CXCL1, 2, and 3 Expression Stimulated by Fibronectin Fragments in Synovial Fibroblasts from Human Temporomandibular Joints

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Abstract

Fibronectin, which is an extracellular matrix component, is broken down by several matrix degradation enzymes. Fibronectin-fragments (FN-fs), with molecular sizes of 29-200 kDa, are present at high levels in synovial fluid from osteoarthritis patients. The roles of FN-fs in temporomandibular joint (TMJ) arthritides were investigated. The 120 kDa FN-f was the most potent inducer of the expression and production of CXCL1, also called growth-related oncogene α (GROα), compared to 30 kDa and 45 kDa FN-fs in synovial fibroblasts (SF) from human temporomandibular joints. Therefore, further investigation was carried out using the 120 kDa FN-f. Microarray analysis indicated that 120 kDa FN-f treatment of SF upregulated the expressions of CXCL1 and chemokines. Real-time PCR analysis showed that the gene expressions of CXCL1, CXCL2, and CXCL3 were significantly higher in 120 kDa FN-f-treated SF than in untreated controls. CXCL1 protein production was increased by the 120 kDa FN-f in a time-dependent manner. Furthermore, signal inhibitor experiments indicated that 120 kDa FN-f-mediated induction of CXCL1 was transduced via activation of NFκB signaling. These results suggest that the 120 kDa FN-f is associated with the inflammatory progression of TMJ arthritides.

Introduction

Cytokines are mediators of cell-cell communication that play important roles in immune and inflammatory responses, wound healing, hematopoiesis, and maintenance of normal homeostasis. Chronic inflammatory diseases are often characterized by cyclic phases of cellular infiltration and tissue breakdown, which appear to be initiated and/or maintained partly by cytokine activities. Chemokines are low-molecular weight cytokines that are involved in regulation of leukocyte accumulation and activation in inflammatory tissues. All chemokines share certain primary structural similarities, including a conserved 4-cysteine motif. Four chemokine subfamilies have been classified based on the positions of certain cysteine residues (CXC, CC, C, and CX3C) (1). CXCL1, also called growth-related oncogene α (GROα), is reported to exert proinflammatory effects, mainly via effects on neutrophils (1, 2).

The synovial membrane of the human temporomandibular joint (TMJ) covers all the intra articular structures except the articular cartilage of eminence, mandibular fossa and head of mandible, and the articular disc (3). And the membrane are composed the synovial fibroblasts (SF). Synovitis, which is often seen with pathological intracapsular conditions, such as disk displacement, internal derangement, and osteoarthritis (OA) of the TMJ, is characterized by chronic inflammatory changes (4, 5). Several mediators of inflammation such as chemokines (6-8) were detected in synovial fluid and tissue with pathological intracapsular conditions. This inflammatory cascade also alters the extracellular matrix (ECM) by depleting matrix substances, such as collagen and fibronectin (FN). It has been shown that FN and collagen fibrils show significant changes within the degenerative disc.

FN, which is an ECM glycoprotein and contains collagen/gelatin, fibrin, heparin, and cell-binding domains, is present
as insoluble cellular FN in the ECM and in a soluble form in blood plasma. FN is localized mainly in the matrix of the surface zone of synovial tissue and cartilage, and an increase in its expression is associated with tissue remodeling and repair (9, 10). FN degradation is a central event leading to joint destruction in many arthritic conditions, including rheumatoid arthritis, OA, and septic arthritis (11). FN-fragments (FN-fs) result from degradation of the FN matrix, mediated by matrix metalloproteinase (MMP)-1, -3, and -13 and by the aggrecanases, such as a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS)-4 and -5 (12, 13). The FN-fs, with molecular sizes of 29-200 kDa, are present at high levels in synovial fluid from OA patients (14), and they induce the expressions of MMP and pro-inflammatory cytokines directly via cell surface receptors. It has been reported in an in vivo experiment that FN-fs induced a progressive degenerative process when injected into rabbit lumbar discs (15).

In the present study, the gene expression profiles and production of CXCL1 in SF when treated with FN-fs were examined. We used to 30 kDa FN-f which contained heparin binding domain, 45 kDa FN-f which contained gelatin binding domain, and 120 kDa FN-f which contained cell binding domain. Furthermore, we examined the effect of kinase inhibitors on the production of CXCL1 in FN-f-stimulated SF. The aim of this study was to investigate the roles of FN-fs in the pathogenesis of TMJ arthritides.

Materials and Methods

Isolation and Culture of SF

Human synovial tissue was obtained from patients who underwent arthroscopy for internal derangement: TMJ1, female, age 34 years, used for the oligonucleotide microarray analysis, real-time PCR, and ELISA; and TMJ2, female, age 26 years, used for ELISA. All patients provided informed consent for the surgery and for the use of their tissue specimens for research purposes. The experimentation with SF was performed according to the guidelines established by the Institutional Review Board of Nihon University School of Dentistry at Matsudo (Ethics Committee Registration Numbers: EC10-037 and EC15-039).

Human SF isolated from the synovial tissues of patients with pathological intracapsular conditions of the TMJ were prepared using the outgrowth method previously reported by Ogura et al (16). In brief, synovial tissue samples were washed with phosphate-buffered saline (PBS), minced, placed in a 35-mm tissue culture dish, and covered with a sterilized glass coverslip. The culture medium used was Ham’s F12 (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Technologies, Gravesano, Switzerland), 100 μg/mL penicillin G (Meiji, Tokyo, Japan), 100 μg/mL kanamycin sulfate (Meiji), and 250 ng/mL Fungizone (Gibco, Grand Island, NY, USA). The medium was changed every 3 days. For the experiments, SF obtained from passages six to nine were used.

Enzyme-Linked Immunosorbent Assay (ELISA)

SF were plated at a density of 5 × 10⁴ cells per well in 24-well plates with Ham’s F12 containing 10% FBS. Confluent cells were cultured for 24 h in the same medium containing 2% FBS. After incubation with or without 150 nM 30 kDa, 45 kDa (Merck KGaA, Darmstadt, Germany), and 120 kDa FN-f (Sigma-Aldrich, St. Louis, MO, USA) for the appropriate length of time in the same medium without FBS, culture supernatants were collected and stored at −80°C until use. The kinetics of CXCL1 protein production were examined in control samples and in SF incubated with 120 kDa FN-f for various lengths of time. Level of the CXCL1 in the conditioned medium were measured using an ELISA kit (R&D Systems, McKinley, MN, USA), according to the manufacturer’s protocol.

Total RNA Extraction

Confluent-stage SF were cultured in medium containing 2% FBS for 24 h and then stimulated in serum-free medium with or without 150 nM 30 kDa, 45 kDa, and 120 kDa FN-fs for various lengths of time. Total RNA from SF was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and then stored at −80°C until use.

DNA Microarray Analysis

Total RNA samples from SF treated with 120 kDa FN-f for 4 h and from untreated control samples were profiled on a SurePrint G3 Human Gene Expression 8×60K v3 Microarray (Agilent Technologies Inc., Santa Clara, CA, USA), according to the manufacturer’s protocols. The array was scanned using an Agilent DNA Microarray Scanner. Gene expression analysis of the DNA microarray was performed using GeneSpring GX software (Agilent). Data were normalized using raw data from each array as a reference. Changes in gene expressions were determined by comparing the normalized intensities for untreated cells
with those of 120 FN-f-treated cells.

**Real-Time Polymerase Chain Reaction (Real-Time PCR)**
Complementary DNA was synthesized from total RNA using a GeneAmp RNA PCR Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Real-time PCR was performed using a TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific Inc.). The PCR mixture (20 μL) contained 2 μL of cDNA. Amplification was performed using a Quantstudio 6 (Thermo Fisher Scientific Inc.). The genes analyzed in this study were examined for their relative expressions to their respective controls using the $^{\Delta \Delta C_{T}}$ method(17).

**Inhibition of IRAK 1/4, PI3K, TAK1, and IKKβ**
SF were plated at a density of $5 \times 10^5$ cells per well in 24-well plates with Ham’s F12 medium containing 10% FBS. Confluent cells were cultured for 24 h in medium containing 2% FBS. The cells were not pretreated (control) or 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-1/4 (IRAK-1/4) inhibitor (20 μM; Merck KGaA), (5z)-7-oxozaenal (transforming growth factor-β-activated kinase 1 (TAK1) inhibitor, 1 μM; Merck KGaA), and PS-1145 (inhibitor of the NFXB kinase β subunit (IKKβ) inhibitor, 10 μL; Cayman Chemical, Ann Arbor, MI, USA) for 30 min, followed by incubation with 120 kDa FN-f. After 24 h, the culture supernatants were collected and stored at −80°C until use. The inhibitor effect was calculated as $100 - \left( \frac{\text{CXCL1 production with 120 kDa FN-f in the presence of the inhibitor}}{\text{CXCL1 production with 120 kDa FN-f}} \right) \times 100$. The CXCL1 levels in the conditioned medium were measured using an ELISA kit (R&D systems).

**Results**

**Effects of 30 kDa, 45 kDa, and 120 kDa FN-f**
The ability of FN-fs to induce an inflammatory response in a SF model was first tested. SF were stimulated for 48 h with the FN-fs (30 kDa, 45 kDa, and 120 kDa) to examine the secretion of CXCL1 protein. The 120 kDa FN-f was the strongest inducer of CXCL1 expression in SF (Fig. 1).

**Microarray Analysis of SF Treated with the 120 kDa FN-f**
The gene expression profiles of SF that were treated with or without the 120 kDa FN-f were then analyzed to determine the mechanisms underlying its effects in pathological conditions of the TMJ. Of the 58,341 genes on the DNA microarray, 23,121 genes were expressed in SF (data not shown), and the expressions of these genes were compared between untreated control cells and the 120 kDa FN-f-treated cells. Genes that showed a greater than 2-fold difference in expression between the 120 kDa FN-f-treated and control cells were further analyzed. A total of 141 genes showed greater than 2-fold changes in expression with the 120 kDa FN-f treatment; the expressions of 102 of these genes were upregulated, and the expressions of 39 of these genes were downregulated (data not shown). Table 1 lists the five most upregulated genes by the 120 kDa FN-f. CXCL1 (GROα) was found to be the 2nd most upregulated gene by the 120 kDa FN-f. In addition, other GRO members were found the top five upregulated genes: CXCL2 (GROβ; rank 5) and CXCL3 (GROγ; rank 3).

![Fig. 1. Effects of FN-fs on CXCL1 protein production by SF. SF were treated with 150 nM 30 kDa, 45 kDa, and 120 kDa FN-fs. The CXCL1 protein levels in the conditioned medium were then assayed using ELISA following culture of the cells with or without 30 kDa, 45 kDa, and 120 kDa FN-fs for 48 h. Data are shown as means±SD (n=4); * P < 0.01.](image-url)
Table 1. Top five upregulated genes by 120 kDa FN-f stimulation for 4 h

| Rank | Gene Symbol | Fold Change | Genbank Accession | Gene Name                        |
|------|-------------|-------------|-------------------|----------------------------------|
| 1    | MT1G        | 1131.4      | NM_001301267      | metallothionein 1G               |
| 2    | CXCL1       | 55.5        | NM_001511         | chemokine (C-X-C motif) ligand 1  |
| 3    | CXCL3       | 45.8        | NM_002090         | chemokine (C-X-C motif) ligand 3  |
| 4    | MT1F        | 43.5        | NM_005949         | metallothionein 1F               |
| 5    | CXCL2       | 43.3        | NM_002089         | chemokine (C-X-C motif) ligand 2  |

![Graphs](image)

Fig. 2  Time course of 120 kDa FN-f induced the mRNA expressions of CXC-type chemokines in SF. The effects of 120 kDa FN-f on (a) CXCL1, (b) CXCL2, and (c) CXCL3 gene expressions in SF were analyzed using real-time PCR following culture of the cells with or without 150 nM 120 kDa FN-f for 4, 8, or 12 h. Data are shown as means±SD(n=3); * P < 0.01.

**Time Course of 120 kDa FN-f-Induced Gene Expression in SF**

The microarray data indicated that the expressions of chemokines were upregulated in SF by the 120 kDa FN-f treatment for 4 h. Therefore, the time course of the 120 kDa FN-f induction of the expression of these genes in SF was examined next. Their expressions were analyzed using real-time PCR following incubation of SF with or without the 120 kDa FN-f for 4, 8, or 12 h. The gene expressions of CXCL1, CXCL2, and CXCL3 were significantly higher in SF treated with the 120 kDa FN-f for 4 h to 12 h than in untreated controls (Fig. 2).

**CXCL1 Protein Production in SF Treated with 120 kDa FN-f**

Next, CXCL1 protein production in SF treated with the 120 kDa FN-f was examined. The 120 kDa FN-f increased the CXCL1 levels in the conditioned media of SF in a time-dependent manner, although there was no significant difference between the cells treated for 6 h with the 120 kDa FN-f and the untreated controls; it was increased by 120 kDa FN-f from 12 h to 48 h (Fig. 3).

**Effects of Signaling Inhibitors on FN-f-Induced CXCL1 Production by SF**

Therefore, the effects of inhibitors of the NFκB and the MAPK signaling pathways on 120 kDa FN-f-induced CXCL1 production by SF were examined. A previous report showed MAPK activation by the FN-f(18). The induction of CXCL1 by the 120 kDa FN-f was decreased in SF by pretreatment with SP600125 (a JNK inhibitor), SB203580 (a p38 inhibitor), and APDC (an NFκB inhibitor); in contrast, its production...
was increased by PD98059 (an ERK1/2 inhibitor). CXCL1 production was inhibited by 52.6% by SP600125, by 48.7% by SB203580, and by 96.8% by APDC (Fig. 4).

Furthermore, the effects of several inhibitors of the NFκB signaling pathway on 120 kDa FN-f induced CXCL1 production by SF were examined. The induction of CXCL1 by 120 kDa FN-f was decreased in SF by pretreatment with an IRAK-1/4 inhibitor, LY294002 (a PI3K inhibitor), (5z)-7-oxozaeanol (a TAK1 inhibitor), and PS-1145 (an IKKβ inhibitor). CXCL1 production was inhibited by 77.6% by an IRAK-1/4 inhibitor, by 65.6% by LY294002, by 98.8% by (5z)-7-oxozaeanol, and by 23.628% by PS-1145 (Fig. 5).

Discussion

CXCL1 is also known as GROα in humans, keratinocyte-derived chemokine (KC) in mice, and cytokine-induced neutrophil chemoattractant type-1 (CINC-1) in rats, where it acts similarly to CXCL8 (interleukin-8) (19–21). CXCL1 is a chemotactic cytokine produced during inflammation and is important for attracting polymorphonuclear cells towards inflammatory sites (22). It has been found that CXCL1 plays an important role in the entry of neutrophils into the rheumatoid joint (23). Therefore, CXCL1 expression was examined to investigate the inflammatory effects of these FN-fs for SF. The 120 kDa FN-f was the most potent inducer of CXCL1 production compared to the 30 kDa and 45 kDa FN-fs in SF from human TMJs.
FN, a 450 kD adhesive glycoprotein made of two subunits linked together by a pair of disulfide bonds at the C-terminus, is found in the ECM of many human tissues (24). This glycoprotein is a substrate for multiple proteinases produced by the host and bacteria (9, 25), and it is then degraded to smaller fragments of FN (30, 45, and 120 kDa). The 120 kDa FN-f contains the central cell-binding domain (26). Previous studies have suggested that FN-fs have different effects on the modulation of inflammatory mediators depending on their size. Based on this result, the changes in gene expressions in SF treated with or without the 120 kDa FN-f were profiled.

Using a high-throughput DNA microarray, a total of 141 genes showed a greater than 2-fold difference in expression intensity between untreated controls and the 120 kDa FN-f-treated SF (data not shown). The upregulated genes contained the numerous chemokine superfamily members that are involved in regulation of leukocyte accumulation and activation in inflammatory tissues. All GRO members were among the top five upregulated genes; CXCL1 (GROα; rank 2), CXCL2 (GROβ; rank 5), and CXCL3 (GROγ; rank 3).

The expression of GRO members was upregulated in SF treated with the 120 kDa FN-f for 4 h to 12 h, and CXCL1 protein production was increased. The 120 kDa FN-f may therefore have a role in inducing inflammation, such as in leukocyte attraction and peripheral pain in TMJ arthritides.

CXCL1, 2, and 3 contain the sequence Glu-Leu-Arg (the ‘ELR’ motif) and are thought to promote angiogenesis (27). Thus, GRO members may lead to both recruitment of inflammatory cells and new small vessels in synovial tissues via CXCR2 expressed on neutrophils. Recent studies have demonstrated that CXCL1 also has a significant role in pain modulation in both peripheral and central systems, because neural cells expressed CXCR1 and CXCR2, which are functional receptors for ELR chemokines such as CXCL1 (19, 28). In addition, CXCL1 contributes to hyperalgesia by stimulating the release of sympathetic amines and prostaglandins within the peripheral inflammatory site (29).

To investigate 120 kDa FN-f-mediated CXCL1 production, the effects of inhibitors of MAPK and NFκB signaling on CXCL1 production were examined in SF treated with the 120 kDa FN-f. The FN-fs are known to increase cytokine and chemokine expressions through MAPK activation and the NFκB-dependent pathway (18, 30). The present data suggest that the induction of CXCL1 production by the 120 kDa FN-f occurs through p38 MAPK, JNK, and NFκB activation in SF derived from TMJs. CXCL1 production was inhibited 96% by ADPC (an NFκB inhibitor) treatment in the 120 kDa FN-f-treated cells. Next, the effects of several inhibitors on CXCL1 production through NFκB activation signaling were examined in SF treated with the 120 kDa FN-f. The 120 kDa FN-f-induced CXCL1 production was inhibited by an IRAK-1/4 inhibitor, LY294002 (a PI3K inhibitor), (5z)-7-oxozaeanol (a TAK1 inhibitor), and PS-1145 (an IKK3 inhibitor). These data suggest that 120 kDa FN-f signal transduction is mediated by NFκB, which activated IL-1 signal transduction.

IRAK is an essential downstream signaling element of both TLRs and IL-1 receptor (IL-1R) pathways. The TLRs were found to be expressed in SF by microarray data. TLR3 and TLR4 were upregulated in SF by the 120 kDa FN-f. In contrast, the levels of IL-1α and IL-1β proteins in the conditioned medium were below detection levels in both cells treated with and without the 120 kDa FN-f using an ELISA (data not shown). Since CXCL1 production was inhibited by an IRAK-1/4 inhibitor, the 120 kDa FN-f signal may be related to TLRs.

The 120 kDa FN-f, which contains the cell-binding RGD sequence, may bind to integrin receptors (31). Integrin-mediated cytokine production is promoted by signaling through p38 MAPK or JNK activation and the NFκB-dependent pathway (32). In this study, CXCL1 production induced by the 120 kDa FN-f was reduced by the inhibitors for p38 MAPK, JNK and NFκB. The SF from human TMJs expressed integrins such as α5, αv, β1, β3, β5, and β8 (data not shown). Thus, CXCL1 production may be induced by the 120 kDa FN-f through not only TLRs receptors, but also integrin.

In the present study, the findings demonstrated 120 kDa FN-f upregulation of the expression of GRO members that is mediated by the NFκB pathway. This may occur through TLRs and integrin receptors and is important in promoting GRO attraction to and invasion of the synovial tissue in TMJ arthritides. Thus, this FN-f cascade is likely to contribute to the promotion of and increase the inflammatory condition and to have a role in pain modulation in TMJ arthritides.

Conclusions

The 120 kDa FN-f induces the mRNA expressions of CXCL1, 2, and 3, as well as the protein production of CXCL1 in SF. The FN-f appears to transduce signals for CXCL1 production via activation of NFκB pathways. The present
data provide insights into the cellular mechanisms by which the FN-f participates in the activation of SF in the inflamed TMJ.

Conflict of Interests

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

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