Rheumatoid arthritis (RA) is an inflammatory joint disorder, whose progression leads to the destruction of cartilage and bone. Chemokines and their receptors are potential therapeutic targets in RA. Among these, it has been suggested that CXC chemokine 4 (CXCR4) and its ligand CXC ligand 12 (CXCL12) are involved in RA pathogenesis. Low-level laser irradiation (LLLI) is currently being evaluated for the treatment of RA; however, the molecular mechanisms underlying its effectiveness remain unclear.

Aim: To understand the anti-inflammatory effect of LLLI, we used the collagen-induced arthritis (CIA) rat as RA model and analyzed the gene expression profile in synovial membrane in the hind-paw joints of control, CIA and CIA + LLLI. Expression of CXCR4 and CXCL12 genes were also studied.

Materials and Methods: Total RNA was isolated from the synovial membrane tissue of CIA rat joints or CIA joints treated with LLLI (830 nm Ga-Al-As diode), and gene expression profiles were analyzed by DNA microarray (41,000 rat genes). The mRNA levels were confirmed by reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR. Immunohistochemical examination to examine CXCR4 protein expression was also carried out.

Results: DNA microarray analysis showed that CXCR4 gene expression was increased in CIA tissue and LLLI treatment significantly decreased CIA-induced CXCR4 mRNA levels. In contrast, CXCL12 did not show any significant changes. The microarray data of CXCR4 mRNA levels were further validated using RT-PCR and real-time PCR. Increased CXCR4 mRNA levels by CIA and its reduction following LLLI was successfully confirmed. CXCR4 production was increased in CIA joints and its production was decreased by LLLI.

Conclusion: Decreased CXCR4 expression may be one of the mechanisms in LLLI-mediated reduction of RA inflammation.

Key Words: Rheumatoid arthritis, rat joint, CXCR4, low-level laser irradiation.
and validate therapeutic targets \(^5\). The pathological features of CIA include proliferative synovitis coupled with infiltration of polymorphonuclear and mononuclear cells, pannus formation, cartilage degradation and bone erosion \(^6\).

Non-invasive physiological therapy such as low level laser irradiation (LLLI) could be important for managing pain. LLLI has been used in the therapy of particular diseases, the application leading to the reduction in the duration of acute inflammation, stimulation of tissue repair, relief from pain, and bone formation \(^7\). Different wavelengths of LLLI include He-Ne, Ga–Al–As and Ga–As. However, there are few studies on the mechanism underlying the anti-inflammatory effects of LLLI. Most of the studies deal only with the analgesic properties of laser and the efficacy of LLLI as a form of anti-inflammatory therapy \(^8\). The interaction between radiation and the biological system occurs at the cellular level but the mechanisms involved are still unknown. Some reports suggest that there are therapeutic advantages in the application of Ga–Al–As LLLI to inflammatory pathologies, when compared to placebos and other treatments \(^9^,^{10}\). LLLI has also been used to treat RA for many years with successful results \(^11\). However, the effect of Ga–Al–As LLLI on CXCR4 and CXCL12 gene expression in the inflamed arthritic joint has not been investigated to date.

In the present study, in order to understand the effectiveness of the Ga-Al-As diode on RA, we constructed CIA rat and examined the effect of LLLI on the gene expression of CXCR4 and CXCL12 as well as their protein expression in the synovial membrane of CIA rat joints.

**Materials and Methods**

**Animals**

Female Lewis (LEW/Crlj) rats 6 weeks old were obtained from Charles River Japan Inc. (Kanagawa, Japan). The rats were allowed free access to food and water available ad libitum at all times and were maintained on 12 h light/dark cycle (lights on 6:00 to 18:00) at 23±1°C, humidity 60±10% environment for a period of 1 week before use. The female rats weighing 151.6±11.9 g were randomly divided into 3 groups: control; type II collagen-induced arthritis (CIA) rat plus vehicle; CIA rat plus low level laser irradiation at therapeutic treatment (days 14), \(n=4\) in each group). 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).

**Induction of arthritis**

Type II collagen-induced arthritis (CIA) rat was induced in rats by multiple intradermal injections, at the base of the tail and into three to five other sites on the back, of 200 \(\mu\)g of bovine type II collagen (Sigma, Tokyo, Japan) in 250 \(\mu\)l 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). Rats were challenged again once weekly for 2 weeks with the same antigen preparation 7 days later. All animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (Somnopentyl®, Kyoritsu Seiyaku, Tokyo, Japan) at 25 mg/kg prior to immunization. CIA rats developed 2 weeks after the third immunization.

**LLL irradiation**

The low-level laser device (Model ZH-M143DJP; Matsushita Industrial Equipment Inc., Osaka, Japan) was used to irradiate the hind paw from knee to toe plated in a density at a wavelength of 830 nm. The laser beam was delivered by optical fibers and was irradiated uniformly in a circular area, 100 mm in diameter, 30 cm above the hind paw from knee to toe. The actual total energy corresponding to an exposure of 8 minutes 20 sec was 5.0 J/cm².

**Methods**

Rats were subdivided into the following groups: (1) control; (2) CIA plus vehicle; (3) CIA plus laser irradiation at therapeutic treatment (days 0-14). Hind paw inflammation was assessed by the increase in hind paw volume. It was performed with the low level laser irradiation at a dose of 5.0 J/cm². The LLL irradiation at therapeutic treatment and its vehicle formulation without LLL irradiation was irradiated to rats for 14 days from the day after arthritis induction. Effect of the LLL irradiation was compared with that of vehicle. LLL irradiations were applied to shaven legs, covering the paw from knee to toe. Thickness of hind paw was measured by calipers at a fixed location using microscope with an accuracy of 0.01 mm. The swelling reaction was recorded at different time points.

**DNA microarray analysis**

For gene expression analysis, on day 7 and day 14, total cellular RNA was isolated from hind-paw joint synovial membranes using the RNeasy Fibrous Tissue Midi Kit Isolation System (Qiagen Ltd.) and a FastPrep machine (FP120; BIO 101). cDNA synthesis was performed using a GeneAmp RNA PCR Kit (Applied
Biosystems, Foster City, California) and the isolated total RNA samples. Labelled cRNA synthesis was conducted using a Quick Amp Labeling Kit (Agilent, Santa Clara, CA) according to the manufacturer’s protocol. For hybridization, 1.65 μg of Cy3-labeled cRNA from LLLI-treated or vehicle-treated cells were combined and hybridized to a Whole Rat Genome Microarray 4×44K (41,000 rat genes and transcripts; Agilent), according to the manufacturer’s protocol. Finally, arrays were scanned using an Agilent Microarray Scanner (Agilent), and scanned images were captured using Agilent Feature Extraction software (Agilent Technologies, Inc Santa Clara, CA).

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). PCR Amplification was started with initial incubation at 95°C for 15 seconds to activate Taq DNA polymerase, and then performed at 95°C for 5 seconds and 56°C for 15 seconds using an adequate number of cycles. RT-PCR products were electrophoresed on a 1.5% agarose gel, followed by ethidium bromide staining to examine the sizes of the PCR products. Real-time PCR was carried out with SYBR Premix Ex Taq™ (Perfect Real-Time PCR, Takara, Japan). To quantitate gene expression 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 expression level 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 are described in the 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’-cgcaaggccccagactac-3’ (the forward primer for CXCR4 gene); 5’-cggagggaggctaggtgatg-3’; (the reverse primer for CXCR4 gene) (predicted size=214 bp); 5’-atccacctttccaggag-3’ (the forward primer for GAPDH); and 5’-atccgacttggtctagatag-3’ (the reverse primer for GAPDH gene) (predicted size=318 bp). Values were calculated as means ± standard deviation (SD). Comparisons were made between two groups using a Student’s t-test.

Immunohistochemical staining

The hindpaws of each rat were dissected on day 7 and day 14 and fixed immediately by immersion in 10% buffered formalin for 6 hours, and decalcified using 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 4 weeks at room temperature. The decalifying solution was changed twice a week. Tissue specimens were processed for paraffin wax embedding and multiple 4 μm sections were prepared from each block for immunohistochemical examination. For immunohistochemical analysis, serial sections were treated in a microwave oven four times with citrate buffer (pH 6.0) for 5 min at 960 W. Sections were hydrated in a humidified chamber with an anti-CXCR4 rabbit polyclonal (IMGENEX) antibody. Subsequently, sections were incubated with biotinylated goat anti-rabbit IgG (H+L) (VECTOR) followed by horseradish peroxidase streptavidin (VECTOR) for 1 hour at room temperature. Colour was developed using 3-3’-diamino benzidine tetrahydrochloride, and sections were counterstained with Mayer’s haematoxylin.

Statistical analysis

All results were expressed as means ± SD. Data was analyzed using one-way ANOVA followed by post hoc tests for pair wise comparisons (Tukey’s test). Differences were considered to be significant for p < 0.05.

**Table 1:** Effects of LLLI on Hind-Paw-Edema in the Collagen-Induced Arthritis Rat model

<table>
<thead>
<tr>
<th>Group</th>
<th>Before 28day</th>
<th>Before 21day</th>
<th>Before 14day</th>
<th>Before 7day</th>
<th>LLLI Start point 0</th>
<th>After 7day</th>
<th>After 14day</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>3.18±0.39</td>
<td>3.15±0.37</td>
<td>3.15±0.42</td>
<td>3.20±0.33</td>
<td>3.14±0.30</td>
<td>3.17±0.32</td>
<td><strong>3.19±0.27</strong></td>
</tr>
<tr>
<td>CIA</td>
<td>3.10±0.28</td>
<td>4.23±0.65</td>
<td>5.25±0.65</td>
<td>5.83±0.78</td>
<td>6.28±0.80</td>
<td>6.65±0.98</td>
<td><em>6.76±0.91</em></td>
</tr>
<tr>
<td>CIA+LLLI</td>
<td>3.24±0.20</td>
<td>4.35±0.74</td>
<td>5.21±0.96</td>
<td>5.80±0.89</td>
<td>6.32±0.98</td>
<td>5.60±0.88</td>
<td><em>5.33±0.70</em></td>
</tr>
</tbody>
</table>

The number of samples in each group were 4 rats. Values were given by the mean±SE (mm). The controlled rats were received sham-laser-irradiation. Significantly different from the value on the day 14 in CIA group than in control group (**p<0.01) and in CIA+LLLI group than in CIA group (*p<0.05).
Results

As shown in Table 1 and Figure 1, CIA significantly increased swelling in the rat hind-paw and LLLI reduced the CIA-induced swelling.

The raw intensity signals of the CXCR4 mRNA level in CIA rat joint tissues with or without LLLI were analysed using DNA microarray analysis. The CXCR4 gene was shown to be “Present” in Flag analysis with more than 2-fold changes. However, CXCL12 was shown to be “Present” in Flag analysis but did not show a big change. A scatter plot of CXCR4 and CXCL12 gene expression profiles are shown in Figure 2.

To further investigate the mRNA level of CXCR4 in samples, RT-PCR was performed. As shown in Figure 3, an amplified DNA band corresponding to CXCR4 in CIA rats was more intense than in the con-

Table 2: Raw intensity signals of CXCR4 and CXCL12 shown in DNA microarray.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genebank ID</th>
<th>Control (Flag*)</th>
<th>CIA (Flag*)</th>
<th>CIA+LLLI (Flag*)</th>
<th>CIA/Control (Fold**)</th>
<th>CIA+LLLI/CIA (Fold**)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR4</td>
<td>BC089804</td>
<td>101.2 (P)</td>
<td>530.8 (P)</td>
<td>271.9 (P)</td>
<td>5.6</td>
<td>-2.0</td>
</tr>
<tr>
<td>CXCL12</td>
<td>AF217564</td>
<td>710.1 (P)</td>
<td>451.3 (P)</td>
<td>717.1 (P)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR4</td>
<td>BC089804</td>
<td>191.2 (P)</td>
<td>445.8 (P)</td>
<td>350.9 (P)</td>
<td>2.3</td>
<td>-</td>
</tr>
<tr>
<td>CXCL12</td>
<td>AF217564</td>
<td>1108.1 (P)</td>
<td>713.2 (P)</td>
<td>603.3 (P)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Flag (P) or (A) indicate the reliability of the data according to present (P) or absent (A) of gene expression. ** Fold, fold change by normalization against the median of the corresponding to control. Genes that were changed signals at least 2.0-fold (up and down) were considered significant.
control, whereas LLLI treatment reduced the intensity of this DNA band after 1 week of treatment. In contrast, the mRNA level of GAPDH, a housekeeping gene used as control, was not different between control, CIA and CIA + LLLI.

<table>
<thead>
<tr>
<th>1-week</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
</tr>
<tr>
<td>CXCR4</td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
</tbody>
</table>

**Figure 3**: End-point PCR analysis. An ethidium bromide-staining pattern of the amplified PCR products using agarose gel electrophoresis.

Another experiment to determine the exact rates of enhancement of CXCR4 gene expression was performed using real-time PCR. The real-time PCR results were converted to mRNA copy units. As shown in **Figure 4**, there was an increase in CXCR4 gene expression in CIA samples when compared to control, and a significant decrease in LLLI samples when compared to that of CIA. Furthermore, this difference was statistically significant.

**Figure 4**: Real-time PCR analysis. Expression levels were normalised to that of the housekeeping gene GAPDH. *p<0.001; N=4.

Finally, the phenotypic expression of CXCR4 was examined using anti-CXCR4-antibody. A significantly positive CXCR4 staining was found in CIA rat joint tissues when compared to control, whereas weak staining was observed in the CIA + LLLI group when compared to the CIA group, suggesting that LLLI reduced CXCR4 protein production.

**Figure 5**: Immunostaining of CXCR4. Representative photomicrographs of sections were stained with polyclonal anti-CXCR4 antibody on day 14 after LLLI. B, Bone; S, synovial membrane.

**Discussion**

RA is caused mainly by CD4+ memory T cell accumulation in the inflamed synovium. CXCR4 is highly expressed on the surface of memory T cells, and CXCL12 concentration is elevated in the synovium of RA patients. CXCL12 couples monogamously with its...
receptor CXCR4. Furthermore, CXCL12 stimulates migration of memory T cells, thereby inhibiting T cell apoptosis. These findings indicate that the CXCR4-CXCL12 interaction plays a crucial role in the accumulation of T cells in the RA synovium and play an important role in the pathogenesis of RA.

CXCR4 antagonists do not prevent but ameliorate arthritis in the CIA murine model. Thus, the chemokine receptor CXCR4 appears to be an attractive therapeutic target for RA in the light of these observations. However, the effect of LLLI on CXCR4 gene expression in the inflamed arthritic joint has not been investigated to date.

In the present study, we constructed CIA rat as an experimental model of RA in rats and examined the effect of LLLI on CXCR4 gene expression. Our results clearly demonstrated that LLLI significantly reduced CXCR4 gene transcription and CXCR4 protein expression in CIA joints. However, CXCL12 did not significantly alter its gene expression in CIA and was not affected by LLLI in our experimental conditions. Right now, we have no hypothesis to explain these results. CXCR4 significantly changed its gene expression at week 1, and such a big change was diminished at week 2, suggesting CXCL12 gene expression changes mediated by LLLI may happen in a few days. Another study examining chemokine signalling events in shorter periods of time may be important.

In conclusion, LLLI decreases the production of CXCR4 by affecting the transcription process in CIA RA synovial membrane tissues. This may be one of molecular mechanisms used to reduce inflammation and pain by LLLI in RA therapy.

Acknowledgements

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