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Secretion of inflammatory factors from chondrocytes by layilin signaling

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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].

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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

were measured by their respective commercially available ELISA kits (Abcam, Cambridge, UK and BD Biosciences, San Jose, CA, USA, respectively), according to the manufacturers' instructions.

2.8. Statistical analysis

Statistical significance was calculated by Student's *t*-test.

3. Result

3.1. TNF-α up-regulates the expression of LAYN in human articular chondrocytes

First, we investigated whether TNF-α affected the expression of LAYN on chondrocytes. Specifically, we treated articular chondrocytes derived from 8 OA patients with 100 ng/ml TNF-α for 24 h. Then we compared the expression of LAYN by Western blotting. As a result, we found that the expression of LAYN was significantly increased by the TNF-α stimulation ($p = 0.025$, Fig. 1).

3.2. Pathological levels of TNF-α up-regulates the expression of LAYN at the mRNA level

We then confirm this phenomenon using OUMS-27, a chondrosarcoma cell line. As a result, the expression of LAYN was up-regulated similarly in OUMS-27 by the same concentration of TNF-α as above (Fig. 2A). This indicates that OUMS-27 would be a useful material to investigate roles and regulation of LAYN in chondrocytes.

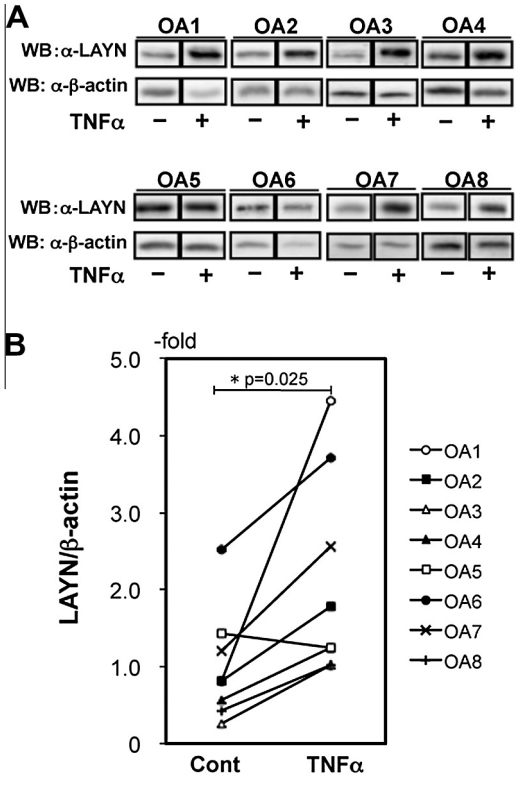

Fig. 1. Effects of TNF-α on the expression of LAYN in articular chondrocytes. (A) Human articular chondrocytes from 8 patients with OA were cultured with or without 100 ng/ml TNF-α for 24 h. Then proteins were extracted and subjected to Western blotting to detect LAYN. Detection of β-actin was used as a control. (B) The intensity of the LAYN bands in the Western blotting, measured by a densitometer, was corrected using that of the β-actin bands. The corrected LAYN intensity was compared between the TNF-α-stimulated and non-stimulated samples in each of the 8 patients. The average of the corrected LAYN intensity in the non-treated samples was defined as 1.0.

Since the concentration of TNF-α in synovial fluid of patients with RA was reported to be increased up to 83–3055 pg/ml [24], we extended our study to test whether the similar levels of TNF-α up-regulated the expression of LAYN using OUMS-27 *in vitro*. As a result, the expression of LAYN was up-regulated by 100–800 pg/ml TNF-α in a dose-dependent manner (Fig. 2B and C).

We next investigated whether the increase of LAYN expression was a result of increased transcription of mRNA for LAYN by quantitative real time PCR. As a result, the mRNA level for LAYN was found increased significantly by the TNF-α stimulation (Fig. 2D). We concluded that TNF-α increased the amount of mRNA for LAYN, which lead to the increased amount of LAYN.

3.3. Activated LAYN signaling promotes secretion of soluble inflammatory factors

Next, we investigated roles of LAYN. Although it was found that LAYN is a receptor for HA, HA binds to other several receptors including CD44. Thus, use of HA could not avoid signaling effects of other HA receptors, thereby, we here used anti-LAYN antibodies to investigate only the LAYN signaling. Specifically, we stimulated OUMS-27 for 48 h with antibodies to the extra-cellular domain of LAYN and then made screening of 36 soluble inflammatory factors in the culture supernatant using a cytokine array (Fig. 3A). As a result, 9 out of the 36 soluble factors were detected in the supernatant and the concentration of 4 out of the 9 soluble factors appeared to be increased. They were interleukin (IL)-8, regulated on activation, normal T cell expressed and secreted (RANTES), soluble intercellular adhesion molecule (sICAM), and complement5 (C5)/C5a with fold-increases of 1.4, 1.6, 1.7, and 3.6, respectively (Fig. 3A and B).

3.4. A human articular chondrocytes as well as OUMS-27 produce C5 and activation of the LAYN signal promotes secretion of C5/C5a

To confirm these result quantitatively, we measured the concentrations of IL-8, RANTES, sICAM, and C5/C5a by their specific ELISA. As a result, as to IL-8, significant increase of the concentration was confirmed by ELISA ($p = 0.002$, Fig. 4A). On RANTES, no increased secretion was evidenced by ELISA (Fig. 4B). The level of sICAM was undetectable by ELISA used here (data not shown). On C5/C5a, we first confirmed that OUMS-27 of a chondrosarcoma cell line and human articular chondrocytes produced C5, since production of C5 in chondrocytes was unexpected. As a result, the existence of mRNA for C5 in OUMS-27 and chondrocytes was demonstrated by PCR, although the amount of the amplified DNA for C5 mRNA from OUMS-27 and chondrocytes was rather small compared to that from HepG2, a hepatocellular carcinoma cell line (Fig. 4C).Thus, OUMS-27 and chondrocytes were evidenced to produce C5. We then measured C5/C5a and C5a alone by their respective ELISA systems. As a result, both of the C5/C5a and C5a alone concentrations were increased significantly by the anti-LAYN antibody stimulation ($p = 0.049$ and $p = 0.016$, respectively) (Fig. 4C). These data indicates that C5 is produced both in OUMS-27 and chondrocytes and the secretion of C5/C5a is promoted by activation of the LAYN signals.

4. Discussion

We here found that TNF-α up-regulated expression of LAYN on chondrocytes and that the LAYN signal enhanced production of IL-8 and C5/C5a.

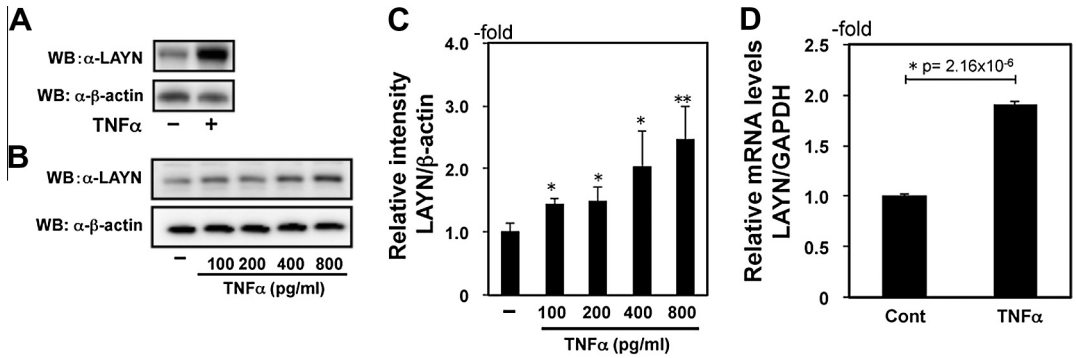

Fig. 2. Effects of TNF-α on mRNA and protein levels of LAYN in OUMS-27. (A) OUMS-27 cells were cultured with or without 100 ng/ml TNF-α for 24 h ($n = 3$). Then proteins were extracted and subjected to Western blotting to detect LAYN. β-actin was detected as a control. (B) OUMS-27 cells were cultured with TNF-α (0, 100, 200, 400, and 800 pg/ml) for 24 h ($n = 3$). Then proteins were extracted and subjected to Western blotting to detect LAYN. β-actin was detected as a control. (C) The LAYN intensity, corrected using that of β-actin, was described. The average of the corrected LAYN intensity in the non-treated samples was defined as 1.0. $^{*}p < 0.05$, $^{**}p < 0.01$ (D) OUMS-27 cells were cultured with or without 800 pg/ml TNF-α for 24 h ($n = 3$). Then, RNA, extracted from the cells, was used for reverse-transcription and the subsequent real time-PCR to estimate amounts of mRNA for LAYN and GAPDH. The LAYN mRNA levels were corrected using those of GAPDH. The average of the corrected LAYN mRNA levels in the non-treated samples was defined as 1.0.

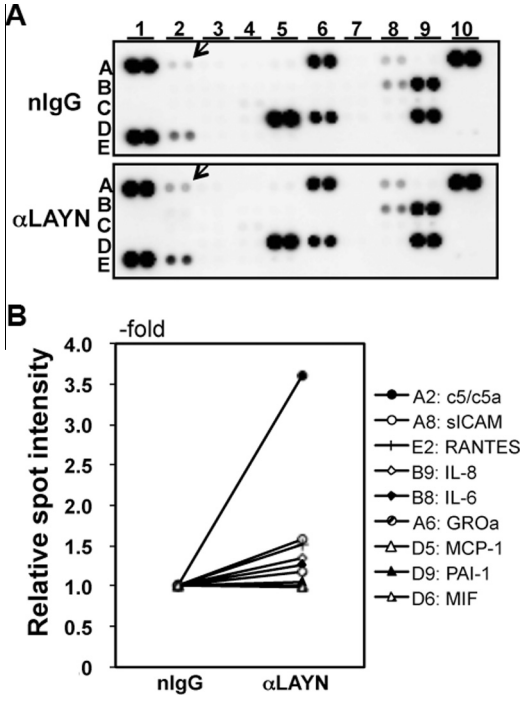

Fig. 3. Screening of soluble inflammatory factors affected by the LAYN signaling. (A) OUMS-27 cells were treated with 10 μg/ml goat anti-LAYN antibodies (αLAYN) or with 10 μg/ml goat normal IgG (nIgG) as a negative control for 48 h. Then the supernatant was subjected to a cytokine array study. A1, A10, and E1: positive controls, E10: negative controls. A2 (arrows): C5/C5a spots. (B) Relative spot intensity was defined by a ratio of the intensity of αLAYN-treated spots to that of nIgG-treated spots in each soluble factor. Results of 9 detected soluble factors are described. The locations in the panels in (A) and names of the 9 soluble factors are shown in the right side (ex. B8: IL-8).

On the first point, it is well known that TNF-α plays a central role in the pathophysiology of RA [15]. In fact, biologics that target TNF-α have been very successful in the treatment of RA [16]. In our study, the expression of LAYN was increased by TNF-α at least in part by up-regulation of the amount of mRNA. Since TNF-α is known to enhance expression of pro-inflammatory cytokines and cyclocxygenase-2 through the activation of NF-κB [25–27], the expression of LAYN is possibly regulated by NF-κB. This point should be investigated in the future.

TNF-α was reported to activate the RhoA/ROCK signal [28]. Recently, it was reported that binding of oligosaccharide HA to LAYN decreased expression of E-cadherin and increased cell permeability through the activation of the RhoA/ROCK signal [14]. Thus, the activation of the RhoA/ROCK signal by TNF-α may be in part due to the increased expression of LAYN.

Again, LAYN has been demonstrated to be a receptor for HA. However, HA binds to other several receptors of CD44, lymphatic vessel endothelial hyaluronan-1 (LYVE-1), hyaluronan receptor for endocytosis (HARE), toll-like receptor 4 (TLR4), and receptor for hyaluronan-mediated motility (RHAMM) [9]. Thereby, it is difficult to examine effects of HA on cells through LAYN alone. We thus here used antibodies to the extra-cellular domain of LAYN to examine the LAYN signaling. Our cytokine array study and the subsequent ELISA revealed that the LAYN signaling enhanced IL-8 and C5/C5a secretion. IL-8 mediates inflammation by displaying a chemotactic activity for neutrophils and inducing degranulation of them [29]. The level of IL-8 was reported to be high in synovial fluid of patients with RA. Thus, the LAYN signal may contribute to the inflammation by the up-regulation of IL-8.

C5a, an anaphylatoxin produced by the cleavage of C5 by C3b, shows a chemotactic activity for monocytes and neutrophils [30]. It was unexpected that C5/C5a was produced in articular chondrocytes. Complements including C5 are produced mainly in hepatocytes and no report has been available that described production of C5 in chondrocytes, to our knowledge. Thereby we first confirmed that the existence of mRNA for C5 by RT-PCR and demonstrated that C5 was really produced in chondrocytes. Since other complements, C1r, C1s, and C2–C4 have been reported to be produced in chondrocytes [31], various complements would be produced in chondrocytes generally even at low levels. Previously, TNF-α was reported to enhance the mRNA levels of C2 and C3 [31]. Taking our data together with the reports, LAYN may participate in the up-regulation of C2 and C3 as well as C5. This point needs further clarification.

From the viewpoint of complements, chondrocytes have been reported to carry a receptor for C5a on their surface [32] and binding of C5a to the receptor leads to secretion of pro-inflammatory cytokines like IL-6 [33]. Thus, it is possible that C5a increased by the LAYN signaling not only shows chemotactic activities for monocytes and neutrophils but also induces secretion of pro-inflammatory cytokines from chondrocytes in an autocrine fashion.

Recently, it was reported that synovial fluid of patients with OA contained high levels of membrane attack complex (MAC)

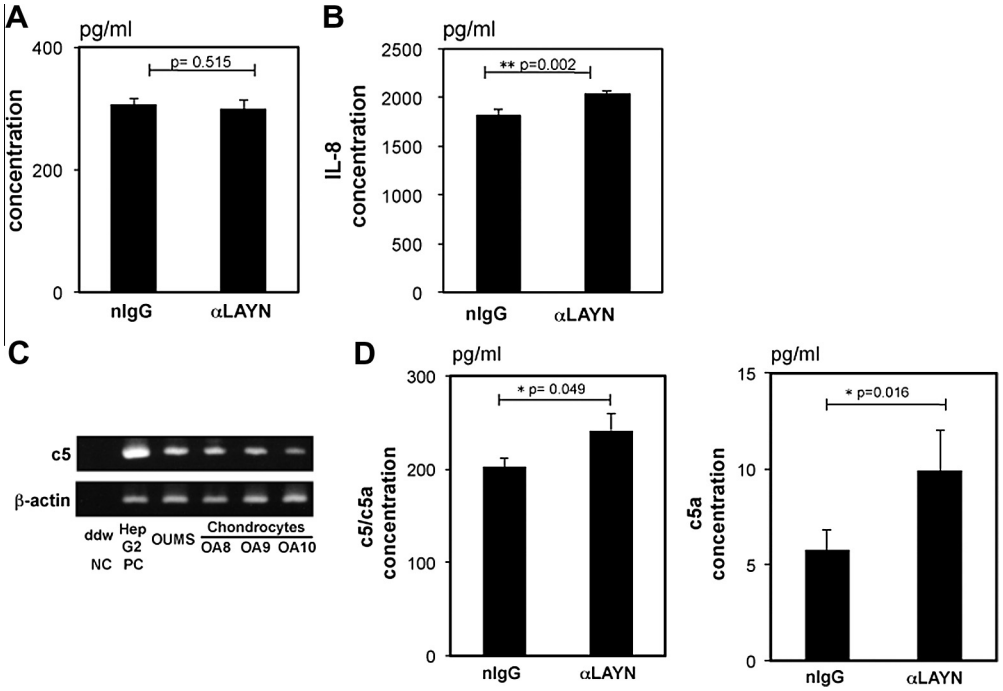

Fig. 4. Detection of IL-8, RANTES, and C5/C5a by ELISA OUMS-27 cells were treated with 10 μg/ml goat anti-LAYN antibodies (αLAYN) or with 10 μg/ml goat normal IgG (nIgG) as a negative control for 48 h ($n = 5$). Then the supernatant was subjected to ELISA to detect IL-8 (A) and RANTES (B). (C) Total RNA was extracted from HepG2 (a positive control, PC), OUMS-27, and 3 OA chondrocyte samples (OA8-10) cultured without anti-LAYN treatment. The extracted total RNA was subjected to RT-PCR for C5 and β-actin. (D) The supernatant from OUMS-27 cells, treated with 10 μg/ml goat αLAYN or with 10 μg/ml goat nIgG for 48 h, was subjected to ELISA to detect C5/C5a and C5a alone ($n = 5$).

consisting of C5b, C6, C7, C8, and C9 and that MAC triggered release of matrix metalloproteinases and inflammatory mediators from chondrocytes, which in turn promoted degradation of cartilage [34]. The enhanced secretion of C5a by the LAYN signaling detected here indicates simultaneous production of C5b, which probably forms MAC on chondrocytes. Thus, the LAYN signaling may lead to chondrocyte death and degradation of cartilage. This possible pathway should be investigated in the near future.

In conclusion, we here demonstrated that TNF-α up-regulated LAYN expression on chondrocytes and that the LAYN signaling enhanced IL-8 and C5/C5a secretion from chondrocytes. Therefore, LAYN would be involved in the enhancement of inflammation and degradation of cartilage in RA and OA.

Conflict of interest statement

This study was partially founded by Chugai Pharmaceutical Co., Ltd.

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