Splice Variants VEGF121 and VEGF165 of the Angiogenic Peptide Vascular Endothelial Cell Growth Factor Are Expressed in the Synovial Tissue of Patients with Rheumatoid Arthritis

THOMAS PUFE, WOLF PETERSEN, BERNHARD TILLMANN, and ROLF MENTLEIN

ABSTRACT. ***Objective.*** To determine the expression of the angiogenic peptide vascular endothelial growth factor (VEGF, also known as vascular permeability factor, VPF) in the synovium of patients with rheumatoid arthritis (RA).

Methods. Expression of VEGF protein from the synovial tissue of 10 patients with RA was monitored by ELISA and visualized by immunocytochemistry, and by double-staining with the VEGFR-1/flt-1. VEGF mRNA and its splice variants were determined by reverse transcriptase polymerase chain reaction (RT-PCR).

Results. VEGF protein was strongly increased in rheumatoid synovium and localized at the synovial surface, whereas the VEGF receptor flt-1 (VEGFR-1) was visualized on microvessels in close vicinity. In synovial tissues from all 10 patients with RA, $VEGF_{121}$ and $VEGF_{165}$ were identified at the mRNA level as the only VEGF splice forms expressed.

Conclusion. Since $VEGF_{165}$ and $VEGF_{121}$ are differently diffusible due to their opposite heparan sulfate-binding properties, they act at different distances. The presence of $VEGF_{121}$ may explain induction of the VEGFR-1 on infiltrating blood vessels near the synovial surface. (J Rheumatol 2001;28:1482–5)

Key Indexing Terms:
RHEUMATOID ARTHRITIS | VASCULAR ENDOTHELIAL CELL GROWTH FACTOR
SYNOVIUM | SPLICE FORMS | VEGF RECEPTOR

Vascular endothelial growth factor (VEGF; alternative term: vascular permeability factor, VPF) was originally identified as a heparin-binding angiogenic peptide secreted by tumor cells. VEGF is a selective endothelial cell mitogen, promotes angiogenesis *in vivo*, and renders the microvasculature hyperpermeable to circulating macromolecules[1,2]. In addition, VEGF is chemotactic for monocytes and is a procoagulant. In normal tissues, VEGF is expressed during embryogenesis and in a limited number of sites in adults, e.g., in the circumventricular organs. In the disease state, apart from its association with tumors, VEGF was detected in the synovial pannus in rheumatoid arthritis (RA) and in keratinocytes during wound healing[3,4].

Five different VEGF isoforms with 121, 145, 165, 189, and 206 amino acids can be generated as a result of alternative splicing from the single VEGF gene. These isoforms differ in their molecular masses and biological properties such as their ability to bind to heparin or heparan sulfate proteoglycans and to different VEGF receptors (VEGFR). The splice forms $VEGF_{121}$, $VEGF_{145}$, and $VEGF_{165}$ are secreted, whereas $VEGF_{189}$ is tightly bound to cell surface heparan sulfate and $VEGF_{206}$ is an integral membrane protein. In contrast to the other forms, $VEGF_{121}$ does not bind to heparin or extracellular matrix proteoglycans. The signaling tyrosine kinase receptors VEGFR-1 (flt-1, fms-like tyrosine kinase 1) bind $VEGF_{121}$ and $VEGF_{165}$ and VEGFR-2 (KDR, kinase domain region/flk-1, fetal liver kinase 1) as well as $VEGF_{145}$ (apart from certain VEGF related peptides). The coreceptors neuropilin 1 and 2 selectively bind the 165 residue VEGF isoform[2].

Because of these different biological activities of VEGF splice forms, it is important to know which of them are produced in a normal or disease state. The occurrence of VEGF has been repeatedly observed in human synovial fluids and synovial tissue of patients with RA[3-5], but there are no reports on the expression of the different VEGF splice variants. We investigated the expression of VEGF in the synovial tissue of patients with RA at the mRNA and protein level and then determined the prevalence of the different VEGF splice variants.

From the Department of Anatomy, University of Kiel, Kiel, Germany.

Supported in part by a grant from the "Forschungsschwerpunkt Muskel- und Skeletsystem" of the Medical Faculty of the University of Kiel.

T. Pufe, MS, Investigator; W. Petersen, MD, Orthopedist; B. Tillmann, MD, Professor, Director, Department of Anatomy; R. Mentlein, PhD, Professor, Research Group Leader.

Address reprint requests to Prof. Dr. R. Mentlein, Anatomisches Institut der Universität zu Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany.

Submitted May 10, 2000; revision accepted January 19, 2001.

MATERIALS AND METHODS

Tissues and cells. Synovial tissues were obtained from necessary synovialectomies at the Lubinus Klinik, Kiel, Germany. Those from patients with RA were from early synovialectomies, generally 1–5 years into the course of the disease (Larsen score 0–2). These patients had been treated with azulfidine (sulfasalazine) and a low dose of cortisone (5 mg/day). Healthy control samples were obtained during arthroscopies. Both types of tissue were immediately frozen in liquid nitrogen and stored at –70°C. The human glioma cell line U343 served as a positive control for VEGF production and was cultivated as described[6].

Immunologic methods. For ELISA, frozen tissue samples were crushed in an achate mortar under liquid nitrogen, homogenized in 150 mM NaCl 20 mM Tris/HCl buffer, pH 7.4, a soluble fraction was obtained by centrifugation (48,000 g, 60 min), and aliquots (100 µl) analyzed by a sandwich ELISA (R&D Systems, Minneapolis, MN, USA) that detects all VEGF splice forms. For immunohistochemistry, surgical samples were fixed in 3% paraformaldehyde, embedded in paraffin, sectioned, irradiated at 750 W in a microwave oven with 3% hydrogen peroxide in 0.01 M sodium citrate buffer, pH 6.0 (twice for 5 min), dewaxed, stained with anti-VEGF (1:40 in Tris buffered saline for 60 min; sc7269 mouse monoclonal IgG2a, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-VEGFR-1 (1:80; sc316-G goat polyclonal antibody; Santa Cruz) followed by biotinylated secondary antibodies and a peroxidase labeled streptavidin-biotin staining technique; nuclei were counterstained with hemalum. Costaining was done according to standard protocols using the Dako envision system (diaminobenzidine and Fast Red as chromogens; Dako, Glostrup, Denmark).

Reverse transcriptase-polymerase chain reaction (RT-PCR) for VEGF splice variants. For RT-PCR, frozen samples (1 g) were crushed in an achate mortar under liquid nitrogen, RNA isolated by the phenol-guanidinium thiocyanate method, purified by isopropanol and repeated ethanol precipitation, and contaminating DNA was destroyed by digestion with RNase-free DNase I (20 min, 25°C; Boehringer, Mannheim, Germany). After inactivation of DNase (15 min, 65°C), cDNA was generated with 1 µl (20 pmol) oligo $(dT)_{15}$ primer (Amersham Pharmacia Biotech, Uppsala, Sweden) and 0.8 µl superscript RNase H^- reverse transcriptase (Gibco, Paisley, UK) for 60 min at 37°C as described[6]. For PCR, 4 µl cDNA were incubated with 30.5 µl water, 4 µl 25 mM $MgCl_2$, 1 µl dNTP, 5 µl 10× PCR buffer, 0.5 µl (2.5 U) Platinum Taq DNA polymerase (Gibco), and the following primers (2.5 µl each containing 10 pmol): (1) Nonselective for all VEGF splice variants 5'-ATG-GCA-GAA-GGA-GGG-CAG-CAT-3' (sense) and 5'-TTG-GTG-AGG-TTT-GAT-CCG-CAT-CAT-3' (antisense) yielding a 255 bp fragment (40 cycles, annealing temperature 55°C); and (2) selective for VEGF splice variants 5'-CCA-TGA-ACT-TTC-TGC-TGT-CTT-3' (sense) and 5'-TCG-ATC-GTT-CTG-TAT-CAG-TCT-3'(antisense) yielding different bp fragments (40 cycles, annealing temperature 55°C[7]).

RESULTS

VEGF mRNA and protein detection in rheumatoid synovial tissue. We verified the strong increase of VEGF protein in synovial tissues from our group of patients with RA. After homogenization of surgical samples, VEGF concentrations were determined by a sensitive ELISA (Figure 1). Whereas in synovial homogenates from healthy controls, VEGF could hardly be detected, high concentrations of VEGF were measured in all samples from patients with RA. The cells producing the elevated VEGF concentrations were identified by staining synovial sections from patients with RA (Figure 2A). In all specimens investigated, VEGF immunoreactivity was predominantly visible in A and B cells near the synovial surface.

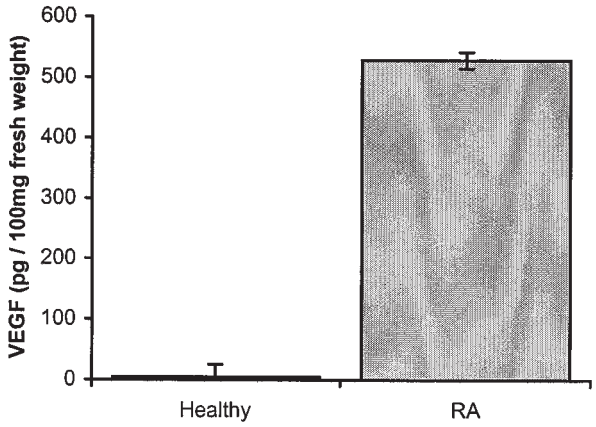

Figure 1. VEGF concentrations are strongly increased in the synovial tissue of patients with RA. Surgical samples from patients and healthy controls were homogenized in buffer, and immunoreactive VEGF determined in the homogenates by an ELISA detecting all splice variants. Mean ± standard deviation, n = 6.

Apart from the VEGF protein, its mRNA could be detected by RT-PCR nonselective for splice variants in synovial samples from patients with RA, but not in healthy controls (not shown). The PCR products from tissue from patients with RA were, however, less intense than those obtained from the same amount of RNA from a glioma cell line that served as a positive control. These experiments prove a high expression of VEGF mRNA and peptide in the synovial tissue of patients with RA.

$VEGF_{121}$ and $VEGF_{165}$ in rheumatoid synovium. In all samples from 10 patients with RA, only 2 PCR products were obtained: one with 526 bp corresponding to $VEGF_{121}$ and one with 658 bp corresponding to $VEGF_{165}$ (Figure 3). The band corresponding to $VEGF_{121}$ was consistently the strongest in samples from patients with RA. Both splice products detected in synovial tissue were also obtained with RNA from human glioma cells (not shown, positive control). However, a sample from human cartilage yielded different splice forms (Figure 3) showing that the method has the resolution necessary to detect other splice products.

Immunostaining VEGFR-1 (flt-1) on endothelial cells of microvessels in rheumatoid synovium. Endothelial cells in microvessels throughout the specimen from RA patients were heavily stained (Figure 2B). Costaining of VEGF and VEGFR-1 revealed that microvessels positive for this receptor are located in close vicinity to the VEGF-producing cells at the synovial surface (Figure 2C). The distance between the VEGF and the VEGFR-1 positive cells are mostly between 5 and 50 µm corresponding to a few cells.

DISCUSSION

Rheumatoid synovium is characterized by chronic inflammatory changes that result in the release of growth factors

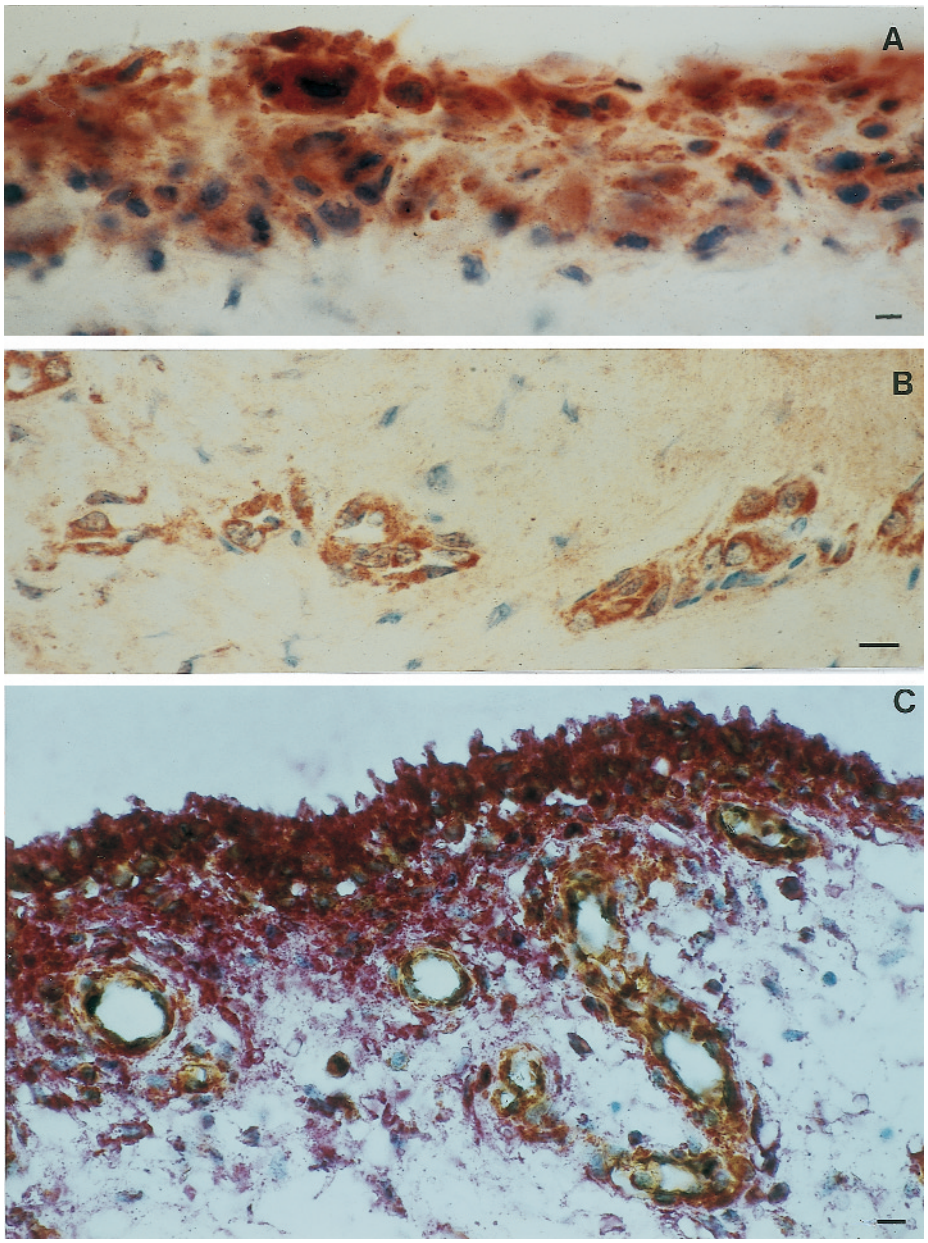

Figure 2. In rheumatoid synovial tissue, VEGF (A) can be immunostained (red) in a population of cells near the synovial surface whereas the VEGFR-1/flt-1 (B) is detected on endothelial cells of microvessels in deeper layers. (C) Costaining of VEGF (red) and VEGFR-1 (brown) shows that the ligand and its receptor are closely adjacent. Cell nuclei are counterstained with Mayer's hemalum (blue). Bars = 10 µm, original magnification 400× (A, C) or 650× (B).

and cytokines that induce proliferation of synovial lining cells and infiltration of leukocytes, fibroblasts, and blood vessels. Several angiogenic factors, including acidic and basic fibroblast growth factor, platelet derived growth factor, and VEGF are produced[3-5,8], but VEGF is the only endothelial cell-specific mitogen. As in other tissues, synovial VEGF expression is induced by hypoxia, growth factors, or inflammatory cytokines/mediators including interleukin 1 and prostaglandin E[9,10]. VEGF production in rheumatoid synovium has been confined to subsynovial macrophages, fibroblasts, vascular smooth muscle cells, and synovial lining cells[5].

The 2 splice variants, $VEGF_{165}$ and $VEGF_{121}$, were detected in rheumatoid synovium. Due to the lack of

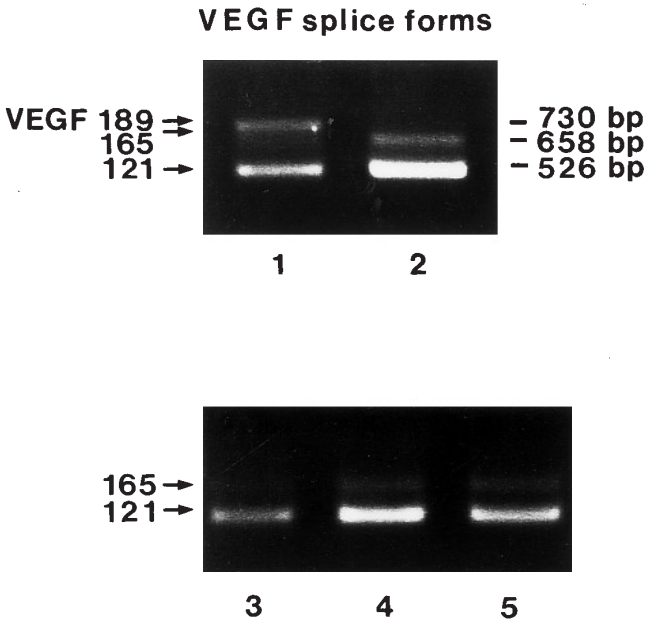

Figure 3. RT-PCR amplification with primers allowing the discrimination of VEGF splice variants detect only $VEGF_{121}$ and $VEGF_{165}$ in synovial tissue from patients with RA (Lanes 2–5), but $VEGF_{189}$ and $VEGF_{121}$ in arthritic human cartilage (Lane 1). RNA from different samples was digested with DNase, reverse transcribed, the cDNA amplified with primers annealing with the VEGF exons 1 (sense) and 8 (antisense), and PCR products were separated on 2% agarose gels stained with ethidium bromide. Two products are detectable in synovial samples: 526 bp product derives from $VEGF_{121}$ with Exons 6 and 7 spliced out, and 658 bp product is from $VEGF_{165}$ with Exon 6 spliced out. In arthritic cartilage $VEGF_{189}$ (expected size 730 bp) can be readily detected and separated from the splice forms in synovial tissue.

commercially available antibodies specific for the VEGF splice variants, we restricted our analysis to the mRNA level, which does not necessarily reflect protein expression. $VEGF_{165}$ is the predominant molecular species produced by a variety of normal and transformed cells[1,2]. However, the expression of the different VEGF splice forms is tissue dependent. In some cell types, multiple VEGF splice forms are detected; for example, human neonatal fibroblasts express $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$[11]. In nonmalignant tissue $VEGF_{145}$ appears to be expressed only in placental and uterine tissue[12]. In general, it appears that the higher residue VEGF splice forms are more frequently expressed in tumors compared to nonmalignant tissue[13].

Native $VEGF_{165}$ is a basic, heparin-binding homodimeric glycoprotein of 45 kDa, whereas $VEGF_{121}$ is a weakly acidic, dimeric polypeptide that fails to bind to heparin[1]. After secretion, $VEGF_{165}$ is restricted partly to the cell surface or to the extracellular matrix, in contrast to $VEGF_{121}$ that is freely diffusible. We localized VEGF immunoreactivity in A/B cells near the synovial surface, whereas the VEGFR-1 was visualized on microvessels in deeper layers of the synovium, but in close vicinity to the ligand (Figure 2C). VEGF splice variants may act at different distances and thus regulate the infiltration of blood vessels into different sites of the rheumatoid synovium.

ACKNOWLEDGMENT

We thank Frank Lichte, Karin Stengel, and Regine Worm for their expert technical assistance.

REFERENCES

1. Ferrara N. Molecular and biological properties of vascular endothelial growth factor. J Mol Med 1999;77:527-43.
2. Neufeld G, Cohen T, Gengrinovitch S, Poltrak Z. Vascular endothelial growth factor and its receptors. FASEB J 1999;13:9-22.
3. Fava RA, Olsen NJ, Spencer-Green G, et al. Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. J Exp Med 1994;180:341-6.
4. Koch AE, Harlow L, Haines GK, et al. Vascular endothelial growth factor: a cytokine modulating endothelial function in rheumatoid arthritis. J Immunol 1994;152:4149-56.
5. Nagashima M, Yoshino S, Ishiwata T, Asano G. Role of vascular endothelial growth factor in angiogenesis of rheumatoid arthritis. J Rheumatol 1995;22:1624-30.
6. Feindt J, Becker I, Blömer U, et al. Expression of somatostatin receptor subtypes in cultured astrocytes and gliomas. J Neurochem 1995;65:1997-2005.
7. Eckhart L, Ban J, Ballaun C, Weniger W, Tschachler E. Reverse transcription-polymerase chain reaction products of alternatively spliced mRNAs form heteroduplexes and heteroduplex complexes. J Biol Chem 1999;274:2613-5.
8. Qu Z, Huang XN, Ahmadi P, et al. Expression of basic fibroblast growth factor in synovial tissue from patients with rheumatoid arthritis and degenerative joint disease. Lab Invest 1995;73:339-46.
9. Ben-Av P, Crofford LJ, Wilder RL, Hla T. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. FEBS Lett 1995;372:83-7.
10. Jackson JR, Minton JAL, Ho ML, Winkler JD. Expression of vascular endothelial growth factor in synovial fibroblasts is induced by hypoxia and interleukin 1ß. J Rheumatol 1996;24:1253-9.
11. Pinney E, Liu K, Sheeman B, Mansbridge J. Human three-dimensional fibroblast cultures express angiogenic activity. J Cell Physiol 2000;183:74-82.
12. Burchardt T, Burchardt M, Chen MW, et al. Expression of VEGF splice variants 144/145 and 205/206 in adult male tissues. IUBMB Life 1999;48:405-8.
13. Okamoto K, Oshika Y, Fukushima Y, et al. Xenografts of human solid tumors frequently express cellular-associated isoform of vascular endothelial growth factor (VEGF) 189. Oncol Rep 1999;6:1201-4.