Effect of a 30 kDa Fibronectin Fragment on GRO Production by Human Temporomandibular Joint Synovial Fibroblasts

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Introduction

The human temporomandibular joint (TMJ) is a bilateral synovial joint formed from the upper temporal bone and mandible. The synovium exists at the surface of the soft connective tissue in the intraarticular structures of the TMJ, excluding the cartilage of the articular eminence, mandibular fossa and head of mandible, and the articular disc (1). The lining layer of synovial tissue is populated by fibroblast-like cells and macrophage-like cells (2, 3). The loose connective tissue of the synovial sublining contains blood vessel sublining fibroblasts and leukocytes. These cells in the synovium play important roles in homeostasis and the progression of pathologic conditions (1). Synovitis, which is characterized by chronic inflammatory changes, such as growth of small new blood vessels and infiltration of inflammatory cells, often accompanies disk displacement (DD)/internal derangement (ID) and osteoarthritis (OA) of the temporomandibular joint (TMJ) and may cause TMJ pain (4, 5). Most inflammatory conditions of the TMJ are non-infectious inflammation, and excessive mechanical stress is considered one reason for the occurrence of inflammation in the TMJ (6). However, pro-inflammatory substances and the mechanisms causing inflammation in the TMJ are poorly understood.
Recently, it has been reported that degradation products of extracellular matrix are induced during chronic inflammation (7). Fibronectin is a typical extracellular matrix protein and has been detected as a component of various tissues including synovial tissue. Fibronectin contains binding sites for fibronectin, collagen, heparin, fibrin, and integrins in the cell surface membrane, and is a constituent of the polymeric aggregate of the extracellular matrix (7). Fibronectin is 450 kDa dimeric glycoprotein that is degraded to 30 kDa, 45 kDa, and 120 kDa fibronectin fragment by extracellular matrix degrading enzymes during metabolic and inflammatory processes (7). High concentrations of fibronectin fragments have been detected in the synovial fluid of OA patients and have been reported to be an exacerbating factor for arthritis (8). These fibronectin fragments have been reported to induce the expression pro-inflammatory cytokines and degrading enzymes (9). It has also been reported that the 30 kDa fibronectin fragment has the strongest effect on cartilage among the fibronectin fragments (10, 11).

This study aimed to elucidate the pathogenic mechanism of temporomandibular joint inflammation, and the effect of the 30 kDa fibronectin fragment on growth-related oncogene (GRO), a subfamily of chemokines, gene expression in synovial cells was determined. Additionally, the signal transduction pathway regulating GRO expression in response to the 30 kDa fibronectin fragment was examined.

**Materials and Methods**

**Isolation and Culture of Synovial Fibroblasts**

Human synovial fibroblasts were isolated using the outgrowth method with synovial tissue samples obtained from two patients (TMJ1, female, age 26 years and TMJ2, male, age 48 years) with internal derangement (ID)/disc derangement (DD) undergoing TMJ arthroscopy. All study participants provided informed consent for the arthroscopy surgery and for the use of their tissue specimens for research purposes.

In brief, synovial tissue samples were washed extensively with phosphate-buffered saline (PBS), minced by scalpel and then cultured under a sterilized glass coverslip in a 35-mm tissue culture dish with Ham’s F12 (Wako, Osaka, Japan) supplemented with 100 µg/mL penicillin G (Meiji, Tokyo, Japan), 100 µg/mL kanamycin sulfate (Meiji), and 250 ng/mL fungizone (Gibco, Grand Island, NY, USA), 20% fetal bovine serum (FBS) (Cell Culture Technologies, Gravesano, Switzerland) in the presence of 95% air and 5% CO2 at 37°C. The medium was changed every 3 days. Confluent synovial fibroblasts were detached with 0.025% trypsin (Gibco) and 0.02% EDTA in PBS, and then subcultured in Ham’s F12 supplemented with 10% FBS and antibiotics. Synovial fibroblasts obtained from passages six to nine were used in the experiments described herein.

The experimentation with synovial fibroblasts from human TMJ patients was performed according to the guidelines established by the Institutional Review Board of Ni- hon University School of Dentistry at Matsudo (Ethics Committee Registration Numbers: EC17–15–039).

**Total RNA Extraction**

Synovial fibroblasts were seeded at a density of 1×10^6 cells per 100-mm dish in Ham’s F12 medium containing 10% FBS and antibiotics. Confluent-stage synovial fibroblasts were cultured in medium containing 2% FBS for 24 h before treatment. Synovial fibroblasts were treated with 300 nM 30 kDa fibronectin fragment (Merck KGaA, Darmstadt, Germany) for various durations, and cells in the control group received no treatment. Total RNA were isolated from synovial fibroblasts using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according the manufacturer’s instruction, and then stored at −80 °C until use.

**Real-Time PCR**

Complementary DNA (cDNA) was synthesized from total RNA using a GeneAmp RNA PCR Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Semi-quantitative real-time PCR (qPCR) analysis was performed with a TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific Inc.), predesigned primers for GRO-α, -β, -γ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Thermo Fisher Scientific Inc.), and 2 µL of cDNA using a Quantstudio 6 (Thermo Fisher Scientific Inc.) with pre-heating at 95 °C for 20 s, followed by 40 cycles of: 95 °C for 1 s, and 60 °C for 20 s. The relative expression ratios were calculated using the 2^{-ΔΔCT} method (12). Three independent experiments were performed in triplicate.

**Quantification of GRO-α**

Synovial fibroblasts were seeded at 5×10^4 cells per
well in 24-well plates with Ham’s F12 containing 10% FBS and antibodies. Confluent-stage cells were cultured for 24 h in the same medium containing 2% FBS, and then cells were culture in serum free medium in the presence or absence of 300 nM 30 kDa fibronectin fragment for the durations indicated in the results. The culture supernatants were collected and stored at -80 °C until use. The concentration of GRO-α protein in the supernatants was measured using an ELISA kit (R&D Systems, McKinley, MN, USA) according to the manufacturer’s protocol.

**Signaling Inhibitor Experiments**

Synovial fibroblasts were plated at a density of $5 \times 10^4$ cells per well in 24 well plates with Ham’s F12 medium containing 10% FBS. Confluent-stage cells were cultured for 24 h in medium containing 2% FBS. The cells were pretreated with PD98059 (ERK1/2 inhibitor, 40 µM; Enzo Life Science, Farmingdale, NY, USA), SP600125 (JNK1/2 inhibitor, 10 µM; Enzo Life Science), SB203580 (p38 inhibitor, 10 µM; Enzo Life Science), or ammonium pyrrolidinedithiocarbamate (APDC) (NFκB inhibitor, 10 µM; Sigma-Aldrich) for 15 min, or with an Interleukin-1 Receptor-Associated-Kinase-1 (IRAK-1) inhibitor (20 µM; Merck KGaA), LY294002 (phosphoinositide 3-kinase (PI3K) inhibitor, 20 µM; Merck KGaA), (5z)-7-oxozeaenol (transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) inhibitor, 1 µM; Merck KGaA), and PS-1145 (inhibitor of the NFκB kinase (IKK) β subunit (IKK β) inhibitor, 10 µL; Cayman Chemical, Ann Arbor, MI, USA) for 30 min, followed by incubation with 30 kDa fibronectin fragment. After 24 h, the culture supernatants were collected and stored at -80 °C until use. The inhibitor effect was calculated as 100 - [(GRO-α production with 30 kDa fibronectin fragment in the presence of the inhibitor) / (GRO-α production with 30 kDa fibronectin fragment) × 100]. GRO-α levels in the conditioned medium were measured using an ELISA kit (R&D systems).

**Statistical Analysis**

The data are expressed as means ± standard deviations (SD) and were analyzed using one-way analysis of variance (ANOVA). Post hoc analyses were carried out using the Student-Newman-Keuls (SNK) Multiple Comparison Test. Values of $P < 0.05$ and $P < 0.01$ were considered to indicate a significant difference.

**Results**

**Gene expression analysis of GRO-α, -β, and -γ**

The expression of the GRO-α, -β, and -γ genes was examined in synovial fibroblasts treated with 30 kDa fibronectin fragment for 6, 12, and 24 h. The gene expression of GRO-α was significantly higher in synovial fibroblasts treated with 30 kDa fibronectin fragment for 6 h to 24 h compared to untreated controls, with peak expression observed following 6 h treatment. In addition, the expression of GRO-β and GRO-γ was significantly higher in synovial fibroblasts treated with the 30 kDa fibronectin fragment for 24 h, relative to untreated controls (Fig. 1).

**Effect of 30 kDa fibronectin GRO-α protein production**

GRO-α protein production was examined in synovial fibroblasts treated with the 30 kDa fibronectin fragment. GRO-α protein production was significantly increased in synovial fibroblasts treated with the 30 kDa fibronectin fragment for 24 h to 48 h, relative to untreated controls (Fig. 2).

**Effects of signaling inhibitors on GRO-α production**

The effects of NFκB and MAPK signaling pathways inhibitors on 30 kDa fibronectin fragment-induced GRO-α production in synovial fibroblasts were examined to evaluate their role in mediating the process. The induction of GRO-α by the 30 kDa fibronectin fragment was decreased in synovial fibroblasts by pretreatment with SP600125 (an JNK inhibitor), SB203580 (a P38 inhibitor), and APDC (an NFκB inhibitor). Notably, GRO-α production was inhibited by 99.8% by APDC (Fig. 3). In contrast, GRO-α production was increased by PD98059 (an ERK1/2 inhibitor). Furthermore, 30 kDa fibronectin fragment-induced production of GRO-α by was significantly decreased in synovial fibroblasts by pretreatment with an IRAK-1/4 inhibitor, LY294002 (a P13K inhibitor), (5z)-7-oxozeaenol (a TAK1 inhibitor), and PS-1145 (an IKKβ inhibitor) (Fig. 4).

**Discussion**

In this study, we investigated whether the 30 kDa fibronectin fragment regulates the expression of GRO chemokines. We previously reported that GRO-α expression was significantly increased by 120 kDa fibronectin fragment stimulation compared to 30 kDa fibronectin.
fragment (13). Previous studies, however, have shown that 30 kDa fibronectin fragment is the most potent molecule by up-regulating catabolic cytokines and MMP family in articular chondrocytes and macrophage. Therefore, we have to examined the effect of the 30 kDa fibronectin fragment on GRO chemokine expression in synovial cells from TMJ. The GRO chemokines (GRO-α, -β, and -γ) belong to the CXC chemokine subfamily and are also called CXCL1, CXCL2, and CXCL3, respectively (14, 15). GRO-α, -β, and -γ exhibit high binding affinity for the CXCR2 chemokine receptor, which is expressed on neutrophils, eosinophil, monocyte/macrophage, and oligodendrocytes, among other cell types. These chemokines contain the sequence Glu-Leu-Arg (The ‘ELR’ motif), which has an angiogenic effect (16–19). The GRO chemokines may lead to recruitment of inflammatory cells expressing CXCR2 as well as the development of new small vessels in synovial tissues (17, 20). We examined the expression kinetics of GRO chemokine genes using real-time PCR. The expression of GRO-α was up-regulated in synovial fibroblasts following treatment with the 30 kDa fibronectin fragment for up to 24 hours, with peak expression observed at 6 hours. Additionally, the expression of the GRO-β and GRO-γ genes was significantly higher in synovial fibroblasts treated with the 30 kDa fibronectin fragment for 24 hours. These data suggest that the 30 kDa fibronectin fragment may be associated with the progression of synovial inflammation in TMJ.

GRO-α is a representative chemokine and is highly expressed in synovial fibroblasts treated with 30 kDa fibronectin fragment for 6 h compared to GRO-β and -γ. Furthermore, the protein concentration of GRO-α in media from synovial fibroblasts was determined. GRO-α protein production also increased in synovial fibroblasts treated with 30 kDa fibronectin fragment. It has been reported that GRO-α plays an important role in rheumatoid arthritis and OA (18, 21, 22). GRO-α binds CXCR2 and is strongly associated with neutrophil attraction and stimulating neovascularization in the rheumatoid synovium (23). Recently, GRO-α was found to promote both nociception and central sensitization via its main receptor CXCR2, which is also expressed in neural cells (24). In addition, GRO-α was found to contribute to hyperalgesia by stimulating the release of sympathetic amines and prostaglandins within peripheral inflammatory sites (25). Increases in GRO-α could possibly cause leukocyte migration and angiogenesis, as well as pathological pain in inflammatory conditions.

We also investigated the signaling pathway involved in the regulation of GRO-α production in synovial fibroblasts following 30 kDa fibronectin fragment treatment. 30 kDa fibronectin fragment-stimulated GRO-α production was examined in the presence of MAPK and NFκB inhibitors because GRO-α is known to be regulated by IL-1β and TNF-α through MAPK and NFκB (26, 27). 30 kDa fibronectin fragment-stimulated GRO-α production was decreased by SP600125, SB203580 and APDC. It was suggested that this effect is mediated by activation of the
JNK1/2, P38 MAPK and NFκB pathways. GRO-α protein production was inhibited by 99.8% by APDC, suggesting that GRO-α production was mainly regulated by NFκB activation. In order to examine the NFκB activation pathway in more detail, we used an inhibitor that acts on the NFκB activation pathway. 30 kDa fibronectin fragment stimulated GRO-α production was significantly reduced by the IRAK-1/4 inhibitors, LY294002, (5z)-7-oxozeaenol, and PS-1145. These data indicated that 30 kDa fibronectin fragment signaling is mediated by NFκB. IRAK is rapidly recruited to the receptor by the adaptor MyD88 upon IL-1R/TLR (Toll-Like Receptor) binding, which leads to further downstream signaling. Since GRO-α production was inhibited by an IRAK-1/4 inhibitor, 30 kDa fibronectin fragment signaling may involve TLRs.

TLR is a membrane protein that recognizes common pathogens and is known as a pathogen receptor or pathogen sensor (28, 29). Humans express multiple TLRs (TLR1 to TLR10), each of which specifically recognize different pathogen components (29). TLR2/4 is well-known as a receptor that recognizes lipoprotein, which is a bacterial membrane component. It can also recognize the products of extracellular matrix degradation of the host and activate transcription factors such as NFκB (28).

This study demonstrated that the 30 kDa fibronectin fragment up-regulates the expression of GRO chemokines mediated by the NFκB pathway. GRO, a CXCL that contains the ELR motif, can potentially cause leukocytes migration and angiogenesis, as well as pathological pain during inflammatory conditions. Therefore, it was suggested that synovial fibroblasts may increase GRO-α production by activating NFκB by TLR-TAK1-IKK following exposure to the 30 kDa fibronectin fragment.
Conclusions
The 30 kDa fibronectin fragment induces the mRNA expressions of GRO-α, -β, and -γ, as well as the protein production of GRO-α in synovial fibroblasts. The 30 kDa fibronectin fragment appears to transduce signals for GRO-α production through activation of the NFκB pathways. Our data contributes insight into the cellular mechanisms by which the 30 kDa fibronectin fragment activates synovial fibroblast during inflammation of the TMJ.

Conflict of Interest
The authors declare that there is no conflict of interests regarding the publication of this paper.

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