Effect of Low Level Laser Irradiation on CXCR3 Gene Expression in Rheumatoid Arthritis Joint

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Abstract: Rheumatoid arthritis (RA) is characterized by the infiltration of leukocytes into the synovial tissue and synovial fluid of joints, ultimately leading to destruction of cartilage and bone. Low-level laser irradiation (LLLI) is evaluated for treating RA, however, the molecular basis mechanism underlying the effectiveness of LLLI is unclear. Chemokines and those receptors play a central role in the progression of RA inflammation. CXCR3 may be recruited directly from the circulation into the synovial sublining regions by its ligand, CXCL9, produced by synovial fibroblasts. The objectives of this study were to determine whether LLLI decreased production of CXCR3 and CXCL9 in RA joints of type II collagen induced RA (CIA) rats. Total RNA was isolated from rat knee joints with or without treatment of 830 nm LLLI irradiation, and gene expression profiles were analyzed by DNA microarray (Affymetrix, 41,000 genes). As results, LLLI significantly reduced CXCR3 mRNA level, but not significantly reduced CXCL9. The reduction of CXCR3 mRNA levels was confirmed by RT-PCR and real-time PCR. Since CXCR3 plays an important role of the progression of RA, the reduction of CXCR3 gene expression may be one of important mechanisms in reduction of inflammation in RA joints by LLLI.

Key Words: Rheumatoid arthritis, Rat joint, CXCR3, Gene expression

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by pain and inflammation, progressive joint destruction, significant disability, systemic manifestations and premature mortality1). This disease is characterized by the infiltration of leukocytes into the synovial tissue and synovial fluid of joints, ultimately leading to destruction of cartilage and bone2). Inflammation of the synovial lining of joints results in further increase in the expressions of inflammatory cytokines and chemokine-mediated recruitment of additional inflammatory cells, as well as activation of B cells with autoantibody production. A vicious cycle of altered cytokine and signal transduction pathways and inhibition of programmed cell death contribute to cartilage and bone destruction by human fibroblast-like synoviocytes and osteoclasts3,4). Collagen-induced arthritis (CIA) is an animal model of RA that is widely used to address questions regarding the pathogenesis of RA and to validate therapeutic targets. The pathological features of CIA include proliferative synovitis with infiltration of polymorphonuclear and mononuclear cells, pannus formation, cartilage degradation and erosion of bone5).

Many treatments are available to relieve RA-associated pain. A non-invasive physiological therapy such as low level laser irradiation (LLLI) could be important for managing pain. The different wavelength laser therapies have been used to the reduction of inflammation and the relief from pain, and bone formation6) including He-Ne, Ga–Al–As (805 or 650 nm) and Ga–As (904 nm). However, there are few studies on the mechanism of anti-inflammatory effects of laser irradiation; most of the studies deal only with the analgesic properties of laser and the efficacy of LLLI as an anti-inflammatory therapy is still controversial7). The interaction of LLLI with the biological system occurs at the cellular level, but the mechanisms involved are yet unknown. Some reports suggest that there exist therapeutic advantages in the application of Ga–Al–As diode laser to inflammatory pathologies compared to placebos and other treatments8,9). In our previous study, Ga–
Al–As diode LLLI significantly reduced the swelling of CIA rat joints and reduced CXCL13 gene expression which plays a key role of the progression of RA, suggesting Ga–Al–As LLLI is effective therapy to RA.

It has been reported that chemokines and those receptors play a central role in the progression of RA inflammation, and CXCR3 may be recruited directly from the circulation into the synovial sublining regions by its ligand, CXCL9, produced by synovial fibroblasts. However, effect of LLLI on CXCR3 gene expression in the inflamed arthritic joint has not been reported. In the present study, effect of LLLI on CXCR3 and CXCL9 gene expression in joint tissues in CIA rats was examined.

Materials and methods

Animal experiment

Female Lewis (LEW/CrlCrlj) rats, 6 weeks old, were injected with type II collagen (Sigma, Tokyo, Japan) in 250 µl of 0.1 M acetic acid emulsified in an equal volume of complete Freund’s adjuvant (Difco Labs, Michigan, USA) containing 2 mg/ml muramyl dipeptide (Wako, Tokyo, Japan) by multiple intradermal injections. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (Somnopentyl®, Kyoritsu Seiyaku, Tokyo, Japan) at 25 mg/kg prior to the injection. All animals were maintained and used in accordance with the guide the Care and Use of Laboratory Animals of Nihon University, School of Dentistry at Matsudo (No. 04-008).

A Ga-Al-As diode laser device (Model ZH-M143DJP; Panasonic Industrial Equipment Inc., Japan) was used as a laser source. This laser therapy system utilizes a wavelength of 830 nm and output power of 100-700 mW, which is variable in a continuous wave (c/w). In the present study, an output power in a c/w of 500 mW was selected. The probe was fixed at 550 mm from the tissue to be irradiated, giving a spot size of 78.5 cm², with an incident power density of approximately 6.4 mW/cm². The irradiation time was 20 min, which equaled an incident energy density of 7.64 J/cm². Each experimental group was carried out by 3 rats.

DNA microarray analysis

Total cellular RNA was isolated from the knee joint synovial membrane using RNeasy Fibrous Tissue Mini Kit Isolation System (Qiagen GmbH, Dusseldorf, Germany.) by a FastPrep machine (FP120; BIO 101). cDNA synthesis was performed using a GeneAmp RNA PCR Kit (Applied Biosystems, Foster, CA, USA) with samples of total RNA. Fluoro-dye labeled cRNA synthesis was used Quick Amp Labeling Kit (Agilent, Santa Clara, CA, USA). For hybridization, 1.65 µg of cy3-labeled cRNA from LLLI and non-LLLI sample was hybridized to a Whole Rat Genome Microarray 4 x 44K (Agilent; 41,000 rat genes and transcripts). Finally, DNA microarrays were scanned using Agilent Microarray Scanner (Agilent), and analyzed using Agilent Feature Extraction software (Agilent) and GeneSpring GX10 software (Agilent).

RT-PCR and real time PCR analysis

RT-PCR and real-time PCR reactions were carried out using a DNA thermal analyzer (RFN-Gene™ 6000; Corbett Life Science, Sidney, Australia). Amplification by PCR was started with an initial incubation at 95 ºC for 15 seconds to activate the Taq DNA polymerase, and then performed at 95 ºC for 5 seconds and 56 ºC for 15 seconds by adequate cycles. RT-PCR products were electrophoresed on 1.5% agarose gel, followed by staining with ethidium bromide to examine the size of PCR products. Real-time PCR was carried out with SYBR Premix Ex Taq™ (Perfect Real-Time PCR, Takara, Japan) and a Green PCR kit (Qiagen). To calculate gene expression fold changes, the initial template concentration was derived from the cycle number at which the fluorescent signal crossed the threshold in the exponential phase of the real-time PCR reaction. The mRNA copy unit was given by the cycle threshold value from the fluorescent signal of all the samples, including the standard curve and target genes, following the method provided by Corbett Life Science Company using RFN-Gene™ 6000 software. Details were described in an operation manual, version 1.7.40, 2006. Each assay was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels.

The DNA primer sequences were 5’-gccttcatcttcacggagag-3’ (the forward primer for CXCR3 gene); 5’-tccttggagctgagagtggt-3’ (the reverse primer for CXCR3 gene), (predicted size=229

Table 1 Raw intensity signals shown in DNA microarray.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genebank ID</th>
<th>Time (-week)</th>
<th>CIA (Flag*)</th>
<th>LLLI (Flag*)</th>
<th>LLLI/CIA (Fold**)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR3</td>
<td>NM_053415</td>
<td>1</td>
<td>17.82 (P)</td>
<td>23.10 (P)</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>60.99 (P)</td>
<td>42.18 (P)</td>
<td>-2.0</td>
</tr>
<tr>
<td>CXCL9</td>
<td>NM_145672</td>
<td>1</td>
<td>7.38 (A)</td>
<td>1.62 (A)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>8.51 (P)</td>
<td>13.70 (P)</td>
<td>-1.1</td>
</tr>
</tbody>
</table>

*Flag, indicates the reliability of the data according to P (present) and A (absent). **Fold, fold change by normalization against the median of the corresponding control sample.
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Results

Table 1 shows the results of raw mRNA signals of DNA microarray analysis of CXCR3 and CXCL9 in CIA rat joint tissues with or without LLLI. The CXCR3 genes which expressed were selected by FFlag analysis as “Present” (P) with more than 2-fold changes on 1-, 2-weeks, but CXCL9 was selected to “Absent” on 1-week and showed less than 2-fold change on 2-week.

<table>
<thead>
<tr>
<th></th>
<th>1-week</th>
<th>2-week</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIA +</td>
<td>CXCR3</td>
<td></td>
</tr>
<tr>
<td>LLLI</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>229 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td>159 bp</td>
</tr>
</tbody>
</table>

A scatter plot of gene expression profile including CXCR3 was shown in Fig. 1. CXCR3 mRNA level was decreased in CIA/LLLI compared to CIA.

Since, CXCR3 was selected as Present with more than 2-fold change, to further investigate the decreased mRNA level of the CXCR3 genes, RT-PCR analysis was performed. As shown in Fig. 2, RT-PCR-amplified DNA band of CXCR3 in the tissue of CIA/LLLI was lower in density than that in CIA tissue and LLLI reduced the density of CXCR3 DNA band in all these samples. In contrast, mRNA levels of GAPDH, the housekeeping control, showed no differences in all these samples.

Another experiment to determine the exact rates of the reduction of CXCR3 gene expression was performed using real-time PCR.
The results in real-time PCR were converted to mRNA and calculated as the copy units. As shown in Fig. 3, CXCR3 gene expressions was increased by CIA, and significantly decreased by LLLI.

**Discussion**

To our knowledge, only one report has been published concerning the effect of LLLI on the reduction of CXCR3 production. Yamaura et al(13) treated the fibroblast-like synoviocytes with 810 nm LLLI, and analyzed CXCR3 gene expression using RT-PCR. As a result, CXCR3 gene expression was reduced, however, the experiment was in cell culture system. So far, there is no report on the effect of 830 nm-LLLI on CXCR3 gene expression in vivo. In the present study, we in first demonstrated that 830nm-LLLI decreased CXCR3 gene expression. CXCR3, a G protein–coupled cell surface receptor, is the unique receptor for chemokines belonging to the CXC group chemokine family, where X is any amino acid and C are two conserved cysteine residues present in the N-terminal part of the molecule, including CXCL9(14). The inflamed synovial tissue of RA patients is characterized by a massive infiltration of immune cells, and chemokines and their receptors are important for lymphocyte trafficking into the inflamed joint. Immunofluorescence staining of RA patient synovial tissue revealed CD3+ lymphocytes in RA synovial tissue intensely immunoreactive for CXCR3, suggesting that this receptor may be particularly important for CD3+ lymphocyte trafficking to the inflamed joint(15). Moreover, Ruschpler et al(16) investigated that gene expression patterns in synovial tissue from RA and osteoarthritis (OA) patients using DNA microarray analysis. The result revealed that CXCR3 mRNAs were significantly upregulated in RA as compared with OA. Using immunohistochemistry, CXCR3 protein was found to be preferentially expressed in synovial tissue from RA patients. These findings also suggest that substantial expression of CXCR3 protein in synovial tissue from RA patients plays a significant role in the pathophysiology of RA. CXCR3-binding chemokines are detected in markedly higher concentrations in RA patients, both in synovial fluid and tissue, and synovial fibroblasts as the main producers of the CXCR3-agonistic chemokine CXCL9 in response to the synergistic effect of IFN-γ and TNF-α(17). Recently, experimental evidence accumulated the concept that IFN-γ inducible chemokines such as CXCL9 and its receptor CXCR3, play an important role in the initial stage of autoimmune disorders. Thus, the involvement of the CXCR3 signal pathway has involved autoimmune endocrine diseases(18).

Taken together with our finding, LLLI may reduce inflammation and pain in RA tissues by reducing the gene expression of CXCR3. This mechanism may be more general and underlie the beneficial effects of LLLI on inflammatory conditions in RA.

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**References**

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