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## Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disease, associated with inflammation of the synovial tissue lining joints and tendons, which leads to degradation of underlying cartilage and bone. Extra-articular manifestations of RA, including depression and anaemia, combine with inflammation and joint destruction to impact significantly on patients' quality of life. RA is also associated with co-morbidities such as increased cardiovascular disease. Successful development of therapies to treat RA requires an understanding of the cellular and molecular events underlying the disease. Angiogenesis is now

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understood to play an important role. Inadequate oxygenation (hypoxia) is believed to drive the increase in synovial angiogenesis which occurs in RA, through expression of hypoxia-inducible molecules, including vascular endothelial growth factor (VEGF). This allows further infiltration of inflammatory cells and production of inflammatory mediators, perpetuating synovitis. In parallel, inflammatory molecules and cells, particularly activated macrophages, are observed in synovial tissue and can also directly affect angiogenesis.

The current chapter describes the importance of angiogenesis in RA and discusses whether angiogenesis may be a potential therapeutic target in RA. Furthermore, we will review how angiogenesis and inflammation may interact to promote, maintain and resolve synovitis in RA, with a particular focus on the functional responses of macrophages in the context of RA. Successful treatment of RA is associated with reduced levels of pro-angiogenic factors such as VEGF, supporting the concept that modulation of blockade could be of therapeutic benefit in RA.

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**Keywords**

Hypoxia • Inflammation • VEGF • TNF $\alpha$  • Anti-angiogenic therapy

**Abbreviations**

ACPA	Antibodies to citrullinated protein antigens
Ang	Angiopoietin
ANGPTL4	Angiopoietin-like 4
CCP	Cyclic citrullinated peptides
CIA	Collagen-induced arthritis
CXCL	CXC chemokine ligand
DMARD	Disease modifying anti-rheumatic drug
DMOG	Dimethylxaloylglycine
EFNA3	Ephrin A3
FGF	Fibroblast growth factor
FIH-1	Factor inhibiting HIF-1
FLS	Fibroblast-like synoviocytes
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
HRE	Hypoxia-response elements
IKK	I $\kappa$ B kinases
IL	Interleukin
iNOS	Inducible nitric oxide synthase
I $\kappa$ B	Inhibitor of NF- $\kappa$ B
LPS	Lipopolysaccharide

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MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MTX	Methotrexate
NF $\kappa$ B	Nuclear factor $\kappa$ B
NRP-1	Neuropilin-1
OA	Osteoarthritis
PDGF	Platelet-derived growth factor
PHD	Prolyl hydroxylase domain-containing enzyme
PI3K	Phosphoinositide 3-kinase
RA	Rheumatoid arthritis
SE	Shared epitope
TAM	Tumour associated macrophages
TH	T helper cell-type
Tie	Tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains
TNF $\alpha$	Tumour necrosis factor $\alpha$
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor

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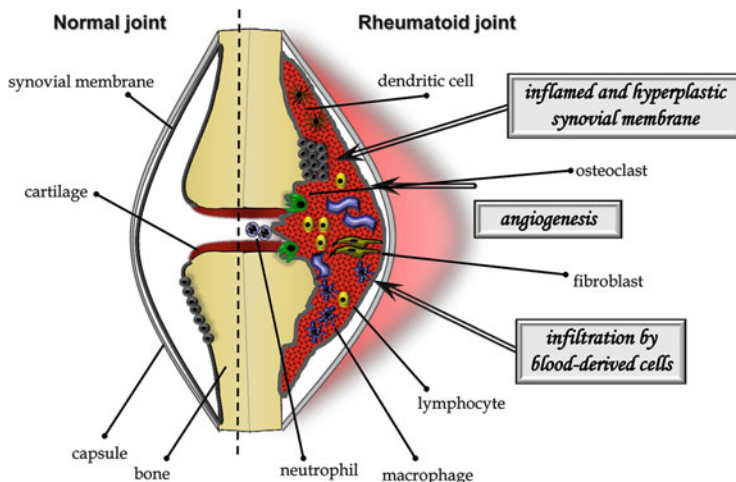
## 16.1 Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease, which affects approximately 1 % of the population worldwide, and is primarily characterised by inflammation of the synovial membrane which lines the joint spaces, leading to the localised invasion and destruction of underlying cartilage and bone. RA may begin at any time from the first few weeks of life until the ninth decade, although the peak time of presentation is 35–45 years of age. Early diagnosis of RA is challenging, due the heterogeneity of its clinical presentation and lack of sufficiently specific and sensitive laboratory tests. Patients present with painful, stiff and swollen joints, predominantly the small joints of the hands and wrists, as well as the metatarsophalangeal joints, ankles, knees and cervical spine. In most patients, symptoms appear over weeks to months, and peri-articular structures such as tendon sheaths may be inflamed. RA is associated with a range of other symptoms such as fatigue, anaemia, weight loss and vasculitis. Depressive symptoms occur in many patients, and there is some evidence that depression may exacerbate pain and disease activity [1, 2]. Indeed more recent clinical trials of new therapeutics in RA include at least one generic patient-reported outcome (PRO) instrument, usually the Health Assessment Questionnaire-Disability Index, to assess function, and/or the so-called ‘SF-36 survey’, to assess Health Related Quality of Life (HRQoL). These instruments have shown validity and sensitivity for assessment of changes in clinical trials of disease modifying anti-rheumatic drugs (DMARD) and biological therapies. Further instruments have been designed

to address problems specific to the RA population. The Arthritis Impact Measurement Scale (AIMS) was one of the first PRO measures developed to evaluate HRQoL in RA [3]. In particular, RA Impact of Disease (RAID) is a new PRO instrument recently developed by EULAR (European League Against Rheumatism) for use in clinical trials, as a measure of the impact of RA on HRQoL, and takes into account pain, functional capacity, fatigue and physical and emotional well-being, together with outcomes such as quality of sleep [4].

Up to 30 % of people with RA become permanently work-disabled within 3 years of diagnosis if they do not have medical treatment [5]. For example, it has been documented in a recent study that at the time of first symptoms of RA, 86 % of men and 64 % of women below 65 years of age were working. More than a third (37 %) of these patients reported subsequent work disability, and the probabilities of continuing to work were 80 % and 68 % at 2 and 5 years, respectively [6]. Furthermore, the standardised mortality ratio for patients with RA is more than 1.5–2.5-fold higher than the ratio for the general population [7]. Although RA patients are at increased risk of dying of urogenital, gastrointestinal, respiratory infections and cancer [8, 9], the major cause of mortality (more than 40 % of deaths) is cardiovascular disease, including ischemic heart disease and heart failure [10]. The odds ratio for RA patients for the risk of all-category stroke was 1.64, with an odds ratio of 2.66 for ischemic stroke [11]. Another study in the USA found that subjects with RA had higher odds ratio for congestive heart failure (3.59) [12]. In a prospective cohort study, which comprised more than 100,000 women free of RA and cardiovascular disease at baseline, the adjusted relative risks of myocardial infarction and stroke in women subsequently diagnosed with RA were 2.00 and 1.48, respectively, when compared to women without RA [13]. A high 10-year risk of cardiovascular disease in newly diagnosed RA patients has been reported, with the absolute cardiovascular risk in RA patients similar to that in non-RA subjects who were 5–10 years older [14]. RA patients also have an increased risk of fatality following myocardial infarction (assessed as the 30-day mortality rates following a first acute cardiovascular event) [15]. A recent study of cardiovascular autopsy findings found that RA patients had more frequent myocardial abnormalities (21 %) than those without RA (12 %) [16].

In terms of disease pathogenesis, interplay between environmental and genetic factors, sex hormones and perhaps an infectious agent or other immune-activating factor is thought to initiate an autoimmune response that culminates in a disease with inflammatory and destructive features [17]. The most common genetic risk factors associated with RA comprise the shared epitope (SE) alleles of the human leukocyte antigen (HLA) gene and a polymorphism of protein tyrosine phosphatase N22 (PTPN22) [18, 19]. Over 80 % of Caucasian RA patients have SE conserved across the HLA-DR1 and HLA-DR4 haplotypes (0101, 0401, 0404 and 1402) [20]. This genetic predisposition has been additionally linked to environmental factors such as smoking [21] and certain autoantibodies. Smoking history, when combined with the presence of HLA-DR SE, increases the risk of RA 21-fold compared with non-smokers carrying no SE genes [20]. Epidemiological and genetic studies of RA have also demonstrated significant differences in subsets of patients with and



**Fig. 16.1** Mechanisms of RA pathogenesis. The normal synovium is generally one to three cell layers thick but in RA becomes thickened (due to fibroblast proliferation) and infiltrated by blood-derived cells, especially lymphocytes and macrophages. Neutrophils accumulate in the synovial fluid within the joint space. This inflamed synovial tissue invades underlying bone and cartilage, particularly as a result of osteoclast activation, leading to joint destruction

without the presence of antibodies to citrullinated protein antigens (ACPA). ACPA are found in approximately 60 % of RA patients but only in 2 % of the normal population, making them highly specific for RA [22–24]. An association between smoking, HLA-DRB1 SE and antibodies to cyclic citrullinated peptides (CCP) has been demonstrated [20]. Furthermore, in anti-CCP-positive individuals, antibodies to the immunodominant citrullinated  $\alpha$ -enolase-1 epitope were detected in approximately 40–60 % of RA patients, and this was linked to HLA-DRB1\*04, suggesting that citrullinated  $\alpha$ -enolase may be an autoantigen linking smoking to genetic risk factors in the development of RA [25, 26].

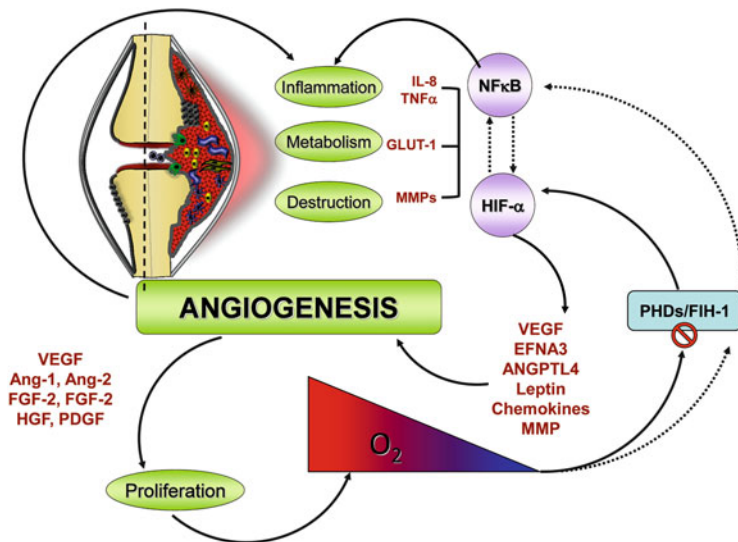
At the tissue and cellular level, RA is characterised by inflammation, hyperplasia of the synovial lining layer and marked infiltration by blood-derived cells, particularly lymphocytes and macrophages (Fig. 16.1). The normal synovium is generally one to three cell layers thick and is composed of loosely associated macrophage- and fibroblast-like cells, as well as vascular endothelial cells. In RA, the synovium is altered to a thickened tissue several cell layers thick, which covers and erodes the adjacent cartilage, bone and tendon. Histologically, the inflamed synovium shows pronounced angiogenesis, cellular hyperplasia and influx of cells, in particular T-cells, macrophages and dendritic cells. The invasive and destructive synovium is responsible for the erosions observed in RA, in that differentiation of monocyte/macrophage cells leads to formation of osteoclasts, which resorb bone matrix. Progressive destruction of the cartilage and bone produces the deformities characteristic of long-standing RA and results in functional deterioration and disability.

In the past, treatment of RA used a pyramidal approach starting with non-steroidal anti-inflammatory drugs at the base of the pyramid and progressing to DMARD such as gold and methotrexate (MTX). However, in spite of such pharmacological interventions, up to 90 % of patients with aggressive synovitis exhibited radiological evidence of bone erosion within 2 years of diagnosis. Major advances in the understanding of the pathogenesis of RA, based on benchside studies of human tissue and animal models of disease, have led to the identification of a number of new biological targets for intervention, the first of which was tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), which mediates many inflammatory and immunoregulatory activities relevant to RA. Other biologicals used in RA include rituximab (which targets B-cells), abatacept (cytotoxic T-lymphocyte antigen 4 fusion protein which prevents T-cell activation) and anti-interleukin (IL)-6 receptor antibody tocilizumab.

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## 16.2 Role of Angiogenesis in RA

RA synovium is a site of active angiogenesis, due to the expression of numerous angiogenic factors, leading to the formation of new blood vessels. Blood vessels fulfil an important role in RA, fuelling synovial expansion and infiltration by cells from the blood, by supplying oxygen and nutrients necessary for cell metabolism and division, as well as by bringing in leukocytes and signalling mediators such as cytokines and growth factors [27–33]. As the synovium expands, more blood vessels are needed to supply poorly perfused and hypoxic areas distant from the pre-existing blood vessels with oxygen and nutrients (Fig. 16.2). The number of synovial blood vessels has been found to correlate with synovial cell hyperplasia and indices of joint tenderness [34]. When affected joints of RA patients were examined using power Doppler ultrasonography, increased synovial blood flow was observed in 81 % of RA patients, but only in less than 10 % of healthy controls, with a correlation between intra-articular microvascular power Doppler flow and clinical synovitis in RA [35]. A number of subsequent studies have confirmed these findings and have further shown that ultrasonographic measures correlate with disease severity in RA and may be useful as a marker of response to therapy [36–39]. Endothelial cells lining blood vessels within RA synovium have been shown to express cell cycle-associated antigens [40], and endothelial proliferation was shown to be increased in synovium from patients with RA [41]. This results in altered synovial blood vessel density [42, 43], with blood vessels of different sizes observed both in areas of diffuse synovitis and in regions of large leukocytic infiltrates with germinal centre-like structures [42, 44]. Despite the formation of blood vessels, their morphology appears similar to that seen in post-capillary venules of lymphoid tissue, and it is thought that there may be a failure to form functional mature vessels. RA synovium contains a significant fraction of neoangiogenic, immature and leaky blood vessels which may be observed from early stages of RA. Comparison of the staining patterns for CD31 and the pericyte marker  $\alpha$ -smooth muscle cell actin revealed a significant fraction of CD31-positive



**Fig. 16.2** Central role of angiogenesis in RA. Angiogenesis plays a key role in perpetuating disease in RA, by fuelling synovial hyperplasia. As cells such as FLS proliferate and become increasingly distant from pre-existing blood vessels, the synovium becomes hypoxic. Reduced oxygen tension and activation of HIF signalling drives expression of angiogenic factors such as VEGF, ANGPTL4, leptin and EFNA3, further promoting angiogenesis. Hypoxia also promotes expression of inflammatory cytokines, chemokines, molecules involved in metabolism (such as the glucose transporter GLUT-1) and MMPs and thus maintains inflammation and synovial destruction. Potential interactions between HIF and NFκB signalling, in part mediated by inhibition of PHD/FIH-1 leading to downstream NFκB activation, also contributes to the destructive and inflammatory synovial milieu

but  $\alpha$ -smooth muscle cell actin-negative cells in RA tissue when compared to osteoarthritis (OA) or control tissue [45, 46]. The presence or density of immature vessels is increased in patients with longer disease duration, higher activity and greater cell infiltration [45].

### 16.2.1 Angiogenic Factors Expressed In RA: A Key Role for VEGF

A range of different factors can promote angiogenesis, directly or indirectly, and many of these factors have been reported to be expressed in RA [47, 48]. Over 30 years ago it was reported that synovial fluids from patients with RA, as well as from those with OA, contained a low molecular weight angiogenesis factor, now termed ESAF (endothelial cell stimulating angiogenesis factor) [49]. In 1994, the groups of Koch and Fava almost simultaneously reported vascular endothelial growth factor (VEGF) expression in RA synovial fluids and tissue [50, 51]. VEGF expression is localised to synovial macrophages, neutrophils and fibroblasts [52, 53], and stimuli

for VEGF release include pro-inflammatory cytokines expressed in RA such as IL-1 [54], TNF $\alpha$  [55] and transforming growth factor  $\beta$  [56], as well as hypoxia, which will be discussed in more detail later. VEGF isoforms VEGF-165 and VEGF-121 appear to be the predominant forms expressed [57]. VEGF receptors (VEGFR) VEGFR1 and VEGFR2 are expressed by RA synovial microvascular endothelial cells [51], and conditioned medium from synovial tissue explants was shown to be mitogenic for endothelial cells, an activity reduced by anti-VEGF antibody [50]. Another study utilised an antibody that selectively recognises VEGFR2 when complexed with VEGF and found that vessel density as assessed using this antibody expression was elevated in RA synovium compared to OA and normal synovium [58]. In addition to synovial expression of VEGF, circulating (serum) levels of VEGF are increased and correlate with inflammatory response markers [54, 59–62]. VEGF levels are increased even in RA patients with a disease duration of less than 2 years [63, 64] and are higher in patients with extra-articular manifestations of RA [65]. Treatment of RA with TNF $\alpha$  inhibitors (alone or with MTX) or anti-IL-6 receptor antibody significantly reduced serum VEGF concentrations [54, 66–70]. Single nucleotide polymorphisms in the VEGF may alter circulating VEGF levels and have been suggested to correlate in RA with both disease activity [71, 72] and onset age [73], although contradictory effects of VEGF polymorphisms on the risk of cardiovascular disease in RA have been reported [74, 75]. In addition to members of the VEGF family, expression of angiopoietin (Ang)-1 and Ang-2 [76, 77] and angiopoietin receptors Tie (Tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains)-1 and Tie-2 [78–80] in RA has been described. Additionally, fibroblast growth factor (FGF)-1 and FGF-2 have been detected in RA synovial tissue [81, 82], together with platelet-derived growth factor (PDGF) [83, 84] and hepatocyte growth factor (HGF) [85].

While VEGF is traditionally considered for its role in angiogenesis, there may be another important function for this growth factor in terms of blood vessel development in RA. In RA, CD133/CD34/VEGFR2-positive endothelial progenitor cells were found close to RA synovial blood vessels [86]. These cells were generated at a higher rate from bone marrow samples taken from RA patients, compared to normal subjects. Furthermore, the capacity of bone marrow-derived cells from RA patients to progress into endothelial cells correlated with synovial microvessel density [87]. Circulating endothelial progenitor cells in patients with active RA have been reported to be lower than in individuals with inactive disease or in healthy controls [88]. Interestingly, a recent study assessed late-outgrowth endothelial progenitor cells and actually found these to be enhanced in RA and suggested that these cells contribute to synovitis perpetuation by promoting blood vessel formation through vasculogenesis [89]. It therefore seems likely that increased synovial vessel density in RA is not just due to angiogenesis but also results from post-natal vasculogenesis, due to increased mobilisation of endothelial progenitors from the bone marrow driven by VEGF and increased endothelial progenitor cell homing to the synovium.

Finally, there is evidence that angiogenesis may be a feature of other joint diseases. In OA, expression of VEGF has been reported, together with chemokines,



HGF and members of the Ang-Tie family [90–93]. Expression of Ang-2 and VEGF is higher in synovium of patients with psoriatic arthritis, relative to RA, whereas Ang-1 levels were more comparable. Psoriatic arthritis and RA exhibited different features in terms of vascular morphology, in that blood vessels in psoriatic synovium were highly tortuous in appearance, compared to the straight and branching vessels seen in RA, suggesting that the balance between Ang and VEGF may affect vessel growth and maturation in arthritic synovium [44].

In summary, a strong pro-angiogenic drive appears to exist in RA. However, increased blood-vessel formation might not necessarily result in improved perfusion and oxygenation, due to the formation of immature vessels with abnormal morphology, and might explain the apparent paradox that although synovial blood vessel density is increased, hypoxia is nevertheless a feature of RA. VEGF is a key pro-angiogenic factor expressed in RA, although its function as a vascular permeability factor may contribute to the enhanced vessel leakiness which is a feature of RA. VEGF may also contribute to synovial vasculogenesis in RA, which may impact detrimentally on vasculogenesis in other organs and could be linked to the increased cardiovascular disease seen in RA patients.

### 16.2.2 Regulation of Angiogenesis by Hypoxia in RA

Oxygen is a key molecular ingredient in life, and hence hypoxia—disruption in O<sub>2</sub> homeostasis caused when oxygen demand exceeds oxygen supply—leads to a number of adaptive changes in cellular responses. The alterations in gene expression induced by hypoxia underlie various physiological processes, including normal embryonic development [94–97] and adaptation to exercise and/or high altitudes [98]. Hypoxia is a feature of certain diseases, in particular solid tumours, but also, with relevance to this chapter, hypoxia plays a key role in RA [33]. The objective of this section is to describe the role of hypoxia in the setting of RA, particularly in terms of angiogenesis, and the potential crosstalk between hypoxia-mediated pathways and inflammatory signalling cascades. As macrophages represent important cellular players in RA, their specific responses to the hypoxic synovial microenvironment will also be considered.

The molecular basis for cellular sensing and adaptation to oxygen-depleted conditions has been extensively described, revealing key roles for members of the family of transcription factors termed hypoxia-inducible factors (HIFs), frequently termed the ‘master regulators’ of the response to alterations in oxygen tension [99]. HIF is a heterodimeric transcription factor composed of two subunits, namely HIF- $\alpha$  (regulated by oxygen levels and post-translational modifications that are sensitive to oxygen levels) and HIF- $\beta$  (expressed constitutively in the nucleus) [99–101]. HIF- $\alpha$  accumulates in the cytoplasm, followed by translocation into the nucleus, where it dimerises with HIF- $\beta$  and binds HIF co-activators, before binding hypoxia-response elements (HRE) in target genes to initiate transcription. The main regulators of HIF- $\alpha$  post-translational modifications are dioxygenases requiring O<sub>2</sub>, 2-oxoglutarate, ferrous iron and ascorbic acid, named HIF prolyl hydroxylase

domain-containing enzymes (PHDs) and factor inhibiting HIF-1 (FIH-1). The PHD enzymes hydroxylate proline residues within HIF- $\alpha$ , thus making HIF- $\alpha$  recognisable by the von Hippel Lindau tumour suppressor, which leads to polyubiquitination and proteasomal degradation [102]. In contrast, hydroxylation of asparagine residues by FIH-1 prevents recruitment of co-activators p300/CBP (CREB-binding protein) [103]. Under conditions of oxygen deprivation, PHD and FIH-1 are inactive, allowing HIF nuclear translocation and induction of transcription of HRE-containing genes.

As mentioned above, hypoxia is a feature of some diseases, particularly when tissue expansion and cellular metabolism result in oxygen demand exceeding supply, as is the case in RA synovium. Both HIF- $\alpha$  isoforms (HIF-1 $\alpha$ , HIF-2 $\alpha$ ) are reported to be expressed in the setting of human RA synovium. In particular, HIF-1 $\alpha$  was expressed abundantly by macrophages in most rheumatoid synovia, predominantly close to the intimal layer but also in sub-intimal zone. Of note there was markedly lower expression of HIF-1 $\alpha$  in OA synovia, and HIF-1 $\alpha$  was absent from healthy synovium [104, 105]. In a subsequent study, using a microelectrode technique in patients having elective hand surgery for RA tendon disease, synovial hypoxia and expression of HIF-2 $\alpha$  were shown in inflammatory infiltrates [106]. Synovial hypoxia is probably the main driving force behind angiogenesis in RA. HIF- $\alpha$  expression in human RA synovium correlates with angiogenesis onset [105] and has been proved to control the expression of a wide range of genes with various roles. VEGF is one of the best characterised HIF-regulated genes, and increased levels of VEGF and VEGFR in RA have been reported in many studies, as mentioned earlier [50, 51, 54, 107]. Importantly, hypoxia increased the angiogenic potential of RA synovium-derived cells, as demonstrated by enhanced blood vessel formation using in vitro angiogenesis assay, together with enhanced synovial cell invasiveness [108]. Hypoxia also drives the expression of other pro-angiogenic players including the chemokines IL-8 (also known as CXC chemokine ligand 8 or CXCL8) [109], CC chemokine ligand 20 [110] and SDF-1 (stromal cell-derived factor 1, CXCL12) [111–113]. We and others have also reported that hypoxia increases expression of angiogenic genes other than VEGF, including angiopoietin-like 4 (ANGPTL4), ephrin A3 (EFNA3) and leptin [114, 115]. Increased levels of pro-inflammatory cytokines such as IL-6 and matrix metalloproteinases (MMP) MMP-1 and MMP-3 [109] have also been reported in response to hypoxia. Synovial hypoxia is, therefore, likely to contribute to RA by promoting inflammation, angiogenesis, cellular infiltration and cartilage degradation.

### 16.2.3 Interplay Between Hypoxia, Angiogenesis and Inflammation

As previously mentioned, low oxygen levels are associated with accumulation of inflammatory cells, enhanced expression of HIF and hypoxia-responsive genes and increased levels of pro-inflammatory cytokines, formally establishing a link between hypoxia and inflammation in the context of many diseases including RA (Fig. 16.2) [116]. Indications that HIF- $\alpha$  could have an important role in

inflammation came from seminal studies utilising mice bearing myeloid-specific deletion of the *Hif- $\alpha$*  gene showing that HIF-1 $\alpha$  allows myeloid cells to generate ATP in oxygen-deprived inflamed tissues, thereby operating energy-requiring processes including aggregation, motility, invasiveness and bactericidal activity [117, 118]. Indeed, the fact that hypoxia and the HIF pathway influence many inflammatory and immune responses relevant to synovitis, including monocyte–macrophage responses, is now well recognised. Pro- and anti-inflammatory cytokines are capable of directly affecting the HIF pathway, thus highlighting the convergence between inflammatory and oxygen-dependent signalling pathways. For instance, studies on gingival fibroblasts and RA fibroblast-like synoviocytes (FLS) have demonstrated that IL-1 $\beta$  and TNF $\alpha$  are capable of activating HIF in normoxia, an effect requiring both mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K) pathways [119, 120]. In contrast to HIF- $\alpha$  protein stabilisation that occurs under hypoxic conditions due to inhibition of PHDs and FIH-1, cytokines have been reported to stimulate HIF- $\alpha$  gene transcription in macrophages and RA FLS [120, 121]. Importantly, it has been demonstrated that T helper cell-type (TH) 1 cytokines can synergise with hypoxia to induce HIF-1 in various cells, including RA FLS [120, 122, 123]. We have also shown that TH1 cytokines in combination with hypoxia are not sufficient to induce angiogenic activity by RA FLS despite inducing HIF-1 and VEGF. In contrast, TH2 cytokines induce pro-angiogenic activity in normoxia and hypoxia, despite their inability to activate HIF-1 in FLS, highlighting the complex relationships between hypoxia, angiogenesis and inflammation in RA [114]. These observations might go some way to explain the apparent paradox of the concurrent presence of hypoxia and angiogenesis in RA synovium, since TH1 cytokines and hypoxia may not lead to formation of fully functional blood vessels, which have been shown to be present in RA [45, 46].

As well as FLS, monocytes and/or macrophages are considered key players within the context of RA, and thus hypoxia inevitably influences critical aspects of their behaviour, namely the transcriptional programme, polarisation status and metabolism, which might determine the perpetuation or resolution of the inflammatory disease. HIF-1 and HIF-2 were shown to be transcriptional effectors regulating macrophage responses to hypoxia [121, 124–126], and hypoxia upregulates a number of HRE-containing genes, including VEGF and ANGPTL4 [125, 127]. With regard to polarisation status, Mantovani and colleagues [128] presented a classification system for macrophage activation in which macrophages are divided into two groups: M1-type macrophages, characterised by enhanced ability of killing intracellular microorganisms and exhibiting pro-inflammatory properties, and M2-type macrophages, which exert anti-inflammatory effects [128]. So-called ‘classically activated’ M1 macrophages stimulate delayed-type hypersensitivity responses and increase surface levels of major histocompatibility complex (MHC) class II molecules, expression of markers such as inducible nitric oxide synthase (iNOS) and secretion of pro-inflammatory cytokines (IL-1 $\beta$ , TNF $\alpha$ , IL-6, IL-12, IL-15). M2 (‘alternatively activated’) macrophages are activated in response to IL-4 or IL-13, synthesise anti-inflammatory cytokines (such as IL-10)

and facilitate TH2 responses. The relevance of HIF factors to macrophage polarisation ability comes from studies involving a specific macrophage subset, primarily expressed in tumour regions, called tumour associated macrophages (TAMs) and exhibiting a M1/M2 mixed phenotype with M2 being slightly more prominent. Werno et al., utilising a tumour-spheroid model, showed that HIF-1 $\alpha$ -deficient macrophages developed a more prominent TAM marker profile (M2-skewed) together with reduced cytotoxicity and also displayed reduced angiogenic potential [129]. The precise role of HIF-2 $\alpha$  during macrophage-mediated inflammatory responses has also been investigated. It was shown that mice lacking HIF-2 $\alpha$  in myeloid cells are resistant to lipopolysaccharide (LPS)-induced endotoxemia and display an inability to mount inflammatory responses to cutaneous and peritoneal irritants. This phenotype was also associated with reduced TAM infiltration in murine hepatocellular and colitis-associated colon carcinoma models, and reduced tumour cell proliferation and progression, probably due to reduced expression of the macrophage-colony stimulating factor receptor and the CXC chemokine receptor 4 [126]. Chemical stabilisation of HIF following treatment with the HIF hydroxylase inhibitor dimethylxaloylglycine (DMOG) has also been described to promote LPS-induced tolerance via expansion of the M2 macrophage population and activation of nuclear factor  $\kappa$  B (NF $\kappa$ B) signalling [130]. Hypoxia-mediated angiogenesis via enhanced blood vessel formation facilitates tissue oxygen delivery, thus contributing to repair of injured tissues, which is a characteristic function of M2 macrophages. On the other hand, hypoxia/HIF induces the expression of pro-angiogenic molecules such as VEGF. A recently published study investigated the differential roles of macrophage-released HIF-1 $\alpha$  and HIF-2 $\alpha$  with regard to angiogenesis progression. In this setting, it was shown that HIF-1 $\alpha$  exhibits pro-angiogenic behaviour via its effects on VEGF, but that HIF-2 $\alpha$  displays anti-angiogenic behaviour through production of the angiogenesis inhibitor soluble VEGFR1 [131]. HIF hydroxylases have also been ascribed different roles in macrophage polarisation. PHD-2 haplodeficient (*Phd-2+/-*) mice displayed preformed collateral arteries that preserved limb perfusion and prevented tissue necrosis in ischemia. Improved arteriogenesis in *Phd-2+/-* mice was due to expansion of tissue-resident, M2-like macrophages, with higher expression of M2-type genes. Conversely, several pro-inflammatory or anti-angiogenic (M1-type) molecules were downregulated, including IL-6, iNOS and IL-12 [132]. On the other hand, PHD-3 was found to be preferentially expressed by M1 macrophages [133]. Modulation of the HIF-PHD-NF $\kappa$ B axis might thus represent a novel approach for targeting inflammatory diseases where a macrophage polarisation switch may be a contributory factor.

The presence of hypoxia and HIF expression against a background of inflammation has prompted the question of whether hypoxia can activate signalling pathways other than the HIF cascade. Of particular relevance in inflammation is the dynamic interplay between the HIF and NF $\kappa$ B pathways. The NF $\kappa$ B pathway is activated in many different cells, including macrophages, dendritic cells, fibroblasts and endothelial cells, important cellular components in the establishment and progression of RA. Activation of this key transcription factor can be induced by a large number of

stimuli, including bacterial products, viral molecules and pro-inflammatory cytokines. Hypoxia has been shown to drive NF $\kappa$ B activation and thereby regulate the release of critical pro-inflammatory mediators [134, 135]. The very first indication that HIF induces NF $\kappa$ B activation came from Walmsley et al. providing data showing that hypoxia promotes human neutrophil survival, an effect requiring HIF $\alpha$ -dependent regulation of inhibitor of NF- $\kappa$ B (I $\kappa$ B)  $\alpha$  and NF $\kappa$ B [136]. Furthermore, the participation of PHD HIF hydroxylases in NF $\kappa$ B activation has been proposed [103, 137–140]. In the canonical pathway of NF $\kappa$ B activation, activity is controlled by I $\kappa$ B kinases (IKK), which mediate serine phosphorylation and degradation of I $\kappa$ B $\alpha$  and allow nuclear accumulation of NF $\kappa$ B. IKK-2 contains an evolutionarily conserved amino acid consensus motif which can potentially be hydroxylated by PHD. Mimicking hypoxia by using small interfering RNA against PHD-1 or PHD-2 resulted in NF $\kappa$ B activation in HeLa cells [137]. Similar studies in other cells also suggest that PHD may negatively regulate IKK via prolyl hydroxylation [138, 139]. Potential candidates for NF $\kappa$ B activation in the setting of RA include pro-inflammatory cytokines, which are likely to cause a vicious cycle of signals that result in chronic inflammation [141]. Activation of NF $\kappa$ B and HIF by converging hypoxic and inflammatory signalling pathways thus probably leads to pathological changes associated with RA, such as inflammation, angiogenesis and bone or cartilage destruction.

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### 16.3 Angiogenesis Inhibition as a Therapeutic Option in RA

The principle underlying angiogenesis blockade for treatment of cancer is to ‘starve’ the tumour of nutrients and oxygen. There is now increasing appreciation that RA can also be classified as an angiogenesis-dependent disease, due to the many parallels with solid tumours, such as hypoxia, inflammation and altered vascularity. We and others have extensively described potential therapeutic applications aiming at inhibiting angiogenesis in RA [27, 29, 31–33, 142]. Therefore in this chapter we will focus on VEGF as a target in RA and review more extensively new potential angiogenesis inhibitors for treatment of RA (Table 16.1).

Over the last decade anti-VEGF biologicals have been approved for the treatment of angiogenesis-related diseases. The best examples are anti-VEGF antibody bevacizumab, used in different forms of cancer including metastatic colon cancer, and ranibizumab and pegaptanib sodium (anti-VEGF antibody and VEGF aptamer respectively) for age-related macular degeneration. The promising clinical outcomes of the use of such biologicals suggested that such treatments may also be an option to treat other angiogenic disorders in which VEGF is over-expressed including RA. Murine collagen-induced arthritis (CIA) is a model widely used for the testing of potential therapeutics for RA and was utilised to develop TNF $\alpha$  inhibitors as a therapeutic modality for RA. In CIA, expression of VEGF and VEGFR has been demonstrated [143–146]. Anti-VEGF antibody in this model delayed disease onset but appeared less effective when administered during the chronic phase of disease [144]. In another study, anti-VEGF inhibited synovitis in

**Table 16.1** Angiogenesis inhibition in models of RA

Target	Inhibitor	Effect	References
VEGF	Anti-VEGF antibody	Delayed onset and reduced disease in mouse model(s)	[144, 147]
	Soluble VEGFR1	Delayed onset and reduced disease in mouse model(s)	[148, 149]
	Anti-VEGFR1 antibody	Delayed onset and reduced disease in mouse model(s)	[150–152]
	Anti-NRP-1 antibody	Reduced disease in mouse model(s)	[145, 152]
IL-17	Anti-CXCL5 antibody	Reduced disease in mouse model(s)	[155]
	Anti-IL-17 antibody	Reduced disease in mouse model(s)	[154]
Ang	Soluble Tie-2	Reduced disease in mouse model(s)	[159]
	Tie-1 splice variant	Reduced disease in mouse model(s)	[160, 161]
Endothelial proliferation	Inhibitors of methionine aminopeptidase 2	Reduced disease in mouse model(s)	[162–164]
	6ODS-LHbD	Reduced disease in mouse model(s)	[165]
	Human plasminogen-related protein B	Reduced disease in mouse model(s)	[168]
Unknown	ICM0301B	Reduced disease in mouse model(s)	[167]

Table summarises angiogenesis inhibitors that have been investigated in in vivo models of RA

CIA, as indicated by a reduction in clinical score and paw swelling relative to untreated mice [147]. A soluble form of VEGFR1 has also been shown to significantly suppress established arthritis [148, 149]. A different strategy to limit angiogenesis via the VEGF pathway was to directly target VEGFR. In a spontaneous model of arthritis, De Bandt et al. observed that treatment with anti-VEGFR1 antibody abrogated bone and cartilage destruction. The antibody delayed the onset of arthritis and attenuated the severity of disease [150]. The group of Carmeliet also demonstrated that treatment with antibody against VEGFR1 reduced the incidence of joint disease, whereas antibody specific for VEGFR2 appeared ineffective [151]. More recently, Kong et al. reported that inhibition of VEGF via neuropilin-1 (NRP-1), an alternative VEGFR, significantly inhibited the survival, adhesion and migration of FLS. The anti-NRP-1 peptide also inhibited the proliferation, capillary tube formation and migration of endothelial cells in vitro, and neovascularisation in vivo, and suppressed experimentally induced arthritis in mice by inhibiting hyperplasia and angiogenesis in the arthritic joints, suggesting that anti-NRP-1 may offer a new approach for the treatment of RA [152]. To provide a more profound understanding of arthritis-associated angiogenesis, we recently evaluated the expression of angiogenesis modulating genes in CIA. One of the important findings of our study was that NRP-1 was a key player in the pathogenesis of CIA. Treatment with anti-NRP-1 antibody significantly reduced disease severity and joint destruction in CIA [145]. Kong et al. further developed a previously investigated hexapeptide which prevents VEGF binding to VEGFR1 by generating a stereochemical D-form of the peptide which was conjugated to PEG to prolong its half-life and found that the new peptide was much more beneficial in the

treatment of angiogenesis in RA than the unmodified peptide. The effect was due to increased stability and delivery, suggesting that further development of current strategies could be applied to treat angiogenesis and RA in humans [153].

In addition to VEGF, pro-inflammatory cytokines have been demonstrated to be important in promoting angiogenesis. In 2010 Pickens et al. documented a novel role for IL-17A (also known as IL-17) in mediating angiogenesis, showing that IL-17 concentrations similar to those found in RA joints were capable of inducing endothelial cell migration and tube formation through the PI3K/AKT pathway. Furthermore, local expression of IL-17 in mouse ankles was able to induce joint inflammation and vascularity and demonstrated that IL-17 was angiogenic and promoted blood vessel formation in mice [154]. In continuation of their previous work the same group has further demonstrated that antibody blockade of CXCL5 ameliorates the angiogenic effect of IL-17 [155]. The ability of IL-17 to stimulate endothelial tube formation and invasiveness was also demonstrated by another recent study, highlighting the potential of targeting IL-17 in treatment of RA [156]. In an endothelial and synovial cell co-culture system, it was shown that IL-6 can also induce angiogenesis [157]. A follow-up study reported that IL-6 stimulation induced endothelial cell growth, by decreasing Ang-1 and increasing VEGF and Ang-2, and destabilised angiogenesis in RA, suggesting that targeting IL-6 signalling in RA (e.g. using tocilizumab) could inhibit angiogenesis [158]. Other approaches have targeted the Ang-Tie pathway [159–161] and the methionine aminopeptidase enzyme involved in endothelial proliferation [162–164].

Chemically modified heparins have also been developed as possible candidates for inhibiting angiogenesis; however, heparin therapy is limited by poor oral bioavailability. A newly synthesised low molecular weight heparin and deoxycholic acid conjugate (6ODS-LHbD) has been described to possess high oral bioavailability in rats. In a murine anti-collagen antibody-induced arthritis model this compound inhibited joint neovascularisation and disease development, suggesting that 6ODS-LHbD may be a promising candidate as an orally active angiogenesis inhibitor for treatment of RA [165]. In another recent study Kumagai and colleagues examined the anti-arthritis effect of ICM0301B, an angiogenesis inhibitor first described in 2004 [166]. They found that in the mouse CIA model ICM0301B inhibited disease development with an efficacy comparable to that of a non-steroidal anti-inflammatory drug, indomethacin, and suggested that the observed anti-arthritis effect of ICM0301B might be partially attributed to its anti-angiogenic activity [167]. Another novel inhibitor of angiogenesis in RA was described by Tanaka in 2011, who tested the effect of human plasminogen-related protein B in mouse CIA and found a significant reduction of disease development, VEGF expression and new blood vessel formation [168]. Chang and colleagues examined the role of macrophage CCAAT/enhancer binding protein delta (CEBPD) in CIA using *Cebpd*<sup>-/-</sup> mice. In the absence of CEBPD, disease development was significantly inhibited, with a decrease in the number of affected paws and reduced angiogenesis. Activation of CEBPD in macrophages was involved in promoting tube formation by endothelial cells and the migration and proliferation of rat synoviocytes [169]. Finally, another study aimed to examine whether



angiogenesis induced by inflammatory agents is mediated via monocyte chemoattractant protein-induced protein (MCP-1). Inflammatory agents, including TNF $\alpha$ , IL-1 $\beta$  and IL-8, were found to induce endothelial tube formation via MCP-1, and moreover MCP-1 was shown to stimulate angiogenesis [170].

### Conclusions

Many new therapies have been developed for treatment of RA, directed at biological responses which play a role in disease pathogenesis, such as B-cell and T-cell responses, or cascades driven by inflammatory cytokines, including IL-6, IL-1 and, notably, TNF $\alpha$ . These important advances offered a targeted strategy, unlike conventional but non-specific traditional treatments such as DMARD. However, in addition to side effects such as injection/infusion site reactions, the most significant side-effect of these therapies is an increase in the risk of all types of infections, including tuberculosis. Indeed, there is a significantly increased risk of reactivation of latent tuberculosis when using TNF inhibitors, and while pre-treatment screening has allowed identification of at-risk individuals; nonetheless, there is a drive to seek alternative treatment approaches. In this regard, angiogenesis represents an attractive option. VEGF is expressed in RA and VEGF inhibitors are already in clinical use for oncology applications. However, up to now VEGF blockade has been intensively used only in animal models of arthritis and no clinical trials of VEGF inhibition have been undertaken for RA. In addition adverse effects of VEGF-targeted biologicals, such as hypertension and gastro-intestinal perforation, coupled with the high cost of such therapies, are major concerns. Nevertheless, there is a valid hypothesis that such therapies can be beneficial in treating angiogenesis in RA and some groups are considering other approaches established in cancer for treatment of RA [171]. Moreover, the presence of immature blood vessels in RA synovium suggests that simply inhibiting VEGF may not be the best option. Interestingly, treatment of RA patients with TNF inhibitors selectively depleted immature vessels, without affecting the mature vasculature [45]. Haploinsufficiency of PHD-2, using an in vivo tumour model in *Phd-2*<sup>+/-</sup> mice, actually resulted in vessel maturation, leading to improved tumour perfusion and oxygenation, and reduced tumour cell invasion, intravasation and metastasis [172]. In a model of colitis, inhibition of HIF hydroxylases in vivo using DMOG reduced disease severity. The possible underlying mechanism may be due to expansion of macrophages [130, 173]. These findings suggest that the best tactic to modulate angiogenesis in RA is at present unclear, and further in vitro and in vivo studies are still needed.



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