Constitutive Expression of Angiopoietin-1 and -2 and Modulation of Their Expression by Inflammatory Cytokines in Rheumatoid Arthritis Synovial Fibroblasts

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ABSTRACT. ***Objective.*** Angiopoietin-1 (Ang-1) and Ang-2 are ligands for the receptor tyrosine kinase, Tie-2. Ang-1, a Tie-2 agonist, may have a vascular stabilizing role in angiogenesis, while Ang-2, an endogenous antagonist of Tie-2, may have an early role in angiogenesis, destabilizing existing vasculature. We show that these ligands are expressed by rheumatoid synovial fibroblasts (RSF) and investigate whether their expression was modulated by proinflammatory cytokines present in the joint in rheumatoid arthritis (RA).

Methods. Using quantitative PCR we determined the level of expression of these 2 ligands in RSF and chronic inflamed synovial tissue. The level of expression of these ligands after treatment with proinflammatory cytokines and hypoxia was also determined.

Results. We observed constitutive expression of Ang-1 and Ang-2 in RSF and chronic inflamed synovial tissue. Ang-1 was the most highly expressed ligand in late stage RA synovial fibroblasts; however, in chronic inflamed synovial tissue, Ang-2 was predominant and was expressed at strikingly high levels (70 to 120-fold increase). We observed that tumor necrosis factor-α (TNF-α) and transforming growth factor-ß (TGF-ß), but not interleukin 1ß or hypoxia, stimulated Ang-1 gene expression in RSF. This was confirmed at the protein level as media from TNF-α treated RSF resulted in increased autophosphorylation of Tie-2. In contrast, TNF-α and TGF-ß had no effect on Ang-2 expression in RSF, but augmented expression of Ang-2 in normal synovial fibroblasts.

Conclusion. The angiopoietins are important angiogenic factors constitutively present in RA, and their expression is modulated by certain cytokines. Ang-2 may have an important role in rheumatoid tissue where vigorous angiogenesis is occurring. (J Rheumatol 2002;29:230–9)

Key Indexing Terms:
ANGIOGENESIS ANGIOPOIETIN ANG-1 ANG-2
TUMOR NECROSIS FACTOR-α RHEUMATOID ARTHRITIS

Angiogenesis is a central process by which new blood vessels are formed and is essential in reproduction, development, and wound repair[1,2]. In these situations angiogenesis is a tightly regulated process. However, many diseases are driven by chronic unregulated angiogenesis[3,4]. An example of such unregulated angiogenesis occurs in rheumatoid arthritis (RA) where angiogenesis is, along with synovial hyperplasia, one of the first pathologies detectable at a histological level in early RA[5]. Angiogenesis contributes to the inflammatory process by increasing tissue perfusion and permitting increased cellular traffic into the synovial tissue[5].

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Angiogenesis is controlled by paracrine signals, many of which are protein ligands that bind and modulate the activity of transmembrane receptor tyrosine kinases (RTK)[2]. The Tie receptors, a family of RTK[6-16], have aroused interest because they are the only RTK, other than those for vascular endothelial cell growth factor (VEGF), that are largely restricted to endothelial cells in their expression[7,14,15,17-21].

Tie-1 receptor is an orphan receptor but ligands for Tie-2 receptor have been identified and cloned[22-24]. These ligands have been termed the angiopoietins and are 75 kDa secreted proteins with considerable sequence homology, containing a coiled coil domain and a fibrinogen-like domain[22-24]. Binding studies reveal that these ligands have similar affinity for the receptor and do not bind Tie-1 receptor[22-24]. Given the similarities between these ligands, it is remarkable that angiopoietin-1 (Ang-1) has been found to cause autophosphorylation of Tie-2 receptor in endothelial cells, whereas angiopoietin-2 (Ang-2) does not[23]. Indeed, Ang-2 is an antagonist, and competitively inhibits Ang-1 induced autophosphorylation of the receptor[23].

It is hypothesized that the role of Ang-1 is to differentiate and stabilize newly formed vessels, recruiting support cells to

encapsulate the vessel, whereas Ang-2 functions to inhibit the effect of Ang-1, causing a relaxation of vessel structure, and allowing the angiogenic process to begin[25-27]. Increased vascularization of the synovial tissue is an obvious prerequisite and continuing necessity to support the inflammation and hyperplasia that occur within the rheumatoid joint[4,28]. Indeed, the RA synovial tissue and fluid is a rich mixture of angiogenic stimuli such as VEGF, platelet derived growth factor, interleukin 1ß (IL-1ß), transforming growth factor-ß (TGF-ß), and tumor necrosis factor-α (TNF-α). In addition the rheumatoid synovial environment is also hypoxic, another key stimulus for angiogenesis[29-36].

Synovial fibroblasts have been implicated in the disease process of RA due to their transformed appearance and proliferation, and because they are the predominant cell type in the joint in early RA[37-39].

It has also been shown that synovial fibroblasts constitutively express VEGF, which is upregulated by angiogenic stimuli within the RA synovial environment such as IL-1ß and hypoxia[40]. Hence it is reasonable to hypothesize a role for the synovial fibroblast in the angiogenesis that occurs in the RA joint.

We investigated the regulation of angiopoietin expression in an inflammatory setting, to determine whether angiopoietins may be modulated by inflammatory factors considered to have an indirect role in regulating angiogenesis. We observed that rheumatoid synovial fibroblast cell lines constitutively express both Ang-1 and Ang-2. We also found that the expression of Ang-1 and Ang-2 is modulated by inflammatory cytokines. These results suggest that the rheumatoid synovial fibroblast is an important source of Ang-1 and Ang-2 in the diseased joint, and may indicate an important role for this cell type in the regulation of angiogenesis in the chronically inflamed joint.

MATERIALS AND METHODS

Cell culture. Patients with RA were diagnosed according to the standards of the American College of Rheumatology[41]. Synovial tissue in culture media (EMEM, minimal essential media with Earle's salts and L-glutamine; Gibco/BRL, Gaitherburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.025 μg/ml amphotericin B (Gibco/BRL) was processed immediately as follows: tissue was digested by the addition of 2000 units of collagenase (Clostridopeptidase A, 17018; Gibco/BRL) and incubated at 37°C in 5% CO_2 for 4 h, followed by addition of an equal volume of 0.05% trypsin, 0.02 mM EDTA in phosphate buffered saline (PBS) and extensive pipetting to break up tissue. This material was incubated at 37°C in 5% CO_2 for 1 h, and then the contents of each dish washed 3 times by centrifugation for 10 min at 400 × g and resuspension in 40 ml PBS (room temperature). The washed material was resuspended in 15 ml of culture media, placed in T-75 flasks, and incubated at 37°C in 5% CO_2 for 24–48 h. Adherent cells were then washed 3 times with warm PBS and fresh medium was added. The adherent cell population is made up mainly of fibroblasts and macrophages, and the macrophages die off by the second passage[42]. We confirmed this by staining for uridine diphosphoglucose dehydrogenase[43], which is specific for the fibroblast-like synoviocytes (data not shown). Once cells were confluent, they were split with trypsin EDTA and maintained in culture media with 10% FBS, penicillin, and streptomycin, and used for study between passage 3 and 9. Cell lines from 4 different patients with RA and 2 different normal synovial samples were used. Origins of the cell lines were as follows: rheumatoid synovial fibroblast-1 (RSF-1), knee arthroscopy — patient had long standing advanced RA and was taking 400 mg/day plaquenil, 15 mg/week methotrexate (MTX) (discontinued 1 week prior to arthroscopy), 5 mg/day prednisone, 1200 mg/day oxaprozin, 1 mg/day folic acid, 400 mg/day etidronate for 2 weeks at 2 week intervals, 500 mg calcium tid; RSF-2, knee arthroscopy — patient had long standing advanced RA and was taking 7.5 mg/week MTX and occasional ibuprofen; RSF-3 and RSF-4, knee synovectomies — both patients had long standing advanced RA, medication is unknown.

Two normal synovial fibroblast lines were established from normal (undamaged) knee synovium of trauma patients (kindly provided by Dr. A. Marchesoni, Ospedale G. Pini, Milano, Italy, and Dr. W. Wester, Monsanto, St. Louis, MO, USA).

Cell treatments. Synovial fibroblasts were plated at 5×10^6 per T-150 flask and grown to confluence (1.5×10^7) in EMEM culture medium. Cells were stimulated (one flask per treatment) for various times with either 1 ng/ml IL-1ß (Genzyme), 2 ng/ml TNF-α (R&D Systems, Minneapolis, MN, USA), 20 ng/ml TGF-ß (R&D Systems), or hypoxia, accomplished by incubating in an environment of 5% CO_2, 94.5% N_2 and 0.5% O_2. This atmosphere was created in a Queue multigas incubator and calibrated and monitored with an oxygen electrode (Microelectrodes Inc., Bedford, NH, USA). Oxygen concentration in culture media was also monitored with the O_2 electrode and typically reached 0.5% after 5 h in the hypoxic atmosphere. None of these stimuli significantly affected cell viability measured by trypan blue exclusion. Treatments were carried out on at least 2 independent cell lines of each type, and a representative experiment is described below.

RNA isolation and cDNA synthesis. Cells were harvested by trypsinization and total RNA was isolated using RNeasy reagents (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol and quantitated by spectrophotometry. cDNA was synthesized from 1 μg of RNA using Superscript II reverse transcriptase (Life Technologies, UK) using the manufacturer's protocols. Origins of the chronic inflamed synovial tissues were as follows: PA-1, wrist arthroscopy — patient had long standing advanced RA and was taking 10 mg/week MTX, 5 mg/day prednisone, 1200 mg/day oxaprozin, 1 mg/day folic acid, calcitonin nasal spray, calcitriol, and omeprazole; PA-2, knee arthroscopy — patient had long standing advanced RA and was taking 15 mg/week MTX, 150 mg/week azathioprine, 7.5 mg/day prednisone, propoxyphene, 1 g/day nabumetone, 50 mg/day hydrochlorothiazide, 180 mg/day verapamil, 200 mg/day troglitazone, and calcium with vitamin D and iron; PA-3, knee arthroscopy — long standing advanced RA, medication unknown.

Quantitative polymerase chain reaction (PCR). Tissue levels of Ang-1 and Ang-2 gene expression were quantified by real-time quantitative Taqman™ PCR using an ABI Prism 7700 (Perkin-Elmer, Foster City, CA, USA). Specific primers and dual labeled fluorescent probes were designed to detect Ang-1 and Ang-2 mRNA using the Primer Express primer design program v.1.01 (Perkin-Elmer). Constitutively expressed GAPDH was used as an internal control. The fluorescent dye 6-carboxy fluorescein (FAM) was used as a reporter and its emission spectra quenched by a second fluorescent dye 6-carboxy-tetramethyl-rodhamine (TAMRA). During the extension phase of PCR, the probe is cleaved by 5' nuclease activity of TaqGold polymerase, resulting in an increase in fluorescent emission at 518 nm. This method allowed us to (1) measure PCR products during the log phase of PCR product accumulation when the fidelity of PCR is greater; and (2) simultaneously measure the expression of different genes, without concern of reaching plateau at different cycles.

The primer and probe sequences were as follows: Ang-1 forward primer 5'-agctgtgatcttgtcttggcc-3', Ang-1 reverse primer 5'-aaagtgtagctgcaggaccca-3', Ang-1 probe 5'-fluor label, 6-FAM-ctttcggaagagcatggacagcatagga-3'. Ang-2 forward primer 5'-tgcaaatgtgccctcatgtt-3', Ang-2 reverse primer 5'-taatcgcttctgacattgcgc-3', Ang-2 probe 5'-fluor label 6-FAM-tgatgcttgtggcccctccaatctaa-3'. GAPDH forward primer 5'-gaaggtgaaggtcggagt-3', GAPDH reverse primer -5'-gaagatggtgatgggatttc-3', GAPDH probe 5'-fluor label JOE-caagcttcccgttctca-3'.

PCR conditions were Taq PCR buffer, $MgCl_2$ 5 mM, dNTPs (dATP, dCTP, dGTP all 300 mM, dUTP 600 mM), 0.01 unit/reaction uracil-N-glycosylase, 1.25 units/reaction TaqMan™ gold. Primer concentrations were 300 nM and probe concentrations were 100 nM except for GAPDH, which were 100 nM for the primers and 50 nM for the probe. The thermal cycling was 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 90 s. All reagents for PCR including primer and probes from Perkin-Elmer.

Data analysis. Data were analyzed using the cycle threshold (C_T) comparative method (Perkin-Elmer ABI Prism 7700 User Bulletin No. 2, 1997). The PCR efficiency of each target (Ang-1 and Ang-2 mRNA) and of the control (GAPDH) were equal over the cDNA concentrations used in these experiments. The amount of mRNA levels was given by

$$(1 + E_{\text{PCR efficiency}})^{-\Delta\Delta CT}$$

where $\Delta\Delta C_T = [C_T \text{ target} - C_T \text{ GAPDH}]_{RA} - [C_T \text{ target} - C_T \text{ GAPDH}]_{control}$ and C_T = PCR cycle at which the amplification plot crosses the baseline threshold. The range (represented in bars in histograms or in parentheses in tables) given for the fold increase in target mRNA relative to control (level in the normal synovial fibroblast NSF–1) is determined by evaluating the expression

$$2^{-\Delta\Delta CT} \text{ with } {}^{\Delta\Delta CT}+s \text{ and } {}^{\Delta\Delta CT}-s$$

where s = standard deviation of the $\Delta\Delta C_T$ value (Perkin-Elmer ABI Prism 7700 User Bulletin No. 2, 1997).

Two sample Student's t test and analysis of variance (ANOVA) were used throughout to assess statistical significance. The trends in expression patterns shown were reproducible in 3 independent experiments.

Autophosphorylation. Serum starved HUVEC cells (CSC System, Seattle, WA, USA) were treated with the conditioned media from the rheumatoid synovial fibroblasts or rheumatoid synovial fibroblasts treated for 24 h with TNF-α (2 ng/ml). The conditioned media was diluted at 25, 50, and 100% into serum-free HUVEC medium (CSC Systems) and applied to the HUVEC. The cells were incubated at 37°C for 10 min and then placed on ice and 500 μl of lysis buffer (RIPA lysis buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% socium dodecyl sulfate (SDS) plus inhibitors: 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EDTA, plus Sigma mammalian cell protease inhibitor cocktail) were added to each well. Then 7 μg of anti-Tie-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to each lysate and incubated 1 h at 4°C. Then 20 μl of protein G-agarose slurry was added and incubated 1 h at 4°C. The agarose/antibody complexes were pelleted and washed with cold lysis buffer and then resuspended in 40 μl 1× SDS-polyacrylamide gel electrophoresis reducing sample buffer; 35 μl of sample was run on a 7.5% SDS/polyacrylamide gel.

The gel was then transferred to a nitrocellulose membrane (Bio-Rad) for Western blotting. The blots were washed with PBS 0.05% Tween-20 and then blocked with 3% BSA/PBS/Tween for 1 h at room temperature. The blots were then incubated with 1 μg/ml antiphosphotyrosine antibody (Santa Cruz Biotechnology) or anti-Tie-2 (Santa Cruz Biotechnology) and horseradish peroxidase conjugated secondary antibodies following manufacturer's protocols. After the final wash, the blot was developed using ECL reagents (Amersham). Using a densitometer and graphics program (ImageQuant; Molecular Dynamics), each blot was scanned and bands quantified.

RESULTS

Ang-1 and Ang-2 expression in rheumatoid synovial fibroblasts (RSF). To investigate the potential contribution of RSF to the angiogenic component of RA, the levels of expression of both Ang-1 and Ang-2 were determined by quantitative PCR in 4 RSF lines. Equal numbers of either normal synovial fibroblasts (NSF) or RSF were plated in a T150 flask and grown to confluency, then RNA was isolated from the trypsinized cells; 1 μg of the RNA was used to synthesize cDNA and this was used to perform Taqman quantitative PCR analysis. The levels of both Ang-1 and Ang-2 were seen to be consistently higher for both ligands in all RSF tested than the levels observed in NSF-1 or NSF-2 (Figure 1). For Ang-1 the levels ranged from a 2-fold increase to a 15-fold increase in expression compared to that observed in NSF-1. For Ang-2 the levels ranged from a 2-fold increase to a 6-fold increase in expression compared to that observed in NSF-1. The data indicate that RSF express both ligands of the Tie-2 receptor at levels significantly higher than those observed in NSF.

Ang-1 and Ang-2 expression in RA synovial tissue. To examine the possibility of differences in the expression of Ang-1 and Ang-2 between RA RSF and that of chronic inflamed synovial tissue where angiogenesis is particularly active, the levels of expression were determined in chronic inflamed synovial tissue from 3 patients with RA (PA-1, PA-2, PA–3). As for RA RSF, in these samples we observed expression of Ang-1 and Ang-2 at levels generally higher than those in NSF-1 (Figure 2). However, unlike RA RSF, in chronic inflamed synovial tissue the level of Ang-2 expression was markedly higher than that of Ang-1. Ang-1 expression in these samples ranged from 1- to 6.5-fold increases, whereas Ang-2 expression ranged from 60- to 120-fold increase. These results indicate that the relative levels of elevated Ang-1 are similar in both RA RSF and chronic inflamed synovial tissue samples, whereas Ang-2 expression is much more elevated in RA chronic inflamed synovial tissue.

Ang-1 and Ang-2 expression in RSF and NSF exposed to angiogenic stimuli. In the inflamed joint there are many angiogenic stimuli, such as IL-1ß, TGF-ß, TNF-α, and hypoxia, all of which are known to induce angiogenesis *in vivo*. It is unknown whether these stimuli are upstream or downstream from the angiopoietins in the angiogenic process. Hence we observed the effect of these angiogenic stimuli present in the synovial environment on the expression of Ang-1 and Ang-2 in synovial fibroblasts, as follows.

Hypoxia. RSF were cultured in hypoxic conditions (0.5% O_2) during a 24 h period and levels of Ang-1 and Ang-2 mRNA were determined at time 0, 8 h, and 24 h. In a representative experiment, the effect of hypoxia on Ang-1 and Ang-2 expression is clearly not stimulatory, as illustrated in Figure 3A. The results show that the effect of hypoxia is to downregulate the mRNA levels of both Ang-1 and Ang-2. There was a significant reduction in Ang-1 and Ang-2 in RSF at 8 h.

IL-1ß. RSF and NSF were cultured in the presence of IL-1ß (1 ng/ml) during a 24 h period and levels of Ang-1 and Ang-2 mRNA were determined at time 0 and 4, 8, and 24 h for RSF-2 and at time 0 and 1, 2, 8, and 24 h for NSF. In representative data from RSF no stimulatory effect of IL-1ß was observed on the levels of Ang-1 or Ang-2 in either RSF or NSF (Figure 3B, 3C).

We observed a possible downregulatory effect of IL-1ß on Ang-1 levels in RSF-2 that was significant at 8 h. The levels of Ang-2 in RSF and Ang-1 and Ang-2 in NSF were unaffected by IL-1ß (Figure 3B, 3C).

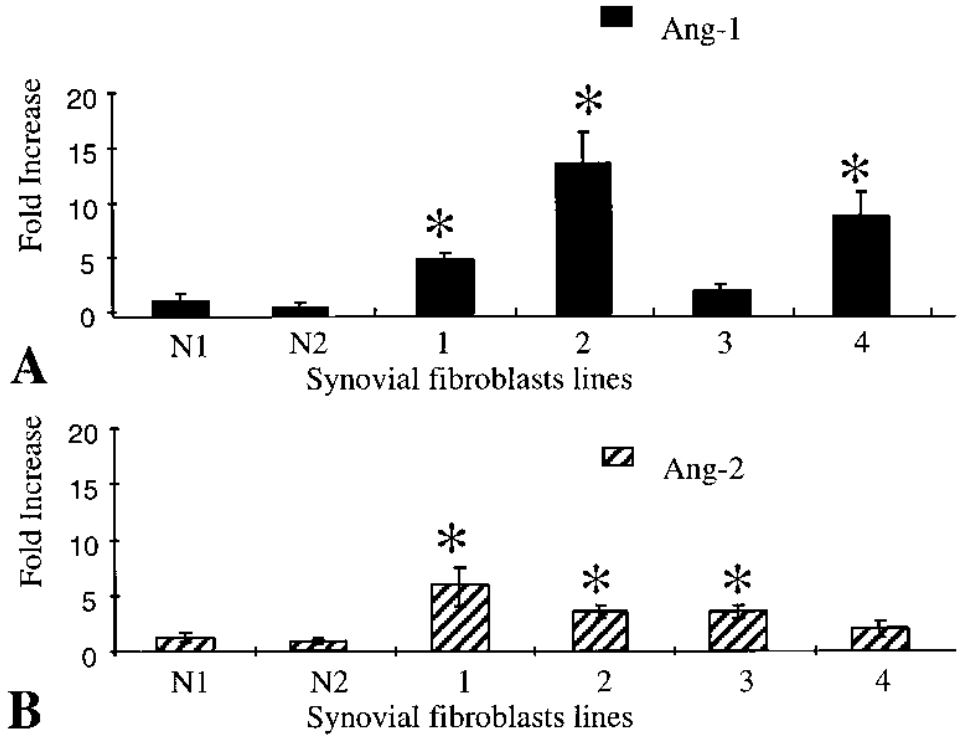

Figure 1. Ang-1 and Ang-2 expression in rheumatoid synovial fibroblasts (RSF). Quantitative determination of the gene expression of Ang-1 and Ang-2 in RSF and normalized to the expression level in normal synovial fibroblasts (N) as measured by quantitative PCR, shown as fold increase in expression. Error bars represent the range given for the fold increase in target mRNA. *Statistically significant increased expression of Ang-1 or Ang–2 in RSF compared to N1; $p < 0.01$. Similar results were obtained in at least 3 independent experiments.

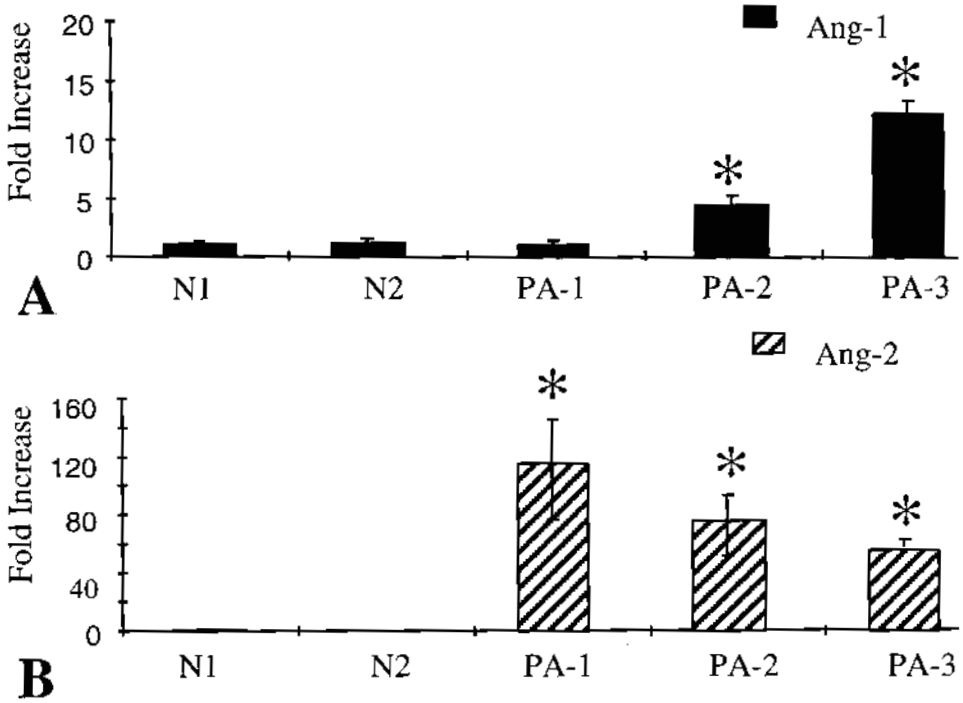

Figure 2. Ang-1 and Ang-2 expression in RA synovial tissue. Quantitative determination of the gene expression of Ang-1 and Ang-2 in chronic inflamed synovial tissue (PA-1, PA-2, and PA-3) and normalized to the expression level in normal synovial fibroblasts (N) as measured by quantitative PCR and shown as fold increase in expression. Error bars represent the range given for the fold increase in target mRNA. *Statistically significant increased expression of Ang-1 or Ang–2 in synovial tissue compared to N1; $p < 0.01$. Similar results were obtained in at least 3 independent experiments.

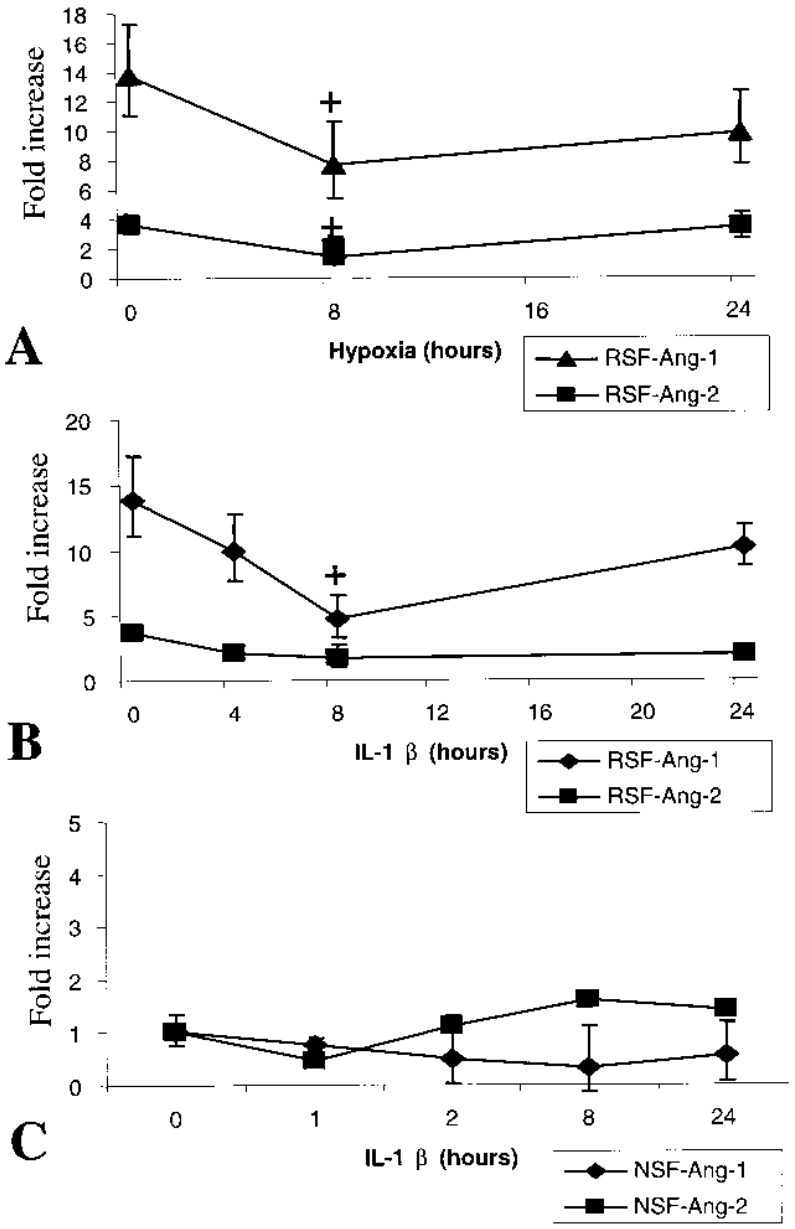

Figure 3. Ang-1 and Ang-2 expression in rheumatoid synovial fibroblasts (RSF) and normal synovial fibroblasts (NSF) exposed to angiogenic stimuli: hypoxia and IL-1ß. Quantitative determination of the expression of Ang-1 and Ang-2 mRNA in RSF exposed to hypoxic culture conditions (0.5% O_2) for 8 and 24 h, over that of the expression in NSF as measured by quantitative PCR and shown as fold increase in expression (A). Quantitative determination of the expression of Ang-1 and Ang-2 mRNA in RSF (B) and NSF (C) treated with IL-1ß (1 ng/ml) for 4, 8, and 24 h, and normalized to the expression level in NSF, at time 0, as measured by quantitative PCR and shown as fold increase in expression. Error bars represent the range given for the fold increase in target mRNA. [+]Statistically significant increased or decreased expression of Ang-1 or Ang–2 in hypoxia or IL-1ß treated RSF at various time points compared to time 0; $p < 0.05$. Similar results were obtained in at least 3 independent experiments.

TGF-β. RSF and NSF were cultured in the presence of TGF-ß (20 ng/ml) for 16–24 h and levels of Ang-1 and Ang-2 mRNA were determined at time 0, 30 min, 2, 8, and 24 h. In representative data from RSF, TGF-ß caused a marked, transient increase in Ang-1 mRNA expression at 30 min, which declined over the 24 h period to be less than the levels of Ang-1 in RSF at time 0. Levels of Ang-2 were unaffected by TGF-ß in RSF (Figure 4A).

In NSF the opposite effect was observed. Levels of Ang-1 were unaffected by TGF-ß; however, upregulation of Ang-2 was observed at 30 min and again at 24 h (Figure 4B).

TNF-α. RSF and NSF were cultured in the presence of TNF-

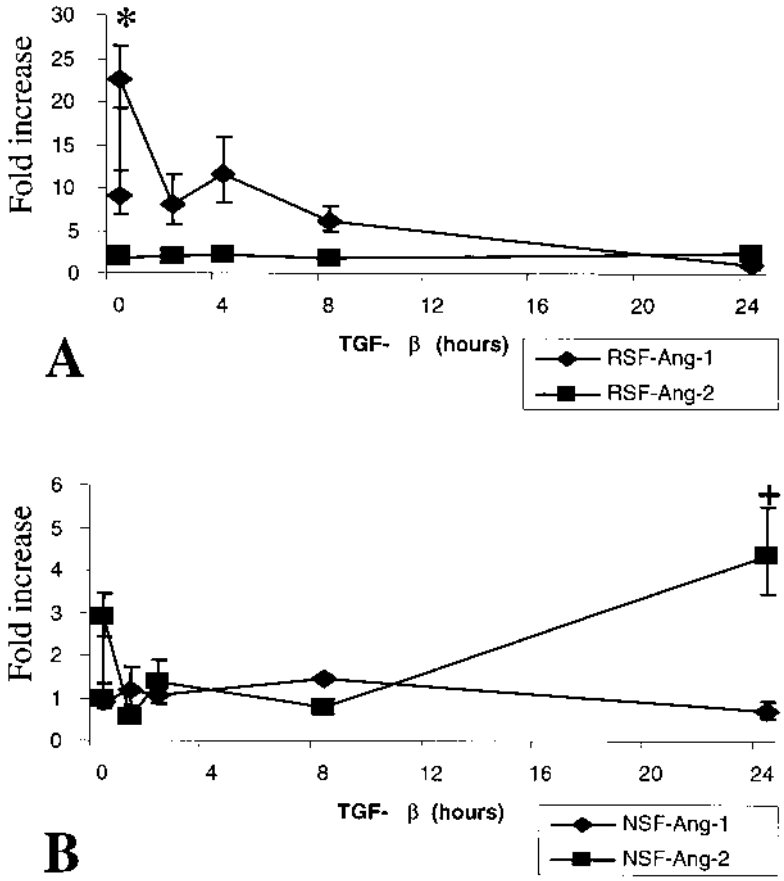

Figure 4. Ang-1 and Ang-2 expression in rheumatoid synovial fibroblasts (RSF) and normal synovial fibroblasts (NSF) exposed to angiogenic stimulus: TGF-ß. Quantitative determination of the expression of Ang-1 and Ang-2 mRNA in RSF (A) and NSF (B) treated with TGF-ß (20 ng/ml) for 30 min, 2, 4, 8, and 24 h, and normalized to the expression level in NSF, at time 0, as measured by quantitative PCR and shown as fold increase in expression. Error bars represent the range given for the fold increase in target mRNA. Statistically significant increased or decreased expression of Ang-1 or Ang–2 in TGF-ß treated RSF at various time points compared to time 0: $^{+}p < 0.05$, $^{*}p < 0.01$. Similar results were obtained in at least 3 independent experiments.

α (2 ng/ml) during a 24 h period and levels of Ang-1 and Ang-2 mRNA were determined at time 0, 30 min, 1, 2, 4, 8, 16, and 24 h for RSF and at time 0, 1, 2, 8, and 24 h for NSF (Figure 5A, 5B). In representative data from RSF an upregulatory effect on Ang-1 was observed, expression increasing up to 24 h ($p < 0.01$, ANOVA). No effect on Ang-2 levels was observed in RSF. As with TGF-ß the effect of TNF-α on Ang-1 and Ang-2 expression in NSF was opposite to that in RSF, where TNF-α induced a marked increase in Ang-2 expression at 8 h.

Autophosphorylation of Tie-2 receptor with media from RSF and RSF treated with TNF-α. To investigate whether changes in expression observed at the mRNA level resulted in changes at the functional protein level we used an assay to measure autophosphorylation of Tie-2 receptor. Autophosphorylation of Tie-2 receptor in HUVEC cells has been shown to be increased in the presence of Ang-1[22].

Measurement of autophosphorylation of Tie-2 receptor was accomplished by immunoprecipitation with Tie-2 antibodies and Western blotting with antiphosphotyrosine (anti P-Y) antibodies (Figure 6). The anti P-Y antibody revealed a low basal level of Tie-2 phosphorylation as evidenced by the intensity of the band in the absence of conditioned media (0%). Treatment of the HUVEC with conditioned media from TNF-α treated RSF revealed a 3.5-fold increase in the intensity of the phosphotyrosine Tie-2 band. Conditioned media from control RSF did not cause a substantial increase in Tie-2 phosphorylation (Figure 6, 25 and 50%); TNF-α alone did not directly cause a change in Tie-2 receptor phosphorylation in HUVEC (data not shown). This indicates that the TNF stimulated increase in Ang-1 mRNA translates to an increase in the level of secreted Ang-1 protein. The right-hand panels of Figure 6 show staining with a Tie-2 antibody to show that the total amount of Tie-2 receptor is equivalent in each lane.

DISCUSSION

The formation of neovasculature results as a consequence of

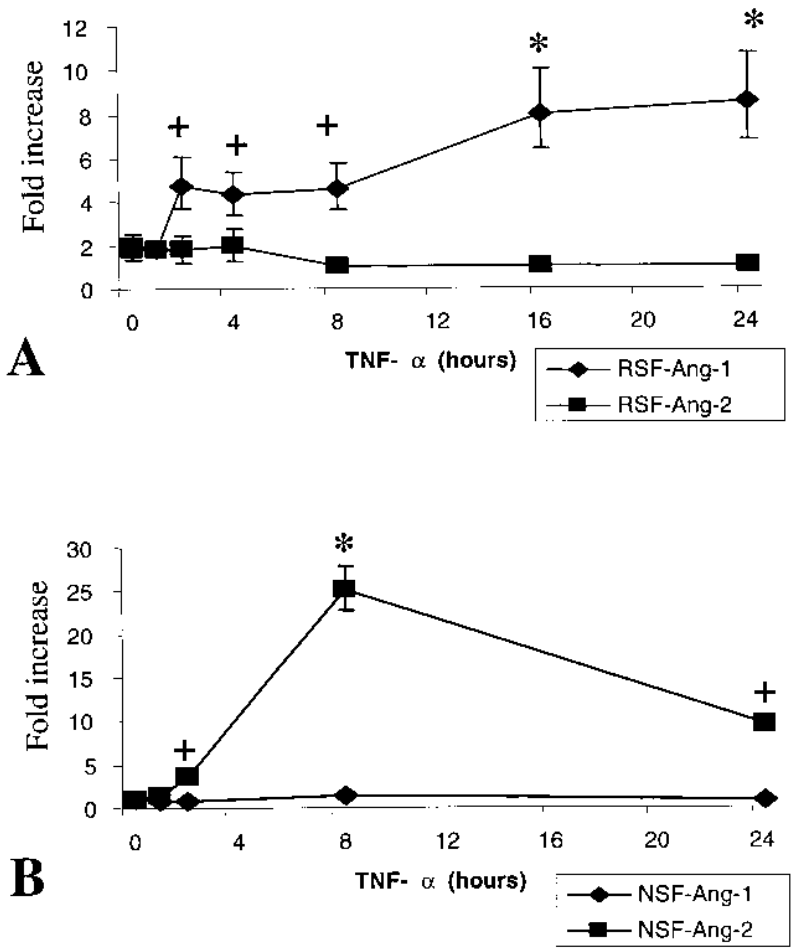

Figure 5. Ang-1 and Ang-2 expression in rheumatoid synovial fibroblasts (RSF) and normal synovial fibroblasts (NSF) exposed to angiogenic stimuli: TNF-α. Quantitative determination of the gene expression of Ang-1 and Ang-2 in RSF (A) and NSF (B) treated with TNF-α (2 ng/ml) for 30 min, 1, 2, 4, 8, 16, and 24 h, and normalized to the expression level in NSF as measured by quantitative PCR and shown as fold increase in expression. Error bars represent the range given for the fold increase in target mRNA. Statistically significant increased expression of Ang-1 or Ang–2 in TNF-α treated RSF or NSF at various time points compared to time 0; $^{+}p < 0.05$, $^{*}p < 0.01$. Similar results were obtained in at least 3 independent experiments.

sprouting from preexisting vessels, a process called angiogenesis[1]. Angiogenesis is a tightly regulated process; however, during the course of chronic inflammatory diseases this tight regulation breaks down and unregulated angiogenesis results[4]. Such unregulated angiogenesis is a major feature of RA. The inappropriate angiogenesis characteristic of RA is most likely a result of changes in angiogenic stimuli expressed by cells surrounding the endothelial cells of the existing vasculature.

The identification of an endothelial cell-specific receptor tyrosine kinase, Tie 2 receptor[6-16], and its ligands[22,23], each with a proven role in angiogenesis[23,24], led us to investigate whether these ligands were expressed by synovial fibroblasts.

We observed that synovial fibroblasts from RA joints constitutively express elevated Ang-1 and Ang-2. This expression was detected in late passage (> passage 10) RSF, indicating that the elevated production of Ang-1 and Ang-2 is constitutive and does not result solely from stimuli within the synovial environment. That diseased synovial fibroblasts constitutively express elevated Ang-1, Ang-2, and VEGF[40] indicates the central role this cell type plays in the angiogenesis that occurs in RA. We did observe variation in the levels of expression of Ang-1 and Ang-2 within the synovial fibroblast cell lines we used, and this was not totally unexpected as each line was generated from single patients and as such is susceptible to the normal variability within a human population.

In the rheumatoid synovial fibroblast lines, all derived from late stage RA, Ang-1 was highly expressed. This finding is consistent with current thinking about the role of Ang-1 in angiogenesis[26,27]. Findings from the Ang-1 gene knockout[24], the fact that Ang-1 is not affected by growth factors released during wound repair[44], the observation that Tie-2 and Ang-1 are expressed to some extent in normal human arteries and veins[26,27], and the recent report of a stabilizing effect on HUVEC network organization[44] all suggest a role for Ang-1 to

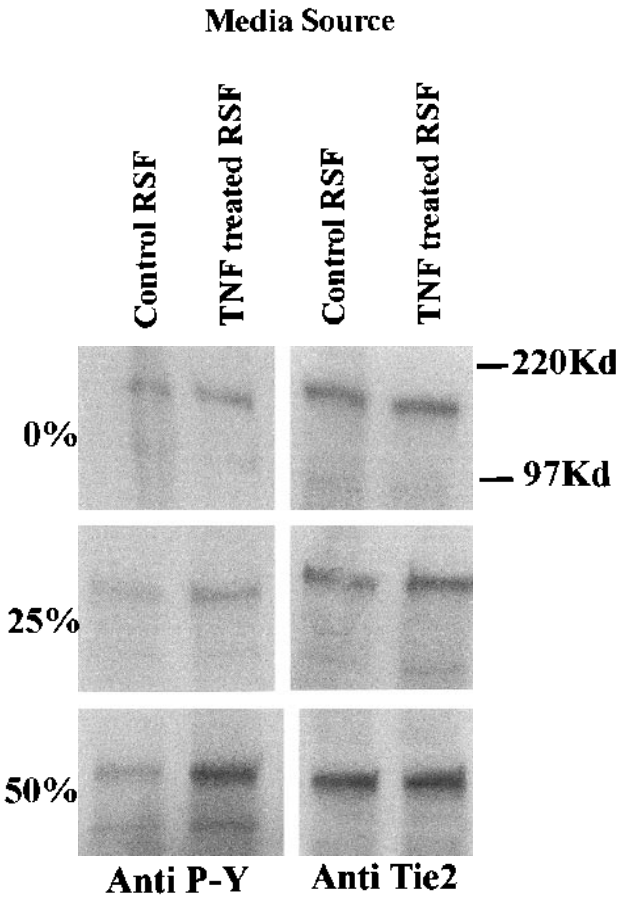

Figure 6. Autophosphorylation of Tie-2 receptor with media from rheumatoid synovial fibroblasts (RSF) and RSF treated with TNF-α. Conditioned media were harvested from RSF cultured in the presence or absence of TNF-α. Media was diluted into HUVEC cultures at 25% and 50%, and the Tie-2 receptor immunoprecipitated, Western blotted, and stained for both phosphotyrosine and Tie-2. Increases in phosphotyrosine staining correlate with an increase in Ang-1 in the conditioned medium. Anti P-Y: antiphosphotyrosine.

induce and maintain the maturation of the newly formed blood vessels. That Ang-1 is highly expressed by synovial fibroblasts derived from patients with late stage RA is in agreement with this hypothesis, in as much as there is an extensive immature neovasculature in late RA and increased Ang-1 expression may be functioning to stabilize these vessels generated earlier in disease.

We observed that the expression of Ang-1 and Ang-2 in chronic inflamed synovial tissue was also constitutive. However, the level of Ang-2 was strikingly higher than that of Ang-1 in a reversal of the observation made in the RA RSF. This suggests that additional cell types may contribute to Ang-2 expression in diseased tissue. The relative increases in Ang-2 gene expression (60- to 120-fold) as compared to the comparatively modest increases in Ang-1 (1- to 6.5-fold) gene expression in these tissues are interesting, in that chronic inflamed synovial tissue is a site of intense angiogenic activity[45]. If the hypothesis (based on observations in transgenic models and the recent report that Ang-2 may promote angiogenesis in glioblastoma blood vessels[46]) suggests that Ang-2 can be involved in the initiation of angiogenesis to destabilize the integrity of existing vessels, allowing responsiveness to other factors such as VEGF and encouraging angiogenic sprouting[26,27], then one might expect Ang-2 to be highly expressed in tissues where angiogenesis is being initiated.

To study the effect of other stimuli known to be associated with RA on the expression of Ang-1 and Ang-2, we cultured RSF or NSF in the presence of several cytokines. Several inflammatory stimuli can promote angiogenesis in animal models[1]. Most of them, including TNF-α and IL-1ß, do not stimulate endothelial cell growth in culture and are thus called indirect angiogenic factors[1]. It is thus reasonable to hypothesize that these factors function via another factor that has a direct effect on endothelial cells, such as VEGF or the angiopoietins. We observed an upregulation of Ang-1 in RSF treated with TGF-ß or TNF-α, whereas hypoxia had a downregulating effect on Ang-1 and Ang-2 expression, and IL-1ß had a downregulating effect on Ang-1 while having little or no effect on Ang-2 expression. No angiogenic stimulus we tested had an upregulating effect on Ang-2 expression in RSF. In addition, no angiogenic stimulus had an effect on Ang-1 expression in NSF; however, Ang-2 was significantly upregulated by TGF-ß and TNF-α. The changes in angiopoietin mRNA expression will not have consequences in the disease if there is not a parallel change in the level of secreted angiopoietin. We tested this by assaying for the biological activity of angiopoietin in conditioned media from TNF-α stimulated RSF. Angiopoietins have been shown to specifically induce autophosphorylation of the Tie-2 receptor in HUVEC cells[22,47]. In our studies, HUVEC incubated with conditioned media from RSF treated with TNF-α had a 3.5-fold increase in autophosphorylation of the Tie-2 receptor over that caused by conditioned media from unstimulated RSF. Although TNF-α can stimulate the expression of many proteins, none (other than the angiopoietins) are known to be capable of directly stimulating autophosphorylation of the Tie-2 receptor. This strongly suggests that the increase in expression of Ang-1 mRNA induced by TNF-α also resulted in increased Ang-1 protein secretion. Nevertheless, these data cannot eliminate the possibility that TNF-α stimulation resulted in the expression of some other novel Tie-2 receptor agonist in the conditioned media.

These results raise a number of intriguing issues. The downregulation of Ang-1 by IL-1ß has been described in human lung fibroblasts[48]. However, most interesting is when we compare these results with observations made in similar experiments in RSF on the effect of various angiogenic stimuli on VEGF expression. VEGF, which is involved in the initiation of angiogenesis, was found to be upregulated in RSF by hypoxia and IL-1ß[40], stimuli that resulted in a downregulation of Ang-1 expression. These findings suggest these 2 ligands, with different roles in angiogenesis, are differentially

modulated by these indirect angiogenic stimuli. The most dramatic effect on Ang-1 expression was the upregulation caused by TNF-α. This is in contrast to VEGF expression, which was not strongly induced by TNF-α in RSF[40].

Different results were obtained when TNF-α or TGF-ß were applied to NSF. It was seen that neither stimuli had an effect on Ang-1 expression, whereas both had an upregulatory effect on Ang-2 expression. This suggests a phenotypic difference between NSF and RSF, and that the cellular mechanisms involved in the response to TNF-α or TGF-ß stimulation are different. This allows a situation, at least in the inflamed RA joint, in which these stimuli modulate angiopoietin expression, and thus angiogenesis, differentially in early and late disease. What specific switch could account for this change in response is unclear, although changes in receptor expression for these cytokines or signal transduction changes seem likely possibilities.

These findings confirm the contribution of the synovial fibroblast to the angiogenesis that occurs in the inflamed RA joint. That the recently identified angiopoietins are constitutively expressed by RSF and modulated by inflammatory cytokines involved in the disease state suggests that interrupting the function of these ligands or their receptor Tie-2 may be beneficial in RA and other chronic inflammatory diseases.

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