Reduction of Matrix Metalloprotease 3 in Rheumatoid Arthritis Rat by Low Level Diode Laser Irradiation

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Abstract

Rheumatoid arthritis (RA) is an inflammatory joint disorder, whose progression leads to the destruction of cartilage and bone. Low-level laser irradiation (LLLI) is currently being evaluated for the treatment of RA ; however, the molecular mechanisms underlying its effectiveness remain unclear. To understand the anti-inflammatory effect of LLLI, we used the collagen-induced RA rat (CIA) model, and analyzed the gene expression profile in synovial membrane in knee joints. 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 laser), and gene expression profiles were analyzed by DNA microarray (41,000 rat genes) and Ingenuity Pathway Analysis system (IPA). The mRNA levels were confirmed by reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR. Proteins expression was also carried out by immunohistochemistry. DNA microarray and IPA network/canonical pathway analysis showed that matrix metalloproteases (MMPs) signaling was involved, among MMPs, MMP3 gene expression was increased in CIA tissue, and LLLI significantly decreased the gene expression. The DNA microarray data of these mRNA levels were further validated and confirmed using RT-PCR and real-time PCR. Immunohistochemical study also demonstrated the reduction of MMP2 protein level by LLLI. Since MMP3 These findings suggest that inhibitory effect of MMP3 expression may be a part of decreased CXCL1 and ICAM1-1 expressions may be a part of the mechanisms in LLLI-mediated reduction of RA inflammation.

Keywords :

rheumatoid arthritis, rat joint, matrix metalloprotease 3, low-level laser irradiation

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 destruction of cartilage and bone (2). Inflammation of the synovial lining of joints results in further increase in the expression 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 osteoclasts (3-5).

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 bone (6).

A non-invasive physiological therapy such as low level laser irradiation (LLLI) could be important for

managing pain. Since the early 1960s with different wavelength laser therapies have been used in the duration of acute inflammation, the stimulation of tissue repair and the relief from pain, and bone formation (7). Different wavelength of laser including Ga-Al-As diode laser. However, there are few studies on the mechanism of anti-inflammatory effects; most of the studies deal only with the analgesic properties of laser and the efficacy of LLLI as an anti-inflammatory therapy is still controversial (8). It is necessary to analyze simultaneous expression levels of thousands of genes. In this context, a powerful way to gain insight into the molecular complexity and pathogenesis of arthritis has arisen from oligonucleotide-based microarray technology, because this platform provides an opportunity to analyze simultaneously the expression of a large number of genes in disease tissues.

In the present study, whole homogenized rat knee joints synovium from normal, CIA and CIA with LLLI rats were analyzed for differential gene expression using the microarray. Furthermore, the genes identified were analyzed for network by ingenuity pathway analysis (IPA) software to identify networks of interacting genes.

Materials and Methods

Animal experiment

Female 6-week-old Lewis (LEW/CrlCrIj) rats 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 in 12 h light/dark cycle (lights on 6:00 to 18:00) at $23 \pm 1^\circ\text{C}$, $60 \pm 10\%$ humidity for a period of 1 week before use. Type II collagen-induced arthritis (CIA) 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 μg of bovine 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). 14 days later the rats were again challenged with the same antigen prepa-

ration. All animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (SomnopenylR, Kyoritsu Seiyaku, Tokyo, Japan) at 25 mg/kg prior to immunization. CIA rats developed 2 weeks after the third immunization. All animals were maintained and used in accordance with the guide to the Care and Use of Laboratory Animals of Nihon University, School of Dentistry at Matsudo (No. 04-008).

LLLI experiment

A Ga-Al-As diode laser device (Model ZH-M143 DJP; Panasonic Industrial Equipment Inc., Japan) was used as a low-power 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 hind-paw to be irradiated, giving a spot size of 78.5 cm^2 , with an incident power density of approximately 6.4 mW/cm^2 . The irradiation time was 20 min, which was equivalent to an incident energy density of 7.64 J/cm^2 . LLI experiment was carried out three times a week for a period of 2 weeks.

DNA microarray analysis

Total cellular RNA was isolated from the knee joint synovial membrane using RNeasy Fibrous Tissue Midi Kit Isolation System (Qiagen Ltd.) by a FastPrep machine (FP120; BIO 101). The cDNA synthesis was performed using a GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, Calif.) with samples of total RNA. Labeled cRNA synthesis was used Quick Amp Labeling Kit (Agilent, Santa Clara, CA) according to the manufacturer's protocol. For hybridization, 1.65 μg of cy3-labeled cRNA from treated and vehicle-treated cells was combined and hybridized to a Whole Rat Genome Microarray $4 \times 44\text{ K}$ (41,000 rat genes and transcripts; Agilent) according to the manufacturer's protocol. Finally, the arrays were scanned using an Agilent Microarray Scanner (Agilent), and the scanned images were captured using Agilent Feature Extraction software

(Agilent) and analyzed using GeneSpring GX10 software (Agilent Technologies, Inc Santa Clara, CA).

Ingenuity Pathways Analysis (IPA)

To facilitate the analysis of the gene microarray data, and to relate gene up- and down-regulation to underlying biological processes, Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com) has proposed using in silico genomics network analysis [8]. IPA version 4.0 (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 their functional annotation and known molecular interactions [9]. This knowledge coming from published, peer-reviewed scientific publications is stored in the Ingenuity Pathways Knowledge Base (IPKB), and is continuously updated. A molecular network of direct or indirect physical, transcriptional, and enzymatic interactions between mammalian orthologs was computed from this knowledge base. By comparing the imported microarray data with the IPKB, the list of genes was transformed in a set of relevant networks, focus genes and canonical pathways were identified, and functional annotation was performed.

Focus genes identified by the IPA program were categorized based on location, cellular components, and reported biochemical, biologic, and molecular functions using the software. The identified genes also were mapped to genetic networks in the IPA database and ranked by score. The score reflects the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone. Canonical pathways analysis identified molecular pathways from the IPA library of canonical pathways (part of the IPKB) that were most significant to the data set. Genes from the data set that were associated with a canonical pathway in the IPKB were considered for the analysis.

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 °C for 15 seconds to activate the Taq DNA polymerase, and then performed at 95 °C for 5 seconds and 56 °C 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 the PCR products. Real-time PCR was carried out with SYBR Premix Ex Taq™ (Perfect Real-Time PCR, Takara, Japan). 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 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'-gtctatcctacc cattgcat-3' (the forward primer for MMP3 gene); 5'-gcttggtgcatcagctccata-3' (the reverse primer for MMP3 gene), (predicted size=219 bp); 5'-atcaccatct tccaggag-3' (the forward primer for gapdh); and 5'-atcgactgtggtcatgag-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.

Immunohistochemistry

The hindpaws of each rat were dissected and fixed immediately by immersion in 10% buffered formalin for 6 hrs and decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 4 weeks at room temperature. The decalcifying solution was changed twice each week. Tissue specimens were processed

for paraffin wax embedding and multiple 4 μ m sections were prepared from each block for immunohistochemical examinations. For immunohistochemistry 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 saturated with 10% BSA in PBS with 0.1% Triton X-100 and incubated overnight at 4 °C in a humidified chamber with the anti-MMP3 rabbit polyclonal antibody (LifeSpan Bioscience, Inc., WA, USA). Subsequent incubations were made with biotinylated goat anti rabbit IgG (H+L) (Vector) followed by horseradish peroxidase streptavidin (Vector) for 1 hr at room temperature.

The color was developed with 3-3'-diamino benzidine tetrahydrochloride. Sections were counterstained with Mayer's hematoxylin.

Results

Gene expression profiles of control, CIA and CIA + LLLI rat joint synovial membrane tissues were analyzed using a high-throughput DNA microarray. Data mining was carried out by cut-off values of ≥ 2.0 for "induction" and ≤ 0.5 for "repression". To further refine the significance of the altered gene identified in DNA microarray data, we investigated possible biological interactions using the IPA tool. We asked

(A) Molecules in net work (CIA vs control)

Molecules in Network	
$\uparrow$ A2M, $\uparrow$ CCL7*, $\uparrow$ DLGAP4, Fgf, $\uparrow$ FN1*, $\uparrow$ GDF5, $\uparrow$ HTRA1, Igf, $\uparrow$ IGFBP5*, Igfbp, $\uparrow$ MMP2*, $\uparrow$ MMP3, $\uparrow$ MMP7, $\downarrow$ MMP8, $\uparrow$ MMP10, $\uparrow$ MMP11, $\uparrow$ MMP12, $\uparrow$ MMP14, $\uparrow$ MMP15, $\uparrow$ MMP19*, $\uparrow$ MMP25, $\downarrow$ MMP27, Mmp, $\uparrow$ MMP23B, $\downarrow$ MUG1* Δ , $\downarrow$ MYOC, $\uparrow$ PARVG*, $\uparrow$ PCOLCE, $\uparrow$ SERPINA3, $\downarrow$ SERPINB2, $\downarrow$ TGFB1*, $\uparrow$ TIMP1, Timp, Trypsin, $\uparrow$ VANGL1*	

(B) Network search of MMP family (CIA vs control)

the network search in the IPA database system using MMP3 as a landmark, and identified a network which included many MMP gene families in network molecules (Fig.1A). The CIA *vs* control networks indicated functional relationships between gene

products based on known interactions reported in the literature including MMPs. The relation of molecules in networks (CIA *vs* control) are illustrated Fig. 1B. The MMP3 gene expression was highly up-regulated (17.7-fold) in CIA rat when compared with

(A) Molecules in network (CIA + LLLI *vs* CIA)

Molecules in Network
↑ALDOA, Ap1, ↓ASGR1, ↑CA3, ↑CA1 (includes EG:759), Carbonic anhydrase, ↓CD34, ↑CTSG, ↓ENG, ↓ETS2*, ETS, ↓FOLR2, ↓FOSB, ↓FOSL1, ↓GLYCAM1, ↓HPGD, Igfbp, ↓MFAP4, ↓MMP3, ↓NFIC, Nuclear factor 1, ↑PADI4, ↑PKLR, ↑PPP2R5A*, ↓PTPRG*, ↓RLN2Δ, ↑SELL, ↓SERPINC1*, ↑SFTPD, ↑STAB2, STAT1/3/5 dimer, ↓TIMP1, ↓TIMP3, Timp, ↓UCN

(B) Network search of MMP family (CIA + LLLI *vs* control)

Fig. 2. Network search of CIA+LLLI *vs* CIA using IPA sytem. (A) Molecules in the network from CIA+LLLI *vs* CIA using MMP3 as a landmark (marked by yellow). (B) The relation of molecules in networks (CIA+LLLI *vs* CIA). Arrow indicates MMP3 gene.

control.

Next we searched a network of effect of LLLI on CIA (CIA+LLLI vs CIA). As we expected, the MMP3 gene expression was down-regulated in LLLI rats when compared with CIA rats (Fig. 2AB).

Gene expression profiles of (CIA vs control) and (CIA+LLLI vs CIA) were summarized as the scatter plots shown in Fig. 3.

The IPA system is not only able to produce a gene network map but also can facilitate the elucidation of canonical pathway analysis. Relationships between networks generated by the IPA system and known pathways were further investigated by IPA canonical pathway analysis. We focused on “Role of Macrophages, Fibroblasts and Endothelial cells in Rheumatoid Arthritis” cytokine signaling pathway. As shown in Fig. 4, CIA highly increased gene expression of MMP3 gene (17.7-fold) with TNFR, IL-1 and IL-1R and c-jun genes, but not MMP1 and MMP13 genes. Interestingly, LLLI suppressed the signaling by decreasing IL-1R and MMP3 gene expression (3.6-fold) by LLLI in CIA rat joint tissues.

In order to validate the results obtained from IPA, we examined the MMP3 gene expression by RT-PCR. As shown in Fig. 5, an amplified DNA band corresponding to MMP3 gene in CIA rats was more intense than in the control rats, whereas LLLI reduced the intensity of this DNA band. In contrast, mRNA levels of GAPDH, a housekeeping control, were not different between the CIA rats and LLLI-treated CIA rats.

To determine the exact rates of enhanced expression for MMP3 gene, we performed real-time PCR. The real-time PCR results were converted to mRNA relative copy units were calculated. As shown in Fig. 6, there was an increase in expression of MMP3 gene in CIA rats, and a significant decrease in expression following LLLI.

Finally, phenotypic expression of MMP3 proteins was examined by immuno-histochemistry. MMP3 proteins could be detected in the synovial tissues of all groups of rats. The synovial tissue from control rat exhibited very weak constitutive expression of MMP3 protein, and was strongly up-regulated in

CIA rats. As we expected, MMP3 protein expression was significantly decreased in CIA rats by LLLI. Staining was negative when the primary antibody was omitted (Fig. 7).

Fig. 3. Canonical pathway analysis of “Role of Macrophages, Fibroblasts and Endothelial cells in Rheumatoid Arthritis”. Red, up-regulated; green, down-regulated. First line under mark is fold change, second line is raw intensity of CIA, and third line is raw intensity of CIA+LLLI.

Fig. 4. Scatter plot of gene expression of MMP gene families. Left, CIA vs control ; right, CIA+LLLI vs CIA.

Discussion

RA is a persistent, systemic inflammatory disease, characterized by inflammation involving multiple cell types, resulting in the progressive destruction of the affected joints. Among dozens of MMPs, MMP3 has been reported to be the major enzyme produced

by fibroblasts and macrophage-like cells in the synovium, and the level of MMP3 has been reported to be significantly higher in synovial fluids from patients with RA (9-11). Destruction of cartilage is a pathological feature in RA, and is a major cause of joint dysfunction in RA patients. Many proteinases

Fig. 5. RT-PCR analysis of mRNA levels of MMP3 gene. An ethidium bromide-staining pattern of the amplified PCR products using agarose gel electrophoresis.

Fig. 6. Real-time PCR analysis of MMP3 gene mRNA levels. Results were expressed as relative mRNA copy unit by normalization to a housekeeping gene (*GAPDH*). * $p < 0.01$, $n = 4$.

are expressed in RA joint tissues of RA patients. MMPs are believed to have a key role in the joint destruction in the arthritides (12).

MMPs, a gene family of neutral Zn^{2+} dependent metalloproteinases, which are classified into five subgroups: (1) collagenases including tissue collagenase (MMP1), neutrophil collagenase (MMP8),

Fig. 7. Immunohistochemical study of MMP3 protein expression in rat synovial tissue. Ab, antibody against MMP3; S, synovial membrane; B, bone. Bar = 100 μm .

and collagenase 3 (MMP13); (2) gelatinases including gelatinase A (MMP2) and gelatinase B (MMP9); (3) stromelysins including stromelysin 1 (MMP3) and stromelysin 2 (MMP10); (4) membrane-type MMPs including MT1-MMP (MMP14), MT2-MMP

(MMP15), MT3-MMP (MMP16), MT4-MMP (MMP17), and MT5-MMP (MMP24); (5) other MMPs including matrilysin (MMP7), stromelysin 3 (MMP11), metalloelastase (MMP12), MMP19, enamelysin (MMP20), and MMP23 (13).

Our gene expression profiles between control and CIA groups using a DNA microarray demonstrated that MMP2, 3, 7, 10, 11, 12, 14, 15, 19, and 25 genes were up-regulated in CIA when compared with control (healthy normal subjects) shown in Fig. 1. Among MMP gene family, MMP1 and MMP3 are produced by synovial lining cells and can be detected in synovial fluid of RA (14), whereas MMP2 is produced by stromal cells in the sublining synovial layer (15). MMP3 is active against cartilage matrix components, such as proteoglycan and fibronectin, and can activate pro-MMPs (16). Our pathway analysis showed that MMP1 gene did not altered by LLLI, whereas only MMP3 gene expression was down-regulated in CIA by LLLI (Fig. 3).

MMP3 is also known as a useful marker for prediction of joint destruction (17). It has been reported that serum MMP-3 levels correlate with MMP3 levels produced by the synovium, and thus reflect the level of activity of rheumatoid synovitis (18). In fact, the serum MMP-3 level decreases in patients who respond to anti-RA drugs (methotrexate and sulfasalazine) treatment (19).

Taken together with these investigation, inhibition of MMP3 expression in CIA synovial membrane tissues by LLLI treatment may be one of important mechanisms and may explain the beneficial effects of LLLI treatment on the RA.

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