Cooperative induction of 15-lipoxygenase in rheumatoid synovial cells by IL-4 and proinflammatory cytokines

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Abstract

Objective

To clarify the role of interleukin-4 (IL-4) in the expression of 15-lipoxygenase (15-LOX), whose metabolites are known to suppress the inflammatory reaction, in freshly prepared rheumatoid synovial cells.

Methods

Adherent synovial cells were prepared by enzymatic digestion of synovia obtained from patients with rheumatoid arthritis (RA). Protein expression of 15-LOX was determined by Western blot analysis. The messenger RNAs of 15-LOX were determined by reverse transcription and the polymerase chain reaction (RT-PCR).

Results

Freshly prepared rheumatoid synovial cells did not express 15-LOX at either the mRNA or protein levels. IL-4 induced the protein expression of 15-LOX after 24 hours of culture. Although interleukin-1α (IL-1α) and tumor necrosis factor α (TNFα), major inflammatory cytokines in rheumatoid synovia, did not induce the expression of 15-LOX, IL-4 and these inflammatory cytokines synergistically enhanced the protein expression of 15-LOX. The synergistic effect was also observed at the level of mRNA.

Conclusions

We demonstrate that IL-4 cooperated with the inflammatory cytokines IL-1α and TNFα to enhance the expression of 15-LOX in rheumatoid synovial cells. Since 15-LOX metabolites have potent anti-inflammatory actions, our data suggest that IL-4 might downregulate rheumatoid inflammation via the induction of 15-LOX and its metabolites.

Key words

Rheumatoid arthritis, 15-lipoxygenase, synovial cell, interleukin-4, tumor necrosis factor α, interleukin-1α.

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Introduction

Interleukin (IL)-4, originally described as a B cell growth factor, is a 20 kDa product of activated T cells (1). Since IL-4 inhibits the ability of macrophage lineage cells to produce a group of inflammatory cytokines such as IL-1, tumor necrosis factor α (TNFα) and IL-6, it is considered to be an anti-inflammatory cytokine (2-4). We previously reported that IL-4 inhibited the production of inflammatory cytokines and prostaglandin E_2 (PGE_2) by freshly prepared rheumatoid synovial cells (5-7). IL-4 also inhibited cartilage destruction (8) and osteoclast differentiation (9). In addition, gene therapy using synovial fibroblasts transfected with the IL-4 gene suppressed the destruction of joints in a collagen-induced animal model (10). These findings suggest that IL-4 might be a promising therapeutic application for RAtreatment (11). However, it is rather unclear how IL-4 exerts these anti-inflammatory actions. It has been reported that the signal transducer and activator of transcription 6 (STAT6), which is activated in IL-4-mediated signal transduction (12), is responsible for its anti-inflammatory action (13). However, we do not know which gene induced by STAT6 activation is involved in these anti-inflammatory actions.

Lipoxygenases (LOX) are classified according to their positional specificity of arachidonate oxygenation (14). Many lipoxygenases (5-LOX, 12-LOX, 15-LOX) have been identified and cloned in humans (15-17). 15-LOX catalyzes oxygenation at C-15 of arachidonic acid, yielding 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) which is transformed by reduction into 15-hydroxyeicosatetraenoic acid (15-HETE). It has been shown that IL-4 up-regulates 15-LOX in a variety of cell types including human monocytes (18-21), and that STAT6 activation is critical for the induction of 15-LOX (22). In addition, 15-LOX metabolites downregulate a number of functions of inflammatory cells (23-31). These findings prompted us to study the regulatory mechanism(s) of 15-LOX expression in rheumatoid synovial cells and the role of IL-4. In the present paper we examine the effect of IL-4 and proinflammatory cytokines on the expression of 15-LOX in rheumatoid synovial cells.

Materials and methods

Reagents and antibodies.

Human recombinant IL-4 (specific activity, 10^6 U/mg protein) was kindly provided by Ono Pharmaceutical Co. (Osaka, Japan). Human recombinant IL-1α and TNFα were kindly provided by Dainippon Pharmaceutical Co. (Osaka, Japan). A polyclonal antibody to 15-LOX was purchased from Cayman Chemical Co. (Ann Arbor, MI).

Synovial cell culture

Rheumatoid synovial cells used in this study were derived from 12 patients with RA who underwent total knee replacement or synovectomy. All patients were diagnosed as having RA according to the 1987 criteria of American College of Rheumatology (32). Informed consent was obtained from each patient. The synovial tissues were cut into fragments 1-3 mm in diameter, and incubated in 0.5–1 mg/ml of collagenase and 5–10 μg/ml of deoxyribonuclease I for 2-3 hours. After digestion, the resulting single cells were washed, filtered through sterile gauze and nylon mesh, and finally resuspended in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin 100 U/ml, gentamicin 60 mg/ml, N-2-hydroxyethlpiperazine-N-2-ethanesulphonic acid 12.5 mM, and L-glutamine 2 mM (DMEM medium). After overnight incubation, non-adherent cells were removed, and the resulting adherent cells were used as adherent rheumatoid synovial cells.

Western blot analysis

After incubation with various stimuli, freshly prepared synovial cells were lysed in a buffer (50 mM Tris-HCl, pH 8.0, 1% NP-40, 0.5% deoxycholic acid, 150 mM NaCl, and 1 mM PMSF). Proteins were separated on a 7.5–10% SDS-PAGE gel, and transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). After blocking the

membrane, it was incubated with the antibody to 15-LOX, then with a rabbit anti-sheep IgG (Zymed Laboratories Inc, San Francisco, CA) conjugated with horseradish peroxidase. Specific bands were visualized by an ECL chemiluminescence detection system (Amersham International, Buckinghamshire, UK), and exposed to Fuji new RX films (Fuji Photo Film, Kanagawa, Japan). The intensity of the bands was analyzed by a NIH image software.

Extraction of RNA, and reverse transcription and polymerase chain reaction (RT-PCR)

Freshly prepared synovial cells were cultured with or without IL-1α and TNFα in the presence or absence of IL-4 for the indicated duration, and their total cellular RNAs were extracted by acid guanidine phenol/chloroform extraction (33) using ISOGEN (Wako Pure Chemical Industries, Ltd.).

Two μg of total RNA was reverse transcribed to cDNA after annealing with 100 pmol of oligo$(dT)_{12\text{-}18}$ primer (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in the presence of 100 U of M-MLV reverse transcriptase (Gibco BRL Life Technologies, Inc., Gaithersburg, MD), 10 U of RNasin (Promega Corporation, Madison, WI), 1 mM DTT, 25 pmol of random primer (Takara Shuzo Co. Ltd. Kyoto, Japan), and 10 pmol of each deoxynucleotide (Takara Shuzo Co.) in a total volume of 10 μl for 1 hr at 37 ºC. Two μl of the resultant cDNA preparation was used directly for each amplification reaction. PCR was carried out in a 50 μl reaction mixture containing 20 pmol of each primer (see below), 20 pmol of each deoxynucleotide and 1.25 U *Taq* DNA polymerase (Takara Shuzo Co. Ltd.). Amplification of a specific PCR product was separately carried out in a different tube. Primers used were: 15-LOX sense primer, 5'-AGAAGGAA GTTGTACCGGTGG-3' (nucleotide 406'-426'), 15-LOX antisense primer, 5-GCTACAGAGAATGACGTTGGC-3' (nucleotide 859'-879'); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense primer, 5'-CAAAA GGGTCATCATCTCTG-3' (nucleotide 408'-427'); GAPDH antisense primer, 5'-CCTGCTTCACCACCTTCTTG-3' (nucleotide 834'-853'). These primer sets yield PCR products of 474 bp and 446 bp for 15-LOX, GAPDH, respectively. Reactions were incubated in a Perkin-Elmer/Cetus DNA Thermal cycler for 25-30 cycles (denaturation for 1 min at 94 ºC; annealing for 2 min at 58-60ºC; extension for 1 min at 72ºC). Aliquots of PCR products were run on a 1.5% agarose gel in TAE buffer, and visualized by ethidium bromide staining. GAPDH was used as a housekeeping gene (34).

Measurement of prostaglandin E_2 (PGE_2)

The levels of PGE_2 in the culture supernatants were measured by an enzyme-linked immunosorbent assay kit (NEOGEN Corporation, Lexington, KY).

Data analysis

Data are presented as the mean ± SD. Statistical analysis was performed by a paired Student's t-test. A value of $P < 0.05$ was considered to be significant.

Results

Effect of IL-4 on the expression of 15-LOX in rheumatoid synovial cells

To clarify the role of IL-4 in the expression of 15-LOX, we used freshly prepared rheumatoid synovial cells. As shown in Figure 1a, these cells spontaneously produced large amounts of PGE_2. The production of PGE_2 was significantly inhibited by IL-4, suggesting that these cells are involved in the inflammatory process in RA, and that IL-4 exerts an anti-inflammatory effect on the synovial cells.

To examine the effect of IL-4 on the expression of 15-LOX, we first carried out a time-course study. Western blot analysis revealed that 15-LOX was not expressed in rheumatoid synovial cells and that IL-4 induced the expression of 15-LOX after incubation of 24-hours (Fig.1b). The induction was consistently observed in 4 different synovial samples (Fig.1c). In addition, IL-4 induced the gene expression of 15-LOX after a culture period of more than 8 hours (Fig.1d). On the other hand, IL-4 did not induce 15-LOX in cultured synovial fibroblasts (data not shown).

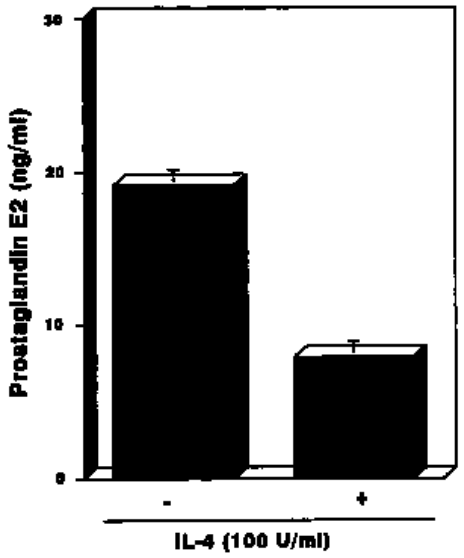
Synergistic effect of IL-4 with IL-1α and TNFα on the induction of 15-LOX in rheumatoid synovial cells

Rheumatoid synovial cells are known to be exposed to large amounts of inflammatory cytokines such as IL-1α and TNFα *in vivo* (35, 36). Therefore, we next examined the effect of IL-4 on the expression of 15-LOX in the presence of IL-1α and TNFα. As shown in Figure 2a, IL-1α and TNFα alone did not induce the protein expression of 15-LOX. On the other hand, IL-4 alone induced small amounts of 15-LOX. Interestingly, the combination of IL-4 and these cytokines strongly enhanced 15-LOX expression. These synergistic effects were consistently observed in 4 different synovial samples (Fig. 2b). The dose-response study revealed that IL-4 induced the expression of 15-LOX at a low concentration of 1 U/ml (Fig. 2c). RT-PCR analysis showed that IL-1α and TNFα alone did not induce 15-LOX mRNA expression and that the synergistic effects of these cytokines with IL-4 were also observed at the level of mRNA(Fig. 2d).

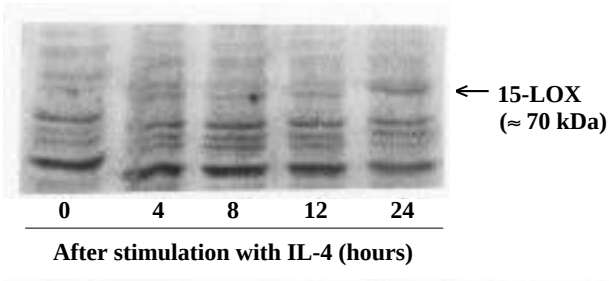
Discussion

Data from studies of experimental arthritis support the beneficial role of IL-4 in prevention of joint destruction in RA. For example, cartilage destruction as a result of collagen-induced arthritis was inhibited by the administration of IL-4 expressing adenovirus vector (10). In addition, we and others demonstrated that IL-4 downregulates the production of a variety of inflammatory cytokines such as IL-1, TNFα and IL-6 by human monocytes (4) or rheumatoid synovial cells (5-7), suggesting that IL-4 may be a promising therapy for RA. However, the mechanism(s) of these effects of IL-4 still remains to be clarified.

IL-4 and/or IL-13 have been previously reported to induce 15-LOX expression in a variety of cell types such as human monocytes (18), alveolar macrophages (19), A549 lung epithelial cells (20), and human tracheo-bronchial epithelial cells (37). We demonstrated here that



a



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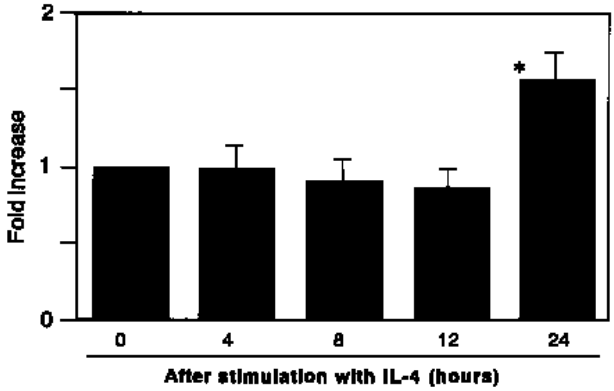

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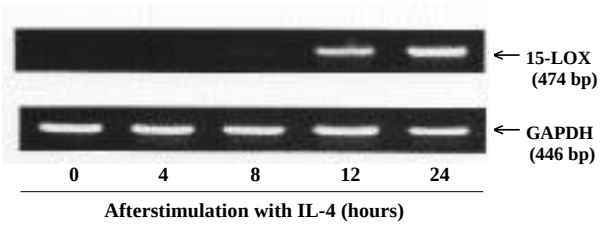

d

Fig 1. Effect of interleukin-4 (IL-4) on the expression of 15-lipoxygenase (15-LOX) in freshly prepared adherent rheumatoid synovial cell. (**a**) The fresh synovial cells (1 x 10^5 cells/well in 24-well plates) were cultured with or without IL-4 (100 U/ml) for 24 hours. After culturing, the levels of prostaglandin E2 in the supernatants were determined by an enzyme-linked immunosorbent assay kit. Data are presented as means ± SD for triplicate determinations. (**b**) The fresh synovial cells were placed in a 6-well plate at a concentration of 1 x 10^6 cells/well and the cells were cultured with IL-4 (100 U/ml) for the indicated duration. After culturing, the cell lysates underwent Western blot analysis using a polyclonal antibody to 15-LOX, as described in Materials and methods. (**c**) The intensity of the 15-LOX bands in the experiments performed on 4 different synovial samples was analysed by NIH image software, and each bar represents an x-fold increase (mean ± SD) (* $P < 0.05$) versus medium alone. (**d**) The fresh synovial cells were cultured with IL-4 (100 U/ml) for the indicated duration. After culturing, the total cellular RNAs were extracted and used for reverse transcription and polymerase reaction (RT-PCR) analysis to determine the gene expression of 15-LOX. GAPGH was used as a housekeeping gene.

freshly prepared rheumatoid synovial cells did not express 15-LOX, and that IL-4 cooperated with IL-1α and TNFα to induce the expression of 15-LOX. To our knowledge, this is the first report showing the synergistic induction of 15-LOX by IL-4 and these inflammatory cytokines. Liagre *et al.* reported that RA type B synoviocytes were able to express the 15-LOX mRNA, as assessed by an *in situ* RT-PCR method, and that IL-4 enhanced the production of 15-HETE by synoviocytes (37). Interestingly, those authors reported that IL-1 also increased the production of 15-HETE, a 15-LOX metabolite, suggesting that IL-1 may be involved in the regulation of 15-LOX expression.

The mechanism(s) of this synergistic effect is unclear. The involvement of STAT6 in IL-4-induced 15-LOX expression was underlined by the fact that no induction of 15-LOX activity was observed in macrophages from homozygous STAT6-deficient mice (38). Analysis of the 15-LOX promoter revealed that a specific STAT6 response element located 952 base pairs upstream of the translational start codon was critical for 15-LOX expression (22). However, the detailed mechanism by which STAT6 acts as a transcriptional factor is complex. For instance, STAT6 has been shown to interact directly with NF-κB, and this interaction lead to synergistic activation of IL-4-induced transcription (39). Therefore it is likely that the synergistic effect of IL-4 and inflammatory cytokines in 15-LOX expression may be related to NF-κB activation by IL-1α and/or TNFα. Alternatively, the sequence analysis of the 15-LOX promoter reveals putative Sp1 and Ap2 binding sites (40). It is possible that the synergistic action of IL-1α and TNFα may take place via the activation of these transcription factors.

Considering the overexpression of IL-1α and TNFα in rheumatoid synovium, our data suggest that 15-LOX may be strongly expressed in rheumatoid synovium after the treatment of IL-4. Although the role of 15-LOX in RA remains to be clarified, it is known that 15-LOX metabolites regulate the inflammatory process. Both 15-HPETE and 15-HETE suppress the production of leukotriene B4 (LTB4), a potent neutrophil chemotactic factor, by rabbit polymorphonuclear leukocytes (23, 24). 15-HETE inhibits T-lymphocyte mitogenesis (25) and human eosinophil LTC4 secretion (26), blocks neutrophil migration across activated endothelium (27), and inhibits superoxide anion pro-

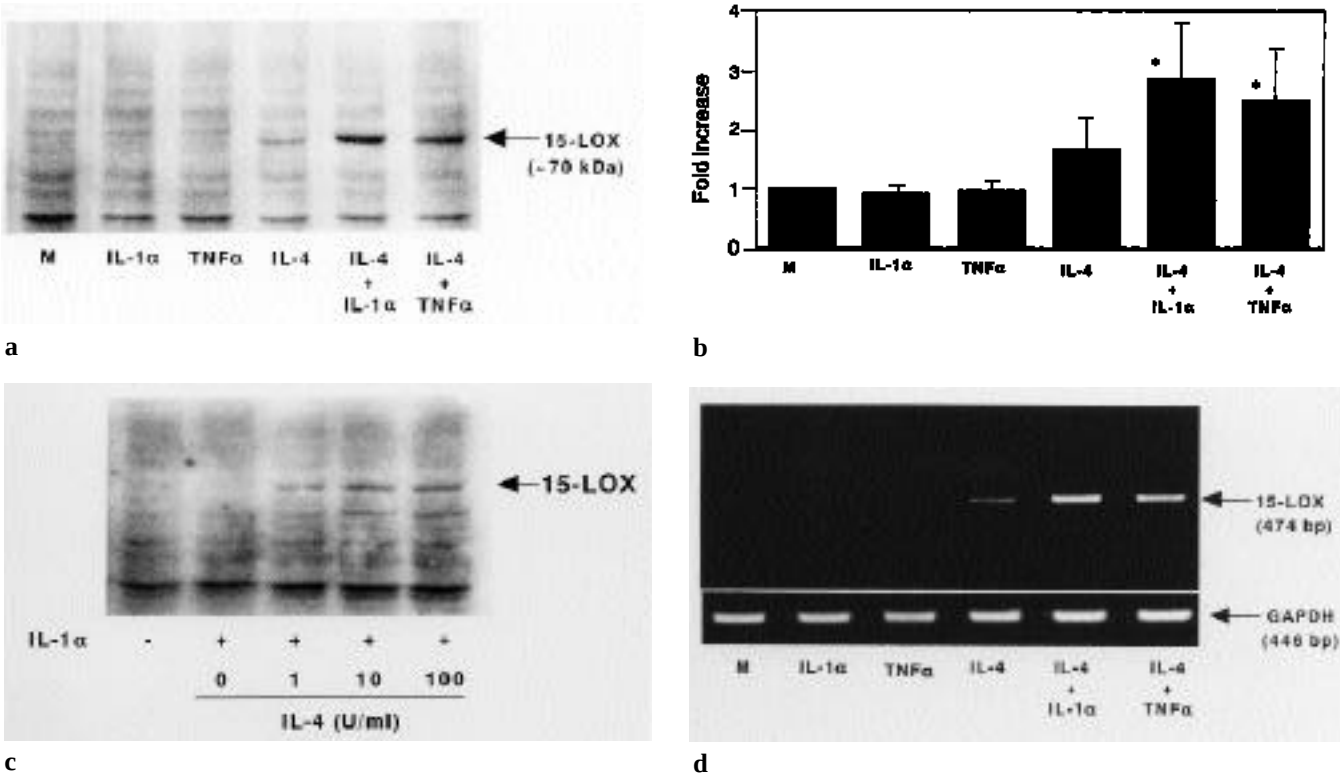

Fig 2. Synergistic effect of interleukin-4 (IL-4) with interleukin-1α (IL-1α) and tumor necrosis factor α (TNFα) on the induction of 15-lipoxygenase (15-LOX) in rheumatoid synovial cells. (**a**) The fresh synovial cells were cultured with IL-1α (10 ng/ml) or TNFα (20 ng/ml) in the presence or absence of IL-4 (100 U/ml) for 24 hours. After culturing, the treated cells were harvested for Western blot analysis using the polyclonal antibody to 15-LOX. (**b**) The intensity of 15-LOX bands in the experiments performed on 4 different synovial samples were analysed by NIH image software, and each bar represents an x-fold increase (mean ± SD) (* P< 0.05 versus medium alone). (**c**) The synovial cells were cultured with the indicated concentrations of IL-4 in the presence of IL-1α (10 ng/ml) for 24 hours. After culturing, the treated cells were harvested for Western blot analysis using the polyclonal antibody to 15-LOX. (**d**) The synovial cells were cultured with IL-1α (10 ng/ml) or TNFα (20 ng/ml) in the presence or absence of IL-4 (100 U/ml) for 8 hours. After culturing, the total cellular RNAs were extracted and used for reverse transcription and polymerase reaction (RT-PCR) analysis for 15-LOX mRNA. GAPGH was used as a housekeeping gene.

duction and degranulation from activated neutrophils (28). 15-HPETE, but not 15-HETE down-regulates Fcγ receptors on human T cells and monocytes (29). In addition, 15-HPETE inhibits important proinflammatory processes including endotoxin-induced TNF production in monocytes and the TNF induced expression of ICAM-1, E-selectin and VCAM-1 via a protein kinase C-dependent pathway (30, 31). These actions of 15-LOX metabolites indicate that IL-4-induced expression of 15-LOX leads to the downregulation of the inflammatory process in rheumatoid joints.

In summary, we demonstrate here that IL-4 cooperates with the inflammatory cytokines IL-1α and TNFα to enhance the expression of 15-LOX in the rheumatoid synovial cell. Our findings provide new insights into the anti-inflammatory actions of IL-4 in RA.

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