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ANGIOGENESIS IN RHEUMATOID ARTHRITIS

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1. ABSTRACT

Endothelial cells lining the lumina of blood vessels are involved in leukocyte extravasation underlying inflammatory states, such as rheumatoid arthritis (RA). New vessel formation, termed angiogenesis, is also crucial for leukocyte extravasation during inflammatory synovitis. The outcome of neovascularization in the RA synovium is highly dependent on the balance or imbalance between angiogenic mediators and inhibitors. There have been several attempts to therapeutically interfere with the cellular and molecular mechanisms underlying RA-associated neovascularization. Most studies have been performed using animal models of arthritis. In addition, a limited number of human clinical trials gave promising results. In this review, authors summarize some relevant information on those angiogenic and angiostatic agents, which have also been studied in context with RA. In addition, further perspectives of anti-angiogenic therapy in arthritis are also discussed. Specific targeting of angiogenesis may be useful in the future management of various inflammatory, as well as malignant, diseases.

2. INTRODUCTION

Angiogenesis is involved in homeostatic processes, such as reproduction, development, and tissue repair, as well as in pathological states including, among others, rheumatoid arthritis (RA), other inflammatory diseases and tumors. The angiogenic process, its mediators and inhibitors, and its clinical relevance for diagnostics and therapy have been discussed extensively in a number of review papers (reviewed in 1-6).

In RA, leukocytes emigrate into the synovium through the vascular endothelium resulting in synovial inflammation and, eventually, joint destruction. The RA synovial tissue is rich in newly formed vessels. Angiogenesis enhances leukocyte extravasation into the synovium and thus the progression of RA. Therefore, RA is considered a member of the family of "angiogenic diseases" (reviewed in 1-9). Numerous angiogenic mediators including growth factors, some cytokines, chemokines, matrix components, hypoxia itself and others have been implicated in capillary formation. In addition, angiostatic agents, such as anti-inflammatory

Table 1. Some mediators of angiogenesis in RA[1]

1. Growth factors	bFGF, aFGF, VEGF, ECGF, PD-ECGF, PDGF, HGF, IGF-I, TGF-β[2]
2. Cytokines	TNF-α[2], IL-1[2], IL-6[2], IL-8, IL-15, IL-18, G-CSF, GM-CSF
3. Chemokines	IL-8, ENA-78, groα, groβ, CTAP-III, SDF-1
4.ECM macromolecules	Type I collagen, Fibronectin, Laminin, Tenascin, Heparin, Heparan sulfate, Fibrinogen
5.Proteolytic enzymes	MMPs, Plasminogen activators
6. Cell adhesion molecules (CAMs)	β_1 and β_3 integrins, E-selectin, VCAM-1, PECAM-1, CD34, Sialyl Lewis-X, Endoglin
7. Other mediators	Angiogenin, Angiotropin, Angiopoietin-1, COX-2/prostaglandin E2, PAF, Histamine, Substance P, Erythropoetin, Prostaglandins, Adenosine, Pleiotrophin, etc.

[1] See text for abbreviations. [2] Exhibits variable stimulatory and inhibitory acivity (see text).

Table 2. Some inhibitors of angiogenesis in RA[1]

1. Growth factors	TGF-β[2]
2. Cytokines	IL-1[2], IL-4, IL-6[2], IL-12, IL-13, IFN-α, IFN-γ, LIF
3. Chemokines	IP-10, PF4, MIG, SLC
4. Heparin-binding factors	Thrombospondin-1, PF4
5. Protease inhibitors	TIMP-1, TIMP-2, PAI-1, PAI-2
6. Antirheumatic drugs	Dexamethasone, Indomethacin, Rofecoxib, Celecoxib, Chloroquine, Sulfasalazine, Methotrexate, Cyclosporine A, Leflunomide, Gold salts, D-penicillamine, Thalidomide, Minocyclin, Infliximab, Anakinra, etc.
7. Antibiotics	Fumagillin, Minocyclin, Deoxyspergualin, Cratithromycin
8. Others	Angiostatin, Endostatin, SPARC, Opioids, Retinoids, Taxol, Troponin I, Anti-rheumatic drugs, Chondromodulin-1, etc.

[1] See text for abbreviations. [2] Exhibits variable stimulatory and inhibitory acivity (see text).

cytokines, some chemokines and others are produced in order to control angiogenesis and thus inflammation (1-9). Angiogenesis research has important clinical relevance. The number of newly formed blood vessels and the amount of angiogenic mediators present may correlate with the extent of inflammation or tumor metastasis formation. In addition, biological therapy may include several anti-angiogenic strategies and thus the suppression of neovascularization may be useful in antirheumatic, as well as in anticancer, therapy (1-9).

The neovascularization process – and thus the net outcome of "angiogenic diseases" – is dependent on the balance or imbalance between angiogenic mediators and angiostatic factors. The suppression of neovascularization by blocking angiogenic mediators, or by the administration of angiostatic agents, may be useful in controlling "angiogenic disorders", such as RA. Neovascularization, the role of angiogenic factors and inhibitors in this process, and the involvement of endothelial cells (ECs), as well as potential intervention strategies, have been discussed in numerous reviews (reviewed in 1-6).

Here we will review those angiogenic mediators and inhibitors, which have been shown to play a role in RA (Table 1 and Table 2). Then we will discuss the regulation of the angiogenic process in RA, including important interactions between ECs, angiogenic mediators and inhibitors. We will also present recent and future therapeutic strategies using angiogenesis-modulating biological agents, as these treatment modalities may be included in antirheumatic, as well as anti-cancer, therapies.

3. THE PROCESS OF ANGIOGENESIS AND ITS EXPERIMENTAL MODELS

Neovascularization is a *program of several distinct steps*. First, angiogenic mediators activate ECs, which then secrete various proteases. These enzymes degrade the endothelial basement membrane, as well as the surrounding extracellular matrix (ECM). The migration of loose ECs results in the formation of primary capillary sprouts followed by further EC proliferation, migration, synthesis of new basement membrane and lumen formation within the sprout. Two sprouts then link to form capillary loops. Finally, the emigration of ECs out of these sprouts results in the development of second and further generation of new vessels (3,6).

Preferential *EC precursors* exist within the population of $CD34^+$ blood stem cells. Some of these stem cells express receptors for the angiogenic vascular endothelial growth factor (VEGF). These EC precursors may, under certain circumstances, develop into ECs (3,10,11). These cells may be important in neovascularization and thus they may also be used for the induction of angiogenesis in future therapeutic trials carried out in various vascular disorders including obliterative atherosclerosis, stroke or coronary heart disease (3,12).

In vitro models of angiogenesis include EC cultures grown on ECM, such as the laminin-containing Matrigel assay, tissue culture systems or EC chemotaxis assays (reviewed in 1-6,13). *In vivo* neovascularization has been studied using the rat, murine, rabbit, and guinea pig corneal micropocket, the chick embryo chorioallantoic membrane, the hamster cheek pouch, the mesenteric assay, the aortic ring, the implanted ECM assay, and other systems (1-6). These models are suitable to study the angiogenic process, and to test soluble or cell-bound angiogenic or angiostatic agents; these studies also exert relevance for angiogenesis targeting studies (1,3).

4. ANGIOGENIC MEDIATORS IN THE RHEUMATOID SYNOVIUM

There is an immense number of soluble and cell surface-bound mediators shown to promote angiogenesis in malignancies, inflammation, tissue repair and other processes. However, here we only discuss mediators involved in RA, including growth factors, cytokines, chemokines, ECM components, cell adhesion molecules (CAMs), proteolytic enzymes and other factors (Table 1). Most of these mediators are produced by ECs and macrophages; cells also present in high quantities in the RA synovium (reviewed in 1-5).

4.1. Heparin-binding growth factors

Basic (bFGF) and acidic fibroblast growth factors (aFGF) are bound to heparin and heparan sulfate in the ECM. During the angiogenic response these mediators are mobilized by EC-derived heparanase and plasmin (1,6). Both FGFs are expressed by macrophages, synovial lining cells and fibroblasts in the RA synovium *in situ* (14-16). Synovial fibroblasts in culture also express bFGF (16).

VEGF is also bound to heparin (1,6). RA synovial fluids contain large amounts of VEGF (17,18). VEGF protein and mRNA are expressed by RA synovial lining cells, macrophages, fibroblasts and smooth muscle cells (18). VEGF has long ago been implicated in RA-associated EC migration, proliferation and chemotaxis (1,17). In addition, the RA synovium contains fenestrated ECs. VEGF is involved in endothelial fenestration (19). The role of VEGF and its receptors in vessel formation from EC precursor stem cells is described above (3,10,11).

Hepatocyte growth factor (HGF)/scatter factor is also a potent heparin-binding angiogenic mediator (20). Large amounts of HGF are detected in RA synovial fluids. RA synovial tissue macrophages and lining cells express HGF (21).

4.2. Other non-heparin binding growth factors

Other angiogenic growth factors in the RA synovium, which do not bind heparin, include platelet-derived growth factor (PDGF), platelet-derived endothelial cell growth factor (PD-ECGF)/gliostatin, epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I) and transforming growth factor-β (TGF)-ß (reviewed in 1-6).

PDGF, unlike FGFs and VEGF, may act indirectly on angiogenesis by inducing the production of other angiogenic factors including bFGF, VEGF and type I collagen (22). PDGF has been found in RA synovial fluids and is expressed by RA synovial tissue macrophages (23). PDGF is also a mitogen for synovial fibroblasts and thus enhances synovial proliferation (22,23).

PD-ECGF/gliostatin is structurally similar to FGFs (1,6). PD-ECGF has been detected in the sera and synovial fluids of RA patients (24). Synovial PD-ECGF levels have been correlated with the release of acute phase proteins in RA (24).

EGF stimulates most steps of angiogenesis; however, it is less potent than heparin-binding factors (1,6). EGF has also been detected in RA synovial fluids (25).

The amount of *IGF-I* is increased in RA compared to normal synovial fluids (1,26). IGF-I mRNA has also been detected in RA synovial tissues (1,26).

TGF-β has dual effects on angiogenesis as well as on arthritis-associated inflammation, which appear to be dose-dependent (1,2,5,6). TGF-β has been suggested as stimulating angiogenesis indirectly by recruiting angiogenic macrophages (27). Furthermore, TGF-β stimulates the secretion of both the angiogenic urokinase-type plasminogen activator (uPAR) and the angiostatic matrix metalloproteinase (MMP) inhibitors by synovial fibroblasts (28). TGF-β has bipolar effects on synovitis as well: it induces neutrophil recruitment into rat joints (1,28), while it suppresses rat adjuvant-induced arthritis (AIA) (29). TGF-β is expressed by synovial macrophages, lining cells and fibroblasts *in situ* (30).

Hypoxia inducible factors (HIF-1α and HIF-2α) have been implicated in angiogenesis as they are essential in regulating the transcription of the VEGF gene (31). Hypoxia itself stimulates VEGF release in arthritis (32). In addition, HIF-mediated inflammatory and angiogenic pathways are also involved in the pathogenesis of RA (31).

4.3. Cytokines

Among pro-inflammatory cytokines involved in the pathogenesis of RA, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-8, IL-15 and IL-18 are involved in angiogenesis (1-6,33-35). The effects of IL-6 on angiogenesis may be, similarly to TGF-ß, dose-dependently disparate (1,5). Interestingly, IL-13, which is considered to be an anti-inflammatory cytokine and generally suppresses angiogenesis under certain circumstances, may also promote EC migration (2,4,36). Other angiogenic cytokines include granulocyte (G-CSF) and granulocyte-monocyte colony-stimulating factors (GM-CSF), as well as oncostatin M (1,2,3,5,6,37). Most of these cytokines are produced by macrophages, and thus they, especially TNF-α, account for the majority of macrophage-derived angiogenic activity in RA (1,6,38-40).

TNF-α is a potent angiogenic cytokine with efficacy comparable to FGFs (38,39). Large amounts of TNF-α have been detected in the sera and synovial fluids of RA patients (40,41). In the RA synovial tissue, macrophages and ECs express antigenic TNF-α (40,41).

The effects of *IL-1* on angiogenesis are somewhat controversial, as some groups showed the angiogenic effects of these cytokines in two different animal models for angiogenesis, while others reported their angiostatic activities in the same systems (1,5). However, recently IL-1 has been described as a potent angiogenic mediator in a Matrigel assay (34). Inhibition of IL-1 by anakinra suppressed neovascularization in rat AIA (42). In RA, synovial tissue macrophages and fibroblasts, as well as peripheral blood monocytes, release significant amounts of IL-1 (43,44).

The role of *IL-6* in angiogenesis is also controversial. It stimulated EC migration (45), but inhibited EC proliferation *in vitro* (46). Therefore, the role of IL-6 in angiogenesis is not fully clear. A significant amount of IL-6 is

found in RA synovial fluid (47). In RA, IL-6 is produced by synovial tissue macrophages and fibroblasts, as well as by lipopolysaccharide-stimulated synovial fluid monocytes (48).

IL-13 is considered an anti-inflammatory cytokine; it inhibits the production of several other inflammatory mediators (49,50). However, IL-13 has been reported to stimulate EC chemotaxis *in vitro* (36). On the other hand, IL-13 gene transfer suppressed neovascularization in rat AIA (51); therefore IL-13 is considered an angiostatic, rather than an angiogenic cytokine. IL-13 has been detected in the sera, synovial fluids and synovial tissues of RA patients (49,50).

IL-15 induces blood vessel growth in nude mice (35). High amounts of IL-15 are present in RA synovial fluids and in the lining layer of RA synovial tissues (52).

IL-18 induces EC migration, EC tube formation on Matrigel *in vitro*, and neovascularization in the murine sponge implant model *in vivo* (33). IL-18 mRNA and protein have been detected in high quantities in RA synovial tissues (53).

Granulocyte- (*G-CSF*) and granulocyte-monocyte colony stimulating factors (*GM-CSF*) exhibit less pronounced angiogenic activities compared to heparin-binding growth factors (1,6). RA synovial tissue macrophages constitutively express GM-CSF. In addition, pro-inflammatory cytokines stimulate synovial fibroblasts to secrete these CSFs (54).

Oncostatin M may act as an angiogenic mediator via bFGF-dependent mechanisms (37). Macrophages produce this cytokine in the RA synovial tissue. High amounts of oncostatin M have been found in RA synovial fluids (55).

4.4. Chemokines and chemokine receptors

A number of C-X-C *chemokines* containing the ELR (glutamyl-leucyl-arginyl-) amino acid sequence, such as IL-8 (CXCL8), epithelial neutrophil activating protein-78 (ENA-78; CXCL5), growth-related oncogene α (groα; CXCL1) and connective tissue activating protein-III (CTAP-III; CXCL6) have been implicated in neovascularization (reviewed in 56-59). In contrast, other C-X-C chemokines lacking the ELR motif are potent angiostatic factors (see below) (56-59). The only ELR-lacking, still angiogenic C-X-C chemokine is stromal cell-derived factor-1 (SDF-1; CXCL12) (3,59) There is relatively less information available on the possible role of other chemokines in angiogenesis. Fractalkine (CX3CL1) is the only known C-X3-C chemokine. It is expressed on cytokine-activated EC and promotes angiogenesis (60). Most of these chemokines play an important role in the recruitment of inflammatory leukocytes into the RA synovium (59).

Regarding ELR^+ C-X-C chemokines, *IL-8 (CXCL8)* induces neovascularization both *in vivo* and *in vitro* (39,57,59). Synovial fibroblasts produce IL-8 mRNA in response to IL-1 (61). In RA, IL-8 is produced by synovial macrophages, chondrocytes and cytokine-activated fibroblasts (59). Large amounts of IL-8 have been detected in RA synovial fluids (59).

ENA-78 (CXCL5) is a potent chemotactic factor for neutrophils and is also angiogenic (56,58,62). Significant amounts of ENA-78 are present in RA synovial fluids (59,63). Synovial lining cells, interstitial macrophages, endothelial cells and fibroblasts abundantly produce ENA-78 (63). TNF-α and IL-1 stimulate RA synovial fibroblasts to produce ENA-78 (63).

Groα (CXCL1) is chemotactic for ECs and stimulates angiogenesis (56,58,59). Synovial tissue fibroblasts and synovial fluid mononuclear cells produce groα upon activation by pro-inflammatory cytokines and mitogens, respectively (59,64). The expression of groα in cultured synovial fibroblasts is induced by IL-1 or TNF-α (61,64). In the RA synovial tissue, groα is present in lining cells and interstitial macrophages (64).

CTAP-III (CXCL6) is a human platelet α-granule derived growth factor. CTAP-III is angiogenic and affects many aspects of ECM metabolism (59,65). RA sera contain high levels of CTAP-III (59,65). CTAP-III stimulates the synthesis of various ECM molecules by synovial fibroblasts (65).

SDF-1(CXCL12) is a C-X-C chemokine lacking the ELR motif which specifically binds to the CXCR4 chemokine receptor (fusin) (3,66). This chemokine induces EC chemotaxis *in vitro* and dermal angiogenesis in mice *in vivo* (66). $CD34^+$/VEGF-2 $receptor^+$ EC precursor stem cells described above express CXCR4 and migrate in response to SDF-1 (10). Thus, SDF-1 may be the first angiogenic C-X-C chemokine that lacks the ELR motif. SDF-1 is expressed by synovial ECs and is involved in synovial T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) (67). SDF-1 is involved in CD4+ T cell recruitment into the RA synovium (68).

There is relatively less data available on the possible role of *C-C chemokines* in angiogenesis. *Monocyte chemoattractant protein-1 (MCP-1; CCL2)* may induce EC chemotaxis *in vitro*, as well as angiogenesis in the chick chorioallantoic membrane assay *in vivo*. MCP-1-induced neovascularization has been associated with abundant EC expression of CCR2 (69). High levels of MCP-1 have been detected in RA synovial fluids (70). RA synovial fibroblasts produce MCP-1 in response to IL-1 or TNF-α (69,70).

Fractalkine (CX3CL1) is a C-X3-C chemokine, which promotes neovascularization (60). High levels of fractalkine have been detected in RA synovial fluid samples. In RA synovial tissue, macrophages, fibroblasts, ECs and dendritic cells express fractalkine (59,60).

Regarding the possible role of *chemokine receptors* in angiogenesis, a number of these receptors may be detected on ECs, thus playing a role in chemokine-derived neovascularization. There is a growing body of evidence that CXCR2 may be the most important EC receptor for ELR-containing angiogenic CXC chemokines: CXCR2 is a major receptor for these chemokines including IL-8, ENA-78 and groα (3,56,58,59,71,72). MCP-1-

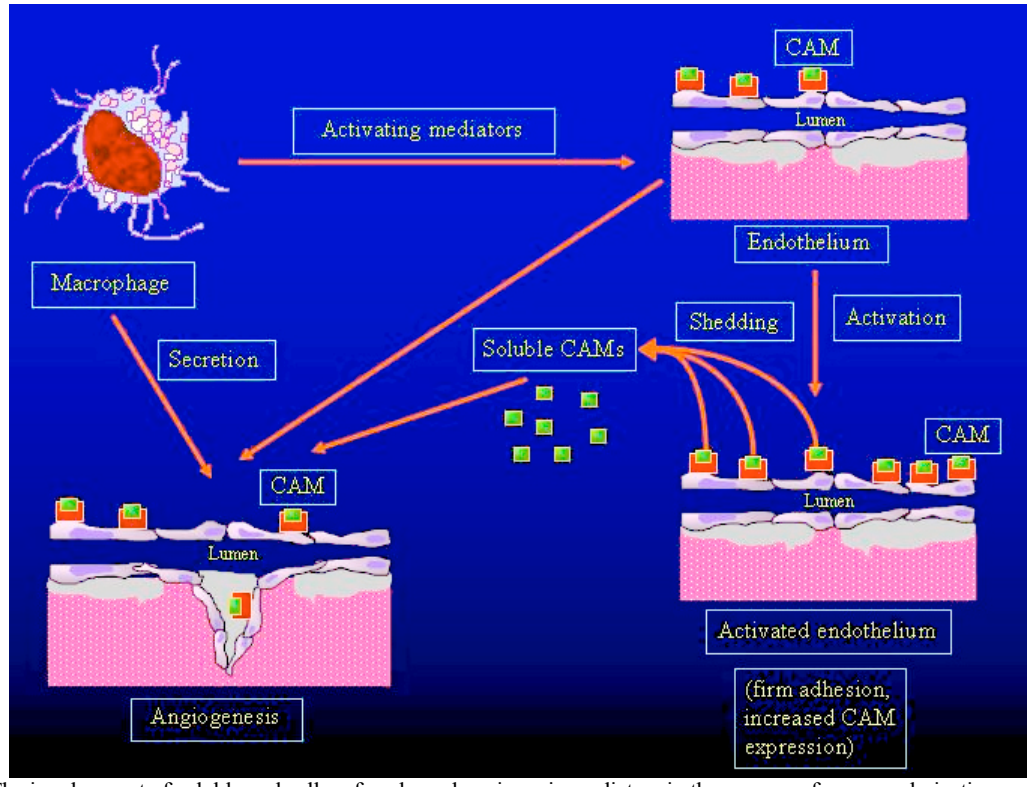

Figure 1. The involvement of soluble and cell surface-bound angiogenic mediators in the process of neovascularization.

induced neovascularization has been associated with abundant endothelial expression of CCR2 (69). Both CXCR2 (73,74), and CCR2 (74,75) have been implicated in the pathogenesis of RA.

4.5. Extracellular matrix components and matrix-degrading proteolytic enzymes

During synovial angiogenesis, ECs migrate into the ECM of the synovial tissue, which contains type I collagen, fibronectin, laminin, vitronectin, tenascin, thrombospondin, proteoglycans and other ECM components (7,76). EC migration and chemotaxis are stimulated by *type I collagen* and, to a lesser extent, *types II, III, IV and V collagen* (1,76). *Fibronectin* is chemotactic for ECs and promotes microvessel elongation in tissue explant cultures (1,77). *Laminin and proteoglycans* are abundantly produced during angiogenesis, and they are involved in EC attachment to the basement membrane and the underlying ECM (1,76). As discussed above, *heparin and heparan-sulphate proteoglycans* are crucial for the action of heparin-binding angiogenic growth factors (1,6). *Tenascin* is also an important component in EC sprouting permissive matrices (78). RA synovial fibroblasts show increased binding to collagen, fibronectin, laminin and tenascin in comparison to normal synoviocytes (79). Thus, all these ECM macromolecules are important in RA-associated neovascularization (1,2,7).

Angiogenesis in RA requires proteolytic enzymes, such as *matrix metalloproteinases (MMPs)*, including collagenase, gelatinase, stromelysin and others, as well as *urokinase- (uPA) and tissue-type plasminogen activators (tPA)* (1,3). A number of MMPs have been found in high quantities in RA synovial fluids and tissues (1,80,81). Synovial fluid MMP concentration is a marker of synovial inflammation (80). In the RA synovial tissue, lining cells are the major producers of collagenase (80). Increased expression of the uPA receptor (CD87) has been detected in RA compared to normal synovial tissues (82). Abundant PA concentrations have been found in RA synovial fluids and synovial tissue extracts (83). RA synovial lining cells express both uPA and tPA (83). A relatively novel family of MMPs termed ADAMTS proteinases includes aggrecanase-1 and –2. These aggrecanases are expressed in inflammation at sites of neovascularization (84).

4.6. Cellular adhesion molecules

CAMs play a crucial role in leukocyte emigration into the RA synovium (reviewed in 85,86). Among CAMs expressed on ECs, most β_1 integrins, the $\alpha_V\beta_3$ integrin, E-selectin, the L-selectin ligand CD34, vascular cell adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1¸CD31), endoglin (CD105), VE-cadherin and some others have been implicated in RA-associated neovascularization (4,41,85,87-91). (Figure 1).

β_1 *integrins,* as well as the $\alpha_V\beta_3$ integrin, which are present on ECs and mediate EC-ECM interactions, stimulate angiogenesis (88,92). Most β_1 integrins are strongly expressed on RA synovial fluid lymphocytes, as well as synovial tissue lining cells, leukocytes, fibroblasts and ECs (79,85,92). The $\alpha_V\beta_3$ integrin has been detected on RA synovial macrophages, lining cells and fibroblasts (79,85). VEGF, at least in part, may stimulate neovascularization via β_1 integrin-dependent mechanisms (93).

Among other EC CAMs, recombinant soluble *VCAM-1* and *E-selectin* may act as proangiogenic factors based on *in vitro* and *in vivo* angiogenesis studies (87). Antibodies to E-selectin inhibited tube formation of bovine capillary ECs (88). High levels of soluble VCAM-1 and E-selectin have been found in the synovial fluids of RA patients (13,94). VCAM-1 and E-selectin have been detected on RA synovial ECs. VCAM-1 is also present in the synovial lining layer (9,95). Antibodies to VCAM-1 and E-selectin partially neutralized RA synovial fluid-mediated EC chemotaxis (87).

Soluble *P-selectin* stimulates EC migration *in vitro* (96). Soluble P-selectin is abundant in RA synovial fluids (97).

The angiogenic effects of *PECAM-1* and the TGF-β receptor *endoglin* have been reported (89-91). Both CAMs have been detected on most RA synovial ECs, as well as on lining cells and macrophages (30,98).

Certain glycoconjugates, such as *Lewis^y/H*, which is structurally related to the E-selectin ligand sialyl Lewis^x, promotes neovascularization and is abundantly expressed in the RA synovium (99). *MUC18* (CD146), a marker for melanoma metastatic potential, has adhesive and angiogenic properties. Synovial fluid levels of MUC18 in RA correlate with synovial angiogenesis (3,100).

Thus, as reviewed in Figure 1, a number of soluble, and possibly also surface-bound CAMs may be involved in the angiogenic process. The synthesis and expression of these CAMs are regulated by soluble angiogenic factors, such as pro-inflammatory cytokines, which are also abundantly produced in the RA synovium (1,4,85).

4.7. Other angiogenic mediators

The cyclooxygenase (COX)/prostaglandin system is also involved in RA-associated angiogenesis. Prostaglandin E2 itself is angiogenic (1,3). COX-2 has been implicated in VEGF-dependent neovascularization (101-103).

Other relevant angiogenic mediators implicated in RA-associated neovascularization include, among some others, *angiogenin, angiotropin, angiopoietin-1, pleiotrophin, platelet-activating factor (PAF), histamine, substance P, erythropoietin,* and *adenosine (*1-6,104,105) (Table 1).

Angiogenin stimulates angiogenesis more effectively than several growth factors; however, its mode of action is not fully clear (1,6). Angiogenin has been detected in the synovia of RA patients (105). It is also secreted by synovial fibroblasts in culture (105).

The role of *receptor tyrosine kinases* in angiogenesis has also been evaluated. The VEGF receptor kinase *flk-1* has been implicated in neovascularization by the discovery of a putative VEGF receptor expressing EC progenitors described above (1-5). *Angiopoietin-1 (Ang-1),* the ligand for the Tie1 tyrosine kinase, exerts no pro-angiogenic activity *in vitro*. Instead, it regulates the assembly of non-endothelial vessel wall components such as smooth muscle cells (106). Ang-1 and the angiostatic Ang-2, as well as their ligands Tie1 and Tie2 are also dominantly expressed in sites of active neovascularization in the RA synovium (107). (Ang-2 will be described among the angiostatic factors.)

PAF stimulates angiogenesis via a heparin-dependent mechanism (108). PAF may be involved in RA synovial fluid-mediated neovascularization (41). A PAF antagonist, BN5730, has been tested in a clinical trial in RA patients, resulting in some clinical improvement in these patients (109).

The neurohormone *substance P* is also a macrophage-derived angiogenic factor (5,6). Increased amounts of substance P have been detected in RA compared to osteoarthritic synovial fluids and tissues (110).

Pleiotrophin is an embryonic growth and differentiation factor with angiogenic properties. It stimulates VEGF synthesis in dermal fibroblasts. In RA, pleiotrophin is expressed by synovial fibroblasts and ECs (104).

5. ANGIOGENESIS INHIBITORS IN RHEUMATOID ARTHRITIS

Angiogenesis inhibitors include some cytokines and growth factors – some of which may also stimulate neovascularization under different circumstances – as well as angiostatic C-X-C chemokines, heparin-binding factors, some antirheumatic drugs, corticosteroids, protease inhibitors, antibiotics, tissue-derived inhibitors, anti-cytoskeletal agents and other compounds (Table 2). These cytokines, chemokines, growth factors and enzymes are naturally produced in the RA synovium and they may control the progression of synovitis. Other synthetic compounds may also influence the progression of RA (reviewed in 1-6).

5.1. Cytokines and growth factors

Some of the growth factors and cytokines, such as *IL-6, IL-13 and TGF-β*, were already discussed above, as these mediators can either stimulate or inhibit neovascularization under various conditions (1,5,36,51,93).

Interferon-α (IFN-α) and IFN-γ block FGF- and VEGF-independent angiogenesis (5,6). The IFN-γ protein, however, is hardly detectable in RA (43). Its mRNA has been found in RA synovial tissues and synovial fluid cells (43).

IL-4 is considered an anti-inflammatory cytokine, which antagonizes several effects of IL-1 and TNF-α in RA (111). IL-4 inhibits angiogenesis both *in vivo* and *in vitro* (112).

IL-12 inhibits neovascularization by inducing the production of two angiostatic mediators, IFN-γ and the IP-10 chemokine (113,114). IL-12 has been detected in RA synovial fluids (115).

RA synovial fluids contain high levels of the angiostatic *leukemia inhibitory factor (LIF)* (116). Synovial fibroblasts produce LIF in response to IL-1 or TNF-α stimulation (116).

5.2. Chemokines and chemokine receptors

As described above, C-X-C *chemokines* lacking the ELR motif, such as platelet factor-4 (PF4; CXCL4), monokine induced by interferon-γ (MIG; CXCL9) and interferon-γ-inducible protein (IP-10; CXCL10) also inhibit neovascularization (56,57,59,117). Very little IP-10 has been detected in the sera of RA patients. On the other hand, significant amounts of IP-10 have been found in RA synovial fluids and tissues (118). MIG has also been detected in RA synovial fluids, as well as in the lining layer of RA synovial tissue (118). A PF4-derived peptide inhibited murine type II collagen-induced arthritis (CIA), a representative model for RA (119). *Secondary lymphoid tissue chemokine (SLC),* is a C-C chemokine; however, it binds to the CXCR3 receptor. SLC showed strong angiostatic and antitumor effects (120).

Chemokine receptors as angiostatic chemokines, such as IP-10, MIG and SLC, all bind to CXCR3; this receptor may play an important role in chemokine-mediated angiogenesis inhibition (3,59). CXCR3 exerts abundant expression in the RA synovium (59,121).

5.3. Protease inhibitors

Protease inhibitors, such as tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2 and TIMP-3) and plasminogen activator inhibitors (PAI-1 and PAI-2) inhibit EC migration during neovascularization (3,5). TIMPs inhibit angiogenesis by preventing the breakdown of ECM components by MMPs (1,3,5). TIMPs have been found in large quantities in RA synovial fluids and tissues (81). PAIs have also been detected in RA synovia. PAI-1 concentrations are increased in RA in comparison to osteoarthritic synovial fluids (83). MMP inhibitors have been tried in several models of angiogenesis (122). Adenovirus-mediated gene transfer of an uPA/uPAR inhibitor inhibited angiogenesis in CIA (123).

5.4. Heparin-binding factors

Heparin-binding molecules inhibit the binding of angiogenic growth factors, such as FGFs, to heparin. Among these compounds, *thrombospondin-1* is an important ECM component, which also serves as a CAM, while *PF4*, a C-X-C chemokine, was described above. Both molecules bind to and block the effects of heparin (5,124). Thrombospondin-1 is expressed on RA synovial ECs and macrophages (124). In addition, in the rat adjuvant-induced arthritis (AIA) model for RA, the introduction of thrombospondin-1 into rat ankles after the induction of arthritis resulted in a biphasic modulation of synovial blood vessel counts (125).

5.5. Antirheumatic drugs

A number of antirheumatic compounds, such as some non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, as well as disease-modifying agents (DMARDs) including gold salts, D-penicillamine, chloroquine, sulfasalazine, methotrexate, azathioprine, cyclophosphamide, leflunomide, thalidomide, corticosteroids, anti-TNF agents and possibly cyclosporine A, may inhibit angiogenesis by various mechanisms (reviewed in 1,5,6,126).

Among *NSAIDs,* indomethacin blocks neovascularization by suppressing the production of the angiogenic prostaglandin E2 (1,5). COX-2 inhibitors, such as celecoxib and rofecoxib, may suppress RA-associated MMP production and angiogenesis (102,103).

Among DMARDs, *methotrexate* blocks blood vessel formation, at least in part by suppressing synovial MMP gene expression (127). *Gold compounds* inhibit angiogenesis *in vitro* and *in vivo* (128). *Cyclosporine A* acts synergistically with fumagillin in suppressing murine collagen-induced arthritis by inhibiting VEGF production (129). Trials with *infliximab* showed that blocking of TNF-α reduced synovial VEGF expression (130). Furthermore, infliximab may also act by increasing serum endostatin levels in RA patients (131).

5.6. Angiostatic steroids

Some corticosteroids already used to control inflammatory diseases, such as dexamethasone, suppress neovascularization (1,5). In addition, a variety of "angiostatic steroids" have been developed. These steroids act by inhibiting basement membrane degradation (5). Among the several known compounds, *2-methoxyestradiol* suppressed CIA in mice (132). A new generation of angiostatic steroids has been produced by the combination of *heparin* and *cortisone*. This complex prevented cartilage degradation in some arthritis models (133).

5.7. Antibiotics

Some antibiotics and their derivatives may suppress angiogenesis by inhibiting the action of angiogenic mediators, such as VEGF or MMPs. The angiostatic analogues of *fumagillin*, such as *AGM-1470*, suppressed rat CIA and AIA (90,129,134). In addition, AGM-1470 ameliorated rat adjuvant-induced arthritis (90). AGM-1470 acts, at least in part, by decreasing serum VEGF levels in arthritis (129).

Minocycline, a tetracycline-derivative, inhibits MMPs, and thus angiogenesis (5). Minocycline also suppressed both AIA and CIA in rats (135). There have been a few trials using this antibiotic drug in RA (136,137).

Deoxyspergualin has angiostatic activity similar to other antibiotics (5). Deoxyspergualin successfully abrogated murine CIA (138).

Clarithromycin, which is widely used against several types of infections, may also inhibit angiogenesis (139). This antibiotic drug has been shown to suppress IL-1, IL-6, IL-8, and GM-CSF production by cultured synovial fibroblasts (140).

5.8. Other angiostatic agents

Additional angiostatic factors include cytoskeleton-disassembling agents including *taxol;* Secreted Protein Acidic and Rich in Cysteine *(SPARC)/osteonectin*; *opioids, Ang-2, angiostatin, endostatin, retinoids, opioids, troponin I, cartilage-derived natural inhibitors including chondromodulin-1,* and others. Many of these agents have been tried in cancer therapy trials and some of them may also be tried in animal models of inflammation, such as arthritis models, and possibly also in humans (see below) (reviewed in 1-6,134,141) (Table 2).

Taxol inhibited rat experimental arthritis (134). Increased *SPARC/osteonectin* levels have been found in synovial fluids of RA patients. In addition, SPARC secretion in chondrocyte cultures is regulated by angiogenic growth factors and cytokines suggesting the existence of a regulatory feedback mechanism in arthritis (5,142). *Opioids* as angiogenesis inhibitors attenuated the progression of rat AIA (5,143,144). The cartilage-derived angiogenesis inhibitor *chondromodulin-1* suppressed antigen-induced arthritis (141).

Angiopoietins (Ang-1 and Ang-2) are described above. *Ang-2* is a naturally occurring inhibitor of Ang-1. This antagonist of the Tie2 receptor tyrosine kinase inhibits neovascularization (145). Ang-2, as well as Ang-1 have been detected in the RA synovium (107).

There is growing amount of data on the inhibitory effects of *angiostatin* and *endostatin* on tumor- and inflammation-associated angiogenesis. Angiostatin, a fragment of plasminogen, was purified from the urine of lung tumor-bearing mice (146), while endostatin, a fragment of collagen type XVIII, is produced by murine hemangioendothelioma cells (147,148). Both compounds have been introduced into angiogenesis inhibition and gene transfer trials in animal models of arthritis (147-151).

6. REGULATION OF ANGIOGENESIS IN THE RHEUMATOID SYNOVIUM

RA is an inflammatory disease characterized by leukocyte ingress into and extensive neovascularization in the synovial tissue leading to pannus formation and eventually cartilage destruction (1,2,7,8). While the normal synovial tissue is relatively poor in microvasculature, the arthritic synovial tissue contains large numbers of vessels with activated high endothelium (8,152). The intense angiogenic activity correlates with clinical and inflammatory scores (1,8).

As already mentioned above, the outcome of angiogenesis, and thus the extent of inflammatory cell invasion through the newly formed vessels into the synovium, depends on the imbalance between angiogenic and angiostatic mediators. Most of these factors are produced by synovial fibroblasts, macrophages, ECs and other cells (reviewed in 1-6). The RA synovium hosts several interactive mechanisms and feedback loops, which regulate neovascularization and thus inflammation. For example, angiogenic mediators may interactively stimulate one another. As described above, some of these factors themselves exert dose-dependent disparate effects on angiogenesis. At the same time, as negative feedback, these factors may induce the production of angiostatic factors, thus down-regulating angiogenesis. In addition to these naturally occuring processes, externally administered therapeutic agents may interfere with both sides of this balance. It is important to understand these interactions so that more effective anti-angiogenic, and thus antirheumatic, therapy can be administered. As these mechanisms cannot be discussed here in their whole complexity, some of these interactions are listed below (Figure 1).

1.*Balance between antagonistic angiogenic and angiostatic couples.* For example, MMPs vs TIMPs, PAs vs PAIs, heparin-dependent growth factors vs heparin-binding antagonists, and ELR^+ vs ELR^- C-X-C chemokines regulate the production of each other (5,56,57,153).

2. *Mediators with disparate effects.* As described above, TGF-ß, IL-1, IL-6 and possibly IL-13, may dose-dependently stimulate or inhibit angiogenesis (29,30,43,44,47,48).

3. *Additive interactions between angiogenic mediators.* Among soluble factors, pro-inflammatory cytokines, especially TNF-α and IL-1, stimulate RA synovial fibroblasts to produce C-X-C chemokines, GM-CSF, HGF and other angiogenic factors (21,54,59,61,63,64). Interactions between integrin CAMs and ECM components are also essential in RA-associated neovascularization (85,86). Pro-inflammatory cytokines can up-regulate the expression of endothelial, angiogenic CAMs (85).

4. *Stimulation of angiostatic factor production by angiogenic mediators.* For example, the angiogenic TNF-α, as well as IL-1, induces the release of the angiostatic LIF (1,6).

5. *Down-regulation of angiogenic mediator secretion by angiogenesis inhibitors.* For example, thrombospondin-1 interferes with bFGF on ECs by inhibiting the chemoattractant activity of this growth factor (154).

6. *Interactions between angiostatic factors.* Cyclosporin A and the fumagillin-derivative AGM-1470 act synergistically in blocking the angiogenic activity of VEGF and thus abrogating murine arthritis (129).

7. *Suppression of angiogenesis by synthetic compounds*. As described above, several antirheumatic drugs inhibit the production of angiogenic factors, and thus control the progression of this disease (5,127).

In conclusion, a regulatory network of inflammatory cells and ECs, as well as soluble angiogenic and angiostatic factors, ECM components, CAMs and several other factors, exists in RA, and likely in other "angiogenic diseases". Angiogenesis inhibitors can interact with these mediators at virtually any step of angiogenesis (Figure 1).

7. CLINICAL RELEVANCE OF ANGIOGENESIS RESEARCH IN RHEUMATOLOGY

7.1. Importance for diagnosis and prognosis

Angiogenesis research has important practical diagnostic and therapeutic relevance. Regarding its possible diagnostic, prognostic value, the number of newly formed blood vessels in biopsy specimens may reflect the progression of the disease, similarly to what we see in malignancies (1). Significantly higher degrees of vascularity have been detected in RA in comparison to osteoarthritic or normal synovial tissues (8). The elevated concentration of the angiogenic soluble CAMs, such as soluble E-selectin and VCAM-1 in the sera and synovial fluid samples of RA patients may also be a useful marker of increased neovascularization, as well as inflammation (13,94).

7.2. Angiogenesis targeting in antirheumatic therapy

Regarding applications for therapy, as discussed above, a number of *antirheumatic drugs* currently used in RA, including corticosteroids, some NSAIDs, classical DMARDs (including methotrexate, leflunomide, gold compounds and others) and anti-TNF biologicals inhibit angiogenesis or the production of macrophage-derived angiogenic mediators (1-5,126,128,129). In recent studies, the selective COX-2 inhibitors celecoxib and rofecoxib suppressed RA-associated MMP production and angiogenesis (102,103). Infliximab treatment may exert angiostatic effects as it reduced synovial VEGF expression (130) and resulted in increased endostatin production in RA patients (131). The IL-1 receptor antagonist biological anakinra suppressed angiogenesis in rat AIA (42). Thus, apart from other modes of action, the angiostatic effects of these drugs need to be taken into consideration during their administration.

Future anti-angiogenic and anti-inflammatory targeting in RA may include the inhibition of *cytokine, growth factor or chemokine* production. As described above, at this moment TNF-α and IL-1 seem to be the main targets for these trials (42,130,131). Other cytokines, such as IL-6 or IL-13, may also be targeted using antibodies or gene therapy (155,156). As seen above, IL-13 may exert disparate effects on angiogenesis. However, in gene transfer studies, IL-13 reduced angiogenesis and synovitis in rat AIA (51). Among angiostatic chemokines, a peptide derived from PF4 was able to abrogate murine arthritis (119). Regarding growth factor targeting, a humanized antibody to VEGF suppressed neovascularization (130). A number of additional synthetic VEGF and VEGF receptor inhibitors are under development (157,158). A soluble VEGF receptor 1 (VEGFR1) chimeric protein dose-dependently suppressed the proliferation of ECs isolated from arthritic synovial tissues (158).

The synthesis and expression of angiogenic *CAMs* could also be inhibited (1,4,85). For example, gold compounds given to RA patients suppressed the expression of synovial E-selectin, an angiogenic CAM, in the synovium (159). Vitaxin (MEDI-522), a humanized antibody to $\alpha_V\beta_3$ integrin blocks the interaction of $\alpha_V\beta_3$ with its ligands vitronectin and osteopontin. Clinical trials with Vitaxin in arthritis are in progress (160).

Other *angiostatic compounds* could also be used to target neovascularization. *MMP inhibitors* have been tried in several models of angiogenesis (122). Adenovirus-mediated gene transfer of an uPA/uPAR inhibitor exerted angiostatic effects in murine CIA (123). Rat CIA was suppressed by the fumagillin-derivative *AGM-1470*, as well as by the cytoskeleton disrupting agent *taxol* (134). *Angiostatin* and *endostatin* also became primary targets. Systemic administration of endostatin abrogated arthritis in various rodent models arthritis (147,148). Endostatin gene transfer inhibited arthritis in human TNF-transgenic mice (149). Angiostatin gene transfer abrogated murine CIA (150). Protease-activated kringles 1-5 (k1-5), an angiogenesis inhibitor related to angiostatin, reduced the severity of murine CIA (151). *Cartilage-derived tissue inhibitors of angiogenesis* have also been tried to use in arthritis (5,141). For example, chondromodulin-1 suppressed antigen-induced arthritis (141). *Opioids* also attenuated rat AIA (143,144). Potentially, most angiogenesis inhibitors described above may undergo further trials in arthritis models and then, possibly, in humans.

8. SUMMARY

In summary, angiogenesis is important in leukocyte extravasation and thus the pathogenesis of RA. The outcome of neovascularization in the rheumatoid synovium depends on the imbalance between angiogenic and angiostatic mediators. Angiogenesis research is important for the better understanding of the pathogenesis of inflammatory arthritis. In addition, existing and potential angiostatic drugs may be useful for future antirheumatic therapies.

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