Reduction of Matrix Metalloproteinase-3 Expression in Rheumatoid Arthritis Rat Joints by Light-emitting Diode

Shu-Guei Wang¹, ², Noboru Kuboyama³, Sheng-Yang Lee², and Yoshimitsu Abiko¹

Departments of ¹Biochemistry and Molecular Biology, ³Pharmacology, Nihon University School of Dentistry at Matsudo, Chiba 271-8587, Japan
²Graduate Institute of Oral Rehabilitation Sciences and School of Dentistry, Taipei Medical University, Taipei, Taiwan

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Abstract
Rheumatoid arthritis (RA) is a systemic autoimmune disease that involves inflammation and joint pain and causes bone resorption. Irradiation with light-emitting diodes (LEDs) is being evaluated for the treatment of RA, but the underlying molecular mechanisms remain unclear. To determine whether anti-inflammatory effects are provided, LED irradiation was provided for rats with collagen-induced RA (CIA), and total RNA was isolated from the synovial tissues of CIA rat joints. Gene expression profiles were then monitored by DNA microarray. LED irradiation decreased the swelling in CIA rat knee joints. DNA microarray analysis demonstrated that expression of many genes was altered in RA, including increased MMP3 mRNA levels, while MMP3 mRNA levels were reduced by LED irradiation. These reductions in MMP3 mRNA levels by LED irradiation were confirmed by RT-PCR and real-time PCR. Immunohistochemical analysis demonstrated strong staining for MMP3 in the CIA group, and LED irradiation significantly reduced this staining. Clinical features of reduced swelling in CIA knee joints suggest that LED irradiation might be useful as RA therapy. Since MMP3 has been identified as an important factor in the pathogenesis of RA, reducing MMP3 expression by LED irradiation might offer an important mechanism for reducing inflammation in RA.

Introduction
Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by pain and inflammation, progressive joint destruction, significant disability, systemic manifestations and premature mortality (1). This disease is characterized by the infiltration of leukocytes into the synovial tissue and synovial fluid of joints, ultimately leading to the destruction of cartilage and bone (2). 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 osteoclasts (3–5).

Many treatments are available to relieve RA-associated pain, such as non-steroidal anti-inflammatory agents and anti-rheumatic steroids. However, some of these agents have side effects when used for a prolonged period and are not effective in all patients. Therapeutic options for RA by reducing the inflammatory mediators have been discussed, and one recent strategy of importance has been to block the actions of IL-6 and TNF-α using neutralizing antibodies (6, 7).

On the other hand, collagen-induced RA (CIA) has been widely used for research into the pathogenesis of RA, because the pathological features are similar to RA, showing proliferative synovitis with infiltration of polymorphonuclear and mononuclear cells, pannus formation, cartilage degradation and erosion of bone (8).

Since the early 1960s, low level irradiation (LLLI) therapies using light at different wavelengths, including He-Ne, Ga-Al-As and Ga-As lasers, have been used in the therapy of specific diseases. These therapies have been aimed at reducing the duration of acute inflammation, stimulating tissue repair, achieving relief from pain, and...
promoting bone formation (9). We have previously examined the effects of a Ga-Al-As (830-nm) diode laser on RA, and reported that this laser irradiation reduced inflammation in CIA rats by reducing gene expression of IL-1β (10), CXCL13 (11), CXCR3 (12), CCL2 (13), and MMP3 (14). Other non-invasive therapies such as irradiation with a light-emitting diode (LED) could also be useful for managing pain, with a recent report showing that using an LED instead of a diode laser instrument as a light source can also achieve anti-inflammatory effects (15). However, little is known regarding the mechanisms by which light irradiation achieves anti-inflammatory effects.

The present study used a CIA rat and employed DNA microarray technology and immunohistochemistry to examine the effects of LED irradiation on RA inflammation.

**Materials and Methods**

**Animal experiment**

Female Lewis (LEW/Crl) rats, 6 weeks old, were obtained from Charles River Japan (Kanagawa, Japan) and injected with type II collagen (Sigma, Tokyo, Japan) in 250μl of 0.1 M acetic acid emulsified in an equal volume of complete Freund’s adjuvant (Difco Labs, Michigan, USA) containing 2mg/ml muramyl dipeptide (Wako, Tokyo, Japan) by multiple intradermal injections. Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (Somnopentyl®; Kyoritsu Seiyaku, Tokyo, Japan) at 25mg/kg prior to the injection. All animals were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals of Nihon University, School of Dentistry at Matsudo (No. 04-008). LEDs (Nisshin LED, Strobo Luminar, PLD-2000-50, Panasonic, Yokohama, Japan) were used to irradiate CIA rat joints. The irradiation time was 20 min, pulse time=50 ms, providing an incident energy density of 7.64J/cm². LED irradiation was performed three times a week for 2 weeks.

**DNA microarray analysis**

Total cellular RNA was isolated using Trizol (Life Technologies GIBCO BRL, Rockville, MD, USA) with a FastPrep machine (FP120; BIO 101, Funakoshi, Tokyo, Japan), and cDNA was synthesized using a Superscript II RNaseH (−) reverse transcriptase system (Invitrogen, Tokyo, Japan) with oligo d(T) (12-18) primers at 42°C for 1 h. Total RNA was extracted using an RNAqueous kit (Ambion, Austin, TX) according to the protocol provided by the manufacturer. Fluoro-dye labeled cRNA was synthesized using a Low RNA Input Fluor Linear Amp kit (Agilent, Santa Clara, CA, USA). For hybridization, 3.5μg of Cy3-labeled cRNA from treated and vehicle-treated cells was combined and hybridized to an Agilent 44K Whole Rat Genome Oligo Microarray (41,000 rat genes and transcripts; Agilent) according to the instructions from the manufacturer. Finally, the arrays were scanned using a GeneArray Scanner (Agilent), and the scanned images were analyzed using GeneSpring version 4.0 software (Silicon Genetics, Redwood City, CA, USA). We set a cut-off of a ≥1.5-fold change to represent “induction” or “repression.”

**Pathways analysis**

Ingenuity Pathways Analysis (IPA) version 4.0 software (Ingenuity Systems, Mountain View, CA, USA) was used to search for possible biological processes, pathways and networks. This web-based entry tool allows for the mapping of gene expression data into relevant pathways based on functional annotations and known molecular interactions. The identified genes were also mapped to genetic networks in the IPA database and ranked by score. This score reflects the probability that a collection of genes equal to or greater than the number in a network could be achieved.

**RT-PCR and real-time PCR analysis**

RT-PCR and real-time PCR reactions were undertaken using a DNA thermal analyzer (RFN-Gene™ 6000; Corbett Life Science, Sydney, Australia). Amplification by PCR was started with an initial incubation at 95°C for 15s to activate the Taq DNA polymerase, then at 95°C for 5s and 56°C for 15s. 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 using SYBR Premix Ex Taq™ (Perfect Real-Time PCR; Takara, Tokyo, Japan) and a Green PCR kit (Qiagen, Dusseldorf, Germany). To calculate fold changes in gene expression, 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 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 the operation manual (version 1.7.40, 2006). Each assay was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. DNA primer sequences were: 5'-gctcatcctacccattgcat-3' (forward primer for MMP3 gene) and 5'-gcttgtgcatcagctccata-3' (reverse primer for MMP3 gene) (predicted size=219bp); and 5'-atcaccatcttccaggag-3' (forward primer for GAPDH) and 5'-atcgactgtggtcatgag-3' (reverse primer for GAPDH gene) (predicted size=318bp). Values are provided as means ± standard deviation (SD). Comparisons were made between two groups using Student’s t-test.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded specimens were subjected to antigen retrieval and endogenous peroxidase blocking (30 min), then rinsed with phosphate-buffered saline (PBS). Immunostaining was performed using Elite ABC kits (Vector, Burlingame, CA, USA) and diaminobenzidine (Kirkegaard & Perry, Gaithersburg, MD, USA) as a chromogen using mouse anti-MMP3 antibody (1:400; Santa Cruz Biotech, Santa Cruz, CA, USA). Peroxidase-conjugated rabbit anti-mouse immunoglobulin diluted 1:10 in PBS supplemented with 2-vol% heat-inactivated normal human serum was used. Peroxidase activity was visualized with 0.06% diaminobenzidine (Walter, Kiel, Germany) and 0.01 vol% H₂O₂.

**Results**

First, we examined the effects of LED irradiation on RA inflammation using the rat CIA model. The hind-paw of CIA rats showed significantly increased swelling, and LED irradiation reduced this swelling (Fig.1). Comparing reduction rates of knee joint swelling between LLLI (830 nm Ga-Al-An diode laser and LED irradiation) suggested that LED irradiation also provided effective relief from inflammation (Fig.2).

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**Fig. 1.** Effects of LED irradiation on swelling in the CIA rat.

**Fig. 2.** Significant reduction of swelling in the CIA rat with LED irradiation. *p<0.05; **p<0.01, N=5.
Fig. 3. IPA gene network search. A) Molecules in the network from CIA vs. control. MMP3 is marked by a yellow box. B) Molecules in the network from CIA+LED vs. CIA. MMP3 is marked by a yellow box. C) Relationship between molecules in the network (CIA+LED vs. CIA). Red arrow indicates MMP3 gene.
Gene expression profiles of control, CIA and CIA + LED synovial membrane tissues of rat joint were analyzed using a high-throughput DNA microarray. Data mining was carried out using cut-off values of ≥2.0 for "induction" or ≤0.5 for "repression". A gene network search of the IPA database system revealed that CIA vs. control networks indicated functional relationships between gene products based on known interactions reported in the literature including MMPs. MMP3 gene expression was up-regulated in CIA rats compared with controls (Fig.3A), and was down-regulated by LED irradiation (Fig.3B). The links from gene expression networks demonstrated that IL-1, IL-1R and NFkB1-RelA were associated with down-regulation of MMP3 gene expression (Fig.3C).

Next, we examined canonical pathways using the IPA system. CIA highly increased gene expression of IL-1, IL-1R, c-fos, MMP13 and MMP3 genes (Fig.4). Interestingly, LED irradiation suppressed TNF-IL1 signaling by decreasing IL-1R, MMK and MMP3 gene expressions in CIA rat joint tissues.

To validate the results obtained from IPA analysis, we focused on MMP3 and examined gene expression by RT-
PCR. An amplified DNA band corresponding to the MMP3 gene was more intense in CIA rats than in control rats, whereas LED irradiation reduced the intensity of this band (Fig.5). In contrast, mRNA levels of GAPDH, a housekeeping control, did not differ between CIA rats and LED-irradiated CIA rats.

Finally, phenotypic expression of MMP3 proteins was examined by immunohistochemistry. MMP3 proteins were detected in synovial tissues of all groups of rats. Synovial tissue from control rats exhibited very weak constitutive expression of MMP3 protein, but this was strongly up-regulated in CIA rats. As we expected, MMP3 protein expression was significantly decreased in CIA rats treated using LED irradiation. Staining was negative when the primary antibody was omitted (Fig.6).

Discussion

Osteoarthritis is a chronic disease of synovial joints. Degradation of the extracellular matrix in articular cartilage is a major cause of joint destruction. Cartilage degradation is mediated by MMPs, principally MMP1, MMP3, and MMP13, and MMP3 cleaves a variety of extracellular matrix components, including proteoglycans, collagens, and procollagens (16). Chondrocytes respond to various stimuli, such as pro-inflammatory cytokines or mechanical load, by inducing production of MMPs. In the MMP gene family, MMP1 and MMP3 are produced by synovial lining cells and can be detected in synovial fluid from individuals with RA; concentrations of MMP3 in synovial fluid from RA patients have been reported as approximately 20-fold higher than those of MMP1 (17). MMP3 levels appear normal in sera...
from individuals with fibromyalgia, osteoarthritis, ankylosing spondylitis, and acute inflammation, but are higher levels in RA patients and appear to correlate with levels of C-reactive protein, suggesting that MMP3 levels might offer a useful predictor of joint destruction (18).

MMP3 actively destroys cartilage matrix components such as proteoglycan and fibronectin, and can activate pro-MMPs (19). Our pathway analysis showed that MMP1 gene expression is unaltered in CIA, whereas MMP3 is up-regulated. Our key finding was that MMP3 expression in CIA was down-regulated following LED irradiation. Serum MMP3 levels reportedly correlate with MMP3 levels produced by the synovium, and thus reflect the level of activity of rheumatoid synovitis (20). Serum MMP3 levels also decrease in patients taking anti-RA drugs administration (21). Taken together with the results of these investigations, the reduction of MMP3 expression in CIA synovial membrane tissues following LED treatment may offer an important explanation of the beneficial effects of LED treatment on RA.

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