

Tryptase enhances release of vascular endothelial growth factor from human osteoarthritic chondrocytes

K. Masuko^{*1,2}, M. Murata¹, Y. Xiang¹, H. Nakamura¹, K. Yudoh¹, K. Nishioka³,
M. Beppu⁴, T. Kato^{1,2}

¹Department of Bioregulation and Proteomics, Institute of Medical Science;

²Dept. of Biochemistry; ³Dept. of Frontier Medicine, Institute of Medical Science;

⁴Dept. of Orthopedic Surgery, St. Marianna University School of Medicine, Kanagawa, Japan.

Abstract

Objective

A contribution of mast cells and its mediators in the pathogenesis of arthritis has been postulated. We aimed to clarify the role of mast cell-derived serine protease tryptase and proteinase activated receptor (PAR)-2-mediated signaling in chondrocytes.

Methods

Human articular cartilage specimens were obtained from patients with osteoarthritis (OA), rheumatoid arthritis (RA) and with traumatic fracture without arthritis (PT; as controls) who underwent joint surgery. Isolated chondrocytes were cultured in vitro by monolayer, and confluent cells were incubated with recombinant human lung β tryptase or with a PAR-2 agonist peptide. The secreted level of vascular endothelial growth factor (VEGF) in culture supernatant was measured using commercially available ELISA kits, and expression of VEGF mRNA was analyzed using real-time PCR.

Results

The tryptase-stimulated chondrocytes from OA or RA, but not from PT patients, produced significantly higher amount of VEGF in their supernatants. The response was blocked by a G-protein receptor inhibitor pertussis toxin, however, was not reproduced by incubation of cells with the PAR-2 agonist, suggesting a presence of non-PAR-2 dependent signals for the VEGF induction. In addition, actinomycin D and cycloheximide did not exert significant inhibition, indicating a regulation of VEGF release by tryptase.

Conclusion

The inflammatory mediator, mast cell-derived protease tryptase may modulate chondrocyte metabolism through induction of VEGF release.

Key words

Tryptase, PAR-2, VEGF.

Kayo Masuko, MD, PhD; Minako Murata, MD, PhD; Yang Xiang, MD, PhD; Hiroshi Nakamura, MD, PhD; Kazuo Yudoh, MD, PhD; Kusuki Nishioka, MD, PhD; Moroe Beppu, MD, PhD; Tomohiro Kato, MD, PhD.

The study was partly supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology; the Japanese Ministry of Health, Labor and Welfare, the Japan Rheumatism Foundation, the Morita Fellowship for the Japanese Association of University Women; and the Japan Medical Women's Association.

Please address correspondence and reprint requests to: Kayo Masuko, MD, PhD, Department of Biochemistry, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki-shi, 216-8511 Kanagawa, Japan. E-mail: kmarianna@mac.com

Received on December 11, 2006; accepted in revised form on June 7, 2007.

© Copyright CLINICAL AND EXPERIMENTAL RHEUMATOLOGY 2007.

Introduction

Mast cells and its mediators have been reported to be a histologically prominent feature in joints of patients with osteoarthritis (OA) (1). For example, Renoux *et al.* (2) reported that OA synovial fluid (SF) contains larger amount of mast-cell mediators than that in SF of rheumatoid arthritis (RA). Also, among the two subtypes, synovial tissue of OA is reported to be rich for mast cells which belong to the tryptase-positive, chymase-negative subset (M_T cells), compared than the MC_{TC} subset which contain both tryptase and chymase; suggesting the major role of tryptase in OA synovitis (1). Tryptase is a trypsin-like serine protease which functions through binding to the receptor protease-activated receptor (PAR)-2, a member of G protein-coupled seven transmembrane receptor (reviewed in (3, 4)). In this regard, we recently reported the expression of PAR-2 in human articular chondrocytes express PAR-2, at a higher level in OA than in normal cells (5). In the present study, we investigated the possible role of synovial mast cells-derived tryptase, possibly via PAR-2, in human chondrocytes.

Materials and methods

Cells

Human articular chondrocytes were obtained from patients with osteoarthritis ($n = 14$, 3 men and 11 women), rheumatoid arthritis (RA; $n = 4$, 2 men and 2 women) who underwent total knee or hip joint replacement, and from patients with no history of joint disease who were undergoing joint surgery after traumatic injury (posttraumatic (PT); $n = 3$, 3 women), at St. Marianna University School of Medicine Hospital. The diagnosis of OA was made according to the criteria of Kellgren and Lawrence. (6) RA was classified according to the criteria of the American College of Rheumatology. (7) Written informed consent was obtained from each patient and the study protocol was approved by the ethics committee of St. Marianna University. The study was performed in compliance with the World Medical Association Declaration of Helsinki (1964).

Chondrocytes were isolated as previous-

ly reported (8). Briefly, after the careful removal of synovial tissue, cartilage was minced, washed and treated with collagenase. Isolated chondrocytes were then washed and cultured *in vitro* by monolayer in DMEM medium supplemented with 10% fetal calf serum (FCS) and antibiotics. The attached cells (P0) were expanded on culture dishes and the cells at subconfluent (P1 cells) were used in the experiments.

In vitro stimulation of chondrocytes

Cultured chondrocytes were serum-starved in medium containing 0.5% FCS for 24h prior to experiment, and stimulated with recombinant human lung β tryptase (0.01-1 μ g/ml; Promega, Madison, WI, USA) or the PAR-2 agonist peptide (SLIGKV; 0.1-1 μ M; synthesized by Peptide Institute, Inc., Osaka, Japan) for up to 72 hours. Cellular viability after stimulation with the reagents was assessed by trypan blue exclusion, showing no significant deterioration under these culture condition (data not shown). Where specified, cells were pretreated with pertussis toxin (PTX) from *Bordetella pertussis* (10 ng/ml; Sigma; Sigma-Aldrich Japan K. K., Tokyo, Japan) for 18h or with Actinomycin D (1 μ M; Medical & Biological Laboratories (MBL) Co. Ltd., Nagoya, Japan) or cycloheximide (10 μ M; MBL) for 1 hour before the addition of tryptase. To avoid the possible effect of trypsin, stimulated chondrocytes were scraped off the dish if necessary using EDTA.

Enzyme-linked immunosorbent assay (ELISA)

Levels of PGE₂, MMP-3, TGF β , nitric oxide (NO) and VEGF were measured using commercially available ELISA kits, according to the manufacturer's instructions: Prostaglandin E₂ EIA Kit, Cayman Chemical CO., Ann Arbor, MI, USA; ProMMP-3 ELISA, MBL, Nagoya, Japan; Human TGF- β 1 Immunoassay, R&D systems, Minneapolis, MN; and Nitrate/Nitrite Colorimetric Assay kit, Cayman Chemical CO.; and VEGF ELISA kit, BioSource International, Inc, Camarillo, CA, USA, respectively. To assess the relative amount of VEGF to cell number, whole

Competing interests: none declared.

cell lysate (WCL) was extracted from cultured chondrocytes and quantified using the Bradford method as previously reported (8), and values of VEGF in the culture supernatant were corrected against the amount of WCL of the same culture dish. Each assay was performed in duplicate. The results are shown by average $\pm$ standard deviation. Statistical significance was calculated using Student's *t*-test.

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from the cells using RNeasyB™ (Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer's instruction, and converted to cDNA using RT Superscript II (Life Technologies, Inc., Rockville, MD, USA). Real-time PCR analysis of VEGF mRNA expression was performed as described elsewhere (8) using a commercial ready-to-use amplification primer mix (Roche Diagnostics Ltd, Tokyo, Japan) and Light-Cycler™-DNA Master SYBR™ Green I mix (Roche Diagnostics).

Results

Tryptase enhances VEGF production in chondrocytes

As a result of the *in vitro* stimulation of chondrocytes, we observed that tryptase enhanced the level of VEGF, a proangiogenic factor, in culture supernatant of chondrocytes either from patients with OA or RA, but not those from traumatic fracture (Fig. 1A). To further confirm the increase of VEGF, relative levels of the produced VEGF were calculated against the amount of protein extracted from the cultured chondrocytes. The results supported the increase of VEGF levels after tryptase stimulation of chondrocytes (Fig. 1B).

The VEGF induction by tryptase was abrogated by pretreatment of PTX, a Gi protein inhibitor (Fig. 1A). Nevertheless, stimulation of chondrocytes with the PAR-2 agonist peptide did not exert the similar effect with tryptase. The VEGF-inducing effect by tryptase was dose-dependent, and there was a slight difference in the VEGF response at the lower concentration; specifically, one RA sample did not show significant

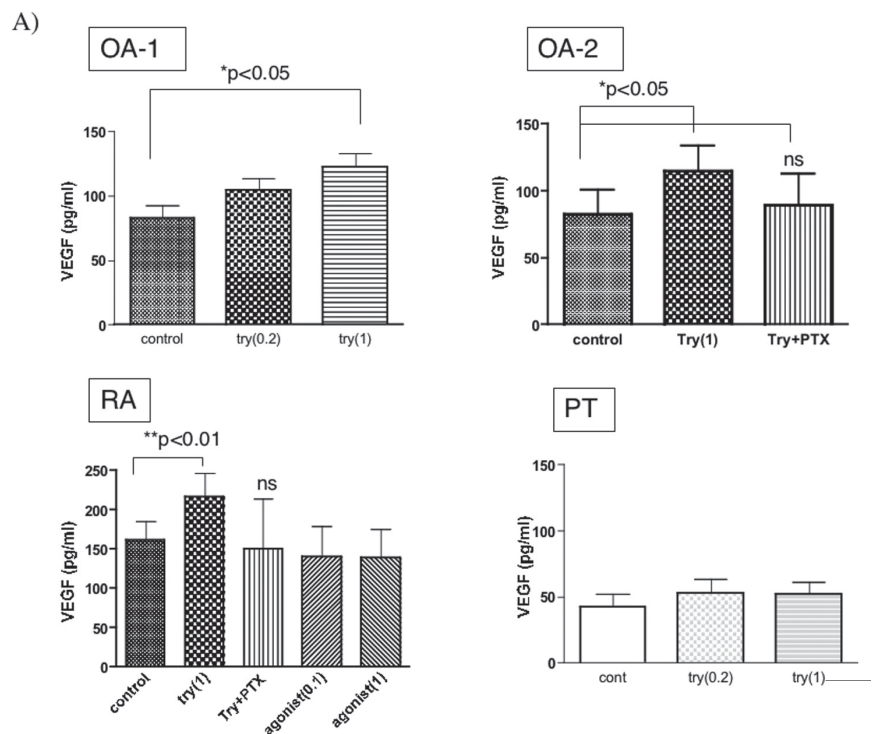


Fig. 1A. Tryptase enhances VEGF secretion from chondrocytes.

ELISA. Cultured chondrocytes were incubated with recombinant human tryptase *in vitro*. Levels of VEGF secreted in culture supernatant were assayed using ELISA. Values were indicated by mean $\pm$ 2SD. OA-1 (n = 5) and OA-2 (n = 5) indicate independent assays employing different panel of OA patients; RA (n = 4), PT (n = 3).

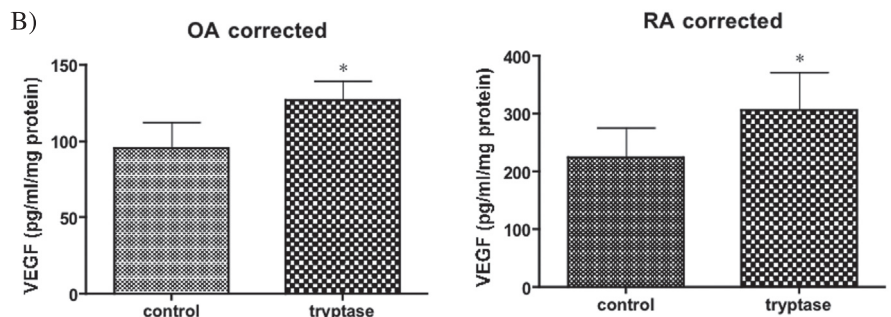


Fig. 1B. Tryptase enhances VEGF secretion from chondrocytes.

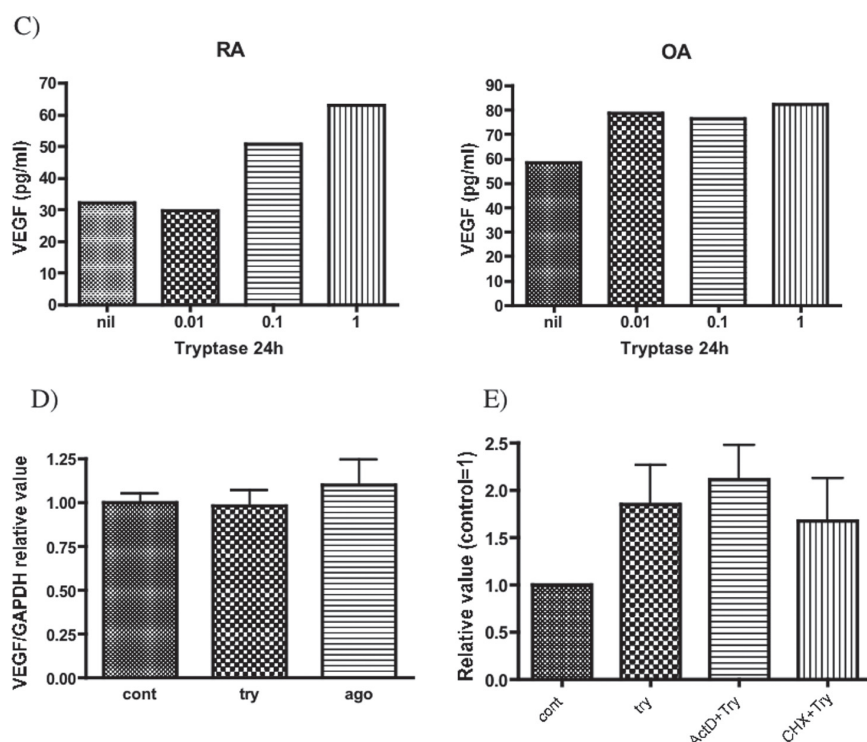
The values of VEGF (of samples from "control" and "try(1)" in Fig. 1A) were corrected against total amount of whole cell lysates and relative levels of VEGF are shown. OA; n = 6, RA; n = 4. **p* < 0.02.

response to 0.01 μ g/ml tryptase, however an OA sample responded to the same concentration of tryptase (Fig. 1C). The increase of VEGF after tryptase stimulation was shown until at least 72 h culture (data not shown).

Tryptase enhances release of VEGF from chondrocytes

To explore at which level the VEGF release is regulated by tryptase, we assessed the effect of tryptase on VEGF mRNA expression using OA

chondrocytes. As a result, unexpectedly, chondrocytes stimulated with tryptase did not show significant increase of VEGF mRNA expression (Fig. 1D). Further, the increased level of VEGF after tryptase stimulation was not blocked by actinomycin D (ActD), nor by cycloheximide (CHX) (Fig. 1E). These findings suggested that tryptase might not regulate the transcription or translation of VEGF in OA chondrocytes; rather, tryptase would mobilize VEGF release.



Figs. 1C, D, and E. Tryptase enhances VEGF secretion from chondrocytes. **C)** Representative results of dose-response to tryptase using one RA and one OA chondrocyte samples. Chondrocytes were stimulated with indicated concentration of tryptase during 24 hours and VEGF levels in supernatants were analyzed. **D)** Real-Time PCR. Levels of VEGF mRNA were analyzed using real-time PCR and the relative expression values (VEGF:GAPDH) were calculated. OA chondrocytes, $n = 3$. The difference did not reach to statistical significance. **E)** Lack of effect of actinomycin D and cycloheximide on the VEGF release from chondrocytes. Relative levels of secreted VEGF from chondrocytes stimulated by tryptase with or without addition of the inhibitors was calculated. Control value was set as 1. ActD: actinomycin D. CHX: cycloheximide. OA chondrocytes, $n = 3$.

We investigated whether tryptase induces expression of other inflammatory mediators and growth factors. As a result, we did not observe significant change of secreted levels of matrix metalloproteinase (MMP)-3, prostaglandin (PG) E_2 , TGF- β , or nitric oxide (NO), after stimulation with tryptase (Fig. 2).

Discussion

To our knowledge, this is the first report to show the interaction between tryptase and VEGF in human articular chondrocytes *in vitro*. The mast cell mediators have been suggested to be involved in the pathogenesis of OA, and our present study may add the serine proteinases tryptase as one of the chondrocyte-modulating mediators released from mast cells upon activation. Tryptase dose-dependently increased VEGF production of chondrocytes, although threshold of the response varied

between samples (Fig. 1C). This might be because of the difference in expression of the receptor PAR-2, or the level of preformed VEGF in chondrocytes. In this regard, the induction of VEGF production from chondrocytes by tryptase was abrogated by the pretreatment with PTX (Fig. 1A). The finding implied an important role of the G-protein coupled receptor, PAR-2, in the VEGF induction as we previously showed the expression of PAR-2 by chondrocytes (5). On the other hand, the PAR-2 agonist peptide did not enhance VEGF levels, suggesting that the PAR-2-mediated signaling is required, but not sufficient, to evoke VEGF production from chondrocytes: a presence of non-PAR-2 dependent pathway is therefore suggested. To support this, it has been reported by Brown *et al.* (9) that tryptase, but not PAR-2-agonist peptides (mouse SLIGRL and human SLIGKV), induced Akt phosphorylation

following tryptase stimulation in dog airway smooth muscle cells, suggesting that tryptase-induced activation of phosphatidylinositol 3-kinase activation may not be mediated via PAR-2 in these cells.

In our study, tryptase did not show enhancing effect on the production of MMP-3, PGE $_2$, TGF- β , or NO. The enhancing effect of tryptase in chondrocytes was therefore rather specific to VEGF. This may be in contrast to fibroblasts, since fibroblasts were reported to proliferate (10, 11) and increase PGE $_2$ synthesis upon stimulation by tryptase via PAR-2 (11). This suggests that tryptase-PAR2 signaling in chondrocytes would follow unique pathway than other cell types with different phenotypes. The present results suggested that tryptase would mobilize VEGF release from chondrocytes. This seems to be in consistent to previous findings on neutrophils, in which ActD and CHX did not exert inhibiting effect on VEGF (12, 13); thus the VEGF release from “pre-existing intracellular VEGF pool” (13) is also suggested in OA chondrocytes. Further, considering the lack of response in normal chondrocyte (Fig. 1A), it could be speculated that the VEGF pool would be preformed at higher level in diseased or inflamed condition (*e.g.* in OA and RA), whereas the level might not have reached to threshold to respond to tryptase in normal cells. The mast cell derived mediator, tryptase, may therefore play a role in cartilage metabolism through regulation of VEGF, as the expression of VEGF and its receptors, and possible role of VEGF in OA have been suggested (8, 14, and Murata M. *et al.* *Osteoarthritis Cart* in press) (Fig. 3). More specifically, higher expression of VEGF and its receptors has been detected in OA cartilage (14, 15), and VEGF is suggested to play a role in osteophyte formation (16), cartilage degradation through proteinase induction (14), and also in modulation of expressions of caspase-3 and cathepsin B (17). We previously reported that hypoxic stress and IL-1 would induce VEGF via distinct signaling pathways, suggesting a difference in machinery VEGF induction between by oxygen tension and by cytokine stimulation (8). In addition, since

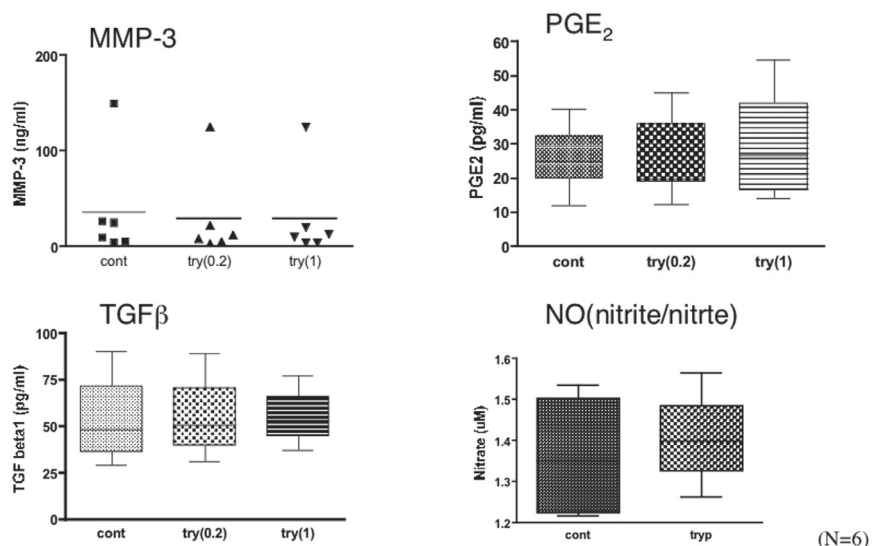


Fig. 2. Tryptase did not show any significant effect on the levels of MMP-3, PGE₂, TGFβ and NO from chondrocytes. See *Materials and methods*.

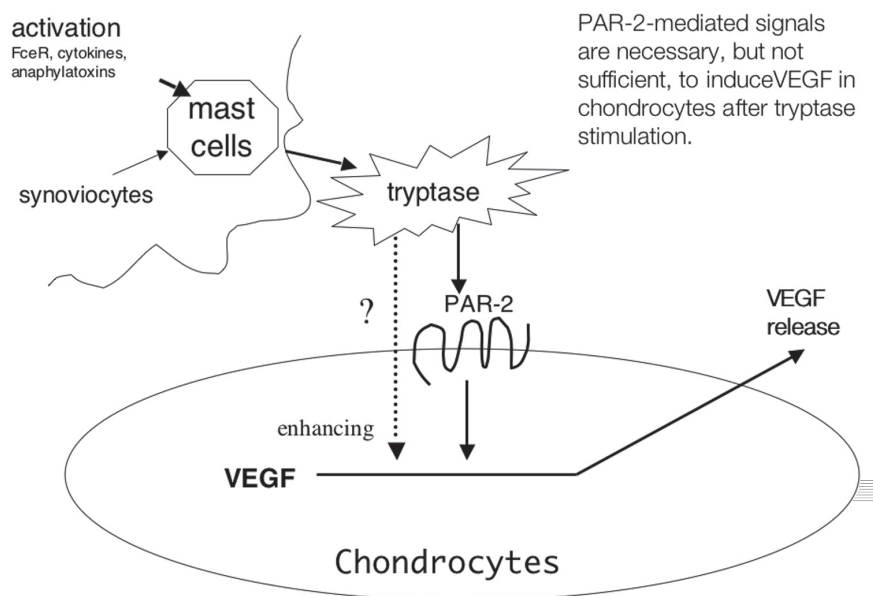


Fig. 3. Suggested signaling pathways of tryptase to induce VEGF production. Tryptase, induced from synovial mast cells by inflammatory stimuli, would deliver distinct signals to chondrocytes: via PAR-2 receptor, and also via as-yet-unidentified pathway(s), to enhance VEGF release.

tryptase itself is reported as a gelatinase which is potent to degrade and regulate turnover of type I collagen (10, 18, 19), it might be involved in de-differentiating process of chondrocytes and/or in matrix degradation. The point should be investigated further.

In conclusion, it has been found that a mast cell derived mediator, tryptase, enhanced VEGF levels produced by human articular chondrocytes. Since tryptase is reported to be present in synovial fluid (2), our finding might bridge

between mast cell derived mediator and angiogenic factors implying a possible involvement in cartilage degradation process in arthropathy.

Acknowledgements

The authors thank Ms Toshiko Mogi and Ms Hiroe Ogasawara for their excellent technical support.

References

1. BUCKLEY M, GALLAGHER P, WALLS A: Mast cell subpopulations in the synovial tissue of

patients with osteoarthritis: selective increase in numbers of tryptase-positive, chymase-negative mast cells. *J Pathol* 1998; 186: 67-74.

2. RENOUX M, HILLIQUIN P, GALOPPIN L, FLORENTIN I, MENKES C: Release of mast cell mediators and nitrites into knee joint fluid in osteoarthritis – comparison with articular chondrocalcinosis and rheumatoid arthritis. *Osteoarthritis Cartilage* 1996; 4: 175-9.
3. TREJO J: Protease-activated receptors: new concepts in regulation of G protein-coupled receptor signaling and trafficking. *J Pharmacol Exp Ther* 2003; 307: 437-42.
4. MACFARLANE SR, SEATTER MJ, KANKE T, HUNTER GD, PLEVIN R: Proteinase-activated receptors. *Pharmacol Rev* 2001; 53: 245-82.
5. XIANG Y, MASUKO-HONGO K, SEKINE T *et al.*: Expression of proteinase-activated receptors (PAR)-2 in articular chondrocytes is modulated by IL-1β, TNF-α and TGF-β. *Osteoarthritis Cartilage* 2006; 14: 1163-73.
6. KELLGREN JH, LAWRENCE JS: Radiological assessment of osteo-arthritis. *Ann Rheum Dis* 1957; 16: 494-502.
7. ARNETT F, EDWORTHY S, BLOCH D *et al.*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
8. MURATA M, YUDOH K, NAKAMURA H *et al.*: Distinct signaling pathways are involved in hypoxia- and IL-1-induced VEGF expression in human articular chondrocytes. *J Orthop Res* 2006; 24: 1544-54.
9. BROWN JK, HOLLENBERG MD, JONES CA: Tryptase activates phosphatidylinositol 3-kinases proteolytically independently from proteinase-activated receptor-2 in cultured dog airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2006; 290: L259-69.
10. ABE M, KUROSAWA M, ISHIKAWA O, MIYACHI Y, KIDO H: Mast cell tryptase stimulates both human dermal fibroblast proliferation and type I collagen production. *Clin Exp Allergy* 1998; 28: 1509-17.
11. FRUNGERI M, WEIDINGER S, MEINEKE V, KÖHN F, MAYERHOFER A: Proliferative action of mast-cell tryptase is mediated by PAR2, COX2, prostaglandins, and PPAR-γ: possible relevance to human fibrotic disorders. *Proc Natl Acad Sci USA* 2002; 99: 15071-7.
12. GAUDRY M, BREGERIE O, ANDRIEU V, EL BENNA J, POCIDALO MA, HAKIM J: Intracellular pool of vascular endothelial growth factor in human neutrophils. *Blood* 1997; 90: 4153-61.
13. SCHRUEFER R, LUTZE N, SCHYMEINSKY J, WALZOG B: Human neutrophils promote angiogenesis by a paracrine feedforward mechanism involving endothelial interleukin-8. *Am J Physiol Heart Circ Physiol* 2005; 288: H1186-92.
14. ENOMOTO H, INOKI I, KOMIYA K *et al.*: Vascular endothelial growth factor isoforms and their receptors are expressed in human osteoarthritic cartilage. *Am J Pathol* 2003; 162: 171-81.
15. PFANDER D, KORTJE D, ZIMMERMANN R *et al.*: Vascular endothelial growth factor in

- articular cartilage of healthy and osteoarthritic human knee joints. *Ann Rheum Dis* 2001; 60: 1070-3.
16. HASHIMOTO S, CREIGHTON-ACHERMANN L, TAKAHASHI K, AMIEL D, COUTTS RD, LOTZ M: Development and regulation of osteophyte formation during experimental osteoarthritis. *Osteoarthritis Cartilage* 2002; 10: 180-7.
 17. PULSATELLI L, DOLZANI P, SILVESTRI T, FRIZZIERO L, FACCHINI A, MELICONI R: Vascular endothelial growth factor activities on osteoarthritic chondrocytes. *Clin Exp Rheumatol* 2005; 23: 487-93.
 18. CAINES J, WALLS A: Mast cell tryptase stimulates the synthesis of Type I collagen in human lung fibroblasts. *J Clin Invest* 1997; 99: 1313-21.
 19. FAJARDO I, PEJLER G: Human mast cell beta-tryptase is a gelatinase. *J Immunol* 2003; 171: 1493-9.