Title: Possible involvement of DExD/H box helicase 60 in synovial inflammation of rheumatoid arthritis: role of Toll-like receptor 3 signaling

（関節リウマチの滑膜炎における DExD/H box ヘリカーゼ 60 関与の可能性：Toll-like receptor 3 シグナルの役割）

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Abstract

Background

Innate immunity is known to be implicated in the etiology of synovitis in rheumatoid arthritis (RA). However, details of the molecular mechanisms have not been fully clarified. DExD/H-box helicase 60 (DDX60), a putative RNA helicase, is of consequence in anti-viral innate immune reactions followed by inflammation. Although DDX60 is involved in the pathogenesis of autoimmune diseases such as systemic lupus nephritis, the role of DDX60 in RA has not been elucidated. The objective of this study was to examine the expression and the role of DDX60 in RA synovial inflammation.

Methods and Results

DDX60 protein expression was investigated by immunohistochemistry in synovial tissues resected from 4 RA and 4 osteoarthritis (OA) patients. We found that synovial DDX60 expression was more intense in RA than in OA. Treatment of human rheumatoid fibroblast-like synoviocytes in culture with polyinosinic-polycytidylic acid, a Toll-like receptor 3 (TLR3) ligand, increased DDX60 protein and mRNA expression. A knockdown experiment of DDX60 using RNA interference revealed a decrease in the expression of poly IC-induced C-X-C motif

chemokine ligand 10 (CXCL10) which induces lymphocyte chemotaxis.

Conclusions

The synovial DDX60 was more expressed in RA patients than in OA. In human RFLS, DDX60 stimulated by TLR3 signaling affected CXCL10 expression. DDX60 may contribute to synovial inflammation in RA.

Keywords: rheumatoid arthritis, synovitis, TLR3, DDX60, CXCL10

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease, which develops in many peripheral joints, causing synovitis, bone erosion, and progressive joint destruction [1]. For the treatment of RA, non-steroidal anti-inflammatory drugs, steroids, and disease-modifying anti-rheumatic drugs have been used [2]. Recently, biological therapies such as anti-tumor necrosis factor-α monoclonal antibody, etc., have been developed. However, the cost and side effects of drugs and opportunistic infections are the problems of conservative treatments. In addition, some patients do not respond to drug therapy, and surgery is also performed for those patients. Therefore, further clarification of the RA pathogenesis and the development of new treatment strategies are necessary.

Although many cell types play crucial roles in the pathogenesis of RA, fibroblast-like synoviocytes (FLS) are one of the key cell types. Rheumatoid FLS (RFLS) produces cytokines and proteases, leading to synovial inflammation and cartilage destruction [3]. However, the mechanisms of inflammatory responses in RFLS have not been fully clarified. Innate immunity is the basic system to protect a host from invading pathogens. The innate immune reaction is initiated upon binding of pathogen-associated molecular patterns to pattern recognition receptors (PRRs), and activation of innate immunity drives the following activation of adaptive

immunity. Although innate immunity is essential to defend the host from infectious microorganisms, dysregulated innate immune reactions are related to the pathogenesis of autoimmune inflammatory diseases including RA [4]. Danger-associated molecular patterns are endogenous molecules that also bind to PRRs, and importantly, are implicated in the inflammation of RA [5]. Therefore, innate immune reactions in RFLS may be important in the initiation and prolongation of inflammation, and details of innate immune reactions in RFLS should be clarified to understand the mechanisms of immunological dysfunction in RA.

Toll-like receptors (TLRs) are PRRs, and activation of innate immunity in synovium by TLR agonists is one of the pathogenic mechanisms of RA. The expression of TLR2, 3, 4, and 5 is increased and related to inflammation in synoviocytes of RA patients [6]. Particularly, TLR3 is increased in synovial fibroblasts and TLR3 signaling induces synovial fibroblasts-mediated activation of Th1 and Th17 lymphocytes [7]. In addition, activation of TLR3-mediated reaction in cultured human RFLS induces the expression of interferon (IFN)-β [8]. IFN-β induces the expression of hundreds of IFN-stimulated genes (ISGs) and ISGs reveal a variety of bioactivities. A chemokine CXCL10, which induces the chemotaxis of lymphocytes, is a member of ISGs and is involved in autoimmune diseases including RA [8, 9]. However, the role of many ISGs in RA is still largely unknown.

The DEAD-box protein family has a conserved motif, Asp/Glu/Ala/Asp (DEAD), and encodes putative RNA helicases [10]. DEAD RNA helicases participate in various RNA metabolisms including RNA binding, transcription, translation, RNA unwinding and genome stability [10], and also in innate immunity against viral infections [11]. DExD/H box helicase 58 (DDX58) is a DEAD box protein and is also known as retinoic acid-inducible gene-I (RIG-I). DDX58/RIG-I encodes a receptor that can recognize viral double-stranded RNA, and the possible involvement of DDX58/RIG-I in synovial inflammation in RA has been reported [12]. DDX60 is another DEAD box protein and is implicated in DDX58/RIG-I-dependent and -independent anti-viral innate immune reactions [13, 14], and both DDX60 and DDX58/RIG-I are included in ISGs. It was reported that TLR3 signaling induces DDX60 in human glomerular endothelial cells in culture, and DDX60 is strongly expressed in glomerular endothelial cells of patients with diffuse proliferative lupus nephritis [15]. This suggests that DDX60 may be involved in the pathological mechanisms of TLR3-related autoimmune diseases. However, to our knowledge, the function of DDX60 in RA is obscure.

In the present study, we hypothesized that DDX60 might be involved in the pathogenesis of RA. This study aimed to elucidate the expression and the role of DDX60 in RA synovitis. We first

examined in vivo DDX60 expression in synovial tissues obtained from RA patients using immunohistochemistry. Second, we investigated DDX60 expression in vitro in cultured RFLS treated with a TLR3 agonist polyinosinic-polycytidylic acid (poly IC). The role of DDX60 in poly IC-induced CXCL10 expression was also examined.

Materials and Methods

Immunohistochemical analysis

Immunohistochemistry was carried out to observe the expression of DDX60 in human RA synovium. Hyperplastic knee synovial tissues resected surgically from 4 RA patients and from 4 osteoarthritis (OA) patients were used. Total knee arthroplasties were operated on all patients because they were classified as end-stage OA or RA, and their knee joints were already destroyed. The ethics committee of Hirosaki University Graduate School of Medicine approved the study, and written informed consent was obtained from all patients. Synovium was collected during the surgery, and immunohistochemical analysis was performed using standard techniques. Briefly, the tissues were fixed with formalin, and the specimens were paraffin-embedded and sliced into 3 μm thick sections. The sections were probed with an anti-human DDX60 rabbit IgG (1:50, GTX32082, GeneTex, Irvine, CA, USA), followed by incubation with

a biotinylated anti-rabbit IgG antibody and horseradish peroxidase (HRP)-conjugated streptavidin. To detect the immunoreaction, a DAB/H_2O_2 system was used. The intensity of the DDX60 protein was evaluated by the pathologist at the Department of Anatomic Pathology, Hirosaki University Hospital.

Cell culture

Human RFLS, obtained from Health Science Research Resources Bank (Sen-nan, Japan), were cultured with Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Poly IC (P1530; Sigma, St. Louis, MO) was added to the medium as indicated. In the experiments examining the concentration-dependent effect of poly IC, RFLS were incubated with 0.08-50 μg/mL poly IC for 24 h (protein analysis) or 16 h (mRNA analysis). When the time course of the poly IC treatment was examined, RFLS were treated with 10 μg/mL poly IC for 2, 4, 8, 16, and 24 h. To examine the role of type I IFN, RFLS were pretreated with a type I IFN neutralizing antibody mixture (1:25 dilution, 39000-1; PBL assay science, Piscataway, NJ, USA) for 1 h before adding poly IC (10 μg/ mL). To perform the RNA interference, RFLS of 40-70 % confluence were transfected with a specific siRNA against human DDX60 (Qiagen, Hilden, Germany) or a negative control non-silencing siRNA (Qiagen) complexed with Lipofectamine RNAi MAX (ThermoFisher, Carlsbad, CA, USA). RFLS were stimulated with

10 μg/ mL poly IC after 48 h of transfection.

Western blotting

After incubating as above, RFLS were washed twice using phosphate-buffered saline (PBS) on ice and scraped with Laemmli buffer. Proteins in the cell lysates were denatured and applied to 5-20 % polyacrylamide gel (ATTO, Tokyo, Japan) electrophoresis. Proteins were blotted onto a polyvinylidene fluoride membrane, followed by probing with an anti-DDX60 antibody (1:1000 dilution), anti-DDX58/RIG-I rabbit antibody [16], or an anti-human actin rabbit IgG (1:5000 dilution, Sigma) overnight. Signals of DDX60 and actin protein were obtained using an HRP-conjugated anti-rabbit IgG gout antibody (1:10000 dilution, Medical & Biological Laboratories, Nagoya, Japan) and an HRP substrate.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis

RNA extraction from RFLS was conducted using a Nucleospin RNA extraction kit (Takara Bio, Kusatsu, Japan). M-MLV reverse transcriptase (ThermoFisher) was used for reverse transcription. Using the synthesized cDNA as a template, PCR amplification of cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), DDX60, CXCL10, and CXCL1 was performed using SsoAdvanced Universal SYBR Green (Bio-Rad, Hercules, CA, USA) and

quantified using a real-time PCR system (Bio-Rad). Expression of DDX60 and CXCL1 mRNA was estimated as a fold increase versus the cells without poly IC treatment. Expression of CXCL10 mRNA was shown as an arbitrary unit because CXCL10 mRNA expression was below the detection limit in cells without treatment. GAPDH was used as an internal control. The sequences of the primers used are as follows: CXCL1-F: 5'- ATGGCCCGCGCTGCTCTCTCC -3', CXCL1-R: 5'- GTTGGATTTGTCACTGTTCAG -3', CXCL10-F: 5'- TTCAAGGAGTACCTCTCTCTAG -3', CXCL10-R: 5'- CTGGATTCAGACATCTCTTCTC -3', DDX60-F: 5'-AAGGTGTTCCTTGATGATCTCC-3', DDX60-R: 5'- TGACAATGGGAGTTGATATTCC -3', GAPDH-F: 5'- GCACCGTCAAGGCTGAGAAC -3', and GAPDH-R: 5'- ATGGTGGTGAAGACGCCAGT -3'.

Lactate dehydrogenase (LDH) assay

Cytotoxicity of ploy IC to the cells is determined by measuring the activity of LDH released from the cells into the culture medium using the LDH assay kit (Dojindo Laboratories, Kumamoto, Japan).

Enzyme-linked immunosorbent assay (ELISA)

After incubation, we collected the culture medium which was then centrifuged. CXCL10 and

CXCL1 protein concentrations in the supernatant were determined using sandwich ELISA kits (R&D systems, Minneapolis, MN, USA).

Immunofluorescence

The cells were seeded onto a gelatin-coated 12 mm glass base dish (IWAKI, Shizuoka, Japan) and cultured for 24 h. Then the cells were stimulated with poly IC for 24 h. After rinsing three times with PBS, the cells were fixed with 10% formaldehyde for 15 min at room temperature. Next, the cells were permeabilized with 100% methanol and blocked with PBS containing 1% bovine serum albumin. Then, the cells were incubated with rabbit anti-DDX60 antibody (1:500, GeneTex) overnight at 4°C. After rinsing, the cells were incubated with fluorescence-labeled goat anti-rabbit IgG (Alexa Fluor 488), (ab150077, Abcam, Cambridge, UK). Finally, the cells were treated with Hoechst 33342 (Dojindo). Immunofluorescence was visualized by laser scanning confocal microscopy (C1si laser microscope; Nikon, Tokyo, Japan).

Statistics

The quantitative data were expressed as mean ± standard deviation (SD) (n=3), and the U-test was used to analyze the differences between the groups.

Results

DDX60 is more strongly expressed in RA synovial tissue than in OA synovial tissue

Immunohistochemically, synoviocytes, fibroblasts, endothelial cells, and plasma cells were positive for DDX60 in synovial tissues from both OA and RA. DDX60 immunoreactivity in those cells was more intense in all 4 RA cases than in OA cases (Fig. 1).

Poly IC induces DDX60 expression in RFLS

In resting conditions, expression of DDX60 protein was observed in cultured RFLS (Fig. 2A). In the experiments using various concentrations of poly IC, DDX60 protein expression was increased in a concentration-dependent manner (Fig. 2A). DDX60 mRNA expression was upregulated with poly IC as well as protein (Fig. 2B). In the condition we tested, release of LDH from cells into the cell culture medium was not changed (Fig. 2C), suggesting that poly IC treatment was not cytotoxic. DDX60 protein (Fig. 2D) and mRNA (Fig. 2E) expression were also upregulated by poly IC in a time-dependent manner. An increase of DDX60 protein and mRNA was found in poly IC-treated cells at 16-24 h (Fig. 2D and E). Poly IC-induced upregulation of DDX60 protein was not changed by pretreatment of cells with type I IFN neutralizing antibody mixture, whereas upregulation of DDX58/RIG-I protein by poly IC was

markedly inhibited (Fig. 2F).

DDX60 plays a role in poly IC-mediated CXCL10 expression in RFLS

Knockdown of DDX60 using RNA interference resulted in a partial decrease of poly IC-induced CXCL10 mRNA expression (Fig. 3A), whereas poly IC-upregulated CXCL1 mRNA level was not changed (Fig. 3B). Partial knockdown of DDX60 mRNA (Fig. 3C) and protein (Fig. 3D) by DDX60 siRNA was confirmed. Knockdown of DDX60 also partially decreased the level of CXCL10 protein (Fig. 3E), but not of CXCL1 protein (Fig. 3F), in medium collected from poly IC-treated cells.

Immunofluorescence

Next, we studied the intracellular localization of DDX60 protein using immunofluorescence. In cultured RLFS, DDX60 protein was localized in the cytoplasmic region before (Fig. 4A) and after (Fig. 4B) poly IC treatment.

Discussion

DDX60 is a putative RNA helicase and is thought to take part in diverse cellular processes involving RNA secondary structure alteration, RNA binding, and gene expression. Therefore,

DDX60 may contribute to the pathogenesis of various pathologic conditions in a complex manner. DDX60 is associated with the pathogenesis of malignant tumors such as oral squamous cell carcinoma [17] and glioma [18], and with an autoimmune disease lupus nephritis [15]. However, there has been no report on the involvement of DDX60 in RA. We first examined, in the present study, DDX60 protein expression in synovial tissues surgically resected from knee joints of patients with RA or OA. DDX60 protein immunoreactivity was found in several cell types including synoviocytes, fibroblasts, endothelial cells, and plasma cells in synovial tissues from patients with RA or OA. These cells in RA showed more intense DDX60 immunoreactivity than those in OA, suggesting that DDX60 may be associated with the pathological process of RA synovitis.

Among these cell types, we focused on synoviocytes and next studied the expression of DDX60 in cultured RFLS. We found that TLR3 signaling induced the upregulation of DDX60 protein and mRNA expression, suggesting that DDX60 may contribute to synovial inflammation related to TLR3. Activation of TLR3 induces the upregulation of several genes in type-I IFN-independent or -dependent manners [19]. We found that neutralization of type I IFN did not change poly IC-induced DDX60 protein expression, while poly IC-induced DDX58/RIG-I expression was completely abolished. This result suggests that TLR3-mediated expression of

DDX60 is type I IFN-independent, whereas TLR3-induced DDX58/RIG-I expression depends on type I IFN. Thus, DDX60 and DDX58/RIG-I may be differentially regulated in RFLS. At 2-8 h after poly IC treatment, DDX60 mRNA expression level showed a temporary decrease. Although we do not have the results that explain this trend of decreasing DDX60 mRNA, there may be an unknown molecular system that regulates DDX60 mRNA expression. Further studies may be necessary.

CXCL10 is a chemokine, and C-X-C motif chemokine receptor 3 (CXCR3) is the receptor for CXCL10 [20]. CXCL10 induces the chemotaxis of CXCR3-expressing cells such as NK cells and T cells and is implicated in various autoimmune diseases including RA [9]. Synovial fluid of RA patients contains much more CXCL10 protein than that of OA patients [21]. In collagen-induced arthritis model mice, knockout of CXCL10 and CXCR3 suppressed macrophage and T cell invasion to the synovium, cartilage degeneration, and bone destruction [22]. In addition, an anti-CXCL10 monoclonal antibody significantly increased rheumatology 20% improvement criteria in RA patients who inadequately responded to methotrexate [23]. This evidence indicates that CXCL10 is an important mediator in RA. In this study, downregulation of DDX60 by siRNA reduced the expression of poly IC-induced CXCL10 in RFLS, whereas the expression of another CXC chemokine CXCL1 upregulated by poly IC was not altered. This suggests that

DDX60 selectively mediates the poly IC-induced CXCL10 upregulation. DDX60 is reported to promote DDX58/RIG-I-mediated signaling [13], and DDX58/RIG-I is implicated in CXCL10 expression induced by IFN-γ in RFLS [12]. Therefore, there is a possibility that DDX60 may increase CXCL10 expression in concert with DDX58/RIG-I. Recently, Chen et al. have shown that DDX60 might be a potential biomarker for systemic lupus erythematosus (SLE) [24], suggesting that DDX60 may be a key molecule of disease activity of SLE and related autoimmune diseases. Therefore, we speculate that DDX60 might be one of the potential targets to develop new therapeutic strategies for RA.

Finally, we examined the intracellular localization of DDX60 protein, and found that DDX60 protein was localized in the cytoplasmic region in unstimulated cells (Fig. 4A), and its localization was not changed by poly IC treatment (Fig. 4B). This result was consistent with a previous study using HeLa cells [13]. DDX60 may regulate TLR3-mediated intracellular signaling in the cytoplasmic region of RFLS, although the details of the molecular system were not clarified in the present study.

There are some limitations in this study. The first limitation is the small sample size of immunohistochemistry. To confirm the results, studies with a larger number of cases should be

performed in the future. Second, molecular mechanisms that regulate DDX60-involved CXCL10 expression were not fully clarified in cultured RFLS. Third, we have not examined the role of DDX60 in RA model mice or genetically modified mouse models. Further studies are needed to elucidate the details of the molecular role of DDX60 in rheumatoid synovitis.

Conclusion

Immunohistochemistry indicated that DDX60 is strongly expressed in synovial tissue from patients affected with RA. Treatment of cultured RFLS with a TLR3 agonist upregulated DDX60 expression. Additionally, knockdown of DDX60 decreased TLR3-mediated CXCL10 expression in RFLS. Taken together, DDX60 may be implicated in RA pathogenesis. Further research is required to clarify the details of molecular mechanisms.

Author contributions

Y. N. contributed to all experiments and manuscript preparation. H. K. I. contributed to all experiments. T. I. contributed to RFLS experiments and manuscript preparation. M.T. contributed to immunofluorescence and western blotting. S. K. and K. S. contributed to qRT-PCR, western blotting, and LDH assay. T. S. contributed to cell culture and collecting samples. A. K., E. S., and Y. I. contributed to immunohistochemistry.

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Statements & Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability Statement

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

Compliance with Ethical Standards

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Figure Legends

Fig. 1

Immunohistochemical detection of DDX60 in knee synovial tissues resected from patients with OA (n = 4, cases 1-4) and RA (n = 4, cases 5-8). DDX60 immunoreactivity was more intense in all 4 RA synovial tissues than in OA.

Fig. 2

Expression of DDX60 in human rheumatoid fibroblast-like synoviocytes (RFLS) in culture. (A) RFLS were incubated with 0.08-50 μg/mL polyinosinic-polycytidylic acid (poly IC) for 24 h. Then, the cells were lysed using Laemmli buffer. Western blotting for DDX60 and actin was carried out. (B) RFLS were incubated with poly IC as in (A) and were incubated for 16 h. RNA was extracted from the cells. Following reverse transcription, cDNA for DDX60 and GAPDH was applied to quantitative real-time PCR (qRT-PCR). (C) Culture medium was collected after incubation as above, and LDH activity in the medium was measured. (D, E) RFLS were incubated with 10 μg/mL poly IC for 2, 4, 8, 16, and 24 h. (D) After incubation, the cell lysates were used for western blotting as in (A). (E) RNA was extracted, and DDX60 mRNA expression was estimated using qRT-PCR. (F) After preincubation with type I IFN neutralizing

antibody mixture for 1 h, poly IC was added to the medium. After 24 h incubation, cellular proteins were applied to western blotting for DDX60, DDX58/RIG-I, and actin.

Fig. 3

Involvement of DDX60 in CXCL10 expression in cultured RFLS. RFLS were transfected with DDX60 siRNA. Forty-eight hours after the transfection, the cells were stimulated with 10 μg/mL poly IC for 16 h. The culture medium, cellular RNA, and cellular protein were collected. CXCL10 (A), CXCL1 (B), and DDX60 (C) mRNA expression were evaluated using qRT-PCR. Expression of DDX60 and actin protein was examined using western blotting (D). CXCL10 (E) and CXCL1 (F) protein concentrations in the culture medium were quantified using specific ELISAs. *$P<0.05$. N.S.: not significant.

Fig. 4

Intracellular localization of DDX60. Cultured human RFLS cells before and after poly IC treatment incubated with rabbit anti-DDX60 antibody. After incubating with fluorescence-labeled goat anti-rabbit IgG (Alexa Fluor 488), the nuclei were stained with Hoechst 3334. Fluorescence was observed using laser scanning confocal microscopy before (A) and after (B) poly IC treatment.