

Relationship of angiogenic factors to disease activity and radiographic damage in rheumatoid arthritis

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Abstract

Objective

To determine the association between angiogenic factor mRNA expression and disease activity and radiographic damage in patients with rheumatoid arthritis (RA).

Methods

We enrolled 42 RA patients and assessed their disease activity (DAS28) and Larsen scores. We used a semi-quantitative reverse transcriptase-polymerase chain reaction to measure levels of angiogenin, endoglin, survivin and angiomin mRNA in peripheral blood mononuclear cells (PBMCs) from 42 patients and in fibroblasts-like synoviocytes (FLS) from 14 RA patients. Then, we compared the angiogenic factor mRNA expression levels and parameters for disease activity and radiographic damage between RA patients and 42 healthy controls. We also compared the mRNA levels from FLS between 14 RA patients and 12 osteoarthritis (OA) patients.

Results

PBMCs from RA patients showed increased expression of survivin and angiomin mRNA compared to controls, while rheumatoid FLS showed increased expression for all genes tested compared to OA FLS. Angiogenin, endoglin, and angiomin mRNA levels of PBMCs did not show any significant correlation with DAS28, but the survivin mRNA level in PBMCs showed a significant positive correlation with DAS28 ($p=0.003$) and Larsen scores ($p=0.012$). Survivin was the only angiogenic factor that showed a significant association with the Larsen score.

Conclusion

The systemic and local production of angiogenic factors are increased in patients with RA and, of the genes tested in this study, survivin gene expression correlated well with disease activity and radiographic damage in patients with RA.

Key words

Rheumatoid arthritis, angiogenic factor, angiogenin, endoglin, survivin, angiomin, disease activity, radiographic damage.

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Introduction

Angiogenesis is recognized as playing an important role in the maintenance and progression of RA. This vascular process is present in early events of synovial proliferation, which can promote cartilage and bone destruction in later stages of RA (1, 2). The importance of angiogenic factors has been reported for a variety of inflammatory autoimmune conditions, with most studies focusing on vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (3, 4). The production of VEGF is increased in patients with RA (5, 6), which is correlated with the disease activity of RA, and radiographic studies have demonstrated that VEGF serum levels correlate with joint damage in RA (7, 8). Previous studies have also shown that FGF augments the proliferation of synovial fibroblasts (SF) and is involved in bone destruction in RA (9, 10). Furthermore, a recent *in vivo* study showed that the anti-angiogenic gene therapy efficiently inhibited the development of rheumatoid synovitis in collagen-induced arthritis mice (11). In this regard, there have been a number of anti-angiogenic drugs undergoing study for the treatment of RA, but several of these studies have shown serious drug-related toxicities, including an increased risk of thromboembolism, hypertension, proteinuria and gastrointestinal perforations (12), which could pose a significant limitation for use in patients with RA.

At present, the association of RA with other angiogenic factors, such as angiogenin, endoglin, survivin, and angiomin, is not clear. To better understand the role of angiogenic processes in RA, it is necessary to examine the differential expression of angiogenic factors in patients with RA. In this study, we investigated mRNA expressions of angiogenic factors such as angiogenin, endoglin, survivin, and angiomin, which might be upregulated in the process of angiogenesis observed in RA (13-16).

Subjects and methods

Study subjects and clinical assessments

Blood samples were obtained from 42 patients with RA who fulfilled the 1987

revised criteria of the American College of Rheumatology (17) and from 42 age- and sex-matched healthy blood donors with no evidence of connective tissue disease. The disease activity of patients with RA was assessed according to the 28 joint count Disease Activity Score (DAS28) (18) and radiographic joint damage was assessed using Larsen scores of hand and foot plain radiography (19). All patients with RA were treated with disease-modifying anti-rheumatic drugs (DMARDs), and 26 of them received glucocorticoids. None of the patients in the study were treated with biological therapeutics prior to sample collection. Synovial tissues for the cultures of fibroblasts-like synoviocytes (FLS) were obtained during total knee replacement surgeries from 14 female patients with longstanding RA, and control synovial tissues were obtained from 12 female patients with osteoarthritis (OA). The demographic, laboratory, and angiographic characteristics of the subjects are summarized in Table I.

This study was approved by our institutional Ethics Committee, and all study subjects provided signed informed consent.

Isolation of PBMCs and FLS

To isolate the peripheral blood mononuclear cells (PBMC), we collected venous blood in tubes containing EDTA. PBMCs were isolated from buffy coats of patients and healthy controls by density gradient centrifugation using the Ficoll-Hypaque method (Pharmacia Biotech AB, Uppsala, Sweden).

Cultures of FLS were performed as follows. Briefly, synovial tissue was rinsed with Hanks's balanced salt solution (GIBCO BRL, Grand Island, NY, USA) supplemented with an antibiotic-antimycotic solution (100 U/ml penicillin, 100 g/ml streptomycin, and 0.25 g/ml amphotericin B; Invitrogen, Merelbeke, Belgium) and digested with 2.5% type VIII collagenase (Sigma Chemical Co., St. Louis, MO, USA) in high glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL) containing 20% fetal bovine serum (FBS; GIBCO BRL) and antibiotics. After a 4-hour incubation at 37°C, cells were collected

Competing interests: none declared.

Table I. Characteristics of patients with RA, OA and controls.

Characteristics	PBMCs			FLS		
	RA patients	Controls	<i>p</i>	RA patients	OA patients	<i>p</i>
n	42	42	-	14	12	-
Age, years	39.2 ± 12.0	39.8 ± 9.8	NS	66.9 ± 6.8	68.0 ± 4.2	NS
Men / women	10/32	10 / 32	NS	0/4	0/2	NS
Disease duration, years	1.9 ± 1.5	-	-	22.0 ± 10.1	18.7 ± 4.9	NS
DAS28	4.9 ± 2.9	-	-	4.9 ± 2.9	-	-
Larsen score	11.9 ± 8.9	-	-	11.9 ± 8.9	-	-
DMARDs / GC	40/26	-	-	4/1	0/0	-

Data were analyzed by independent t-test or χ^2 test.

PBMCs: peripheral blood mononuclear cells; FLS: fibroblast-like synoviocytes; DAS: disease activity score; DMARDs: disease-modifying anti-rheumatic drugs; CS: glucocorticoids; NS: not significant.

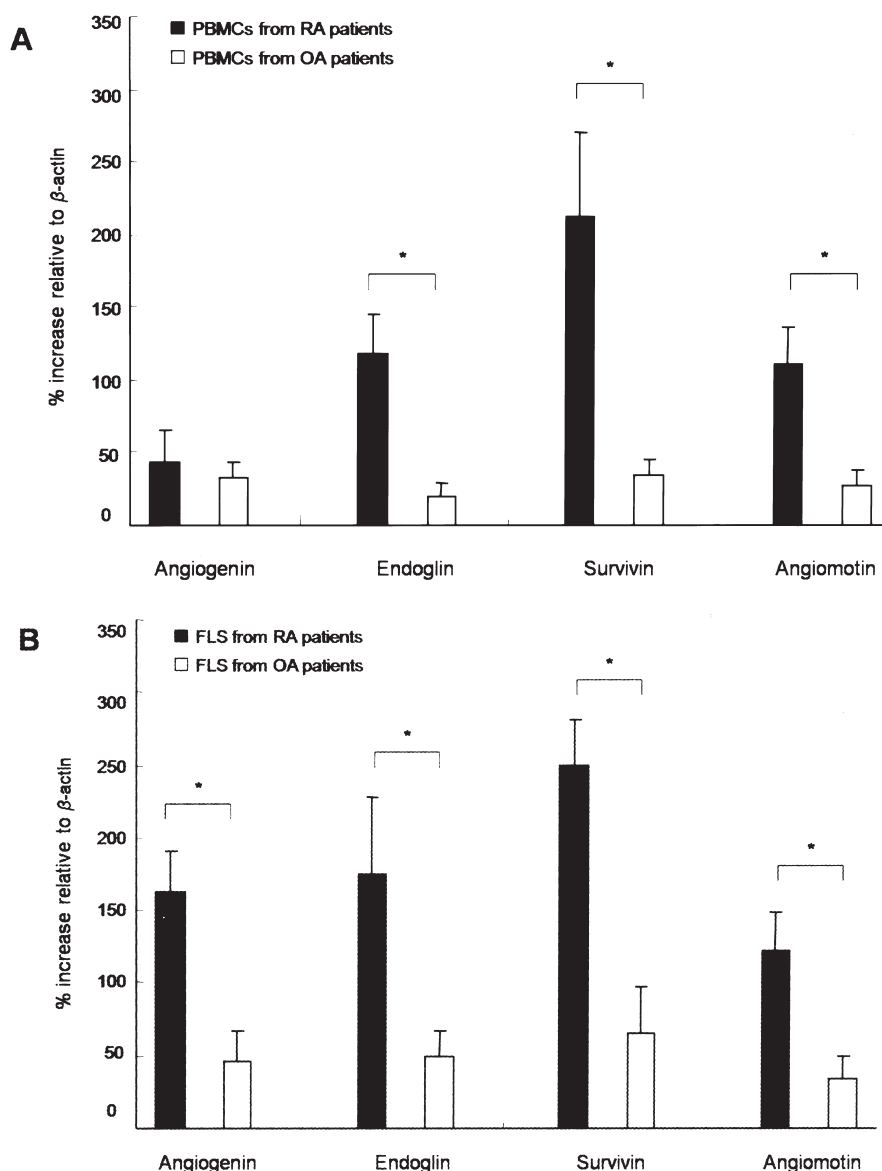


Fig. 1. Expression of angiogenin, endoglin, survivin, and angiominin mRNA in peripheral blood mononuclear cells (A) and synovial fibroblasts (B). Angiogenin mRNA level in PBMCs of RA patients was not different from that of healthy controls. There were significant increases in levels of endoglin, survivin and angiominin mRNA in PBMCs from RA patients compared to controls. In FLS, angiogenin, endoglin, survivin and angiominin were significantly higher in RA patients compared to OA patients. **p*<0.01.

by centrifugation, washed twice, resuspended in high glucose DMEM growth media supplemented with 20% FBS and antibiotics, plated in a T75 flask for passage 0, and allowed to attach for 72 hours. Non-adherent cells were removed through changing the growth media. For expansion, FLS were cultured as a monolayer in growth media at 37°C in a humidified atmosphere of 5% CO₂. The media were replaced twice a week. Upon reaching confluence, FLS were washed twice with calcium and magnesium-free phosphate buffered saline, harvested by treatment with trypsin-EDTA (0.25% trypsin, 1 mM EDTA; GIBCO BRL), and then replated in a T75 flask for passage 1. FLS were passaged in the same way with a 1:3 dilution. Population doubling time was mathematically derived by dividing the number of days in culture starting from passage 5 until 9 by the theoretical number of population doublings, assuming that after a 1:3 dilution a population tripling is required to reach confluence in a similar flask. This number was subsequently multiplied by two to determine the population doubling time.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 5×10⁶ cells, using RNeasy (Qiagen, Valencia, CA, USA), according to the manufacturer's recommendations. RNA was homogenized, using Qias shredder columns (Qiagen). The obtained RNA pellet was dissolved in 40 µl of RNase-free water and stored at -80 °C. Each sample had approximately 5 µg of total RNA, as determined by spectrophotometry. cDNA was prepared by reverse transcription of 1 µg of total RNA sample, using a cDNA synthesis kit, in accordance with the manufacturer's instructions (Bioneer, Daejeon, Korea). The sense and antisense primer pairs used were as follows: angiogenin, 5'-GTG CTG GGT CTG GGT CTG AC-3' and 5'-GGC CTT GAT GCT GCG CTT G-3'; endoglin, 5'-TGC CAC TGG ACA CAG GAT AA-3' and 5'-GAT GAG GAC GGC ATC GAG AT-3'; survivin, 5'-GGA CCA CCG CAT CTC TAC AT-3' and 5'-GCA CTT TCT TCG

Table II. Relationship of angiogenesis factor expressions PBMCs from patients with RA to disease activity and radiographic damage.

Angiogenic factors	DAS28		Larsen score	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Angiogenin	0.211	0.103	0.275	0.121
Endoglin	0.269	0.091	0.134	0.172
Survivin	0.599	0.012	0.599	0.012
Angiomotin	0.320	0.088	0.188	0.101

Values are correlation coefficients and *p*-values, calculated using Spearman's rank correlation test. DAS: disease activity score; PAF: platelet activating factor.

CAG TTC C-3'; angiomotin, 5'-CCG AGG GAC TGA ACT AGC-3' and 5'-CGA TGT GTC TCC GAT GGT -3'. The conditions for amplification were as follows: denaturation at 94 °C for 2 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 50 °C for 30 seconds, and extension at 72 °C for 1 minute. PCR was performed in 50-µl volumes, and each PCR sample underwent a 30-cycle amplification to ensure that the reactions had not reached the plateau phase of amplification. PCR products were electrophoresed on a 2% agarose gel (ImageMaster VDS; Amersham Biosciences). The intensity of each band was quantified, using the Tina 2.1 program and compared with a β-actin control (intensity=1).

Statistical analysis

All statistical analyses were performed using SPSS version 11.5 and results were expressed as the mean ± standard deviation (SD). A comparison of demographic data between patients and controls was performed using the independent t-test or chi-squared test, and the intensity of a given mRNA relative to the β-actin density in each sample was compared using the Mann-Whitney U-test. The Spearman's rank correlation test was used for correlations. A *p*-value of less than 0.05 was considered statistically significant.

Results

Gene expression of angiogenic factors

We examined mRNA expression levels of angiogenin, endoglin, survivin, and angiomotin compared to that of β-actin in PBMCs from patients with RA

and healthy controls, and the results are shown in Figure 1A. The mRNA expression level of angiogenin in PBMCs of patients with RA was not different from that of healthy controls (43.0±22.0% vs. 32.6±11.7%, respectively). But mRNA levels of endoglin (118.4±27.0% vs. 19.9±9.7%, *p*<0.01), survivin (211.7±58.9% vs. 35.2±10.3%, *p*<0.01) and angiomotin (111.5±23.7% vs. 26.5±11.2%, *p*<0.01) in PBMCs of patients with RA were significantly increased compared with those of controls.

We next compared the mRNA expression of angiogenic factors between FLS from patients with RA and patients with OA (Fig. 1B). The mRNA levels of angiogenin (163.5±27.4% vs. 46.4±21.0%, *p*<0.01), endoglin (174.2±53.6% vs. 49.0±18.9%, *p*<0.05), survivin (249.2±31.1% vs. 66.2±30.5%, *p*<0.01) and angiomotin (121.6±27.4% vs. 33.6±15.2%, *p*<0.01) were significantly higher in RA patients compared to OA patients.

Relationship to disease activity and radiographic damage

The correlations between angiogenic factor expression levels in PBMCs from patient with RA and clinical parameters for disease activity (DAS28 score) and radiographic damage (Larsen score) were evaluated, and the results are shown in Table II. The mRNA levels of angiogenin, endoglin, and angiomotin did not show any significant correlation with DAS28, but the level of survivin gene expression showed a significant positive correlation with DAS28 (*r*=0.662, *p*=0.003). Survivin mRNA level also correlated well with Larsen scores (*r*=0.599, *p*=0.012), but

there were no significant correlations between the expressions of other angiogenic factors with the Larsen score. The use of DMARDs and glucocorticoids was not significantly associated with expression of angiogenesis factors (data not shown).

Discussion

In this study, we determined the systemic and local expression of angiogenic factors in RA and found that endoglin, survivin, and angiomotin gene expressions are significantly increased in both PBMCs and FLS from RA patients. Angiogenin gene expression is increased in rheumatoid FLS, but there is no evidence of systemic upregulation in this study. Liote et al reported a similar result showing that the production of angiogenin in synovial fluids from patients with RA in remission was similar to those from patients with OA, and levels decreased with the resolution of acute gout (20). Along with our observations, this result suggests a local and nonspecific role of angiogenin in joints exhibiting inflammation.

On the other hand, we also found increased expression of endoglin and angiomotin mRNA in both PBMCs and SFs from RA patients, but there was no correlation between the gene expression and disease activity or radiographic damage. Endoglin, which is present at high levels in human endothelial cells of small vessels in various tissues (14), has been shown to be upregulated in myeloid elements and the endothelium of rheumatoid synovial tissue (21). However, endoglin expression is also increased in OA compared to normal synovial tissue lining cells (21), suggesting it may function in OA and normal synovial tissue endothelia. Angiomotin, which was recently identified for its ability to bind to angiostatin using a yeast two-hybrid screen (22), induces migration and tubule formation from endothelial cells and promotes angiogenesis (16). Similar to results for endoglin, there was a lack of correlation between angiomotin levels and clinical variables for RA in this study. This result suggests that not all angiogenic mediators are strongly related to pathogenesis of RA, and some may

play a role in the physiology of normal synovium or endothelium.

Suppression of apoptosis has been suggested as a key mechanism supporting selection and accumulation of distinct lymphocyte subsets in chronically inflamed joint tissues (23). Survivin, along with its potent angiogenic activity, is known as a multipotent inhibitor of apoptosis, neutralizing several caspases at the final steps of the apoptosis cascade (15). The results from this study provide evidence that survivin may promote synovial proliferation by stimulating angiogenesis. Previous studies have shown a pathogenic role of survivin in RA and other autoimmune diseases (24, 25). Bokarewa *et al.* reported significantly increased plasma and synovial fluid survivin levels in patients with destructive RA as compared with in patients with non-erosive disease. They also found that anti-rheumatic treatment using DMARDs significantly lowered survivin levels, suggesting a strong association with disease activity of the disease (24). We also that survivin is highly expressed in PBMCs and FLS from RA patients and further, that the level of survivin gene expression is correlated with disease activity and radiographic damage of RA. These data, together with the report that survivin enhances angiogenesis and inhibits apoptosis (15), suggests that survivin is linked to the angiogenic and pro-inflammatory nature of RA. However, additional work utilizing a more precise quantification method is necessary to determine if survivin is a reliable surrogate marker for angiogenesis.

In this study, we investigated the gene expressions of various angiogenic factors in RA and found their correlations with the validated clinical criteria for disease activity and radiographic damage, such as DAS28 and Larsen score. However, our study has several limitations. First, we used semi-quantitative reverse transcriptase PCR for mRNA expression assay. Although the results are able to show the relative increases of given genes compared to that of housekeeping gene, a more precise quantification would yield more accurate information on the expressions

of tested genes. Second, only mRNAs have been tested in both PBMCs and FLS. The protein productions of these angiogenesis factors may vary from the findings on of mRNA expressions due to specific posttranscriptional regulation and secretion. And additional histologic localization for their expressions could show more valuable information on the pathogenic role of these factors. Further investigation including precise protein quantification and localization would provide their correlations with clinical variables and their causative role in RA.

The majority of novel therapeutic strategies that are currently being tested and introduced into clinical practice target distinct molecules and mechanisms of disease within the inflamed joints of patients with arthritis. Thus, it is of utmost interest to precisely identify the appropriate target molecules. We found a significant increase in the systemic and local expression of the survivin gene and identified a strong association with disease activity and radiographic damage in RA patients. This finding suggests that survivin plays an important role in the pathogenesis of RA and is a novel therapeutic target for the treatment of RA.

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