# Vascular endothelial growth factor activities on osteoarthritic chondrocytes

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## Abstract

### Objective

*Evaluation of the role of VEGF in cartilage pathophysiology.*

### Methods

*VEGF release from chondrocytes in the presence of IL-1β, TGFβ and IL-10 was detected by immunoassay. VEGF receptor -1 and -2 expression and VEGF ability to modulate caspase-3 and cathepsin B expression were detected by immunohistochemistry on cartilage biopsies and cartilage explants. VEGF effects on chondrocyte proliferation was analysed by a fluorescent dye that binds nucleic acids.*

### Results

*VEGF production by osteoartritis (OA) chondrocytes was significantly reduced by IL-1β while it was increased in the presence of TGFβ. Cartilage VEGFR-1 immunostaining was significantly downregulated in 'early'OA patients compared to normal controls (NC). VEGFR-2 expression was negligible both in OA and in NC. VEGF decreased the expression of caspase-3 and cathepsin B, whereas it did not affect proliferation.*

### Conclusion

*VEGF is able to down-modulate chondrocyte activities related to catabolic events involved in OA cartilage degradation.*

### Key words

Vascular endothelial growth factor, osteoarthritis, chondrocytes.

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## Introduction

Vascular endothelial growth factor (VEGF) is a dimeric glycoprotein that was originally considered to be an endothelial-specific factor. As a result of its activities as a promoter of angiogenesis *in vivo* and *in vitro* [i.e. mitogen for endothelial cells and inducer of permeabilization of blood vessels (1)], VEGF plays a very important role in physiological and pathological angiogenesis. Alternative splicing of the mRNA produces five VEGF isoforms identified by the different number of constitutive amino acids: 121, 145, 165, 189 and 206 (1).

VEGF actions are mediated by its binding to two tyrosine-kinase receptors: the fms-like tyrosine kinase Flt-1, also called VEGFR-1, and the kinase domain region KDR, also called VEGFR-2 (2).

In addition, neuropilin-1, a receptor of the collapsin/semaphorin family, has been identified as specific receptor for $VEGF_{165}$ isoform, and acts as a coreceptor of VEGFR-2 (2).

In the last few years much work has focused on the ability of VEGF production and/or VEGF receptor expression by non-endothelial tissue (3-6). As far as osteo-chondrogenic cell lineage is concerned, VEGF production by hypertrophic chondrocytes (7,8) and by osteoblasts has been described (9). Recent studies on physiological processes involved in endochondral bone formation have stressed a key role of VEGF in mediating the critical signals for growth plate morphogenesis and cartilage remodeling. VEGF has been shown to be involved in coupling cartilage resorption, angiogenesis and bone formation during endochondral ossification (7, 8).

A large amount of evidence has been reported regarding increased VEGF production in synovial tissue in joint diseases (10-15). Recent evidence has been reported about VEGF production and expression of its receptors in OA cartilage (16-19). These studies, in particular, have shown an increased expression of VEGF by OA chondrocytes, while conflicting results have been described concerning VEGF receptor expression. Pufe *et al.* (17) showed that only VEGFR-2 was expressed by articular OA chondrocytes. On the other hand, Enomoto *et al*. (18) demonstrated the presence of both receptors in OA cartilage. Furthermore, in the same study, they reported the ability of VEGF to induce an increased production of metalloproteinase by cultured OAchondrocytes.

In order to gain new insights into the role of VEGF in cartilage pathophysiology, our experiments were aimed at: a) evaluating the ability of isolated human OA chondrocytes to produce VEGF both in basal conditions and upon stimulation by catabolic (IL-1β) and anabolic (TGFβ and IL-10) mediators; b) analyzing the expression of the two VEGF receptors, VEGFR-1 and VEGFR-2, in different phases of disease progression, and c) investigating the effect of VEGF on chondrocytes, by focusing on the modulation of specific metabolic activity whose modifications are associated with degenerative chondrocyte phenotype, that have a role in pathogenic events in OAcartilage.

## Materials and methods

*VEGF release by articular chondrocytes*

*Specimens.* Articular cartilage specimens were obtained from a total of 24 OApatients (mean age: 66 years, range 38-86; mean disease duration: 64 months, range 24-120) undergoing joint replacement surgery. The diagnosis of OA was based on clinical, laboratory and radiological evaluations (20, 21). Disease severity was classified by radiological parameters. Kellgren-Lawrence radiological score (22) for all OA patients was 3-4. Normal human cartilage was obtained from femoral condyles removed following trauma in 10 subjects with no history of joint pathology (PTcases) (mean age 73, range 59-84).

Informed consent from the patients and approval by the ethical committee of the hospital were obtained.

*Chondrocyte isolation*. Chondrocytes were isolated from articular cartilage by sequential enzymatic digestion: 30 min with 0.1% hyaluronidase (Sigma, St. Louis, MO, USA), 1 hour with 0.5% pronase (Sigma) and 1 hour with

0.2% collagenase (Sigma) at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) with 25 mM HEPES (Sigma), 100 units/ml penicillin (Biological Industries, Israel), 100 μg/ml streptomycin (Biological Industries), 50 μg/ml gentamicin (Flow, Biaggio, Switzerland), 2.5 μg/ml amphotericin B (Biological Industries). The isolated chondrocytes were filtered by 100 μm and 70 μm nylon meshes, washed and centrifuged. The pellet was seeded at high density ($2x10^5$ cells/$cm^2$) and cultured in DMEM supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% $CO_2$. For all experiments only primary cultures were used in order to ensure the stability of chondrocyte phenotype.

*Chondrocyte stimulation*. Cell monolayers were incubated for 72h with or without rhIL-1β (2 ng/ml) (specific activity 5.0 x $10^7$ U/mg) or rhTGFβ (1 ng/ml) or IL-10 (10 ng/ml) (Boehringer, Mannheim, Germany). The cytokine concentrations and incubation time were selected based on results obtained in previous dose-dependence and kinetic experiments performed to assess the optimal conditions for detecting chemokine production (Data not shown).

After 72 h the supernatants were collected, aliquoted and stored at -80°C until analysis. Cell viability was assessed by the eosin dye exclusion test

*Measurement of VEGF release by chondrocyte cultures*. VEGF concentrations in cell supernatants were evaluated by immunoassay using a commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Results are expressed as pg/ ml/ $2x10^5$ cells.

*VEGF and VEGF receptor expression*

*Patients*. VEGF receptor expression was investigated by immunohistochemical analysis performed on cartilage biopsies obtained by arthroscopic examination of the knee from 11 patients with OA (Kellgren-Lawrence radiological score was 2-3) (mean age: 62 years; range: 57 – 72) and on cartilage explants obtained from 7 OA patients (mean age: 66 years; range: 58 – 74) undergoing joint replacement surgery (Kellgren-Lawrence radiological score: 3-4). VEGF expression was analyzed only on cartilage explants. Normal cartilage was obtained from 4 donors (NC) who had undergone organ explantations (mean age: 46 years; range 42 – 48).

*Knee arthroscopy*. Knee arthroscopy was performed in patients under local anaesthesia, by introducing a Hamou-Storz microarthroscope (Karl Storz, Tuttlingen, Germany) into the joint cavity of the knee, with the knee held at 30° flexion. A cartilage biopsy sample was taken from a non-weight bearing area of medial condyle, as previously described (23).

*Immunohistochemistry*. All biopsy samples were snap-frozen in liquid nitrogen and stored at –80°C. Serial cryostat sections, 4 μm thick, were air dried, and stored at –80°C until analyzed. Re-hydrated sections were fixed in 4% paraformaldehyde 30 minutes at room temperature. Non-specific binding was blocked in PBS supplemented with 10% of normal serum of the species from which the secondary antibodies were obtained for 30 minutes at room temperature. Sections were incubated overnight at 4°C with goat polyclonal antibody to human Flt-1 (VEGF receptor-1) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:100 and with mouse anti-human VEGF (R&D Systems, Minneapolis, MN, USA) (10 μg/ml). Detection of VEGFR-2 was performed utilizing three different antibodies to human Flk-1 (VEGF receptor-2): mouse anti-human VEGF receptor-2 (clone KDR-1) (Sigma) (25 μg/ml), mouse anti-human VEGF receptor-2 (clone KDR-2) (Sigma) (25 μg/ml), mouse anti-human VEGF receptor-2 (Santa Cruz Biotechnology, Inc.) (10 μg/ml). VEGF receptor-1 and VEGF, VEGF receptor-2 staining was developed respectively by an hour's incubation with a biotinylated rabbit antigoat immunoglobulins (PIERCE, Rockford, Illinois, USA) diluted 1:200 and biotinylated goat anti-mouse immunoglobulins (KPL, Gaithersburg, MD, USA) diluted 1: 200, followed by an hour's incubation with streptavidin – alkaline phosphatase (AP) conjugate (Boehringer Mannheim) diluted 1:2000. All reactions were developed using a fuchsin substrate solution (Dako, Glostrup, Denmark) supplemented with 5 mM levamisole (Sigma) to block endogenous alkaline phosphatase. After nuclear counterstaining with hematoxylin, sections were mounted in glycerol gel and stored for subsequent analysis.

Negative staining control experiments were performed according to the above-described procedure, with omission of the primary antibody.

Specificity control was carried out using a normal goat IgG (R & D Systems), mouse IgG1 control isotype (R & D Systems) and mouse IgG2b control isotype (R & D Systems) at the same concentrations as goat anti-human VEGFR-1, mouse anti-human VEGFR-2, and mouse anti-human VEGF were used.

*Quantification of VEGF receptor–positive cells*. Results of immunohistochemical analysis were expressed as percentage of VEGF, VEGFR-1 and VEGFR-2 expressing chondrocytes by counting in whole sections the number of positive chondrocytes respectively for VEGF, VEGFR-1 or VEGFR-2 and the total number of chondrocytes

*Analysis of chondrocyte metabolic pathway activated by VEGF*

*Cultures of articular cartilage explants and stimulation by VEGF*. Full-thickness cartilage explants obtained from 7 OA patients were cultured in DMEM supplemented with 10% FCS; after 24 hours, the culture media was replaced by serum-free DMEM containing different concentrations of VEGF (5 – 25 – 50 ng/ml) (24). After one week, cartilage explants were recovered, frozen in liquid nitrogen and stored at –80°C. Supernatants were aliquoted and maintained at –80°C.

*Immunohistochemistry*. Serial cryostat sections, 4 μm thick, were air dried, fixed in 4% paraformaldehyde 30 minutes at room temperature. Non-specific binding was blocked in PBS supplemented with 10% of normal serum of the species from which the secondary antibodies were obtained for 30 min-

utes at room temperature. Sections were incubated overnight at 4°C with rabbit polyclonal antibody to human activated form of caspase-3 (1 μg/ml) (R&D Systems), and mouse monoclonal antibody to human cathepsin B (10 μg/ml) (Oncogene Research Products, Boston, MA). Staining was performed by avidin-biotin technique: biotinilated goat anti-rabbit immunoglobulins (Dako) diluted 1:350 were utilized for detection of caspase-3, biotin-labeled goat anti-mouse immunoglobulins (KPL, Gaithersburg, MD, USA) diluted 1:200 for cathepsin B. All reactions were developed by one hour's incubation in streptavidin–alkaline phosphatase (AP) conjugate (Boehringer Mannheim) diluted 1:2000 and using a fuchsin substrate solution (Dako) supplemented with 5 mM levamisole (Sigma) to block endogenous alkaline phosphatase. After nuclear counterstaining with hematoxylin, sections were mounted in glycerol gel and stored for subsequent analysis.

Negative staining control experiments were performed according to the above-described procedure, with omission of the primary antibody. Specific staining control was carried out using normal rabbit IgG (R & D Systems) or mouse IgG2a (R & D Systems) respectively at the same concentration as rabbit anti-human caspase 3 and mouse anti-human cathepsin B.

*Chondrocyte proliferation assay*. Isolated chondrocytes were seeded in 96-well plates (20 x $10^4$/well) and cultured for one week. Cultures were then incubated with different concentrations of VEGF (5 – 25 – 50 ng/ml) for 72 hours. The proliferation of chondrocytes was assessed by the CyQuant Cell Proliferation Kit (Molecular Probes, Leiden, The Netherlands) according to the manufacture's protocol. Briefly, cells were visualized utilizing a green fluorescent dye, which shows strong fluorescence enhancement when bound to cellular nucleic acid. On the basis of a standard curve, fluorescence intensity was converted into cell number. Fluorescence intensity was measured by a fluorescence microplate reader with excitation at 485 nm and emission at 520 nm.

*Statistical analysis*

Statistical analysis was carried out by non-parametric tests. The Kruskal-Wallis test was utilized to perform multiple comparison of unpaired data and the non-parametric Mann-Whitney U test to compare the variables between groups. The non-parametric Friedman test was used for multiple comparison of paired data and the Wilcoxon test was applied when the Friedman test was significant. The analyses were performed using Statistica for Windows package (Statsoft Inc., Tulsa, USA).

## Results

*VEGF production by chondrocytes*

Isolated chondrocytes spontaneously produced detectable amounts of VEGF. In the presence of IL-1β, VEGF production by OA chondrocytes was significantly reduced ($p < 0.01$). IL-10 did not decrease VEGF production by chondrocytes but, on the contrary, showed a trend to increase production. VEGF release was significantly upregulated in the presence of TGFβ ($p < 0.01$) (Fig. 1). In the control group, VEGF production was significantly modulated only in the presence of TGFβ ($p < 0.05$) (Figure 1).

These results were confirmed by an *in vivo* study, that showed VEGF immunostaining in OA cartilage explants with a mean percentage of positive chondrocytes of 47% (range 32-69%). VEGF positive chondrocytes were mainly localized in the middle and deep zones of articular cartilage

*VEGF receptor expression*

In order to identify differential cartilage expression of VEGF receptors related to OAin different stages of disease, we compared receptor expression in cartilage biopsies obtained by arthroscopic examinations ('early' OA) to samples obtained from patients at the time of joint replacement surgery ('late'OA).

VEGFR-1 cartilage expression was detected both in cartilage biopsies (percent of chondrocyte positive ranged from 3% to 53%) and in cartilage explants (positive chondrocyte range 13-44%). Percentage of chondrocytes expressing VEGFR-1 in NC was significantly higher than those in all OA patients ('early'and 'late'OA) ($p < 0.05$) (Fig. 2). This statistical difference is mainly due to the contribution of the 'early'OA group. Indeed, a significant difference was reached only between NC and 'early OA' ($p < 0.02$) (Fig. 2). No difference in VEGFR-1 expression was shown between 'early' and 'late'

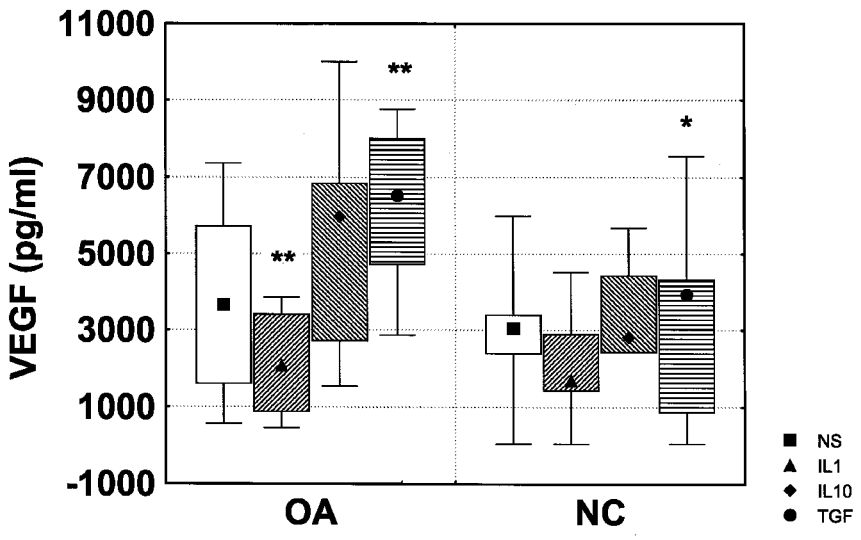



**Fig. 1.** Vascular endothelial growth factor production by human chondrocytes obtained from patients with osteoarthritis (OA) and by normal controls (NC). VEGF was detected in chondrocyte culture supernatants in unstimulated condition (NS) and in the presence of IL-1β (2 ng/ml), IL-10 (10 ng/ml), TGFβ (1 ng/ml). Results are represented as median values. Boxes show 25th and 75th percentiles. Vertical lines below and above boxes show 10th and 90th percentiles.
*$p < 0.05$; ** $p < 0.01$ compared to VEGF production obtained in unstimulated conditions (NS).

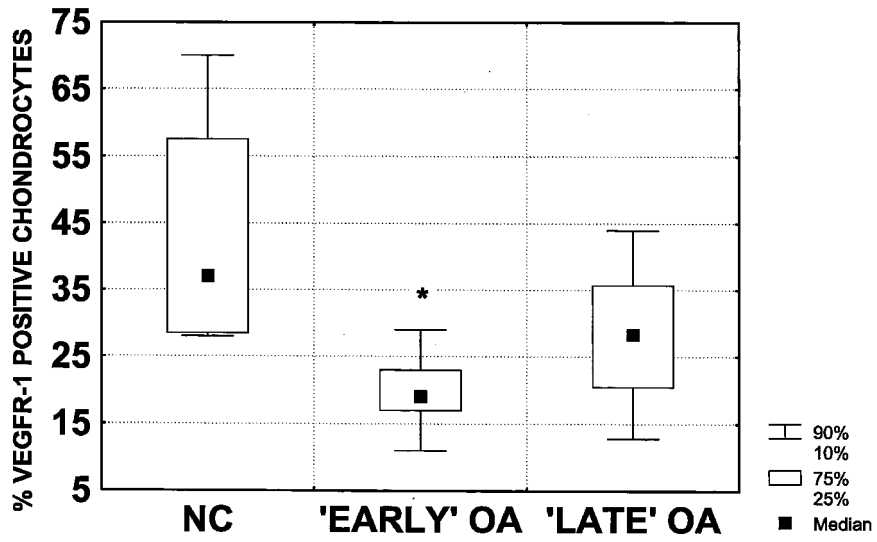



**Fig. 2.** Percentage of VEGF receptor-1 positive cells in cartilage sections obtained from normal controls (NC) and from patients with osteoarthritis (OA). In OApatients, VEGFR-1 expression was investigated by immunohistochemistry in arthroscopic biopsy sections ('early' OA) and cartilage explant sections ('late'OA). Results are represented as median values. Boxes show 25$^{th}$ and 75$^{th}$ percentiles. Vertical lines below and above boxes show 10$^{h}$ and 90$^{th}$ percentiles.
* $p < 0.02$ compared to VEGFR-1 expression in normal controls cartilage (NC).

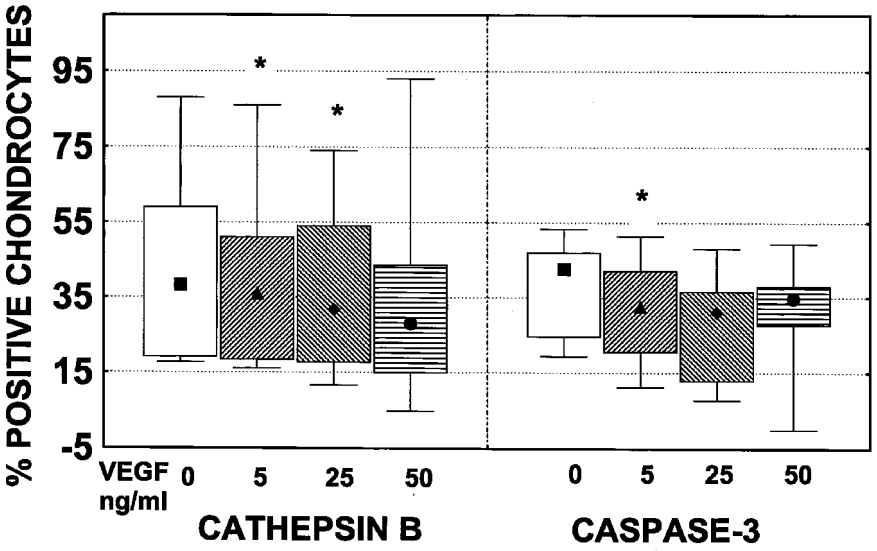



**Fig. 3.** Percentage of cathepsin B and active caspase-3 positive cells in sections of cartilage explants obtained from patients with osteoarthritis (OA). Caspase-3 and cathepsin B immunostaining was detected in cartilage explants cultured in unstimulated condition (NS) and in the presence of different concentrations (5-25-50 ng/ml) of VEGF. Results are represented as median values. Boxes show 25$^{th}$ and 75$^{th}$ percentiles. Vertical lines below and above boxes show 10$^{h}$ and 90$^{th}$ percentiles.
* $p < 0.05$ compared to cartilage expression in unstimulated condition (NS).

OA. VEGFR-2 expression was negligible both in OAand in NC samples (data not shown).

In cartilage explants obtained from OA patients, VEGF receptor expression by chondrocytes was not significantly modified by VEGF pretreatment, and lack of substantial VEGFR-2 expression was confirmed (data not shown).

*Effect of VEGF on caspase-3 and cathepsin B expression*

The effect of VEGF on the expression of activated form of caspase-3 and cathepsin B was investigated by immunoistochemistry in articular cartilage explants obtained from 7 OA patients.

Caspase-3 expression in cartilage explant treated with VEGF 5 ng/ml was significantly reduced ($p<0.05$) compared to untreated samples; this trend was confirmed in the presence of 25 ng/ml and 50 ng/ml VEGF even though it did not achieve statistical significance (Fig. 3).

In the presence of 5 ng/ml and 25 ng/ml of VEGF, the number of chondrocytes expressing cathepsin B in cartilage explants was lower compared to those observed in the sample without VEGF ($p < 0.05$) (Fig. 3).

Caspase-3 and cathepsin B positive chondrocytes were mainly localized in the middle and deep zones of articular cartilage (data not shown).

*Effect of VEGF on chondrocyte proliferation*

The mitogenic effect of different concentrations of VEGF was investigated on isolated chondrocytes by proliferation assay. The presence of VEGF (5-25-50 ng/ml) in culture medium did not induce an increase in fluorescence emission of treated samples compared to untreated controls, showing that VEGF did not stimulate chondrocyte proliferation.

**Discussion**

The main findings of our study were as follows: a) proinflammatory cytokine IL-1β down-modulated chondrocyte production of VEGF; on the other hand, anabolic factor, TGFβ and, to a lesser extent, antiinflammatory cytokine IL-10 enhance VEGF release by chondrocytes; b) chondrocytes expressed VEGFR-1 to a variable extent, but only negligible amounts of VEGFR-2; VEGFR-1 expression were down-modulated in 'early' OA; c) investigations of the metabolic pathways involved in chondrocyte degradation activities showed that VEGF was able to reduce cartilage expression of cathepsin B and active form of casapase-3.

Recently, evidence has been provided, by *in vivo* studies, about VEGF production in cartilage (16-18). These studies showed an increased expression of VEGF by OAchondrocytes. We did not find a significant difference between normal and OA chondrocyte VEGF production. This discrepancy might be due to the different experimental condi-

tions of our study; indeed, we investigated *in vitro* VEGF production by evaluating VEGF release by cultured isolated chondrocytes. We chose this kind of experimental design because our main goal was to evaluate the modulation of chondrocyte VEGF production by specific factors and not the absolute amount of VEGF production *in vivo*.

Several studies have evaluated VEGF expression modulation in different cell-types. In most settings, VEGF production could be enhanced by factors such as fibroblast growth factor, PDGF, TNFα, TGFβ, and keratinocyte growth factor, insulin-like growth factor I, IL-1, IL-6 (1). Other cytokines, such as IL-10 and IL-13 can reduce the release of VEGF (1). Our study showed that chondrocyte VEGF release was modulated in a peculiar way. Indeed, while TGFβ, for chondrocytes, was a strong inducer of VEGF release, IL-1β was shown to reduce chondrocyte VEGF production and IL-10 did not significantly affect VEGF production. TGFβ promotes chondrocyte anabolism *in vitro* and *in vivo* (enhancing matrix production, cell proliferation, osteochondrogenic differentiation) (25); IL-10, exerts an inhibitory effect on cellular inflammatory cytokine production (26). Therefore chondrocyte production of VEGF appears to be stimulated by anabolic and anti-inflammatory stimuli. On this basis we were led to hypothesize a metabolic activity to counteract cartilage degradation for VEGF.

In normal subjects we found higher cartilage expression of VEGF receptor-1 than in OA patients. This result suggests a role of VEGF in physiological condition, underlining that in joint diseases, chondrocyte modification leads to a decreased expression of VEGF receptor. In pathologic conditions VEGF production could be greatly enhanced in synovial microenvironment (10-15), and chondrocytes could be stimulated by VEGF produced both by chondrocytes themselves, in an autocrine/paracrine manner, and by synovial tissue. A decrease in chondrocyte VEGF receptor expression, as shown in our study, might represent a mechanism to balance over-production of VEGF. Alternatively, we should underline that our normal cartilage donors were younger than OA patients, thus suggesting a possible role of aging in down-modulation of VEGFRs.

In addition, we showed that VEGFR-2 is expressed only in a negligible amount. This result is not in line with the findings reported by other authors (17, 18), but we confirmed it via the use of different antibodies to VEGFR-2. These discrepancies might be due to different techniques utilized to treat tissue for immunohistochemichal staining. We performed analysis on cartilage frozen-paraformaldehyde fixed samples, whereas in the other studies the cartilage tissue utilized for immunohistochemical techniques was treated with a procedure that can affect antigen expression and immunostaining results (i.e.: decalcification, antigen unmasking by proteolytic enzymes etc.).

Another possible explanation for the low VEGFR-2 expression might be its internalization following receptor-ligand interaction. Indeed, such evidence was provided by Duval *et al*. (27) in a study performed on endothelial cells. In that study the authors showed that sustained stimulation of endothelial cells by VEGF induced a down-regulation of VEGFR-2. This down-regulation was a consequence of internalization of receptor after engagement with VEGF, that was then degraded by the ubiquitination mechanism.

It has been demonstrated that VEGF-induced cell proliferation is mediated via VEGFR-2 (6). It is noteworthy that the lack of VEGFR-2 chondrocyte expression is in keeping with our negative results regarding chondrocyte proliferation.

We then investigated whether VEGF was able to modulate chondrocyte metabolic pathways typically involved in cartilage damage and repair occurring in OA.

Since modifications of metabolic activity in OA chondrocytes have been extensively reported and a phenotype shift of osteoarthritic chondrocytes toward a dedifferentiated phenotypes have been described (28-32), we evaluated the modulation of markers typically expressed during catabolic events (such as apoptosis markers and cathepsin B expression).

Chondrocyte expression of caspase-3, a member of the family of cysteine protease which has a primary role in the execution of the apoptotic program (33), was significantly decreased in the presence of certain dosage of VEGF. Our results confirmed previous data showing the ability of VEGF to prevent apoptotic cell death of other cell populations, such as hematopoietic cells and endothelial cells by blocking pathways leading to caspase activation (34, 35).

VEGF reduced chondrocyte expression of cathepsin B significantly. This cysteine peptidase was recognized as a mediator of pathological proteolysis in articular cartilage, and high cathepsin B activity was shown at sites of matrix degradation in OA cartilage (36, 37). Furthermore, *in vitro* studies have demonstrated that expression of cathepsin B marked dedifferentiated chondrocyte phenotype that closely resembles modification of OA chondrocytes and *in vivo* may participate in osteophyte formation by promoting cartilage neovascularization and mineralization (38).

In conclusion, VEGF, in addition to the documented enhanced activity on metalloproteinase production (18), down-modulates cartilage activities typically expressed during catabolic events. Therefore, it might also counteract degradative activities of OA chondrocyte metabolism.

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**References**

1. NEUFELD G, COHEN T, GENGRINOVITCH S, POLTORAK Z: Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 1999; 13: 9-22.
2. ORTEGA N, HUTCHINGS H, PLOUET J: Signal relays in the VEGF system. *Front Biosci* 1999; 4: d141-d152
3. CLAUSS M, WEICH H, BREIER G *et al*.: The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem* 1996; 271: 17629-34.
4. WANG H, KEISER JA: Vascular endothelial growth factor upregulates the expression of

matrix metalloproteinases in vascular smooth muscle cells. Role of flt-1. *Circ Res* 1998; 83: 832-40.

5. DANKBAR B, PADRÒ T, LEO R *et al.*: Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. *Blood* 2000; 95: 2630-6.
6. DUNK C, AHMED A: Vascular endothelial growth factor receptor-2-mediated mitogenesis is negatively regulated by vascular endothelial growth factor receptor-1 in tumor epithelial cells. *Am J Pathol* 2001; 158: 265-73.
7. GERBER HP, VU TH, RYAN AM, KOWALSKI J, WERB Z, FERRARA N: VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* 1999; 5: 623-8.
8. CARLEVARO MF, CERMELLI S, CANCEDDA R, DESCALZI CANCEDDA F: Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. *J Cell Sci* 2000; 113: 59-69.
9. DECKERS MML, KARPERIEN M, VAN DER BENT C, YAMASHITA T, PAPAPOULOS SE, LÖWIK CWGM: Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. *Endocrinology* 2000; 141: 1667-74.
10. JACKSON JR, MINTON JAL, HO ML, WEI N, WINKLER JD: Expression of vascular endothelial growth factor in synovial fibroblasts is induced by hypoxia and interleukin 1β. *J Rheumatol* 1997; 24: 1253-9.
11. IKEDA M, HOSODA Y, HIROSE S, OKADA Y, IKEDA E: Expression of vascular endothelial growth factor isoforms and their receptors Flt-1, KDR, and neuropilin-1 in synovial tissues of rheumatoid arthritis. *J Pathol* 2000; 191: 426-33.
12. LU J, KASAMA T, KOBAYASHI K *et al.*: Vascular endothelial growth factor expression and regulation of murine collagen-induced arthritis. *J Immunol* 2000; 164: 5922-7.
13. LEE S, JOO YS, KIM WU *et al.*: Vascular endothelial growth factor levels in the serum and synovial fluid of patients with rheumatoid arthritis. *Clin Exp Rheumatol* 2001; 19: 321-4.
14. ETHERINGTON PJ, WINLOVE P, TAYLOR P, PALEOLOG E, MIOTLA JM: VEGF release is associated with reduced oxigen tensions in experimental inflammatory arthritis. *Clin Exp Rheumatol* 2002; 20: 799-805.
15. HAYWOOD L, MCWILLIAMS DF, PEARSON CI *et al.*: Inflammation and angiogenesis in osteoarthritis. *Arthritis Rheum* 2003; 48: 2173-7.
16. PFANDER D, KÖRTJE 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.
17. PUFE T, PETERSEN W, TILLMANN B, MENTLEIN R: The splice variants $VEGF_{121}$ and $VEGF_{189}$ of the angiogenic peptide vascular endothelial growth factor are expressed in osteoarthritic cartilage. *Arthritis Rheum* 2001; 44: 1082-8.
18. 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.
19. HONORATI MC, CATTINI L, FACCHINI A: IL-17, IL-1β and TNFα stimulate VEGF production by dedifferentiated chondrocytes. *Osteoarthritis Cartilage* 2004; 12: 683-91.
20. ALTMAN R, ASCH E, BLOCH D *et al.*: Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. *Arthritis Rheum* 1986; 29: 1039-49.
21. ALTMAN R, ALARCON G, APPELROUTH D *et al.*: The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hip. *Arthritis Rheum* 1991; 34: 505-14.
22. KELLGREN JH, LAWRENCE JS: Radiological assessment of osteo-arthritis. *Ann Rheum Dis* 1957; 16: 494-502.
23. PASQUALI-RONCHETTI I, FRIZZIERO L, GUERRA D *et al.*: Aging of the human synovium: an *in vivo* and *ex vivo* morphological study. *Semin Arthritis Rheum* 1992; 21: 400-14.
24. MAZZETTI I, MAGAGNOLI G, PAOLETTI S *et al.*: A role for chemokines in the induction of chondrocyte phenotype modulation. *Arthritis Rheum* 2004; 50: 112-22.
25. GRIMAUD E, HEYMANN D, RÉDINI F: Recent advances in TGF-β effects on chondrocyte metabolism. Potential therapeutic roles of TGF-β in cartilage disorders. *Cytokine Growth Factor Rev* 2002; 13: 241-57.
26. SUGIYAMA E, KURODA A, TAKI H *et al.*: Interleukin 10 cooperates with interleukin 4 to suppress inflammatory cytokine production by freshly prepared adherent rheumatoid synovial cells. *J Rheumatol* 1995; 22: 2020-6.
27. DUVAL M, BÉDARD-GOULET S, DELISLE C, GRATTON JP: Vascular endothelial growth factor-dependent down-regulation of Flk-1/KDR involves Cbl-mediated ubiquitation. *J Biol Chem* 2003; 278: 20091-7.
28. VON DER MARK K, KIRSCH T, NERLICH A *et al.*: Type X collagen synthesis in human osteoarthritic cartilage. *Arthritis Rheum* 1992; 35: 806-11.
29. AIGNER T, DUDHIA J: Phenotypic modulation of chondrocytes as a potential therapeutic target in osteoarthritis: a hypothesis. *Ann Rheum Dis* 1997; 56: 287-91.
30. SANDELL LJ, AIGNER T: Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* 2001; 3: 107-13.
31. BERARDI S, LANG A, KOSTOULAS G, HÖRLER D, VILEI EM, BAICI A: Alternative messenger RNA splicing and enzyme forms of cathepsin B in human osteoarthritic cartilage and cultured chondrocytes. *Arthritis Rheum* 2001; 44: 1819-31.
32. TERKELTAUB RA: What does cartilage calcification tell us about osteoarthritis? *J Rheumatol* 2002; 29: 411-5.
33. DONEPUDI M, GRÜTTER MG: Structure and zymogen activation of caspases. *Biophys Chem* 2002; 101-102: 145-53.
34. KATOH O, TAKAHASHI T, OGURI T *et al.*: Vascular endothelial growth factor inhibits apoptotic death in hematopoietic cells after exposure to chemotherapeutic drugs by inducing MCL1 acting as an antiapoptotic factor. *Cancer Res* 1998; 58: 5565-9.
35. MUNSHI N, FERNANDIS AZ, CHERLA RP, PARK IW, GANJU RK: Lipopolysaccharide-induced apoptosis of endothelial cells and its inhibition by vascular endothelial growth factor. *J Immunol* 2002; 168: 5860-6.
36. BAICI A, HÖRLER D, LANG A, MERLIN C, KISSLING R: Cathepsin B in osteoarthritis: zonal variation of enzyme activity in human femoral head cartilage. *Ann Rheum Dis* 1995; 54: 281-8.
37. BAICI A, LANG A, HÖRLER D, KISSLING R, MERLIN C: Cathepsin B in osteoarthritis: cytochemical and histochemical analysis of human femoral head cartilage. *Ann Rheum Dis* 1995; 54: 289-97.
38. KOSTOULAS G, LANG A, NAGASE H, BAICI A: Stimulation of angiogenesis through cathepsin B inactivation of the tissue inhibitors of matrix metalloproteinases. *FEBS Lett* 1999; 455: 286-90.