T cells can also induce osteoclastogenesis in cocultured autologous monocytes [14]. These observations suggest that IL-15 mediates inflammatory bone destruction and actively stimulates osteoclast differentiation. However, it is not clear whether IL-15 is involved in osteoclastogenesis through its effect on rheumatoid synovial fibroblasts such as osteoblastic cells or which molecules are involved in osteoclast activation by IL-15.

Phospholipase D (PLD) is an enzyme that hydrolyzes the phosphodiester bond in phosphatidylcholine (PC), yielding choline and phosphatidic acid (PA) [15,16]. In mammalian cells, two isoforms of PLD have been identified: PLD1 and PLD2 [17]. PLD is involved in physiological and cellular signaling pathways primarily through the production of second messengers such as PA and, indirectly, diacylglycerol. Activation of PC–PLD is thought to play a key role in the signal transduction that regulates a range of physiological processes including membrane trafficking and cytoskeletal reorganization [18–22], mitogenesis [23], neuronal and cardiac stimulation [24,25], phagocytosis [26], the respiratory burst in neutrophils [27], inflammation, and diabetes [28].

Only a few studies have reported on the effects of PLD in bone cells. PLD is involved in induction of the proresorptive cytokine IL-6 by prostaglandin or thrombin in osteoblasts [29]. PLD1 is induced by RANKL early in the culture of mouse bone marrow mononuclear cells [30]. IL-8 secreted by human lung cancer cells has been shown recently to increase PLD activation and consequently promote osteoclast differentiation [31]. These reports suggest that PLD can mediate the inflammatory response and induce bone resorption by stimulating osteoclast differentiation.

We hypothesized that PLD plays a role in osteoclastogenesis by targeting osteoclast precursors, either indirectly, via rheumatoid synovial fibroblasts such as osteoblastic cells, or directly. In the present study, we investigated the role of IL-15 in osteoclast differentiation and the signaling molecules that control this process. We found that, in rheumatoid synovial fibroblasts, IL-15 induces the expression of RANKL through the activation of PLD1, mitogen-activated protein kinases (MAPKs), and NF-κB, and that these activation increase osteoclastogenesis. PLD1 is required for osteoclast formation from osteoclast precursor cells treated with RANKL and IL-15. Our data suggest that PLD1 plays an important role in bone destruction and that inhibition of PLD1 or its signal transduction pathway might be an efficient therapeutic strategy for preventing bone and cartilage destruction in rheumatoid arthritis.

2. Materials and methods

2.1. Reagents

Human recombinant proteins used in the various treatments, such as IL-15, recombinant human macrophage colony-stimulating factor (M-CSF), and soluble RANKL, were purchased from R&D Systems (Minneapolis, MN) or PeproTech (London, UK). PA, the activator protein-1 (AP-1) inhibitor curcumin, and 1- and 3-butanol and anti-β-actin were purchased from Sigma (St Louis, MO). The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, extracellular signal-regulated kinase (ERK) inhibitor PD98059, and NF-κB inhibitor parthenolide were purchased from A.G. Scientific, Inc. (San Diego, CA). STAT5 inhibitor ($C_{16}H_{11}N_3O_3$), horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse IgG were purchased from Santa Cruz Biotechnology (San Diego, CA). The Jun N-terminal kinase (JNK) inhibitor SP600125 and Janus-activated kinase (JAK) inhibitor AG490 were purchased from Calbiochem (San Diego, CA) and Tocris Bioscience (Ellisville, MO), respectively. Rabbit anti-ERK-1/2, anti-phospho-ERK-1/2, anti-phospho-JNK, anti-phospho-inhibitor of κB (IκB), anti-PLD1 and anti-phospho-PLD1 (Thr147) were from Cell Signaling Technology (Frankfurt, Germany).

2.2. Patients

Human serum and synovial fluid were obtained from patients with an established diagnosis of RA (serum, $n = 13$; fluid, $n = 20$) or osteoarthritis (OA) (serum, $n = 14$; fluid, $n = 20$) after informed consent was given. The patients were diagnosed with RA according to the American College of Rheumatology criteria. Serum from healthy controls ($n = 10$), who turned out to have no medical disorders, was used as the control.

2.3. Isolation of synovial fibroblasts

RA synovial tissue samples were collected from the knee joints of patients who underwent primary total joint replacement surgery. To isolate the fibroblasts, synovial tissues were diced into small pieces and digested overnight with 1 mg/ml type I collagenase (Worthington, Lakewood, NJ). The suspended fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS), and 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate (all from Invitrogen, Carlsbad, CA). The fibroblasts were passaged after reaching 90% confluence and used for experiments after 5–6 passages. More than 98% of synovial cells were $CD90^+$ (BD Biosciences, San Diego, CA), and no macrophages ($CD11b^+$ cells) (BD Biosciences) were detected using a FACSCalibur (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies.

2.4. Quantitative polymerase chain reaction (PCR)

Total cellular RNA from human synovial fibroblasts was extracted with TRIzol reagent (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's specifications. Two micrograms of total RNA was reverse transcribed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) using random hexameric primers according to the manufacturer's protocol. PCR was performed with primers specific for human RANKL, PLD1, PLD2, and the housekeeping gene β-actin. The PCR started with 1 μl of reverse transcription mixture (cDNA), 1.5 μl of 10× PCR buffer, 25 pmol of primers, and double-distilled water to a total volume of 17.5 μl, and the solution was incubated at 95 °C for 1 min. The temperature was held at 80 °C as 1.5 U of *Taq* DNA polymerase (1 μl; 5 U/μl) (Invitrogen) was added. The PCR continued for 22 cycles (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final elongation step at 72 °C for 7 min) for β-actin, 35 cycles for RANKL, and 32 cycles for PLD1 and PLD2; the annealing temperature was 60 °C. The primers were as follows: β-actin upstream, GGA CTT CGA GCA AGA GAT GG and downstream, TGT GTT GGC GTA CAG GTC TTT G; RANKL upstream, ACC AGC ATC AAA ATC CCA AG and downstream, CCC CAA AGT ATG TTG CAT CC; PLD1 upstream, TGT CGT GAT ACC ACT TCT GCC A and downstream, AGC ATT TCG AGC TGC TGT TGA A; and PLD2 upstream, CGT CCA GGC CAT TCT GCA C and downstream, GTG CTT CCG CAG ACT CAA GG. Real-time quantification of messenger RNA was performed using Roche LightCycler 1.5 capillary-based system real-time PCR system (Roche Diagnostics) in 20 μl of reaction mixture containing 10 μl of SYBR Green PCR Master Mix (Takara, Osaka, Japan), 500 nM of each primer, and 2 μl of sample DNA. The results were expressed as the threshold cycle (Ct) and then calculated as the ratio of the number of molecules of the target gene to the number of molecules of β-actin. The primer efficiencies for the test genes were the same as for the GAPDH gene. Standard curves were generated with the same plasmids used for the target sequences.