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REVIEW PAPER

Angiogenesis as a therapeutic target in arthritis: learning the lessons of the colorectal cancer experience

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Abstract The idea of a therapeutic modality aimed at 'starving' a tissue of blood vessels, and consequentially of oxygen and nutrients, was born from the concept that blood vessel formation (angiogenesis) is central to the progression and maintenance of diseases which involve tissue expansion/invasion. In the first instance, solid malignancies were the target for anti-angiogenic treatments, with colorectal cancer being the first disease for which an angiogenesis inhibitor—anti-vascular endothelial growth factor antibody bevacizumab—was approved in 2004.

Our understanding of the pathogenesis of rheumatoid arthritis (RA) has lead to many parallels being drawn between this chronic inflammatory disease and solid tumours, in that both involve tissue expansion, invasion, expression of cytokines and growth factors and areas of hypoxia/hypoperfusion. As a result, angiogenesis blockade has been touted as a possible treatment for RA. The lessons learnt during the progression of eventually successful therapies such as bevacizumab should undoubtedly guide us in the future development of comparable treatments for RA.

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Introduction

A review published in 1998 focused on the role of the vasculature in rheumatoid arthritis (RA) and on the prospects for developing vascular-targeted therapies for RA [1]. Nearly 10 years have elapsed since that particular review was written, and while considerable progress has been made, there are many questions which still remain unanswered in this field. There is no doubt that the vasculature plays a pivotal role in RA pathogenesis, and indeed since that review was written, a number of publications have reported that targetting angiogenesis in different models of arthritis modulated disease severity. However, we are still some way from clinical trials of angiogenesis inhibitors in RA. One reason for this may be the fact that it is actually only just over 3 years since an angiogenesis inhibitor - anti-vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab - was approved for colorectal cancer. This is despite the fact that it is now nearly 40 years since Judah Folkman first proposed that formation of new blood vessels ('angiogenesis') was critical to tumour growth and development [2, 3], and indeed 20 years since the first descriptions of VEGF as an angiogenic factor [4–6].

The present review is aimed as an update of our understanding of the role of the vasculature in RA, discussing the relative contribution of angiogenesis and vasculogenesis, and the prospects for anti-angiogenic therapy in RA, building upon the experiences gained from the development of bevacizumab for metastatic colorectal cancer (and more recently for non-small cell lung cancer).

Angiogenesis and RA

RA is a chronic inflammatory disease characterised by inflammation of the synovial lining of joints and tendon sheaths, together with hyperplasia of the synovium and infiltration of the synovium by blood derived cells, in particular, memory T cells and macrophages. Destruction of underlying cartilage, bone and soft tissues by the invading synovium leads to eventual loss of function. For example, inflammation in RA of the synovium overlaying tendons has been shown to be associated with the formation of tendon adhesions, and if left untreated, the inflamed tenosynovium can erode into the tendon substance itself. This can ultimately result in tendon rupture, equating to a poorer prognosis for long-term hand function [7, 8].

Angiogenesis in the synovial membrane is considered by many investigators to be an important early step in pathogenesis of RA and in the perpetuation of disease [9, 10]. Histologically, luxuriant vasculature is a prominent feature of RA synovitis, evident on microscopic examination of synovial biopsies from the earliest stages of disease evolution [11, 12]. Indeed, synovial angiogenesis may precede other pathological features of RA. For example, in a patient with very early RA, changes in vascular density were demonstrated without either lining cell proliferation or mononuclear cell infiltration [13]. The number of synovial blood vessels has been found to correlate with synovial cell hyperplasia, mononuclear cell infiltration and indices of joint tenderness [14]. Endothelial cells lining blood vessels within RA synovium have been shown to express cell cycle-associated antigens, including Ki67, as well as integrin $\alpha V\beta 3$, which is associated with vascular proliferation [15, 16]. These changes in vascular supply are thought to be necessitated by the hyperplastic nature of RA synovial tissue, which as it expands, requires a compensatory increase in the number of synovial blood vessels. The new blood vessels supply nutrients and oxygen to the augmented inflammatory cell mass. In addition, delivery of inflammatory cells and molecules is also maintained, thus perpetuating the synovitis.

Many pro- and anti-angiogenic factors have been reported to be expressed in RA synovium (reviewed in [17–20]). Members of the fibroblast growth factor (FGF) family, FGF-1 and FGF-2, have been detected in human RA synovial tissue [21, 22], as has platelet-derived growth factor (PDGF), a potent mitogen for many cell types including fibroblasts [23]. Hepatocyte growth factor (HGF), or scatter factor, so called due to its ability to disperse cohesive epithelial colonies, has been found at significant levels in RA synovial fluids [24–26]. Other molecules which may exert angiogenic activity in RA include epidermal growth factor [27, 28]. Furthermore, expression of both angiopoietin-1 and angiopoietin-2 in RA synovial tissue has been described [29, 30], together with expression of the angiopoietin receptors Tie-1 and Tie-2 [31, 32].

Importantly, the most widely studied pro-angiogenic factor, VEGF (or VEGF-A), is expressed in RA. VEGF was originally described as a vascular permeability factor produced by tumour cells that promoted accumulation of ascites fluid [5]. The gene for human VEGF is organised into eight exons, and the resultant RNA undergoes alternative splicing events to generate at least five transcripts encoding VEGF proteins containing 121, 145, 165, 189 and 206 amino acids. The various VEGF isoforms exhibit different heparin-binding properties, which govern whether the proteins are secreted or remain cell-associated [33]. VEGF protein levels are elevated in the serum and synovial fluids of RA patients [34–36], and correlate with levels of C-reactive protein, a marker of inflammation and disease activity [35, 37–40]. VEGF isoforms VEGF-165 and VEGF-121 appear to be the predominant forms expressed [41]. VEGF levels are increased even in RA patients with disease duration of less than 2 years, and predict subsequent joint destruction, further supporting the concept that angiogenesis may be a very early event in RA progression [42–44]. Treatment of RA, for example with inhibitors of tumour necrosis factor (TNF) α (alone or with methotrexate), with other disease modifying anti-rheumatic drugs or with anti-interleukin (IL)-6 receptor antibody, significantly reduced serum VEGF concentrations [37, 42, 45–49]. Serum VEGF levels are also apparently higher in patients with extra-articular manifestations of RA [50]. VEGF-induced effects are mediated through receptor tyrosine kinases with seven extracellular immunoglobulin-like domains [33], expressed predominantly, though not exclusively, on endothelial cells. VEGF (in particular VEGF-165), placental growth factor (PlGF) and VEGF-B are the primary ligands for Flt-1 (VEGFR1), whereas the mitogenic effects of VEGF are mediated through an alternative VEGF receptor, KDR/Flk-1 (VEGFR2), which also binds VEGF-C, although with a reduced affinity compared to VEGF. A further sub-set of VEGF binding molecules are the semaphorin receptors neuropilin (NRP)-1 and NRP-2. NRP-1 has been shown to bind VEGF-165 and thereby enhance VEGFR2-mediated signal transduction. In RA synovium, VEGFR1, VEGFR2 and NRP-1are all expressed by synovial endothelial cells [36, 51]. Another study utilised an antibody which recognises VEGFR2 when complexed with VEGF, and found that its expression was higher in RA synovium relative to control tissue [52].

To summarise, a strong pro-angiogenic drive appears to exist in RA synovium. It is not yet known which (if any) is the predominant angiogenic stimulus in RA, since blockade of many of the above factors appears to decrease angiogenic activity in in vitro assays. The subsequent sections will discuss how angiogenesis may be stimulated in RA

synovium, and will review studies on angiogenesis inhibition in in vivo models of disease, which could go some way to answering the question of which factor governs angiogenesis in RA.

Hypoxia and cytokines: the driving forces for angiogenesis in RA

The pro-angiogenic profile that characterises the RA synovium is thought to be the result of at least two major driving forces, namely local tissue hypoxia and the presence of inflammatory cytokines. Hypoxic RA synovial fluids were first described in 1970 by Lund-Olesen, who found that the mean synovial fluid oxygen tension in RA patients was 27 mmHg (3.6% O_2), compared to 63 mmHg (8% O_2) in controls and 43 mmHg (5.6%) in osteoarthritis patients [53]. Since this publication, no further reports on synovial pO_2 measurements in humans were released for many years. A similar study, however, was conducted in a murine arthritis model, which showed that the pO_2 measured with microelectrodes in the hind limb knee joints was significantly lower in arthritic animals compared to control animals [54]. In a recent brief report, in vivo oxygen measurements were taken intra-operatively in RA patients undergoing elective hand surgery, where the measurements were performed in the inflammatory and invasive synovial tissue (rather than fluid) using a microelectrode, and oxygen tensions of 18–33 mmHg (2.4–4.4% O_2) were observed [55]. In comparison, control measurements from healthy individuals showed a pO_2 range of 69–102 mmHg (8.5–13.5% O_2) in the same study, thus supporting the notion that RA synovitis is characterised by the presence of hypoxia.

The cellular response to hypoxia and the potential role of the hypoxic response in RA has been largely extrapolated from studies of tumours. The main feature of hypoxia is the rapid protein stabilisation and accumulation of hypoxia inducible transcription factors (HIF)-1 or HIF-2, known to induce a large variety of genes involved in restoring tissue oxygen tensions, including those involved in angiogenesis, such as VEGF, stromal derived factor-1 and angiopoietins, all of which are present in the RA synovium [29, 34, 56]. In the presence of oxygen, the HIFα subunit (which together with HIF-β forms part of HIF-1 and HIF-2 heterodimers) is hydroxylated by specific prolyl hydroxylases, a step which is required for the interaction with the von Hippel-Lindau tumour suppressor protein, ubiquitination and subsequent proteasomal degradation [57, 58]. Accumulation of HIFs is induced when the oxygen levels decrease below 5–7%, as has been shown in vitro in a variety of cell types, including RA fibroblasts [59, 60]. In addition to oxygen-dependent regulation of HIFs, levels of these transcription factors are also affected by receptor mediated signals under normoxic conditions [61–65]. Inflammatory cytokines present in the RA synovium, such as TNFα and IL-1β, act via such receptor mediated pathways and have been reported to induce changes in HIF-1α levels and/or transcriptional activation in a number of cell types [61, 63, 64, 66]. In vitro studies have shown that inflammatory cytokines can augment hypoxia mediated upregulation of HIF activity and VEGF secretion in cultures of RA fibroblasts [67]. HIFs may therefore act as the convergence point that integrates the cellular response of the RA synovium to low oxygen tension and inflammatory cytokines and thus contribute to the pro-angiogenic profile in the RA synovium.

Local tissue hypoxia is thought to arise in the RA synovial lining when the resident fibroblast population expand in a hyperplastic fashion as is characteristic for RA. As the arthritic tissue expands and invades the intra-articular space, the metabolically active tissue furthest from the underlying synovial vessels is thought to experience perfusion insufficiency and thus hypoxia. This has been confirmed by immunohistochemical studies showing expression of HIFs in RA synovial tissue subjected to 1% oxygen, where the staining appeared to be confined to the hyperplastic fibroblasts in the synovial lining of RA tissue [56, 68]. In an animal model of arthritis, HIF-1α was shown to be associated with areas of hypoxia in inflamed joints [69]. More recently, expression of HIF-2α has also been reported [70]. The generation of a hypoxic synovial micro-environment in RA is also driven by the accumulation of synovial fluid, which is thought to apply pressure on existing vessels, thereby further compromising oxygen flow to the synovium. In support of this, Richman et al have shown that oxygen tensions in the synovial fluid vary inversely with volumes of synovial fluid [71]. A recent study investigated the contribution of synovial hyperplasia to synovial fluid pO_2 and found that synovial proliferation had a significant impact on fluid pO_2 in RA patients, but interestingly, this correlation was not seen in OA patients [72].

To date a lot of research effort has focused on understanding the mechanisms driving synovial hypercellularity, and there is evidence for the involvement of at least three individual factors: dysregulated proliferation and apoptosis of fibroblasts, and increased migration into the tissue of inflammatory cells. Increased proliferation of RA fibroblasts is a concept supported by the increased presence of growth factors and markers of proliferation in the synovial fluids, such as FGF-2 and transforming growth factor β [73, 74]. Regulators of the cell cycle and of transcription are also abundantly expressed in the RA synovium, such as c-fos, Ras, Myc and macrophage inhibitory factor (MIF) [75–77]. MIF is found in synovial fluid and has been shown to induce RA fibroblast proliferation in vitro at concentrations similar to those found

in synovial fluids [77]. More recent findings concern serum amyloid A (SAA), a major acute phase reactant and a marker for a variety of inflammatory diseases. SAA is present in the synovial fluid and the RA synovium and has been shown to induce RA fibroblast proliferation in vitro by increasing levels of intracellular calcium and subsequent activation of ERK and Akt, leading to expression of Cyclin D1 and of the anti-apoptotic protein Bcl-2 [78]. As in various malignancies, the RA fibroblasts are characterised by being resistant to apoptosis. This phenomenon could be explained by an increased expression of anti-apoptotic molecules by the RA fibroblasts such as Bcl-2 [78, 79]. Besides Bcl-2, other molecules which regulate apoptosis have been found to be over-expressed by RA fibroblasts, with examples including NFκB and FLICE [80, 81]. Amongst the most recent publications on the subject however, is one on small ubiquitin-like modifier (SUMO)-1 which was shown to alter the resistance of RA fibroblasts to Fas-induced apoptosis by increasing the recruitment and retention of the pro-apoptotic adaptor molecule DAXX to nuclear bodies whereby its effects are inhibited [82]. Interestingly, VEGF-165 is also reported to act as an inhibitor of fibroblast apoptosis, and is reported to do so by binding to NRP-1 thus inducing ERK and Akt signalling and expression of Bcl-2 [79]. Controversial findings, however, raise questions about the relative contribution of proliferation and apoptosis of RA fibroblasts to synovial hyperplasia. First of all, some studies have shown that RA fibroblasts do not proliferate more in vitro than normal fibroblasts [83], and that there are low mitotic counts in the synovial lining as assessed by tritiated thymidine incorporation into cultured synovial explants [84]. Similarly, in vitro experiments have shown a considerable variability in the sensitivity of RA fibroblasts to apoptosis [85].

Whether the expansion of the RA synovium occurs via decreased apoptosis and/or increased proliferation in addition to the incorporation and retention of inflammatory cells, the highly metabolically active tissue is consuming oxygen at a high rate, leading to the generation of a hypoxic environment in the RA joint. Low oxygen tensions in conjunction with inflammatory cytokines activate HIFs and ensure the production of angiogenic factors with concomitant angiogenesis. The pathological process involving tissue hypoxia, inflammation, angiogenesis and synovial invasion is set in train, where each step in the process sustains the next in a cyclical fashion, eventually leading to joint destruction in RA.

Role of endothelial progenitor cells in RA synovial blood vessel formation

In addition to angiogenesis, vasculogenesis (formation of the primordial vascular network from precursor cells) is also important in formation of the vasculature. As is the case for angiogenesis, the VEGF:VEGFR1/VEGFR2 system is intimately involved in regulation of embryonic vasculogenesis [33, 86]. More recently, vasculogenesis has also been shown to contribute to blood vessel formation in adults. Endothelial progenitor cells were isolated from human peripheral blood by selection for cells expressing CD34, which is shared by angioblasts and haematopoietic stem cells [87]. These cells were found to differentiate into endothelial cells, express classic endothelial cell markers, including CD31 and VEGFR2 and exhibit endothelial cell properties, such as expression of the endothelial-specific isoform of nitric oxide synthase and the adhesion molecule E-selectin. Crucially, these cells also incorporated into sites of angiogenesis in vivo [87]. These findings were expanded upon in studies which demonstrated that VEGF, which was well described as playing a central role in many disease states associated with alterations in vessel density, can increase the number of endothelial progenitor cells in the circulation by mobilising these cells from the bone marrow [88–91]. In RA synovium, CD34/VEGFR2-positive cells have been described, suggesting that in addition to angiogenesis, VEGF-mediated vasculogenesis may contribute to synovial vessel formation [92]. Conversely, endothelial progenitor cell numbers are lower in the peripheral blood of patients with active RA (assessed using the disease activity score) than in individuals with inactive disease or in healthy controls [93]. Another study demonstrated reduced migration of endothelial progenitor cells from RA patients in response to VEGF, suggesting that the functional capacity of these cells may also be attenuated in RA. Endothelial progenitor cells from RA patients exhibited only modest adhesion to endothelial cells stimulated with TNFα, compared with cells from healthy subjects, despite comparable levels of adhesion to unstimulated endothelial cells or matrix proteins such as fibronectin or laminin [94]. Subsequently, bone marrow-derived CD34-positive cells were expanded into CD31- and von Willebrand factor (vWf)-expressing cells. 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 the synovial microvessel density [95]. Treatment of RA patients with active disease with TNFα inhibitors (etanercept or infliximab) resulted in a restoration of circulating endothelial progenitor cell levels to those seen in healthy control subjects. This effect was not seen in patients with active RA but receiving conventional disease-modifying drugs [93]. A more recent study showed a significant increase in endothelial progenitor cell levels and adhesion (to fibronectin), after 2 weeks of anti-TNFα antibody infliximab treatment. Interestingly, a correlation was seen between the

extent of clinical response and the degree of increase in endothelial progenitor cell numbers [96].

Many studies have reported that RA patients have an increased mortality when compared to the general population, most probably due to a higher frequency of cardiovascular disease [97, 98], and that this may in part be due to endothelial progenitor cell recruitment to RA synovium, depleting the circulating pool of endothelial progenitors which would function to restore vascular supply to areas of ischaemic myocardium. The number and functional activity of peripheral blood endothelial progenitor CD34/VEGFR2-expressing cells was found to be reduced in patients with coronary artery disease compared to healthy volunteers, and correlated inversely with the total number of risk factors for coronary artery disease, suggesting that the decreased endothelial progenitor cell numbers and activity may contribute to impaired vascularisation in such patients [99–102]. The above data suggest that enhanced recruitment from peripheral blood of endothelial progenitor cells to RA synovium might then lead to increased RA synovial blood vessel formation, perpetuating disease. Furthermore, increased endothelial progenitor cell trafficking to the synovium would be paralleled by reduced peripheral blood endothelial progenitors in RA, which could be a significant factor in the increased cardiovascular mortality seen in RA. The likelihood that TNFα, which is a strategic player in RA, also contributes to the reductions in endothelial progenitor cell numbers and hence potentially to the cardiovascular co-morbidity, is further underlined by the observation that TNFα reduces endothelial progenitor cell numbers and function [103, 104]. Conversely, restoration of endothelial progenitor cell numbers following TNFα blockade may in part account for the reduction in cardiovascular events.

It appears, therefore, that not only angiogenesis, but also vasculogenesis, may contribute to the vascular changes observed in RA synovium, and indeed that synovial vasculogenesis may be one of the factors underlying cardiovascular co-morbidity in RA.

Angiogenesis inhibition in cancer: what have we learnt?

At first glance, it may seem that RA and cancer are two separate disease entities sharing little or no similarities. On the contrary, the basis of the two disease processes revolves around highly metabolically active cells undergoing uncontrolled proliferation and invasion within an altered pro-inflammatory micro-environment. The aggressive invasion of proliferating synovium causes joint destruction and deformities in rheumatoid disease, and in cancer, results in local spread and distant metastasis. Certainly these observations have lead to suspicions that like cancer, rheumatoid synovium would also harbour hypoxic regions [105], and the involvement of angiogenesis has made both cancer and RA a potential target for anti-angiogenic therapy. In particular, studies on the molecular and cellular mechanisms underlying cancer of the colon and rectum have made this type of cancer the first to be treated with angiogenesis inhibitors.

Colorectal cancer is the third most common cancer worldwide, with 307,432 new cases diagnosed in 2006 in the European Union alone [106]. In the UK, colorectal cancer is the second leading cause of all cancer related deaths [107]. The physical, psychological and financial impact of the disease is significant, particularly when an estimated 55% of malignancies present at advanced stages with established lymph node involvement or distant metastases [108]. Although the mainstay of treatment involves surgery with the intent of resecting the tumour to achieve a margin free of tumour, the majority of patients with advanced malignancies may only be amenable to chemo- and/or radiotherapy or palliative care. The systemic and local adverse reactions coupled with eventual resistance of tumours to chemo- or radiotherapy were clear indications for the need for breakthrough treatments with novel therapeutic targets. The ultimate aim of such agents would be to slow or inhibit tumour growth, and ideally cause tumour regression. In 1971, Judah Folkman described the critical role of tumour angiogenesis to potentiate tumour growth and metastasis [2, 3], and it is now accepted that VEGF, expression of which is upregulated in numerous solid malignancies including primary and metastatic colon cancer, is a pivotal promoter of tumour angiogenesis [109]. This therefore provided the impetus to develop the first anti-angiogenic therapy in the form of a VEGF antagonist for the treatment of advanced colorectal cancer.

Tumour angiogenesis is crucial for tumour growth and metastasis since growth is limited to 2–3 mm^3 in the absence of neo-vascularisation [2]. This is supported by clinical studies showing a positive correlation between tumour angiogenesis and tumour stage [110], and, in the case of colorectal cancer, by immunohistochemical studies confirming higher VEGF expression and microvessel density in resected colorectal cancer tissue containing high concentrations of HIF-1 [111]. However, despite the expansion of blood supply through the development of tumour microvessels, solid tumours ironically harbour hypoxic regions. This is thought to be due to two reasons, first the process of angiogenesis results in disproportionate and inadequate development of vasculature to the tumour and second, the formed vessels are structurally and functionally different from those in normal tissues [112, 113]. Unlike RA, hypoxia is a well-recognised phenomenon in solid tumours such as carcinomas of the cervix, breast, colon and rectum, and in melanoma [114]. Intra-tumoural

hypoxia has been confirmed in colorectal cancers by oxygen electrode measurements and immunohistochemical techniques that rely on the accumulation of an exogenous imidazole-based hypoxia marker or expression of endogenous carbonic anhydrase or hypoxia-inducible proteins [115]. Nevertheless, the high rate of endothelial cell proliferation confers a unique property of tumour neovasculature thus offering a selective novel therapeutic target for inhibiting angiogenesis.

The regulation of angiogenesis occurs at multiple levels, with the balance between pro- and anti-angiogenic mediators controlling the pathways leading to extracellular matrix degradation, endothelial cell proliferation and migration. Thus the target for anti-angiogenic drugs currently in development include matrix metalloproteinase inhibitors, cell proliferation and migration inhibitors, agents that inhibit endothelial cell-specific integrin signalling and agents that are antagonists to angiogenic promoters such as VEGF [116]. Of the possible targets mentioned, only two major classes of agents which target VEGF signalling have shown some promise, namely anti-VEGF neutralising monoclonal antibody bevacizumab, and vatalanib, a small molecule inhibiting the downstream signals mediated by tyrosine kinase on activation of the membrane-bound VEGFR.

Bevacizumab [117] is a recombinant humanised IgG_1 monoclonal antibody which binds to VEGF-A and its isoforms, thereby blocking its interaction with receptors. Although the original concept was to inhibit outgrowth of new tumour vessels, bevacizumab also suppresses tumour growth by causing regression and normalisation of existing tumour vasculature and recruitment of bone marrow derived progenitor cells [117]. The hypothesis of vessel normalisation leading to improvement of the interstitial fluid pressure (which is commonly elevated in tumour micro-environment) underlies the greater penetration of chemotherapy into the tumour thus resulting in further damage of the vasculature and subsequent tumour regression [118–120]. A clinical study by Willett et al showed that treatment of human rectal cancer with VEGF-specific antibody produced a decrease in tumour perfusion, vascular volume, microvascular density, increased pericyte coverage of the vasculature and a significant decrease in interstitial fluid pressure [119]. Furthermore, bevacizumab may exert a pro-apoptotic effect by provoking tumour cells to enter cell-death pathways as a response to oxygen and nutrient deprivation resulting from regression of tumour vasculature [121].

Bevacizumab is licensed in the European Union for the first-line treatment of patients with metastatic cancer of the colon or rectum in combination with fluorouracil (5-FU) and folinic acid (leucovorin) with or without irinotecan. A combination of bevacizumab with standard chemotherapy regimes has been shown to significantly improve survival of patients with metastatic colorectal cancer [122]. In a Phase III clinical trial of 813 patients with untreated metastatic colorectal cancer, patients were randomised to receive irinotecan, 5-FU and leucovorin (IFL) alone, or in combination with bevacizumab at 5 mg/kg every 2 weeks. The group receiving additional bevacizumab had a longer median duration of survival (20.3 versus 15.6 months), progression-free survival (10.6 versus 6.2 months) and an improved response rate to treatment (44.8 versus 34.8% respectively) [123]. The addition of bevacizumab to 5-FU and leucovorin also benefited patients with previously untreated metastatic colorectal cancer [124]. Other Phase III trials using newer chemotherapeutic regimes such as 5-FU, leucovorin and oxaliplatin (FOLFOX)-4 have further confirmed improved median overall survival, progression survival time and higher response rates in patients receiving chemotherapy in combination with bevacizumab [125]. In late 2006, bevacizumab was approved by the FDA in combination with carboplatin and paclitaxel for the initial treatment of patients with unresectable, locally advanced, recurrent or metastatic, non-squamous, non-small cell lung cancer.

Furthermore, the new family of tyrosine kinase inhibitors such as vatalanib attracted great attention during its early stages of development. Unlike bevacizumab which inhibits VEGFR1 and VEGFR2 signalling by binding VEGF, vatalanib blocks both angiogenesis and lymphangiogenesis by inhibiting downstream signalling of all three receptors for VEGF family members, and also signals mediated by PDGF and FGF-2 [126]. Enthusiasm for molecules such as vatalanib was dampened by results from two Phase III trials, CONFIRM-1 [127] and -2 [128]. CONFIRM-1 randomised patients without prior treatment for metastatic disease to conventional FOLFOX-4 regimen alone or with additional vatalanib, and CONFIRM-2 included patients who had failed first line chemotherapy with IFL. Both trials showed no overall significant benefit in progression-free survival except for a sub-group of patients with elevated lactate dehydrogenase (LDH) who demonstrated a significant benefit in progression-free survival. These trials have highlighted several dilemmas: first, that LDH is a non-specific tumour marker, and hence its role in selecting patients for such treatments remains unclear [129], second, that the once daily dosing may be inadequate to sustain a sufficiently high level of the drug to ensure complete inhibition of signalling through VEGFR [130] and finally, that inhibition of another kinase associated signalling pathway may be the cause of the attenuated drug effect.

While bevacizumab has been a major step forward in the treatment of colorectal cancer, translating this advance into a chronic inflammatory disease such as RA may be

complex. VEGF has a critical pathophysiological role in RA, as the most potent angiogenic factor in RA synovium, causing increased vascular permeability, potentiating the chronic oedema and swelling typical of RA as well as producing the chondrolytic and osteolytic fragments that can be found in RA joint effusions [131]. However, VEGF is essential for maintenance and survival of the endothelial cell and furthermore is believed to exert homeostatic control over the systemic blood pressure by modulating vascular tension. If anti-VEGF therapies are to be used in RA, the anticipated adverse effects of the treatment would be expected to mirror those reported in colorectal patients receiving bevacizumab treatment. Apart from hypertension, bevacizumab was generally well tolerated with no significant difference in the incidence of adverse events between the groups receiving IFL alone versus IFL with bevacizumab in the Phase III trial conducted by Hurwitz et al [123]. There was no significant difference in adverse events leading to hospitalisation, discontinuation of study treatment or affecting the 60-day rate of death from any cause. However out of the 402 patients assigned to receive IFL and bevacizumab, six patients (1.5%) developed perforation of the gastrointestinal tract as opposed to none in the IFL alone control arm. A special consideration may have to be applied to RA patients who may be at a higher risk of gastrointestinal perforations as a result of their multiple concurrent medications which may include non-steroidal anti-inflammatory drugs. Similar results were also observed in the First BEAT trial [132], a study established to evaluate the safety profile of bevacizumab, in which gastrointestinal perforations were reported in 1.2% of patients receiving bevacizumab. Bevacizumab administered in combination with 5-FU/leucovorin-based chemotherapy did increase wound healing complications in patients who had major surgery during bevacizumab therapy, although the majority of bevacizumab-treated patients experienced no complications [133]. Other known adverse effects include arterial thromboembolic events and proteinuria.

Additionally, since naturally occurring anti-angiogenic factors exist to oppose formation of new blood vessels, current work is ongoing to examine the direct effect these factors have on the cellular regulatory pathways of endothelial cells. The attraction of targetting genetically stable endothelial cells is the theoretical risk reduction of patients developing drug-induced resistance. Angiostatic agents which may have future therapeutic roles include (1) endostatin, the internal fragment of collagen type 18, (2) angiostatin, a cleavage fragment of plasminogen, (3) tumstatin, (4) platelet factor-4, a platelet derived chemokine, (5) thrombospondin-1 and (6) 16-kDa N-terminal fragment of prolactin [134]. There is emerging evidence that these agents have multiple complex intra- and extracellular effects, capable of inhibiting matrix metalloproteinases and integrins therefore limiting endothelial cell migration. In addition angiostatic treatments have been shown to arrest the endothelial cell cycle, thereby attenuating endothelial cell proliferation, and the anti-angiogenic effect is further enhanced by the activation of both extrinsic and intrinsic pathways to promote endothelial cell apoptosis. Our understanding of how these inhibitors work is still at its infancy but these therapeutic approaches could potentially have larger implications for the treatment of other cancer cell types and also non-cancer related diseases such as RA, age-related macular degeneration, diabetic eye disease and psoriasis.

The first generation of angiogenesis inhibitors have clearly revolutionised our approach in the management of advanced colorectal cancer. The success story of bevacizumab is an indication that novel therapies can benefit patients with advanced colorectal cancer and crucially provides direction and encouragement for the development of other treatments, and for the potential use of angiogenesis blockade in other diseases.

Prospects for anti-angiogenic therapy in RA

The proliferative and invasive nature of arthritic synovium has frequently led to comparisons with tumour development. Both the arthritic synovium and the growing tumour exhibit the apparently paradoxical features of hypoperfusion and concomitant angiogenesis, and thus it is possible in theory at least to extrapolate from the bevacizumab experience in colorectal cancer to the potential effectiveness of angiogenesis blockade in RA (Fig. 1).

Rodent models have been used extensively to study the mechanisms underlying the angiogenic process in arthritic diseases and to develop new therapeutic interventions, including those based on inhibition of angiogenesis. Arthritis can be induced in genetic susceptible mouse strains by immunisation with type II collagen, resulting in an autoimmune response against autoantigens in the articular cartilage and eventually leading to a destructive polyarthritis. The arthritis starts approximately 3–4 weeks after immunisation, usually in a limited number of joints, gradually spreading to multiple joints. The classical way is to use heterologous (e.g. bovine, chicken) collagen for the immunisation [135]. Heterologous collagen-induced arthritis in mice shares many features with RA, including linkage to the major histocompatibility region, infiltration of synovium by blood-derived cells, synovial hyperplasia, pannus formation, angiogenesis, as well as destruction of cartilage and bone. This model has been widely used to study mechanisms involved in the arthritic process and to identify new strategies for RA treatment, such as TNFα inhibitors. However, induction of arthritis in rodents using

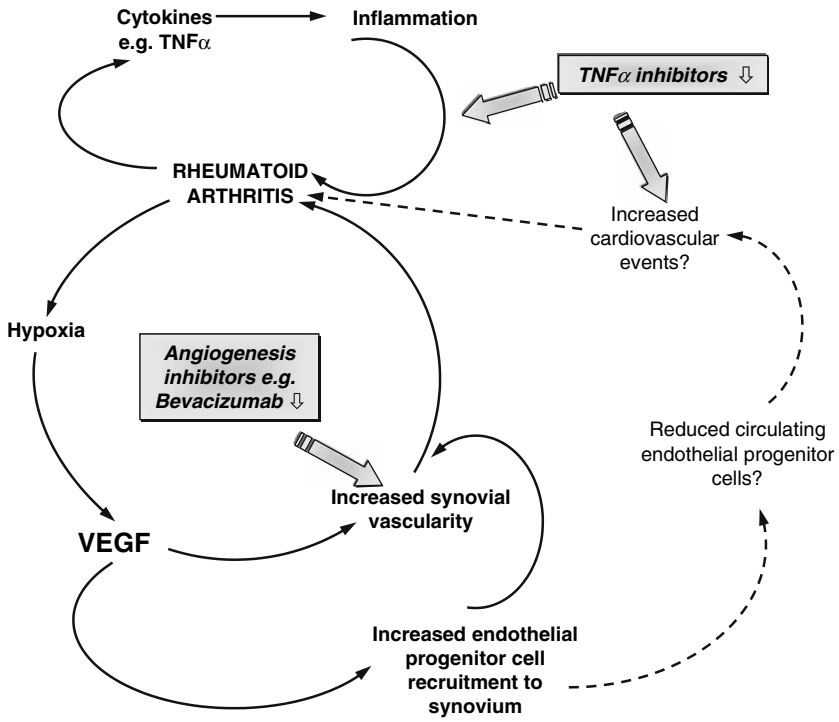

Fig. 1 Schematic representation of the interactions between VEGF, hypoxia, vasculogenesis and angiogenesis during the pathogenesis of RA. The potential effects of TNF and VEGF blockade are shown

heterologous collagen tends to follow an acute, self-limiting disease course, and as such it could be considered less relevant to human RA. On the other hand, immunisation with homologous collagen (i.e. mouse collagen) results in a chronic and relapsing arthritic disease involving an increased number of joints with time [136, 137]. While no animal model of disease is ideal, heterologous collagen-induced arthritis has been used extensively to investigate new therapeutic targets, in part due to the success of this model in predicting the success of TNFα blockade [138–140].

VEGF inhibition has been the focus of considerable clinically oriented research, and interestingly angiogenesis blockade has been shown to be effective in different in vivo models of arthritis, including collagen-induced arthritis. Lu and colleagues showed that VEGF and its receptors are expressed during the development of murine collagen-induced arthritis. In this model (induced using chicken collagen), the level of VEGF expression correlated with disease severity and the degree of neovascularisation as assessed by the expression of vWf. Neutralisation of VEGF activity by administration of anti-VEGF antibody delayed disease onset, but appeared less effective when administered during the chronic phase of disease [141]. In another study, application of anti-VEGF treatment in established bovine collagen-induced arthritis inhibited synovitis, as indicated by a reduction in clinical scores and paw swelling relative to untreated mice [142]. A soluble form of VEGFR1 (soluble Flt-1), a naturally occurring antagonist of VEGF, has been shown to significantly suppress established arthritis. Mice that received adenoviral vectors expressing human soluble VEGFR1 after the onset of arthritis showed reductions in the extent and severity of disease (assessed as the clinical score), and decreased paw swelling and joint destruction, when compared to control animals. Furthermore, decreased levels of VEGF and vWf could be observed in ankle lysates of these animals [143, 144]. A different strategy to limit angiogenesis via the VEGF pathway was to directly target VEGFR. In a spontaneous model of arthritis in KRN/NOD mice, De Bandt et al observed that treatment with anti-VEGFR1 (but not anti-VEGFR2) antibody abrogated bone and cartilage destruction. The antibody delayed the onset of arthritis and attenuated the severity of disease [145]. The group of Carmeliet also compared different approaches targetting VEGF (using anti-VEGFR1 and anti-VEGFR2 antibodies) in chicken collagen-induced arthritis in mice. Treatment with anti-VEGFR1 reduced the incidence of joint disease by 60%, and suppressed the development of clinical symptoms by 85%, whereas anti-VEGFR2 appeared ineffective [146]. The VEGFR tyrosine kinase inhibitor PTK787/ZK222584 has also been shown to be effective in arthritis models [147]. It is interesting to speculate at this point whether targetting VEGF or VEGFR1 actually translates to angiogenesis inhibition in vivo. The expression of VEGFR1 on monocytes has been reported, and has been suggested to mediate VEGF-induced monocyte migration [148, 149]. This might imply that part of the observed effects of anti-VEGF,

anti-VEGFR1 or soluble VEGFR1 could be mediated by attenuation of monocyte migration. In terms of dissecting out the mechanism of action of VEGF blockade in vivo, reduced angiogenesis is likely to result in decreased inflammatory cell trafficking, and vice versa, complicating our understanding of the mode of action of soluble VEGFR1 or anti-VEGF antibodies. Although mice lacking either VEGFR1 or VEGFR2 die in the embryonic stage, mice lacking just the tyrosine kinase domain of VEGFR1 (*VEGFR1* $tk^{-/-}$) are viable. The human T-cell leukaemia virus-1 *pX* transgenic Balb/c mouse model of arthritis is a model of spontaneous destructive progressive arthritis. When back-crossed onto this background, *VEGFR1* $tk^{-/-}$ mice developed milder arthritis, with reduced synovitis. *VEGFR1* $tk^{-/-}$ bone marrow cells displayed suppression of multi-lineage colony formation, and macrophages showed reduced cytokine and VEGF production, suggesting that the VEGF:VEGFR1 axis may be important in haematopoietic and inflammatory cell function, as well as (or maybe rather than?) in angiogenesis [150]. This might explain why VEGFR2-targeted therapies have been so ineffective [145, 146], despite the importance of VEGF:VEGFR2 in the endothelial angiogenic response. The dual activities of VEGF in regulating both angiogenesis and inflammatory cell function clearly complicate our interpretation of the effects of VEGF inhibition in a disease such as RA, where angiogenesis and inflammation are closely linked.

VEGF inhibition in vivo is associated with side-effects, such as impaired wound healing, haemorrhage and gastrointestinal perforation. This is not surprising, perhaps, given the heterozygous lethal phenotype of VEGF knockout mice [151], which suggests a strategic physiological role for this molecule. As a consequence, other members of this family have been targeted. VEGF-B is a member of the VEGF family which binds to both VEGFR1 and NRP-1, and whose exact function still remains unclear. Knockout of the VEGF-B gene also reduced angiogenesis in *Vegf-B*$^{-/-}$ mice, and attenuated both collagen-induced and adjuvant induced arthritis, suggesting that VEGF-B contributes to the angiogenic process in RA [152]. PlGF, like VEGF-A and VEGF-B, binds to VEGFR1 (and to soluble VEGFR1), but, in contrast to VEGF, PlGF does not bind VEGFR2 [153, 154]. PlGF appears not only to induce distinct signalling events via VEGFR1, but also to amplify VEGF-driven activation through VEGFR2 and to complex with VEGF/VEGFR2 forming heterodimeric complexes which transphosphorylate each other [155]. Interestingly, PlGF-deficient mice are viable, and do not display major vascular abnormalities. Moreover, loss of PlGF did not impair reproduction, suggesting that this molecule is not essential during physiological angiogenesis, for example during development, skeletal bone growth or in the female reproductive cycle [156]. Instead, PlGF may play a more pronounced role in pathological angiogenesis, as evidenced by impaired tumour growth and vascularisation in mice lacking this molecule. PlGF is expressed in RA synovial fluid [157]. In contrast to VEGF blockade, there should be few, if any, side effects of PlGF inhibition in terms of disruption of physiological angiogenesis. Moreover, blocking PlGF would avoid the consequences of inhibition of the neuroprotective effects of VEGF, which were highlighted by a study showing that a targeted reduction in VEGF expression in the mouse spinal cord was associated with adult-onset progressive motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis [156]. In summary, while the role of PlGF in angiogenesis has still not fully been clarified, PlGF has been proposed to be involved in *pathological* angiogenesis via VEGFR1, inducing cross-talk between VEGFR1 and VEGFR2 and hence enhancement of responses to VEGF and upregulating expression of VEGF itself. This evidence raises the interesting question of whether PlGF blockade might reduce disease severity and angiogenesis in murine in vivo arthritis models.

Other positive regulators of angiogenesis highly expressed in the RA synovium include HGF, which may contribute to endothelial migration and angiogenesis in RA, since anti-HGF partially neutralised the chemotactic activity for endothelial cells found in RA synovial fluids [24]. Patients with RA have elevated levels of HGF in the synovial fluid and serum, and these correlate with disease activity [25, 26]. A competitive HGF antagonist, NK4, which binds to the c-Met receptor, but does not induce tyrosine phosphorylation of c-Met, has been described [158]. Interestingly, NK4 is able to inhibit angiogenesis not only induced by HGF, but also by other pro-angiogenic factors like FGF-2 and VEGF [159–161], but its efficacy in RA has not been assessed.

An additional approach to suppress angiogenesis in arthritis is to use physiological occurring angiostatic mediators, like angiostatin or endostatin. Angiostatin is a plasminogen derived inhibitor of angiogenesis that down-regulates endothelial cell proliferation and migration. Kim and colleagues transplanted NIH/3T3 cells carrying angiostatin expressing retroviral vectors into knees of mice before disease onset. Local expression of the human angiostatin gene controlled the pathology of disease, which was reflected in reduction of pannus formation and cartilage destruction. The therapeutic effect was accompanied by reduced angiogenesis. The knee joints of mice treated with angiostatin revealed a decreased vascularity compared to knees of control mice [162]. Suppression of disease has also been demonstrated using adeno-associated adenovirus-mediated and human immunodeficiency virus vector-mediated transfer of angiostatin [163, 164]. Sumariwalla

et al assessed the effect in collagen-induced arthritis of K1-5, protease-activated kringles 1-5, an angiogenesis inhibitor that is related to angiostatin but shows enhanced anti-angiogenic activity. Mice received a daily treatment with K1-5 when first macroscopic signs of arthritis occurred. Daily treatment with K1-5 reduced paw swelling and clinical score compared to untreated animals. Additionally, histological assessment of arthritic paws revealed a reduction in joint inflammation and destruction [165]. Endostatin has been shown to also be effective in both murine arthritis models, rat adjuvant-induced arthritis and in the SCID model, in which human RA tissue is grafted into SCID mice (SCID/HuRAg) [166–169].

Treatment of arthritis in rodents with broadly acting angiogenesis inhibitors such as AGM-1470 or Taxol prevented disease and significantly suppressed established arthritis. Administration of these drugs reverted synovial expansion and inhibited neovascularisation [170–173]. TNP-470 (AGM-1470) is a synthetic analogue of fumagillin, a naturally occurring fungal antibiotic from *Aspergillus fumigatus*. TNP-470 was proven to be effective in the treatment of arthritis in different animal models, including rat collagen-induced and adjuvant-induced arthritis [172–175], and the KRN/NOD mouse model of spontaneous arthritis [170]. The mechanism of action was suggested to be inhibition of angiogenesis since peripheral blood VEGF levels were reduced by TNP-470 [175]. In the SCID/HuRAg model, TNP-470 treatment reduced the number of blood vessels and synovial lining cells [176]. Interestingly, TNP-470 and fumagillin both selectively and irreversibly inhibit the enzyme methionine aminopeptidase (MetAP) type 2 isoform (MetAP-2), involved in protein maturation. A recent report has described an irreversible inhibitor of MetAP-2 which not only inhibited proliferation of endothelial cells and fibroblasts, but also showed significant in vivo activity in a rat model of peptidoglycan-polysaccharide-induced arthritis [177, 178].

Further compounds with anti-angiogenic effects include the endogenous oestrogen metabolite 2-methoxyoestradiol, which was shown to be a potent inhibitor of endothelial cell proliferation [179] and to exhibit anti-angiogenic activity in vivo in tumour models [180]. Interestingly, the severity of RA is reduced during pregnancy, supporting a possible role for oestrogen in arthritis. The biologically active oestrogen molecule, 17β-oestradiol, has been reported to reduce the severity of murine arthritis [181]. The effect of 2-methoxyoestradiol 2 was also studied in collagen-induced arthritis, using murine collagen to induce disease. Treatment with 2-methoxyoestradiol markedly reduced disease and suppressed proliferation of endothelial cells [182], which may be attributable to attenuation of angiogenesis. This may be of future relevance, since 2-methoxyoestradiol is in clinical trials for different malignancies, and trials in RA are being considered.

These studies support the role of angiogenesis in RA development and implicate angiogenesis and VEGF as being important in this process. It is likely that the blockade of angiogenesis holds the potential for considerable therapeutic benefit in the future for RA, most likely through combination with existing treatments such as cytokine inhibitors.

Conclusions

Angiogenesis—excessive or insufficient—underlies many pathological situations, such as RA, malignancies or cardiovascular disease. Targetting angiogenesis should yield new therapeutic options in the future, expanding upon already successful treatments such as bevacizumab. When targeting the vasculature in RA, it will be important to consider both angiogenesis and vasculogenesis, although blocking bi-functional molecules such as VEGF may affect both processes. However, the reduced number and activity of these cells in patients with coronary artery disease suggest that it will be important to ensure these cells are measured following anti-angiogenic interventions in arthritis, to ensure no further reductions occur, and hence that cardiovascular morbidity/mortality are not further affected in these already vulnerable patients.

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