Expression of proinflammatory cytokines in osteoarthritis of the temporomandibular joint

Rolando Vernal a,b,*, Edgar Velásquez c, Jorge Gamonal b, Jose A. García-Sanz a, Augusto Silva a,1, Mariano Sanz c,1

a Cellular and Molecular Physiopathology Department, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain
b Conservative Dentistry Department, Dentistry School, Universidad de Chile, Santiago, Chile
c Stomatology III Department, Dentistry School, Universidad Complutense de Madrid, Madrid, Spain

A B S T R A C T

Keywords:
Temporomandibular joint
Temporomandibular disorders
Osteoarthritis
Cytokines
Inflammation

Objective: This study reports the expression of proinflammatory cytokines in temporomandibular joint (TMJ) of patients affected with temporomandibular osteoarthritis (OA).

Design: In twelve OA of the TMJ (OA-TMJ) affected patients and in six healthy volunteer subjects studied as control, the expression of IL1β (interleukin-1β), IL2, IL4, IL5, IL6, IL10, IL12p35, IL12p40, IL17, IFNγ (interferon-gamma), TNFα (tumor necrosis factor-alpha), and TNFβ mRNAs was evaluated. Using quantitative real-time RT-PCR technique, the cytokine levels, reported as Ct (cycle threshold), ΔCt (Ct cytokine – Ct 18S rRNA) and RQ (relative quantification), in patient and control groups were compared.

Results: Expression of IL1β, IL2, IL12p35, IL12p40, IL17, TNFα, TNFβ, and IFNγ mRNAs was significantly higher in patients as compared with controls. In particular, IL12 was the predominant cytokine expressed in patients (IL12p35 RQ = 30.2 and IL12p40 RQ = 29.0). Conversely, IL10 mRNA levels were higher in controls (RQ = 1.8).

Conclusions: These data suggest that not only IL1β, IFNγ, and TNFα but also IL10, IL12, and IL17 are involved in the OA-TMJ pathogenesis. Furthermore, an inflammatory response characterised by the predominant expression of IL12 mRNA and down-regulated expression of IL10 mRNA is associated with the degenerative changes observed in OA-TMJ.

1. Introduction

Temporomandibular disorders (TMD) are a group of conditions affecting the masticatory muscles, the temporomandibular joints (TMJ), and their associated anatomic structures, with a different aetiology and clinical presentation. Osteoarthritis (OA) is a degenerative disease affecting the TMJ. It is the most common TMD and shows a higher prevalence in women and older age groups.1–3 OA of the TMJ (OA-TMJ) is characterised by variable degrees of inflammation and abrasion of the articular cartilage and bone.4,5 Various inflammatory mediators, bone-destruction associated cytokines, and metallo-proteinases (MMPs) have been considered as possible markers of active OA-TMJ, including interleukin (IL) 1β, IL6, interferon-gamma (IFNγ), tumor necrosis factor-alpha (TNFα), prostaglandin E2 (PGE2), receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG), MMP-2, MMP-9, aggrecanase-1, and aggrecanase-2.6–11

* Corresponding author at: Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain Tel.: +34 918 373112; fax: +34 915 360432.
E-mail address: rvernal@uchile.cl (R. Vernal).
1 Both authors must be considered senior authors.
Although inflammatory-bone and/or cartilage destruction diseases, such as rheumatoid arthritis (RA) and chronic periodontitis (CP), have been traditionally categorized as Th1-type diseases, Th1 cells do not have osteoclastogenic characteristics activity, indicating that the osteoclastogenic cells might belong to an as-yet unknown Th subset. Recent data have indicated that an IL17 producing Th cell subset is responsible of the bone damage observed in autoimmune arthritis and it has been reported that IL17, rather than IL12 or IFNγ, is critical in the onset phase of the disease. Moreover, it has been reported that IL17 is detectable in the synovial fluid from RA patients and enhances bone destruction after induction of RANKL expression. In OA–TMJ, Th1 cytokines are not always highly expressed in the lesion, thus, not only IL1β, IL12 or IFNγ but also IL17 producing cells could be related to its pathogenesis.

The cytokine balance, their receptors, and receptor antagonists are determinants not only for initiation and progression, but also for the clinical expression of these diseases. Recent advances in the biochemical analysis of synovial fluid from affected patients have provided new insights into the pathophysiology of the TMJ disorders; however, the molecular basis of OA etiopathogenesis remains still unclear. Association of IL12 with TMJ disorders has been scarcely studied and IL17 expression in OA–TMJ has not been previously described. This study aimed to determine the cytokine expressions from cells sampled from the TMJ of OA affected patients.

2. Materials and methods

Samples of TMJ synovial fluid were collected from twelve patients (four males and eight females, aged 41.4 ± 2.4) diagnosed of OA, based on their clinical symptomatology and ratified by magnetic resonance and radiographic images. As control, six healthy volunteers (two males and four females, aged 39.0 ± 2.2) without a history of TMD and lack of TMD-related symptoms were studied. All subjects signed an informed consent approved by the Institutional Review Board of the University Complutense de Madrid. Inclusion criteria for patient enrolment were the presence of TMJ soreness, impaired joint mobility, and presence of images characteristic of degenerative changes of the condyle, such as flattening and erosion. Exclusion criteria were diagnosis of chronic systemic inflammatory diseases or previous treatment of TMJ disorders. Evaluation and data registration were performed by two independent oral surgeons. Patients were given no medication for at least 2 weeks before the synovial fluid sampling was performed.

2.1. Sample collection

After extracapsular local anesthesia, the synovial fluid was collected by puncture with a 21-gauge needle inserted inferolaterally into the superior joint space. Two milliliters of normal saline solution was injected and, after 1 min mixing by repeated opening and closing of the patient’s mouth, 2 mL of synovial fluid and saline solution mixture was aspirated. Samples were devoid of blood contamination. The cells present in these samples were recovered by centrifugation at 300 × g for 10 min and subsequently resuspended in 5 mL of sterile culture medium RPMI 1640, supplemented with 50 UI/mL penicillin, 50 μg/mL streptomycin, 200 mM l-glutamine (Sigma Chemical Co, St. Louis, MO, USA) and 10% foetal bovine serum (Gibco Invitrogen, Grand Island, NY, USA). Cell counting was performed on a Neubauer chamber using a phase contrast microscope and cell viability was calculated by Trypan blue dye exclusion. In the analysed samples, cell viability was always equal to or greater than 90%. The cell suspensions obtained from the synovial fluid were then used to prepare the RNAs used for this study.

2.2. Cytoplasmic RNA isolation

Cytoplasmic RNA was isolated as previously described. For each subject, 10⁶ cells were washed twice in PBS for 10 min at 300 × g at room temperature, transferred to a microcentrifuge tube in 1 mL of PBS, centrifuged for 10 s at 14 000 × g at room temperature and lysed in 400 μL of ice-cold lysis buffer containing 0.5% Nonident P-40, 50 mM Tris HCl pH 8, 100 mM NaCl and 5 mM MgCl₂, supplemented with 10 mM VRC 40 (Gibco). Complete cell lysis was obtained by pipetting up–down 10 times, and the nuclei were removed by centrifugation for 10 s at 14 000 × g. Four hundred microliters of the supernatant were digested with 25 μg/mL proteinase K (Roche Ltd., Basel, Switzerland), in the presence of 1% SDS and 15 mM EDTA (Fluka) for 30 min at 37°C. Subsequently, RNA was extracted with an equal volume (400 μL) of chloroform (phenol:chloroform:isoamylalcohol 25:24:1) and centrifuged for 10 min at 14 000 × g at room temperature (Fluka). The RNA present in the aqueous solution was precipitated for 1 h at −20°C with 0.3 M Na-acetate, 2.5 volumes 100% EIOH and 1 μL glycogen 20 μg/μL (Roche), recovered by centrifugation for 30 min at 14 000 × g, and resuspended in 20 μL H₂O RNase-free. RNA quality was determined in a bioanalyser (Agilent 2100B, Agilent Tech., Palo Alto, CA, USA). Cytoplasmic RNA was quantified using a spectrophotometer (Nanodrop ND-1000, Nanodrop Tech., Wilmington, ND, USA) and stored at −80°C at a final concentration of 1 μg/μL.

2.3. First-strand cDNA synthesis

Reverse Transcription (RT) was performed using a Transcriptor First Strand cDNA synthesis kit (Roche) following the manufacturer’s recommendations. Briefly, 20 μL reaction containing: 4 μL 5× RT buffer, 0.5 μL RNase inhibitor (20 U), 2 μL deoxynucleotide mix (1 mM each), 2 μL random hexamer primer (60 μM), 0.5 μL reverse transcriptase (10 U), 10 μL RNA-grade H₂O, and 1 μL RNA sample (1 μg) were retrotranscribed under the following conditions: 10 min at 25°C and 1 h at 50°C (Primus 96 plus, MWG Biotech AG, Ebersberg, Germany). The reverse transcriptase activity was subsequently inactivated by incubating the sample at 85°C for 5 min.

2.4. Quantitative real-time PCR

The mRNA expression levels of the following cytokines: IL1β, IL2, IL4, IL5, IL6, IL10, IL12p35, IL12p40, IL17, IFNγ, TNFα, and TNFβ were quantified using real-time quantitative PCR. Fifty
nanograms of cDNA were amplified using a FastStart Taqman Probe Master (Roche), as previously described.21 Forward and reverse primers were designed and specific FAM dye-labelled probe was selected (Table 1) using the Roche web-site (www.roche-applied-science.com). Twenty microliters volume reaction containing: 0.2 μL PCR probe (250 nM), 0.2 μL forward primer (900 nM), 0.2 μL reverse primer (900 nM), 10 μL FastStart master, 8.4 μL PCR-grade H2O, and 1 μL cDNA were analysed under the following conditions: 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C, in a ABI PRISM 7900 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). As endogenous control assay 18S rRNA levels expression are shown in Table 2. Since the amplification efficiency of each cytokine as compared to the 18S rRNA control was similar and no differences were detected in the Ct of 18S rRNA between the patient and control groups, ΔCt (Ct cytokine – Ct 18S rRNA) was comparable and the fold-change of RQ expression of each cytokine could be represented by the 2−ΔΔCt method.22 Significantly lower Ct and ΔCt levels for IL1β, IL2, IL12, IL17, TNFα, TNFβ, and IFNγ were found in patients affected with OA–TMJ as compared to those determined in the healthy control group. The Ct data, but not the ΔCt data, of IL5 was significantly lower in OA than in the healthy control group.

RQ analysis confirmed the Ct and ΔCt differences. The fold-change and the estimated error range of RQ for each studied cytokine are shown in Table 3. IL12p35 and IL12p40 were the highest over-expressed mRNAs detected within the OA group, with RQ of 30.2 (11.2–81.0) and 29.0 (9.9–85.0)-fold change, respectively. Over-expression of IL17 and IFNγ were approximately 15-fold, and IL1β, IL2, TNFα, and TNFβ levels were higher than 6-fold. IL10 was down-regulated in the patient group. When the RQ was re-calculated considering the adjusted IL10 expression in the patient group as 1, IL10 expression in the controls was 1.8 (0.6–5.9)-fold higher than the levels detected in the OA subjects.

### 4. Discussion

During OA–TMJ, a complex inflammatory response is developed, involving the synthesis of different cytokines by synovial and inflammatory cells that infiltrate the joint tissues.4,5 During the disease initiation and progression, there is a local imbalance between the expression of specific cytokines, their receptors, and regulatory soluble receptors, which may be critical in the biological activity of the cytokine network.5 Under these conditions, both fibroblast and synovial cells are activated to express MMPs and bone-associated cytokines that control the formation/destruction of articular cartilage and bone, determining the clinical outcome of the OA–TMJ.9–11,23
controls, using quantitative real-time RT-PCR analysis. Real-time RT-PCR is the most sensitive method allowing quantification of low expression levels and it is a fast and reliable screening.\textsuperscript{55} For normalisation, 18S rRNA expression levels were selected due to their small fluctuations in nucleated cells.\textsuperscript{26} Indeed, no differences were detected in the 18S rRNA expression between patient and control groups. Our data demonstrate lower Ct and ΔCt values for IL1β, IL2, IL12, IL17, TNFα, TNFβ, and IFNγ in patients affected with OA–TMJ as compared to the corresponding levels from the control group. Since Ct and ΔCt values decrease linearly with increasing input target quantity,\textsuperscript{24} the selection of patients and controls have been suggested as the scores from a visual analog scale of pain and host-defence response, their over-expression and association to the RANKL/OPG activity, however, has been demonstrated.

This study has determined the mRNA expression levels of IL1β, IL2, IL4, IL5, IL6, IL10, IL12, IL17, TNFα, TNFβ, and IFNγ in the TMJ of patients affected with OA–TMJ and healthy controls, using quantitative real-time RT-PCR analysis. Real-time RT-PCR is the most sensitive method allowing quantification of low expression levels and it is a fast and reliable screening.\textsuperscript{55} For normalisation, 18S rRNA expression levels were selected due to their small fluctuations in nucleated cells.\textsuperscript{26} Indeed, no differences were detected in the 18S rRNA expression between patient and control groups. Our data demonstrate lower Ct and ΔCt values for IL1β, IL2, IL12, IL17, TNFα, TNFβ, and IFNγ in patients affected with OA–TMJ as compared to the corresponding levels from the control group. Since Ct and ΔCt values decrease linearly with increasing input target quantity,\textsuperscript{24} the selection of patients and controls have been suggested as the scores from a visual analog scale of pain and host-defence response, their over-expression and association to the RANKL/OPG activity, however, has been demonstrated.

Cytokine level differences (Ct and ΔCt data) between TMJ Osteoarthritis Patients and Healthy Controls were determined using the two-sample unpaired Student’s t-test: *p < 0.01; **p < 0.05; ***p < 0.001.

\textsuperscript{a} = Cycle threshold.
\textsuperscript{b} = ΔCt = Ct cytokine – Ct 18S rRNA.

### Table 2 – Ct and ΔCt of IL1β, IL2, IL4, IL5, IL6, IL10, IL12p35, IL12p40, IL17, IFNγ, TNFα and TNFβ in temporomandibular joint osteoarthritis patients and healthy controls evaluated by real-time RT-PCR and represented as mean ± standard deviation

<table>
<thead>
<tr>
<th></th>
<th>TMJ osteoarthritis patients</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td><strong>Ct\textsuperscript{a}</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1β</td>
<td>33.198 ± 1.04*</td>
<td>36.250 ± 1.02*</td>
</tr>
<tr>
<td>IL2</td>
<td>32.985 ± 1.20*</td>
<td>35.750 ± 1.39*</td>
</tr>
<tr>
<td>IL4</td>
<td>36.424 ± 1.48</td>
<td>36.625 ± 1.27</td>
</tr>
<tr>
<td>IL5</td>
<td>35.895 ± 1.45**</td>
<td>37.218 ± 0.68*</td>
</tr>
<tr>
<td>IL6</td>
<td>37.748 ± 2.04</td>
<td>38.270 ± 1.35</td>
</tr>
<tr>
<td>IL10</td>
<td>37.247 ± 1.62</td>
<td>36.465 ± 0.96</td>
</tr>
<tr>
<td>IL12p35</td>
<td>23.946 ± 1.52*</td>
<td>14.967 ± 2.61</td>
</tr>
<tr>
<td>IL12p40</td>
<td>25.035 ± 2.34*</td>
<td>1.666 ± 1.42*</td>
</tr>
<tr>
<td>IL17</td>
<td>29.822 ± 1.42***</td>
<td>7.542 ± 2.31***</td>
</tr>
<tr>
<td>TNFα</td>
<td>32.477 ± 1.51*</td>
<td>10.197 ± 2.56**</td>
</tr>
<tr>
<td>TNFβ</td>
<td>31.977 ± 1.65***</td>
<td>9.697 ± 2.25*</td>
</tr>
<tr>
<td>IFNγ</td>
<td>23.893 ± 1.94*</td>
<td>1.613 ± 2.49***</td>
</tr>
<tr>
<td>18S</td>
<td>22.288 ± 1.94</td>
<td>22.349 ± 1.34</td>
</tr>
</tbody>
</table>

### Table 3 – Relative quantification (RQ) of IL1β, IL2, IL4, IL5, IL6, IL10, IL12p35, IL12p40, IL17, IFNγ, TNFα and TNFβ in temporomandibular joint osteoarthritis patients and healthy controls evaluated by real-time RT-PCR and represented as fold-change mean and estimated range error

<table>
<thead>
<tr>
<th></th>
<th>TMJ osteoarthritis patients</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td><strong>RQ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1β</td>
<td>7.9</td>
<td>1.0</td>
</tr>
<tr>
<td>IL2</td>
<td>6.5</td>
<td>1.0</td>
</tr>
<tr>
<td>IL4</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>IL5</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td>IL6</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>IL10*</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>IL12p35**</td>
<td>30.2</td>
<td>1.0</td>
</tr>
<tr>
<td>IL12p40**</td>
<td>29.0</td>
<td>1.0</td>
</tr>
<tr>
<td>IL17</td>
<td>14.7</td>
<td>1.0</td>
</tr>
<tr>
<td>TNFα</td>
<td>6.0</td>
<td>1.0</td>
</tr>
<tr>
<td>TNFβ</td>
<td>6.4</td>
<td>1.0</td>
</tr>
<tr>
<td>IFNγ</td>
<td>16.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

RNA cytokine expression in healthy controls was considered as 1 to relative quantification, and used as reference for fold-change of RNA cytokine expression in temporomandibular joint osteoarthritis patients.

To determine the cytokines more over-expressed on each group the unpaired ANOVA test and the Tukey post hoc test were used: *p < 0.01; **p < 0.001.
clearly related to the pathogenesis of many disease states. Thus, the role of IL1β and TNFα in the OA–TMJ pathological changes is a reasonable possibility. They both can participate directly, or mediated by RANKL/OPG induction, in the differentiation and activation of cartilage and bone resorbing cells.

According our data, IL12 was the highest over-expressed cytokine and IL10 was down-regulated in OA patients. Association of IL12 and TMJ disorders have been scarcely studied. No-significant higher concentration of IL12 was detected in patients with chronic closed lock of the TMJ showing clinical improvement after TMJ irrigation when compared with non-improved subjects. IL12 was, however, elevated in synovial fluid and was correlated with disease activity in rheumatoid arthritis patients. Additionally, IL12 levels were decreased in rheumatoid arthritis patients who improved their clinical symptoms after treatment with anti-rheumatic drugs. Subsequent studies have shown that a Th1 IL2 and IL12 production and Th2 IL5 and IL6 production can be directly inhibited by IL10. IL10 inhibits both monocyte/macrophage synthesis of IL1α, IL1β, IL6, IL8, IL12, TNFα, reactive oxygen and nitrogen intermediates, and dendritic cell and NK production of IFNγ. The ability of IL10 to inhibit synthesis of proinflammatory cytokines such as IL1β and TNFα, as well as to induce the production of anti-inflammatory cytokines such as IL1RA, suggests that IL10 may inhibit the inflammatory response in diseases characterised by inflammatory mediated tissue degeneration.

The rate and concentration of IL10 were significantly higher in patients affected by chronic closed lock under irrigation treatment with successful clinical outcome when compared with those in unsuccessful clinical outcome. Our data demonstrate that IL10 mRNA was down-regulated in patients affected of OA–TMJ and was the predominant cytokine expressed in healthy controls. It can be speculated that, in OA–TMJ, IL10 has a protective role and its presence in the synovial fluid may be used as a predictor of a successful treatment outcome.

Several clinical studies have indicated a pivotal role for IL17 in the pathogenesis of rheumatoid arthritis. IL17 induces secretion of IL6 by rheumatoid synoviocytes and both TNFα and IFNγ augment the IL17 activity. IL17 has been shown as inducer of synovial inflammation and cartilage degradation, inhibitor of chondrocyte proliferation, and stimulator of NO and osteoelasticogenous cytokine production, such as IL1β and IL6. This study is the first to report on the detection of IL17 in the synovial fluid of TMJ and it may indicate that its presence may be important in the pathophysiology of OA–TMJ. In autoimmune arthritis, bone destruction is attributable to osteoclast activity which is directly and indirectly regulated by CD4+ T cells. Direct regulation is mediated by RANKL and indirectly is mainly mediated by inflammatory cytokines produced by synovial cells, such as IL1β and TNFα, which induce RANKL expression on synovial fibroblasts. Recently, it has been reported that IL17, rather than IL12 or IFNγ, is critical for the onset of autoimmune arthritis. IL17 has been involved in the induction of proinflammatory cytokines, chemokines and MMPs, and it has been directly involved in diseases characterised by bone and/or cartilage destruction.

In summary, these results allow us to possibly attribute a role of IL12 and IL17 in the OA–TMJ pathophysiology. In affected joints, an over-expression of these cytokines by synovial cells could, either directly or through the expression of RANKL, participate in the degeneration of the articular cartilage and bone. Furthermore, taking into account the wide range of molecular and cellular interactions described between the immune and skeletal systems, it is plausible to speculate the involvement of cytokines from both IL17 and IL12/IFNγ axes in the pathologic events leading to OA–TMJ. In this context, the observed down-regulation of IL10 expression may also be implicated in the pathophysiology of this condition, since these low levels would lead to the amplification of IL12 and IL17 activity.

Acknowledgments

The work in the author’s laboratories has been supported by grant from the Spanish Ministry of Health (FIS-060181 to MS), Spanish Ministry of Science and Education (SAF2006-4826 and SAF2006-04903 to AS and JAGS, respectively), and Chilean Government (CONICYT-2680046 to RV).

References