**EXPRESSION OF VEGF ISOFORMS AND THEIR RECEPTORS IN HUMAN OSTEOARTHRITIC CARTILAGE**

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**Introduction**

Vascular endothelial growth factor (VEGF) is an angiogenic mitogen and plays a major role in the angiogenesis under physiological and pathological conditions such as normal embryonic development, wound healing and solid tumor growth. VEGF has five major isoforms (VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206), resulting from alternative splicing from a single gene. VEGF binds to its respective tyrosine kinase receptors, Flt-1 (VEGF-R1) and KDR (VEGF-R2). Neuripilin-1 is considered to function as a VEGF165 specific receptor when co-expressed with VEGF-R2. VEGF had been thought to be an endothelial-specific growth factor, mostly based on the predominant distribution of its receptors. However, attention has been recently focused on the expression of VEGF and receptors in non-endothelial tissues. It has been demonstrated that VEGF and VEGF-R2 colocalize in the hypertrophic cartilage of growth plate, and VEGF is associated with the neovascularization in hypertrophic cartilage during endochondral bone formation. To assess the possible involvement of VEGF in osteoarthritis (OA), we examined the expression of VEGF isoforms and their receptors in OA and also measured the production levels of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in OA chondrocytes stimulated with VEGF.

**Materials and Methods**

**Cartilage samples.** With approval of informed consent, non-osteophytic articular cartilage samples were obtained at arthroplasty from patients with OA. Normal (NOR) samples were also obtained from patients with femoral neck fracture. **RNA extraction and RT-PCR analyses.** Total RNA was extracted directly from the cartilage using ISOGEN (NIPPON GENE) according to the manufacturer’s protocol. RT-PCR was performed using specific primers corresponding to cDNA sequences for VEGF or its receptors. As a positive control, RT-PCR for β-actin was carried out. The fidelity of each amplified cDNA was confirmed by dye terminator cycle sequencing. **In situ hybridization.** Using the template DNA encoding human VEGF121 cDNA which was cloned in pBluescript KS vector, single-strand sense and antisense digoxigenin-labeled RNA probes were generated by in vitro transcription of the cDNA with T3 or T7 RNA polymerase using the DIG Labeling Kit (Boehringer Mannheim). Hybridization was performed at 50°C for 16 hours and followed by washing under stringent conditions. After blocking endogenous peroxidase activity and nonspecific bindings, the sections were incubated with a mouse monoclonal antibody to digoxigenin (Boehringer Mannheim), and the antibody bound to the antigen was complexed with biotinylated horse anti-(mouse IgG) IgG (Vector Laboratories). Color was developed by the avidin-biotin-peroxidase complex method.

**Immunolocalization of VEGF.** Sections from the PLP-fixed samples were treated with polyclonal antibodies to VEGF (5 µg/ml) (Santa Cruz Biotechnology) or the antibodies absorbed with the blocking peptide (Santa Cruz Biotechnology). Subsequently, the sections were incubated with goat antibodies against rabbit immunoglobulin conjugated to peroxidase-labeled dextran polymer (DAKO). Color was developed with 3, 3′-diaminobenzidine tetrahydrochloride.

**Cultures of cartilage slices and detection of VEGF.** Full-thickness cartilage slices with or without OA changes were cultured in serum-free DMEM + F12 containing 0.2% lactalbumin hydrolysate. After 3-day cultures in a 5% CO₂ incubator, supernatants and cartilage tissues were separately obtained. The cartilage tissues were weighed after lyophilization. VEGF concentrations in culture media were measured by ELISA kit (R&D). **Chondrocyte cultures.** OA chondrocytes were obtained by digestion of the minced cartilages with 0.4% (w/v) Prounase followed by 0.025% (w/v) collagenase type I (Worthington Biochemical Corporation). The isolated cells were plated on 24-well culture plates at 5x10⁵ cells/well in DMEM with 50 µg/ml ascorbic acid and 10% fetal bovine serum and cultured for 24 hours. After cells were starved of serum for 24 hours, the media were exchanged for serum-free DMEM + F12 supplemented with 50 µg/ml ascorbic acid and further incubated for 3 days with or without recombinant VEGF165 (R&D) (10, 50 ng/ml). The concentrations of MMP-1, 2, 3, 7, 8, 9, 13 and TIMP-1, 2 in the supernatants were measured by the corresponding EIA systems (Fuji Chemical Industries Ltd.). **Statistical analysis.** Mann-Whitney U test was used to compare the data of OA and NOR samples. Spearman’s rank correlation was used for analyses of relationship between parameters recorded in this study.

**Results**

mRNAs for three VEGF (VEGF121, 165, 189) isoforms were detected in all OA (n=10) and NOR (n=7) cartilage samples. The mRNA expression of VEGF receptors was recognized only in OA cartilage samples; VEGF-R1 (60%, 6/10 samples), VEGF-R2 (70%, 7/10 samples) and Neuripilin-1 (100%, 10/10 samples). By in situ hybridization, chondrocytes in the superficial and transitional zones of OA cartilage were labeled with the antisense RNA. Immunohistochemistry demonstrated that VEGF localizes to the chondrocytes, mainly in the superficial and transitional zones in 95% of the OA samples (39/41 samples). The chondrocytes located in the radial zone were stained when the cartilage had deep fissures reaching the zone. Clustered chondrocytes close to fissures were frequently labeled. When the percentage of the immunostained chondrocytes to the whole cells was calculated, 25.4 ± 20.0% of the total chondrocytes were positively immunostained in the OA samples. VEGF staining was also found in 50% of the NOR samples (6/12 cases), but only a few chondrocytes in the superficial zone (2.5 ± 2.6%) were immunostained. The percentage of the VEGF positive chondrocytes was significantly higher in the OA cartilages than in the normal samples (p < 0.0001). There was a linear correlation between degree (%) of the immunostained chondrocytes and Mankin scores (p = 0.822, p < 0.001). The level of VEGF production in the media of OA cartilage slices (166.0 ± 93.1 pg/ml/mg tissue, n=12) was significantly higher than that in NOR samples (39.1 ± 22.9 pg/ml/mg tissue, n=7) (p < 0.01). The production levels of MMP-1 and MMP-3 by cultured OA chondrocytes were upregulated by VEGF (Table), while the effects of VEGF on the other MMPs and TIMPs were not significant (data not shown).

<table>
<thead>
<tr>
<th></th>
<th>MMP-1 (ng/ml)</th>
<th>MMP-3 (ng/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>997 ± 324</td>
<td>35345 ± 15431</td>
</tr>
<tr>
<td>VEGF (10 ng/ml)</td>
<td>1173 ± 330*</td>
<td>40455 ± 15053*</td>
</tr>
<tr>
<td>VEGF (50 ng/ml)</td>
<td>1240 ± 368*</td>
<td>42478 ± 16071*</td>
</tr>
</tbody>
</table>

**Table.** Effects of VEGF on MMP production by OA chondrocytes. (*p < 0.05 vs. control)**

**Discussion**

In this study, we have demonstrated for the first time that NOR and OA cartilage express three types of VEGF isoforms (VEGF121, VEGF165, VEGF189). Since chondrocytes are highly differentiated cells and cartilage is avascular, it is unexpected that NOR and OA chondrocytes spontaneously produce VEGF, which is a strong angiogenic growth factor. The predominant production of VEGF in the OA cartilage was confirmed by the ELISA assay in the media of OA cartilage slices. VEGF immunoreactivities in the chondrocytes of the OA cartilage also showed a direct correlation with Mankin scores. In addition, VEGF receptors were expressed only in OA cartilage, and the production of MMP-1 and MMP-3 in OA chondrocytes was stimulated by VEGF. Thus, these data suggest the possibility that VEGF is involved in the destruction of OA through upregulation of MMP-1 and MMP-3 by chondrocytes in an autocrine and/or paracrine manner.

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