Tumor Necrosis Factor-α Induces Vascular Endothelial Growth Factor-C Expression in Rheumatoid Synoviocytes

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ABSTRACT. ***Objective.*** To determine the expression of vascular endothelial growth factor-C (VEGF-C) in the synovial fluid of patients with rheumatoid arthritis (RA) and to investigate the regulation of VEGF-C production by major proinflammatory cytokines in fibroblast-like synoviocytes (FLS).

Methods. The concentrations of VEGF-C, tumor necrosis factor-α (TNF-α), and interleukin 1ß (IL-1ß) were measured using an ELISA method in synovial fluids obtained from 20 patients with RA and 20 with osteoarthritis (OA). Primary cultured RA FLS were stimulated with TNF-α or IL-1ß, and the expression levels of VEGF-C mRNA and protein were assessed by quantitative real-time polymerase chain reaction and ELISA.

Results. Significantly higher levels of VEGF-C were found in RA synovial fluids compared to OA synovial fluids. VEGF-C levels showed a highly significant correlation with the levels of both TNF-α and IL-1ß in the synovial fluid of patients with RA. TNF-α stimulation significantly increased VEGF-C mRNA and protein expression in RA FLS in a dose-dependent manner. A tendency to increased expression of VEGF-C was also observed after IL-1ß stimulation in FLS.

Conclusion. Overexpression of VEGF-C in FLS by stimulation with TNF-α may play an important role in the progression of synovial inflammation and hyperplasia in RA by contributing to local lymphangiogenesis and angiogenesis. (J Rheumatol 2007;34:16–19)

Key Indexing Terms:
RHEUMATOID ARTHRITIS | VASCULAR ENDOTHELIAL GROWTH FACTOR-C
TUMOR NECROSIS FACTOR | SYNOVIOCYTES | SYNOVIAL FLUID

Although the role of angiogenesis in rheumatoid arthritis (RA) pathogenesis is now well established, the distribution and role of lymphatic vessels is less well defined[1]. Earlier studies reported few if any normal lymphatic structures in rheumatoid synovial tissues, and this defect of lymphatic vessels has been proposed as a cause of the villous proliferation of synovial tissues and the accumulation of synovial fluid[2,3]. However, a recent report indicates that numerous lymphatic vessels are present in RA synovium, and that their number is increased where there is edema and accumulation of inflammatory cells[4]. Moreover, lymphatic endothelium in RA synovium expressed high levels of chemokines[5], and this aberrant expression of chemokines might be driving the development of the lymphoid-like structures seen in the inflamed synovium. Thus, lymphangiogenesis may play an important role in the perpetuation of synovial hyperplasia and inflammation.

Vascular endothelial growth factor-C (VEGF-C), a VEGF family member, is the major lymphangiogenic factor[6]. Recent studies report that VEGF-C is overexpressed in RA synovial tissues, when compared to osteoarthritis (OA) or normal synovial tissues[7,8]. However, the inducing factors for VEGF-C production in RA synovium are not yet known. We investigated the regulation of VEGF-C production by major proinflammatory cytokines in fibroblast-like synoviocytes (FLS). We also examined the expression of VEGF-C in synovial fluid (SF) of patients with RA and OA, and correlated the levels of VEGF-C with the presence of inflammatory cytokines in SF.

MATERIALS AND METHODS

The experimental protocol was approved by Samsung Medical Center Institutional Review Board and signed informed consent form was obtained from each patient. SF was collected from the knee joints of 20 patients with RA (18 women, 2 men, mean age 56.4 yrs; range 32–70) and 20 with OA (19 women, one man, mean age 63.5 yrs; range 40–86). All patients had chronic swollen knee joints of at least 3 months' duration. Patients with RA were diagnosed according to the standards of the

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American College of Rheumatology[9]. Medications of RA patients at the time of SF aspiration were methotrexate (75%), hydroxychloroquine (65%), sulfasalazine (20%), cyclosporine (15%), low-dose prednisolone (55%), and nonsteroidal antiinflammatory drugs (95%). No patient with RA had been treated with biologic agents such as tumor necrosis factor-α (TNF-α) or interleukin 1ß (IL-1ß) inhibiting agents. Aspirated SF was centrifuged and the supernatant was stored at –20°C until the experiments were performed. FLS used for experiments were prepared from enzymatically dispersed RA synovial tissues as described[10].

Total RNA was isolated from FLS as described[10]. The VEGF-C mRNA expression level was measured by quantitative real-time polymerase chain reaction (PCR) using an ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Real-time PCR amplification was performed using the predeveloped assay-on-demand gene expression set for VEGF-C gene (Assay ID Hs00153458-m1, Gene bank accession number NM-005429, Applied Biosystems) and Human ACTB (ß-actin) Endogenous Control (VIC/MGB Probe, Applied Biosystems) in combination with the TaqMan® Universal PCR Master Mix. Quantitation of VEGF-C mRNA expression was calculated with the absolute method provided by the manufacturer (Applied Biosystems).

Synovial fluids or culture supernatants were assayed for TNF-α, IL-1ß (R&D Systems, Minneapolis, MN, USA), and VEGF-C (Bender Medsystems GmbH, Vienna, Austria) by enzyme linked immunosorbent assay (ELISA), as per the manufacturer's instructions.

Data are expressed as mean ± SEM. Relationships among variables were determined by Spearman's test. Differences between 2 groups were compared using Mann-Whitney U-test or paired Student's t test. P values less than 0.05 were considered significant.

RESULTS

Initial studies were performed to assess VEGF-C, TNF-α, and IL-1ß expressions in SF samples obtained from 20 patients with RA and 20 with OA (Figure 1). Significantly higher levels of VEGF-C were found in RA SF compared to OA SF. The levels of TNF-α and IL-1ß were also significantly increased in RA SF compared to OA SF. VEGF-C levels showed highly significant correlations with the levels of TNF-α and IL-1ß in RA SF (Figure 2). However, VEGF-C levels in OA SF did not correlate significantly with the levels of TNF-α ($r_s = 0.354$, $p = 0.13$) or IL-1ß ($r_s = 0.409$, $p = 0.08$).

We next investigated whether these proinflammatory cytokines could induce VEGF-C production in FLS (n = 4). TNF-α treatment led to significantly increased VEGF-C mRNA ($p < 0.05$) and protein ($p < 0.01$) expression, in a

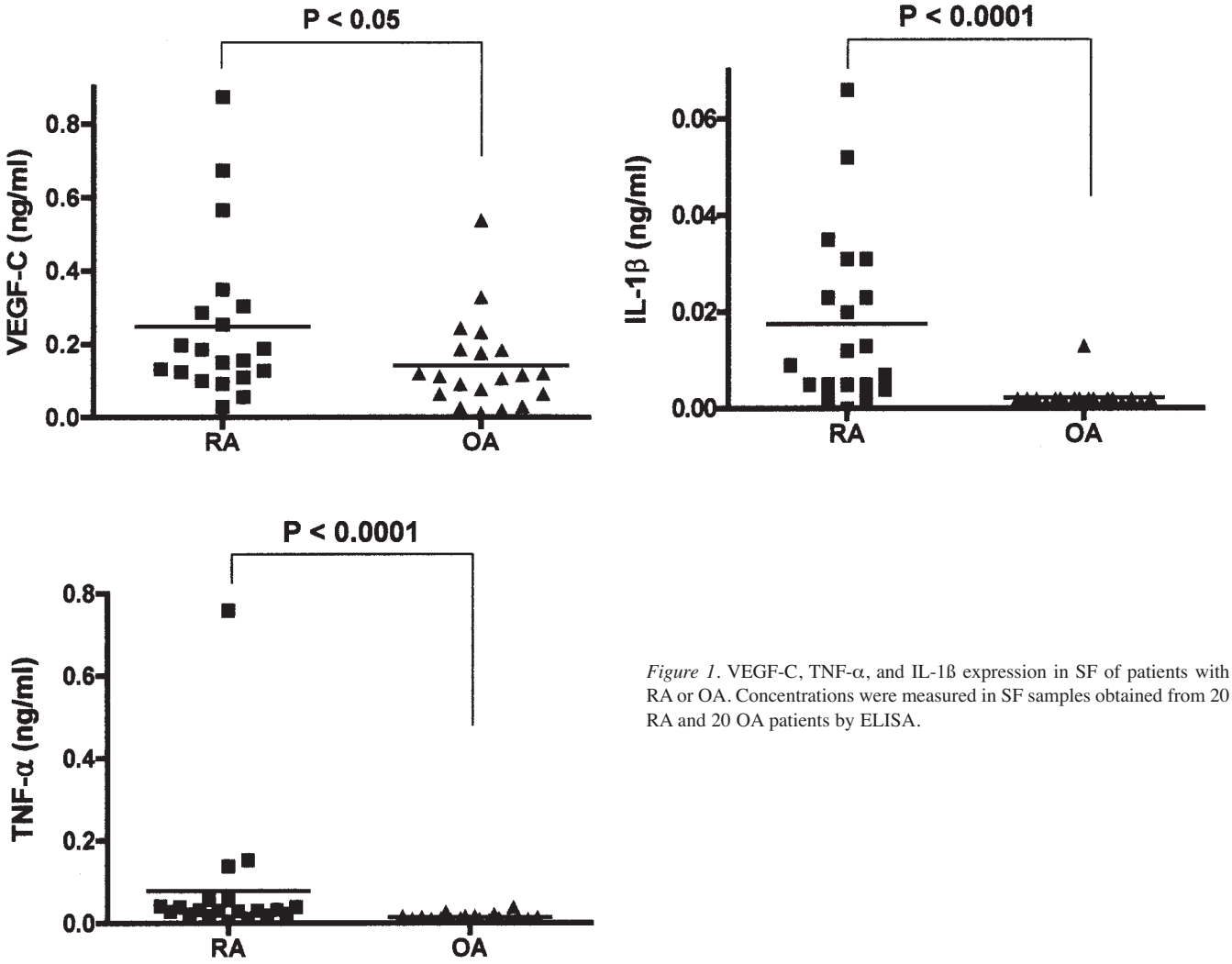

Figure 1. VEGF-C, TNF-α, and IL-1ß expression in SF of patients with RA or OA. Concentrations were measured in SF samples obtained from 20 RA and 20 OA patients by ELISA.

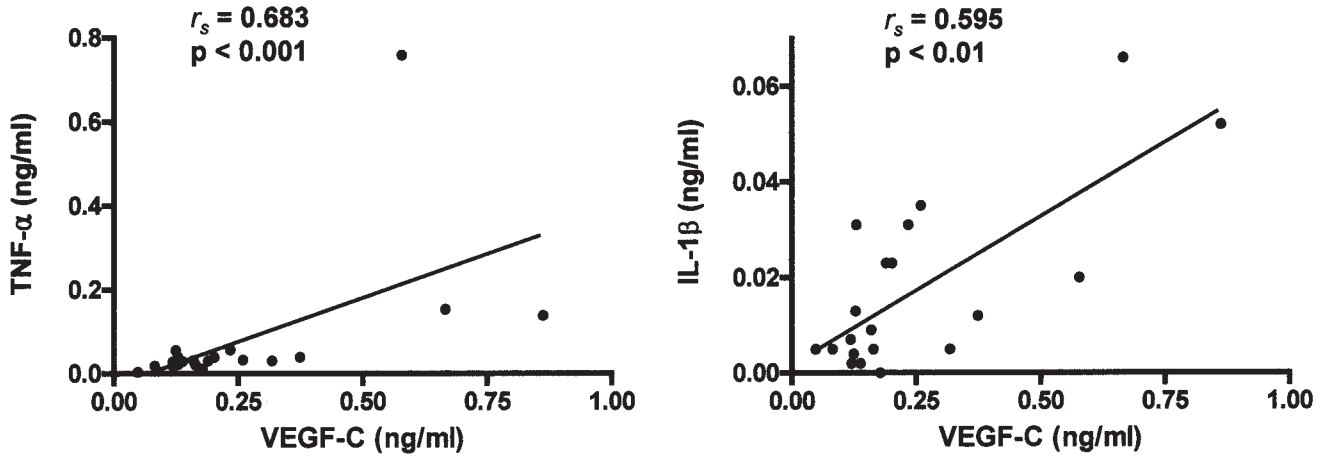

Figure 2. Correlation of VEGF-C level in RA SF with TNF-α or IL-1ß level. SF from 20 patients with RA were assayed for TNF-α, IL-1ß, and VEGF-C by ELISA. r_s: Spearman's correlation coefficient.

dose-dependent manner (Figure 3). IL-1ß did not significantly induce VEGF-C expression, although a tendency to increased expression was observed at higher IL-1ß concentration (2 ng/ml).

DISCUSSION

We found that VEGF-C levels were significantly increased in SF of patients with RA when compared to those with OA. This result is consistent with a report showing that the mature VEGF-C protein was detected in RA, but not in OA synovial tissues[8], and suggests that VEGF-C may play a role in the pathogenesis of RA. In addition, VEGF-C levels in RA SF showed a highly significant correlation with SF levels of TNF-α and IL-1ß, the central proinflammatory cytokines in RA. These results suggest that increased TNF-α and IL-1ß, which are secreted by many inflammatory cells during the disease process, might induce VEGF-C production in RA synovial tissue. Such speculation is supported by the fact that proinflammatory cytokines stimulate VEGF-A secretion by FLS[11], and that TNF-α and IL-1ß upregulate VEGF-C expression in human lung fibroblasts[12]. Further, VEGF-C colocalized with TNF-α and IL-1ß in RA synovium, especially in the synovial intimal lining area, and FLS were the major cells identified by the presence of VEGF-C[7,8]. Therefore, FLS are likely to be the primary source of VEGF-C production resulting from stimulation by proinflammatory cytokines. Indeed, in our observations, although IL-1ß did not show significant effect on VEGF-C production, TNF-α upregulated VEGF-C expression at both the transcriptional and protein level in RA FLS.

VEGF-C has been seen to promote angiogenesis in the setting of tissue ischemia[13]. VEGF-C might also contribute to angiogenesis in the synovium because its main receptor, VEGF receptor-3, was detected in most of the blood vessels in synovial tissues[7], and because it can also bind to VEGF receptor-2, which is crucial for vascular endothelial cell survival and growth[14,15]. VEGF-D also binds to VEGF receptor-2 and VEGF receptor-3, and has angiogenic and lymphangiogenic effects[6]. However, compared to VEGF-C, only a minimal amount of VEGF-D expression was detected in RA synovium[7]. Therefore, we studied VEGF-C expression only in RA SF and FLS.

Our data show that VEGF-C, which is overexpressed in RA, is inducible from FLS by stimulation with TNF-α. Increased expression of VEGF-C may play an essential role in the progression of synovial inflammation and hyperplasia by contributing to local lymphangiogenesis and angiogenesis.

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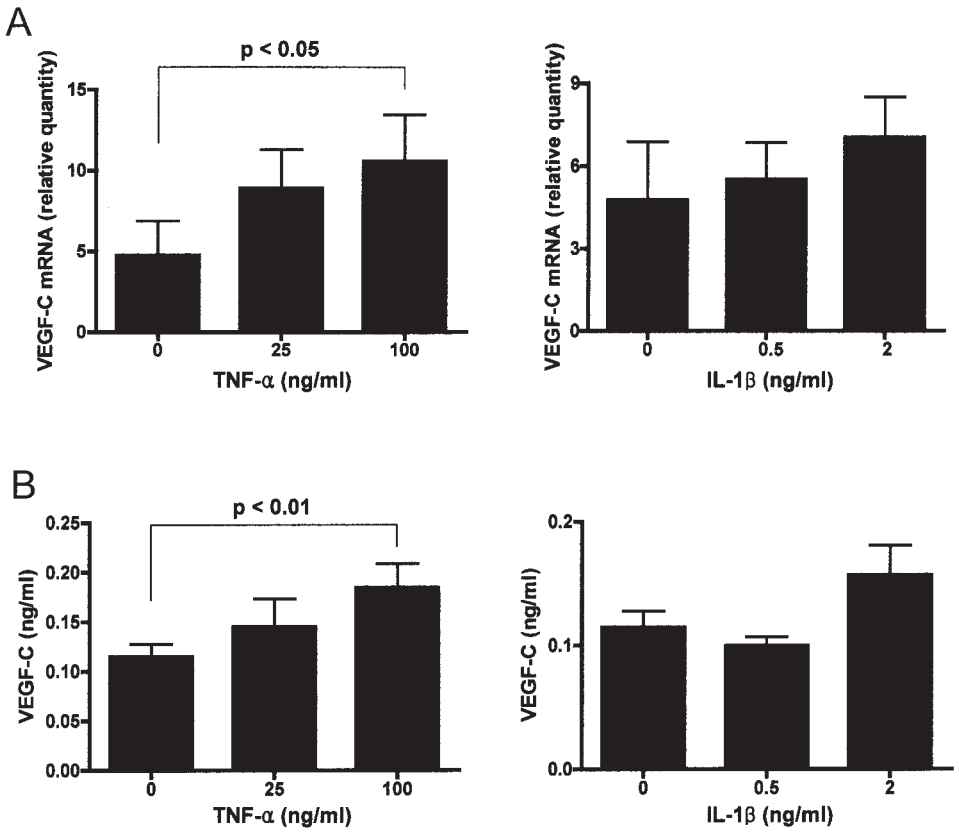

Figure 3. Induction of VEGF-C expressions by proinflammatory cytokines in RA FLS. A. RA FLS were stimulated with TNF-α (0, 25, 100 ng/ml) or IL-1ß (0, 0.5, 2 ng/ml) for 6 hours. Total RNA was extracted and quantitative real-time PCR was performed to measure VEGF-C expression (n = 4). Data are mRNA levels normalized by ß-actin mRNA expression. B. RA FLS were treated with TNF-α (0, 25, 100 ng/ml) or IL-1ß (0, 0.5, 2 ng/ml) for 18 hours. Culture supernatants were collected, and the protein level of VEGF-C was measured by ELISA (n = 4).

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