Vascular Endothelial Growth Factor Isoforms and Their Receptors Are Expressed in Human Osteoarthritic Cartilage

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To assess the possible involvement of vascular endothelial growth factor (VEGF) in the pathology of osteoarthritic (OA) cartilage, we examined the expression of VEGF isoforms and their receptors in the articular cartilage, and the effects of VEGF on the production of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in OA chondrocytes. Reverse transcriptase-polymerase chain reaction analyses demonstrated that mRNAs for three VEGF isoforms (VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉) are detectable in all of the OA and normal (NOR) cartilage samples. However, the mRNA expression of their receptors (VEGFR-1 = Flt-1, VEGFR-2 = KDR and neuropilin-1) was recognized only in the OA samples. The protein expression of VEGFR-1 and VEGFR-2 in OA chondrocytes was also demonstrated by immunohistochemistry of the OA cartilage tissue and cultured OA chondrocytes. *In situ* hybridization and immunohistochemistry indicated that VEGF is expressed in the chondrocytes in the superficial and transitional zones of OA cartilage. A linear correlation was obtained between VEGF immunoreactivity and Mankin scores in the cartilage ($r = 0.906$, $P < 0.001$). The production levels of VEGF determined by enzyme-linked immunosorbent assay were significantly 3.3-fold higher in OA than in NOR samples ($P < 0.001$). Among MMP-1, -2, -3, -7, -8, -9, and -13, TIMP-1 and -2 measured by their sandwich enzyme immunoassay systems, the production of MMP-1 and MMP-3 but not TIMP-1 or TIMP-2 was significantly enhanced by the treatment of cultured OA chondrocytes with VEGF ($P < 0.05$), whereas no such effect was obtained with cultured NOR chondrocytes. These results demonstrate that VEGF and its receptors are expressed in OA cartilage, and suggest the possibility that VEGF is implicated for the destruction of OA articular cartilage through the increased production of MMPs. (*Am J Pathol* 2003, 162:171–181)

Cartilage is composed of highly differentiated chondrocytes and extracellular matrix (ECM), and essentially an avascular tissue. However, during endochondral ossification in a developing bone, neovascularization from subchondral bone takes place in the growth plate of cartilage, resulting in resorption of cartilage ECM and replacement by bone matrix.¹ In pathological conditions such as rheumatoid arthritis (RA) and osteoarthritis (OA), damaged articular cartilage is frequently covered with and invaded by the granulation tissue with high vascularity, ie, pannus tissue. These findings observed in the pathophysiological conditions suggest the involvement of angiogenic factors in the process. In fact, a number of angiogenic molecules including basic fibroblast growth factor,² vascular endothelial growth factor (VEGF),³ and transforming growth factor- β ⁴ are present in growth plate cartilage. Among them, VEGF, which is produced from hypertrophic chondrocytes, is considered to be a coordinator of ECM remodeling, angiogenesis, and bone formation in the growth plate.³ VEGF is also known to be expressed in the cells of RA synovial tissue and the expression is related to angiogenesis in the synovium.⁵ However, limited information is so far available for the VEGF expression in the articular cartilage under the pathophysiological conditions.

VEGF has a strong angiogenic activity with specific mitogenic and chemotactic actions on endothelial cells.^{6,7} Alternative splicing of VEGF mRNA generates the five different isoforms with 121, 145, 165, 189, and 206 amino acid residues, which are named VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆, respectively. VEGF was originally recognized in a tumor-conditioned medium,⁸ but recent studies have demonstrated that it is expressed by various types of cells including vascular smooth muscle cells, monocytes, mesangial cells, and megakaryocytes,⁹ in some of which the expression is constitutive. Thus, it is conceivable that the biological function of VEGF is dictated mainly by the expression of

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its receptors on the cells in various tissues. There are two types of well-known receptors of VEGF, ie, fms-like tyrosine kinase, Flt-1 (VEGFR-1)¹⁰ and kinase insert domain-containing receptor, KDR (VEGFR-2).^{11,12} Neuropilin-1 (NRP-1) is an isoform-specific co-receptor of VEGFR-2, and enhances the bioactivity of VEGF₁₆₅ by increasing the binding affinity of the molecule to VEGFR-2.¹³ Because the primary function of VEGF was considered to be at angiogenesis, previous studies on the receptors have focused on endothelial cells, and demonstrated the expression in the vascular endothelial cells in the pathological tissues with angiogenesis.¹⁴ Interestingly, binding of VEGF to its receptors on the endothelial cells stimulates not only their proliferation and chemotaxis but also production of ECM-degrading metalloproteinases, ie, matrix metalloproteinases (MMPs).^{15–17} These MMPs are thought to play a key role in the degradation of basement membrane of blood vessels and their surrounding ECM, facilitating endothelial cell migration.¹⁸ However, little is known about the expression of VEGF receptors or biological activity of VEGF in articular cartilage.

In the present study, we examined the expression of VEGF isoforms and their receptors in OA and normal (NOR) articular cartilage, and the effect of VEGF on the production of MMPs and their common inhibitors [tissue inhibitors of metalloproteinases (TIMPs)] in cultured OA and NOR chondrocytes. Our results demonstrate that VEGF and its receptors are expressed in OA cartilage and VEGF stimulates OA chondrocytes to produce MMP-1 and MMP-3 without affecting the levels of TIMPs.

Materials and Methods

Clinical Samples and Histology

Nonosteophytic cartilage samples were obtained at arthroplasty from 3 hip joints and 46 knee joints (49 samples) with OA (72 ± 8 years old, mean age ± SD) diagnosed according to the American Rheumatism Association Criteria.¹⁹ NOR control cartilage samples without macroscopic changes were taken from 17 hip joints (17 samples) with femoral neck fracture (79 ± 9 years old). All of the samples were cut into slices (~3-mm thick), fixed with periodate-lysine-paraformaldehyde or 4% paraformaldehyde fixatives for ~24 hours at 4°C, and embedded in paraffin wax after decalcification with 0.5 mol/L of ethylenediaminetetraacetic acid, pH 7.4. Periodate-lysine-paraformaldehyde-fixed paraffin sections (4 μm thick) were stained with hematoxylin and eosin or toluidine blue, and subjected to histological/histochemical grading according to Mankin and colleagues.²⁰ For the experimental use of the surgical samples, informed consent was obtained from the patients according to the hospital ethical guidelines.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Sequencing Analysis

Total RNA was extracted directly from articular cartilage samples (13 OA and 5 NOR samples) by the acid gua-

nidium-phenol-chloroform method using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. By using a random oligonucleotide hexamer (Takara, Otsu, Japan), randomly primed cDNAs were prepared from 2 μg of total RNA by Superscript II reverse transcriptase (Life Technologies, Inc., Rockville, MD). A 1-μl aliquot of the reaction product was subjected to RT-PCR analysis on the expression of VEGF, VEGFR-1, VEGFR-2, NRP-1, and β-actin at 30 cycles. PCR was performed in a 50-μl reaction volume containing 800 nmol/L of each primer, 220 μmol/L of dNTPs, and 1 U of Ex TaqDNA polymerase (Takara, Otsu, Japan). The thermal cycle was 1 minute at 94°C, 1 minute at 64°C for VEGF and VEGFR-1, 63°C for VEGFR-2 and NRP-1, or 65°C for β-actin, and 1 minute at 72°C, followed by 3 minutes at 72°C for the final extension. The nucleotide sequences of the PCR primers were 5'-TGCCTTGCTGCTCTACCTCC-3' (forward, on exon 1) and 5'-TCACCGCCTCGGCTTGTCAC-3' (reverse, on exon 8) for VEGF; 5'-GATGTTGAGGAAGAGGAGGATT-3' (forward) and 5'-AAGCTAGTTTCCTGGGGGTATA-3' (reverse) for VEGFR-1; 5'-GATGTGGTCTGAGTCCGTCT-3' (forward) and 5'-CATGGCTCTGCTTCTCCTTTG-3' (reverse) for VEGFR-2; 5'-CAACGATAAATGTGGC-GATACT-3' (forward) and 5'-TATACTGGGAAGAAGCTGTGAT-3' (reverse) for NRP-1; 5'-TGACGGGGTCACCCCACTGTGCCCCATCTA-3' (forward) and 5'-CTAGAA-GCATTTGCGGTGGACGATGGAGGG-3' (reverse) for β-actin. The above RT-PCR analysis enabled us to differentiate each isoform of VEGF by the difference in size of the amplified DNA fragments. The expected sizes of the amplified cDNA fragments of VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆, VEGFR-1, VEGFR-2, NRP-1, and β-actin were 0.41, 0.48, 0.54, 0.61, 0.66, 1.1, 0.56, 0.82, and 0.66 kb, respectively. An aliquot of the PCR product was electrophoresed in a 2% agarose gel, and stained with ethidium bromide. To confirm the specific amplification from the target mRNAs, the RT-PCR products were subcloned into the pBluescript KS vector (Stratagene, La Jolla, CA) and analyzed by sequencing with fluorescent T7 primer (Amersham Pharmacia Biotech, Buckinghamshire, UK) using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech) and ALF DNA sequencer II (Amersham Pharmacia Biotech).

In Situ Hybridization

Paraffin sections from the paraformaldehyde-fixed samples (seven OA and five NOR cartilage samples) were treated with proteinase K (5 μg/ml; Sigma Chemical Co., St. Louis, MO) in 10 mmol/L of Tris-HCl, pH 8.0, and 1 mmol/L of ethylenediaminetetraacetic acid at 37°C for 15 minutes, and postfixed in 4% paraformaldehyde at room temperature for 10 minutes. They were then rinsed in 0.1 mol/L of phosphate buffer and incubated in 0.2 mol/L of HCl at room temperature for 10 minutes. After washing in 0.1 mol/L of phosphate buffer, they were dehydrated in ethanol and air-dried. Single-stranded sense and anti-sense digoxigenin-labeled RNA probes were generated by *in vitro* transcription of the cDNA with T3 or T7 RNA polymerase using the DIG RNA labeling kit (Boehringer

Mannheim, Mannheim, Germany) following the protocol from manufacturer. Template DNA was a 517-bp cDNA encoding human VEGF₁₂₁, which was cloned in pBlue-script KS vector. This cDNA clone was kindly provided by Dr. Herbert A. Weich (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany). Hybridization with the digoxigenin-labeled RNA probes was performed at 50°C for 16 hours in 40 µl of buffer containing 50% formamide, 10 mmol/L Tris-HCl, pH 7.6, 0.2 µg/µl tRNA, 1× Denhardt's solution (0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 10% dextran sulfate, 600 mmol/L NaCl, 0.25% sodium dodecyl sulfate, and 1 mmol/L ethylenediaminetetraacetic acid. After hybridization, the sections were washed in a buffer containing 50% formamide and 2× standard saline citrate at 50°C for 30 minutes, followed by digestion with ribonuclease A (10 µg/ml; Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 30 minutes. After washing in 2× standard saline citrate at 50°C for 20 minutes and twice in 0.2× standard saline citrate at 50°C for 20 minutes, they were treated with 0.3% hydrogen peroxide and 0.1% sodium azide in distilled water for 30 minutes at room temperature to block endogenous peroxidase activity. After blocking nonspecific binding with 10% normal horse serum, they were incubated with mouse anti-digoxigenin antibody (1/750 dilution; Boehringer Mannheim) at room temperature for 90 minutes, then incubated with biotinylated horse antibodies against mouse immunoglobulin (IgG) (1/200 dilution; Vector Laboratories, Burlingame, CA) for 30 minutes, and finally reacted with an avidin-biotin-peroxidase complex solution (1/100 dilution; DAKO, Glostrup, Denmark) for 30 minutes. Color was developed with 0.2 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride in 50 mmol/L Tris-HCl, pH 7.6, containing 0.003% hydrogen peroxide, and the sections were counterstained with hematoxylin.

Immunohistochemistry

Sections from the periodate-lysine-paraformaldehyde-fixed samples (44 OA and 12 NOR cartilage samples) were treated with 0.3% H₂O₂ and 10% normal horse serum to block endogenous peroxidase and nonspecific binding, respectively. The sections were then treated with rabbit polyclonal antibodies against human VEGF (1/50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 90 minutes. After the reactions with goat antibodies against rabbit IgG conjugated to peroxidase labeled-dextran polymer (no dilution; En Vision+ Rabbit; DAKO) at room temperature for 30 minutes, the color was developed with 3,3'-diaminobenzidine tetrahydrochloride in 50 mmol/L of Tris-HCl, pH 7.6, containing 0.006% H₂O₂. Counterstaining was performed with hematoxylin. As for a control, sections were reacted by replacing the first antibodies with nonimmune rabbit IgG (DAKO) or with the anti-VEGF antibodies that were incubated with the blocking peptide for VEGF (Santa Cruz Biotechnology) at room temperature for 2 hours before the immunostaining.

For immunohistochemistry of VEGFR-1 and VEGFR-2, paraffin sections of OA (five samples) and NOR cartilage

(five samples) were reacted with goat polyclonal antibodies against VEGFR-1 (1/50 dilution; R&D Systems, Minneapolis, MN) or mouse monoclonal antibody against VEGFR-2 (1/50 dilution; Santa Cruz Biotechnology), and then treated with biotinylated rabbit IgG to goat IgG and horse IgG to mouse IgG (Vector Laboratories), respectively. As for a control, primary antibodies were replaced with nonimmune goat or mouse IgG (Santa Cruz Biotechnology). After the reactions, the sections were incubated with avidin-biotin-peroxidase complex (DAKO) and the color was developed with 3,3'-diaminobenzidine tetrahydrochloride as described above.

Cultures of Articular Cartilage Explants and Detection of VEGF Protein

Full-thickness cartilage slices (18 OA and 12 NOR samples) were cultured in serum-free Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (Life Technologies, Inc.) containing 0.2% lactalbumin hydrolysate for 3 days. After centrifugation at 1500 rpm for 5 minutes, the culture media were stored at -20°C until used for assay. The levels of VEGF (ng/ml) were measured using the enzyme-linked immunosorbent assay (ELISA) system for human VEGF, which recognizes VEGF₁₂₁ and VEGF₁₆₅ isoforms (R&D Systems). The values were expressed as ng/g dry tissue weight.

Isoforms of VEGF were also analyzed by immunoblotting. The media (2 ml/lane) from OA and NOR cartilage slices (two samples each), which showed high and negligible levels of VEGF by ELISA, were first concentrated with 3.3% trichloroacetic acid and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% total acrylamide) under the reducing condition. The proteins separated in the gels were transferred onto nitrocellulose filters, and the filters were incubated for ~12 hours at room temperature with rabbit polyclonal antibodies against human VEGF (1/100 dilution; Santa Cruz Biotechnology). They were reacted with biotinylated goat IgG to rabbit IgG and color was developed with 3,3'-diaminobenzidine tetrahydrochloride as described above. As for a control, culture media (2 ml/lane) of OA chondrocytes (three samples) were subjected to immunoblotting for VEGF according to the above-mentioned method.

Chondrocyte Cultures and Immunohistochemistry

Chondrocytes were isolated from OA (seven samples) and NOR cartilage (five samples) by digestion of the minced cartilage with 0.4% (w/v) Actinase E (Kaken, Tokyo, Japan) for 1 hour at 37°C and then with 0.025% (w/v) bacterial collagenase type I (Worthington Biochemical Corp., Freehold, NJ) for ~16 hours at 37°C. Isolated cells were plated on 24-well culture flasks at a density of 5×10^5 cells/well in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 24 hours. The culture media were replaced with serum-free Dulbecco's

modified Eagle's medium containing 0.2% lactalbumin hydrolysate, and then treated with recombinant VEGF₁₆₅ (0, 10, or 50 ng/ml) (R&D Systems) for 3 days. After the treatment, the media were harvested and centrifuged at 3000 rpm for 5 minutes. The supernatants were stored at -20°C until used for assays of MMPs and TIMPs.

To show the expression of VEGFR-1 and VEGFR-2 and exclude the possibility of the endothelial cell contamination in the isolated OA and NOR chondrocytes, the cells were cultured on Lab-Tek chamber slides (Nalge-Nunc International, Tokyo, Japan) and subjected to immunohistochemistry for the receptors and endothelial cell markers (CD31 and von Willebrand factor). They were reacted with antibodies against VEGFR-1 (1/50 dilution; R&D Systems) and VEGFR-2 (1/50 dilution; Santa Cruz Biotechnology), mouse monoclonal antibody against CD31 (1/200 dilution; DAKO), rabbit polyclonal antibodies against von Willebrand factor (1/200 dilution; DAKO), or nonimmune goat, mouse and rabbit IgG (DAKO). As for a control, human umbilical vein endothelial cells in culture were also subjected to the immunostaining with these antibodies. A chondrocytic phenotype of the cultured OA and NOR cells was confirmed by the positive immunostaining of aggrecan and type II collagen by using the mouse monoclonal antibody against human aggrecan (1/10 dilution; Abcam Limited, Cambridge, UK) and rabbit polyclonal antibodies against human type II collagen (1/20 dilution; Monosan, Am Uden, Netherlands) (data not shown).

Measurement of MMPs and TIMPs

Concentrations (ng/ml) of MMP-1, -2, -3, -7, -8, -9, and -13 and TIMP-1 and -2 secreted into the culture media of OA and NOR chondrocytes were determined according to the corresponding sandwich enzyme immunoassay systems as we have described previously.²¹ The sandwich enzyme immunoassay systems for MMP-1, -3, -8, and -13 measure both precursor and active forms of the MMPs, but those for MMP-2, -7, and -9 detect only their latent forms (proMMPs). The EIA for TIMP-1 determines the whole amount of TIMP-1 including free TIMP-1 and the complexed forms with active MMPs and proMMP-9. However, the sandwich enzyme immunoassay for TIMP-2 detects free TIMP-2 and TIMP-2 complexed with active MMPs, but not the complex with proMMP-2.

To study activation of proMMP-2 and proMMP-9, the culture media of OA chondrocytes were subjected to gelatin zymography according to our method.²² After sodium dodecyl sulfate-polyacrylamide gel electrophoresis using gelatin-containing gels, the gels were washed in 2.5% Triton X-100 to remove sodium dodecyl sulfate, incubated for 22 hours at 37°C in 50 mmol/L Tris-HCl, pH 7.4, containing 0.15 mol/L NaCl, 10 mmol/L CaCl₂, and 0.02% Na₂S₂O₃, and then stained with 0.1% Coomassie Brilliant Blue R250. Activation ratios were estimated by computer-assisted densitometric scanning.

Figure 1. RT-PCR for the expression of VEGF isoforms and their receptors (VEGFR-1, VEGFR-2, and NRP-1) in OA and NOR cartilage. Total RNA was extracted from OA and NOR cartilage and reverse-transcribed into cDNA followed by a PCR reaction using specific primer pairs for VEGF and VEGF receptors as described in Materials and Methods.

Chondrocyte Proliferation Assay

The proliferation of chondrocytes was assayed by the cell proliferation ELISA system (Amersham Pharmacia Biotech) according to the manufacture's protocol. In brief, chondrocytes were plated on 96-well culture flasks at a density of 2×10^5 cells/well in Dulbecco's modified Eagle's medium containing 0.2% lactalbumin hydrolysate for 24 hours. The culture media were replaced with the same media and treated with recombinant VEGF₁₆₅ (0, 10, 50, and 100 ng/ml) (R&D Systems) for 48 hours. After adding 5-bromo-2'-deoxyuridine (BrdU) to give a final concentration of 10 μ mol/L, the cells were incubated for additional 2 hours at 37°C. Incorporation of BrdU to the chondrocytes was detected using peroxidase-labeled anti-BrdU antibody by the immunoperoxidase method and the optical density was determined in a microplate reader (Bio-Rad Laboratories, Hercules, CA).

Statistics

Mann-Whitney *U*-test was used to compare the data of the OA and NOR samples. Simple linear regression or Spearman's rank correlation was used for analysis of relationship between different parameters recorded in this study, and one-way repeated-measures analysis of variance was used for comparison between more than three parameters.

Results

Expression of VEGF Isoforms and Their Receptors in OA and NOR Cartilage

The expression of VEGF isoforms and their receptors was examined by RT-PCR using total RNA extracted directly from OA and NOR cartilage. As shown in Figure 1, three isoforms of VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ were expressed in all of the samples from both OA and NOR cases, whereas VEGF₁₄₅ and VEGF₂₀₆ were not detected in the samples. On the other hand, mRNA expression of their receptors was recognized only in OA cartilage: VEGFR-1 (92%, 12 of 13 cases), VEGFR-2 (69%, 9 of 13 cases), and NRP-1 (100%, 13 of 13 cases). Interestingly, the samples expressing VEGFR-2 were also positive for both VEGFR-1 and NRP-1, the latter of which is a co-

Figure 2. *In situ* hybridization of VEGF in NOR and OA cartilage. Paraffin sections were reacted with digoxigenin-labeled anti-sense or sense RNA probes as described in Materials and Methods. Negligible signals are present in NOR cartilage with the anti-sense and sense probes (**A** and **B**). Note a positive signal in the OA chondrocytes (**arrows**) with the anti-sense probe (**C**), whereas only a background signal with the sense probe (**D**). Scale bar, 50 μ m.

receptor of VEGFR-2 (Figure 1). The specific amplification from the target mRNAs was confirmed by sequencing the amplified DNA products (data not shown).

In Situ Hybridization of VEGF in OA and NOR Cartilage

Cells expressing VEGF mRNA were identified by *in situ* hybridization. Chondrocytes in the superficial and transitional zones of the OA cartilage were labeled with the anti-sense RNA probe, and some clustered chondrocytes were also positive (Figure 2C). The signal in the chondrocytes of the NOR cartilage was negligible (Figure 2A). The sense probe gave only a background signal in the chondrocytes of OA (Figure 2D) and NOR cartilage (Figure 2B).

Immunolocalization of VEGF in OA and NOR Cartilage

The 44 cartilage specimens from nonosteophytic areas of OA cartilage (44 cases) had the typical OA changes such

as surface irregularities, fibrillation, and fissuring. Mankin scores of the samples ranged from 2 to 12 (5.9 ± 2.4 , mean $\pm$ SD; $n = 44$). The 12 control samples from NOR articular cartilage (12 cases) had little or no microscopic changes with Mankin scores 0 or 1 (0.6 ± 0.5 , $n = 12$). Immunohistochemistry demonstrated that VEGF localizes to the OA chondrocytes mainly in the superficial and transitional zones in 95% of the samples (42 of 44 samples) (Figure 3, B and C). The chondrocytes located in the radial zone were stained, when the cartilage had deep fissures reaching the zone. Clustered chondrocytes close to the fissures were frequently labeled (Figure 3C). When the percentage of the immunostained chondrocytes to the whole cells was calculated, $\sim 25\%$ of the total chondrocytes on average ($28.6 \pm 23.0\%$) were immunostained positively in the OA samples. VEGF staining was also found in 50% of the NOR cartilage samples (6 of 12 samples), but only a few chondrocytes in the superficial zone ($2.5 \pm 2.6\%$) were immunostained (Figure 3A). The percentage of the VEGF-positive chondrocytes was significantly higher in the OA than in the NOR cartilage ($P < 0.001$). The specificity of the VEGF immunostaining was

Figure 3. Immunolocalization of VEGF in the NOR and OA cartilage. Immunostaining was performed as described in Materials and Methods. **A:** NOR cartilage, Mankin grade 0. Only a few chondrocytes in the superficial zone (arrows) are stained. **B:** OA cartilage, Mankin grade 4. Chondrocytes in the superficial and transitional zones are stained (arrows). **C:** OA cartilage, Mankin grade 8. Clustered chondrocytes in the transitional and radial zones are stained (arrows). **D:** OA cartilage, Mankin grade 8. The cartilage was immunostained with the antibodies absorbed with the VEGF peptide. No staining is observed. Scale bar, 100 μ m.

assured, because the staining was abolished with the antibody absorbed with the neutralization peptide (Figure 3D) or no staining was observed with nonimmune rabbit IgG (data not shown). A linear correlation was found between the percentage of immunostained chondrocytes and Mankin score ($r = 0.906$, $n = 56$, $P < 0.001$) (Figure 4).

Immunostaining of VEGF Receptors in OA and NOR Cartilage and Cultured Chondrocytes

The protein expression of VEGFR-1 and VEGFR-2 in OA and NOR cartilage specimens was examined by immunohistochemistry. As shown in Figure 5, individual and clustered chondrocytes in the superficial and transitional zones of the OA cartilage were immunostained with the antibodies against VEGFR-1 (Figure 5C) and VEGFR-2 (Figure 5D), whereas NOR cartilage showed negligible staining (Figure 5, A and B). No staining was obtained with nonimmune IgG (data not shown).

In addition, cultured OA chondrocytes showed positive immunostaining for VEGFR-1 and VEGFR-2 (Figure 6, A and B), but the staining was negative with NOR chondro-

cytes (data not shown). These cultured OA chondrocytes were negatively stained with the antibodies against CD31 and von Willebrand factor or nonimmune IgG (Figure 6; C, D, and E), although human umbilical vein endothelial cells were immunostained with these antibodies (Figure 6F for von Willebrand factor and data not shown for CD31). These demonstrate that the cultured OA chondrocytes are not contaminated by endothelial cells.

Production of VEGF in OA and NOR Cartilage Explants

To measure the amounts of VEGF produced by OA and NOR cartilage, the ELISA system was used for conditioned media of the cartilage slices. The level of VEGF in culture media of OA cartilage (282.1 ± 164.0 ng/g dry weight, $n = 18$) was significantly 3.3-fold higher than that in NOR cartilage (86.8 ± 41.5 ng/g dry weight, $n = 12$; $P < 0.001$) (Figure 7A). Immunoblotting analysis for VEGF in the culture media showed that OA culture media contain positive bands of 20, 22, and 23 kd, which correspond to VEGF₁₂₁, VEGF₁₆₅ and glycosylated VEGF₁₂₁,

Figure 4. Correlation of VEGF immunostaining with Mankin score. A direct positive correlation between VEGF immunoreactivity and Mankin score is found by Spearman's rank correlation ($r = 0.906$, $n = 56$, $P < 0.001$). Open and closed circles indicate NOR and OA cartilage samples, respectively.

and glycosylated VEGF₁₆₅, respectively²³ (Figure 7B). Similar VEGF species were also observed in the culture media of OA chondrocytes (Figure 7B).

Effect of VEGF on the Proliferation of OA Chondrocytes

To study the mitogenic effect of VEGF on OA chondrocytes in culture, the proliferation assay was performed. However, there was no increase in the incorporation of BrdU by the cells treated with VEGF (10, 50, or 100 ng/ml) compared to the control untreated cells (Figure 8).

Effect of VEGF on the Production of MMPs and TIMPs in OA and NOR Chondrocytes

The effects of VEGF on the production of seven different MMPs (MMP-1, -2, -3, -7, -8, -9, and -13) and two TIMPs

(TIMP-1 and -2) by cultured OA and NOR chondrocytes were evaluated by measuring their concentrations in the media of OA and NOR chondrocytes incubated with or without VEGF. VEGF significantly enhanced in a dose-dependent manner the production levels of MMP-1 and MMP-3 in OA chondrocytes ($P < 0.05$), but not in NOR chondrocytes (Table 1). The VEGF effects on the production of MMP-2, -9, -13, TIMP-1, and TIMP-2 were not evident, and MMP-7 and MMP-8 were undetectable in the media (Table 1). By gelatin zymography, gelatinolytic bands of 92, 68, and 62 kd, which correspond to proMMP-9, proMMP-2, and active MMP-2, respectively^{22,21} were detected in the OA culture media, but neither production of these proMMPs nor activation ratio of proMMP-2 was changed by the VEGF treatment (data not shown).

Discussion

The present studies demonstrate that VEGF is synthesized by chondrocytes of both OA and NOR articular cartilage. First, RT-PCR analyses indicated that OA and NOR cartilage express the mRNA. Also, *in situ* hybridization with RNA probes for VEGF demonstrated the expression in the chondrocytes of OA cartilage. The production of VEGF protein was shown by ELISA assay in the culture media of OA and NOR cartilage, and by immunoblotting in the media of OA cartilage. In addition, immunohistochemistry demonstrated the production of VEGF in the chondrocytes of the OA and NOR cartilage.

Human VEGF mRNA is known to have five different splicing variants encoding the isoforms, ie, VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆. An important biochemical property that distinguishes the larger VEGF isoforms from VEGF₁₂₁ is their ability to bind to heparin and heparan-sulfate.^{24,25} Because of the binding capability, VEGF₁₄₅ and VEGF₁₆₅ can be localized to the cell surface and ECM, and VEGF₁₈₉ and VEGF₂₀₆ are almost completely sequestered in ECM, whereas VEGF₁₂₁ is a soluble form without binding to ECM.^{25,26} In the present study, we have demonstrated that the chondrocytes in OA and NOR cartilage express the transcripts for VEGF₁₂₁, VEGF₁₆₅ and VEGF₁₈₉. During preparation of the present article, Pufe and colleagues²⁷ have reported that VEGF is expressed in OA cartilage. They claimed that the PCR products are VEGF₁₂₁ and VEGF₁₈₉, although their sequences were not examined in the study.

Table 1. Production Levels of MMPs and TIMPs in Culture Media from OA and NOR Chondrocytes

Chondrocytes	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-13	TIMP-1	TIMP-2
OA									
VEGF, 0 ng/ml	996.7 ± 323.6	206.1 ± 86.4	35344.7 ± 15431.4	ND	ND	6.5 ± 7.0	27.8 ± 21.9	473.4 ± 131.3	7.5 ± 8.3
VEGF, 10 ng/ml	1137.0 ± 330.7*	212.0 ± 78.8	40455.7 ± 15052.8*	ND	ND	6.5 ± 7.7	30.1 ± 25.3	468.5 ± 139.6	6.7 ± 7.3
VEGF, 50 ng/ml	1240.0 ± 368.3*	213.2 ± 77.8	42477.7 ± 16070.9*	ND	ND	7.3 ± 7.9	32.3 ± 27.0	456.0 ± 121.9	6.7 ± 7.9
NOR									
VEGF, 0 ng/ml	506.4 ± 306.9	54.9 ± 28.6	16525.0 ± 5197.2	ND	ND	ND	12.9 ± 0.7	510.0 ± 126.8	32.0 ± 1.1
VEGF, 10 ng/ml	511.4 ± 318.7	54.4 ± 27.6	16616.5 ± 1732.1	ND	ND	ND	12.8 ± 1.1	527.0 ± 121.9	28.9 ± 1.5
VEGF, 50 ng/ml	539.4 ± 323.9	56.4 ± 31.3	17800.0 ± 4680.8	ND	ND	ND	14.1 ± 1.1	517.2 ± 87.8	30.9 ± 0.2

OA and NOR chondrocytes were treated with VEGF (0, 10, or 50 ng/ml) for 3 days, and MMPs and TIMPs were measured by the corresponding assay systems as described in Materials and Methods. Values (ng/ml) are the mean ± SD of three separate cultures. ND, not detectable.

*, $P < 0.05$ versus without VEGF treatment.

Figure 5. Immunolocalization of VEGFR-1 and VEGFR-2 in NOR and OA cartilage. Immunostaining of VEGFR-1 (**A** and **C**) and VEGFR-2 (**B** and **D**) was performed as described in Materials and Methods. **A** and **B**: NOR cartilage, Mankin grade 0. **C** and **D**: OA cartilage, Mankin grade 8. Note that OA chondrocytes in the transitional and radial zones are positively immunostained for VEGFR-1 (**C**, arrows) and VEGFR-2 (**D**, arrows). Scale bar, 100 μ m.

Our data are different in that besides the two isoforms, VEGF₁₆₅ was detected in both OA and NOR cartilage samples. The reason for the difference is unclear. However, it is definite that such three VEGF isoforms were expressed in our cartilage samples, because the PCR fragments showed the identical sequences to those of the isoforms.

The results of our ELISA indicated that VEGF is synthesized and secreted from the cartilage explants, although it did not provide the information of VEGF isoforms in the media. However, we could demonstrate by immunoblotting that the VEGF₁₂₁ and VEGF₁₆₅ isoforms, but not VEGF₁₈₉, are present in the culture media of both OA cartilage explants and OA chondrocytes. Thus, it seems likely that VEGF₁₈₉ protein secreted by the chondrocytes may be trapped within the ECM macromolecules of the cartilage, which include heparan-sulfate proteoglycans. VEGF is produced in the local tissues under various pathological conditions such as cancers, wound healing, ischemic myocardium, diabetic retinopathy, and RA.²⁸ In these tissues, VEGF₁₂₁ and VEGF₁₆₅ are commonly expressed, and the expression of VEGF₁₈₉ is also fre-

quently observed.²⁹ In contrast, VEGF₁₄₅ expression is restricted to the cells derived from reproductive organs,²⁶ and VEGF₂₀₆ is a rare form expressed in placental tissue.³⁰ Thus, VEGF isoforms of VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ expressed in the cartilage appear to be a common combination.

When VEGF functions as a ligand of growth factor to certain cells, the expression of its receptors is essential. Thus, we examined the expression of VEGF receptors in the cartilage by RT-PCR, and found that VEGFR-1, VEGFR-2, and NRP-1 are expressed specifically in the OA cartilage in at least 70% of the samples. By immunohistochemistry, the protein expression of VEGFR-1 and VEGFR-2 was also demonstrated in chondrocytes of OA cartilage tissue and cultured OA chondrocytes, which were not contaminated by endothelial cells. Among the receptors, NRP-1 is unique in that it acts as a co-receptor specific to VEGFR-2 and enhances the signal of VEGF₁₆₅.¹³ In our study, all of the OA cartilage samples expressing VEGFR-2 also expressed NRP-1. VEGFR-1 and VEGFR-2 were originally thought to be expressed predominantly in endothelial cells, but recent studies

Figure 6. Immunohistochemistry of VEGFR-1, VEGFR-2, CD31, and von Willebrand factor in cultured OA chondrocytes. OA chondrocytes (A–E) and human umbilical vein endothelial cells (F) were cultured on chamber slides and immunostained with antibodies against VEGFR-1 (A), VEGFR-2 (B), CD31 (C), and von Willebrand factor (D and F) or nonimmune mouse IgG (E) as described in Materials and Methods. Note that OA chondrocytes are positively stained with the antibodies to VEGFR-1 (A) and VEGFR-2 (B), but negative for CD31 (C) and von Willebrand factor (D). Human umbilical vein endothelial cells (a positive control) are stained with the antibody to von Willebrand factor (F). Scale bar, 25 μ m.

have indicated that other types of cells also express one or both of the receptors: VEGFR-1 is expressed in trophoblasts,³¹ monocytes,³² and mesangial cells,³³ and VEGFR-2 is expressed in hematopoietic stem cells, megakaryocytes,³⁴ and retinal progenitor cells.³⁵ The present data add OA chondrocytes in the list, and suggest the possible direct actions of VEGF on the articular chondrocytes in OA. The expression of VEGFR-2 and NRP-1 in OA cartilage contrasts with the finding in our previous studies that OA synovium expresses none of the molecules.⁵ The regulation mechanism of the receptor expression in OA joint tissues is not clear at the present time. However, because cartilage is under the hypoxic condition³⁶ and hypoxia is reported to be an inducer of VEGFR-1 and VEGFR-2 in endothelial cells of various origins,^{37–40} it might be possible to speculate that hypoxia is partly involved in the selective expression of the receptors in OA cartilage.

Contrary to our expectation, VEGF did not stimulate the proliferation of OA chondrocytes. Thus, we further examined the effects of VEGF on the production of MMPs and

TIMPs in cultured OA and NOR chondrocytes. The data indicated that without changing the production levels of TIMPs, VEGF stimulates OA chondrocytes to produce increased amounts of MMP-1 and MMP-3 in a dose-dependent manner. The gene expression of MMP-1 and MMP-3 is regulated by various cytokines and growth factors,⁴¹ and the enhanced expression of these MMPs by VEGF is reported with endothelial cells,^{15,17} and vascular smooth muscle cells.⁴² However, the present study is the first to demonstrate the stimulation of the MMP production by OA chondrocytes with VEGF. Because of the expression of MMP-1 and MMP-3 in OA cartilage and their proteinase activities to cartilage ECM macromolecules, these MMPs are believed to play a central role in the cartilage destruction through the degradation of cartilage ECM.⁴¹ In the present study, our immunohistochemical analyses indicated that the immunoreactivity of VEGF in the cartilage correlates directly with Mankin score, the degree of the cartilage destruction. Therefore, this observation, together with the stimulated production of the MMPs by the VEGF-treated OA chondrocytes,

Figure 7. Production of VEGF in the culture media of cartilage explants. **A:** The media were obtained from the cultured explants of OA and NOR cartilage, and VEGF was measured by the ELISA assay as described in Materials and Methods. Bars indicate mean values. *, $P < 0.001$. **B:** Culture media from OA cartilage (lane 1), NOR cartilage (lane 2), and OA chondrocytes (lane 3) were immunoblotted with antibody to VEGF as described in Materials and Methods.

suggests that VEGF contributes, at least in part, to the OA cartilage destruction through the up-regulation of MMP-1 and MMP-3 in an autocrine and/or a paracrine manner.

Among various biological actions of VEGF, the angiogenic effects on blood vessels may be the primary function of VEGF. Although NOR articular cartilage is a highly differentiated avascular tissue and prevents from being invaded by blood vessels,⁴³ the cartilage in RA and OA are frequently covered by pannus tissue, which contains numerous small blood vessels at least in the early stage.⁴⁴ Thus, the present data suggest that VEGF expressed and produced by chondrocytes is an important determinant to facilitate the endothelial cells in the pannus tissue to invade the damaged cartilage in OA and RA, in the latter of which VEGF isoforms are also produced by articular cartilage (Enomoto and Okada, unpublished data). However, one puzzling and interesting finding is that VEGF is also expressed in NOR cartilage. It may be possible that the amount of VEGF produced in NOR cartilage is too little to function as an angiogenic factor. However, another possibility is that the activity of VEGF produced in the cartilage is blocked at the local tissue. To identify the inhibitor protein of VEGF, we screened the chondrocyte cDNA library by yeast two-hybrid system using VEGF₁₆₅ as bait, and found that connective tissue growth factor (CTGF), which is expressed in chondrocytes,⁴⁵ binds to VEGF₁₆₅ and blocks its angiogenic activity.²³ In addition, we have recently demonstrated that MMPs selectively digest CTGF in the VEGF₁₆₅/CTGF complex and reactivate angiogenic activity of VEGF₁₆₅ by releasing VEGF₁₆₅ from the complex.⁴⁶ Thus, it is tempting to speculate that CTGF blocks the VEGF angiogenic activity by forming complexes in the NOR cartilage and its degradation by MMPs expressed in OA and RA cartilage reactivate VEGF activity, which enable the vascular pannus tissue to invade the articular

Figure 8. Effect of VEGF on the proliferation of OA chondrocytes. OA chondrocytes in culture were treated with VEGF₁₆₅ (0, 10, 50, or 100 ng/ml) for 48 hours and incorporation of BrdU was detected as described in Materials and Methods.

cartilage. This hypothesis, however, remains to be elucidated at the cellular and tissue levels.

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