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RhoA/ROCK-dependent pathway is required for TLR2-mediated IL-23 production in human synovial macrophages: Suppression by cilostazol

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ABSTRACT

IL-23 is produced by antigen presenting cells and plays critical roles in immune response in rheumatoid arthritis. In this study, we investigated whether the RhoA/Rho-kinase pathway is required to elevate TLR2-mediated IL-23 production in synovial macrophages from patients with rheumatoid arthritis (RA), and then examined the suppressive effect of cilostazol on these pathways. IL-23 production was elevated by lipoteichoic acid (LTA), a TLR2 ligand, and this elevation was more prominent in RA macrophages than in those from peripheral blood of normal control. LTA increased the activation of RhoA in association with increased the nuclear translocation of NF-κB and its DNA-binding activity. Pretreatment of RA macrophages with the pharmacological inhibitors exoenzyme C3 (RhoA), Y27632 (Rho-kinase) or BAY11-7082 (NF-κB) inhibited IL-23 production by LTA. Inhibition of the RhoA/Rho-kinase pathway by these drugs attenuated NF-κB activation. Cilostazol suppressed the TLR2-mediated activation of RhoA, decreased NF-κB activity with down-regulated IL-23 production, and these effects were reversed by Rp-cAMPS, as an inhibitor of cAMP-dependent protein kinase. The expression of IL-23, which colocalized with CD68(+) cells in knee joint of CIA mice, was significantly attenuated by cilostazol along with the decreased severity of arthritis. Taken together, the RhoA/Rho-kinase pathway signals TLR2-stimulated IL-23 production in synovial fluid macrophages via activation of NF-κB. Thus it is summarized that cilostazol suppresses TLR2-mediated IL-23 production by suppressing RhoA pathway via cAMP-dependent protein kinase activation.

1. Introduction

Rheumatoid arthritis (RA) is a common autoimmune and chronic inflammatory joint disease characterized by increased infiltration of macrophages, proliferation of synovial fibroblast with joint destruction [1]. When abundant monocytes/macrophages in the synovial fluid of RA patients are activated, they produce high levels of cytokines and chemokines, such as interleukin-1β (IL-β), tumor necrosis factor α (TNFα), IL-6, and MCP-1, which contribute to chronic inflammation and joint destruction [2,3].

Toll-like receptors (TLRs) are conserved receptors that recognize pathogen-associated molecular patterns, and play important roles in innate and adaptive immunity [4]. TLR2 are mainly expressed on cells, such as macrophages and dendritic cells; and act as primary sensors by recognizing diverse ranges of stimuli [5]. The lipoteichoic acid (LTA) and peptidoglycan are recognized mainly by TLR-2 [6]. It has been reported that TLR2 stimulation causes the preferential induction of IL-8 and IL-23 p19 [7]. IL-23 is involved in autoimmune diseases like RA and psoriasis, in which the cellular function of IL-23 is associated with the self-reactive productions of IL-17, IL-6, and TNF-α, and thus IL-23 plays a critical role in development of autoimmune inflammation [8]. Furthermore, they reported that mice deficient in IL-23 ($p19^{-/-}$) were relatively resistant to the development of joint and bone inflammation in a collagen-induced arthritis (CIA) model.

The Rho-GTPase family of monomeric RhoA, Rac1 and Cdc42 is known to cycle between the inactivated GDP-bound state and

Abbreviations: CIA, collagen induced arthritis; IL-23, interleukin-23; LTA, lipoteichoic acid; RA, rheumatoid arthritis; TLR2, toll like receptor 2.

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the activated GTP-bound state [9]. In the active state, Rho is implicated in various cellular processes, such as the cell cycle, cytoskeletal regulation, cellular growth and apoptosis [10,11]. Furthermore, it has been reported that RhoA is a key regulator of transcription factors, NF-κB [12], and that inhibition of Rho-kinase reduces the severity of synovial inflammation in rats with CIA [13].

Although it has been demonstrated that IL-23 production plays a crucial role in inflammatory reactions associated with rheumatoid arthritis [14], the signal pathway by which TLR2 induces IL-23 production in RA synovial macrophages has not been defined. On the other hand, cilostazol, a type-III phosphodiesterase inhibitor, has been reported to have anti-inflammatory effects due to the cAMP-dependent protein kinase activation-coupled suppression of NF-κB gene transcription [15].

Therefore, in the present study, we undertook to investigate the signal transduction pathways responsible for TLR2-mediated IL-23 production in synovial fluid macrophages from RA patients: in particular, the present study highlighted implication of the RhoA/ROCK signal pathway in the regulation of TLR2-mediated IL-23 production in RA macrophages. We found that increased IL-23 production by TLR2 involves the activation of NF-κB via a RhoA/ROCK pathway. Further, cilostazol was found to inhibit TLR2-mediated IL-23 production by suppressing RhoA activity via the activation of cAMP-dependent protein kinase, and to suppress the expression of IL-23 in the knee joints of CIA mice.

2. Materials and methods

2.1. Reagents and antibodies

Lipoteichoic acid (LTA), BAY11-7082 and Y27632, were obtained from Sigma (St. Louis, MO). Rp-cAMPS was purchased from Alexis (San Diego, CA). Clostridium botulinum exoenzyme C3 transferase (exoenzyme C3) was from Upstate Biotechnology (Lake Placid, NY. Anti-TLR2 antibody was from Abcam (Cambridge, MA). NF-κB p65, IκBα, Histone H1and RhoA antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). TLR2 neutralizing antibody was from eBioscience (San Diego, CA). IgG isotype control antibody (R&D systems, Minneapolis, MN). Cilostazol (OPC-13013), [6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinolinone, >98.5% purity by HPLC, mean particle size, 14–28 (mean, 20) μm] was donated by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan), and was dissolved in dimethyl sulfoxide to produce a 10 mM stock solution.

2.2. Cell preparations

Synovial fluid samples were obtained from the knees of 20 RA patients. All patients fulfilled the 1987 revised criteria of the American College of Rheumatology at the time of therapeutic arthrocentesis. Informed consent was obtained from all patients and the study protocol was approved by the ethics committee of Dong-A University Hospital (Busan, South Korea). Peripheral blood was donated by five healthy volunteer donors. Mononuclear cells (MC) from the peripheral blood (PB) or synovial fluid (SF) were isolated by Ficoll Paque (Sigma) density gradient centrifugation and cultured in RPMI 1640 containing 10% FBS for 1 h, when non-adherent cells were washed out, and adherent SFMC were incubated in culture medium. Adherent PBMC were allowed to differentiate into normal control macrophages, as previously described by Liu et al. [16]. Cell differentiation was determined by using monocyte/macrophage markers by anti-CD14 antibody FACS. More than 90% of cells were CD14-positive.

2.3. RhoA activation assay

The levels of active GTP-bound RhoA were determined by pull-down GTP-bound RhoA with GST-Rhotekin-RBD coupled to glutathione agarose beads. Cells were stimulated with IGF-1 for 5 min, and lysed with lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged, and supernatants were incubated with beads coupled to GST- Rhotekin-RBD for 2 h at 4 °C. Beads were then washed with lysis buffer and GTP-bound RhoA was eluted with the sample buffer. Amounts of active RhoA were determined by Western blot analysis.

2.4. Enzyme-linked immunosorbent assay

Levels of IL-23 in culture supernatants from synovial fluid were measured using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, incubated with anti-IL-23 (Abcam, Cambridge, MA) for 1 h, and then with Cy3-conjugated secondary antibody for 30 min. Fluorescent images were obtained using a confocal microscope (OLYMPUS FV-1000, Tokyo, Japan).

2.6. Western blotting assay

Proteins (30 μg) were resolved in a 10% SDS-polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes (Amersham Biosciences, Inc., Piscataway, NJ). Blocked membranes were then incubated with antibodies. Protein bands were visualized with a chemiluminescent reagent from the Supersignal West Dura Extended Duration Substrate Kit (Pierce Chemical, Rockford, IL). Signals from bands were quantified using the UN-SCAN-IT gel 5.1 software (Silk Scientific, Inc., Orem, UT). Results are expressed as relative densities.

2.7. Small interfering RNA preparation and transfection

TLR2 siRNA oligonucleotides (GenBank accession number: NM_003264.3) was synthesized by Bioneer (Daejeon, Korea). siRNA negative control duplex was used as a control oligonucleotide. Cells were transfected with TLR2 siRNA using Lipofectamine 2000 (Invitrogen).

2.8. NF-κB transcription factor assay

To quantify NF-κB transcription factor levels in nuclear extracts, cell lysates were extracted using a nuclear extraction kit (Chemicon International, Temecula, CA) according to manufacturer's protocol. DNA binding activities of NF-κB p65 were determined using a colorimetric NF-κB p65 transcription factor assay kit (Rockland Immunochemicals, Gilbertsville, PA) according to the manufacturer's protocol.

2.9. Animals

Male DBA/1J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were used for experiments when 8–12 weeks old. All experimental procedures were conducted in accordance with the Animal Care Guidelines of the Animal Experimental Committee of the College of Medicine, Pusan National University.