Clinical and Experimental Rheumatology 2001; 19: 321-324.

Vascular endothelial growth factor levels in the serum and synovial fluid of patients with rheumatoid arthritis

S.-S. Lee[1], Y.-S. Joo[2], W.-U. Kim[3], D.-J. Min[3], J.-K. Min[3], S.-H. Park[3], C.-S. Cho[3], H.-Y. Kim[3]

[1]Department of Internal Medicine, Chonnam National University Medical School, Kwangju; [2]Department of Internal Medicine, Eulji Medical College, Seoul; [3]Department of Internal Medicine, The Center for Rheumatic Diseases, Kangnam St. Mary's Hospital, The Catholic University of Korea, Seoul, Korea.

Shin-Seok Lee, MD; Yeong-Shil Joo, MD, PhD; Wan-Uk Kim, MD; Do-Jun Min, MD; Jun-Ki Min, MD, PhD; Sung-Hwan Park, MD, PhD; Chul-Soo Cho, MD, PhD; Ho-Youn Kim, MD, PhD.

This study was supported by grant no. 2000-2-21200-001-3 from the Basic Research Program of the Korea Science & Engineering Foundation.

Please address correspondence and reprint requests to: Dr. Chul-Soo Cho, Department of Internal Medicine, The Center for Rheumatic Diseases, Kangnam St. Mary's Hospital, The Catholic University of Korea, no. 505 Banpo-Dong, Seocho-Ku, Seoul, 137-040, Korea.
E-mail: chocs@cmc.cuk.ac.kr

Received on April 11, 2000; accepted in revised form on December 20, 2000.

Key words: Vascular endothelial growth factor, rheumatoid arthritis, disease activity.

ABSTRACT

Objective

To determine the vascular endothelial growth factor (VEGF) concentrations in serum and synovial fluid (SF) from patients with rheumatoid arthritis (RA) and to search for relationships between VEGF levels and clinical and laboratory variables.

Methods

We measured VEGF levels using an enzyme-linked immunosorbent assay. Serum samples were obtained from 99 RA patients, 49 osteoarthritis (OA) patients, and 80 normal controls. Paired samples of serum and SF were collected from 32 patients with RA and 15 with OA.

Results

The mean serum VEGF concentration was 590.1 pg/ml for RA patients, 286.7 pg/ml for OA patients, and 265.8 pg/ml in controls. The serum VEGF concentration was significantly higher in the RA patients than in the OA patients or the controls (both $p < 0.001$). Furthermore, the VEGF levels in SF from RA patients were significantly higher than in SF from OA patients ($p = 0.017$). However, there was no correlation between VEGF levels in serum and SF from the same RA patients. The serum VEGF concentration was correlated with the ESR, serum CRP concentration, serum rheumatoid factor, number of tender and swollen joints, Modified Health Assessment Questionnaire, and patient and physician global assessments of disease activity in RA patients.

Conclusion

These results suggest that VEGF level is related to RA disease activity, suggesting that VEGF may play some role in the pathogenesis of RA.

Introduction

Angiogenesis is not only necessary for maintaining physiologic functions, such as wound healing and corpus luteum development, but also plays an important role in the development of pathologic phenomena, such as tumor growth and pannus formation in rheumatoid arthritis (RA) (1-3). Histologically, RA is characterized by synovial cell hyperplasia, infiltration by plasma cells, lymphocytes, and macrophages, and the overgrowth of a fibrovascular granulation tissue, known as pannus, which proliferates like tumor cells and destroys cartilage and bone within the joint (4). Since angiogenesis is necessary for the continual proliferation of synovial tissue, it is believed to play an important role in the development and progression of RA. When angiogenesis inhibitors were given to rats with collagen-induced arthritis, they prevented the development of arthritis and significantly suppressed established disease, in parallel with the marked inhibition of pannus formation (5,6). Currently, more than 10 different angiogenic factors related to RA have been reported; of these, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor- (TGF-), and vascular endothelial growth factor (VEGF) are the most important (7).

Unlike the other growth factors, VEGF selectively acts on vascular endothelial cells, causing the proliferation and migration of endothelial cells to form new blood vessels. In addition, it induces malignant effusion in cancer patients by increasing vascular permeability (8). Hypoxia, which has been shown to induce VEGF expression, is present in rheumatoid joints. Many mediators, including prostaglandins E_1 and E_2 (PGE_1, PGE_2), tumor necrosis factor- (TNF-), and interleukin 1, 6, and 8 (IL-1,IL-6, IL-8), have also been implicated in the induction of angiogenesis (9-11). VEGF has been detected in synovial fluid (SF) from RA patients and VEGF mRNA expression is increased in synovial lining cells and macrophages (12, 13). Immunohistochemical staining of RA synovial tissues demonstrated that VEGF protein is localized to macrophages within the synovial lining layer, and to the endothelial cells lining small blood vessels within the pannus that are the putative target of VEGF (14). Moreover, endothelial cells near VEGF-positive macrophages expressed mRNA for both FLT1 (VEGF-receptor-1) and FLK1 (VEGF-receptor-2). These findings imply that VEGF is produced in the synovial lining cells and macrophages, and induces angiogenesis after binding

with its receptors in vascular endothelial cells.

Although VEGF is known to play a role in the proliferation of synovial tissue through angiogenesis in RA, the clinical significance of this cytokine has been little studied. Therefore, we measured VEGF levels in serum and SF from RA patients and tried to determine the clinical significance of VEGF by examining the relationship between VEGF levels and disease activity variables.

Materials and methods

Patients

Patients were recruited from the Center for Rheumatic Diseases in Kangnam St. Mary's Hospital. Patients were diagnosed with RA and osteoarthritis (OA) according to the American College of Rheumatology criteria (15, 16). People who visited our hospital for regular health check-ups and showed no abnormalities on physical examination and laboratory tests were chosen for the control group.

Serum samples were obtained from 99 patients with RA (11 men and 88 women; mean age 46.6 ± 1.3 yrs, range 21-80, mean duration of disease of 9.3 ± 0.8 years), 49 patients with OA (4 men and 45 women; mean age 59.6 ± 1.1 yrs, range 40-73), and 80 normal controls. In RA, 82% of the patients were being treated with one or more of methotrexate, hydroxychloroquine, sulfasalazine, cyclosporine, and azathioprine. Paired serum and SF samples were collected simultaneously from 32 patients with RA and 16 patients with OA to determine the relationship between the two compartments.

After obtaining blood samples from the patients and normal controls, the blood samples were left at room temperature for 1 h and centrifuged. Separated serum samples were kept at -70°C until the VEGF level was measured. SF samples were aspirated from knee joints by arthrocentesis,and also centrifuged and kept frozen until measured.

VEGF ELISA

The VEGF levels in serum and SF were measured by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates were coated with 100 l of 0.4 g/ml goat anti-human VEGF antibody (R&D Systems, Minneapolis, MN) buffered with 50 mM of sodium carbonate (pH 9.6). After incubation overnight at 4°C, the plates were blocked 1% BSA in PBS for 1 h at room temperature. The human recombinant $VEGF^{165}$ (R&D Systems) or test samples were added to the wells and then reacted with the plate for 2 h at room temperature. The plates were incubated with 0.2 g/ml biotinylated goat anti-human VEGF antibody (R&D Systems) at room temperature for 2 h. Peroxidase-labeled extravidin (Sigma, St. Louis, MN), diluted 1:1000, was added to react with the plates at room temperature for 1 h. Color reaction was induced by addition of substrate solution (TMB/H_2O_2) and was stopped by addition of 1 M phosphoric acid. An automated microplate reader (Vmax, Molecular Devices, Palo Alto,CA) was used to measure the OD at a wave length of 450 nm. Between each step, the plates were washed four times with PBS containing 0.1% Tween 20. The limit of sensitivity of the VEGF assay was 14.0 pg/ml. No significant cross-reactivity or interference was observed.

Clinical variables

When the samples were collected, each patient provided a medical history and underwent a physical examination and laboratory assessment. Clinical variables included counts of tender and swollen joints (out of 68 and 66 joints, respectively), patient and physician global assessments of disease activity (on a visual analog scale [VAS] of 0-100 mm), patient assessment of pain (VAS, 0-100 mm), a Modified Health Assessment Questionnaire (MHAQ) score (17), Westergren ESR, and C-reactive protein (CRP) and rheumatoid factor levels.

Statistical analysis

Comparisons between patient groups and healthy controls were made using the Mann-Whitney U test and Kruskal-Wallis test. Spearman's correlation coefficient was used to analyze the relationships between VEGF level and clinical variables. The relationship between serum and SF VEGF levels in RA patients was examined using the same method. Statistical analyses were performed using the SPSS software (SPSS, Chicago, IL). $p < 0.05$ was considered statistically significant.

Results

The mean serum VEGF levels were 590.1 ± 51.9 pg/ml in RA patients, 286.7 ± 30.0 in OA patients, and 265.8 ± 19.8 pg/ml in controls. Although there was no difference in the serum VEGF levels of OA patients and controls, the serum VEGF levels in RA patients were significantly higher than those in OA patients and controls (both $p < 0.001$, Fig. 1). The mean SF VEGF levels were also significantly higher in RA patients than in OA patients (3,235.2 ± 451.3 vs. 1,520.0 ± 268.3 pg/ml, $p = 0.017$, Fig. 2).

The serum VEGF levels showed significant positive correlations with the ESR, CRP, and rheumatoid factor (r =

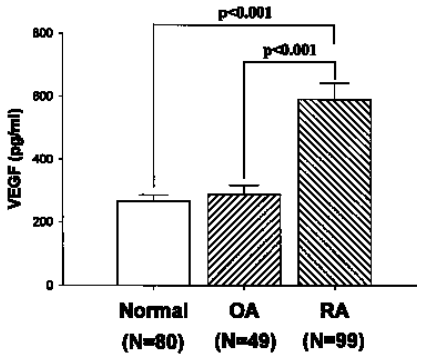

Fig. 1. Serum concentration of vascular endothelial growth factor (VEGF) in healthy controls and patients with osteoarthritis (OA) and rheumatoid arthritis (RA).

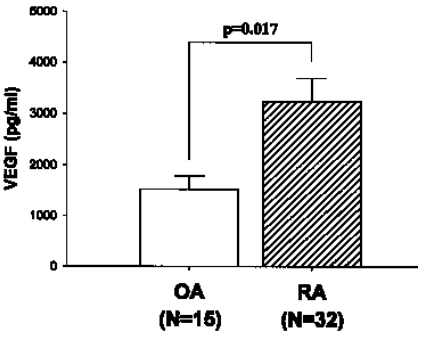

Fig. 2. Synovial fluid concentration of vascular endothelial growth factor (VEGF) in patients with osteoarthritis (OA) and rheumatoid arthritis (RA).

0.278, p = 0.012; r = 0.294, p = 0.008; r = 0.261, p = 0.02). Serum VEGF levels also showed significant correlations with counts of tender and swollen joints, MHAQ, and patient and physician global assessments of disease activity (r = 0.283,p = 0.036; r = 0.408, p = 0.002; r = 0.330, p = 0.013; r = 0.336, p = 0.011; r = 0.358, p = 0.007, Fig. 3). Patient assessment of pain was not correlated with the serum VEGF levels. In contrast to the serum VEGF, the SF VEGF levels did not show a significant correlation with any clinical variables.

VEGF levels were determined for paired serum and SF samples collected simultaneously from 32 patients with RA. The serum VEGF did not show a significant correlation with the SF VEGF (r = 0.142, p = 0.455).

Discussion

VEGF, also known as vascular permeability factor and vasculotropin, is produced by cells near endothelial cells and acts on endothelial cells via interaction with its receptors (8). Four forms of VEGF (VEGF-A, B, C, and D) exist; of these, VEGF-A is the best studied and most potent VEGF (18-20). VEGF-A is further divided into 4 types (VEGF-$A^{121, 165, 189, 206}$) by alternative splicing of mRNA. VEGF-A^{165} is the most abundant factor in the majority of cells and tissues (21). In this study, we measured VEGF levels using ELISA to detect VEGF-A^{165}.

The major role of VEGF is related to blood vessel formation. VEGF stimulates endothelial proliferation and reduces endothelial death (22). It can stimulate endothelial migration through effects on mobility, proteolysis,cell adhesion, and permeability. Another important role of VEGF is its ability to increase vascular permeability, possibly via the formation of capillary fenestrations, the assembly of vesiculo-vacuolar organelles, or by opening intercellular junctions or transcellular gaps (23). These actions cause malignant effusions in cancer patients and joint swelling in RA patients (24).

Fava *et al*. (12) and Koch *et al*. (13) showed that there was more VEGF in SF from RA compared with OA or other forms of arthritis and that the main producers of this cytokine were macrophages and synovial lining cells. Recently, Paleolog *et al*. (9) reported that serum VEGF levels were elevated in RA patients and that treatment of RA patients with anti-TNF monoclonal antibody significantly decreased serum VEGF. In our study, the serum and SF VEGF concentrations were higher in RA patients than in OA patients and normal controls. Furthermore, in paired samples, the VEGF concentration was higher in SF than in serum, suggesting that VEGF is generated in the joints. Combined, VEGF was increased at both local and systemic levels in RA patients, showing its close relationship with the disease.

The serum VEGF concentration was significantly correlated with the ESR, CRP, rheumatoid factor, numbers of tender and swollen joints, and other clinical variables. In a study of polyarticular juvenile RA patients, a significant correlation was found between serum VEGF levels and ESR, CRP, and the number of joints with active arthritis (25). Harada *et al*. (26), found that serum VEGF was correlated with CRP, but not with ESR or rheumatoid factor. The difference between the studies may be related to sample size. In this study, the serum VEGF showed the highest correlation with the number of swollen joints. Since VEGF causes malignant effusion in cancer patients, the relationship between VEGF and swollen joint counts can be explained. We cannot explain the correlation between VEGF and rheumatoid factor. In our study, a single serum sample from each patient was tested, not multiple samples collected over time. Despite these weaknesses, our study suggests that the serum VEGF level indicates the disease activity of RA.

Although SF VEGF levels were also elevated in RA patients, they were not correlated with ESR, CRP, the number of tender and swollen joints or other clinical variables. Furthermore, the SF

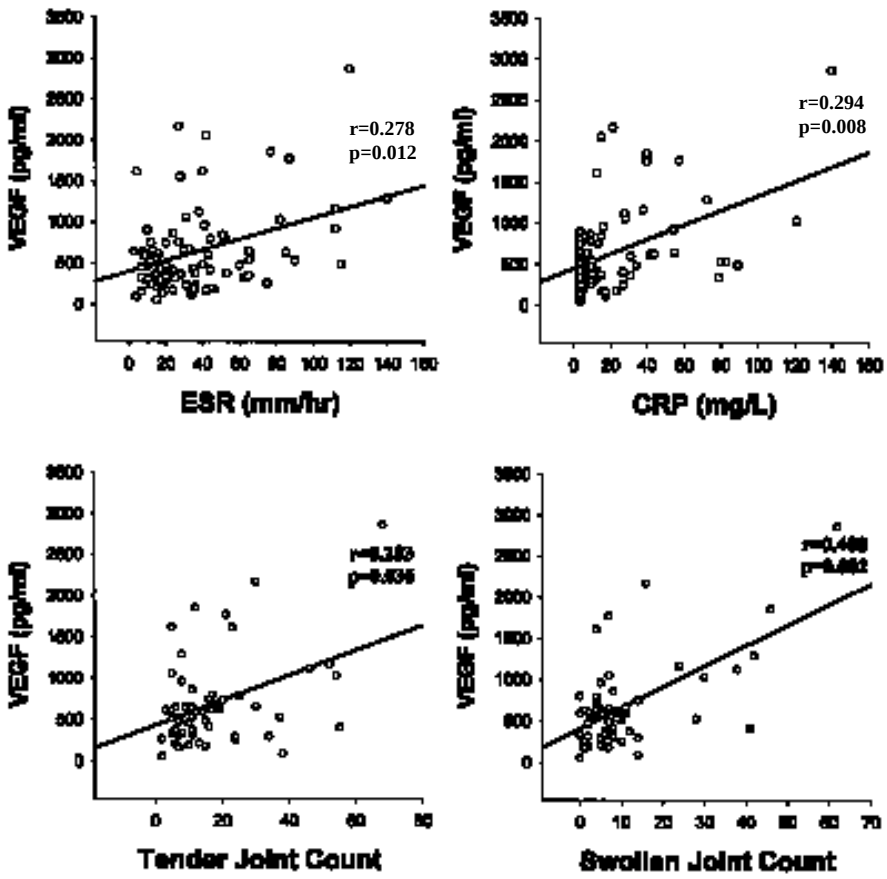

Fig. 3. Relationship between serum concentration of vascular endothelial growth factor (VEGF) and ESR, CRP, tender and swollen joint counts in patients with rheumatoid arthritis (RA).

VEGF level was not correlated with the simultaneous serum VEGF level. Therefore, SF VEGF is constantly elevated and might predict the severity of RA. If we were to measure the radiographic grade of bony erosion and this study was performed longitudinally, we could draw some conclusions about the relationship between SF VEGF and disease severity. Further investigation is needed to elucidate the role of SF VEGF.

In summary, there is a correlation between the serum VEGF level and RA disease activity. The relative ease of these assays makes measuring serum VEGF a useful laboratory method to assess disease activity. Whether the SF VEGF reflects the severity of disease requires further study.

References

1. FOLKMAN J: How is blood vessel growth regulated in normal and neoplastic tissue ? *Cancer Res* 1986; 46: 467-73.
2. COLVILLE-NASH PR, SCOTTDL: Angiogenesis and rheumatoid arthritis: pathogenic and therapeutic implications. *Ann Rheum Dis* 1992; 51: 919-25.
3. DVORAK HF, DETMAR M, CLAFFEY KP, NAGY JA, VAN DE WATER L, SENGER DR: Vascular permeability factor/vascular endothelial growth factor: An important mediator of angiogenesis in malignancy and inflammation. *Int Arch Allergy Immunol* 1995; 107: 233-5.
4. PALEOLOGEM: Angiogenesis:A critical process in the pathogenesis of RA - A role for VEGF ? *Br J Rheumatol* 1996; 35: 917-9.
5. PEACOCK DJ, BANQUERIGO ML, BRAHN E: Angiogenesis inhibition suppresses collagen arthritis. *J Exp Med* 1992; 175: 1135-8.
6. OLIVER SJ, BANQUERIGO ML, BRAHN E: Suppression of collagen-induced arthritis using an angiogenesis inhibitor, AGM-1470, and a microtubule stabilizer, taxol. *Cell Immunol* 1994; 157: 291-9.
7. KOCH AE: Angiogenesis: Implications for rheumatoid arthritis. *Arthritis Rheum* 1998; 41: 951-62.
8. CARMELIET P, COLLEN D: Vascular development and disorders:Molecular analysis and pathogenic insights. *Kidney Int* 1998; 53: 1519-49.
9. PALEOLOG EM, YOUNG S, STARK AC, MCCLOSKEY RV, FELDMANN M, MAINI RN: Modulation of angiogenic vascular endothelial growth factor by tumor necrosis factor alpha and interleukin-1 in rheumatoid arthritis. *Arthritis Rheum* 1998; 41: 1258-65.
10. 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.
11. JACKSON JR, MINTON JA, HO ML, WEI N, WINKLER JD: Expression of vascular endothelial growth factor in synovial fibroblasts is induced by hypoxia and interleukin 1beta. *J Rheumatol* 1997; 24: 1253-9.
12. 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.
13. KOCH AE, HARLOW LA, HAINES GK, *et al.*: Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. *J Immunol* 1994; 152: 4149-56.
14. 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.
15. ARNETT FC, EDWORTHY SM, BLOCH DA, et al.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
16. 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.
17. FRIES JF, SPITZ PW, YOUNG DY: The dimensions of health outcomes: The health assessment questionnaire, disability and pain scales. *J Rheumatol* 1982; 9: 789-93.
18. OLOFSSON B, PAJUSOLA K, KAIPAINEN A, *et al.*: Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci USA* 1996; 93: 2576-81.
19. JOUKOV V, PAJUSOLA K, KAIPAINEN A, *et al.*:A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996; 15: 290-8.
20. YAMADA Y, NEZU J, SHIMANE M,HIRATA Y: Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. *Genomics* 1997; 42: 483-8.
21. NEUFELD G, COHEN T, GITAY-GOREN H, *et al.*: Similarities and differences between the vascular endothelial growth factor (VEGF) splice variants. *Cancer Metastasis Rev* 1996; 15: 153-8.
22. ALON T, HEMO I, ITIN A, PE'ER J, STONE J, KESHET E: Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med* 1995; 1: 1024-8.
23. ROBERTS WG, PALADE GE: Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J Cell Sci* 1995; 108: 2369-79.
24. FENG D, NAGY JA, PYNE K, HAMMEL I, DVORAK HF, DVORAK AM: Pathways of macromolecular extravasation across microvascular endothelium in response to VPF/VEGF and other vasoactive mediators. *Microcirculation* 1999; 6: 23-44.
25. MAENO N, TAKEI S, IMANAKA H, *et al.*: Increased circulating vascular endothelial growth factor is correlated with disease activity in polyarticular juvenile rheumatoid arthritis. *J Rheumatol* 1999; 26: 2244-8.
26. HARADA M, MITSUYAMA K, YOSHIDA H, *et al.*: Vascular endothelial growth factor in patients with rheumatoid arthritis. *Scand J Rheumatol* 1998; 27: 377-80.