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Production of the chemokine eotaxin-1 in osteoarthritis and its role in cartilage degradation

Key Words: Chemokine• Eotaxin-1• Osteoarthritis• Matrix metalloproteinase• Chondrocytes

Abstract

We have examined the expression of chemokine eotaxin-1 and its receptor in human osteoarthritic chondrocytes, and their roles implicated in cartilage degradation. In addition to the chemokines RANTES and MCP-1α, the plasma concentration of eotaxin-1 was also higher in the patients with osteoarthritis (OA) than those in normal human. Stimulation of chondrocytes with interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α) significantly induced eotaxin-1 expression by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) analysis. The production of eotaxin-1 induced it own receptors, CCR3, and CCR5 expression on cell surface by flow cytometric analysis, suggesting an autocrine/paracrine pathway involved in eotaxin-1 action in OA. In addition, the production of eotaxin-1 markedly increased matrix metalloproteinase-3 (MMP-3) and MMP-13 expressions but had no effect on tissue inhibitor of metaloproteinase-1 (TIMP-1) expression in chondrocytes. These results first demonstrate that human chondrocytes can express chemokine eotaxin-1, and that expression is induced by treatment of IL-1β and TNF-α. The cytokine-triggered induction of eotaxin-1 further resulted in enhancing the expressions of its own receptor and MMPs, suggesting eotaxin-1 play an important role in cartilage degradation in OA.

Introduction

Osteoarthritis (OA) is a chronic degenerative joint disease, characterized by degradation of articular cartilage and inflammation of synovium (1-3). In OA, the normal balance between the catabolic and the anabolic function of the chondrocytes is biased toward catabolic function, and it is well know that these functions are markedly influenced by cytokines (4-6). The productions of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) have been suggested that play major roles in the progressive cartilage disruption by suppressing the synthesis of proteoglycans, type II and IX collagen, and tissue inhibitor of metalloproteinases (TIMPs), and inducing the expression of plasminogen activator and proteinases, such as those of the matrix metalloproteinase (MMP) family (7-10). However, IL-1β and TNF-α also induce the production of other cytokines and certain chemokines, such as IL-8, growth regulated oncogene-α (GRO)-α, regulated upon activation normal T cell expressed and secreted (RANTES), and monocyte chemoacctractant proteins (MCP-1).

Chemokines are a family of small heparin-binding cytokines that are primely involved in the recruitment of leukocytes to site of inflammation. According to the juxtaposition of cysteine residues in the chemokine protein's N-terminus, they can be distinguished four subfamilies and named C, CC, CXC, and CX3C (11, 12). Among them, the CC and CXC chemokines represent

two major subgroups. The CC chemokines include eotaxin-l, eotaxin-2, MCPs, macrophage inflammatory protein-1α (MIP-lα), MIP-1β, and RANTES. The CXC chemokines include IL-8, GRO-α, and platelet factor 4 (13, 14). Several chemokines have been shown that overproduced in arthritic joint, including IL-8, MCP-1, MIP-1α, MIP-1β and RANTES. Eotaxin-1 was first isolated from lung lavage fluid of sensitized guinea-pigs following allergen exposure (15). Eotaxin-1 has been demonstrated to selectively induce eosinophils recruitment to the airways and to the skin in vivo (16, 17), and activate eosinophils to release granule content. Several cell types expressed eotaxin-1, including epithelial, endothelial cells, T-lymphocytes, macrophages, eosinophils, and fibroblast (18-20). Recent reports indicated that chondrocytes respond to CC and CXC chemokines, such as RANTES and MCP-1, by releasing MMP-3 and N-acetyl-β-D-glucosaminidase, thus contributing to the cartilage matrix catabolism. However, it is not known whether eotaxin-1 is produced in cartilage or involves in chondrocyte activation.

In the present study, we found that eotaxin-1 is induced in the cytokine-activated chondrocytes. The production of eotaxin-1 further stimulated its receptors CCR3, CCR5, MMP-3, and MMP-13 expressions, and those are associated with joint inflammation and cartilage degradation.

Materials and Methods

Patients: Patients with OA were selected according to the American College of Rheumatology criteria (21, 22). Plasma samples were obtained from 50 normal donors and 40 patients with OA.

Chondrocyte isolation and cell culture: OA knee cartilage was obtained from patients undergoing total joint replacement surgery. Cartilage slices were cut into pieces (2-3 mm^3) and chondrocytes were released from articular cartilage by sequential enzymatic digestion as described: 0.1% hyaluronidase (Sigma Chemical, St. Louis, MO) 30 min and 0.2% type II collagenase (Sigma) 3 h at 37℃ in Dulbecco's modified Eagle's medium (DMEM) with antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin). After filtration through nylon meshes, chondrocytes were washed and seeded at high density in DMEM supplemented with 10% fetal calf serum (FCS, Gibco BRL, Grand Island, NY) and antibiotics at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. All experiments were performed with cells between the second and fourth passages. Human SW1353 chondrosaroma cells were cultured in the same medium as primary cultured chondrocytes.

*Reverse Transcription-Polymerase Chain Reaction (RT-PCR):*Total RNA was isolated from both of control and tested cultured cells and RT-PCR was performed as described previously (23). In brief, complementary DNA was synthesized in a 25-μl reaction mixture containing 5 μg of total RNA, 2.5 mM of each dNTP, 1 mM of random hexamer primers, and 10 U of M-MLV reverse transcriptase (Epicentre, Madison, WI), by incubation at 37℃ for 90 minutes. The resulting cDNA (2 μl) was subjected to PCR using Taq DNA polymerase (Epicentre) and specific primers for the eotaxin-l, CCR, MMPs, TIMP-1, and GAPDH (Table 1). PCR for eotaxin-1 was performed for 35 cycles at 94℃ for 30 seconds, 56℃ for 30 seconds, and 72℃ for 30 seconds, and CCR were amplified in a protocol of 94℃ for 1 minute, 60℃ for 1 minutes and 72℃ for 1 minutes. For MMP and TIMP, the PCR protocol was 35 cycles at 94℃ for 1 minute, 56℃ for 1

minute, and 72℃ for 1 minute. In each experiment, amplification of cDNA for the housekeeping gene GAPDH was used as internal standard. PCR products were analyzed on 2% agarose gels.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of CC chemokines in the plasma and cultured medium were determined by commercially available ELISA kits according to the instructions of the manufacturer (R&D systems Inc, Minneapolis, MN) (23).

Flow Cytometric Analysis

For detection of cell surface expression of CC chemokine receptor, cells were placed in 15-ml polyethylene tubes on a rocker plate form at 37℃ for 6 h to allow the re-expression of receptors cleaved during enzymatic treatment. For premeabilized cell staining, chondrocyte were fixed with freshly prepared 4% paraformaldyhyde in PBS at room temperature for 30 minutes, than washed twice with PBS. Cells (10^5) were processed in 0.05 ml of PBS-10% FCS, and incubated with anti-CCR3 or anti-CCR2 conjugated with phycoerythin (PE) and anti-CCR5 conjugated with fluorescein isothiocyanate (FITC) monoclonal antibodies and isotype-control mouse IgG conjugated with PE or FITC (R&D systems) for 45 minutes at 4℃ in dark. After washing, the cells were suspended in 4% paraformaldehyde and immediately analyzed by FACS.

Results

The Patients with OA Have High Level of Chemokine Eotaxin-1 in Plasma

To investigate whether CC chemokine eotaxin-1 was up-regulated in the patient with OA, plasma samples from patients with OA and normal donors were evaluated for the CC chemokines concentration by ELISA analysis. As shown in Fig. 1, the average concentration of cheokines RANTES, MCP-1α, and eotaxin-1 were higher in the patients with OA than normal donors. Similar to previous reports, the CC chemokines RANTES and MCP-1α markedly up-regulated in OA articular cartilage. In addition, we first found that chemokine eotaxin-1 also increased in OA samples, and showed that mean concentration of eotaxin-1 were 24.11 pg/ml and 93.84 pg/ml in normal donors and OA patients, respectively. These results suggest that up-regulation of eotaxin-1 might play a role in OA.

Induction of Eotaxin-1 Expression by IL-1β And TNF-α in Articular Chondrocytes

To determine whether chondrocytes express eotaxin-1 in response to stimulation of IL-1β or TNF-α. Chondrocytes were stimulated with IL-1β or TNF-α, and detected the expression of eotaxin-1 by RT-PCR analysis. As shown in Fig. 2A & 2B, IL-1β and TNF-α significantly induced eotaxin-1 expression in a time-dependent manner. The sequences of eotaxin-1 RT-PCR products were confirmed and verified by DNA sequencing (Data not shown). Low level of eotaxin-1 was detected in the absence of IL-1β or TNF-α. In addition, ELSIA analysis of cultured supernatants revealed chondrocytes secrete eotaxin-1 protein in the presence of IL-1β or TNF-α (Fig. 2C).

Chemokine Receptor Expression in Chondrocyte

Previous studies have indicated that eotaxin-1 can activate cells through the CC chemokine receptors CCR2, CCR3, and CCR5. To understand whether chondrocytes have the potential to response to eotaxin-1, CC chemokine receptor expression on chondrocyte was analyzed. In human chondrosacroma cells SW1353, 10 ng/ml of eotaxin-1 stimulated CCR3 and CCR5 expressions on cell surface by flow cytometric analysis (Fig. 3). However, CCR2 expression has no change in the treatment of eotaxin-1 (Data not shown). Both of cytokines IL-1β and TNF-α also induced the chemokine receptors expression of CCR3 and CCR5 on cell surface. The expression pattern of CCR3 and CCR5 also identified with the results from primary cultured chondrocytes (Data not shown).

Modulation of MMP-3 and MMP-13 Productions by Eotaxin-1

It well known that MMP and TIMP are involved in cartilage degradation process. To question whether eotaxin-1 modulate expressions of MMPs and TIMPs, RT-PCR analysis was performed to detect the mRNA expressions of MMP-3, MMP-13, and TIMP-1. As shown in Fig. 4, eotaxin-1 significantly stimulated MMP-3 and MMP-13 expressions in chondrocytes. In contrast, no significant alteration in the levels of TIMP-1 was found after eotaxin-1 stimulation.

Discussion

Several studies implicated that chondrocytes could express chemokines, such as RANTES, IL-8, GROα, and MCP-lα, as well as chemokine receptors, include CCR1, CCR2, CCR3, CCR5, CXCR1, and CXCR2. Our study is the first to report on the eotaxin-1, which affected catabolic function of cartilage expression by chondrocytes.

OA is a non-inflammatory arthritis, and the lyphocytes infiltration was not significant while the concentrations of several chemokines include IL-8, GROα, RANTES, and MCP-1α were higher than those of normal. It implied that chemokines not always go along with inflammation. Differ from other chemokines, eotaxin-1 constitutively expressed in some animal tissues without eosinophils infiltration (24-26). These findings suggested that eotaxin-1 not only regulate immune system but also influence other biological function. Eotaxin-1, a CC chemokine, closely resembles MCP family in protein and DNA sequence, and another CC chemokine RANTES expression was induced by IL-1β treatment in chondrocytes. In the present study, we found that the concentrations of eotaxin-1 in OA patients' sera were higher than those in normal, and eotaxin-1 was up-regulated by IL-1β and TNF-α stimulation in chondrocytes. Therefore, we proposed that eotaxin-1 might influence the progression of OA.

To cartilage homeostasis, the balance between catabolic and anabolic function of chondrocytes is very important, which influenced by lots of cytokines and growth factors. It is well established that cartilage-derived MMPs involved in the degradation of cartilage collagen and proteoglycan in OA. According to their structural and functional properties, the classical MMP family can be subdivided into four major groups: (i) the collagenases (MMP-1, -8, -13), (ii) the gelatinases (MMP-2, -9), (iii) the stromelysins (MMP-3, -10, -11), (iv) a heterogeneous

subgroup including matrilysin (MMP-7), enamelysin (MMP-20), macrophage metalloelastase (MMP-12) and MMP-19 (27). Cartilage degradation led by MMPs is irreversible. MMP-3 is particularly effective, since it cleaves a wide variety of matrix molecules, such as proteoglycans, fibronectins, and several collagens (28, 29). On the other hand, MMP-13 has been shown to degrade type II collagen more rapidly and effectively than MMP-1 or MMP-8 (30). Animal experiments have also demonstrated that blockage of MMP-13 activity resulted in decreasing degradation of type II collage (31). The results strongly suggested that MMP-13 play a important role in cartilage degradation cascade. In the present study, eotaxin-1 markedly increased MMP-3 and MMP-13 expression in response to IL-1β or TNF-α in chondrocytes, suggesting that eotaxin-1 involved in the cartilage degradation. Moreover, eotaxin-1 significantly increased its own receptors CCR3, and CCR5 expressions, indicating that exist a positive feedback regulation loop to amplify eotaxin-1 action, thus enhancing MMP expression and cartilage degradation.

Overall, chondrocytes increased the expressions of chemokine (eotaxin-1), chemokine receptor (CCR3), and MMPs in response to the stimulation of cytokine, such as IL-1β or TNF-α. The production of eotaxin-1 further stimulated the expressions of its own receptor (CCR3) and MMPs, thus amplifying the destruction of articular cartilage (Fig. 5).

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Table 1. Primer sequences used for reverse transcriptase-polymerase chain reaction.

Primer sequences*	Fragment size, bp
Eotaxin-1	
sense 5'-CCCAACCACCTGCTGCTTTAACCT-3'	
antisense 5'-TGGCTTTGGAGTTGGAGATTTTTGG-3'	208
MMP-3	
sense 5'-CCTCTGATGGCCCAGAATTGA-3'	
antisense 5'-GAAATTGGCCACTCCCTGGGT-3'	440
MMP-13	
sense 5'-GACTTCACGATGGCATTGCTG-3'	
antisense 5'-GCATCA CCTGCTGAGGATGC-3'	491
TIMP-1	
sense 5'-CACCCACAGACGGCCTTCTGCAAT-3'	
antisense 5'-AGTGTAGGTCTTGGTGAAGCC-3'	345
CCR3	
sense 5'-ATGCTGGTGACAGAGGTGAT-3'	
antisense 5'-AGGTGAGTGTGGAAGGCTTA-3'	354
CCR5	
sense 5'-CGTCTCTCCCAGGAATCATCTTTAC-3'	
antisense 5'-TTGGTCCAACCTGTTAGAGCTACTG-3'	356
GAPDH	
sense 5'-CCACCCATGGCAAATTCCATGGCA-3'	
antisense 5'-TCTAGACGGCAGGTCAGGTCCACC-3'	598

*MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinase; CCR: C-C chemokine receptor.

Figure legend

Fig. 1 Plasma levels of CC chemokines in the patients with osteoarthritis. Plasma samples obtained from patients with osteoarthritis (OA, n=40), or from normal donors (Normal, n=50) were analyzed the concentrations of CC chemokine RANTES, MCP-1α, and eotaxin-1 by ELISA as described in "Materials and Methods." Values are represented the mean ± S.E. *$p < 0.05$ compared with normal donors by Student's *t*-test.

Fig. 2 Effect of IL-1β and TNF-α on the eotaxin-1 expression in chondrocytes. Cells were treated with IL-1β (A) or TNF-α (B) for indicated times and determined the mRNA levels of eotaxin-1 and GAPDH by RT-PCR as described in "Materials and Methods." The positive control was obtained from human A549 cells treated with IL-1β for 6 h. Band intensities were quantified by densitometry (IS-1000 Digital Imaging System) and shown the relative fold of eotaxin-1/GAPDH. (C). Cells were treated with IL-1β or TNF-α for 24 h and determined the eotaxin-1 levels in cultured medium by ELISA analysis as described in "Materials and Methods."

Fig. 3 Effect of eotaxin-1 on the chemokine receptor expression in human SW1353

chondrosaroma cells. Cells were treated with IL-1β (1 ng/ml), TNF-α (3 ng/ml), or various concentrations of eotaxin-1 for 24 h and determined the levels of CCR3 and CCR5 chemokine receptors by flow cytometry as described in "Materials and Methods." Shaded histograms represent the basal fluorescence levels after staining with isotype control (anti-CCR antibodies). Open histograms represent the expression of chemokine receptor after stimulation of IL-1β, TNF-α, or eotaxin-1.

Fig. 4 Effect of eotaxin-1 on the expressions of MMP-3 and MMP-13 in chondrocytes. Cells were treated with IL-1β, TNF-α, or eotaxin-1 for 6 h and determined the mRNA expressions of MMP-3, MMP-13, TIMP-1, and GAPDH by RT-PCR as described in "Materials and Methods." Band intensities were quantified by densitometry (IS-1000 Digital Imaging System) and shown the relative fold of eotaxin-1/GAPDH.

Fig. 5 Hypothesis for positive feedback regulation of MMP and chemokine receptor expressions by eotaxin-1 in osteoarthritis. Stimulation of the chondrocytes by cytokines (IL-1β or TNF-α) directly and indirectly activates productions of eotaxin-1, CCR, and MMPs. The production of Eotaxin-1, in turn, further activates the expressions of it own receptors, which results in amplification of Eotaxin-1 signal, and MMPs productions, which results in destruction of articular cartilage.

Fig. 1

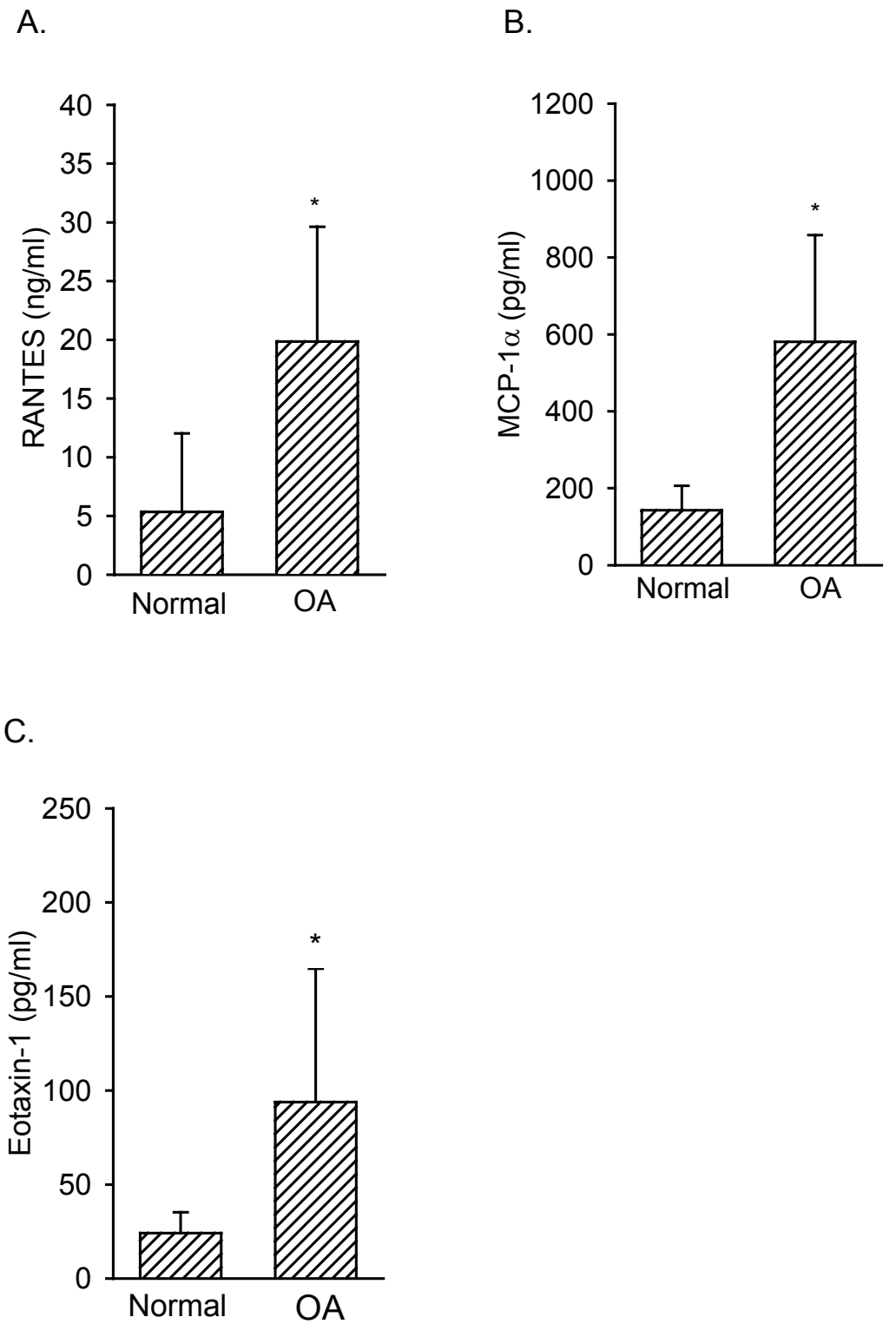
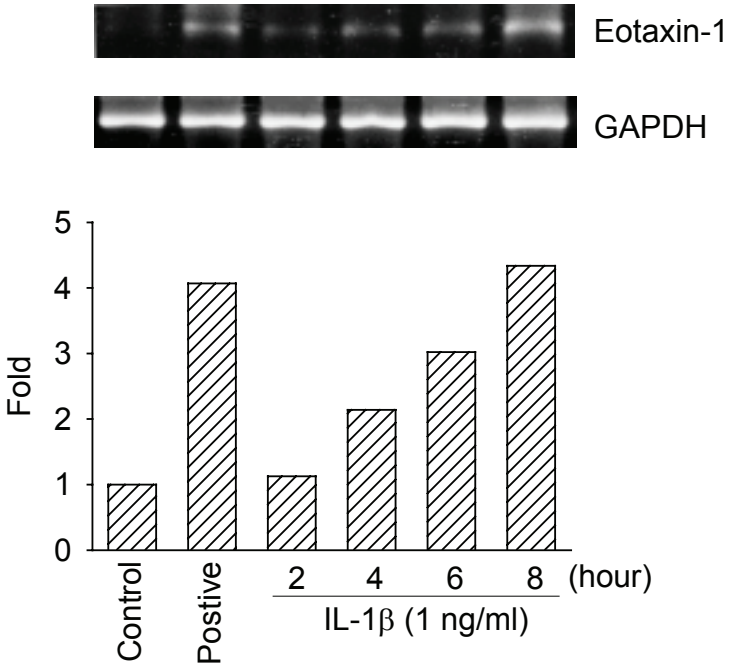

A.
RANTES (ng/ml)
40
35
30
25
20
15
10
5
0
*
Normal
OA
B.
MCP-1α (pg/ml)
1200
1000
800
600
400
200
0
*
Normal
OA
C.
Eotaxin-1 (pg/ml)
250
200
150
100
50
0
*
Normal
OA

Fig. 2

A.


Eotaxin-1
GAPDH
Fold
0
1
2
3
4
5
Control
Postive
2
4
6
8
(hour)
IL-1β (1 ng/ml)

B.

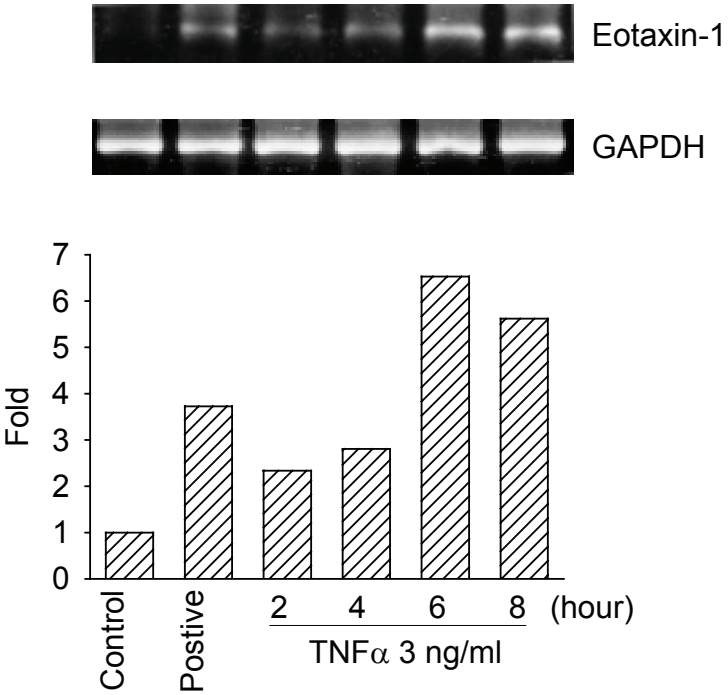

Eotaxin-1
GAPDH
Fold
0
1
2
3
4
5
6
7
Control
Postive
2
4
6
8
(hour)
TNFα 3 ng/ml

Fig. 2

C.

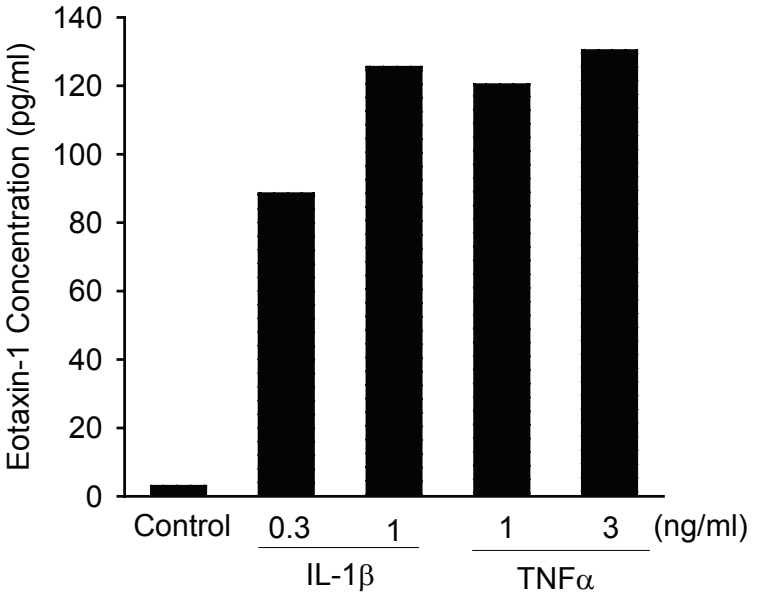

Eotaxin-1 Concentration (pg/ml)
140
120
100
80
60
40
20
0
Control
0.3
1
1
3
(ng/ml)
IL-1β
TNFα

Fig. 3

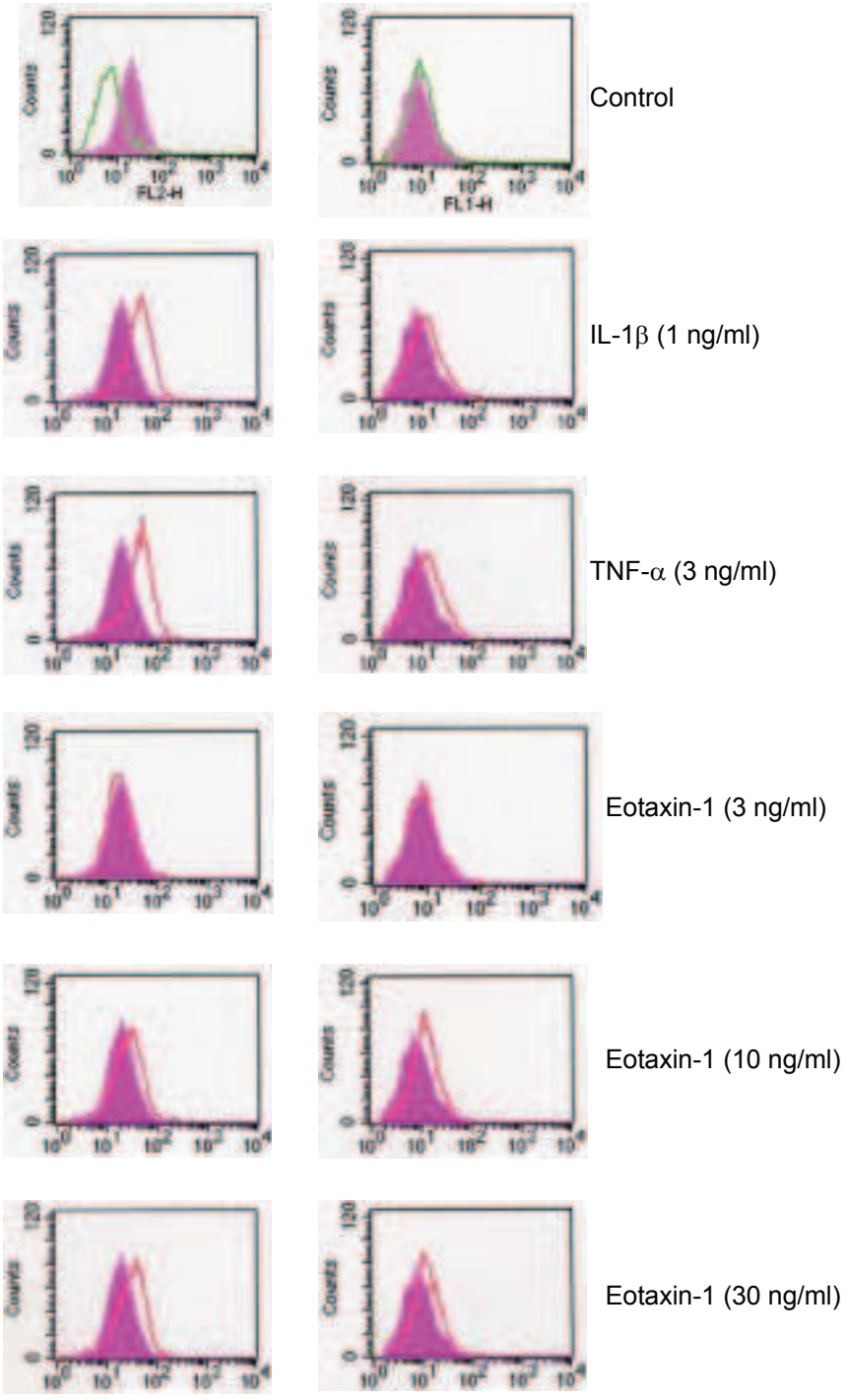

Control
IL-1β (1 ng/ml)
TNF-α (3 ng/ml)
Eotaxin-1 (3 ng/ml)
Eotaxin-1 (10 ng/ml)
Eotaxin-1 (30 ng/ml)
CCR3
CCR5

Fig. 4

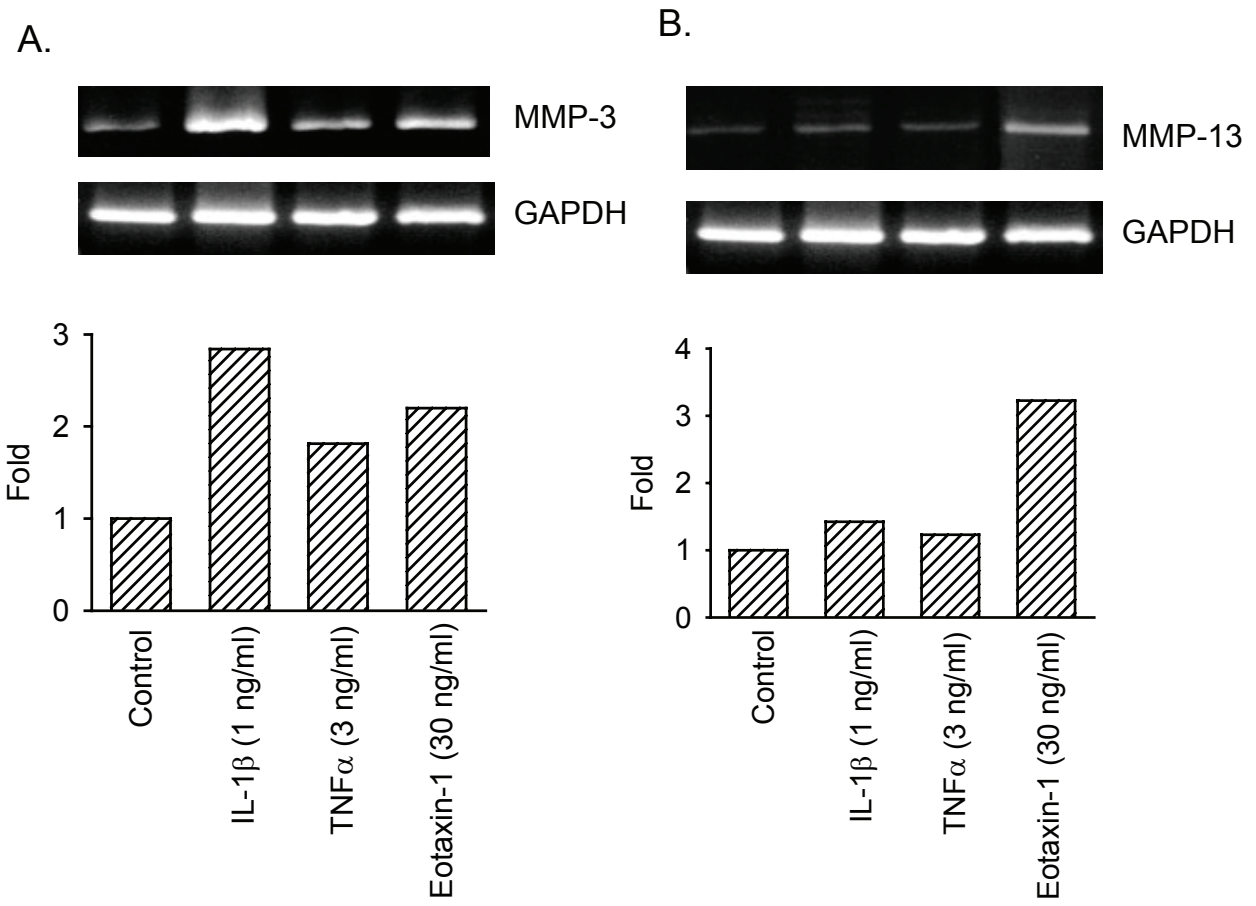

A.
MMP-3
GAPDH
Fold
3
2
1
0
Control
IL-1β (1 ng/ml)
TNFα (3 ng/ml)
Eotaxin-1 (30 ng/ml)
B.
MMP-13
GAPDH
Fold
4
3
2
1
0
Control
IL-1β (1 ng/ml)
TNFα (3 ng/ml)
Eotaxin-1 (30 ng/ml)

Fig. 4

C.

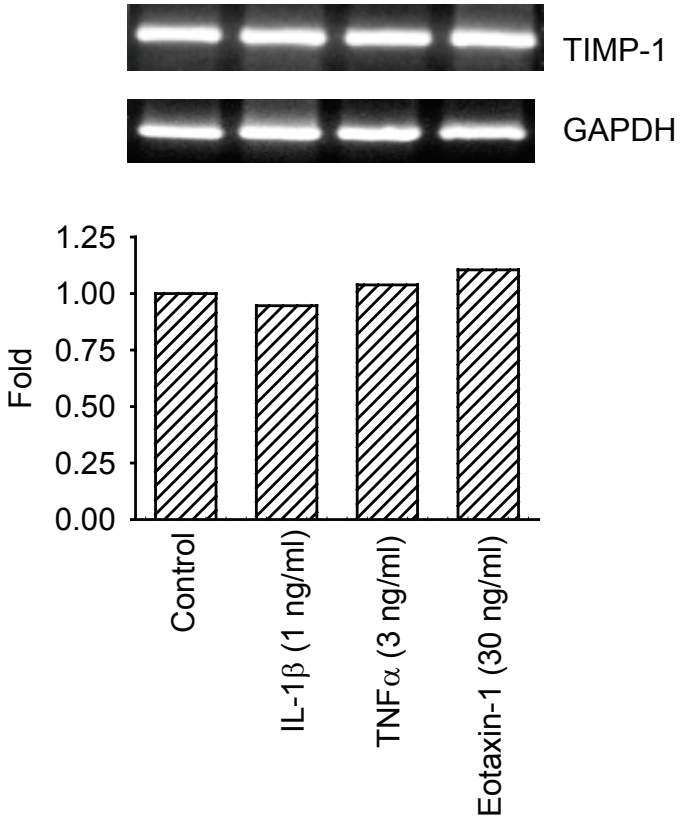
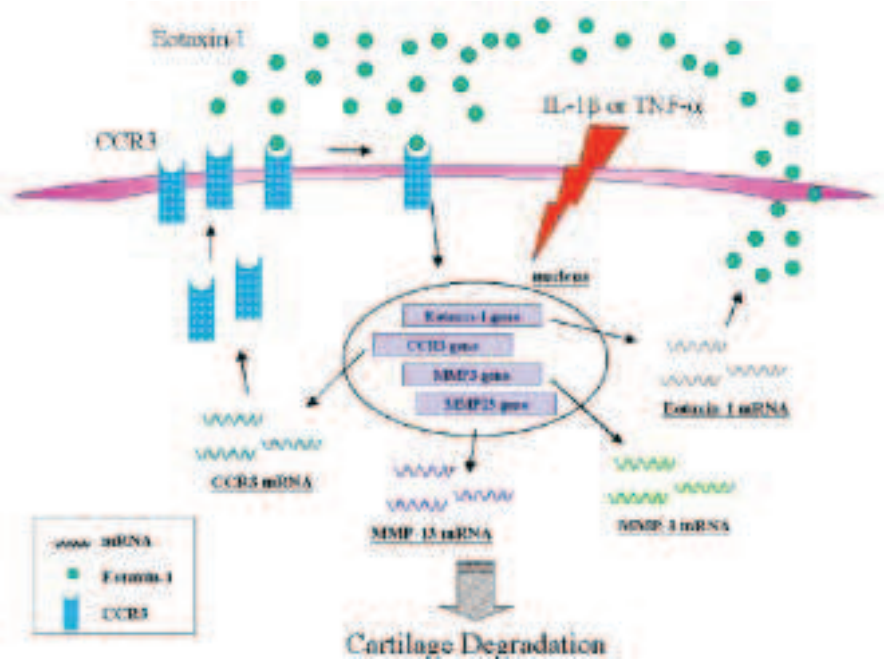

TIMP-1
GAPDH
Fold
1.25
1.00
0.75
0.50
0.25
0.00
Control
IL-1β (1 ng/ml)
TNFα (3 ng/ml)
Eotaxin-1 (30 ng/ml)

Fig. 5


Eotaxin-1
IL-1β or TNF-α
CCR3
nucleus
CCR3 mRNA
MMP-13 mRNA
Eotaxin-1 mRNA
MMP-3 mRNA
Cartilage Degradation