REDUCTION OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 EXPRESSION IN RHEUMATOID ARTHRITIS RAT JOINTS WITH LIGHT-EMITTING DIODE PHOTOTHERAPY

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Background: Rheumatoid arthritis (RA) is a systemic autoimmune disorder that involves inflammation and pain of the joints. Light-Emitting Diode (LED) irradiation is being evaluated for treating RA; however, the mechanism is unclear. Monocyte chemotaxis protein (MCP)-1 is a key chemokine in the inflammatory status of RA, and MCP-1 levels in plasma are described as a marker for joint inflammation in RA.

Aim: To understand the mechanism of the anti-inflammatory effect of LED irradiation on RA, the expression of MCP-1 was examined in the knee joints of collagen-induced arthritis (CIA) rats.

Materials and methods: The rats were immunized with type II collagen and CIA development was confirmed. CIA rat joints were irradiated with LED energy (3 sessions/week, 2 weeks. 840 nm, 2 J/cm²). Total RNA was isolated from the rat knee joint tissues and the MCP-1 mRNA levels were monitored with the reverse transcription polymerase chain reaction (RT-PCR) technique and real-time PCR. MCP-1 production in the rat knee joints was analyzed immunohistochemically.

Results: RT-PCR analysis demonstrated that MCP-1 mRNA levels had increased in CIA animals when compared to controls, and LED irradiation significantly reduced the gene expression in CIA rats. Real-time PCR analysis confirmed a significant reduction in MCP-1 gene expression. The immunohistochemical analysis demonstrated strong MCP-1 staining in CIA rat joint synovial membrane tissue, and LED irradiation significantly reduced the staining.

Discussion: Since MCP-1 has been identified as an important chemokine in the pathogenesis of RA, the reduction of MCP-1 expression would appear to be one of the mechanisms in the reduction of inflammation by LED irradiation.

Conclusion: LED irradiation reduced RA-related inflammation through the reduction of MCP-1 gene expression in CIA rat knee joint synovial tissue.

Key words: Rheumatoid arthritis, rat joint, LED, MCP-1.

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).

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Many treatments are available to relieve RA-associated pain, such as non-steroidal anti-inflammatory agents (NSAIDs) and anti-rheumatic steroids. However, the prolonged use of some of these agents is associated with side effects and are not effective in all patients. Since the early 1960s, phototherapy with laser energy at different wavelengths, including He-Ne, Ga-Al-As and Ga-As lasers, has been used in the treatment of specific diseases. These treatments have been aimed at reducing the duration of acute inflammation, and stimulating tissue repair, achieving relief from pain, and promoting bone formation. More recently irradiation with non-laser sources such as new-generation light-emitting diodes (LEDs) has attracted attention in the management of inflammation and pain, with a recent report showing that using an LED instead of a diode laser system as phototherapeutic source could also achieve anti-inflammatory effects. However, little is known regarding the mechanisms by which light irradiation achieves anti-inflammatory effects. Recently, it has been reported that the induction of monocyte chemoattractant protein 1 (MCP-1) signaling is important in the progression of RA. So this could be a potential target for phototherapy intervention in the treatment of RA.

Collagen-induced arthritis (CIA) has been widely used for research in 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. However, the effect of LED irradiation on the CIA rat has not yet been elucidated. In the present study, we examined the effect of LED irradiation on MCP-1 expression in CIA rat knee joints.

Materials and Methods

Animal experiment

Female Lewis (LEW/CrlCrj) rats, 6 weeks old, were obtained from Charles River Japan (Kanagawa, Japan) and underwent multiple intradermal injections 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). Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (Somnopen, Yokohama, Japan) at 25 mg/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. 04-0008). LEDs (PLD-2000-50, 840 nm, Panasonic, Yokohama, Japan) were used as the light source. The LEDs were set up 8 cm from the target tissue, delivering 840 nm in the near infrared wavelength, at an incident irradiance of 4 mW/cm² for 8 min 20 s to give an energy density of 2 J/cm². LED irradiation was performed three times a week for 2 weeks.

Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR analysis

The RT-PCR and real-time PCR techniques were performed with 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 15 s to activate the Taq DNA polymerase, then at 95°C for 5 s and 56°C for 15 s. RT-PCR products were electrophoresed on 1.5% agarose gel, followed by staining with ethidium bromide to examine their size. Real-time PCR was carried out using a SYBR Premix Ex Taq™ system (Perfect Real-Time PCR; Takara Co. Ltd, Tokyo, Japan) and a Green PCR kit (Qiagen, Dusseldorf, Germany). To calculate the 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). DNA primer sequences were: 5’-gtgctgaccc-caaataggg-3’ (forward primer for MCP-1 gene) and 5’-tgaggtgtggttgaaagaga-3’ (reverse primer for MCP-1 gene) (predicted size=185 bp); and 5’-atcacatctctcaggg-3’ (forward primer for GAPDH) and 5’-atcgaatggtagctag-3’ (reverse primer for GAPDH gene) (predicted size=318 bp). Values are provided as means ± standard deviation (SD). Comparisons were made between two groups using the 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 with rabbit polyclonal anti-MCP-1 antibody.
(ABCAM, CA, USA). Peroxidase-conjugated goat anti-rabbit immunoglobulin (Funakoshi, MP Biomedicals Inc., Tokyo, Japan) 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

As shown in the RT-PCR analysis results, an amplified DNA band corresponding to the MCP-1 gene was more intense in CIA rats than in control rats, whereas LED irradiation reduced the intensity of gene expression (Figure 1). In contrast, mRNA levels of GAPDH, a housekeeping control, did not differ in control, CIA or LED-irradiated CIA rats.

In relative quantitation of gene expression analysis, we performed real-time PCR to examine MCP-1 mRNA levels. The real-time PCR results were converted to mRNA relative copy units. As shown in Figure 2, there was an increase in the expression of the MCP-1 gene in CIA rats, and a significant decrease in expression after LED irradiation.

Finally, phenotypic expression of MCP-1 proteins was examined with immunohistochemical analysis. Synovial tissue from control rats exhibited very weak constitutive expression of MCP-1 protein, but this was strongly up-regulated in CIA rats. As expected, MCP-1 protein expression was significantly decreased in CIA rats following LED irradiation (Figure 3).

Discussion

Rheumatoid arthritis is a persistent, systemic inflammatory disease, characterised by inflammation involving multiple cell types and resulting in the progressive destruction of the affected joints. An essential component of the switch to persistence that underlies this joint destruction is the production of chemokines, which recruit mononuclear cells, such as lymphocytes and monocytes, to the inflamed joint.

Chemokines are relatively small proteins (8–10 kDa) with a similar three-dimensional structure. Based on the presence of a cysteine (C) containing a signature motif at the amino terminal, chemokines have been classified into four subfamilies, C, CC, CXC, and CX3C.

MCP-1 is a chemokine belonging to the CC subfamily, which is produced by macrophages, endothelia, synovial fibroblasts, and chondrocytes in RA joints. MCP-1 exerts its effects through binding to G-protein-coupled receptors (CCR2/5) on the surface of the targeted leukocytes. MCP-1 receptors (CCR2) belong to
the family of heptahelical, pertussis-sensitive, G-protein-coupled receptors\(^ {10}\). These receptors, once activated, trigger a set of cellular signaling processes that result in activation of phospholipase C (PLC) together with inositol triphosphate (IP\(_3\)) and diacylglycerol (DAG) formation, intracellular calcium release, and protein kinase C (PKC) activation\(^ {11}\). MCP-1 binds to CCR2 receptors and increased intracellular calcium mobilization, which is essential for chemotaxis\(^ {12}\). The MAP kinases (MAPK), Janus kinase (JUNK), and p38, phospholipase C (PLC) and two isoforms of PI3-kinase have all been implicated in MCP-1 signal transduction\(^ {13-15}\). **Figure 4** shows a schematic of the MCP-1 signal pathway; other genes in this pathway may need to be proved in future, and such experiments are now in progress in our laboratory.

Elevated MCP-1 levels have been reported in plasma, synovial fluid, and the synovial tissue of patients with RA. MCP-1 levels in plasma have been proposed as a marker for joint inflammation in RA\(^ {16}\). Thus, MCP-1 may be a key chemokine in the inflammatory progression of RA. In support of this, MCP-1 antagonist treatment was found to ameliorate disease severity in CIA by decreasing macrophage infiltration\(^ {17}\). Furthermore, when etanercept was administered to RA patients, the soluble tumor TNF-alpha receptor lowered the MCP-1 serum values\(^ {18}\). Moreover, Xia et al\(^ {19}\) investigated the mechanism for the regulation of inflammation of RA by infliximab *in vivo* and found that MCP-1 levels in RA patients significantly decreased. Recently, microRNAs (MRNAs) are known to be involved in many diseases. To elucidate the role of MRNAs in the pathogenesis of RA, Nakamachi et al\(^ {20}\) found that mRNA miR-124a levels significantly decreased in RA synoviocytes when compared with OA synoviocytes. Transfection of precursor miR-124a into RA synoviocytes significantly suppressed their proliferation and arrested the cell cycle at the G1 phase. They succeeded in identifying a putative consensus site for the miR-124a binding site in the 3'-untranslated region of MCP-1 mRNA, and induction of miR-124a in RA synoviocytes significantly suppressed the production of MCP-1 proteins\(^ {20}\).

When these reports are taken together, a reduction of MCP-1 production in RA patients is an important strategy in a therapeutic regimen for RA therapy, thus the reduction of MCP-1 gene expression observed following LED irradiation is an important anti-inflammatory mechanism in therapy for RA.

**Conclusion**

Our study indicates that CIA increased MCP-1 levels, which were significantly reduced by LED irradiation. In particular, it is important to address the relationship between LED irradiation and MCP-1 levels, because MCP-1 is critical to the development of rheumatoid synovitis in RA. We have validated the increased expression of MCP-1 protein in CIA rat tissue by immunohistochemistry. It is evident that LED irradiation reduced CIA-induced MCP-1 expression in CIA rat joint tissue, and in turn diminishing leukocyte infiltration and may also suppress bone destruction.

**Figure 4**: Schematic representation of MCP-1 signaling.
References


