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Tie2 Receptor Tyrosine Kinase, a Major Mediator of Tumor Necrosis Factor α–Induced Angiogenesis in Rheumatoid Arthritis

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***Objective*. Rheumatoid arthritis (RA) is an inflammatory disease and an angiogenic disease. However, the molecular mechanisms promoting angiogenesis in RA are not clearly identified. Our objective was to study the role of an endothelium-specific receptor tyrosine kinase, Tie2, in angiogenesis of inflammatory arthritis.**

***Methods*. Expression of Tie2 and its ligand, angiopoietin 1 (Ang1), in human synovium was examined by immunohistochemistry and Western blot. A novel synovium vascular window model was established to study the role of Tie2 in angiogenesis in vivo. Primary cultured endothelial cells and synoviocytes were used to study tumor necrosis factor α (TNFα)–induced Tie2 and Ang1 expression.**

***Results*. Tie2 was implicated in pathologic angiogenesis. We observed that Tie2 and Ang1 were elevated in human RA synovium. Using a novel collagen-induced arthritis synovial window model, we demonstrated that Tie2 signaling regulated arthritis angiogenesis in vivo. We also showed that Tie2 mediated TNFα-induced angiogenesis in a mouse cornea assay. In addition, we observed that TNFα can regulate Tie2 activation in multiple ways that may involve interactions between endothelial cells and synoviocytes. TNFα up-regulates Tie2 in endothelial cells through nuclear factor κB, and it up-regulates Ang1 in synoviocytes. These findings suggest paracrine regulation of angiogenesis between endothelial cells and synoviocytes.**

***Conclusion*. This study demonstrates that Tie2 regulates angiogenesis in inflammatory synovium. Tie2 signaling is an important angiogenic mediator that links the proinflammatory cytokine TNFα to pathologic angiogenesis.**

Inflammation and angiogenesis are two of the fundamental processes that underlie pathologic disorders. Tissue injury induces inflammation, and inflammation triggers angiogenesis, which in turn, initiates tissue repair and tissue growth (1). Rheumatoid arthritis (RA) is an inflammatory disease as well as an angiogenic disease (2–5). The joint in RA contains massive proliferating synovium, which forms an invading tissue termed pannus. The formation of pannus is central to joint erosion and results in the destruction of cartilage and bone. Angiogenesis is an important component of most inflammatory reactions and subsequent repair/growth processes (3,4,6,7). Persistent angiogenesis is critical both to maintaining the chronic architectural changes in the RA synovium via delivery of nutrients and inflammatory cells and to providing an important source of cytokines and protease activity (8).

Tie2 is an endothelium-specific receptor tyrosine kinase required for embryonic vascular development (9,10). Disruption of Tie2 function in transgenic mice results in embryonic death due to vascular defects (9,10). Angiopoietin 1 (Ang1) is an agonist ligand for Tie2. It stimulates Tie2 phosphorylation and activates Tie2 signaling (11). In Ang1 knockout mice, embryonic death

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occurs because of vascular defects resembling those in Tie2 knockout mice. Tie2 also regulates pathologic angiogenesis (12). Tie2 and Ang1 have been shown to be up-regulated in psoriasis (13) and in choroidal neovascular membranes (14). Levels of Tie2 are elevated in breast cancer tissue (15), and indeed, Tie2 activation regulates tumor angiogenesis. Blocking Tie2 action by a soluble Tie2 protein (ExTek) inhibits tumor angiogenesis and tumor growth in vivo (16). Systemic delivery of ExTek by an adenoviral vector has been shown to inhibit the growth of both well-established primary tumors and tumor metastases of a mammary tumor and a melanoma (17). Similar findings of Tie2-mediated pathologic angiogenesis have been reported in hepatocellular carcinoma (18), melanoma (19), and in retinal and choroidal neovascularization (20). Tie2 protein and Ang1 messenger RNA (mRNA) have also been detected in RA synovium (21,22).

Tumor necrosis factor α (TNFα) plays a major role in regulating inflammation and angiogenesis in RA synovium (23–25). Synovial fluids from RA patients induce angiogenesis partly through the TNFα pathway (26). Anti-TNFα treatment in RA patients inhibits vascularity in synovium (23,27,28). It has been suggested that the angiogenic properties of TNFα may be mediated through secondary angiogenic mediators, such as vascular endothelial growth factor (VEGF), interleukin-8, basic fibroblast growth factor (bFGF), VEGF receptor 2 (VEGFR-2) and its coreceptor neuropilin-1, and ephrine A1 (29–32). Recently, Willam et al reported that TNFα regulates Tie2 expression in endothelial cells (33). Scott et al reported that TNFα stimulates Ang1 mRNA expression in synoviocytes (34). However, the role of Tie2 signaling in RA has not been determined.

Although the importance of angiogenesis in arthritis progression has been well recognized, the molecular mechanisms promoting angiogenesis in RA have not been clearly identified. Both synovial tissue and synovial fluid are enriched in angiogenesis factors (2,35). How these factors are regulated in RA synovium is not clear. Little research has been done to examine possible communication between endothelial cells and synoviocytes. In this study, we examined the role of Tie2 signaling in the angiogenesis of inflammatory arthritis. We observed Tie2 and Ang1 expression in human RA synovium; TNFα regulated Tie2 signaling, and Tie2 mediated TNFα-induced angiogenesis in collagen-induced arthritis (CIA). Using a novel synovium vascular window model, we demonstrated that blockade of Tie2 activation inhibited angiogenesis in vivo. This study demonstrates an important role of Tie2 signaling in angiogenesis of inflammatory arthritis.

MATERIALS AND METHODS

Materials. Recombinant human TNFα was purchased from R&D Systems (Minneapolis, MN). The mouse monoclonal antibody against Tie2, 33.1, was prepared in our laboratory (15). Polyclonal antibodies against Ang1 (H-98 and N-18) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant replication-defective adenovirus AdIκBα, a mutated inhibitor of nuclear factor κBα (IκBα), was a gift from Dr. D.A. Brenner, University of North Carolina at Chapel Hill (36). Adenoviral vectors directing the expression of ExTek (AdExTek [17]) or a soluble TNFα receptor (AdsTNFR [37]) were used to block Tie2 activation and TNFα function, respectively. An adenoviral vector directing the expression of green fluorescent protein (AdGFP) was used as a control vector in the experiments. The adenoviral vectors were propagated in 293 cells and purified by CsCl gradients as described elsewhere (17). Virus titers were determined by optical densitometry, and recombinant viruses were stored in 10% glycerol at -80°C.

Synovium vascular window model. The animal studies were approved by the Institution Animal Care and Use Committee (IACUC) at Vanderbilt University Medical Center. An in vivo synovium vascular window model was adapted from our tumor window as previously described (16). This consists of a metal frame applied to the back skin fold on syngeneic DBA/1j mice (The Jackson Laboratory, Bar Harbor, ME). Briefly, one 0.8-cm diameter hole was dissected in one side of the epithelial surface of the dorsal skin flap. The underlying tissue was dissected away until a fascial plane with associated vasculature remained. A 0.1-mm^3 piece of RA synovium isolated from a CIA mouse paw joint was then placed onto the fascial plane. Saline solution was added and the chamber was then sealed with a glass coverslip to form a semitransparent chamber. RA synovium in the window chambers (200-μm thick) was photographed using a microscope for vascular length density measurement.

CIA model. The animal studies were approved by the IACUC at Vanderbilt University Medical Center. Disease-susceptible DBA/1j mice developed polyarthritis ~3–4 weeks after primary immunization with bovine type II collagen (BII). Arthritis was induced following the standard immunization protocol by intradermal injection at the base of the tail with 100 μl of emulsion containing 100 μg of BII in Freund's complete adjuvant. After 21 days, the mice were boosted with 100 μg of BII in Freund's incomplete adjuvant. The signs of arthritis were monitored every other day from this time point (38).

Measurement of synovium vascular length density. Synovium vascular length density was measured and used as an indicator of arthritis-induced angiogenesis. It was measured from photographs of 10-day-old RA synovium within the window as previously described (16). From 3 to 5 areas inside the synovium were randomly selected for measurement. The vascular length density in mm/mm^3 was calculated using the following formula: length density = N/(4gdt), where N is the average number of intersections between vessels and grid per sheet, g is the number of blocks in the grid (54 blocks), d is the length of one grid square calibrated by a micrometer image at the same magnification (0.1333 mm), and t is the measured depth of field through which microvessels could be discerned (0.2 mm).

Statistical analysis. Results are reported as the mean ± SEM for synovium vascular length density for each group. Student's 2-tailed *t*-test was used to analyze statistical differences between the control-treated group and the ExTek-treated group. *P* values less than 0.05 were considered statistically significant.

Mouse cornea micropocket assay. The animal studies were approved by the IACUC at Vanderbilt University Medical Center. Eight-week-old C57BL mice were used for all experiments. The assay was performed as described previously (16). Briefly, a hydron pellet containing 5 ng of TNFα was implanted into a surgically created micropocket at 0.5–1 mm from the limbus. The mice were then divided into two groups. Mice in the control group were injected intravenously (IV) with AdGFP. Mice in the other group received IV injections of AdExTek (17). On day 7 after pellet implantation, mice were killed, and corneas were perfused intraventrically with India ink. The corneas were dissected and examined under a Provis microscope (Olympus, Lake Success, NY) configured for digital imaging. Images were analyzed using image analysis software (NIH Image, National Institutes of Health, Bethesda, MD; online at: http://rsb.info.nih.gov/nih-image/) to determine the circumference area of neovascularization as an index of corneal angiogenesis.

Cell culture. Primary human synoviocytes were isolated as described previously (39) from patients who underwent joint replacement. Synoviocytes were grown on 0.1% gelatin–coated plates in RPMI (BioWhittaker, Walkersville, MD) plus 20% fetal bovine serum and 1% antibiotic/antimycotic solution (Gibco, Grand Island, NY) in a humidified incubator with 5% CO_2 at 37°C. Prior to incubation with TNFα, cells were cultured to 80% confluence and then serum-starved in RPMI for 12 hours. Human umbilical vein endothelial cells (HUVECs) were generously provided by Dr. Douglas Vaughan, Vanderbilt University Medical Center. HUVECs were grown on 0.1% gelatin–coated plates in endothelial growth medium (Clonetics, San Diego, CA) in a humidified incubator with 5% CO_2 at 37°C. HUVECs at passages 3–7 were used in this study. Prior to incubation with TNFα, the cells were cultured to 80% confluence and then starved in endothelial basal medium (Clonetics) for 12 hours. For gene delivery using adenoviral vector, the cells were infected with adenovirus for 12 hours prior to serum starvation, followed by stimulation with TNFα.

Immunohistochemistry. Synovium was recovered from patients who underwent joint replacement. The tissue was freshly embedded in OCT compound (Sakura Finetek, Torrance, CA). Frozen sections (7μ) were cut, fixed in ice-cold acetone for 10 minutes, and blocked with 1% horse serum and avidin/biotin blocking reagents (Vector, Burlingame, CA). The sections were then incubated with monoclonal antibody 33.1, antibody against Ang1, or IgG control in a humidified chamber for 1 hour at room temperature. A biotinylated secondary antibody was applied for 30 minutes, followed by another 30-minute incubation with streptavidin-conjugated horseradish peroxidase (HRP). Peroxidase activity was localized with diaminobenzidine (DAB) and was enhanced by DAB-enhancing solution (Vector).

Western blotting. Synovial tissues were harvested from patients, then lysed in radioimmunoprecipitation assay buffer plus proteinase inhibitors and vanadate (17). Cellular proteins were collected, and the protein content was measured using a BCA protein assay kit (Bio-Rad, Hercules, CA). Proteins (20 μg per sample) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Immunoblotting was performed with an anti-Tie2 antibody for 1 hour at room temperature. The membrane was washed and incubated with HRP-conjugated goat anti-rabbit IgG. The membrane was developed using enhanced chemiluminescence Western blotting detection reagents. The same membrane was stripped and reblotted with an anti-Ang1 antibody, then restripped and reblotted with an anti–β-tubulin antibody.

RESULTS

Expression of Tie2 and its ligand Ang1 in human RA synovium. Tie2 signaling plays important roles in tumor angiogenesis (16,17). To determine whether this pathway contributes to angiogenesis in RA, we first examined Tie2 and Ang1 expression in human RA synovium. High levels of Tie2 and Ang1 were detected in RA synovium (Figure 1). As expected, Tie2 staining was localized to the vascular endothelium (Figures 1B and b). Ang1 was expressed in synoviocytes and infiltrating inflammatory cells throughout the RA synovium and was highly expressed in cells that surround the endothelium (Figures 1C and c). The presence of Tie2 and Ang1 proteins was also confirmed by Western blot analysis. RA synovium expressed higher levels of both Tie2 and Ang1 than those observed in a tissue sample from a normal individual who had undergone joint replacement because of an injury (Figure 1D). These data provide evidence suggesting that Tie2/Ang1 signaling is elevated in RA synovium and that it may contribute to angiogenesis in RA.

Angiogenicity of arthritic synovium and regulation of angiogenesis in arthritis by Tie2 activation. To determine the role of Tie2 in arthritis-related angiogenesis, we established a novel in vivo synovium vascular window model. The vascular window model allowed us to directly examine angiogenesis noninvasively during the evolution of arthritis. Synovium was collected from the paw of a mouse CIA. The CIA model is a widely accepted animal model that has histologic and clinical manifestations resembling those of RA (38).

The in vivo semitransparent synovium vascular window was established on the back skin fold of a DBA/1j mouse. As expected, CIA synovium was highly

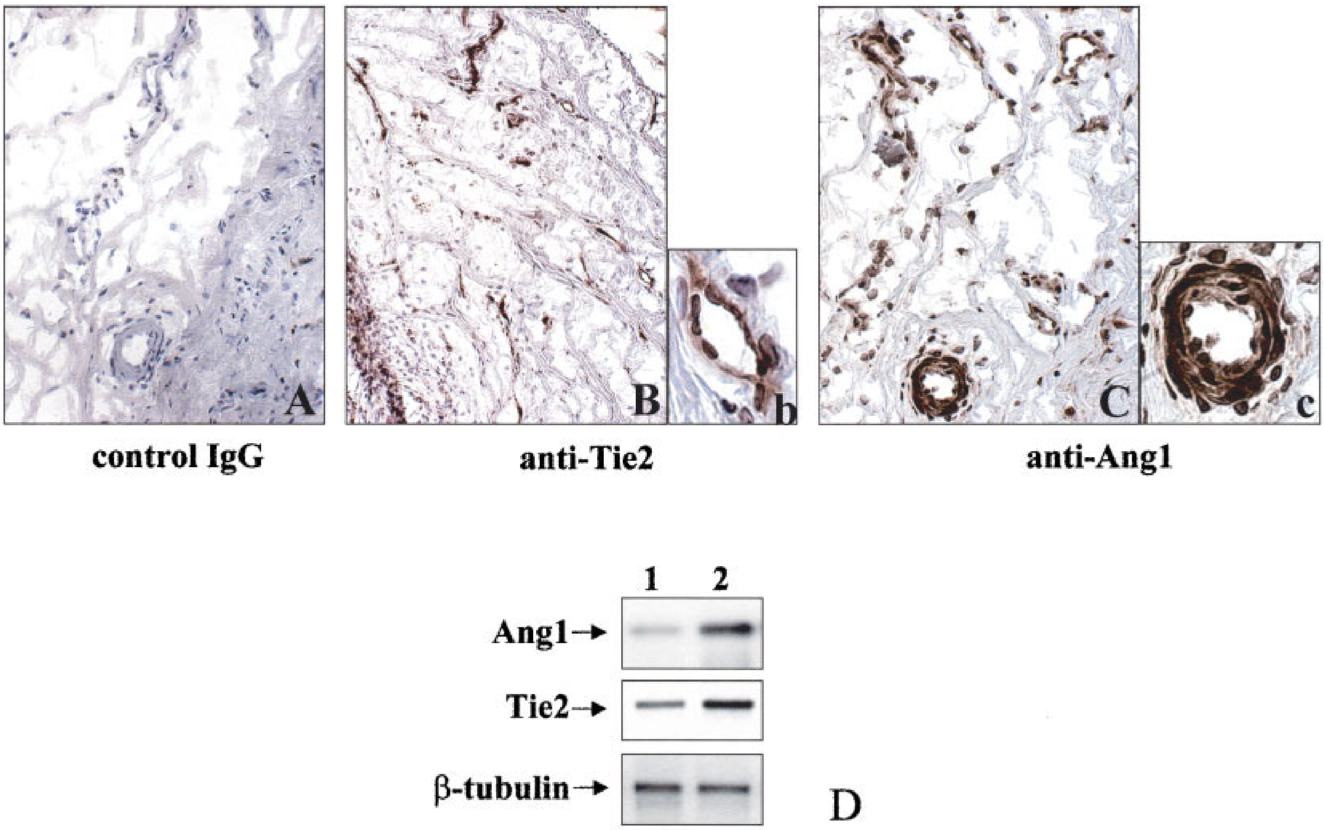

Figure 1. Expression of Tie2 and angiopoietin 1 (Ang1) in human rheumatoid arthritis (RA) synovium. Synovium was obtained from an RA patient and immediately embedded in OCT compound. Frozen sections were subjected to immunohistochemical analysis and staining with **A**, control IgG, **B** and **b**, anti-Tie2 antibody, and **C** and **c**, anti-Ang1 antibody. (Original magnification × 100 in **A**, **B**, and **C**; × 600 in **b** and **c**.) **D,** Synovial tissues obtained from a normal individual undergoing joint replacement because of injury (lane 1) and from an RA patient (lane 2) were lysed, and the tissue lysates were analyzed by Western blotting for reactivity to anti-Tie2 antibody, anti-Ang1 antibody, and control anti–β-tubulin antibody.

angiogenic, since it produced high levels of angiogenic factors. Implantation of synovium isolated from a CIA mouse paw into the window chamber induced a dramatic angiogenesis within 8 days, and the synovium survived. In contrast, implanted normal joint tissue failed to induce angiogenesis, and the tissue died within days (Figure 2A).

To determine whether Tie2 signaling plays a role in angiogenesis in CIA, mice were divided into two groups 1 day after establishment of synovium windows. One group received IV AdGFP (1×10^9 plaque-forming units [PFU]); the other group received IV AdExTek (1×10^9 PFU). Control-treated synovium developed numerous vessels within 10 days of synovium implantation. In contrast, the AdExTek-treated group developed significantly fewer blood vessels. The vessels were not well formed, and we often observed hemorrhage within the treated synovium window (Figure 2B). Compared with the control-treated group, a significant reduction of vascularity in the AdExTek-treated group was demonstrated after measuring the vascular length density as an index of vascularity (Figure 2C). These data strongly support an important role of Tie2 signaling in arthritis-induced angiogenesis.

Tie2 as an important mediator of TNFα-induced angiogenesis in vivo. TNFα plays an essential role in the development of RA, and it is found in large quantities in RA synovium. TNFα also regulates angiogenesis in vivo (25,40,41). Anti-TNFα treatment in RA patients reduces vascularity in the rheumatoid synovium (23,27,28). However, studies of the angiogenic properties of TNFα have yielded contradictory results, and it has been suggested that its angiogenic function is mediated through secondary angiogenic factors (25,29–32,41,42).

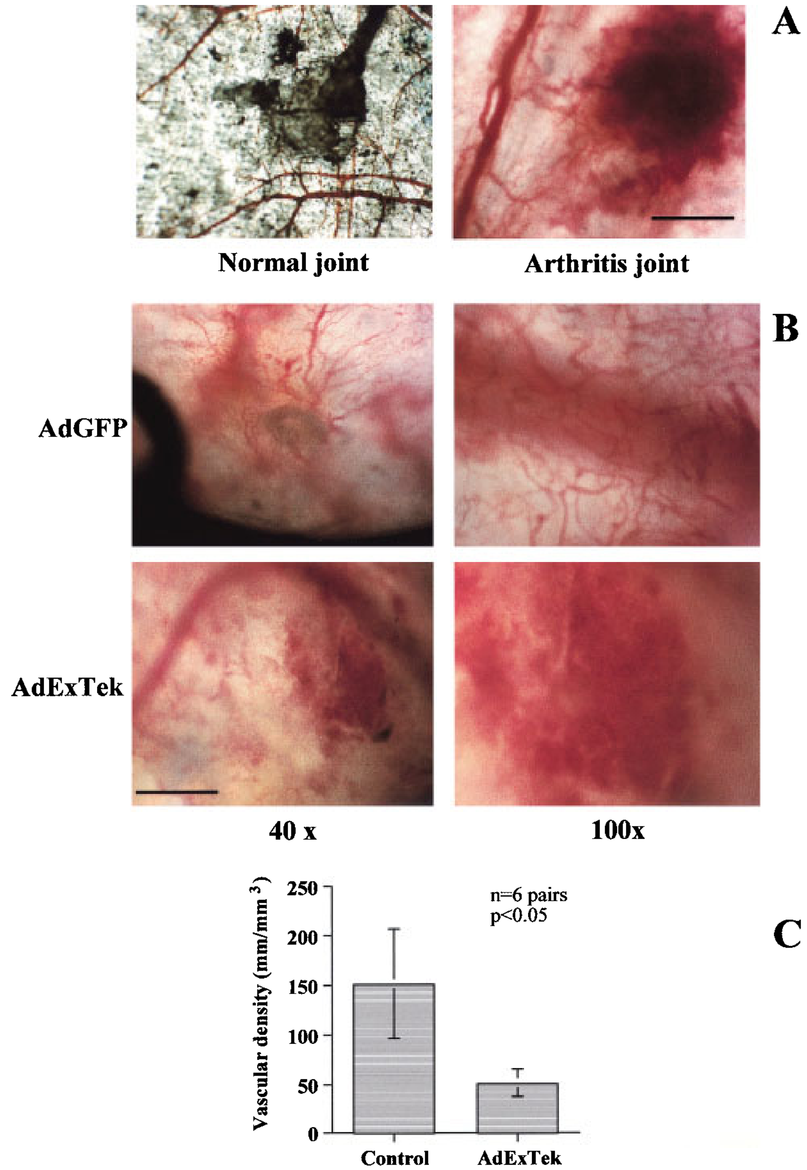

Figure 2. Regulation of angiogenesis by activation of Tie2 in synovium from mice with collagen-induced arthritis (CIA). **A,** CIA synovium induced strong angiogenesis. A novel synovium vascular window model was established on the back skin fold of a DBA/1j mouse to study arthritis-induced angiogenesis. Synovium samples were isolated from a donor CIA mouse paw joint. A small piece of CIA synovium (right panel) or normal joint tissue (left panel) was implanted into the vascular window. Photographs show synovium windows in live mice. Bar = 1 mm. **B,** Blocking Tie2 signaling significantly inhibited angiogenesis in CIA synovium. Synovium vascular windows were established with implantation of CIA synovial tissues. The mice were then divided into 2 groups (n = 6 per group). One group received an intravenous (IV) injection (1×10^9 plaque-forming units) of an adenoviral vector directing the expression of soluble Tie2 protein (AdExTek) to block Tie2 activation. The other (control) group received an IV injection (same dose) of an adenoviral vector directing the expression of green fluorescent protein (AdGFP). Live window photographs were taken 10 days after tissue implantation. The AdGFP-treated group developed numerous blood vessels around the synovial tissue (upper panels). In contrast, the AdExTek-treated group exhibited significantly fewer angiogenic vessels and more hemorrhage (lower panels). Bar = 1 mm. (Original magnifications are shown across the bottom.) **C,** CIA synovium vascular length density, an indicator of synovium angiogenesis, was measured from photomicrographs of the synovium window chambers as shown in **B**. Blocking the action of Tie2 by AdExTek resulted in an ~60% reduction in synovium vascular length density ($P < 0.05$ by paired *t*-test). Values are the mean ± SEM.

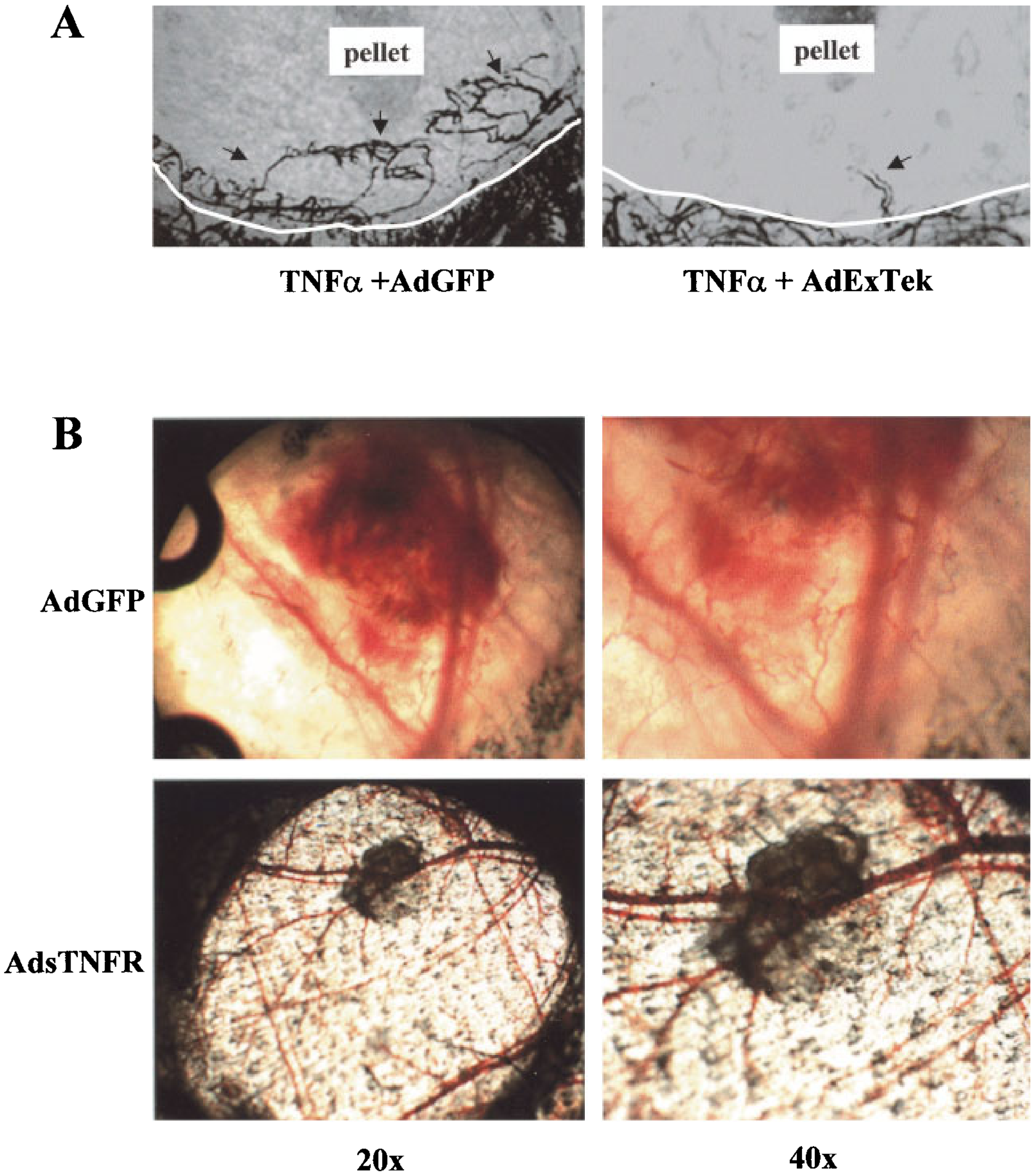

Figure 3. Tie2 as an important mediator of tumor necrosis factor α (TNFα)–induced angiogenesis in vivo. **A,** Blocking Tie2 signaling by ExTek inhibited TNFα-induced angiogenesis in a cornea model. A TNFα pellet was implanted into a surgically created micropocket on the cornea of mice. The next day, the animals were divided into two groups (n = 4 per group). One group received AdExTek by IV injection, and the other (control) group received AdGFP by IV injection. On day 7 after pellet implantation, the animals were killed, and the corneas were perfused with India ink and then excised to obtain the record of the vascular pattern of growth. Representative photographs are shown. TNFα induced corneal angiogenesis (TNFα + AdGFP) that was significantly inhibited when Tie2 function was blocked (TNFα + AdExTek). **Arrows** above the white lines indicate vessels newly formed in response to TNFα. The vessels below the white lines are original limbus vessels. **B,** Neutralizing TNFα function inhibited angiogenesis in CIA synovium. Synovium vascular windows were established with implantation of CIA synovial tissues. The mice were divided into two groups (n = 3 per group). One group received an IV injection of an adenoviral vector directing the expression of a soluble TNFα receptor (AdsTNFR; 1×10^9 plaque-forming units) to block TNFα function. The other (control) group received AdGFP (same dose) by IV injection. Live window photographs were taken 10 days after tissue implantation. The AdGFP-treated group developed numerous blood vessels around the synovial tissue (upper panels). In contrast, neutralizing TNFα function by systemic expression of a soluble TNFα receptor blocked rheumatoid arthritis angiogenesis (lower panels). See Figure 2 for other definitions. (Original magnifications are shown across the bottom.)

Since we observed that both Tie2 and Ang1 were expressed in RA synovium, we used a mouse cornea micropocket assay to examine whether Tie2 signaling contributed to TNFα-induced angiogenesis. A weak angiogenic response was seen within 7 days of implanting a TNFα pellet (5 ng/pellet/eye) (Figure 3A). To block Tie2 function, we simultaneously injected 1×10^9 PFU of AdExTek at the initiation of the cornea assay. ExTek protein is a soluble form of Tie2 receptor and functions as a Tie2 inhibitor (16). ExTek protein was detected 1 day after virus injection, and its production lasted through the course of the experiment (data not shown). Blocking Tie2 action by this approach almost completely inhibited TNFα-induced angiogenesis compared with the AdGFP-treated control group (Figure 3A). These data support the notion that TNFα indirectly regulates angiogenesis and that Tie2 is a major mediator of TNFα-induced angiogenesis in vivo. The data indicate that Tie2 may mediate TNFα-induced angiogenesis in RA synovium.

In addition, we examined the role of TNFα in angiogenesis associated with inflammatory arthritis. We implanted a small amount of CIA synovial tissue into the windows in DBA/1j mice. The mice received an IV injection of either a control virus (AdGFP) or a soluble TNFα receptor adenoviral vector (AdsTNFR [37]) at a dose of 1×10^9 PFU per mouse. Soluble TNFα receptor binds TNFα and neutralizes its function. Control-treated CIA synovium developed numerous vessels within 10 days of synovium implantation. In contrast, systemic expression of a soluble TNFα receptor using AdsTNFR blocked angiogenesis induced by CIA in vivo (Figure 3B). The data show that the proinflammatory cytokine TNFα induces angiogenesis in inflammatory arthritis.

TNFα-induced Tie2 up-regulation in endothelial cells mediated through the transcription factor nuclear factor κB (NF-κB). We showed that Tie2 was expressed in RA synovium (Figures 1 and 2) and that Tie2 functioned as an important mediator of TNFα-induced angiogenesis (Figure 3). To determine whether TNFα regulates angiogenesis through Tie2 signaling, we examined Tie2 expression in cultured endothelial cells. Stimulation with TNFα up-regulated Tie2 expression in HUVECs in a dose-dependent manner (Figure 4A). A time-response curve was obtained in HUVECs using 3 ng/ml of TNFα as a stimulator (Figure 4B). We observed that up-regulation started at 1 hour and peaked at 4 hours poststimulation. The same membrane was reprobed with an anti–β-tubulin antibody as a loading control. The data were consistent with our early findings reported above that Tie2 signaling mediates TNFα-induced angiogenesis in vivo, indicating that TNFα may induce angiogenesis through the Tie2 pathway by increasing Tie2 expression in endothelium.

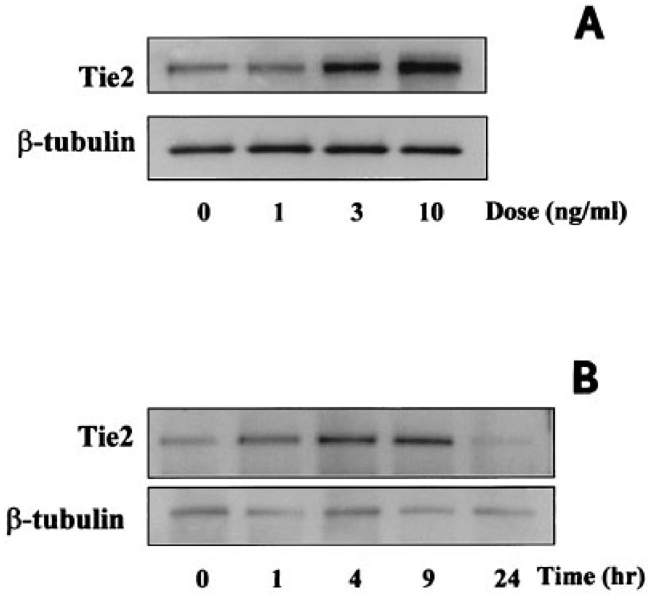

Figure 4. Tie2 expression up-regulated by tumor necrosis factor α (TNFα) in cultured endothelial cells. Serum-starved human umbilical vein endothelial cells were stimulated with **A,** different doses of TNFα for 4 hours and **B,** 3 ng/ml of TNFα for various amounts of time as indicated. Cells were then lysed, and the cell lysates were subjected to Western blot analysis and probed with an anti-Tie2 antibody. The experiments were repeated 3 times.

TNFα is known to act through the NF-κB pathway. Therefore, we tested whether NF-κB regulated TNFα-induced Tie2 expression. Prior to the TNFα stimulation, we infected HUVECs with AdIκBα, which expresses a mutated IκBα to block NF-κB function. Expression of IκBα in HUVECs completely blocked Tie2 up-regulation induced by TNFα (Figure 5A) in a dose-dependent manner (Figure 5B). Overexpression of a control protein, GFP, had no effect on TNFα-induced Tie2 expression. The data suggest that the TNFα-induced Tie2 expression is mediated through NF-κB.

Ang1 expression in human synoviocytes up-regulated by TNFα. Tie2 is predominantly expressed in vascular endothelium, but Ang1 is mainly produced by other cell types surrounding the endothelium. In addition to the presence of Tie2, there are high levels of Ang1 in RA synovium (Figure 1). Therefore, we studied Ang1 expression in human synoviocytes. Four lines of synoviocytes were established from 4 patients who either

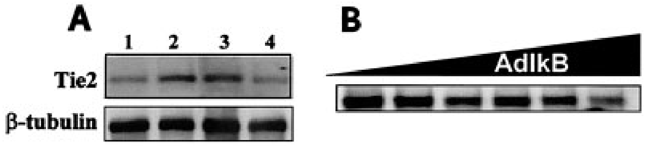

Figure 5. Tumor necrosis factor α (TNFα)–induced Tie2 up-regulation in endothelial cells mediated through nuclear factor κB (NF-κB). Cultured human umbilical vein endothelial cells (HUVECs) were infected with recombinant replication-defective adenovirus AdIκBα (a mutated inhibitor of NF-κBα) or with adenoviral (control) vector directing the expression of green fluorescent protein (AdGFP) for 24 hours followed by serum starvation for 12 hours. Cells were then stimulated with TNFα at 3 ng/ml for 4 hours. Cell lysates were subjected to Western blot analysis and probed with an anti-Tie2 antibody. **A,** Lane 1, without stimulation; lane 2, with TNFα stimulation; lane 3, infected with AdGFP and then treated with TNFα; lane 4, infected with AdIκBα and then treated with TNFα. **B,** AdIκBα inhibited Tie2 expression in response to TNFα stimulation in a dose-dependent manner. HUVECs were infected with increasing doses of AdIκBα virus (to increase IκBα expression) prior to TNFα stimulation, yielding gradual decreases in Tie2 expression.

had RA or had undergone joint replacement (non-RA patients). Cultured human synoviocytes were lysed, and the cell lysates were analyzed by Western blotting and probed with an antibody against Ang1. High levels of Ang1 were easily detected in all 4 lines of synoviocytes compared with the control NIH3T3 cells, which had little or no detectable Ang1 (Figure 6A). The data suggest that paracrine regulation may be present between endothelial cells and synoviocytes in the inflamed synovium. Accordingly, synoviocytes may produce Ang1, and Ang1 may activate Tie2 on the endothelium to induce angiogenesis.

Since we observed that Tie2 signaling is a mediator of TNFα-induced angiogenesis (Figure 3), we examined the effects of TNFα treatment on Ang1 production in synoviocytes. Cultured synoviocytes from an RA patient (CT line) were serum-starved and then stimulated with TNFα at 3 ng/ml for various amounts of time in order to obtain time-response curves (Figure 6B) or with various amounts of TNFα for 24 hours in order to obtain dose-response curves (Figure 6C). The same membrane was reprobed with an anti–β-tubulin antibody as a loading control. Stimulation of human synoviocytes with TNFα up-regulated Ang1 expression in a time- and dose-dependent manner (Figures 6B and C).

DISCUSSION

RA progression depends on angiogenesis. Study of the molecular mechanism of RA angiogenesis offers the promise of developing better and more specific inhibitors for RA treatment. Tie2 signaling regulates pathologic angiogenesis, which includes tumor (15–17,19,43), psoriasis (13), and choroidal (14) neovascularization. Recently, Tie2 and Ang1 were detected in RA synovium (21,22). However, the function of Tie2 signaling in RA has not been determined. We report here that Tie2 signaling is a major mediator of TNFα-induced angiogenesis and plays an important role in RA angiogenesis. We observed that both Tie2 and Ang1 proteins were elevated in human RA synovium. We further demonstrated that Tie2 signaling mediated synovial angiogenesis. Blockade of Tie2 action significantly inhibited angiogenesis in CIA synovium, as demonstrated with the use of a novel synovium vascular window model.

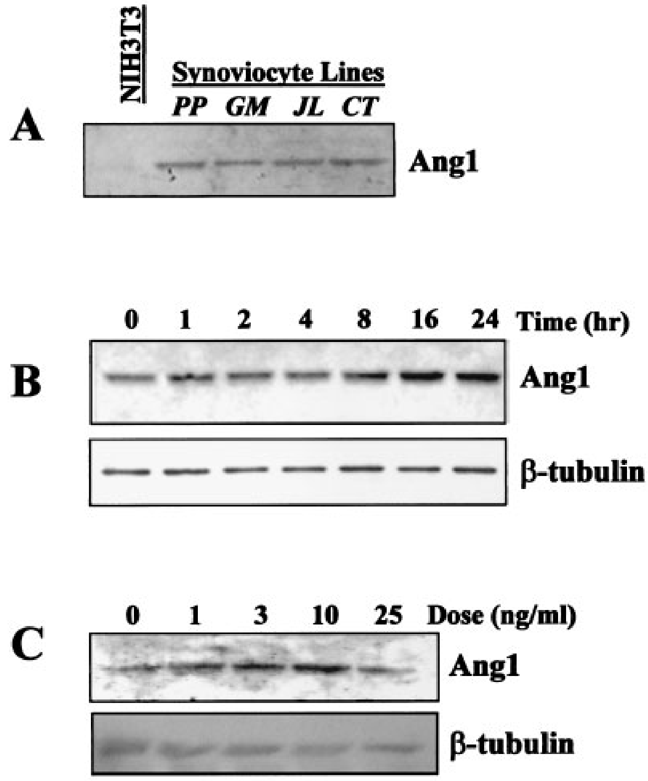

Figure 6. Angiopoietin 1 (Ang1) expression in synoviocytes up-regulated by tumor necrosis factor α (TNFα). **A,** Four lines of synoviocytes (PP, GM, JL, and CT) and control NIH3T3 cells were lysed and subjected to Western blot analysis and probed with an anti-Ang1 antibody. Synoviocytes (CT line) were stimulated with **B,** TNFα at 3 ng/ml for various amounts of time to obtain a time-response curve or with **C,** various amounts of TNFα for 24 hours to obtain a dose-response curve. **C,** Cell lysates were subjected to Western blot analysis and probed with an anti-Ang1 antibody. The experiments were repeated 3 times.

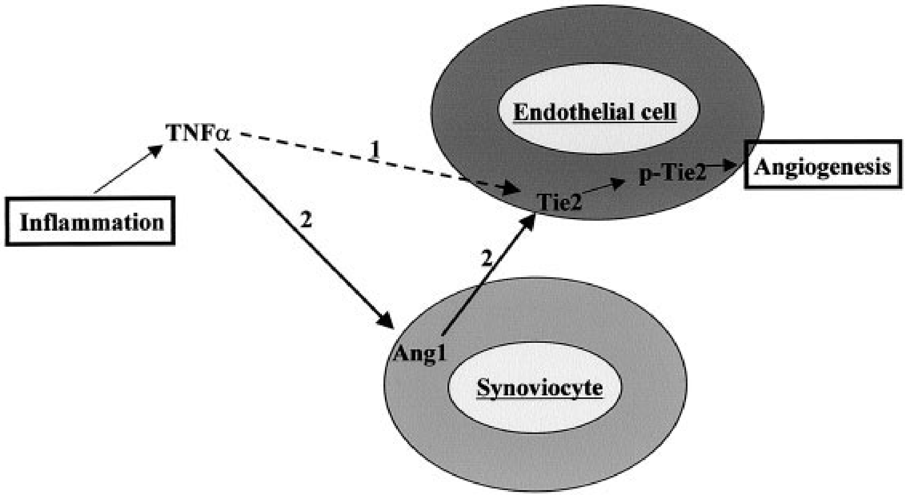

Figure 7. Model of the possible molecular mechanism of TNFα-mediated angiogenesis through the Tie2/Ang1 pathway. The inflammatory cytokine TNFα increases Tie2 activation by two different mechanisms involved in cell–cell communications between endothelial cells and synoviocytes. In mechanism 1, TNFα up-regulates Tie2 in endothelial cells. In mechanism 2, TNFα up-regulates the expression of the Tie2 ligand, Ang1, in synoviocytes. p-Tie2 = phosphorylated Tie2 (see Figure 6 for other definitions).

TNFα plays important roles in the development of arthritis and induces angiogenesis. However, it has been suggested that TNFα indirectly regulates angiogenesis through secondary factors (29–32). Our data confirm that TNFα mediates angiogenesis. In addition, we identified Tie2 signaling as an important mediator of TNFα-induced angiogenesis in vivo. Blocking the action of Tie2 was also shown to inhibit TNFα-induced angiogenesis in a corneal angiogenesis model.

To understand the molecular mechanism that induces Tie2 signaling, we investigated Tie2 and Ang1 expression in response to TNFα treatment. We observed that TNFα could regulate Tie2 activation in multiple ways that may involve interactions between endothelial cells and synoviocytes (Figure 7). TNFα up-regulated Tie2 in endothelial cells (Figure 4), and the induction of this up-regulation was mediated through NF-κB (Figure 5). Further, TNFα up-regulated Ang1 in synoviocytes (Figure 6). These data confirm the finding by Willam et al that TNFα regulates Tie2 expression (33), and extend this finding to show that Tie2 induction is mediated through NF-κB. Collectively, our data support an important role of Tie2 in mediating angiogenesis in inflammatory arthritis, suggesting that targeting the Tie2 pathway may offer the potential of developing more specific inhibitors for disease therapy.

RA synovium contains many angiogenic factors, including bFGF and VEGF (8,35), and this is consistent with the highly vascularized nature of RA synovium. In the present study, we established a novel, noninvasive synovium vascular window model, which allows the direct visualization of synovium angiogenesis in vivo. Using this approach, we demonstrated that blocking Tie2 activation significantly inhibits angiogenesis in CIA synovial tissues even in the presence of other angiogenic factors. This result is consistent with earlier findings in neoplasms, in which blocking Tie2 activation inhibited tumor angiogenesis even when the tumor produced other angiogenic factors, such as VEGF (16,17,19,43).

TNFα plays important roles in RA pathogenesis. Targeting TNFα action has proved to be very effective in treating RA patients, and it is associated with reduced angiogenesis. However, studies of the angiogenic properties of TNFα have yielded contradictory results. On the one hand, TNFα induces angiogenesis in vivo (40), and anti-TNFα treatment in RA patients results in reduction of vascularity in the synovium (23). On the other hand, TNFα inhibits the action of mitogens such as bFGF and VEGF on endothelial cell growth in vitro and down-regulates the expression of VEGFR-2 (41,44,45). Therefore, it has been suggested that the angiogenic property of this cytokine might be mediated through secondary angiogenic factors (29,31,32). Our study reveals that Tie2 and Ang1 are important factors that may mediate TNFα-induced angiogenesis in arthritis. These findings link a potent proinflammatory cytokine to a novel receptor tyrosine kinase pathway that may function in pathologic angiogenesis.

In conclusion, our data connect inflammation to angiogenesis through the TNFα–Tie2/Ang1 pathway. Understanding the molecular mechanisms whereby inflammation induces angiogenesis holds tremendous potential for understanding disease progression as well as for developing more specific inhibitors for therapy.

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