Stimulation of Gro-α Gene Expression and Production by IL-1β in Synovial Fibroblasts from Human Temporomandibular Joints

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Abstract
Elevated levels of IL-1β have been found in synovial fluid from patients with temporomandibular disorders (TMD) and are thought to be involved in pain and progression of TMD. Synovial cells in the temporomandibular joint have important roles in progression of synovitis; however, their biological roles are poorly understood. In this study, we examined the effect of IL-1β on gene expression in synovial fibroblast cultures from 5 different patients. Gene expression was monitored by Affymetrix GeneChip analysis and real-time PCR. Gro-α protein level was measured by ELISA. Among the genes with altered expression, GeneChip analysis detected increased Gro-α mRNA in fibroblasts from all 5 synovial specimens. This increased Gro-α gene expression was confirmed by real-time PCR. Gro-α protein was preferentially increased by IL-1β in synovial fibroblasts. Gro-α has chemotactic effects on lymphocytes and monocytes, and it also regulates endothelial cell proliferation and stimulates angiogenesis. When these established effects are taken together with the results presented herein, increased gene expression and protein production of Gro-α in synovial fibroblasts in response to IL-1β appears to play an important role in recruitment of inflammatory cells into the synovium and in progression of synovitis.

Keywords:
temporomandibular joint, synovial fibroblasts, Gro-α, IL-1β, GeneChip

Introduction
Histologically, destructive changes in articular cartilage and/or adjacent joint connective tissue have been demonstrated in the temporomandibular joint (TMJ); such degenerative damage of the TMJ resembles osteoarthritis (OA) of synovial joints (1). Synovitis is characterized by chronic inflammatory changes such as hyperplasia of the cells lining the synovium (2, 3), and is frequently associated with infiltration by inflammatory cells (4, 5).

Elevated levels of IL-1β have been reported in synovial fluid from patients with temporomandibular disorders (TMD) (6, 7), and are thought to be involved in pain and progression of TMD (8). TMJ synovial cells have important roles in the progression of synovitis, however, their biological roles in TMD are poorly understood. Growth-related gene product alpha (Gro-α), a member of the chemokine superfamily, plays an important role in the ingress of neutrophils into rheumatoid arthritis joints (8). However, production of Gro-α in synovial cells of the TMJ has not been well characterized. Recently, there has been much interested in the possibility of using transcriptomic approaches such as GeneChip technology to rapidly identify a large number of genes (9).

In this study, using the Affymetrix GeneChip system, we examined the effect of IL-1β on gene expression in synovial fibroblasts primarily cultured from TMJ of patients. Among the genes with altered expression, we focused on chemokine Gro-α and confirmed mRNA levels by real-time polymerase chain reaction (PCR). Furthermore, amounts of Gro-
protein in cultured medium from synovial fibroblasts were also examined.

**Materials and Methods**

**Cell culture**

Human synovial tissue was obtained from patients with internal derangement of the TMJ who had undergone arthroscopy or arthroscopic synovectomy of this joint. All patients gave complete informed consent for the surgery and use of tissue in research, and the study was performed according to the guidelines established by the Institutional Review Board of Nihon University School of Dentistry at Matsudo (EC03-003).

Using a previously described method (10), cultures of synovial fibroblasts from the TMJ were prepared from five patients, three female and two male between 17 and 27 years old, and were designated TMJ 1–6. TMJ 5 and TMJ 6 were prepared from the right and left TMJs of the same patient. Synovial fibroblasts were primarily cultured and the 6th to 8th doubling passages of cells were used in experiments. Synovial fibroblasts were plated at $5 \times 10^4$ cells per well in 24-well plates with Ham’s F12 medium supplemented with 10% FCS and antibiotics. The confluent-stage cells were cultured for 24 h in Ham’s F12 medium containing 2% FCS, and were then either treated with or without IL–1β (Cistron Biotechnology, Pine Brook, NJ, USA).

**RNA extraction**

Total cellular RNA was isolated by an acid guanidinium thiocyanate–phenol–chloroform extraction method (11) using a FastPrep machine (FP120; BIO 101, Vista, CA, USA). Seven $\mu g$ of total RNA was mixed with T7-(dT) 24 primer containing a T7 RNA polymerase promoter site (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England), reverse–transcribed to cDNA and synthesized to the double–stranded cDNA using a SuperScript Choice System (Gibco BRL, Grand Island, NY, USA) according to the manufacturer’s protocol. In vitro transcription using double–stranded cDNA as a template with Bio–11–CTP and Bio–16–UTP (Enzo Diagnostics, Farmingdale, NY, USA) was carried out using a T7 Megascript kit (Ambion, Austin, TX, USA). The cRNAs were purified and fragmented to approximately 50–100 nucleotides following the protocol described in the Affymetrix manual, and stored at $-20 ^\circ C$.

**GeneChip expression analysis**

Twenty $\mu g$ of the cRNA were hybridized to GeneChip (Human Genome Focus Array; Affymetrix, Santa Clara, CA, USA, human 8,793 genes), and this sample was washed and stained with streptavidin–phycoerythrin (Molecular Probes, Eugene, OR, USA) using the Midi-euk2 protocol in an Affymetrix fluidics station. Fluorescence intensities were scanned with an Affymetrix GeneArray Scanner. Data analysis was performed with Affymetrix Genechip expression analysis software and GeneSpring™ software (Silicon Genetics, Redwood, CA, USA).

**Real–time PCR**

cDNA synthesis and amplification by reverse transcription–polymerase chain reaction (RT–PCR) were performed using a GeneAmp RNA PCR kit (Perkin–Elmer, Norwalk, CT, USA). The primers, designed by referring to sequences of Gro–α (12) and GAPDH (13) cDNA in previous reports; were as follows: Gro–α forward primer, 5′–TAC ACC AGT GGC AAG TGC TC–3′; Gro–α reverse primer, 5′–GAA GCC TCC CAA GCT AGG AC–3′; GAPDH forward primer, 5′–ATC ACC ATC TCC CAG GAG–3′; and GAPDH reverse primer, 5′–ATC GAC TGT GGT CAT GAG–3′.

Real–time PCR was performed using a DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The PCR mixture, containing 20 pmol of forward and reverse primers and 2 $\mu l$ of cDNA, was subjected to amplification with a DNA Engine Opticon 1 (MJ Research, San Francisco, CA), with preheating at 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 sec, 55 °C for 30 sec and 72 °C for 30 sec. The amplicons were detected directly by measuring the increase in fluorescence caused by binding of SYBR Green I dye to gene–specific, amplified, double-
stranded DNA, using a DNA Engine Opticon 1. After the PCR reaction was complete, the temperature was raised from the annealing temperature to 95 °C for melting curve analysis.

The initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction (C<sub>T</sub>-value). The number of transcripts was determined based on the threshold cycles of Gro-α and GAPDH. ΔC<sub>T</sub> (C<sub>T</sub>-Gro-α minus C<sub>T</sub>-GAPDH) was designated the relative abundance of the Gro-α transcript. ΔΔC<sub>T</sub> (ΔC<sub>T</sub>-treated minus ΔC<sub>T</sub>-control), representing the relative n-value compared to the control. The value 2<sup>−n</sup> represented the difference in Gro-α expression between IL-1β treated cells and control cells.

**ELISA**

The cultured supernatants of synovial fibroblasts were collected, centrifuged and kept at −80 °C until ELISA measurement. Gro-α levels in conditioned media were measured using an ELISA kit and specific antibody against human Gro-α (PIERCE En- dogen, Rockford, IL, USA).

**Statistical analysis**

Data are expressed as mean±S.D. Differences between groups were analyzed using Student’s t-test.

**Results**

The gene expression profiles of synovial fibroblasts TMJ 1–5 from 5 patients with or without IL-1β treatment were examined using the Affymetrix GeneChip system. Fig. 1 shows changes in mRNA levels in each of the 5 synovial fibroblast cultures. Expression of many genes was altered, including Gro-α. Among these genes, Gro-α mRNA levels were preferentially enhanced by treatment with IL-1β in all 5 synovial fibroblast cultures. The degree to which this occurred differed between the various fibroblast cultures. Fig. 2 shows scatter plots from average data of the 5 synovial fibroblast cultures and summarizes the difference in gene expression between samples treated with IL-1β and controls. Table 1 summarizes how treatment with IL-1β enhanced Gro-α mRNA levels in 5 different synovial fibroblast cultures.

Since we found that IL-1β stimulated Gro-α gene expression level in all 5 synovial fibroblasts, we selected synovial fibroblast culture of TMJ 6 follow-
Table 1. GeneChip analysis of Gro-α gene expression in synovial fibroblasts TMJ 1–5 from human TMJ stimulated with IL-β.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Normalized Intensity</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>IL-1β</td>
</tr>
<tr>
<td>TMJ 1</td>
<td>8.69</td>
<td>83.41</td>
</tr>
<tr>
<td>TMJ 2</td>
<td>1.68</td>
<td>74.65</td>
</tr>
<tr>
<td>TMJ 3</td>
<td>2.17</td>
<td>76.88</td>
</tr>
<tr>
<td>TMJ 4</td>
<td>12.51</td>
<td>57.68</td>
</tr>
<tr>
<td>TMJ 5</td>
<td>5.81</td>
<td>68.06</td>
</tr>
<tr>
<td>Average</td>
<td>6.17</td>
<td>72.14</td>
</tr>
</tbody>
</table>

Table 2. Effect of IL-β on Gro-α gene expression in synovial fibroblasts from human TMJ 6 using real-time PCR.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gene</th>
<th>C_{T}</th>
<th>ΔC_{T}</th>
<th>ΔΔC_{T}</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Gro-α</td>
<td>16.427</td>
<td>3.760</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
<td>12.667</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Gro-α</td>
<td>11.417</td>
<td>-1.865</td>
<td>-5.625</td>
<td>49.3</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
<td>13.282</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C_{T}*, the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction; ΔC_{T***}, C_{T}−Gro-α minus C_{T}−GAPDH; ΔΔC_{T****}, ΔC_{T}−IL-1β treated minus ΔC_{T}−control.

Next, we examined the production of Gro-α protein in response to IL-1β. TMJ 6 synovial fibroblasts were incubated in the presence of increasing concentrations of IL-1β (0.01 to 10.0 units/ml). As shown in Fig. 3, IL-1β enhanced Gro-α production in a dose-dependent manner. Fig. 4 shows the time course of Gro-α levels in conditioned media from synovial fibroblasts treated with IL-1β and from controls. A significant, time-dependent increase in Gro-α production was observed with IL-1β treatment.

**Discussion**

Gro-α was initially described as melanoma growth...
stimulatory factor and characterized by its growth stimulatory activity on malignant melanoma cells (12). Gro-α is also produced by a variety of normal cells such as monocytes (14), endothelial cells (15), fibroblasts, and synovial cells (16). Gro-α is a 73–amino acid, 8–kDa protein that is highly chemotactic for neutrophils (17). Subsequently, two additional Gro gene products, Gro-β and Gro-γ, were discovered (18). These 3 Gro families have 90% sequence identity and similar neutrophil–activating properties (19–21), and interestingly, the Gro gene products have 30% homology to IL-8 (22). The potent chemotactic activities of Gro-α described in these reports suggest that it may play an important role in the pathogenesis of inflammatory diseases.

Inflammatory cytokines including IL-1β have been detected in synovial fluid from patients with TMD (6, 7, 23, 24). Alstergren et al (23), demonstrated that IL-1β in synovial fluid was associated with pain and hyperalgesia in the TMJ, suggesting that it is a warning sign of tissue destruction.

In this study, using the Affymetrix GeneChip system, we examined the effect of IL-1β on gene expression in 5 individual synovial fibroblast cultures from TMJ patients. In all TMJ synovial fibroblast cultures, TMJ 1–5 Gro-α mRNA levels were significantly enhanced with different degrees by treatment with IL-1β. To confirm Gro-α mRNA level changes, real-time PCR were performed using TMJ 6 synovial fibroblasts. The level of Gro-α mRNA in synovial fibroblasts was increased approximately 49.3-fold by treatment with IL-1β when compared to the untreated cells. We cultured synovial fibroblasts in the presence of 0.01 to 10.0 units/ml of IL-1β, which is the range of IL-1β concentrations detected in TMJ synovial fluid in previous studies, and examined Gro-α production. The stimulatory effect of IL-1β on Gro-α production was apparent at a concentration of 0.1 unit/ml, and a plateau was reached at a concentration of approximately 1.0 unit/ml. Furthermore, when synovial fibroblasts were treated with 1 unit/ml of IL-1β, Gro-α production was increased in a time–dependent manner. These findings suggest that proinflammatory cytokine IL-1β enhances gene expression and production of Gro-α in TMJ synovial cells in TMD.

Rheumatoid arthritis is an inflammatory disorder characterized by infiltration of leukocytes into the synovial tissue and synovial fluid of joints and is thought be similar to TMD. Koch et al (8), reported that Gro-α possessed as potent neutrophil–stimulating activity as the chemokine superfamily, and that it played an important role in the ingress of neutrophils into rheumatoid joints. It is noteworthy that antagonists of Gro-α caused a significant reduction of ankle swelling in arthritis model mice (25). These findings suggest that inflammatory cytokines such as IL-1β in synovial fluids stimulate synovial fibroblasts to produce excessive Gro-α and thereby contribute to the emigration and accumulation of neutrophils in progression of TMD. Hence, antagonist therapy against Gro-α may be useful in TMD.

Acknowledgments

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