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Interleukin-4 Antagonizes Oncostatin M and Transforming Growth Factor Beta-Induced Responses in Articular Chondrocytes

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Abstract Oncostatin M (OSM) stimulates cartilage degradation in rheumatoid arthritis (RA) by inducing matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS; a disintegrin and metalloproteinase with thrombospondin motif). Transforming growth factor beta (TGF-β1) induces cartilage repair in joints but in excessive amounts, promotes inflammation. OSM and TGF-β1 also induce tissue inhibitor of metalloproteinase-3 (TIMP-3), an important natural inhibitor of MMPs, aggrecanases, and tumor necrosis factor alpha converting enzyme (TACE), the principal proteases involved in arthritic inflammation and cartilage degradation. We studied cartilage protective mechanisms of the antiinflammatory cytokine, interleukin-4 (IL-4). IL-4 strongly (MMP-13 and TIMP-3) or minimally (ADAMTS-4) suppressed OSM-induced gene expression in chondrocytes. IL-4 did not affect OSM-stimulated phosphorylation of extracellular signal-regulated kinases (ERKs), protein 38 (p38), c-Jun N-terminal kinase (JNK) and Stat1. Lack of additional suppression with their inhibitors suggested that MMP-13, ADAMTS-4, and TIMP-3 inhibition was independent of these mediators. IL-4 also downregulated TGF-β1-induced TIMP-3 gene expression, Smad2, and JNK phosphorylation. Additional suppression of TIMP-3 RNA by JNK inhibitor suggests JNK implication. The cartilage protective effects of IL-4 in animal models of arthritis may be due to its inhibition of MMPs and ADAMTS-4 expression. However, suppression of TIMP-3 suggests caution for using IL-4 as a cartilage protective therapy. J. Cell. Biochem. 103: 588–597, 2008.

Key words: rheumatoid arthritis; oncostatin M; interleukin-4; joint damage; proteases

The integrity of cartilage depends on balance between matrix degrading matrix metalloproteinases (MMPs)/aggrecanase (ADAMTS's) and tissue inhibitors of metalloproteinases (TIMPs) which in turn is controlled by the levels of proinflammatory and antiinflammatory cytokines. These homeostatic balances are altered in arthritic joints leading to cartilage collagen and aggrecan degradation by these proteases [Goldring, 2000]. Oncostatin M (OSM), a major cytokine produced by macrophages and activated T cells with both pro- and antiinflammatory activities in vivo, is elevated in the patients with rheumatoid arthritis (RA) [Manicourt et al., 2000]. Although human and bovine OSM share 58% identity [Malik et al., 1995], little is known about bovine OSM receptors. Nevertheless, bovine cells respond very well to human OSM. In human cells, OSM uses a gp130/OSM receptor beta complex for signal transduction rather than gp130/leukemia inhibitory factor receptor complex leading to activation of JAK/STAT and mitogen-activated protein kinases (MAPK) pathways [reviewed in Chen and Benveniste, 2004]. OSM by itself and by synergizing with interleukin-1, TNF-α, and IL-17 contributes to cartilage and bone loss in RA by elevating active ADAMTS-4 (aggrecanase-1) and MMP-13, the principal enzymes responsible for cartilage aggrecan

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and collagen breakdown, respectively [Richards, 2004; Porter et al., 2005]. Adenoviral overexpression of OSM in mouse joints induces periosteal bone apposition and causes synovial inflammation, hyperplasia, and resorption of cartilage and bone as in RA patients [de Hooge et al., 2002; Rowan et al., 2003]. Blocking antibodies to OSM in mouse models of RA ameliorate arthritis [Plater-Zyberk et al., 2001]. However, in other studies, bacterial lipopolysaccharide (LPS)-induced TNF-production, inflammation, and tissue damage was reduced by human OSM [Wallace et al., 1999].

Transforming growth factor beta (TGF-β1), a pleiotropic growth factor, is a potent inducer of chondrogenesis during development and cartilage ECM synthesis and regeneration [Lotz et al., 1995]. Excessive TGF-β is also implicated in the formation of osteophytes in osteoarthritis (OA) [Scharstuhl et al., 2002], synovial hyperplasia, and inflammation in RA [Hamilton et al., 1991]. Inhibition of endogenous TGF-β in a murine arthritis model resulted in prevention of osteophyte formation and impaired cartilage repair, suggesting its role in these pathological and physiological processes [Scharstuhl et al., 2002]. TGF-β inhibits the expression of MMPs but induces TIMP-1 and TIMP-3 in articular chondrocytes [Su et al., 1996]. TIMP-3 is associated with extracellular matrix (ECM) where it binds with proteoglycans [Yu et al., 2000] and is the major inhibitor of MMP-13 and ADAMTS-4, the principal enzymes implicated in cartilage degradation in arthritis [Hashimoto et al., 2001; Kashiwagi et al., 2001]. It also inhibits TNF-α converting enzyme (TACE or ADAM-17), which activates proarthritic cytokine, TNF-α [Lee et al., 2001]. TIMP-3 overexpression induces apoptosis in certain cell types [Bond et al., 2002]. Thus, TIMP-3 is an important therapeutic protein in arthritis [Lee et al., 2002; Mohammed et al., 2003]. This notion is supported by the prevention of TIMP-3 overexpressing human rheumatoid synovial fibroblasts to invade cartilage in a mouse model [van der Laan et al., 2003]. Interleukin-4 (IL-4) is an antiinflammatory cytokine as its neutralization with specific antibody initiates arthritis [Yoshino et al., 1998; van Roon et al., 2001]. It also inhibits IL-1-induced MMP-3 production in human chondrocytes and suppresses human and bovine cartilage degradation [Shingu et al., 1995; Yeh et al., 1995; Cawston et al., 1996; Nemoto et al., 1997]. However, this inhibition is observed in dedifferentiated and not in primary human chondrocytes due to altered levels of IL-4 receptor and signal transduction [Guicheux et al., 2002]. IL-4 also reduced IL-1-induced nitric oxide production and bovine chondrocyte apoptosis [Schuerwegh et al., 2003]. Despite increasing synovial inflammation, IL-4 inhibited collagen-induced bone and cartilage degradation in mouse models [Lubberts et al., 1999, 2000a; Watanabe et al., 2000]. IL-4 also reduced IL-17-induced suppression of proteoglycan synthesis in cartilage explants and in vivo [Lubberts et al., 2000b]. Thus, IL-4 is considered an important cartilage and bone protective agent [van de Loo and van den Berg, 2002]. Both IL-4 and TGF-β increased weight of the engineered cartilage [Blunk et al., 2002]. Here we investigated the mechanisms of anticatabolic activities of IL-4 by studying its impact on OSM- and TGF-β-induced responses in chondrocytes including MMP-13, ADAMTS-4, and TIMP-3 gene expression.

MATERIALS AND METHODS

Culture of Chondrocytes and Treatments

Normal bovine articular cartilage was obtained from the knee and hip joints of normal adult, 30-month-old animals from a local abattoir. Chondrocytes were released by 90 min pronase and collagenase (type II) (Sigma Chemical Company, St. Louis, MO) digestion for 9 h in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Sciences, Inc., Burlington, ON). The cells were washed with phosphate buffered saline (PBS) and grown in DMEM with 10% fetal calf serum (FCS) as high-density primary monolayer cultures until confluent growth. Cells were distributed in 6-well plates (Becton Dickinson, Franklin Lakes, NJ), grown to confluence, washed with PBS and kept in serum-free DMEM for 24 h. Chondrocytes were pretreated either with the indicated doses of human IL-4 alone for 60 min or stimulated further for 24 h with human recombinant OSM (50 ng/ml) or TGF-β (10 ng/ml) (R & D Systems). In some experiments, ERK, p38, and JNK pathway inhibitors, U0126, SB203580, SP600125 (all from Calbiochem at 5 μM dose final) and STAT1 inhibitor, fludarabine (Calbiochem; at 15 μM dose final) were added simultaneously with IL-4 (10 ng/ml).

RNA Extraction and Northern Hybridization Analysis

Total RNA was extracted by a rapid procedure [Chomczynski and Sacchi, 1987] and aliquots of 3–5 μg analyzed by electrophoretic fractionation in 1.2% formaldehyde–agarose gels. The integrity and quantity of applied RNA were verified by ethidium bromide staining of the gels and photography of the 28S and 18S ribosomal RNA bands. The RNA was electroblotted onto Zetaprobe nylon membrane (Bio-Rad, Mississauga, ON) using a Bio-Rad Transblot in the presence of 1X TAE buffer at a current of 40 mA overnight. MMP-13 and ADAMTS-4 probes were described before [Sylvester et al., 2004]. Northern blots were hybridized with a bovine TIMP-3 cDNA probe [Su et al., 1996]. The human 28S ribosomal RNA plasmid (from ATCC, Manassas, VA) was digested with XbaI and a probe synthesized using T7 polymerase. All probes were labeled to high-specific activity (1×10^8 cpm/μg) with [α-^{32}P] CTP (3,000 Ci/mmol, Perkin Elmer Canada, Inc., Woodbridge, ON) with the RNA labeling kit from Promega Biotech (Madison, WI) according to their protocols.

Western Blot Analysis

The activation of ERK, p38, and JNK MAP kinases, STAT-1 and Smad2 was analyzed by Western immunoblotting by using antibodies against phosphorylated or total proteins (Cell Signaling Technology, Beverley, MA) as described before [Li et al., 2001]. For measuring TIMP-3 protein levels, total cellular protein extracts from bovine chondrocytes (20 μg) were subjected to fractionation by a 15% SDS–PAGE mini gel (Bio-Rad system), transferred to PVDF (Pall Corporation, Anne arbor, MI) by electroblotting and reacted with the 1:400 dilution of human TIMP-3 polyclonal antibody (Chemicon International, Temecula, CA). Subsequently, membranes were incubated with the antirabbit secondary horseradish peroxidase-conjugated antibody (Promega) and the TIMP-3 protein bands revealed with the chemiluminescence detection system of Roche Biochemicals (Laval, QC) with their protocols.

Statistical Analysis

RNA and protein band intensities from multiple blots were quantified by NIH ImageJ 1.32j (National Institute of Health, USA) software. Results are reported as means ± SEM of at least three different experiments and were compared by ANOVA, followed by a post hoc Tukey's multiple comparison or student's t-test. $P < 0.05$ was considered significant.

RESULTS

Interleukin-4 Down-Regulates OSM-Induced MMP-13 and ADAMTS-4 Gene Expression

To study the effect of OSM and IL-4 on MMP-13 and ADAMTS-4 gene expression in primary bovine chondrocytes, cells were pretreated with different doses of IL-4 and then stimulated with OSM for 24 h. OSM induced MMP-13 and ADAMTS-4 mRNA expression above basal levels and IL-4 strongly (MMP-13; 57–62%) or minimally (ADAMTS-4) downregulated the induction without changing the 28S ribosomal RNA internal control levels (Fig. 1).

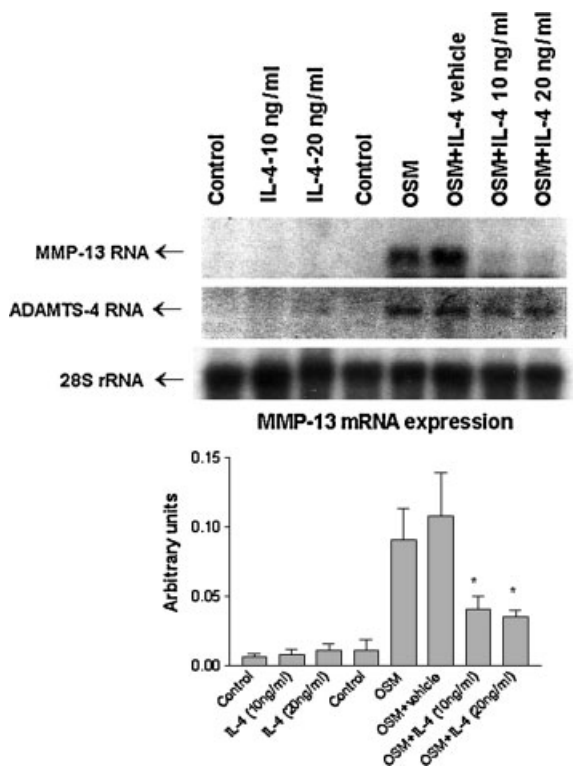

Fig. 1. Downregulation of oncostatin M (OSM)-induced MMP-13 and aggrecanase-1 gene expression by interleukin-4 (IL-4). Bovine articular chondrocytes were grown to confluence, kept in serum-free medium for quiescence, treated with vehicles (control) or pretreated with the indicated doses of human IL-4 and then stimulated with human OSM (50 ng/ml) for 24 h. A representative Northern blot depicting MMP-13 and ADAMTS-4 mRNAs and 28S ribosomal RNA (rRNA) is shown. **Bottom panel** shows quantitative analysis of MMP-13 bands from three experiments. $^*P < 0.05$ and $^{**}P < 0.01$ IL-4 versus OSM + vehicle.

Interleukin-4 Reduces OSM-Induced TIMP-3 Gene Expression

To examine the effect of IL-4 on TIMP-3 (the major inhibitor of MMP-13 and ADAMTS-4) gene expression, chondrocytes were pretreated with IL-4 and then stimulated with OSM. IL-4 downregulated basal and OSM-induced expression of TIMP-3 RNA without significantly affecting the constant levels of 28S ribosomal RNA (Fig. 2). Concomitant ADAMTS-4 mRNA inhibition by IL-4 was also observed.

Interleukin-4 Does not Affect OSM-Induced Signal Transduction

To investigate the mechanism of this suppression, we measured the levels of different signal transduction protein mediators implicated in OSM-induced MMP-13 and TIMP-3 expression [Li et al., 2001]. OSM-stimulated ERK, p38, JNK, and STAT1 phosphorylation was not significantly affected by IL-4 (Fig. 3).

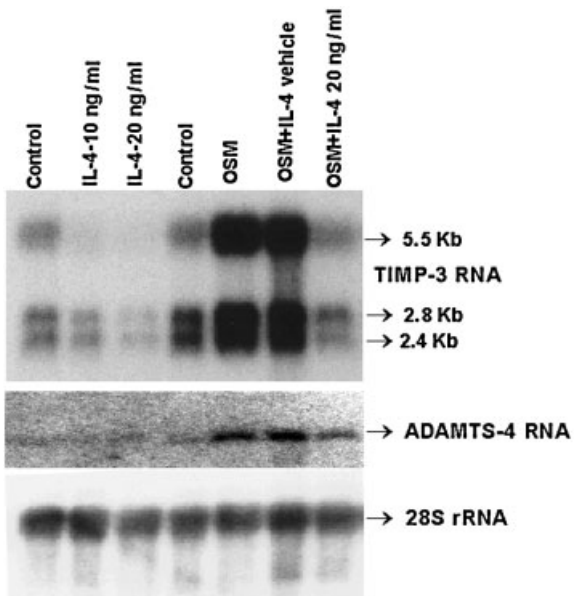

Fig. 2. Suppression of basal and oncostatin M (OSM)-induced tissue inhibitor of metalloproteinases-3 (TIMP-3) gene expression by interleukin-4 (IL-4). Confluent cultures of chondrocytes were made quiescent by maintaining in serum-free medium for 24 h, treated with IL-4 alone or pretreated with human IL-4 and then stimulated with OSM for 24 h. A representative Northern blot depicting TIMP-3 (three transcripts due to differential choice of 3′ polyadenylation sites) and ADAMTS-4 (for comparison) mRNA and 28S rRNA are shown.

MAPK and STAT1 Inhibitors do not Further Suppress IL-4-Driven MMP-13, ADAMTS-4, and TIMP-3 Expression

To further explore the mechanism of suppression by IL-4, cells were treated with low doses of IL-4 and pharmacological inhibitors of the above mediators. ERK, JNK1/2, and p38 pathway inhibitors, U0126, SP600125, SB203580, and STAT1 inhibitor, fludarabine suppressed OSM-induced MMP-13, ADAMTS-4, and TIMP-3 expression but did not cause additional suppression of their expression (Fig. 4).

Interleukin-4 Downregulates TGF-β-Induced TIMP-3 Gene Expression

TGF-β is a potent inducer of TIMP-3 in chondrocytes [Su et al., 1996]. To study the effect of IL-4 on TGF-β-inducible TIMP-3 gene expression, chondrocytes were pretreated with IL-4 and then exposed to TGF-β for 24 h. IL-4 dose-dependently downregulated TGF-β-induced expression of TIMP-3 RNA transcripts without affecting constant levels of 28S ribosomal RNA. Similarly, the levels of TIMP-3 protein expression were also reduced by IL-4 (Fig. 5).

Interleukin-4 Downregulates TGF-β-Induced Smad2 and JNK Phosphorylation

To investigate the mechanism of this suppression, we measured the levels of different protein mediators implicated in TGF-β signal transduction. As reported earlier, TGF-β treatment led to increased phosphorylation of Smad2, ERK, p38, and JNK MAPKs within 20 min [Li et al., 2004; Qureshi et al., 2005]. IL-4 slightly downregulated Smad2 and JNK phosphorylation but did not affect the ERK and p38 MAPK phosphorylation. The total levels of these proteins were not affected by these treatments (Fig. 6).

JNK Inhibitor Further Suppresses IL-4-Driven TIMP-3 Expression

To further explore the mechanism of suppression, chondrocytes were co-treated with the above-mentioned MAPK inhibitors. Smad2-specific inhibitors have not been described. Quantitative analysis of several experiments revealed that JNK inhibitor further reduced (37.9%) IL-4-driven TIMP-3 RNA suppression while ERK and p38 inhibitors had no further effect (Fig. 7).

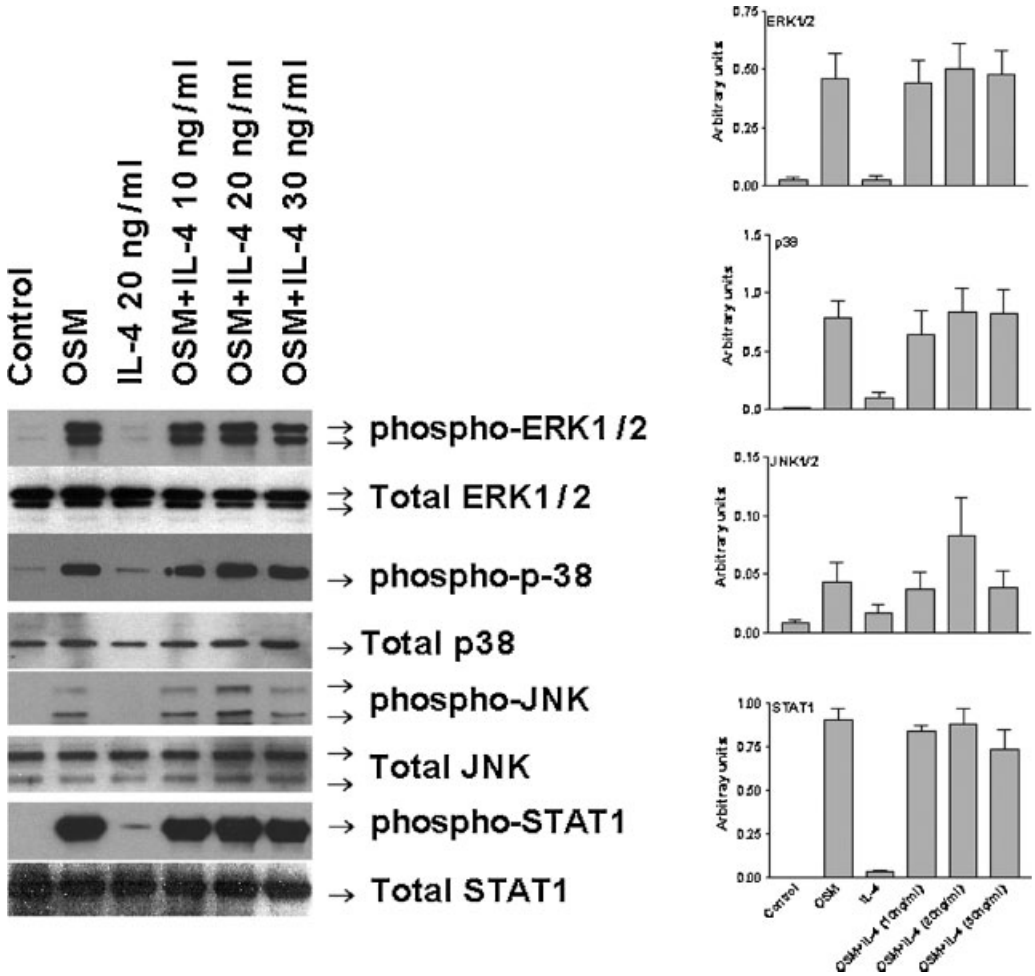

Fig. 3. Impact of oncostatin M and Interleukin-4 on mitogen-activated protein kinases and STAT-1 phosphorylation. Quiescent chondrocytes were either treated with vehicles (control) or pretreated with IL-4 and then stimulated with OSM for 20 min. Phosphorylation of ERK1/2, p38, JNK1/2, and STAT-1 mediators as well as respective total proteins measured by Western blotting are shown. In the **right panels**, quantification of bands by densitometry from two experiments is depicted.

DISCUSSION

OSM is a major proinflammatory cytokine that by itself and in synergy with IL-1, IL-17, and TNF-α promotes cartilage degradation. Here we have shown that the antiinflammatory cytokine, IL-4 can inhibit the OSM-induced expression of major proteases responsible for aggrecan and collagen loss as well as their inhibitor, TIMP-3. Induction of both ADAMTS-4 and MMP-13 by OSM in bovine chondrocytes supports a catabolic role for this cytokine. Previously, OSM was reported to induce MMP-13 and ADAMTS-4 mRNA in synergy with IL-1 in a human chondrocyte cell line [Koshy et al., 2002]. However, in our experience, OSM alone is able to induce genes of these proteases. Induction of TIMP-3, the major inhibitor of two proteases is in agreement with our previous studies [Li and Zafarullah, 1998; Li et al., 2001]. Despite TIMP-3 induction, OSM promoted cartilage degradation in vivo [de Hooge et al., 2002], suggesting that the multiple proteases-driven catabolic activities may overwhelm the protective ability of TIMP-3.

IL-4 is regarded as one of the major antiinflammatory cytokines that could potentially antagonize the proinflammatory cytokine-induced catabolism and inflammation in arthritic joints. Indeed, IL-4 gene transfer by plasmid or an adenovirus gave protection against collagen-induced arthritis, a human RA-like disease model [Joosten et al., 1997;

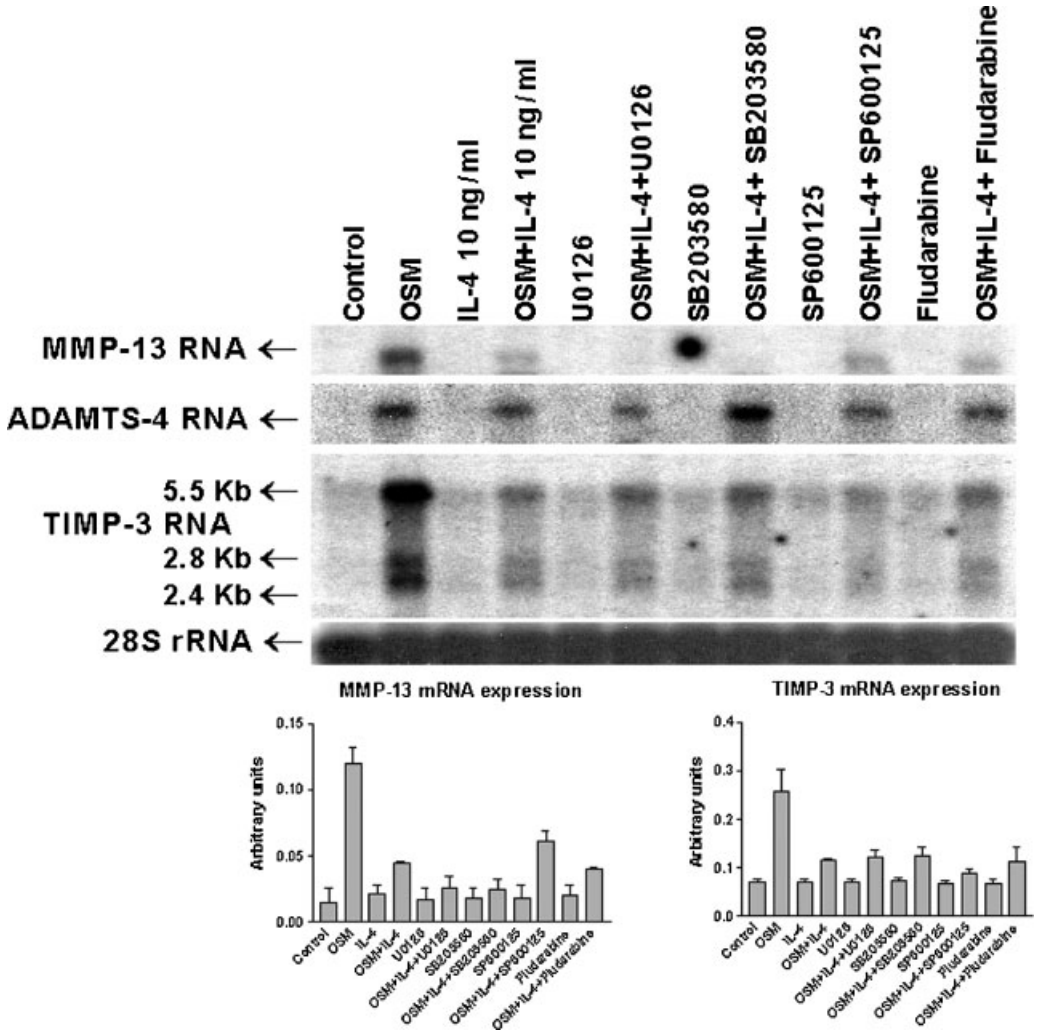

Fig. 4. Impact of MAPK or STAT1 pharmacological inhibitors and IL-4 on oncostatin M-induced MMP-13, ADAMTS-4, and TIMP-3 gene expression. Quiescent bovine chondrocytes were either treated with vehicles (control) or pretreated with IL-4 and U0126, SB203580, SP600125 (all at 5 μM), or fludarabine (15 μM) and then stimulated with OSM for 24 h. Representative of three MMP-13, TIMP-3, and 28S Northern blots and graphic quantification of their bands are depicted in the upper and **lower panels**.

Lubberts et al., 1999; Ho et al., 2004]. The observed inhibition of proinflammatory cytokine-stimulated bovine cartilage explant degradation by IL-4 [Yeh et al., 1995; Cawston et al., 1996; Salter et al., 1996] may be due to its ability to completely or partially inhibit MMP-13 and ADAMTS-4 demonstrated here. However, our results suggest that besides its reported beneficial effects of inhibiting MMP-13 and ADAMTS-4, it also inhibits the expression of the most important natural MMP and aggrecanase-1 inhibitor, TIMP-3. Thus, it is not surprising that IL-4 had limited effectiveness in clinical trials with RA patients [van Roon et al., 2001].

We also studied the mechanisms of OSM response inhibition. As reported previously, OSM induced the phosphorylation of major MAPK pathways and STAT-1 within 20 min [Li et al., 2001] and IL-4 did not significantly affect their activation within 20 min. Their involvement was also not supported by pharmacological inhibitor treatments suggesting that the inhibitory effect may not be mediated by ERK, p38, JNK, and Stat1. These target genes might be inhibited by other unidentified molecular signaling pathways. Transduction of IL-4 signal takes place via JAK1, JAK3, and STAT6 [Losman et al., 1999; McGaha et al., 2003]. Interestingly, OSM also

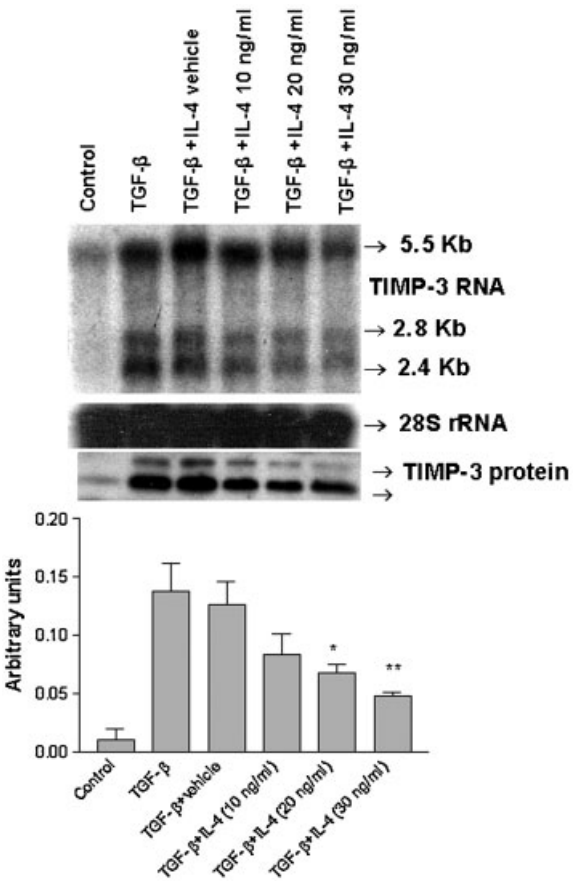

Fig. 5. Suppression of transforming growth factor beta (TGF-β1)-induced tissue inhibitor of metalloproteinases-3 gene expression by interleukin-4 (IL-4). Confluent quiescent cultures of chondrocytes were pretreated with different doses of human IL-4 for 1 h and then stimulated further with TGF-β1 (10 ng/ml) for 24 h. A representative of three Northern blots depicting TIMP-3 mRNA and 28S rRNA are shown in the **upper panels**. The respective TIMP-3 protein levels measured by Western blotting are shown in the **middle panel**. Quantification of TIMP-3 RNA bands by densitometry is shown in the **bottom panel**. $^{*}P < 0.05$ and $^{**}P < 0.01$ IL-4 versus TGF-β alone.

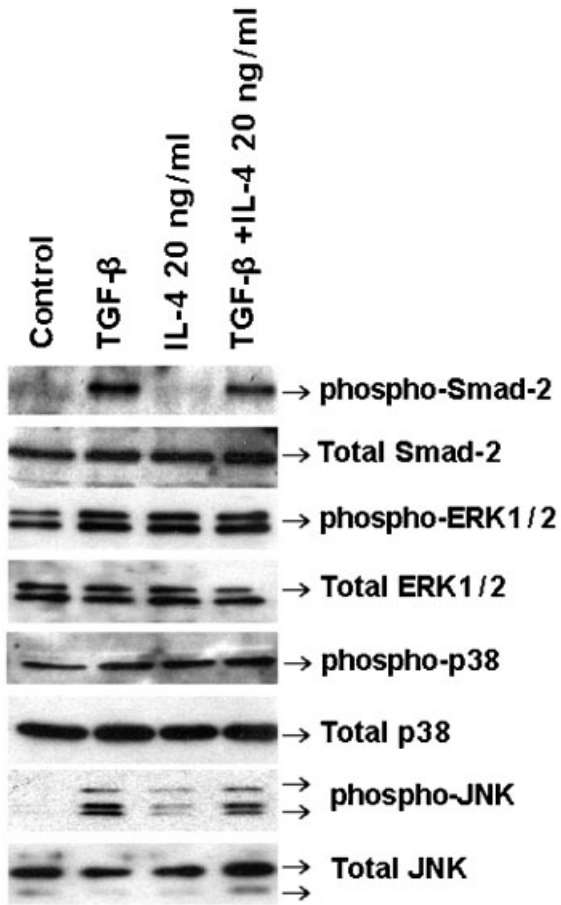

Fig. 6. Effect of Interlekin-4 (IL-4) on transforming growth factor beta (TGF-β1)-induced signaling mediators. Quiescent bovine chondrocytes were either treated with vehicles (control) or pretreated with IL-4 and then stimulated with TGF-β1 for 20 min. Phosphorylation of Smad-2, ERK1/2, p38, and JNK1/2 mediators as well as respective total proteins measured by Western blotting are shown.

induces JAK/STAT pathway in chondrocytes [Li et al., 2001]. Whether STAT6 is inhibitory to OSM-induced STAT-1 or STAT3, remains to be studied. IL-4 is known to inhibit prostaglandin E2 (PGE2) production [Corcoran et al., 1992; Mehindate et al., 1996; Alaaeddine et al., 1999] and may also inhibit OSM-stimulated PGE2 production [Repovic et al., 2003] and downregulate these genes.

TGF-β induces TIMP-3 gene in articular chondrocytes by activating multiple pathways including Smad and MAPKs [Li et al., 2004; Qureshi et al., 2005]. Our studies suggest that reduction of TIMP-3 by IL-4 may be mediated partly by interference with Smad2 and JNK phosphorylation. JNK inhibitor studies further support this notion. This mechanism appeared to be specific as the phosphorylation of ERK and p38 MAPKs was not affected. The interference of IL-4 with TGF-β signaling could be analogous to that of interferon gamma (IFN-γ), which via Janus kinase and STAT pathway induces antagonistic Smad7, which in turn inhibits TGF-β-induced Smad3 phosphorylation and downstream signaling events [Ulloa et al., 1999]. Similarly, IFN-γ inhibits TGF-β-induced collagen I gene via antagonistic interaction of JAK/STAT and Smad pathways for binding with a common cofactor, p300/CREB binding protein [Ghosh et al., 2001].

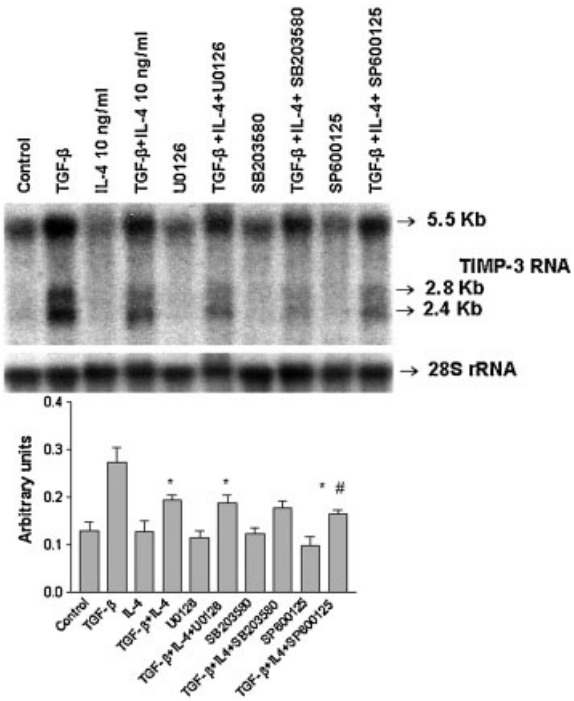

Fig. 7. Impact of IL-4 and MAPK pharmacological inhibitors on TGF-β-inducible TIMP-3 gene expression. Quiescent chondrocytes were either treated with vehicles (control) or pretreated with IL-4 and U0126, SB203580, SP600125 (all at 5 μM), and then stimulated with TGF-β for 24 h. Representative of three TIMP-3 and 28S rRNA Northern blots and graphic quantification of their bands are depicted in the upper and **lower panels**. $^{*}P < 0.05$ versus TGF-β alone (Tukey's test). $^{\#}P < 0.05$ versus TGF-β + IL-4 + SP600125 (student's *t*-test).

Besides inhibition of catabolic proteases and their TIMP-3 inhibitor gene observed here, IL-4 has several features of particular interest in arthritis. IL-4 displayed antiapoptotic activities against sodium nitroprusside-induced apoptosis for synovial fibroblasts and synovial membrane explants [Relic et al., 2001], which is a potential disadvantage particularly in the pathogenesis of RA. However, such antiapoptotic activity may be beneficial for cartilage maintenance. Antiinflammatory activity of IL-4 was also attributed to its ability to induce apoptosis in monocytes [Mangan et al., 1992]. Interestingly, mutations of IL-4 receptor have been recently linked to increased susceptibility to hip osteoarthritis in Caucasian females [Forster et al., 2004].

In conclusion, antiinflammatory cytokine, IL-4 was shown to suppress OSM-induced MMP-13, ADAMTS-4, and TIMP-3 and TGF-β-induced TIMP-3 gene expression, latter with partial involvement of JNK and Smad pathways. Suppression of proteases by IL-4 may be of therapeutic value in arthritis while inhibition of TIMP-3 may be a potentially disadvantageous side effect.

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