# Expression and Localization of Vascular Endothelial Growth Factor-C in Rheumatoid Arthritis Synovial Tissue

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*ABSTRACT.* ***Objective.*** Vascular endothelial growth factor-C (VEGF-C), a member of the VEGF family, induces lymphangiogenesis through VEGF receptor-3 (VEGFR-3/Flt-4). We examined the expression and localization of VEGF-C to clarify its role in synovial tissues in rheumatoid arthritis (RA).

***Methods.*** Reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis, immunohistochemical staining, and *in situ* hybridization for VEGF-C were performed on synovial tissue specimens obtained from 10 patients with RA and 4 with osteoarthritis (OA). VEGFR-3 expression was determined using Western blot analysis.

***Results.*** RT-PCR analysis showed that VEGF-C mRNA was expressed in all RA and OA synovial tissues. Based on Western blot analysis, the mature form of VEGF-C was found in RA synovial tissues, but not in OA synovial tissues, and VEGFR-3 was detected in RA and OA synovial tissues. Immunohistochemical staining showed that the VEGF-C protein was localized in many synovial lining cells, endothelial cells, and stromal cells in RA synovial tissues. In OA synovial tissues, the VEGF-C protein was localized in synovial lining cells and endothelial cells. A large number of synovial lining cells and stromal cells surrounding microvessels in RA synovial tissues expressed VEGF-C mRNA, as determined by *in situ* hybridization.

***Conclusion.*** Mature VEGF-C and VEGFR-3 expression may contribute to lymphangiogenesis in RA. (J Rheumatol 2002;29:34–38)

*Key Indexing Terms:*
VASCULAR ENDOTHELIAL GROWTH FACTOR-C RHEUMATOID ARTHRITIS SYNOVIUM
VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-3 OSTEOARTHRITIS

Angiogenesis, the formation of new blood vessels from vascular endothelium, is a key event in several biological processes, including wound healing and tumor growth, invasion, and metastasis[1,2]. Vascular endothelial growth factor (VEGF), which belongs to the platelet derived growth factor family, is currently known as the major inducer of angiogenesis and vascular permeability[3]. Angiogenesis and synovial cell proliferation are considered to be essential underlying processes in chronic and destructive arthritis as well as in rheumatoid arthritis (RA). We have reported that VEGF mRNA is expressed in macrophages, synovial lining cells, and fibroblasts of RA synovial tissues, and that the expression of VEGF protein in cases of RA is significantly higher than in osteoarthritis (OA). This growth factor might participate in angiogenesis in RA synovial tissues and also indirectly in synovial cell proliferation[4].

Recent studies also reported that VEGF including VEGF type B, C, D, and E are novel regulators of endothelial cell proliferation[5-11]. Cell surface receptors for VEGF-C and -D were reported to be VEGF receptor-3 (VEGFR-3/Flt-4)[12] and VEGFR-2 (Flk-1/KDR). VEGFR-2 has an affinity for VEGF in addition to VEGF-C and -D, whereas VEGFR-3 binds specifically to VEGF-C and –D[13]. VEGFR-2 is expressed on vascular endothelial cells and leads to angiogenesis[3]. On the other hand, the distribution of VEGFR-3 is highly restricted to the lymphatic endothelial cells in adult tissues. Moreover, the affinity of VEGF-C to VEGFR-2 in proliferating endothelial cells is considered to be decreased in adult tissues[14,15]. Therefore, the main receptor of VEGF-C is considered to be VEGFR-3 in adults, and the function of VEGF-C appears to extend to the lymphatic system, where it serves as a ligand for VEGFR-3. Chronic inflammatory changes including infiltration of lymphocytes, plasma cells, and macrophages are observed in RA synovial tissues. Lymphangiogenesis in RA synovial tissues, however, has not been documented.

We examined the expression and the localization of VEGF-C mRNA and its protein in RA and OA synovial tissues using reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis, immunohistochemical techniques, and

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*in situ* hybridization. Further, we examined the expression of VEGFR-3 protein in RA and OA synovial tissues using Western blot analysis.

## MATERIALS AND METHODS

*Tissue samples*. Tissue specimens were obtained from 10 patients with RA who fulfilled the diagnostic criteria of the American College of Rheumatology, with a disease duration of 10–15 years. For comparative analysis, we also obtained tissues from 4 patients with OA. After obtaining their informed consent, synovial tissue samples were obtained from patients with RA and OA during total knee joint arthroplasty. The tissue samples were frozen in liquid nitrogen and stored until use for protein and RNA extraction. For histological examination, the tissues were fixed in 4% paraformaldehyde (PFA) and then embedded in paraffin; paraffin sections were cut at a thickness of 3–4 $\mu$m. The study was approved by the Human Ethics Committee of the Nippon Medical School.

*RT-PCR*. Total RNA was extracted from synovial tissues with Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Then cDNA synthesis and PCR were performed using the Takara RNA PCR kit (Takara, Tokyo, Japan). The primer pair used for VEGF-C corresponded to nucleotides (nt) 590–609 (5′-TGT-ACA-AGT-GTC-AGC-TAA-GG-3′) and 753–772 (5′-CCA-CAT-CTA-TAC-ACA-CCT-CC-3′) of the human VEGF-C cDNA (199 bp)[6]. PCR was carried out in a Takara PCR thermal cycler MP (Takara) for 35 cycles, each consisting of 1 min at 95˚C, 1 min at 55˚C, and 2 min at 72˚C. As a positive control, expression of ß-actin mRNA was determined, and as a negative control, RNA without being reverse transcribed was used as the template for PCR.

*Immunohistochemistry*. The anti-VEGF-C polyclonal antibody (C-20) and the blocking peptide for this antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-VEGF-C antibody was an affinity purified goat polyclonal antibody mapped at an amino acid sequence corresponding to the amino terminus of the precursor form of human VEGF-C. This antibody reacts with human VEGF-C as determined by Western blotting and immunohistochemistry, but does not react with any other members of the VEGF/placenta growth factor (PlGF) family (Santa Cruz Biotechnology). Paraffin embedded sections (3 $\mu$m) were subjected to immunostaining using the streptavidin-peroxidase technique. Tissue sections were heat treated with citric acid buffer (pH 6.0) using an autoclave for 15 min at 121˚C. Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol. Tissue sections were incubated 10 min at room temperature with 10% normal rabbit serum (Vector Laboratories, Burlingame, CA, USA), and then for 16 h at 4˚C with a VEGF-C antibody (1:100 dilution) in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). Bound antibodies were detected with biotinylated rabbit anti-goat IgG (Organon Teknika, West Chester, PA, USA) and the streptavidin-peroxidase complex, using diaminobenzidine tetrahydrochloride as substrate. Sections were counterstained with Mayer's hematoxylin. An immunoabsorption test using a VEGF-C blocking peptide was performed as a negative control.

*Western blot analysis*. The VEGF-C antibody used for immunohistochemistry and the VEGFR-3 antibody (R&D Systems, Minneapolis, MN, USA) were employed for Western blotting. The anti-VEGFR-3 antibody was an affinity purified goat polyclonal antibody mapped at an amino acid sequence corresponding to the extracellular domain of human VEGFR-3. Synovial tissues were solubilized in lysis buffer containing 75 mM potassium phosphate buffer (pH 7.4), 75 mM KCl, and 100 $\mu$M Pefabloc SC (Merck, Darmstadt, Germany). Then the concentration of all samples was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). The samples (20 $\mu$g/lane) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and separated proteins were electrophoretically transferred onto an IPVF membrane (Nihon Millipore Ltd., Yonezawa, Japan). Membranes were blocked overnight with 3% skim milk in TBS (0.2 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 0.05% Tween 20 (TBST), and incubated with the anti-human VEGF-C antibody (1:10,000) or the anti-human VEGFR-3 antibody (1:20,000) for 2 h at room temperature. Membranes were then washed for 1 h with TBST, and incubated with the anti-goat IgG secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescent substrate (Pierce).

*Probe preparation*. A 199 bp BamHI-EcoRI cDNA fragment corresponding to nucleotides 581–779 of the human VEGF-C cDNA sequence was generated by PCR amplification of single stranded cDNA that was reverse transcribed from human placental RNA. The VEGF-C cDNA fragment was subcloned into the pGEM-T vector, and authenticity was confirmed by sequencing. The probes were labeled with digoxigenin-UTP by SP6 or T7 RNA polymerase using the DIG RNA labeling kit (Boehringer Mannheim, Germany).

*In situ hybridization*. *In situ* hybridization was performed as reported[16]. Tissue sections (4 $\mu$m thick) were placed on MAS coated slides (Matsunami Glass Ind, Ltd., Osaka, Japan), deparaffinized, and incubated at 23˚C for 20 min with 0.2 N HCl and at 37˚C for 15 min with 70 $\mu$g/ml proteinase K. The sections were then postfixed for 5 min in PBS containing 4% PFA, incubated briefly twice with PBS containing 2 mg/ml glycine and once in 50% (vol/vol) formamide/2× SSC for 1 h prior to the hybridization reaction initiated by adding of 100 $\mu$l of the hybridization buffer. Hybridization was performed in a moist chamber for 16 h at 45˚C, using a probe concentration of 100 ng/ml. The sections were then washed sequentially with 2× SSC for 20 min at 45˚C, and 0.2× SSC for 20 min at 45˚C. For immunological detection, the DIG nucleic acid detection kit was used. The sections were washed briefly with buffer 1 solution (100 mM Tris-HCl and 150 mM NaCl, pH 7.5) and incubated with 1% (wt/vol) blocking reagent in the same buffer 1 solution for 60 min at 23˚C. These were then incubated 30 min at 23˚C in a 1:2000 dilution of alkaline-phosphatase conjugated polyclonal sheep anti-digoxigenin Fab fragment containing 0.2% Tween 20. The sections were then washed twice for 15 min at 23˚C with buffer 1 solution containing 0.2% Tween 20 and equilibrated with buffer 3 solution (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min. The sections were incubated with a staining solution containing nitroblue tetrazolium and X-phosphate in a dark box for 1–2 h. After the reaction was stopped with TE buffer (10 mM Tris-HCl,1 mM EDTA, pH 8.0), the sections were mounted in an aqueous mounting medium (Mutoh Chemical Co., Tokyo, Japan).

## RESULTS

*RT-PCR analysis*. To investigate whether VEGF-C mRNA is expressed in synovial tissues of patients with RA, RT-PCR analysis was performed. A positive band corresponding to VEGF-C mRNA was observed at 199 base pairs in all synovial tissues from RA and OA patients and human placental tissues (Figure 1).

*Western blot analysis*. To compare the level of VEGF-C protein synthesis in RA and OA, Western blot analysis was performed. In RA synovial tissues, 4 bands corresponding to the VEGF-C protein were observed at 21, 29, 31, and 58 kDa, as reported in human gastric cancer tissues[17] (Figure 2). In contrast, only 29, 31, and 58 kDa bands were observed for OA synovium samples and the 21 kDa band corresponding to mature VEGF-C was not detected in 4 OA samples (Figure 2), and the intensities of all the bands were weaker than those for RA synovial tissues samples. VEGFR-3 was detected in RA synovial tissues at close to 60 kDa and 80 kDa, whereas it was weakly expressed in OA synovial tissues (Figure 3).

*Immunohistochemistry*. Immunohistochemical staining was performed to determine the localization of VEGF-C in RA and OA synovial tissues. VEGF-C was moderately abundant in

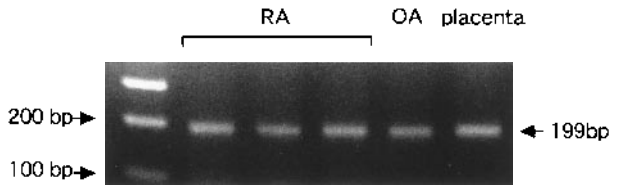


*Figure 1*. RT-PCR analysis for VEGF-C mRNA in synovial tissues from patients with RA and OA. A band of 199 bp indicating VEGF-C mRNA in 3 RA and one OA synovial tissue samples, and also in a human placental tissue (positive control).

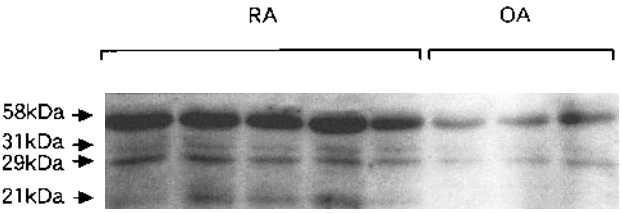


*Figure 2*. Western blot analysis for VEGF-C protein on RA and OA synovial tissues. In RA synovial tissues, 4 bands corresponding to VEGF-C protein were observed at 21, 29, 31, and 58 kDa. In OA synovial tissues, only 29, 31, and 58 kDa bands were detected.

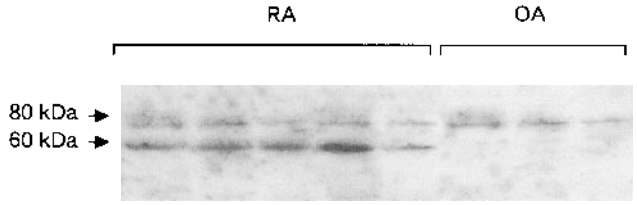


*Figure 3*. Western blot analysis for VEGFR-3 protein on RA and OA synovial tissues. The extracellular domain of VEGFR-3 protein was observed at 60 and 80 kDa in RA synovial tissues, and was weakly expressed in OA synovial tissues.

proliferated synovial lining cells (Figure 4A), endothelial cells (Figure 4B), and stromal cells in RA synovial tissues. On the other hand, in OA synovial tissues, VEGF-C was localized in a small number of synovial lining cells (Figure 4C) and endothelial cells (Figure 4C).

*In situ hybridization*. To determine which cells overexpressed VEGF-C mRNA in RA synovial tissues, *in situ* hybridization was performed. Positive signals for VEGF-C mRNA were detected in many synovial lining cells (Figure 5B) and stromal cells surrounding microvessels (Figure 5A), but not in endothelial cells. *In situ* hybridization using the sense probe did not reveal any positive signals (Figure 5C).

## DISCUSSION

VEGF-C was found to be expressed in the human heart, placenta, muscle, ovary, and small intestine[6]. There have been several studies on the relationship between VEGF-C and malignant diseases such as neuroblastoma, malignant mesothelioma, Kaposi's sarcoma, thyroid carcinoma, gastric cancer, breast cancer, and prostatic carcinoma[16-22]. These reports suggest that VEGF-C is functionally associated with diseases with lymphatic involvement and lymph node metastasis. Patients with RA show tumor-like proliferation of synovial connective tissue, which is composed primarily of fibroblast-like cells and new blood vessels[4,23].

Using immunohistochemistry, we determined the VEGF-C protein was localized on synovial lining cells, stromal cells, and vascular endothelial cells in RA synovial tissues. On the other hand, in OA synovial tissues, the VEGF-C protein was expressed weakly on synovial lining cells and stromal cells. These differences between RA and OA synovial tissues seem to lie in the predominant synthesis of the VEGF-C protein by synovial lining cells and stromal cells.

The expression and localization of VEGF-C mRNA in synovial tissues from patients with RA and OA were detected using RT-PCR and *in situ* hybridization. In RA, although VEGF-C mRNA was found to be expressed in synovial lining cells and fibroblasts surrounding microvessels, it was not detected in vascular endothelial cells. Thus, VEGF-C is particularly produced in large amounts by stromal cells surrounding microvessels and synovial lining cells in RA.

VEGF-C is synthesized as a precursor protein that requires proteolytic cleavages for full activity. During proteolytic processing, VEGF-C acquires the ability to bind to and activate VEGFR-2, and increases its affinity and activating properties toward VEGFR-3[24]. The proteolytic processing would release mature VEGF-C and/or the secreted form of VEGF-C, which are able to signal via VEGFR-3 in the extracellular step. Thus the extracellular processing introduces an additional level of regulation of the VEGF-C activity. It has been suggested that the 170 kDa glycosylated precursor of VEGFR-3 matures to the 190 kDa form, and the molecular weight of the extracellular domain is reported to be roughly 60 to 80 kDa[25].

In our Western Blot analysis, the mature VEGF-C ($M_r$ 21,000) was found in RA synovial tissues. On the other hand, it was not detected in OA synovial tissues. The extracellular domain of VEGFR-3 was more strongly expressed in RA synovial tissues than OA synovial tissues. These results suggest that VEGF-C may be proteolytically processed by several inflammatory cytokines or activating enzymes, and mature VEGF-C may have a stronger potential for lymphangiogenesis via VEGFR-3 in RA synovial tissue than in OA synovial tissue.

Recently it was reported that VEGF-C is upregulated by interleukin 1α (IL-1α), IL-1ß, or tumor necrosis factor-α (TNF-α) *in vitro*[26]. The inflamed synovium in RA produces many cytokines including IL-1, IL-6, and TNF-α, and proteolytic enzymes that promote articular destruction[27]. Considering these reports, VEGF-C may be induced by these cytokines, and may be overexpressed in the inflammatory synovial tissues as in RA, compared to OA synovial tissues.

It is believed that liquid, macromolecules, and migrating

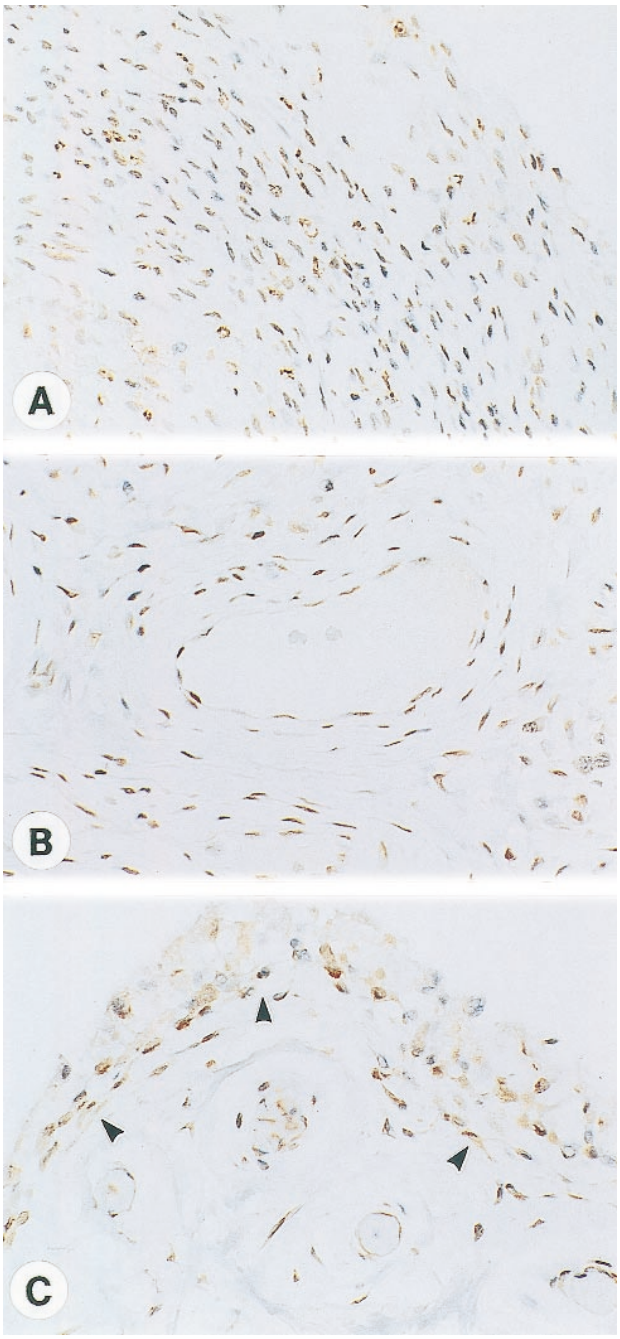


*Figure 4*. Immunohistochemical staining for VEGF-C on RA synovial tissue specimens (A, B) and OA specimens (C). VEGF-C protein was localized in synovial lining cells (A), endothelial cells (B), and stromal cells in RA synovial tissue. In OA synovial tissue, the protein was localized in synovial lining cells (C, arrowheads) and in a small number of endothelial cells (C). (A, B, C, original magnification ×400.)

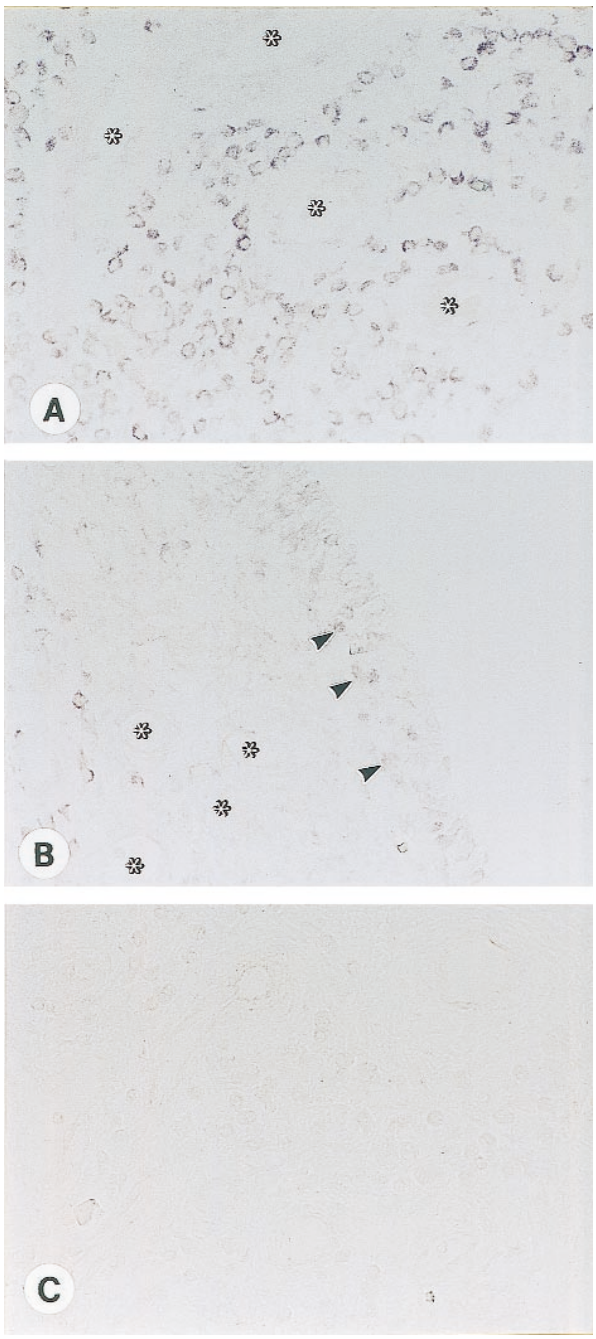


*Figure 5*. *In situ* hybridization for VEGF-C mRNA in RA synovial specimens. A, B: Positive signals are shown in stromal cells surrounding microvessels (asterisks) and synovial lining cells (arrowheads) with antisense probe. C: No positive signals are seen with sense probe. (A, B, C, original magnification ×200.)

cells pass through the blood capillary-endothelial interface, enter the tissues and are gradually absorbed into the lymphatic system, percolate through lymph nodes, and eventually return to the blood circulation via connection with the central veins[28]. On the other hand, Wilkinson, *et al* reported that lymphatic vessels were identified in normal synovium, but normal lymphatic vessels were not detected in RA synovium. They proposed that any lymphatic vessels may be activated to express markers not normally present and may also change their morphology[29]. Although we could not directly clarify lymphangiogenesis, our results may contribute to understanding lymphangiogenesis in RA.

We determined the expression and localization of VEGF-C mRNA and its protein in RA and OA synovial tissues. The mature VEGF-C and VEGFR-3 were more highly expressed in RA than in OA synovial tissues. These results suggest that expression of mature VEGF-C and VEGFR-3 may contribute to lymphangiogenesis in RA synovial tissues.


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