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Upregulation of Toll-like Receptor 2 by TL1A Stimulation in Synovial Fibroblasts

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Background: TNF-like molecule 1A (TL1A) is a ligand of the tumor necrosis factor (TNF) superfamily that mediates apoptosis and inflammation via its effective receptor DR3 and the activation of NF-$\kappa\beta$. Toll-like receptors (TLRs) are receptors that recognize microbes and activate the immune response. Endogenous ligands such as interleukin 1β (IL-1β) and fibronectin fragments also trigger the TLR2-mediated immune response. Recently, the DR3 receptor was found in the synovia of patients with rheumatoid arthritis and osteoarthritis. In this study, we examined the influence of TL1A on the expression of TLRs 1-5 in synovial fibroblasts. **Methods:** Synovial fibroblasts were isolated from rat knee joints and treated with TL1A for 0, 6, 12, or 24 h. Changes in TLR 1-5 expression were determined by RT-PCR. The expression of TLR2 protein was detected by immunocytochemistry and western blotting. **Results:** The expression of the *Tlr2* was significantly upregulated by TL1A in the first six hours of stimulation. TLR2 protein was also upregulated. The expression of *Tlr1* and *Tlr3-5* was not significantly affected by TL1A. **Conclusions:** We have demonstrated for the first time that TLR2 expression is upregulated by TL1A in synovial fibroblasts. DR3 expression in rheumatoid arthritis and osteoarthritis and our findings suggest that TL1A plays a role in the process of articular inflammation and somehow influences the expression of TLR2.

Key words: toll-like receptor, TNF-like molecule 1A, synovium

INTRODUCTION

TNF-like molecule 1A (TL1A) was first identified in human endothelial cell cDNA libraries. The TL1A cDNA encodes a 252-amino-acid protein with high sequence similarity to tumor necrosis factor (TNF)[1]. It belongs to the TNF ligand superfamily but lacks both a transmembrane domain and the first conserved β strand that occurs in the other TNF family ligands. Like other TNF ligands, TL1A can be expressed on the cell membrane or released as a soluble factor by proteolytic cleavage[1,2]. Studies have shown that two members of the TNF receptor superfamily interact with TL1A, death receptor 3 (DR3) and soluble decoy receptor 3 (DcR3)[1,3]. DR3 contains a conserved cytoplasmic death domain, which mediates both the apoptotic cascade and the activation of NF-$\kappa\beta$[4,5]. DcR3 interacts with Fas ligand (FasL), LIGHT, and TL1A and neutralizes the biological effects of these ligands by inhibiting the interactions between FasL-Fas[6], LIGHT-LTβ[7], and TL1A-DR3[1].

TL1A plays an important role in the inflammation process. Previous studies have shown that the interactions between TL1A and DR3 are involved in T-cell activation, differentiation, and enhanced T-cell expansion[1,8,9]. The binding of TL1A to its receptor DR3 on the mucosa in inflammatory bowel disease dominants the Th1 immune response by increasing the secretion of interferon γ (IFN-γ)[8,10]. The upregulated expression of TL1A and DR3 in carotid atherosclerotic plaque macrophage/foam cells induces proinflammatory cytokine/chemokine secretions and decreases plaque stability by inducing extracellular-matrix-degrading enzymes[11].

Toll-like receptors (TLRs) occur in mammals and are defined as pathogen sensors. In infectious diseases, TLRs bind to specific ligands, thus initiating microbial recognition and immediate intracellular signaling to induce the appropriate defense responses[12]. TLR signaling associated with the activation of NF-$\kappa\beta$ and mitogen-activated protein kinase induces the production of mediators of the innate immune system, such as interleukin-1 (IL-1), IL-6,

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IL-8, and TNF-α[13,14]. Further studies have also revealed that TLRs recognize endogenous ligands induced by the inflammatory response in the absence of infection, such as stimulation with IL-1 and TNF-α[15-17]. In our previous study, we demonstrated that TLR2 is expressed in human articular cartilage and is upregulated by endogenous agents such as IL-1β and fibronectin fragments[18].

Because TL1A is a TNF-like molecule and shares a similar role in the inflammatory processes of immune diseases, we examined changes in the expression of TLRs during stimulation with TL1A, to explore the potential role of TL1A in arthritis. Our novel finding is that TLR2 is upregulated by TL1A in synovial cells.

METHODS

Synovium Isolation and Culture

Male Sprague Dawley (SD) rats weighing 280-300 g were obtained from the National Applied Research Laboratories and National Laboratory Animal Center (Taiwan). All the experiments were approved by the local Institutional Review Board and were performed in adherence to the National Institutes of Health Guidelines for the treatment of laboratory animals. The synovia of the knee joints were aseptically removed from six normal SD rats aged eight weeks. The synovia were cut into small fragments and incubated with antimicrobial solution for 1 h. After they had been washed with sterile phosphate-buffered saline (PBS), the synovial fragments were digested with 3 mg/mL collagenase (Sigma, St Louis, MO, USA) at 37 °C for 12 h. The cell suspension was centrifuged at 1200 g for 10 min. The supernatant was discarded and the pellet resuspended in PBS to wash off the collagenase. After centrifugation at 200 g for 10 min, the cells were resuspended in 20 mL of Ham's F12 medium (Sigma) containing 10% fetal bovine serum (Sigma) and 100 μL/mL penicillin/streptomycin (Gibco), then seeded in a 75 cm^2 flask. The synovial fibroblasts were cultured in a humidified 5% CO_2 atmosphere at 37 °C. When the synovial cells were confluent in the flask, they were detached with 0.05% trypsin/0.53 mM EDTA (Gibco™ Invitrogen, Burlington, Canada) and counted with a hemocytometer. They were then seeded at a density of 5×10^5 cell/mL in 55 mm Petri dishes for further experimental procedures.

Experimental Protocol

Dishes of synovial fibroblasts were treated with serum-free medium overnight, then incubated with 0.1 μg/mL TL1A (R&D Systems Inc., Minneapolis, MN, USA) at 37 °C for 0, 6, 12, or 24 h. The TL1A concentration used here was determined in a previous study of TL1A stimulation of human T cells and NK cells[20].

Tetrazolium Assay

TL1A cytotoxicity was assessed with a tetrazolium (MTT) assay. 1×10^4 primary cultured synovial fibroblasts were seeded on 96-well microtiter plates in 0.2 mL of the growth medium described above. TL1A (0.1 μg/mL) was added to the culture wells and incubated for 0, 6, 12, or 24 h, with triplicate samples for each set of conditions. Tetrazolium (50μL) was added to each well and incubated for 3 h at 37 °C. After aspiration of the medium and tetrazolium, 25 μL of Sorenson's glycine buffer (0.1 mol/L glycine, 0.1 mol/L NaCl, pH 10.5) and 200μL of dimethyl sulfoxide were added to each well. The plate was shaken for 5 min. The optical density at 540 nm was determined on a microplate reader (μQuant, BIO-TEK Instruments, Inc., USA) and analyzed with KCjunior ver. 1.5 for Windows (BIO-TEK Instruments, Inc.).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from cells with the easy-BLUE? Total RNA Extraction Kit (iNtRON Biotechnology, Korea). Total RNA (3 μg) was used in a single-round RT reaction, containing 0.75 μg of oligo$(dT)_{14}$ primer, 1 mM dNTPs, 1$\times$ first-strand buffer, 4 mM DTT, 40 units of RNaseOut recombinant ribonuclease inhibitor, and 200 units of SuperScript II reverse transcriptase (Invitrogen®) in a total volume of 25 μL. RT was performed at 42 °C for 2 h and 95 °C for 5 min. PCR was performed using 0.9μL of the cDNA as template, 1 mM gene-specific primers, 1$\times$ PCR buffer, 0.25 mM dNTPs, and 1.5 units of KlenTaq DNA polymerase (Ab Peptides Inc., St Louis, MO, USA). For TLRs, the cDNA mixture was heated at 94 °C for 2 min, then amplified for 30 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified for 25 cycles of 94 °C for 2 min, 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 45 s. All PCR products were size fractionated by 1.5% agarose gel electrophoresis, and the DNA bands were visualized by staining the gel with ethidium bromide. GAPDH was used as the internal control. The specific primers used for PCR are shown in Table 1.

Immunocytochemistry

Ice-cold fixative (50% methanol/50% ethanol; Merck & Co., USA) was added to the Petri dishes for 2 min at room temperature. The dishes were then immediately

Table 1 Specific rat PCR primers

Primer	Sequence
r-TLR1F	5'-AAACGGTCTCATCCACGTTC
r-TLR1R	5'-GAGCAATTGGCAGCACACTA
r-TLR2F	5'-GGCCAGCAAATTACCTGTGT
r-TLR2R	5'-TTCTCCACCCAGTAGGCATC
r-TLR3F	5'-TGCCTTGGTCCCAAGCCTTCAACGA
r-TLR3R	5'-TGGCCCGAAAACCTTCTTCTCAACGGA
r-TLR4F	5'-CGCTTTCAGCTTTGCCTTCATTAC
r-TLR4R	5'-TGCTACTTCCTTGTGCCCTGTGAG
r-TLR5F	5'-AAGAGGGAAACCCCACAGAA
r-TLR5R	5'-GGGGACTAAGCCTCAACTCC,
r-GAPDHF	5'-CCCTTC ATTGACCTCAACTA
r-GAPDHR	5'-GCCAGTGAGCTTCCCGTTCA

washed twice with PBS for 5 min each. The cells were covered with 1% horse serum in 10% bovine serum albumin (BSA) diluted with antibody diluent (DakoCytomation, CA, USA) and incubated for 30 min at room temperature. After they had been washed twice with PBS, the cells were gently covered with primary antibody directed against either CD68 (NeoMarkers Inc., Fremont, CA, USA) or TLR2 (Santa Cruz Biotechnology Inc., Europe) diluted in 1% BSA in antibody diluent, for 1 h at room temperature. The negative controls were covered with PBS. After the primary antibody reaction, the cells were washed three times with PBS, and then incubated for 45 min with biotin-linked anti-mouse/rabbit secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA) diluted in 1% BSA, followed by washing in PBS. Streptavidin/horseradish peroxidase (HRP; DakoCytomation) and AEC^+ substrate chromogen (DakoCytomation) were used for enzyme-mediated detection. The cells were counterstained with hematoxylin for 5 min, covered with cover slips, and observed under a microscope.

Western Blotting

Cells were lysed in lysis buffer composed of 1×PBS, 1 mM Na_3VO_4, 1% Igepal (Sigma) and 1×proteinase cocktail inhibitor (Roche, Germany). Protein concentrations were determined with the Lowry method. Whole protein extracts (20 μg) were separated electrophoretically on 10% SDS-PAGE, transferred onto poly(vinylidene difluoride) membrane, and blocked overnight at 4 °C with 2% BSA in TBST (12.5 mM Tris/HCl [pH 7.6], 137 mM NaCl, 0.1% Tween 20). Proteins were detected with rabbit anti-human-TLR2 antibody (Zymed, San Francisco, CA, USA) or mouse anti-human-tubulin antibody (Lab Vision Co., USA) and the corresponding HRP-conjugated secondary antibodies, followed by detection with the Enhanced Chemiluminescence Plus western blotting detection system (Amersham Biosciences, UK), according to the manufacturer's instructions. The membranes were washed extensively with TBST for 30 min (six washes, 5 min each) between each reaction.

Scanning System and Data Analysis

The DNA bands on the agarose gel were analyzed with a gel documentation system (BioLight, Bio-Profil ver. 2000, Vilber-Lourmat, France). The values are expressed as the ratio of the intensity of the target gene band to that of the control *Gapdh* gene. Semiquantified data on TLR expression levels were also collected as described above. Variance and *P* values were analyzed by Graphpad Prism ver. 4.0, 2003 (paired t test) for Windows. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Characterization of Synovial Fibroblasts

Morphologically, the synovial fibroblasts were spindle-shaped or polygonal in shape. The cells exhibited a histiocytic nature and were immunoreactive to anti-CD68 antibody. Basal expression of TLR2 was also detected (Fig. 1).

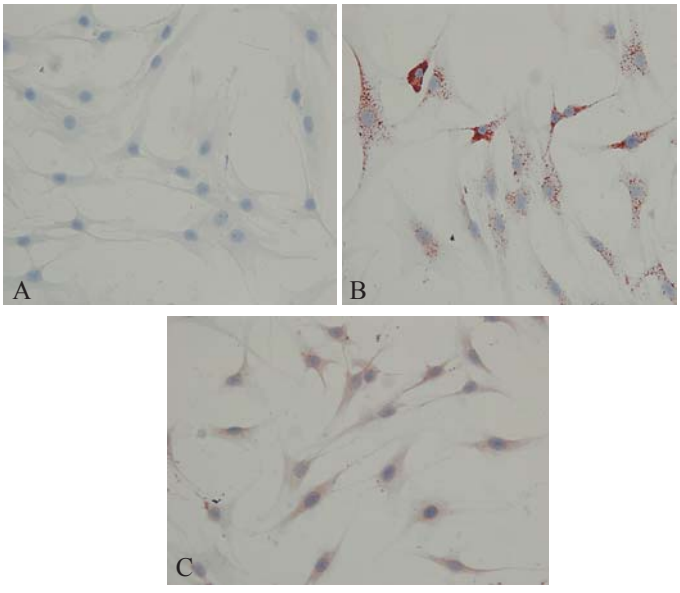

Fig.1 Immunocytochemistry for CD68 and TLR2 of synovial fibroblasts (400X). The synovial fibroblasts isolated from SD rat knee joints were cultured in 60 mm petri dish and were immunostained for CD68 and TLR2. (A) Negative immunostaining control which omitted primary antibody. (B) Synovial fibroblasts showed characteristic spindle or polyglonal shape with positive stain by anti-CD68 indicating histiocytic nature. (C) The expression of TLR2 was also detected.

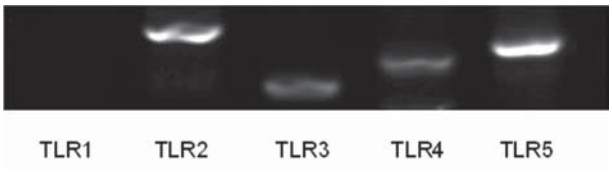

Fig. 2 The expression pattem of toll-like receptors 1-5 in synovial fibroblasts isolated from normal rat knee joint.

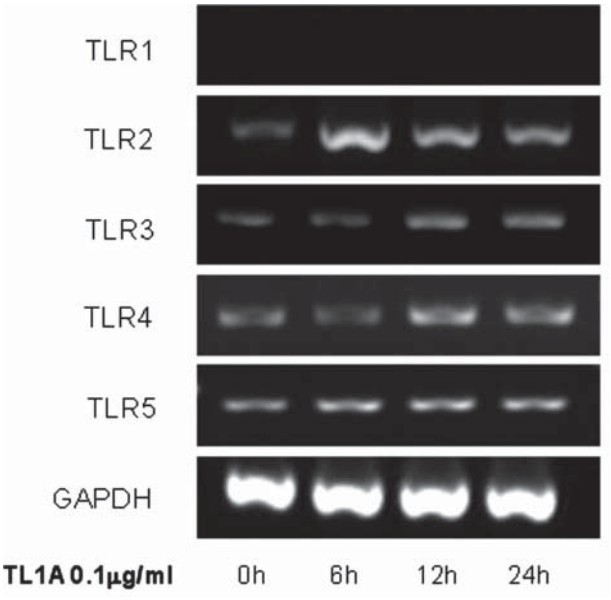

Fig. 3 A representative agarose gel for TLRs 1-5 expression with TL1A incubation of the synovial fibroblasts in a concentration of 0.1 μg/ml was shown. GAPDH served as internal control.

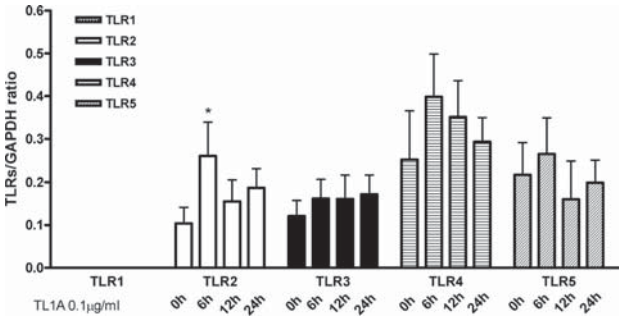

Fig. 4 The semiquantitative data of TLRs 1-5 expression (TLRs/GAPDH ratio) with TL1A stimulation were demonstrated. There was no expression of TLR1. TLR3, 4, and 5 showed no significant change in the experimental conditions tested. Only significant upregulation of TLR2 in 6 hours was identified (n=6, *p<0.05).

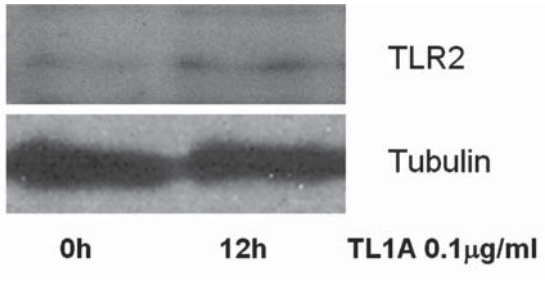

Fig. 5 Western blot of TLR2 protein expression with TL1A stimulation was demonstrated. Increased protein level of TLR2 after 12 hours of TL1A stimulation was identified. Tubulin served as internal control.

TLR2 was Upregulated by TL1A Stimulation

The expression patterns of TLRs 1-5 in normal rat synovial cells are shown in Fig. 2. There was no basal expression of *Tlr1* in the primary synovial fibroblasts. The concentration of TL1A used in this experiment had no cytotoxic effect, according to an MTT assay. The changes in the expression of *Tlr1*-*Tlr5* induced by TL1A stimulation in cultured rat synovial fibroblasts were then examined by RT-PCR. *Tlr1* was expressed in neither untreated cells nor TL1A-stimulated cells. The expression of *Tlr2* was significantly upregulated in response to TL1A stimulation at 6 h ($P < 0.05$). However, after 6 h, the expression of *Tlr2* decreased and had returned to approximately basal levels at 12 h and 24 h after TL1A treatment. The expression pattern of *Tlr5* was similar to that of *Tlr2* and no statistically significant difference was noted. The changes in *Tlr3* and *Tlr4* expression were also not statistically significant. The changes in the gene expression of TLRs 1-5 in rat synovial fibroblasts after TL1A stimulation in one representative test are summarized in Fig. 3. The semi-quantitative data are shown in Fig. 4. The regulation of protein expression was also examined by western blotting. Total cellular proteins were extracted after incubation with TL1A for 12 h and subjected to western blotting and immunodetection. TLR2 protein expression was upregulated by TL1A stimulation (Fig. 5).

DISCUSSION

The TNF family ligands and their corresponding receptors play important roles in immune regulation, including in lymphoid development, cell proliferation, differentiation, activation, and death[19]. Over the last few years, there have

been many additions to the ever-increasing TNF family ligands and their receptors. TL1A, also known as TNFSF15, was identified in human umbilical endothelial cells in 2002. Little TL1A expression is seen in human aortic endothelial cells, fibroblasts, B cells, T cells, NK cells, monocytes, or dendritic cells. Although the cells that express TL1A are generally immune-related cells, TL1A mRNA can be detected in many systemic organs, such as the kidney, prostate, placenta, stomach, intestine, lung, and thymus. TL1A acts as a costimulator of T-cell activation via its death-domain-containing receptor, DR3, causing increases in the secretion of IL-2, INF-γ and granulocyte-macrophage colony-stimulating factor, both in vitro and in vivo[1]. Other studies have also revealed that TL1A cooperates with IL-12 and IL-18 to increase IFN-γ secretion in human T cells, NK cells, and other immune cells[20,21]. Recently, it has been shown that DR3 is downregulated in rheumatoid arthritis relative to in the osteoarthritic synovium[22]. This phenomenon is thought to be associated with promoter hypermethylation and an apoptosis defect.

The inner intima of the synovium is now recognized to be a mixture of bone-marrow-derived macrophages (histiocytes) and specialized fibroblast-like cells[23]. It is generally accepted that the synovial macrophage-like inner layer cells retain several distinctive characteristics of monocyte/macrophage cells in terms of the immune responses[24]. The synovial fibroblasts used in this study also expressed CD68, a macrophage/histiocyte marker.

Toll-like receptors recognize conserved pathogen-associated molecules expressed by a wide spectrum of infectious microorganisms. Mammalian TLR4 is adapted to recognize lipopolysaccharide, the major component of the Gram-negative bacterial cell wall[25]. TLR2 recognizes a broad spectrum of pathogens, ranging from viruses even to parasites. The molecules recognized include peptidoglycan and lipoteichoic acids from Gram-positive bacteria[26,27], lipoproteins from Gram-negative bacteria[28], and lipopeptides from mycoplasma[29]. TLR2 also senses zymosan, a yeast cell wall component[30], and even glycolipids derived from *Trypanosoma cruzi*[31]. TLR2 can activate human monocytic cells induced by the measles virus and induces the secretion of proinflammatory cytokines such as IL-6[32]. TLR2 also mediates the inflammatory cytokine response to herpes simplex virus 1 and cytomegalovirus envelope proteins[33,34]. TLR3 recognizes dsRNA, and induces the secretion of type I interferons (IFNs) and proinflammatory cytokines[35]. Similar to TLR4, TLR3 also recognizes endogenous ligands. Endogenous heterologous mRNA released from necrotic cells stimulates human dendritic cells via TLRs and induces IFN-α secretion[36]. Natural killer cells express TLR3 and upregulate TLR3 mRNA upon stimulation with polyinosinic-polycytidylic acid/poly(I:C), a synthetic double-stranded polyribonucleotide that elicits immune responses analogous to those observed during viral infection. This significantly augments their cytotoxicity and induces the expression of proinflammatory cytokines, such as IL-6 and IL-8, as well as IFN-γ[37]. TLR5 is characterized by its ability to recognize flagellin from both Gram-positive and Gram-negative bacteria. Activated TLR5 induces the secretion of proinflammatory cytokines such as TNF and IL-8 via the activation of NF-κB[38].

In this study, we have demonstrated for the first time that recombinant TL1A protein can stimulate the upregulation of TLR2 expression in rat synovial cells. The most obvious increase in TLR2 mRNA was observed in the first 6 h, with statistical significance. This short-term effect might be associated with an acute response to synovial damage, in a reaction to synovial matrix degradation[18]. Like other toll-like receptors, we found no statistically significant increase in the expression of TLR3-5 after stimulation with TL1A similar to that shown for TLR2. The possible relationships between TL1A, DR3, and TLR2 might be such that the binding of TL1A and DR3 initiates the activation of NF-κB to upregulate TLR2 gene and protein expression, thus inducing the inflammatory response. However, the significance of the upregulation of TLR2 in arthritis requires further clarification. Because TL1A is a member of the TNF superfamily and induces the activation of substantial downstream signaling, our findings suggest that TL1A plays a role in articular inflammation.

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