Angiopoietins, Growth Factors, and Vascular Morphology in Early Arthritis

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ABSTRACT. *Objective.* To examine angiogenic growth factors in patients with early, untreated inflammatory arthritides and controls.

Methods. Synovial membrane (SM) infiltrate and Ang1, Ang2, and vascular endothelial growth factor (VEGF) mRNA and protein expression were examined using immunohistochemistry and *in situ* hybridization. Synovial fluid (SF) VEGF, transforming growth factor-ß (TGF-ß1), and tumor necrosis factor-α (TNF-α) protein were measured by ELISA. Vascular morphology was assessed at arthroscopy.

Results. Ang2 mRNA and protein expression was observed in early psoriatic arthritis (PsA) and rheumatoid arthritis (RA) SM. Expression of Ang2 and VEGF was significantly greater in early PsA SM and correlated strongly. SF VEGF and TGF-ß1 concentrations were also significantly higher in early PsA compared to RA. Distinct vascular morphology, with tortuous vessels in PsA, correlated with microscopic vascular scores (r = 0.54, p = 0.005) and VEGF levels (r = 0.51, p = 0.01). Ang1 mRNA and protein expression was observed, but concentrations were markedly lower than for Ang2 and VEGF. Clinical disease activity, SM infiltration, and SF TNF-α concentrations were similar in both groups.

Conclusion. This is the first report of angiopoietin expression in early inflammatory arthritis. There is a close relationship between angiopoietins, VEGF, TGF-ß, and vascular morphology. There is differential angiogenesis at an early stage of inflammation, with major pathogenic and therapeutic implications. (J Rheumatol 2003;30:260–8)

Key Indexing Terms:
ANGIOPOIETINS
VASCULAR MORPHOLOGY
VASCULAR ENDOTHELIAL GROWTH FACTOR
EARLY ARTHRITIS

Angiogenesis is the formation of new vessels by sprouting of capillaries from existing vessels[1-3]. It is usually inactive in adults except in the female reproductive process and under pathologic conditions such as in diabetes, cancer, and inflammatory arthritides[4-7]. Angiogenesis is tightly controlled by a balance of pro- and antiangiogenic stimuli, which promote/inhibit generation and proliferation of new endothelium cells (EC)[7-14]. Key angiogenic factors include vascular endothelial growth factor (VEFG), transforming growth factor (TGF-ß), fibroblast growth factors (FGF-1 and -2), platelet derived growth factor (PDGF), tumor necrosis factor (TNF-α), interleukin 8 (IL-8) and IL-12, and the chemokine–interferon inducible protein 10. EC proliferation is mediated

through the expression of a specific tyrosine kinase receptor, Tie 2, which is bound by a novel family of proteins, the angiopoietins (Ang), that are critical in the formation of vasculature[15-19]. Binding of Ang1 is thought to induce stabilization of maturing vessels, whereas Ang2 is thought to destabilize the vessel, preparing for new sprout formation[5,15-19]. These data suggest that the relationship of Ang2 and VEGF, in particular, may be critical in determining blood vessel morphology.

Angiogenesis is fundamental in inflammatory arthritis, of which rheumatoid (RA) and psoriatic arthritis (PsA) are the most common clinical types[20]; however, they have distinct pathogenic features[21-24]. RA synovial membrane (SM) is rich in blood vessels[25], and numerous factors have been implicated in increased SM vascularization including VEGF, TGF-ß, and FGF[7]. Expression of VEGF, TGF-ß, and their receptors has been described in RA peripheral blood, synovial fluid (SF), and SM[7,12,26-30]; however, all previous reports studied patients with long established disease, often after treatment such as steroids.

In contrast, little is known about angiogenesis in PsA — studies have described distinct micro- and macroscopic vascular changes in PsA SM, suggesting a possible basis for a different pathogenesis than RA[21-24]. The most striking difference appears at macroscopic assessment, which we recently described with distinct blood vessel morphology[23]. Micro-

scopically, there are increased blood vessel numbers and differential adhesion molecule expression in PsA SM and psoriasis skin[22,31-36]. Macroscopic vascular morphology and increased expression of growth factors have also been describd in the skin[36] and the nail-fold capillaries[37] of patients with psoriasis.

Studies have examined angiogenesis and growth factors in the SM of patients with long-standing established RA who have already received disease modifying therapy[7,10,12,13,38-40]. To date, angiopoietins have not been described in RA or PsA SM tissue. Recent evidence from knockout mice and *in vitro* mechanistic studies using animal tissue and/or cell lines suggests that the agonist effects of angiopoietins and VEGF are fundamental to vessel structure and function[18,19,41-44]. Thus we examined expression of the novel angiogenic factors Ang1 and Ang2 in addition to VEGF and TGF-ß and macrovascular morphology at a very early stage of human disease, before immunomodulatory therapy, in patients with PsA and RA and in disease controls.

MATERIALS AND METHODS
Twenty-six consecutive patients (RA, n = 14; PsA, n = 12) with early arthritis (< 12 mo since onset of symptoms) and active knee involvement (defined as current pain, swelling, and/or heat) were recruited from the outpatient clinic. All patients gave informed consent, and the procedure was approved by the local ethics committee. Patients with RA and PsA were matched for disease activity including Health Assessment Questionnaire (HAQ), C-reactive protein (CRP), and swollen joint count (SJC). No patient had received a disease modifying antirheumatic (DMARD) or corticosteroid drug at any time prior to the study. Diagnosis of RA was based on the 1987 American College of Rheumatology criteria[45], for PsA the criteria included inflammatory arthritis, psoriasis, and negative rheumatoid factor. Patients underwent arthroscopy under local anesthesia; a 2.7 mm diameter needle arthroscope was inserted into the knee joint, SF was aspirated, and SM biopsies were obtained under full visualization, a technique that is safe and well tolerated[46,47]. SF and SM were obtained from patients with knee osteoarthritis (OA) who had established radiographic changes and negative serology (n = 12). SF was also obtained from patients with long-standing disease (n = 21; 13 RA, 8 PsA) with paired SM (n = 10) with active knee involvement (> 18 mo) who served as disease controls.

Arthroscopy macroscopic analysis. The vascularity of the SM was assessed macroscopically using a visual analog scale (VAS: 0–100 mm) for the intensity of vascularization, by 2 blinded observers using a validated method[23,48]. Vascular morphology was defined at arthroscopy by the pattern of blood vessels, with tortuous, bushy vessels = 1 or straight branching vessels = 0, as described[23].

Microscopic analysis: immunohistochemistry. SM biopsies were snap frozen in liquid nitrogen, wrapped in foil, and stored at –70°C. Cryostat sections 6 µm thick were cut and dried overnight at 37°C. Sections were fixed in acetone for 10 min. Before staining, endogenous peroxidase was quenched by treatment with 3% H_2O_2 for 5 min, followed by a pretreatment in 3% normal serum for 30 min. A routine 3 stage immunoperoxidase staining technique incorporating avidin-biotin-immunoperoxidase complex (ABC) was used. Sections were incubated 1 h at 37°C with goat polyclonal Ang1, Ang2, and a mouse monoclonal VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To examine synovial infiltration, SM sections were stained with specific monoclonal antibodies (Mab) for CD3, CD4, and CD68 as T cell and macrophage markers (Dako, Glostrup, Denmark) for 1 h at 37°C. SM sections were also incubated with an irrelevant isotype matched Mab as a negative control. Color was developed in solution containing diaminobenzidine

tetrahydrochloride (Sigma), 0.5% H_2O_2 in Tris buffer without saline (pH 7.6). Slides were counterstained with hemotoxylin and mounted. Cells were assessed by 2 blinded observers using an established and validated[49,50] semiquantitative scoring method (0–4 scale), where 0 = no stained cells, 1 = < 24% stained cells, 2 = 25–50% stained cells, 3 = 50–75% stained cells, and 4 = 75–100% stained cells. Lining layer thickness was also measured as the number of cells in depth at 3 points in each high powered field, and expressed as the mean value.

In situ hybridization. SM biopsies were embedded in paraffin wax. DNA probes for VEGF, Ang1, and Ang2 (in-house) were *in vitro* transcribed using DIG RNA labeling mix (Roche Biochemicals, East Sussex, UK) according to the protocol provided by Roche Biochemicals. All solutions were DMPC treated and the glassware baked at 200°C overnight. The sections were dewaxed and rehydrated through a graded series of ethanol (100–30%). They were then washed in PBS, incubated in PBS containing 0.3% Triton X-100 (15 min), and permeabilized in 20 mg/ml Proteinase K solution in PBS and acetylated in 0.1 M triethanolamine buffer containing 10 ng DIG labelled probe in 3× SSC, 25% deionized formamide, 0.1% polyvinylpyrolidine, 0.1% Ficoll, 1% bovine serum albumin; 250 mg/ml salmon sperm DNA and 250 mg/ml yeast t-RNA was applied to the appropriate sections, which were then covered with cover slips and hybridized in a humid chamber at 42°C overnight. Cover slips were removed in 4× SSC and sections washed twice in 3× SSC and finally washed in 0.1 M Tris, 0.15 M NaCl buffer, pH 7.5. Immunostaining was performed using sheep anti-DIG antibody and visualization using NBCBCIP with 10 mM levamisole. Tumor tissue was used as a positive control. Semiquantification scoring (0–4 scale) was also used to assess expression of Ang1, Ang2, and VEGF in synovial tissue assessed by 2 blinded observers. Different regions were examined and scored separately: perivascular cells, interstitial regions, and lining layer cells[51].

ELISA. SF was obtained immediately before arthroscopy: supernatants were collected after centrifugation and aliquoted into cryotubes and stored at –70°C until analyzed. SF protein concentrations of VEGF, TGF-ß1 (human quantikine, R&D Systems, Abingdon, UK), and TNF-α (Biosystems, Manchester, UK) were measured by ELISA using commercial kits.

Statistics. The Wilcoxon signed-rank test for nonparametric data on SPSS9 for Windows was used to analyze differences in semiquantitative immunohistology, *in situ* hybridization of SM tissue, vascular scores, and SF protein levels. A Spearman rank correlate and a regression analysis model on SPSS9 were used to examine the relationship between macroscopic, microscopic, and molecular analysis.

RESULTS
Clinical and demographic data. SM and SF were obtained from 26 patients with knee synovitis. The cohort consisted of patients with early PsA (10 men, 2 women) with (mean ± SE) disease duration of 7.3 ± 1.43 months and median age 39 years (range 20–72) and patients with early RA (6 men, 8 women) with disease duration 7.9 ± 1.42 months and median age 43 years (range 27–79). Patients with early arthritis were matched for measures of clinical disease activity including total HAQ score, CRP, and SJC (Table 1). Importantly, no patient had received corticosteroids or DMARD at any time prior to study. OA control patients (4 men, 8 women) had disease duration > 24 months and median age 72 years (range 56–93). A further 21 patients with late inflammatory arthritis (8 men, 13 women) with active knee involvement served as disease controls; they had disease duration > 18 months and median age of 45 years (range 27–75).

Macroscopic scoring. The macroscopic SM vascularity showed higher scores for early PsA patients (74 ± 6.2 mm)

Table 1. Clinical demographics and arthroscopic macroscopic vascular scoring in patients with early psoriatic (PsA) and rheumatoid arthritis (RA). Clinical measures of disease activity (mean ± SE) include modified Health Assessment Questionnaire (HAQ) (total score), C-reactive protein (CRP, mg/ml), and swollen joint count (range 0–66). Arthroscopy assessed blood vessel morphology and macroscopic vascular scores. Macroscopic vascular scores were lower in early RA compared to early PsA. There was a significant difference in blood vessel morphology between early PsA and early RA (*p = 0.0001). Clinical variables were similar in both groups.
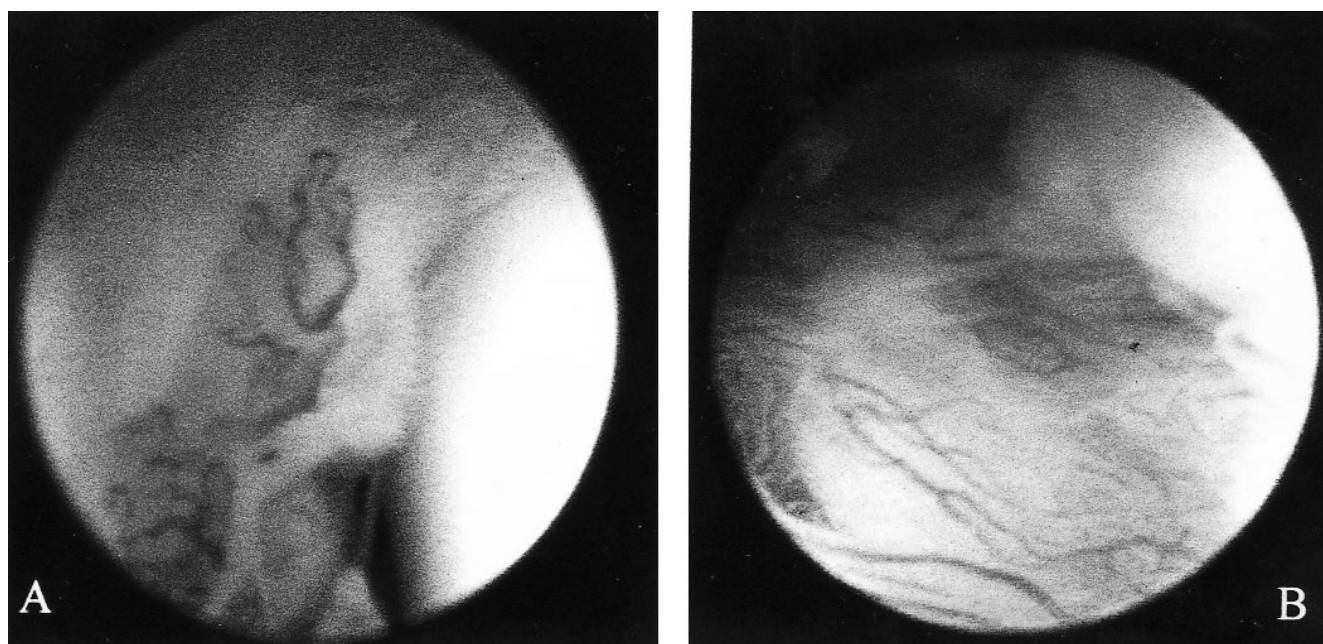
	Early PsA	Early RA
Age, yrs, median (range)	39 (20–72)	43 (27–79)
Disease duration, mo mean (SD)	7.3 (1.43)	7.9 (1.42)
HAQ	8.0 (1.0)	10.1 (1.7)
CRP	12.0 (1.8)	16.0 (5.7)
Swollen joint count	4.2 (0.8)	5.4 (1.6)
Macroscopic vascular scores, 0–100 mm, mean (SE)	74.1 (6.28)	54.1 (7.6)
Tortuous bushy vessels, %	91*	—
Straight, branching vessels, %	9	84*
Mixed vascular pattern, %	—	16

compared to early RA (54 ± 7.8 mm) (Table 1). Vascular patterns showed a significant difference in early PsA (tortuous and bushy vessels) compared to early RA (p < 0.0001), where blood vessels were straight and branching (Figures 1A, 1B), Table 1). The tortuous, bushy vascular pattern was found in 91% of patients with PsA, with 84% of early RA patients having straight branching vessels; the remaining patients had mixed patterns.

*Microscopic scoring*. Ang1, Ang2, and VEGF protein expression was determined in both early PsA and RA SM. Expression of Ang2 and VEGF was localized to the perivascular cells, lining layer cells, and to a lesser extent substromal cells (Table 2, Figure 2). In the perivascular regions Ang2 and VEGF expression (mean ± SE) were higher in the early PsA compared to the early RA patients (3.0 ± 0.3 vs 2.0 ± 0.3, p < 0.05; 2.6 ± 0.2 vs 1.8 ± 0.2, p < 0.05, respectively; Table 2, Figure 2). Ang2 and VEGF expression was higher in the lining layer of patients with early PsA compared to early RA; however, this did not reach statistical significance. VEGF and Ang2 expression was significantly higher in the lining layer and perivascular regions in the patients with early PsA compared to OA tissue (p < 0.05), and while concentrations were higher in patients with early RA compared to OA this did not reach significance. Ang1 expression was also determined in the perivascular and lining layer region of the SM (Table 2, Figure 2), but to a much lesser extent compared to Ang2 and VEGF. In addition, there was no significant difference in expression between early PsA and RA.

The SM infiltrate (early RA vs PsA; mean ± SE) was similar in both groups for CD4 (2.3 ± 0.3 vs 1.8 ± 0.2), CD68 sublining (2.4 ± 0.3 vs 2.1 ± 0.3), and CD68 lining layer (2.7 ± 0.3 vs 2.1 ± 0.4), with a slight increase in CD3+ T cells (1.6 ± 0.7 vs 0.9 ± 0.56; p < 0.05) in early RA compared to PsA. Lining layer thickness was increased in early RA compared to PsA (5.1 ± 2.1 vs 3.1 ± 0.9; p < 0.01). Level of growth factor expression in comparison to the amount of mononuclear cell infiltration and lining layer hyperplasia was increased in the SM of early PsA patients compared to early RA. VEGF, Ang2, and Ang1 were increased in a subgroup of patients with long-

Figure 1. Synovium visualized by videoarthroscopy shows the distinct patterns of (A) highly tortuous bushy vessels in early PsA; and (B) the straight, branching vessels in patients with early RA.
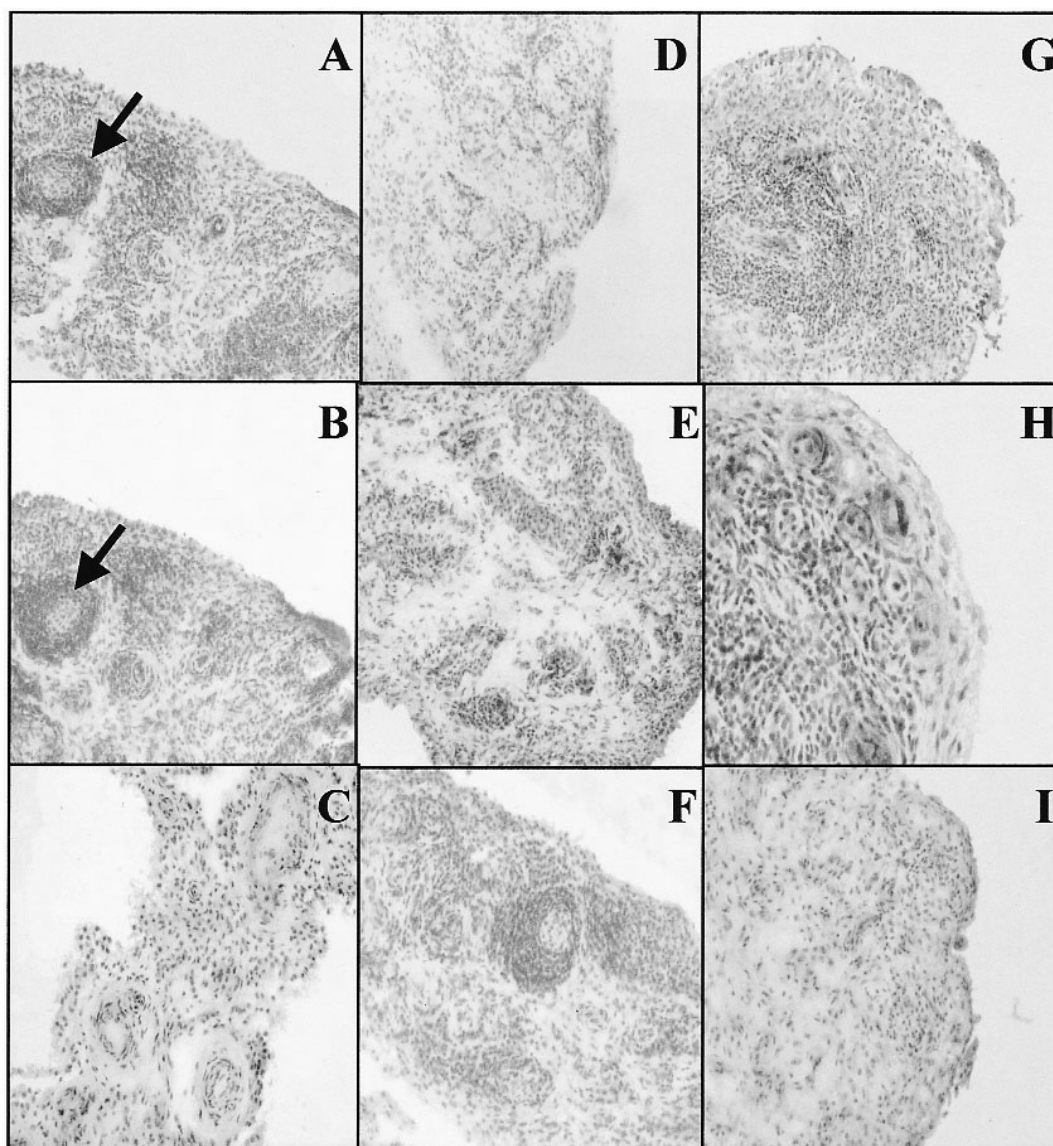
Figure 2. Immunohistology for VEGF, Ang1, and Ang2 protein in SM of patients with early PsA and RA, late inflammatory arthritis (LIA), and OA. Ang2 protein expression in PsA SM (A), colocalization of VEGF in early PsA SM (B), Ang2 expression in OA SM (C), Ang1 expression in early PsA SM (D), Ang2 expression in LIA SM (E), mouse IgG control (F), Ang2 expression in early RA SM (G), VEGF expression LIA SM (H), goat IgG control (I). Colocalization of Ang2 and VEGF around blood vessels in early PsA is indicated by arrows in panels A and B.

standing disease (n = 10) (Table 2, Figure 2) compared to the patients with early RA; however, growth factor levels were similar to the early PsA group, suggesting that differential expression of these angiogenic factors may be seen only in early disease before any treatment with DMARD.

In situ hybridization of VEGF, Ang1, and Ang2. VEGF mRNA expression was determined in all PsA and RA SM; however, intensity of expression was greater in early PsA compared to early RA (Figures 3A, 3B, Table 3). VEGF expression was localized to perivascular cells, lining layer cells, and substromal cells. VEGF was significantly higher in early PsA compared to early RA in the perivascular region (mean ± SE 3.2 ± 0.2 vs 2.2 ± 0.2; p < 0.05). Ang2 expression was localized to perivascular cells, lining layer cells, and substromal cells (Figures 3C, 3D, Table 3). Ang2 mRNA levels were significantly higher in early PsA compared to early RA in the perivascular region (mean ± SE 3.0 ± 0.4 vs 1.5 ± 0.3; p < 0.05). Expression of VEGF and Ang2 mRNA was higher in the lining layer and interstitial tissue in early PsA compared to early RA; however, this was not statistically significant. Ang2 mRNA strongly correlated with VEGF mRNA in the perivascular region (r = 0.7, p < 0.001). Low expression of Ang1 mRNA was found in early PsA and RA SM.

VEGF, TGF-ß1, and TNF-α measurements in SF. SF levels (mean ± SE) of VEGF and TGF-ß1 were significantly greater in early PsA patients compared to early RA patients (1769.1 ±

Table 2. Semiquantitative scores for VEGF, Ang1, and Ang2 protein expression by immunohistology in perivacular region, lining layer, and interstitial tissue in early PsA, early RA, OA, and late inflammatory arthritis (LIA) synovial membrane. Expression of VEGF, Ang1, and Ang2 protein scored by semiquantitative analysis (0–4), where 0 = no positive cells and 4 = 75–100% cells positive. Values are expressed as mean ± SE.

	Early PsA	Early RA	OA	LIA
VEGF				
Perivascular	2.6 (0.2)*	1.8 (0.2)	1.2 (0.2)[#†]	2.6 (0.2)
Lining layer	1.8 (0.2)	1.4 (0.2)	0.8 (0.4)[#†]	1.9 (0.3)
Interstitial tissue	1.4 (0.2)	1.0 (0.3)	0.6 (0.2)[†]	0.9 (0.2)
Ang2				
Perivascular	3.0 (0.3)*	2.0 (0.3)	1.0 (0.3)[#†]	2.8 (0.3)**
Lining layer	1.8 (0.2)	1.4 (0.2)	0.8 (0.2)[#†]	2.2 (0.2)
Interstitial tissue	1.6 (0.2)	1.4 (0.2)	0.8 (0.4)	1.7 (0.3)
Ang1				
Perivascular	1.2 (0.2)	1.0 (0.3)	0.8 (0.2)*	1.4 (0.2)
Lining layer	0.6 (0.2)	0.8 (0.4)	0.6 (0.4)	1.1 (0.2)
Interstitial tissue	1.0 (0.0)	0.6 (0.2)	0.6 (0.2)	0.7 (0.3)

* $p < 0.05$, early PsA significantly different from early RA. ** $p < 0.05$, LIA significantly different from early RA. [#] $p < 0.05$, OA significantly different from early PsA. [†] $p < 0.05$, OA significantly different from LIA.
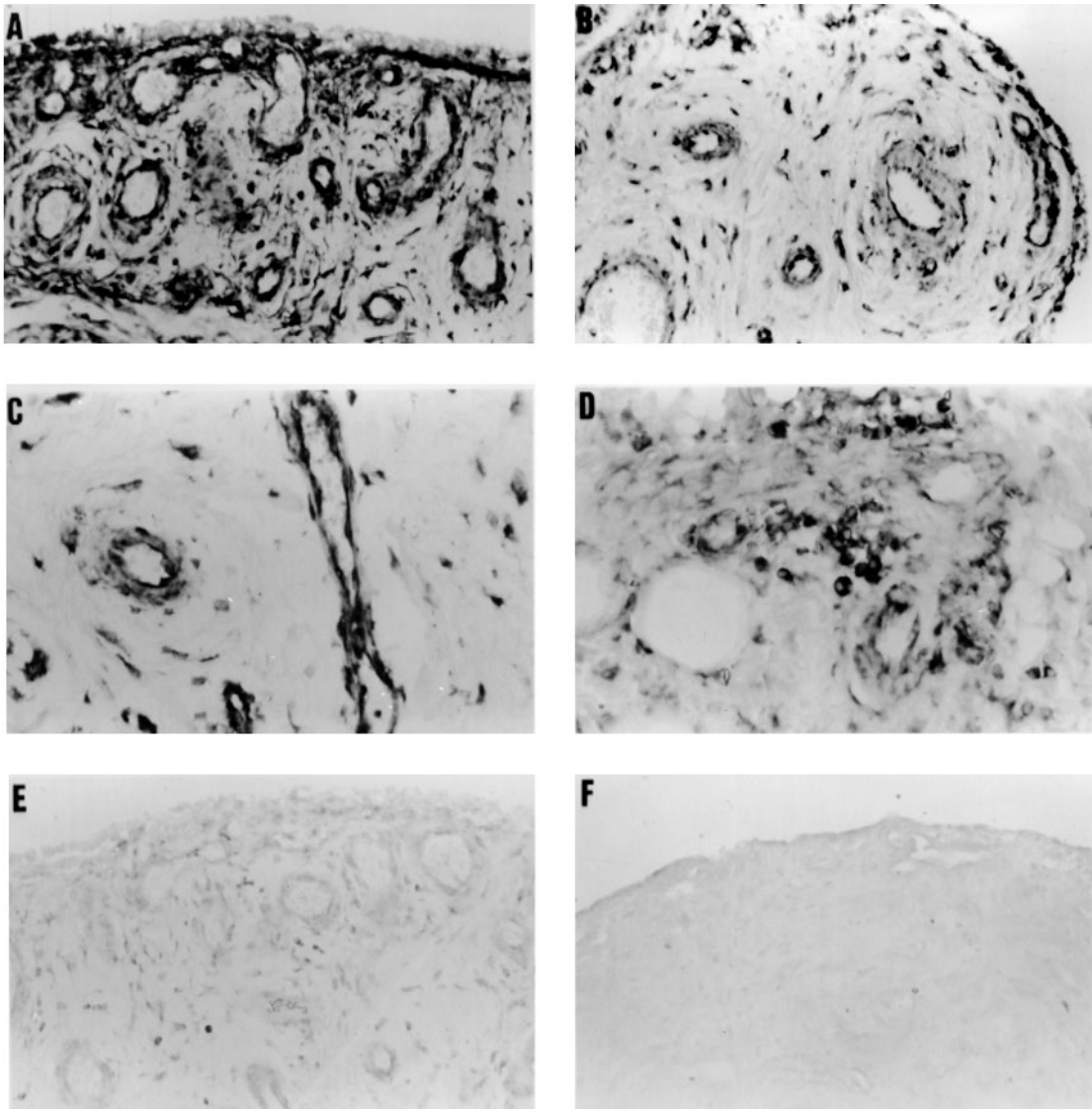

Figure 3. In situ hybridization for VEGF and Ang2 mRNA in SM of patients with early PsA and RA. VEGF mRNA expression in early PsA SM (A), VEGF mRNA expression in early RA SM (B), Ang2 mRNA expression in early PsA SM (C), Ang2 mRNA in early RA SM (D). Negative–sense probe in early PsA (E) and early RA (F).

Table 3. Semiquantitative scores of expression of VEGF and Ang2 mRNA by *in situ* hybridization in perivascular region, lining layer, and interstitial tissue in early PsA and RA SM. Expression of VEGF and Ang2 mRNA was scored by semiquantitation analysis (0–4), where 0 = no positive cells and 4 = 75–100% cells positive. Values are expressed as mean ± SE.

	Early PsA	Early RA
VEGF mRNA		
Perivascular/endothelial	3.2 (0.2)	2.2 (0.2)*
Lining layer	2.2 (0.4)	1.8 (0.5)
Interstitial tissue	1.8 (0.2)	1.25 (0.2)
Ang2 mRNA		
Perivascular/endothelial	3.0 (0.4)	1.5 (0.3)**
Lining layer	2.5 (0.8)	1.2 (0.4)
Interstitial tissue	1.0 (0.3)	0.25 (0.1)

* $p < 0.05$, VEGF expression significantly greater in the perivascular region in early PsA compared to early RA. ** $p < 0.05$, Ang2 expression significantly greater in the perivascular regions in early PsA compared to early RA.

279 vs 799 ± 179 pg/ml, p = 0.003; and 3718.4 ± 780 vs 1730 ± 303 pg/ml, p = 0.014, respectively) and OA patients (700 ± 104; 1262 ± 213 pg/ml, p = 0.006 and p = 0.002) (Figure 4). A positive correlation was observed between VEGF and TGF-ß1 for the whole group (r = 0.62, p = 0.0001), and in the early patients (r = 0.61, p = 0.007). VEGF and TGF-ß1 were increased significantly in the established group compared to the patients with early RA (2404 ± 30, 3147 ± 566, p < 0.00, respectively); however, levels were similar to the early PsA group. In contrast, levels of the cytokine TNF-α were not significantly different between early RA and PsA (19 ± 5.0 vs 23 ± 11 pg/ml). TNF-α levels were significantly higher in the early PsA and RA group compared to OA (p = 0.022, p = 0.025, respectively). The highest levels for TNF-α were found in the group with established disease (43 ± 14 pg/ml), where

they were significantly greater than in the early PsA (p = 0.04) and OA (p = 0.001) groups.

Correlation of macroscopic, microscopic, and molecular analysis. Strong correlations were found between macroscopic synovial vascular scores and blood vessel pattern (r = 0.5, p = 0.005). A significant correlation was found between SF VEGF and macroscopic vascular score (r = 0.4, p = 0.004) and the macroscopic blood vessel pattern (r = 0.51, p = 0.01). Ang2 mRNA strongly correlated with VEGF mRNA expression in the perivascular region (r = 0.7, p = 0.001). TGF-ß1 strongly correlated with VEGF (r = 0.62, p = 0.000) and with the macroscopic vascular score (r = 0.3, p = 0.028).

DISCUSSION

Ang1 and Ang2 are ligands for the receptor kinase (Tie-2) and play an important role in angiogenesis and vascular stabilization/destabilization. This study describes the expression of these novel vascular growth factors in the synovium of patients with PsA and RA with early disease before any treatment. Expressions of SM Ang2 and VEGF mRNA and protein, along with SF protein levels of VEGF and TGF-ß1, were significantly higher in the patients with early PsA compared to early RA. Significant correlations were observed between blood vessel pattern, the macroscopic vascularity score, and growth factor expression (VEGF and TGF-ß1). Ang 1 expression was also observed in both PsA and RA synovium; however, the level was markedly less than that of Ang2 and VEGF.

Several studies suggest distinct mechanisms of inflammation exist in PsA and RA[21-24,52-54]. The earliest reports on vascular morphology in PsA using electron microscopy defined ultrastructural abnormalities of blood vessels[21]. Some reports on SM in ankylosing spondylitis suggest similarities to RA[55]; however, we and other investigators have reported prominent vascular changes showing quantifiable differences in vascu-
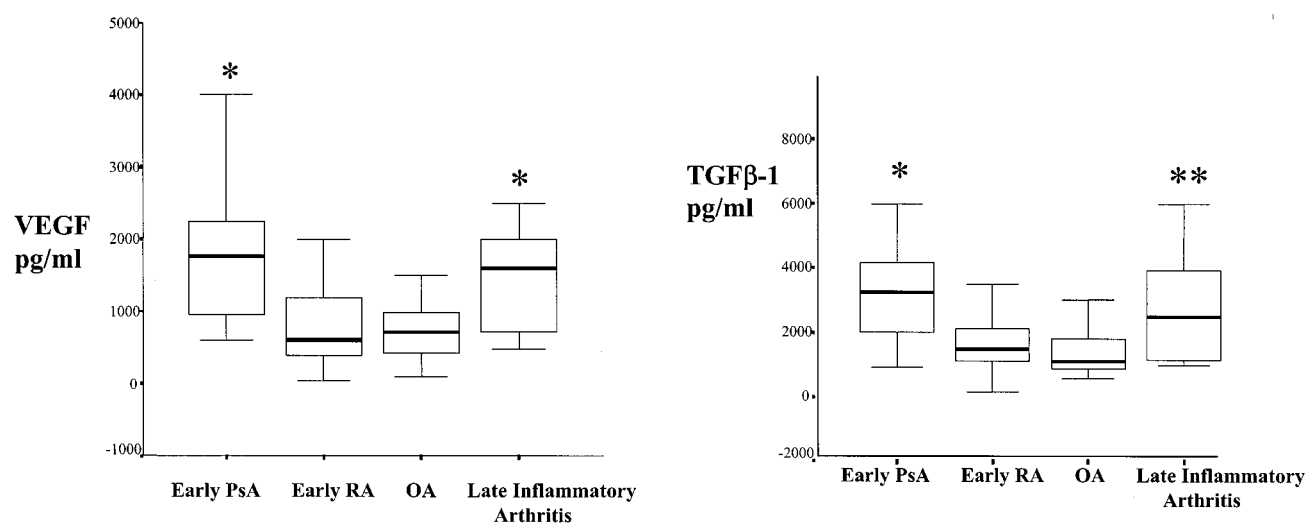
Figure 4. Levels of VEGF and TGF-ß1 in synovial fluids from patients with early PsA (n = 12), early RA (n = 14), OA (n = 12) and late established arthritis (n = 21). Synovial fluids were assayed using a specific linked immunosorbent assay (R&D Systems, Abingdon, UK). *p < 0.05, significantly different from early RA and OA. **p < 0.05, significantly different from OA. Concentrations are expressed as pg/ml. Boxes show the median and 25th and 75th percentiles whiskers indicate the 10th and 90th percentiles.

larity and differential adhesion molecule expression in PsA SM[21-23,31,52-54,56,57]. In addition we described distinct macroscopic blood vessel morphology from arthroscopic assessment of the SM in early seronegative arthritis, including PsA, compared to early RA[23]. The data from the present study provide further evidence supporting the hypothesis that the pathogenesis of PsA is different from RA and may result from a primary dysregulation of angiogenesis. As patients were matched for clinical disease activity, and had similar histological SM infiltration and SF TNF-α protein levels, the differential expression of VEGF and Ang2 does not merely represent different levels of inflammation.

Angiogenesis is an important process in inflammatory arthritis. At the macroscopic level this can be seen as the capillary hyperemia and increased vascularity within the advancing pannus, the new vessels invading the normally avascular cartilage[26]. At a microscopic level this is represented by increased blood vessels in the sublining layer stroma, consistent with a response to a hypoxic stimulus[58-60], which may stimulate VEGF production[61]. In addition, TGF-ß production is upregulated in late RA, is a potent stimulus of angiogenesis[62], and has been localized at the cartilage–pannus junction[12,26]. Dysregulation of angiogenesis with increased expression of VEGF, TGF, and FGF-1 and 2 has been reported in SM of patients with established RA, many of whom have received drug therapy[7,12,27-30]. In the present study, however, we found that differential expression of angiogenic factors and dysregulation of angiogenesis is already well established in early disease. More interestingly, this is the first description of Ang2 and Ang1 expression in early rheumatoid disease; these angiopoietins were recently identified as key factors in animal and mechanistic studies that showed that coexpression of VEGF and angiopoietins are critical to vascularization and blood vessel stability[16-19,41-44]. The increase in VEGF and Ang2 expression in PsA at the earliest stage suggests a primary event with a more significant shift towards proangiogenic factors and "destabilization" of blood vessels in comparison to early RA.

Several studies have reported VEGF is expressed by SM endothelial cells and lining layer cells in late stage RA[7,28,29] and acts by binding one of 2 possible receptor kinases, Flt-1 and Flk-1[61]. It is thought that Flk-1 mediates differentiation of endothelial cells, while activation of Flt-1 mediates a later stage of vasculogenesis[63-66]. VEGF has been shown to be expressed on endothelial cells, fibroblasts, macrophages, vascular smooth muscle cells, neutrophils, and chondrocytes. Ikeda, *et al* demonstrated increased mRNA expression of VEGF and its receptors in RA synovium, where VEGF was predominantly expressed on lining and sublining cells[67]. The results of our study are in agreement with the findings of Ikeda, *et al*; however, in addition, we found VEGF expressed on cells infiltrating into the synovium in the perivascular regions. The perivascular expression of VEGF in tissue from our early disease cohort may be explained by the minimal disease duration

and the fact that these patients were DMARD naive.

Angiopoietins are an important family of proteins, interacting with VEGF, which are thought to be responsible for destabilization of blood vessels. Proliferation and organization of EC into tubules is critical to angiogenesis, and thus EC-specific receptors and their ligands are key in this process. One such specific tyrosine kinase receptor is Tie-2, and its ligands are Ang1 and Ang2. The importance of these ligands and receptor in angiogenesis has been demonstrated in genetically engineered mice[16,18,19,68]. Binding of Ang1 and Ang2 can lead to vascular events, depending on the presence or absence of growth factors. Downstream of the actions of VEGF, Ang1 acts via Tie-2 in the presence of platelet derived growth factor and becomes important in the maturation or stabilization of vessels, resulting in inhibition of EC proliferation[5], while Ang2 expression and binding to Tie-2 in the presence of VEGF result in destabilization and increased angiogenesis[5,41]. In an *in vivo* model (corneal implants), Ang1 plus suboptimal levels of VEGF caused an increased number of new vessels with evidence of a "stable" vascular system, while Ang2 plus high levels of VEGF caused destabilization resulting in the formation of longer, wider vessels[41]. These results, together with *in vivo* mouse data, suggest that cooperation of Ang2 with VEGF, in the absence of Ang1, results in destabilizing cell–cell interactions and vessel sprouting, which in turn blocks endothelial–periendothelial cell interactions for formation of mature vessels by pericyte recruitment. In our study we observed increased expression of both VEGF and Ang2 in early PsA and RA; however, expression of both were higher in the perivascular and lining layer regions in PsA SM. Thus high levels of Ang2/VEGF with low levels of Ang1 in the PsA joint suggest stabilization of the new vessels is blocked, resulting in the formation of more "plastic" vessels, which are readily able to respond to sprouting signals by VEGF. Such high levels of Ang2 and VEGF in early PsA along with the distinct vascular morphology observed macroscopically suggest that these vessels are in a hypervascularized state. Further, the coexpression of Ang2 and VEGF in the perivascular regions suggests they are directly involved in the leading edge of blood vessel sprouts. We observed low expression of Ang1 in the patients with early disease, and while its expression was higher in SM of the subgroup with well established disease, levels were much lower than that of Ang2. This is in agreement with a recent study by Scott, *et al*, who demonstrated increased levels of Ang1 and 2 in RA synovium compared to healthy controls by quantitative polymerase chain reaction; however, they also found that the Ang2 levels were strikingly high compared to those of Ang1, and found Ang2 to be the predominant Ang in the inflamed synovium[69]. Such low levels of Ang1, in contrast to high expression of Ang2/VEGF, suggest that blood vessels are in a destabilized state, signaling VEGF and other factors to promote angiogenesis. Ang2 levels were also significantly higher than those of Ang1 in the group with late inflammatory arthritis, suggesting that the extensive neovasculature in late disease is still in an immature destabilized state.

Several studies examining angiopoietins in embryonic or tumor vascularization suggest that Ang1 plays an angiogenic role, distinct from VEGF, at a later stage. Suri, *et al*, demonstrated that transgenic overexpression of Ang 1 in the skin of mice produced larger and more stable vessels[42]. In contrast, Ang2 is thought to play an important role at an earlier stage of vascularization, where it has been shown to be involved in vessel invasion[43,44,70]. It is thought that Ang2 acts in the presence of abundant VEGF on invading vascular sprouts by blocking the stabilization process[19]. This causes vessels to be more responsive to a sprouting signal provided by VEGF[19,42]. This hypothesis is consistent with our study, where blood vessel growth was irregular, leading to angiogenic dysregulaton in both early PsA and early RA synovial membranes. The high expression of VEGF and Ang2 in PsA, along with the distinct vascular morphology (elongated, tortuous), in comparison to RA further supports studies that indicate increased vascularization may be pivotal in the differential pathogenesis of PsA and RA.

We have demonstrated for the first time expression of Ang2 and Ang1 at a very early stage of disease in synovium of patients with PsA and with RA. Coexpression of VEGF and Ang2 appears to be a marker of early vascularization, which may be responsible for the differential vascular morphology observed in PsA compared to RA. We have also identified a close relationship between macroscopic synovial membrane vascular changes and angiogenic growth factors. These results emphasize the important implications of early treatment, not only to prevent further angiogenesis, but also to promote regression of fragile new blood vessels in the invading synovium.

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