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Endogenous conversion of n-6 to n-3 polyunsaturated fatty acids attenuates K/BxN serum-transfer arthritis in *fat-1* mice

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Abstract

It is suggested that n-3 polyunsaturated fatty acids (PUFAs) can be used in the preventive or therapeutic management of rheumatoid arthritis (RA); however, controversial results have been reported. Here, we examined the effects of a decrease in the n-6/n-3 PUFA ratio on RA using *fat-1* transgenic mice. First, we tested whether *fat-1* expression modulated signaling pathways in fibroblast-like synoviocytes (FLSs) stimulated with tumor necrosis factor α (TNF-α). TNF-α activated p38 mitogen-activated protein kinase and increased phosphorylation of the signal transducer and activator of transcription 3 in wild type (WT) FLSs but not in *fat-1* FLSs. Arthritis was induced by injection of K/BxN serum. Based on clinical scores, ankle thickness and pathological severity, we showed that WT mice developed clinically overt arthritis, whereas *fat-1* mice showed attenuated arthritis. Moreover, *fat-1* mice exhibited down-regulated local and systemic levels of inflammatory cytokines. Lastly, bone marrow-derived macrophages (BMMs) of WT mice differentiated into tartrate-resistant acid phosphatase-positive multinucleated osteoclasts, whereas the osteoclastogenenic process was suppressed in BMMs of *fat-1* mice. The endogenous conversion of n-6 to n-3 PUFAs *via* *fat-1* plays a key role in attenuation of RA; therefore, dietary supplementation of n-3 PUFAs may have therapeutic potential for the management of RA.

Keywords: n-3 PUFA; RA; *fat-1*; IL-6; K/BxN

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by chronic inflammation and progressive joint destruction. Fibroblast-like synoviocytes (FLSs) are the leading cells in joint erosion and contribute actively to inflammation [1]. RA is predominantly mediated by excessive production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 [2]. These cytokines activate mitogen-activated protein kinase (MAPK) and stimulate production of matrix metalloproteinases (MMPs) and proinflammatory cytokines and cellular proliferation in RA FLSs [1]. Consistent with these findings, MAPK inhibitors decrease joint destruction and synovial inflammation in animal models of arthritis [3,4]. In addition, studies have demonstrated the expression of Janus-activated kinase and signal transducer and activator of transcription (JAK-STAT) signaling components in RA FLSs [5], and modulation of this pathway provides an effective therapeutic strategy in RA [6,7].

The production of proinflammatory cytokines is regulated by various factors including eicosanoids. Dietary n-6 and n-3 polyunsaturated fatty acids (PUFAs) are incorporated into cell membranes where they are used as eicosanoid precursors. In general, these two types of PUFAs have opposite effects on inflammatory responses; n-6 PUFAs, especially arachidonic acid (AA), are the precursors of inflammatory eicosanoids, whereas the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) give rise to antiinflammatory eicosanoids [8,9]. Experimental animal studies have shown that increasing the amount of n-3 PUFAs in the diet suppresses cytokine synthesis [10]. Consistent with these reports, several clinical studies have shown that consumption of n-3 PUFA-rich fish oil leads to

Abbreviations: RA, rheumatoid arthritis; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; MMP, metalloproteinase; FLS, fibroblast-like synoviocyte; JAK, janus activated kinase; STAT, signal transducer and activator of transcription; PUFA, polyunsaturated fatty acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; BMM, bone marrow-derived macrophage; TRAP, tartrate-resistant acid phosphatase; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand.

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the down-regulation of proinflammatory cytokines and the up-regulation of antiinflammatory mediators and has benefits in RA prevention and treatment [11,12]. However, some human studies have reported controversial results [13–15].

This study used *fat-1* mice, which contain the *fat-1* gene from *Caenorhabditis elegans* and are able to convert n-6 to n-3 PUFAs *in vivo* [16]. As a result of the reduced ratio of n-6/n-3 PUFAs in blood and tissues, these mice exhibit antiinflammatory effects in various inflammatory disease models such as pancreatitis [17], diabetes [18] and asthma [19]. In contrast to feeding studies, studies using *fat-1* mice could eliminate confounding factors of diet such as nutrient composition, total caloric intake, duration of feeding and contamination of trace elements. Furthermore, a recent study reported a delay of osteoarthritis development in *fat-1* mice, suggesting favorable effects of endogenous n-3 PUFAs on the experimental arthritis model [20]. To our knowledge, however, there are no reports using *fat-1* mice to study RA.

Given the above background, we considered whether inflammation and joint destruction observed in a K/BxN serum-transfer arthritis model would be inhibited in a *fat-1* mouse background. We also compared intracellular signaling pathways in FLSs isolated from wild type (WT) and *fat-1* mice.

2. Materials and methods

2.1. Isolation and culture of mouse FLSs

Mouse FLSs were derived from *fat-1* and WT mouse knee and ankle joints by microdissecting the synovium and enzymatically dispersing the cells [21]. Mouse FLSs from Passages 4 to 5 and at 80–90% confluence were used. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in high glucose-containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2-mM glutamine, 100-units/ml penicillin, 100-μg/ml streptomycin and 2.5-μg/ml amphotericin B. All treatments were performed in serum-free medium. TNF-α was obtained from Invitrogen (Carlsbad, CA, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

2.2. Osteoclast differentiation

Bone marrow-derived macrophages (BMMs) were prepared as described previously [22]. BMMs were seeded at 5×10^4 cells/well in 48-well plates and cultured with macrophage colony-stimulating factor (M-CSF, 20 ng/ml) and receptor activator of NF-κB ligand (RANKL, 30 ng/ml) for 4 days. Osteoclasts were visualized by staining for tartrate-resistant acid phosphatase (TRAP) activity. TRAP-positive cells were counted as osteoclasts.

2.3. Animals

Dr. J. X. Kang at the Harvard Medical School (Boston, MA, USA) kindly provided the *fat-1* transgenic mice. Mice were housed in a laminar flow cabinet and maintained on AIN-93 M diets containing 5% corn oil provided *ad libitum*. Mice were aged 7–8 weeks at the start of each experiment. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011). In addition, the Institutional Animal Care and Use Committee of Chonbuk National University (Approval No. CBU 2014-00047) approved the current study protocol.

2.4. Gas chromatography

Fats were extracted from tissues or sera with a solvent mixture (chloroform: methanol, 2:1) using a blade type homogenizer (IKA T10 basic). The methylation of fatty acids was conducted with 0.25-N sodium methoxide in methanol at 70 °C for 20 min. The fatty acid methylesters (FAMEs) were extracted with hexane and separated by gas chromatography as described previously [23]. FAME samples were injected into a gas chromatograph equipped with a flame ionization detector. The column used was a highly polar cyanopropylsiloxane phase (SP2560, 100 m×0.25 mm i.d.) fused silica capillary column (Supelco Inc., Bellefonte, PA, USA). Helium was used as a carrier gas with a head pressure of 300 kPa. Injector and detector temperatures were 230 °C and 260 °C, respectively. The initial oven temperature was held at 170 °C for 2 min and then increased at a rate of 1.5 °C/min to 220 °C. Peaks were identified by comparison with PUFA standards, and the integrated peak area of each resolved peak was used to calculate the percentage of each PUFA.

2.5. K/BxN serum transfer arthritis and clinical evaluation

KRN TCR-transgenic mice were a gift from D. Mathis and C. Benoist (Harvard Medical School, Boston, MA, USA) and were maintained in a B6 background. To induce K/BxN serum-transfer arthritis, *fat-1* and WT mice were intraperitoneally injected with K/BxN serum (100 μl) pooled from arthritic K/BxN mice on Days 0 and 2 as described previously [24]. Clinical arthritis scores were evaluated using a scale of 0–4 for each limb (0=*no swelling*; 1=*slight swelling and erythema*; 2=*moderate swelling and erythema*; 3=*severe swelling and erythema*; and 4=*maximal inflammation with joint rigidity*). The maximum possible score for each mouse was 16.

Ankle thickness was measured using an electric caliper placed across the ankle joint at the widest point. An increase in diameter of the arthritic ankle at specific time points over 8 days was defined as the change in thickness. On Day 8, the mice were sacrificed, and joint tissues were harvested from each animal for end-point histology.

2.6. Histopathologic examination

Mouse joint tissues were fixed with 10% formalin, decalcified for 3 weeks in 10% EDTA, dehydrated and embedded in paraffin. Sections (4 μm) were stained with H&E and safranin-O for light microscopy. The joint sections were scored on a scale of 0–5 for changes in inflammation, bone erosion and cartilage degradation. To examine the osteoclastic bone resorption activity in arthritic mice, sections were stained using a TRAP staining kit (Sigma-Aldrich). The total number of TRAP-positive multinucleated cells containing three or more nuclei was counted in 10 areas of each ankle (magnification ×100).

2.7. IL-6 assay

IL-6 levels in the cell culture supernatants and sera of arthritic mice were determined using Quantikine ELISA kits (Invitrogen).

2.8. Preparation of cytoplasmic and nuclear protein extracts

FLSs were washed twice by centrifugation in PBS and then pelleted at 500 *g* for 5 min. The pellets were lysed in CytoBuster protein extraction buffer (Novagen, Madison, WI, USA). Protein extracts from ankle joints were isolated by homogenization of the joints (50 μg tissue/ml) in lysis buffer (T-PER Tissue Protein Extraction Reagent; Thermo, Rockford, IL, USA) containing a proteinase inhibitor cocktail. Cytoplasmic and nuclear extracts were prepared from cells or tissues using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA).

2.9. Western blot analysis

FLSs or joint tissues were homogenized with protease and phosphatase inhibitors in a protein extraction solution (Pro-Prep, Intron Biotechnology, Sungnam, Korea). Homogenates containing 30-μg protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The blot was probed with 1-μg/ml primary antibody against p-p38, p38, p-ERK, ERK, p-JNK, JNK, STAT-3 and p-STAT-3 (Cell Signaling Technology, Beverly, MA, USA), proliferating cell nuclear antigen (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Bioworld Technology, Minneapolis, MN, USA). Horseradish peroxidase-conjugated antirabbit or antimouse IgG (Enzo Life Sciences, Farmingdale, NY, USA) was used as a secondary antibody.

2.10. RNA isolation and real-time RT-PCR

RNA was isolated from FLSs or tissues using Trizol reagent (Invitrogen) and then precipitated with isopropanol and dissolved in DEPC-treated distilled water. Total RNA (2 μg) was treated with RNase-free DNase (Invitrogen), and first-strand cDNA was generated using a random hexamer primer with a first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Specific primers for each gene were designed using primer express software (Applied Biosynthesis; Table 1). GAPDH was used as an invariant control. Real-time RT-PCR mixtures consisted of 10-ng reverse transcribed total RNA, 167 nmol/l forward primer, and 2× PCR master mix in a final volume of 10 μl. Reactions were carried out in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

2.11. Statistical analysis

Data are expressed as the mean±SEM. The significant differences between groups were determined using Student's unpaired *t*-tests. *P*-values less than 0.05 were considered statistically significant.