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Suppression of matrix metalloproteinase production from synovial fibroblasts by meloxicam in-vitro

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Abstract

The aim of this study was to evaluate the influence of meloxicam on the production of both matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) from human synovial fibroblasts by TNF-α stimulation in-vitro. Synovial fibroblasts (2×10^4 cells/mL) derived from patients with osteoarthritis were stimulated with 20.0 ng mL^{-1} TNF-α in the presence of various concentrations of meloxicam. After 24 h, the culture supernatants were obtained and assayed for MMP-1, MMP-2, MMP-3, MMP-13, TIMP-1 and TIMP-2 by ELISA. mRNA expression for MMPs and TIMPs in 4-h-cultured cells were examined by real-time polymerase chain reaction. Transcriptional factor (NF-κB and AP-1) activation in 2-h-cultured cells was also examined by ELISA. Meloxicam could suppress MMP production in a dose-dependent manner. The minimum concentration of the agent that showed significant suppression was 0.6×10^{-6} M for MMP-1, MMP-2 and MMP-3, and 1.3×10^{-6} M for MMP-13. The ability of synovial fibroblasts to produce TIMPs was also suppressed by meloxicam as in the case of MMP production. Addition of meloxicam into synovial fibroblast cultures inhibited dose-dependently mRNA expression for MMPs and TIMPs, which were increased by TNF-α stimulation, through the suppression of NF-κB and AP-1 activation. The suppressive effect of meloxicam on the production of MMPs and TIMPs may partly be involved in attenuation of the clinical conditions of osteoarthritis and rheumatoid arthritis.

Introduction

Osteoarthritis (OA) is characterized by loss of articular cartilage and secondary bone as well as changes to the synovium, including marginal osteophyte formation and synovitis (Altman 1997). The two main macromolecules of articular cartilage extracellular matrix are the large aggregating proteoglycans, aggrecan and type II collagen (Gendron et al 2003). Type II collagen fibrillar network forms the backbone of cartilage and provides it with stability and tensile strength, while the proteoglycan component is highly hydrated, absorbs loads and provides cartilage with compressive stiffness. Destruction of these components has been observed to occur in OA cartilage (Gendron et al 2003; Kafienah et al 2003).

It has been shown that both biochemical and mechanical changes may contribute to the development of OA. Chondrocytes may respond to excessive loading by elaboration of degradative enzymes and inappropriate repair responses (Grodzinsky et al 2000; Jin et al 2000). Furthermore, a number of studies have also demonstrated that inflammatory cytokines, such as interleukin (IL)-1 and tumour necrosis factor (TNF), as well as functional changes of chondrocytes themselves, play potential roles in the process of deterioration by inducing expression of proteinases, such as those of the matrix metalloproteinase (MMP) family (Shinmei et al 1991; Westacott & Sharif 1996; Yuan et al 2001). MMPs are members of a unique family of zinc-binding endopeptidases that are responsible for degradation of collagen, elastin and other macromolecules of the extracellular matrix (Sadowski & Steinmeyer 2001; Kafienah et al 2003). As expected from their degradative nature, the uncontrolled expression of MMPs has been reported to be associated with many pathological conditions in articular cartilage of both rheumatoid arthritis and OA (Yamada et al 1996; Kafienah et al 2003), whereas their controlled expression is essential for normal tissue remodelling and wound healing (Nagase & Woessner 1999).

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Non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are frequently used in OA therapy. Despite their therapeutic efficacy in modulating clinical status, especially pain, of OA, their influence on cartilage metabolism is not fully understood (Yamada et al 1996; Sadowski & Steinmeyer 2001). Several lines of evidence clearly showed that inflammatory processes, such as the intense infiltration of inflammatory cells in synovial fluid, joint swelling or synovial effusion in the affected joints, are one of the most common clinical features in OA (Jasin 1989; Specter et al 1996; Nakamura et al 1999). It is accepted that these inflammatory responses require the alteration of basement membrane composed of fibroblasts, which is controlled by MMPs. Furthermore, the importance of synovial fibroblasts as producers of MMPs, in addition to chondrocytes, is reported (Yamada et al 1996; Sadowski & Steinmeyer 2001; Masuhara et al 2002; Kafienah et al 2003; Pillinger et al 2003). Therefore, this study was undertaken to examine the influence of meloxicam, a preferential cyclooxygenase (COX)-2 inhibitor (Fenner 1998; Pairet & van Ryn 1998; Sadowski & Steinmeyer 2001), on MMP generation from synovial fibroblasts in response to TNF-α stimulation in-vitro.

Materials and Methods

Reagents

Meloxicam was donated by Nippon Boehringer Ingelheim Co. Ltd (Hyogo, Japan) as a preservative-free pure powder. This was firstly dissolved in dimethyl sulfoxide (DMSO; Sigma Pure Chemical Ind., St Louis, MO) at a concentration of 5.0 mg mL^{-1} and was then diluted with antibiotic-free RPMI-1640 medium (Sigma Pure Chemical Ind.) supplemented with 10% heat-inactivated fetal calf serum (RPMI-FCS; Irvine, Santa Ana, CA) at appropriate concentrations just before use. Human recombinant TNF-α, preservative-free, was purchased from CHEMICON International, Inc. (Temecula, CA). This was dissolved in normal saline at a concentration of 50.0 μg mL^{-1} and then diluted with RPMI-FCS at 80.0 ng mL^{-1} just before use.

Cell source

Synovial tissues were obtained from OA patients at the time of joint replacement, under informed consent, which was confirmed by the Ethics Committee of Showa University. The experimental protocols were also approved by the Ethics Committee of Showa University. Donors were all female, aged 70–78 years. They were not medicated, including NSAID administration, two weeks before surgical operation. The tissues obtained were cut into small pieces (approximately 1.0 mm^2) and then rinsed several times with normal saline containing 500 U penicillin, 500 μg mL^{-1} streptomycin and 5.0 μg mL^{-1} amphotericin B. The diced tissues were plated at a density of 10 pieces per dish in 100-mm tissue culture dishes and covered with microscope slides, which were stuck to the dish with sterile Vaseline. They were then stored at 37°C in a humidified atmosphere containing 5% CO_2. When a monolayer of fibroblast-like cells was found to be confluent, the explanted tissues were removed. The cells were then trypsinized and re-plated at a concentration of 5×10^5 cells/mL. The medium was changed every 3 days for 2–3 weeks until confluence was attained. Subsequently, the cells were split into 1:2 at confluency and passaged. The cells were then characterized (Asano et al 2003) and used as synovial fibroblasts.

Cell culture

Synovial fibroblasts, 5th–7th generation, were introduced into each well of 24-well culture plates at a concentration of 2×10^5 cells/mL and allowed to adhere for 2 h. After gently washing twice to remove un-attached and dead cells, the attached cells were cultured with or without 20.0 ng mL^{-1} TNF-α in the presence of various concentrations of meloxicam. After 24 h, the culture supernatants were removed and stored at −40°C until used. To prepare cells for examining mRNA expression and transcriptional factor activation, synovial fibroblasts were cultured in a similar manner for 4 h and 2 h, respectively. In all cases, meloxicam was added to cell cultures at the start of TNF-α simulation.

Assay for MMP-1, MMP-2, MMP-3, MMP-13, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2

MMP and TIMP levels in culture supernatants were examined by commercially available human MMP-1, MMP-2, MMP-3, MMP-13, TIMP-1 and TIMP-2 ELISA assay kits (Amersham Biosciences Corp., Piscataway, NJ) according to manufacturer's recommendations. The sensitivity of these ELISA kits was 1.7 ng mL^{-1} for MMP-1, 0.37 ng mL^{-1} for MMP-2, 0.6 ng mL^{-1} for MMP-3, 0.094 ng mL^{-1} for MMP-13, 51.0 ng mL^{-1} for TIMP-1 and 3.0 ng mL^{-1} for TIMP-2. The activity of MMP-1, MMP-2, MMP-3 and MMP-13 in culture supernatants was also examined by ELISA test kits (Amersham Biosciences Corp.) according to the manufacturer's instructions. The sensitivity of these ELISA kits was 3.0 ng mL^{-1} for MMP-1, 0.5 ng mL^{-1} for MMP-2, 5.0 ng mL^{-1} for MMP-3 and 0.5 ng mL^{-1} for MMP-13.

Assay for transcriptional factors (NF-κB and AP-1)

NF-κB activity was analysed by a commercially available ELISA test kit (Active Motif, Co. Ltd, Carlsbad, CA), which contained sufficient reagents and monoclonal antibodies against P50 and P65 according to the manufacturer's recommendation. In brief, nuclear extract (5.0 μg protein) from synovial fibroblasts was introduced into each well of 96-well microtitre plates pre-coated with oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) in a volume of 20.0 μL, and incubated for 1 h at 25°C. After washing three times, 100 μL of monoclonal antibody against P50 or P65 was added to the appropriate wells, and incubated for a further 1 h at 25°C. Anti-IgG horseradish-peroxidase conjugate in a volume of 100 μL was then added and incubated for 1 h at 25°C. Absorbance at 450 nm was measured after the addition of tetramethylbenzidine solution. AP-1 activity was also examined by a commercially available ELISA test kit

(Active Motif Co. Ltd) that contained sufficient reagents and monoclonal antibodies to Fra 1 and Jun B in a similar manner. According to manufacturer's data sheets, the amount of transcription factors, NF-κB and AP-1, which bind to DNA can be measured by the ELISA system.

Real-time polymerase chain reaction (PCR)

mRNA was extracted from synovial fibroblasts using μMACS mRNA isolation kits (Milteny Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The first-strand cDNA synthesis from 1.0 μg mRNA was performed using the SuperScript Preamplification System for cDNA synthesis (GIBCO BRL, Gaithersburg, MD). PCR was then carried out using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR mixture consisted of 2.0 μL of sample cDNA solution (10.0 ng μL^{-1}), 25.0 μL of SYBR-Green Mastermix (Applied Biosystems), 0.3 μL of both sense and anti-sense primers and distilled water to give a final volume of 50 μL. The reaction was conducted as follows: 4 min at 95 °C, followed by 40 cycles of 15 s at 95°C and 60 s at 60 °C. β-Actin was amplified as an internal control. mRNA levels were calculated by using the comparative parameter threshold cycle (Ct) and normalized to β-actin. The primers used for RT-PCR were: 5′-AAAG GGAATAAGTACTGGG-3′ (sense) and 5′-GTTTTTCCAGT GTTTTCCTCAG-3′ (anti-sense) for MMP-1 (Kobayashi et al 2005); 5′-CTGTCCTGACCAAGGATATAGCCT-3′ (sense) and 5′-ACCTGTGGGCTTGTCACGTGGTGT-3′ (anti-sense) for MMP-2; 5′-CTGTCCAGACCAAGGGTACAGCCT-3′ (sense) and 5′-GTGGTATAGTGGGACACATAGTGG-3′ (anti-sense) for MMP-9; 5′-CCTTATACCAGCCGTTATAAGATCAA GAT-3′ (sense) and 5′-GTCCACAAACAGTGAGTGTCACTC-3′ (anti-sense) for TIMP-1; 5′-GCAATGCAGACGTAGTGAT CAGAG-3′ (sense) and 5′-GATCATGGGACAGCGAGT GATCTT-3′ (anti-sense) for TIMP-2 (Uchida et al 2000); and 5′-CGGAACCGCTCATTGCC-3′ (sense) and 5′-ACCCACACT GTGCCCATCTA-3′ (anti-sense) for β-actin (Asano et al 2003; Kanai et al 2004).

Statistical analysis

The results were expressed as the mean ± s.e. of five different subjects. The statistical significance of the difference between the control and experimental data was analysed using analysis of variance followed by Fisher's PLSD test. $P < 0.05$ was considered statistically significant.

Results

Suppressive activity of meloxicam on MMP and TIMP production from synovial fibroblasts after TNF-α stimulation

The first set of experiments was undertaken to examine whether meloxicam could suppress the ability of synovial fibroblasts to produce MMP and TIMP. The influence of meloxicam on spontaneous MMP production was firstly examined. Synovial fibroblasts at 2×10^4 cells/mL were cultured with either 0, 0.3, 0.6, 1.3, 2.0 or 2.5×10^{-6} M meloxicam for 24 h. MMP levels in culture supernatants were assayed by ELISA. As shown in Table 1, meloxicam could suppress the spontaneous production of MMP-1, MMP-2, MMP-3 and MMP-13, when the cells were treated with meloxicam at 0.6×10^{-6} M and more. The next experiments were undertaken to examine whether meloxicam could also suppress MMP production induced by TNF-α stimulation. Cells were cultured with 20.0 ng mL^{-1} TNF-α in the presence of various concentrations of meloxicam for 24 h. MMP levels in culture supernatants were also assayed by ELISA. Addition of meloxicam to cell cultures suppressed the ability of synovial fibroblasts to produce MMP-1, MMP-2 and MMP-3 in a dose-dependent manner (Table 2). The minimum concentration of the agent that caused significant suppression of production of these MMPs was 0.6×10^{-6} M. The data in Table 2 also show the clear inhibitory action of meloxicam on TNF-α-induced MMP-13 production from synovial fibroblasts when the cells were treated with meloxicam at 1.3×10^{-6} M or more. The third experiment was undertaken to examine the influence of meloxicam on TIMP production. As shown in Table 3, meloxicam could suppress the spontaneous production of both TIMP-1 and TIMP-2. The minimum concentration of meloxicam that caused significant suppression was 1.3×10^{-6} M and 0.6×10^{-6} M, respectively. The fourth experiment was designed to examine whether meloxicam could also suppress TNF-α-induced TIMP production. The data in Table 3 show that meloxicam could exert a suppressive effect on

Table 1 Influence of meloxicam on spontaneous MMP production from synovial fibroblasts in-vitro

Treatment	Dose of agent ($\times 10^{-6}$ M)	MMP level (ng mL^{-1})			
		MMP-1	MMP-2	MMP-3	MMP-13
Cells alone		20.5 ± 3.4	20.5 ± 1.4	0.66 ± 0.07	0.56 ± 0.10
Meloxicam	0.3	19.4 ± 2.6	21.4 ± 3.5	0.60 ± 0.07	0.60 ± 0.10
	0.6	10.8 ± 1.4*	11.8 ± 3.4*	0.42 ± 0.05*	0.30 ± 0.08*
	1.3	9.4 ± 2.5*	10.4 ± 1.5*	0.30 ± 0.07*	0.30 ± 0.09*
	2.0	8.2 ± 2.1*	9.2 ± 2.1*	0.30 ± 0.07*	0.20 ± 0.08*
	2.5	5.2 ± 1.1*	9.2 ± 1.1*	0.25 ± 0.09*	0.21 ± 0.13*

Data are means ± s.e., n = 5. Human synovial fibroblasts (2×10^4 cells/mL) obtained from patients with OA were cultured with various concentrations of meloxicam for 24 h. *$P < 0.05$ compared with cells alone (analysis of variance, PLSD test).

Table 2 Influence of meloxicam on MMP production from synovial fibroblasts in response to TNF-α stimulation in-vitro

Treatment	Dose of agent ($\times 10^{-6}$ M)	MMP level (ng mL^{-1})			
		MMP-1	MMP-2	MMP-3	MMP-13
Cells alone		25.7 ± 10.2	21.9 ± 3.6	N.E.	N.E.
TNF-α alone		93.8 ± 21.1	78.9 ± 13.6	19.1 ± 2.6	1.6 ± 0.3
TNF-α + meloxicam	0.3	90.6 ± 17.4	75.5 ± 16.7	17.4 ± 1.8	1.6 ± 0.5
	0.6	57.6 ± 14.2*	55.3 ± 5.9*	11.9 ± 1.5*	1.4 ± 0.3*
	1.3	49.6 ± 14.9*	47.6 ± 4.8*	8.5 ± 1.2*	0.9 ± 0.1*
	2.0	42.1 ± 12.8*	46.2 ± 5.5*	6.70 ± 1.27*	0.8 ± 0.2*
	2.5	40.1 ± 10.5*	35.2 ± 4.2*	6.2 ± 0.9*	0.7 ± 0.3*
	3.0	30.9 ± 11.6*	34.5 ± 2.4*	3.7 ± 1.3*	0.5 ± 0.2*

Data are means ± s.e., n = 5. Human synovial fibroblasts (2×10^4 cells/mL) obtained from patients with OA were stimulated with 20.0 ng mL^{-1} TNF-α in the presence of various concentrations of meloxicam for 24 h. N.E., not examined. *$P < 0.05$ compared with TNF-α alone.

Table 3 Influence of meloxicam on TIMP production from synovial fibroblasts in-vitro

Treatment	Dose of agent ($\times 10^{-6}$ M)	TIMP level (ng mL^{-1})			
		TIMP-1[a]	TIMP-2[a]	TIMP-1	TIMP-2
Cells alone		100.0 ± 5.0	12.5 ± 0.3	146.8 ± 16.0	8.3 ± 0.7
TNF-α alone		N.E.	N.E.	228.7 ± 18.4	29.9 ± 2.3
Meloxicam	0.3	90.0 ± 7.5	13.5 ± 0.25	211.9 ± 42.1	29.8 ± 2.7
	0.6	92.5 ± 2.5	8.00 ± 0.35*	201.4 ± 31.1	23.3 ± 1.3*
	1.3	60.0 ± 2.0*	6.5 ± 0.3*	156.8 ± 21.1*	22.4 ± 1.9*
	2.0	60.0 ± 2.5*	5.00 ± 0.25*	157.7 ± 19.6*	17.6 ± 1.4*
	2.5	50.0 ± 2.5*	5.0 ± 0.3*	146.7 ± 10.3*	14.5 ± 0.6*
	3.0	N.E.	N.E.	132.1 ± 10.1*	11.1 ± 0.7*

Data are means ± s.e., n = 5. Human synovial fibroblasts (2×10^4 cells/mL) obtained from patients with OA were stimulated with (or without[a]) 20.0 ng mL^{-1} TNF-α in the presence of various concentrations of meloxicam for 24 h. N.E., not examined. *$P < 0.05$ compared with TNF-α alone (analysis of variance, PLSD test).

Table 4 Influence of meloxicam on MMP activity in culture supernatants from synovial fibroblasts in response to TNF-α stimulation in-vitro

Treatment	Dose of agent ($\times 10^{-6}$ M)	MMP level (ng mL^{-1})			
		MMP-1	MMP-2	MMP-3	MMP-13
Cells alone		20.3 ± 3.9	20.9 ± 3.7	N.E.	N.E.
TNF-α alone		78.9 ± 8,3	65.4 ± 4.8	15.3 ± 0.6	1.0 ± 0.1
TNF-α + meloxicam	0.3	78.1 ± 7.7	65.5 ± 3.5	14.2 ± 0.5	1.0 ± 0.2
	0.6	50.0 ± 7.0*	45.3 ± 2.5*	10.1 ± 0.6*	0.7 ± 0.1*
	1.3	48.7 ± 5.5*	44.6 ± 2.1*	7.8 ± 0.5*	0.60 ± 0.07*
	2.0	32.5 ± 4.3*	37.6 ± 3.2*	6.5 ± 0.7*	0.50 ± 0.09*
	2.5	33.3 ± 2.8*	34.7 ± 5.7*	5.5 ± 0.5*	0.4 ± 0.1*

Data are means ± s.e., n = 5. Human synovial fibroblasts (2×10^4 cells/mL) obtained from patients with OA were stimulated with 20.0 ng mL^{-1} TNF-α in the presence of various concentrations of meloxicam for 24 h. N.E., not examined. *$P < 0.05$ compared with TNF-α alone.

TIMP-1 and TIMP-2 production from synovial fibroblasts, and that this suppressive activity was firstly noted at 1.3×10^{-6} M for TIMP-1 and 0.6×10^{-6} M for TIMP-2. The final experiments in this section were carried out to examine the influence of meloxicam on MMP activity. As shown in Table 4, addition of meloxicam at 0.6×10^{-6} M and more into cell cultures could suppress the activity of the MMPs examined.

Influence of meloxicam on MMP and TIMP mRNA expression

The second set of experiments was undertaken to examine the possible mechanisms by which meloxicam suppressed MMP and TIMP production from synovial fibroblasts in response to TNF-α stimulation. Synovial fibroblasts were cultured with 20.0 ng mL^{-1} TNF-α in the presence of several doses of meloxicam for 4 h and mRNA expression was examined by real-time RT-PCR. The data in Table 5 clearly show the suppressive effects of meloxicam on mRNA expression for MMP-1, MMP-2, MMP-3 and MMP-13 when the agent was added to cell cultures at 0.6×10^{-6} M and more. As in the case of MMP mRNA expression, meloxicam could also suppress the enhancement of TIMP mRNA expression induced by TNF-α stimulation in synovial fibroblasts (Table 5), and the minimum concentration of the agent that caused significant suppression of TIMP mRNA expression was 0.6×10^{-6} M (Table 5).

Suppression of the activation of NF-κB and AP-1 by meloxicam

The final set of experiments was designed to examine the influence of meloxicam on transcriptional factor (NF-κB and AP-1) activation by TNF-α stimulation. Synovial fibroblasts were cultured with 20.0 ng mL^{-1} TNF-α in the presence of various concentrations of meloxicam for 2 h and factor activation was measured by ELISA. Addition of meloxicam at 0.3×10^{-6} M into cell cultures did not affect NF-κB activation. Optical density at 450 nm in experimental groups was similar (not significant) to those in appropriate controls (Table 6). However, meloxicam at 0.6×10^{-6} M and more caused significant suppression of P50 and P65 activation (Table 6). The data in Table 6 also clearly showed the suppressive activity of meloxicam on AP-1, Fra 2 and Jun B activation in synovial fibroblasts after TNF-α stimulation.

Table 5 Influence of meloxicam on mRNA expression for MMPs and TIMPs in synovial fibroblasts in-vitro

Treatment	Dose of agent ($\times10^{-6}$ M)	MMP level (ng mL^{-1})					
		MMP-1	MMP-2	MMP-3	MMP-13	TIMP-1	TIMP-2
Cells alone		0.29 ± 0.09	0.32 ± 0.10	0.60 ± 0.10	0.29 ± 0.10	0.41 ± 0.08	0.44 ± 0.06
TNF-α alone		1.08 ± 0.22	0.95 ± 0.14	1.09 ± 0.10	1.18 ± 0.25	0.47 ± 0.08	0.53 ± 0.02
TNF-α + meloxicam	0.3	1.15 ± 0.13	0.90 ± 0.10	1.10 ± 0.10	1.15 ± 0.22	0.49 ± 0.07	0.49 ± 0.04
	0.6	0.49 ± 0.10*	0.42 ± 0.05*	0.67 ± 0.12*	0.59 ± 0.15*	0.21 ± 0.06*	0.31 ± 0.08*
	1.3	0.47 ± 0.06*	0.39 ± 0.05*	0.57 ± 0.12*	0.37 ± 0.06*	0.28 ± 0.07*	0.34 ± 0.05*

Data are means ± s.e., n = 5. Human synovial fibroblasts (2×10^4 cells/mL) obtained from patients with OA were stimulated with 20.0 ng mL^{-1} TNF-α in the presence of various concentrations of meloxicam for 24 h. *$P < 0.05$ compared with TNF-α alone (analysis of variance, PLSD test).

Table 6 Influence of meloxicam on the activation of NF-κB and AP-1 in synovial fibroblasts after TNF-α stimulation in-vitro

Treatment	Dose of agent ($\times10^{-6}$ M)	OD at 450 nm			
		NF-κB		AP-1	
		P50	P65	Fra-1	Jun B
Cells alone		0.27 ± 0.02	0.17 ± 0.01	0.14 ± 0.04	0.13 ± 0.04
TNF-α alone		0.78 ± 0.06	1.33 ± 0.20	0.98 ± 0.12	1.26 ± 0.12
TNF-α + meloxicam	0.3	0.82 ± 0.05	1.39 ± 0.13	0.98 ± 0.10	1.24 ± 0.10
	0.6	0.52 ± 0.03*	0.71 ± 0.11*	0.89 ± 0.14	1.15 ± 0.13
	1.3	0.45 ± 0.07*	0.53 ± 0.16*	0.51 ± 0.09*	0.81 ± 0.12*
	2.0	0.33 ± 0.02*	0.28 ± 0.11*	0.43 ± 0.07*	0.61 ± 0.07*
	2.5	0.23 ± 0.03*	0.16 ± 0.04*	0.43 ± 0.08*	0.39 ± 0.05*

Data are means ± s.e., n = 5. Human synovial fibroblasts (2×10^4 cells/mL) obtained from patients with OA were stimulated with 20.0 ng mL^{-1} TNF-α in the presence of various concentrations of meloxicam for 24 h. *$P < 0.05$ compared with cells alone (analysis of variance, PLSD test).

Discussion

MMPs are reported to be efficient in cleaving many components of cartilage extracellular matrix and to activate pro-MMPs (Sadowski & Steinmeyer 2001; Kafienah et al 2003). Elevated levels of MMPs and imbalance between MMP activity and TIMP inhibition have been found in synovial fluid from patients with OA (Dean et al 1989; Pelletier et al 1990; Woessner 1991; Masuhara et al 2002). It is also observed that chondrocyte and synovial fibroblasts from OA patients could produce significantly higher levels of MMPs than those from healthy control subjects when these cells were stimulated with inflammatory cytokines in-vitro (Masuhara et al 2002; Roy-Beaudry et al 2003). These reports may suggest the implied involvement of MMPs in the pathogenesis of cartilage destructive diseases, such as OA and rheumatoid arthritis, and that the modulation of the ability of cells in joints to produce MMPs may be of great therapeutic value for the treatment of patients with OA. However, there is little evidence showing the influence of drugs such as NSAIDs, which are frequently used for OA treatment, on MMP production (Yamada et al 1996; Sadowski & Steinmeyer 2001).

These results clearly show that meloxicam could suppress the production of MMPs and TIMPs from synovial fibroblasts, induced by TNF-α stimulation, in a dose-dependent manner. The minimum concentration of meloxicam that suppressed the production of the MMPs and TIMPs examined was 0.6×10^{-6} M. After oral administration of meloxicam at a dose of 15.0 mg, which is a recommended therapeutic dose, to man, the blood levels of the agent gradually increased and attained plateau at 1.1 μg mL^{-1} (2.4×10^{-6} M) (Fenner 1998; Pairet & Ryn 1998). It is also reported that the maximum synovial fluid concentration of meloxicam is 0.55 μg mL^{-1} (1.4×10^{-6} M), when 15.0 mg meloxicam was orally administered to man (Fenner 1998). From these reports, our results should reflect the biological function in-vivo.

The MMPs comprise an enzyme superfamily of at least 21 members, which can be classified into subgroups of collagenases, stromelysins, gelatinases, membrane-type MMPs and other MMPs (Uchida et al 2000; Sadowski & Steinmeyer 2001; Gendron et al 2003). MMP-1 and MMP-13 are known to cleave type I and II collagens, the most abundant protein present in the osteroid layer, into characteristic 3:1 length fragments, which subsequently denature into random coiled polypeptide chains (Knauper et al 1996). The digestion of type I and II collagens by MMP-1 and MMP-13 thus appears to be the initial steps of the entire bone resorption process. Subsequently, denatured collagen fragments can be degraded further by two gelatinases, MMP-2 and MMP-9. MMP-3, on the other hand, has a broad substrate specificity and can cleave other bone matrix proteins, such as proteoglycans, glycoproteins and native types IV, VII and IX collagens, in the osteoid layer (Nagase 1995). MMP-3 is also accepted to be essential for full activation of pro-MMP-2 and pro-MMP-13 (Knauper et al 1996; Uchida et al 2000). From these reports, our results may suggest that meloxicam decreases the ability of synovial fibroblast to produce MMPs induced by cytokine stimulation, and indirectly protects degeneration of osteoid layer covering the mineralized bone matrix, resulting in prevention of destruction of joint cartilage and subchondral bone. Apart from a pivotal role in cartilage degradation, MMPs are reported to enhance microvascular permeability (Herouy et al 2001), which is responsible for oedema and cell migration into the location of the diseases, suggesting that the attenuating effect of meloxicam on MMP production may contribute to the inhibition of infiltration of inflammatory cells in synovial fluid, joint swelling or synovial effusion in the affected joints. Besides MMP inhibitory activity, TIMPs are well known to have several unique functions, which are independent of their inhibitory action. TIMP-1 and TIMP-2 are reported to act as growth factors for a wide range of cells, including mouse embryo fibroblasts and human gingival fibroblasts (Hayakawa et al 1994; Wingfield et al 1999). TIMP-3 also has a potent growth-promoting activity in the human fibroblast cell line Hs 68 (Yang & Hawkes 1992). Histological observation of OA joints clearly showed the development of fibrosis on the surface of cartilage (Nishida et al 2003). From these reports, administration of meloxicam to patients may prevent the growth of fibroblasts in OA joints and favorably modify the clinical condition of the affected joints.

Indometacin, the best-known NSAID, could significantly increase TIMP-1 production from human chondrocytes induced by IL-1β stimulation in-vitro when the agent was added to cell cultures at a concentration comparable with that found in the synovial fluid of patients orally administered with the drug, as assessed by protein level and mRNA expression (Yamada et al 1996). On the other hand, our results clearly show that meloxicam, at a therapeutic dose, could not increase in TIMP-1 production from synovial fibroblasts by TNF-α stimulation in-vitro. This discrepancy may be explained by the differences in cell types, culture condition and stimulation.

MMP gene expression requires translocation of the nuclear transcription factors NF-κB and AP-1 to the nucleus and binding to promoter regions, which causes the induction of mRNA expression (Sakai et al 2001). It is reported that NSAIDs could inhibit the activation of transcriptional factors NF-κB and AP-1 (Joussen et al 2002; Kundu et al 2004), suggesting that meloxicam inhibits the activation of NF-κB and AP-1 induced by TNF-α stimulation and results in suppression of MMP production. This suggestion may be supported, in part, by our observation of the suppressive activity of meloxicam, at a therapeutic synovial fluid concentration, on NF-κB (P50 and P65) and AP-1 (Fra 1 and Jun B) activation induced by TNF-α stimulation. In addition to transcription factors NF-κB and AP-1, prostaglandins, especially prostaglandin E2 (PGE2), have been reported to regulate the production of MMPs in synovial fibroblasts and tumour cells by cytokine stimulation (Martel-Pelletier et al 2003). This suppressive effect of PGE2 on MMP production was further confirmed by experiments using NS-398, a specific COX-2 inhibitor, in gingival fibroblasts in-vitro (Domeij et al 2002). It is also reported that meloxicam, at more than 1.4×10^{-6} M, could suppress COX-2 activation (Pairet & van Ryn 1998), with the capacity to inhibit PGE2 biosynthesis in-vitro and in-vivo (Engelhardt et al 1996; Ogino et al 1997). Together with our results showing that meloxicam, at 0.6×10^{-6} M, exerted suppressive effects on MMP production, it is reasonable to

speculate that PGE2-independent mechanism(s) may be responsible for the suppressive activity of meloxicam on MMP production from synovial fibroblasts induced by TNF-α stimulation. In addition to the COX-2- and PGE2-dependent signal pathway to produce MMPs, the c-Jun N-terminal kinase (JNK)-dependent signal pathway, which is activated by TNF-α stimulation, is involved in MMP production in synovial fibroblasts (Pillinger et al 2003). p38 kinases were also found to play an important role in the regulation of collagenase (Reunanen et al 1998) and gelatinase (Simon et al 1998) gene expression in cultured cells. These reports may suggest that meloxicam suppresses the activation of both JNK and p38 kinases induced by TNF-α stimulation and results in inhibition of MMP production in synovial fibroblasts. This suggestion may be supported by the observation that COX-2 inhibitors could suppress the activation of JNK and p38 kinases, which are involved in MMP production in articular chondrocytes (Liacini et al 2002), induced by inflammatory stimulation in-vitro (Shishodia et al 2004; Kim et al 2005).

In conclusion, these results clearly demonstrate that meloxicam acts not only against the degenerative joint disease symptoms of pain and inflammation but can also interfere with the collagenase activity that underlies the destruction of articular cartilage during OA. These effects may contribute, in part, to the favourable modification of clinical status and quality of life of patients with OA.

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