Biochemical and Biophysical Research Communications 452 (2014) 85–90

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Secretion of inflammatory factors from chondrocytes by layilin signaling

Kota Asano [a], Mitsumi Arito [a], Manae S. Kurokawa [b], Kazuki Omoteyama [a], Kazuki Okamoto [a], Naoya Suematsu [a], Kazuo Yudoh [c], Hiroshi Nakamura [d], Moroe Beppu [e], Tomohiro Kato [a,*]

[a] Clinical Proteomics and Molecular Medicine, St. Marianna University Graduate School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, Kanagawa 216-8511, Japan
[b] Disease Biomarker Analysis and Molecular Regulation, St. Marianna University Graduate School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, Kanagawa 216-8511, Japan
[c] Institute of Medical Science, St. Marianna University Graduate School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, Kanagawa 216-8512, Japan
[d] Department of Orthopedic Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan
[e] Department of Orthopaedic Surgery, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, Kanagawa 216-8511, Japan

ARTICLE INFO

Article history:
Received 9 August 2014
Available online 19 August 2014

Keywords:
Chondrocytes
Complement5
Inflammation
Layilin
Rheumatoid arthritis
TNF-α

ABSTRACT

Layilin (LAYN) is thought to be involved in reorganization of cytoskeleton structures, interacting with merlin, radixin, and talin. Also, LAYN is known to be one of the receptors for hyaluronic acid (HA).

In rheumatoid arthritis (RA), inflammatory cytokines like tumor necrosis factor α (TNF-α) have been known to play pathological roles. HA with low molecular weight is speculated to exacerbate inflammation in RA. In this context, differences of quantity and functions of HA receptors would affect the severity of inflammation in RA. Chondrocytes, which play critical roles in maintaining articular cartilage and are affected in RA, express at least kinds of HA receptors like CD44 and LAYN. However, roles and regulation of LAYN in articular chondrocytes have been poorly understood.

To clarify regulation of LAYN in chondrocytes, we here investigated whether TNF-α affected expression levels of LAYN in human articular chondrocytes. Next, to clarify LAYN-specific roles in chondrocytes, we investigated whether binding of antibodies to the extracellular domain of LAYN affected secretion of inflammatory cytokines using a chondrosarcoma cell line. As a result, we found that TNF-α up-regulated expression levels of LAYN in the chondrocytes. Further, the LAYN signaling was found to enhance secretion of inflammatory factors, IL-8 and complement5 (C5)/C5a, from the cells. Our results indicate that LAYN would be involved in the enhancement of inflammation and degradation of cartilage in joint diseases such as RA and OA.

1. Introduction

Layilin (LAYN), a transmembrane protein with a C-type lectin-like domain [1,2], is thought to be involved in reorganization of cytoskeleton structures, interacting with merlin, radixin, and talin [3–5]. The reorganization of cytoskeleton structures is critical for cytoskeletal–plasma membrane interactions. In fact, down-regulation of LAYN inhibited invasion and metastasis of cancer cells [6,7]. On the other hand, LAYN was reported as a novel receptor for hyaluronic acid (HA). Since LAYN does not contain a common HA-binding module, LAYN is thought to bind to HA through the C-type lectin domain [8].

HA is a tandem repeat of a disaccharide that consists of glucronic acid and N-acetyl-glucosamine. Numbers of disaccharides in HA have huge diversity, thereby some HA shows molecular weights (MW) of more than 1000 kDa, while other HA shows MW of less than 50 kDa [9]. HA with high MW (HMW-HA) was demonstrated to have anti-inflammatory activities and inhibit angiogenesis [9]. Thereby, intra-articular administration of HMW-HA has been used as an effective therapy in patients with rheumatoid arthritis (RA) [10]. Further, in osteoarthritis (OA), HMW-HA was demonstrated to suppress osteophyte formation and progression of the disease [11]. Accordingly, intra-articular administration of HMW-HA has been used in the treatments of OA as well as RA [12]. On the other hand, HA with low MW (LMW-HA) rather enhances inflammation and promote angiogenesis [9]. Instantly, binding of LMW-HA to TLR4 was reported to up-regulate arachidonic acid and activate COX2 and PGE2 in human monocytes and murine macrophages [13]. In bronchial epithelial cells, it was reported that binding of oligosaccharide HA and LMW-HA to LAYN increased cell permeability by suppressing E-cadherin expression [14].

* Corresponding author. Fax: +81 44 976 7553.
E-mail addresses: ysmwk987@yahoo.co.jp (K. Asano), m-ari@marianna-u.ac.jp (M. Arito), manae@marianna-u.ac.jp (M.S. Kurokawa), omoteyama@marianna-u.ac.jp (K. Omoteyama), k2oka@marianna-u.ac.jp (K. Okamoto), n2sue@marianna-u.ac.jp (N. Suematsu), yudo@marianna-u.ac.jp (K. Yudoh), nakamura@nms.ac.jp (H. Nakamura), mbortho7@marianna-u.ac.jp (M. Beppu), t3kato@marianna-u.ac.jp (T. Kato).

http://dx.doi.org/10.1016/j.bbrc.2014.08.053
0006-291X/

In RA, inflammatory cytokines like tumor necrosis factor α (TNF-α) have been known to play pathological roles [15]. In fact, the blockage of the cytokines is used as effective therapies in RA [16]. Recently, increased levels of LMW-HA in sera and synovial fluid of patients with RA were reported and thus LMW-HA was speculated to exacerbate inflammation in RA [17–19]. In this context, in addition to the difference of MW of HA, differences of quantity and functions of HA receptors would affect the severity of inflammation in RA, since several receptors for HA have been identified until now [9]. As to chondrocytes, which play critical roles in maintaining articular cartilage and are affected in RA as well as OA, express at least 2 kinds of HA receptors, CD44 and LAYN [9,20,21]. However, roles and regulation of LAYN in articular chondrocytes have been poorly understood.

To clarify regulation of LAYN in chondrocytes, we here investigated whether TNF-α affected expression levels of LAYN in chondrocytes, since TNF-α is the major pro-inflammatory cytokine involved in the pathophysiology of RA [15]. Next, to clarify LAYN-specific effects in chondrocytes, we investigated whether binding of anti-LAYN antibodies to LAYN affected secretion of inflammatory cytokines using a chondrosarcoma cell line.

2. Materials and methods

2.1. Clinical samples and preparation of chondrocytes

Human articular chondrocytes were obtained from articular cartilage of 10 female patients with OA (OA1-10, mean age 73 years [range 61–81 years]) who underwent arthroplasty of hip (OA1-6) or knee (OA7-10) joints. The diagnosis of OA was made according to the criteria of Kellgren and Lawrence [22]. Written informed consent was obtained from each of the patients and this study protocol was approved by the ethics committee of St. Marianna University School of Medicine. The study was performed in compliance with the World Medical Association Declaration of Helsinki.

After careful removal of synovial tissue, cartilage was minced, washed, and treated with collagenase (Wako Pure Chemical Industries, Osaka, Japan). Isolated chondrocytes (Passage 0, P0) were washed and grown *in vitro* in monolayer culture in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Carlsbad, CA) supplemented with 10% fetal calf serum (Wako), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma–Aldrich, St. Louis, MO, USA) on type I collagen-coated culture dishes.

In addition to the articular chondrocytes, a human chondrosarcoma cell line of OUMS-27 cells, obtained from Health Science Research Resource Bank (Osaka, Japan), was used. OUMS-27 cells were cultured in DMEM similarly prepared as above. The cells were cultured at 37 °C in 5% CO_2.

2.2. Treatment of chondrocytes and OUMS-27 cells with reagents

The prepared articular chondrocytes (P1-P3, 1.0×10^7 cells/φ100 mm dish) and OUMS-27 cells were cultured in the starvation condition of DMEM supplemented with 10% charcoal dextran-treated FBS (Wako), 100 units/ml penicillin, and 100 μg/ml streptomycin for 24 h. The cells were treated with or without 0.1–100 ng/ml human TNF-α (PROSPEC, Ness Ziona, Israel) for 24 h and subjected to Western blotting and real time PCR. Further, OUMS-27 cells were treated with 10 μg/ml goat anti-human LAYN polyclonal antibodies (R&D, Minneapolis, MN, USA) or 10 μg/ml goat normal IgG (R&D) as a control for a cytokine array study and ELISA.

2.3. Western blotting

Proteins were extracted from the chondrocytes into a lysis buffer (20 mM Tris–HCl, 250 mM NaCl, 1% NP-40, 1 mM dithiothreitol) including protease inhibitor cocktail (Roche, Basel, Switzerland) for Western blotting. Goat polyclonal antibodies to human LAYN (R&D) and mouse monoclonal antibodies to β-actin (Wako) were used as the 1st antibodies. Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG and anti-mouse IgG antibodies (Invitrogen/Zymed) were used as the 2nd antibodies, respectively. The bound antibodies were visualized using an enhanced chemiluminescence detection system (GE healthcare, Buckinghamshire, UK).

2.4. RNA extraction and reverse transcription (RT)-PCR

Extraction and purification of RNA from the cells and reverse-transcription of the RNA samples were performed using RNeasy® (Qiagen, Venlo, the Netherlands) and High Capacity cDNA Reverse Transcription Kits® (Life Technologies, Rockville, MD, USA), respectively. Then, 2 μg of total RNA-derived cDNA was mixed with 1 μM each of the forward and reverse primers and Premix Taq™ (Ex Taq™ Version 2.0, Takara, Shiga, Japan) and subjected to PCR. Nucleotide sequences of the primers for the amplification of a C5 DNA fragment and a β-actin fragment, determined from a previous report [23], are as follows. For C5: 5′-GTTGAAGCCCGAGAGAACAG and 5′-AGGGAAAGAGCATACGCAAGA, and for β-actin: 5′-AGGC ACCAGGGCGTGAT and 5′-TGCTCCCAGTTGGTGACGAT. The thermal cycle conditions were as follows: 98 °C for 5 min, followed by 40 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s.

2.5. Quantitative real time PCR

Real time PCR was performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster city, CA, USA), according manufacturer's instructions. 1 μg of total RNA-derived cDNA was mixed with 300 nM each of the forward and reverse primers and Power SYBR® Master Mix (Applied Biosystems) and then was subjected to real time PCR. The thermal cycle conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Nucleotide sequences of the primers for the amplification of a LAYN DNA fragment and a GAPDH fragment are as follows. For LAYN: 5′-CACAGCCTGCCAGGACCTTTA and 5′-TG CACCGGTCATCATTCCA, and for GAPDH: 5′-TGGTATGGTGGAAGG ACTCA and 5′-ATGCCAGTGAGCTTCCCGTT.

2.6. Human cytokine array

The relative protein levels of 36 different cytokines or soluble factors in the supernatant of each cell culture were simultaneously detected by a commercially available kit of Human Cytokine Array, Panel A (R&D), according to the manufacturer's instructions. The levels of the followings were measured: complement component (C5)/C5a, CD40 ligand, G-CSF, GM-CSF, GROα (CXCL1), I-309 (CCL1), sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8 (CXCL8), IL-10, IL-12, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32a, IP10 (CXCL10), I-TAC (CXCL11), MCP-1 (CCL2), MIF, MIP-1a/b, serpin E1 (PAI-1), RANTES (CCL5), SDF-1 (CXCL12), TNF-α, and sTREM-1.

2.7. ELISA

Concentrations of IL-8, RANTES and sICAM in the culture supernatant of OUMS-27 were measured by their respective commercially available ELISA kits (R&D), according to the manufacturer's instructions. Similarly, concentrations of C5/C5a and C5a alone