Histol Histopathol (2002) 17: 961-972

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

The role of the angiogenic molecule VEGF in the pathogenesis of rheumatoid arthritis

A.O. Afuwape*, S. Kiriakidis* and E.M. Paleolog

Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science Technology and Medicine, London, UK

* YA and SK contributed equally to this manuscript

Summary. The expansion of the synovial lining of joints in rheumatoid arthritis (RA), and the subsequent invasion by the pannus of underlying cartilage and bone, necessitates an increase in the vascular supply to the synovium, to cope with the increased requirement for oxygen and nutrients. New blood vessel formation - 'angiogenesis' - is now recognised as a key event in the formation and maintenance of the pannus in RA. Although many pro-angiogenic factors have been demonstrated to be expressed in RA synovium, the potent pro-angiogenic cytokine vascular endothelial growth factor (VEGF) has been demonstrated to have a central involvement in the angiogenic process in RA. The additional activity of VEGF as a vascular permeability factor may also increase oedema and hence joint swelling in RA. Several studies, including those from the Kennedy Institute of Rheumatology Division, have shown that targeting angiogenesis in animal models of arthritis ameliorates disease. Inhibition of angiogenesis, as an adjunct to existing therapy of RA, or even as a stand-alone treatment, would not only prevent delivery of nutrients to the synovium, but could also lead to vessel regression and possibly reversal of disease.

Key words: Angiogenesis, Rheumatoid arthritis, VEGF

Introduction

Musculoskeletal disorders are more prevalent and a more frequent cause of disability than either heart disease and cancer. The focus of our research is rheumatoid arthritis (RA), a chronic, inflammatory and destructive disease, which typically affects the peripheral joints, but may affect any synovial joint in the body (Harris, 1986). In a recent study, RA patients of working age were found to be 32 times more likely to stop work on health grounds than controls matched for age, gender and employment status at baseline (Barrett et al., 2000). In addition to imposing a large social and economic burden, due to loss of earnings and medical expenses, such conditions are frequently associated with increased morbidity and mortality. For example, RA patients have been found to have a higher prevalence of angina pectoris and stroke, and the mortality of patients with severe RA is equivalent to that of individuals with triple vessel coronary artery disease (McEntegart et al., 2001; Pincus et al., 2001). Research into the mechanisms underlying musculoskeletal disorders such as RA, and into the development of newer and more effective therapeutics, is thus desirable.

With a prevalence of 1%, RA usually presents as a symmetrical polyarthritis, predominantly involving the small joints of the hands and wrists, the metatarsophalangeal joints, ankles, knees and cervical spine. Peri-articular structures, such as bursae and tendon sheaths, are commonly inflamed, and in addition a variety of non-articular features may be seen in RA. These include inflammatory nodules, vasculitis and pericarditis, together with involvement of the lungs and nervous system, as well as certain haematological abnormalities. Many extra-articular features are potentially life threatening, most probably due to the high frequency of associated heart disease.

RA is thought to be T cell-mediated, although the initiating agent has not yet been defined. The T lymphocyte-mediated inflammatory response leads to

Offprint requests to: Dr Ewa Paleolog, Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science Technology and Medicine, Arthritis Research Campaign Building, 1, Aspenlea Road, Hammersmith, London W6 8LH, United Kingdom. e-mail: e.paleolog@ic.ac.uk

Abbreviations. AIA: adjuvant induced-arthritis; CIA: collagen-induced arthritis; CRP: C-reactive protein; DMARDs: disease-modifying anti-rheumatic drugs; FGFs: fibroblast growth factors; IL: interleukin; MAPK: mitogen-activated protein kinase; MTX: methotrexate; OA: osteoarthritis; OIA: ovalbumin-induced arthritis; PDTC: pyrrolidine dithiocarbamate; PI 3-kinase: phosphatidylinositol 3-kinase; RA: rheumatoid arthritis; TGFß: transforming growth factor ß; TNFα: tumour necrosis factor α; VEGF: vascular endothelial growth factor; vWF: von Willebrand Factor

production of cytokines, and activation and recruitment of cells to the synovial lining of the joints. The synovium in RA becomes inflamed and increases greatly in mass, due to oedema and hyperplasia, from a thickness of just 1-2 cells, to over 10 cells. Blood-derived cells, including T cells, B cells and macrophages, infiltrate the sub-lining of the synovium, and lymphocytes frequently form aggregates similar to those seen in lymph nodes. The lymphocytes infiltrating the synovium are predominantly CD4+ (helper/inducer) T cells, with high expression of CD45RO antigens and adhesion molecules of the VLA and LFA super-family.

Although RA shares these histological features (namely infiltration and hyperplasia) with other inflammatory arthritides, for example seronegative spondylo-arthropathies such as ankylosing spondylitis, a very characteristic feature of RA is the propensity for the synovium to become locally invasive at the synovial interface with cartilage and bone. This invasive and destructive front is termed 'pannus', and is responsible for the marginal erosions observed in RA. Progressive destruction of the articular cartilage, sub-chondral bone and peri-articular soft tissues eventually combine to produce deformities and eventually functional deterioration and profound disability in the long term.

An attribute of RA, which has long been recognised but has only recently risen to prominence, due to an increased understanding of the underlying mechanisms, is the role of the vasculature in these invasive and destructive processes. It is now clear that the vasculature - and in particular formation of new blood vessels in the synovium - is central to maintaining and promoting RA. This review will focus on the key molecules involved, in particular on vascular endothelial growth factor, and on the potential for development of new vascular-targeted therapies for the treatment of RA.

The synovial vasculature: a key role in the development of RA

A review in 1982 suggested that in RA "microcirculatory compromise, concomitant with an increase in metabolic needs of synovial tissue, may initiate tissue injury" (Rothschild and Masi, 1982). It is now becoming increasingly clear that alterations in the endothelial cell lining of synovial blood vessels contribute to the development and maintenance of RA.

The synovial vasculature in RA displays several unusual features. For example, endothelial cells lining synovial blood vessels resemble those lining high endothelial venules which control lymphocyte migration into lymphoid tissues (Jalkanen et al., 1986). The basement membrane is also thickened. In a study by Bresnihan and colleagues, endothelial cells in synovial tissue from patients with active RA appeared taller than in control synovium. Interestingly, endothelial cell tallness in tissues obtained from patients with advanced disease was greater than controls only when vessels located in focal lymphoid aggregates were assessed, suggesting different pathogenic mechanisms as RA progresses (Yanni et al., 1993). In addition to an altered appearance, proliferation of endothelial cells, and changes in synovial blood vessel density, also occur during the course of RA. Vascular proliferation has been reported in tissues from inflamed joints of patients with RA (FitzGerald et al., 1991). Endothelial cells lining blood vessels within RA synovium have been shown to express cell cycle-associated antigens such as PCNA and Ki67, and integrin $\alpha v\beta 3$, which are associated with vascular proliferation (Walsh et al., 1998). A recent detailed study demonstrated that endothelial proliferation and cell death indices were increased in synovia from patients with RA compared with non-inflamed controls or individuals with osteoarthritis (OA). In contrast, endothelial fractional areas did not differ significantly among disease groups (Walsh et al., 1998). A morphometric study has suggested that capillaries are distributed more deeply in RA synovium, compared to normal tissue, although the blood volume fraction was greater in normal knees than in RA (Stevens et al., 1991). The number of synovial blood vessels has been found to correlate with synovial cell hyperplasia, mononuclear cell infiltration and indices of joint tenderness (Rooney et al., 1988).

A consequence of the synovial hyperplasia which occurs in RA is an increase in the distance between the proliferating cells and the nearest blood vessels, leading to local hypoxia and hypoperfusion. It has also been shown the oxygen consumption of the RA synovial membrane is elevated, and that glucose is oxidised via an anaerobic, rather than aerobic, pathway. This is not unexpected, given the increased metabolic demand imposed by the proliferating synovial cells. As a result, synovial oxygen tension (PO_2) is reduced. It has been reported that PO_2 is low in aspirated synovial fluid samples taken from human knee joints, with the lowest PO_2 reported in RA patients (Lund-Olesen, 1970). Resting intra-articular pressure in chronically inflamed joints has been found to be raised relative to normal joints, and this effect would be compounded during movement of joints, inducing acute ischemia in the synovial environment (Jawed et al., 1997). The increased pressure of the fluid accumulating within the arthritic joint may obliterate synovial capillary flow, further exacerbating the reduction in perfusion.

In vivo, increased metabolic demand - for example, during tissue expansion - results in a compensatory increase in new blood vessel formation of ('angiogenesis'). Disregulated angiogenesis contributes to the pathology of a number of disease states, during which tissue proliferation outstrips the supply of nutrients and oxygen. These include tumour formation, diabetic retinopathy and psoriasis. In the case of RA, angiogenesis is a pathogenic response to the increased metabolic demand and local hypoxia imposed by the proliferating synovial cells, and hence contributes to the maintenance and development of the synovial tissue mass. Subsequent sections will discuss the pro-

angiogenic signals which have been detected in RA synovium, potential mechanisms for their induction, and new approaches targeting angiogenesis which might be applied for treatment of RA.

Expression of angiogenic stimuli in RA

Brown and colleagues reported as long ago as 1980 that synovial fluids from patients with RA contained a low-molecular-weight angiogenesis factor apparently identical with that derived from tumours (Brown et al., 1980). Subsequently it was shown that RA synovial fluids induced morphological changes in endothelial cell cultures, including the formation of tubular networks morphologically resembling capillaries (Semble et al., 1985). The stimuli which control the formation of blood vessels in RA have been the subject of a large number of studies, leading to the demonstration that several growth factors play a key role in RA (Paleolog and Fava, 1998; Paleolog and Miotla, 1998; Ballara et al., 1999; Walsh and Pearson, 2001).

Vascular endothelial growth factor (VEGF) is the most endothelial cell-specific angiogenic factor characterised to date (Neufeld et al., 1999). The ever-increasing VEGF family now contains at least 6 related cytokines, although the original member, VEGF, remains the most extensively studied. VEGF is secreted by various cell types *in vitro*, including fibroblasts, osteoblasts, macrophages, platelets and lymphocytes, and expression is elevated in a range of angiogenesis-associated disease states (Brown et al., 1997). Two VEGF receptor tyrosine kinases have been identified, Flt-1 and KDR/Flk-1. A key feature of VEGF is the upregulation of this growth factor by hypoxia (Shweiki et al., 1992). Several distinct molecular mechanisms are involved in hypoxia-induced upregulated of VEGF expression. A number of oxygen-regulated genes, such the hypoxia-inducible transcription factor HIF-1, promote VEGF gene transcription by binding to hypoxia-responsive elements in the VEGF promoter. Additionally, the discrepancies observed between the increase in VEGF mRNA levels under hypoxic conditions and the change in the transcription rate suggest that post-transcriptional mechanisms may operate, including increased VEGF mRNA stability (Levy et al., 1995, 1998).

The dual activities of VEGF as an endothelial cell and a modulator of changes in vascular permeability are of relevance in the pathogenesis of RA. VEGF levels are markedly elevated in the serum and synovial fluids of RA patients, relative to either patients with OA or normal controls (Koch et al., 1994; Paleolog et al., 1998; Nagashima et al., 2000a; Lee et al., 2001). Serum VEGF concentrations in RA patients correlate with levels of C-reactive protein (CRP), a marker of inflammation and disease activity (Paleolog et al., 1998). Expression of VEGF mRNA by RA lining layer cells has been reported, and immunohistochemical analyses of RA synovial biopsies revealed VEGF expression by synovial lining layers and endothelial cells lining small blood vessels within the pannus (Fava et al., 1994; Koch et al., 1994; Nagashima et al., 1995; Pufe et al., 2001) (Fig. 1). Moreover, microvascular endothelial cells in the vicinity of VEGF-positive cells express mRNA for VEGF receptors (Fava et al., 1994; Ikeda et al., 2000). Using an antibody which binds preferentially to the VEGF/KDR complex, and recognises an epitope induced by conformational changes after VEGF binding to KDR, a recent study showed that the density of synovial vessels expressing KDR complexed with VEGF was significantly higher in RA than in OA and normal tissues (Giatromanolaki et al., 2001). Perhaps the most relevant property of VEGF in the context of angiogenesis and RA is the upregulation of this growth factor by hypoxia. The hypoxic state in the RA joint suggests that formation of new blood vessels in the pannus may be driven by hypoxia-induced expression of VEGF. We have reported that dissociated RA synovial membrane cells respond to hypoxia by upregulating VEGF production, indicating that a component of the new blood vessel formation observed in RA may result from hypoxia-driven induction of VEGF (Paleolog et al., 1998).

We have also recently demonstrated that VEGF is important in the development of joint destruction in RA. We observed that at first presentation serum VEGF concentrations were markedly higher in patients with early RA than in patients with long-standing treated RA. A key finding within the group of patients in whom the diagnosis of RA was confirmed at 1 year was the significant correlation of serum VEGF at presentation with the magnitude of radiological deterioration within the first year. In this study, the rate of radiological deterioration in the hands and feet was comparable with rates reported over the first year after presentation of RA by other investigators. Patients with radiological deterioration less than the median rate had lower circulating VEGF concentrations than those with greater than the median rate of radiological deterioration (Fig. 2) (Ballara et al., 2001). These results suggest that high serum VEGF levels at an early stage of disease are associated with the increased subsequent damage to joints observed by radiography.

Other pro-angiogenic molecules expressed in RA include the fibroblast growth factors (FGFs), which stimulate DNA synthesis and cell division in a variety of cell types, including endothelial cells and fibroblasts. Members of the FGF family - FGF1 and FGF2 - have been detected in human RA synovial tissue (Sano et al., 1993). Similarly, platelet derived growth factor, a potent mitogen for many cell types including fibroblasts and smooth muscle cells, is expressed in RA synovium (Remmers et al., 1991). The heparin-binding cytokine hepatocyte growth factor has been found at significant levels in RA synovial fluids in a biologically active form, with levels higher in RA compared to OA (Koch et al., 1996). The potential role of transforming growth factor ß (TGFß) during the course of RA, and in synovial angiogenesis, is unclear. TGFß was shown to

induce VEGF expression in human synovial fibroblasts (Fava et al., 1995). Indeed, TGFß is by far the most powerful inducer of VEGF secretion by human synovial fibroblasts, when compared with other cytokines associated with the pathogenesis of RA, such as interleukin (IL)-1 or platelet derived growth factor. Thus, it seems likely that in RA, TGFß exerts its angiogenic effects predominantly through the induction of VEGF secretion by fibroblasts. The pro-inflammatory cytokine tumour necrosis factor α (TNFα), which is a key player in RA pathogenesis, also has effects on angiogenesis, although its actions are both stimulatory and inhibitory, depending on the system. For example, TNFα inhibits basal and FGF2-stimulated endothelial cell proliferation and migration *in vitro*, but promotes neovascularisation in the rabbit cornea (Frater-Schroder et al., 1987). Exposure of endothelial cells to TNFα has been reported to induce release of VEGF and FGF2

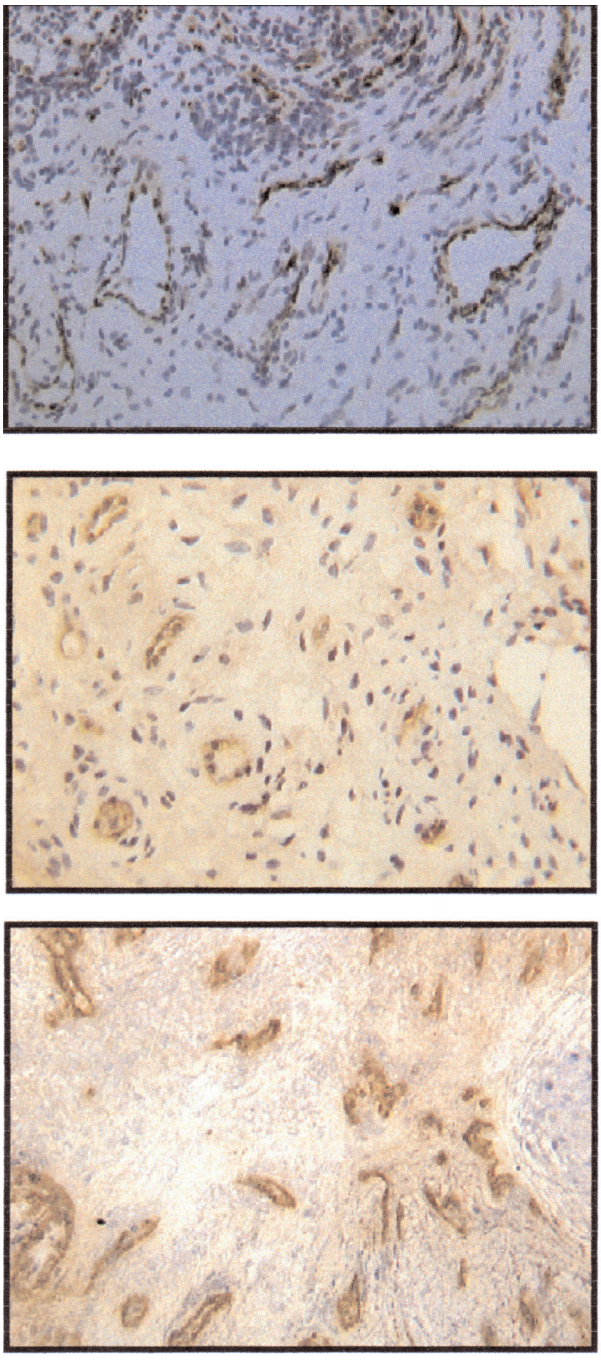

Fig. 1. Expression of CD34, VEGF and von Willebrand factor in RA synovium. Frozen or paraffin embedded sections were stained using antibodies against human CD34, VEGF or von Willebrand Factor (vWf). Subsequently, samples were incubated with biotinylated anti-mouse or anti-goat immunoglobulin, followed by streptavidin-horseradish peroxidase. Immune complexes were detected using 3,3'-diaminobenzidine. x 200

(Yoshida et al., 1997). Production by synovial joint cells of angiogenic cytokines such as VEGF is at least in part induced by TNFα, as was demonstrated in our study showing reduced synovial cell VEGF release in the presence of anti-TNFα antibody (Paleolog et al., 1998). The role of TNFα in the angiogenic process in RA is therefore very complex.

In summary, the invasive pannus in RA is highly vascularised, and numerous growth factors are expressed, which might promote new blood vessel formation. Subsequent sections will examine the signalling mechanisms involved in the induction of VEGF expression in the context of RA, and the development of new therapies targeting blood vessels in RA.

Effects of therapy on angiogenesis

The findings of elevated expression of angiogenic factors in RA suggest that reducing synovial vascularity may be a desirable component of anti-RA therapies. Certain disease-modifying anti-rheumatic drugs (DMARDs) have been shown to inhibit angiogenesis in experimental systems. These include drugs such as methotrexate (MTX) (Hirata et al., 1989), sulphasalazine (Madhok et al., 1991) and penicillamine (Matsubara et al., 1989). Combinations of DMARDs also affect production of VEGF by synovial cells *in vitro*. For example, bucillamine and gold sodium thiomalate inhibited VEGF production (Nagashima et al., 2000b).

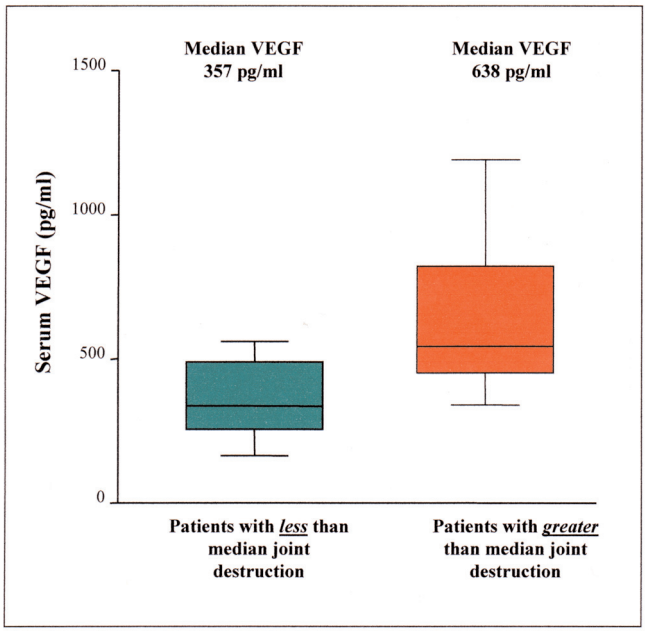

Fig. 2. Serum VEGF levels at presentation with RA correlate with joint destruction. The diagnosis of RA was assigned according to American College of Rheumatology classification criteria in 28 patients presenting less than 2 years from symptom onset. Hand and feet radiographs were taken at initial presentation and at 1 year follow-up, and were scored as pairs in chronological order, by two independent observers who were unaware of the identity of the patients. Patients were sub-divided into those with less than the overall median change in joint destruction (n=14, median change 1.6) and greater than the median change in joint destruction (n=14, median change 11.9). Serum VEGF levels were measured by ELISA in both sub-groups. Results were analysed using Student's unpaired t-test: $p<0.001$.

Further insights into the importance of reduced angiogenesis in RA were gained from clinical trials at the Kennedy Institute of anti-TNFα antibody infliximab (Remicade™), a chimeric mouse Fv, human IgG1, κ antibody of high affinity. From the earliest trials in 1992, infliximab has shown remarkable therapeutic efficacy, reducing both clinical and laboratory indices of disease activity (Feldmann and Maini, 2001). Since we have shown that serum VEGF levels are elevated in RA, and that TNFα can modulate synovial VEGF expression (Paleolog et al., 1998), we postulated that part of the benefit of anti-TNFα antibody in RA was through a reduction in synovial vascularity. To examine this hypothesis, we measured serum VEGF levels in RA patients treated with anti-TNFα antibody, and observed significant reductions in circulating concentrations of this angiogenic cytokine (Paleolog et al., 1998). In a more recent study, the effects of infliximab on synovial angiogenesis, vascularity and VEGF expression were investigated (Taylor et al., 1998). Patients with active RA received a single infusion of anti-TNFα antibody. Synovial biopsies were taken during arthroscopic examination of the knee joint 1 day before and 2 weeks after treatment, and synovial vascularity was assessed by immunohistochemistry followed by quantitative image analysis. Anti-TNFα therapy was found to reduce synovial vascularity as assessed by CD31 and von Willebrand factor (vWF) immunostaining. Additionally, a significant reduction in the number of αvß3 integrin-positive vessels was found. The reduced expression of CD31, vWF and αvß3 integrin following TNFα blockade is in agreement with the concept that the balance of new vessel growth and regression is altered such that a net loss of microvessels occurs.

These observations suggest that TNFα regulates production of VEGF *in vivo*, and that part of the beneficial effect of anti-TNFα in RA may be a down-modulation in blood vessel formation. Since the endothelial surface plays a key role in mediating cell traffic and nutrient delivery, such alterations in vascular density may also contribute to therapeutic efficacy. Currently we are in the process of using power colour Doppler to examine the effects of anti-TNFα antibody treatment on synovial vascularity.

Signalling pathways involved in the induction of VEGF in RA

Although VEGF is known to play a role in RA, and despite the increased belief that therapies such as TNFα blockade act at least in part through the downregulation of VEGF-mediated angiogenesis, the production of VEGF in the context of RA has not been extensively

studied. Indeed, although many of the signalling events downstream of VEGF receptor activation have been elucidated, the stimuli and mechanisms involved in the production of VEGF are still relatively controversial. However, most of the studies on the origin of VEGF in pathological conditions are mainly focused on cancer, and very limited for other VEGF-related diseases. In this section, we will firstly summarise the studies describing the signalling mechanisms regulating the production of VEGF, and then focus on studies describing mechanisms of induction of VEGF in rheumatoid synovium.

Ras represents a junction in the transmission of signals from growth factor receptors and from exposure of cells to stress-inducing agents. In many different cell types, activated Ras triggers a kinase cascade involving Raf-1, which phosphorylates and activates the mitogen-activated protein kinase (MAPK) kinase MEK, and then p44/p42 MAPK. The Ras pathway appears to regulate VEGF secretion, in human and rodent tumour cell lines (Milanini et al., 1998; Feldkamp et al., 1999; Okajima and Thorgeirsson, 2000; Rak and Kerbel, 2001). A role for phosphatidylinositol 3-kinase (PI 3-kinase) and Akt has also been suggested in a variety of cells including endothelial cells (Wang et al., 1999; Jiang et al., 2000) and fibroblasts (Miele et al., 2000). For example, over-expression of a constitutively active form of PI 3-kinase stimulated angiogenesis in the chorioallantoic membrane of the chicken embryo. In contrast, over-expression of dominant-negative constructs of PI 3-kinase inhibited angiogenesis. Levels of VEGF mRNA were found to be elevated in chicken embryo fibroblasts and endothelial cells expressing activated PI 3-kinase. Interestingly, the PI-3 kinase inhibitor LY294002 prevented VEGF expression in endothelial-like cells but not in fibroblasts (Jiang et al., 2000).

The Src family of non-receptor protein tyrosine kinases has also been implicated in the induction of VEGF (Mukhopadhyay et al., 1995; Fredriksson et al., 2000). Targets of Src include PI-3 kinase, thus feeding into the pathways regulating apoptosis/survival. Hypoxia was found to increase the kinase activity of pp60c-Src, without affecting Fyn or Yes. Expression of a dominant-negative mutant form of c-Src markedly reduced VEGF induction, although there was a compensatory activation of Fyn (Mukhopadhyay et al., 1995). Several reports have also indicated that activation of protein kinase C up-regulates VEGF expression in a variety of cell types (Shih et al., 1999; Kozawa et al., 2000; Pal et al., 2001; Suzuma et al., 2002). MAPK are also thought to play a significant role in the induction of VEGF under hypoxic conditions. This was shown in several different tumour, as well as primary, cell types (Berra et al., 2000; D'Angelo et al., 2000; Pilch et al., 2001).

We have had a long-standing interest in the role of the NFκB pathway in the induction of cytokines and growth factors. NFκB is a transcription factor that controls the expression of many genes involved in immune and inflammatory responses including TNFα, IL-1ß and IL-6 (Tak and Firestein, 2001). However the importance of the NFκB in the context of VEGF expression is relatively unknown and remains controversial. Inhibition of NFκB activation using pyrrolidine dithiocarbamate (PDTC) did not suppress LPS-induced upregulation of the VEGF mRNA in ventricular myocytes (Sugishita et al., 2000). In contrast interruption of IκB kinase, which phopsphorylates the endogenous NFκB inhibitor IκBα, thereby allowing NFκB activation resulted in partial inhibition of expression of IL-8 release but not VEGF (Bancroft et al., 2001). NFκB has been shown to be partly responsible for the upregulation of VEGF mRNA and development of vessel-like structures by human microvascular endothelial cells in response to TNFα (Yoshida et al., 1997). Nevertheless in two recent studies on human cancer cells transfected with a mutated IκBα, blockade of NFκB signalling significantly inhibited expression of VEGF (Huang et al., 2000, 2001). Our own preliminary data suggest that NFκB plays an important role in the upregulation of VEGF release by activated macrophages, and that complex upstream events, involving not only IκB kinases, but also other signals, are important.

In the context of this review, it is important to address the issue of signalling pathways involved in the induction of VEGF in RA. As we previously mentioned, synovial fibroblasts, monocytes from peripheral blood and synovial tissue macrophages are all significant potential sources of VEGF. Various pro-inflammatory cytokines, such as IL-1, TNFα, IL-6, IL-8 and M-CSF, are present in the synovial fluid and tissue of RA patients and are known to induce VEGF production (Paleolog and Miotla, 1998; Paleolog et al., 1998; Koch, 2000; Koch et al., 2001). Recently placental growth factor was reported to induce VEGF secretion from mononuclear cells isolated from RA patients (Bottomley et al., 2000). However, signalling pathways triggered by these stimuli to induce expression of VEGF in these cells are still unknown.

In 1995 Ben-av and co-workers demonstrated that inflammatory mediators such as prostaglandin E2 and IL-1 induce the expression of VEGF by rheumatoid synovial fibroblast cells. Activators of protein kinase A pathway stimulated the expression of VEGF whereas down-regulation of protein kinase C did not influence the prostaglandin E2-mediated effect (Ben-Av et al., 1995). Tsuji and colleagues investigated the effects of MAPK inhibitors or phosphodiesterase inhibitors on IL-1 induced cytokine production in primary fibroblast-like cells derived from the inflamed synovium of RA patients. Fibroblast-like synovial cells stimulated by IL-1ß produced various cytokines, including VEGF. SB202190 or SB203580, both inhibitors of p38 MAPK, inhibited all cytokine production including VEGF. In contrast, quazinone, an inhibitor of cyclic GMP-inhibited phosphodiesterase, scarcely affected cytokine production in fibroblast-like synovial cells. These results suggested that activation of the MAPK cascade may be important for IL-1-induced VEGF production in

synovium-derived cells (Tsuji et al., 1999, 2000).

NFκB is activated in RA synovium and plays a role in the induction of TNFα release (Aupperle et al., 1999, 2001). A study on human rheumatoid synovial fibroblasts showed that IL-1ß-induced IL-8 production, which is known to be a NFκB-regulated event, was inhibited by hymenialdisine. Hymenialdisine is a marine natural product and was characterised as an inhibitor of NFκB activation. Interestingly, in this study IL-1-induced production of VEGF was not affected by exposure to hymenialdisine (Roshak et al., 1997). Recently it was reported that ligation of CD40 induces the expression of VEGF by rheumatoid synovial fibroblasts. In this study it was also shown that dexamethasone completely abrogated CD40 ligand-induced VEGF production. The hypothesis was that dexamethasone may block activation of NFκB through stimulation of the transcription of IκBα, the inhibitor of NFκB. In addition, it was shown that PDTC partially down-regulated CD40 ligand-induced VEGF production, suggesting that the NFκB pathway was partly involved in the signalling by CD40 ligand leading to VEGF production and that additional elements may be involved in VEGF upregulation in synovial fibroblasts (Cho et al., 2000).

Summarising the above studies, we can conclude that many questions regarding the signalling mechanisms leading to VEGF production in RA remain still to be addressed. An increased molecular understanding of those mechanisms is vital for the design and development of specific inhibitors of VEGF production, and hence angiogenesis, in RA.

Angiogenesis as a target in RA: lessons from animal studies

Although many of the pathways involved in VEGF-mediated angiogenesis in RA still remain to be elucidated, the vasculature has long been considered as a potential target for therapy in arthritis. There is agreement amongst many observers that the proliferative nature of the invasive pannus within the arthritic joint closely resembles that of a growing tumour. There is constant remodelling of the pannus, somatic mutations in regulatory genes (Ha-ras and p53), expression of angiogeneic cytokines and growth factors, including FGF2, IL-8 and VEGF, as well as soluble adhesion molecules (Koch, 1998), characteristics that are shared with solid tumours. This allows for the overlapping between cancer and RA of many therapeutic strategies targeting angiogenesis.

The most characterised animal model for RA is the collagen-induced arthritis (CIA) model which is triggered by the intra-dermal immunisation of animals with bovine type II collagen in complete Freund's adjuvant (Trentham et al., 1977). It was initially described in rats but can be induced in other species, particularly in rodents such as mice (Staines and Wooley, 1994). One significant difference from RA is that the resulting arthritis can go into remission and resolve itself over a period of time, and so the disease is effectively acute in nature. However it shares many features with the clinical disease such as synovitis, pannus formation, bone/cartilage erosions. It is T cell dependent, can be adoptively transferred to naïve animals and has been very useful in developing biological therapies (Williams et al., 1992). Brahn and colleagues were the one of the first groups to explore the inhibition of angiogenesis as a potential therapy for RA. They chose to use AGM-1470 (TNP-470) a synthetic derivative of fumagillin, a natural fungal antibiotic and a potent endothelial cell toxin. TNP-470 was previously shown to be highly effective in suppressing solid tumours in animal models in addition to *in vitro* human tumour studies. In rat CIA, sub-cutaneous injection of TNP-470 both before disease onset and once disease was established, resulted in prevention and reduction of severity respectively (Peacock et al., 1992). Radiological and histological assessments indicated significant suppression in bone destruction and pannus formation in the treatment groups. Furthermore when measuring specific antibodies and delayed type hypersensitivity responses of the rats to assess immunity to collagen, there were no differences between the treatment regimes, indicating that AGM-1470 had no anti-proliferative or immunosuppressive activity. In a separate study when administering this agent into the rat adjuvant induced-arthritis (AIA) model, prevention and suppression of disease was observed (Peacock et al., 1995). Brahn and co-workers suggested that combination therapy for RA encompassing an anti-angiogenic agent with one that is either anti-inflammatory and/or anti-neoplastic was inevitable, because of the toxic side effects of existing DMARDs (Oliver and Brahn, 1996). To this end, in the rat CIA model, TNP-470 was combined with taxol, a microtubule stabiliser (Oliver et al., 1994), and cyclosporin A, the immunomodulatory agent that inhibits cytokine induced T cell activation (Oliver et al., 1995). In each study TNP-470, when combined with either taxol or cyclosporin A, was more effective both clinically and radiologically than any of the agents used in isolation, though only taxol, as a single agent, had any therapeutic action. The administration of TNP-470, whether in combination with cyclosporin A or alone, decreased mean serum VEGF levels while leaving TNFα elevated at levels observed in arthritic controls. This gave an tentative indication of the proposed mechanism of TNP-470 in arthritic rodent models and the pathophysiological role that VEGF may play, particularly during the early induction phase of neo-vascularisation and pannus formation of the joint (Oliver et al., 1995).

Another model of RA is the KRN/NOD transgenic mouse, which spontaneously develops polyarthritis that closely resembles the clinical disease. The T cell receptor of this transgenic KRN strain is Vß6 (Mangialaio et al., 1999) and when crossed with NOD mice results in T cell recognition of an NOD derived

MHC II protein, which serves as the trigger for the disease without the need for any immunisation. Anti-angiogenic intervention by TNP-470 both prevented disease onset and ameliorated established disease (de Bandt et al., 2000). Significant improvement was observed in the histological scores of the joints taken from TNP-470-treated KRN/NOD mice and correlated with a reduction in circulating VEGF serum levels and arthritic index. Moreover high levels of VEGF were measured in the synovial fluids of control KRN/NOD mice that even exceeded serum concentrations. However, due to improvement or absence of arthritis, no VEGF was assessed in the TNP-470-treated KRN/NOD mice because these animals possessed little or no synovial fluid and joint effusions. These data concurred with previous interpretations in that lymphocyte proliferation and histological analysis of germinal centres and draining lymph nodes was comparable between treatment groups, suggesting that TNP-470 did indeed target angiogenesis.

The teratogenic action of thalidomide had been attributed to its anti-angiogenic properties (D'Amato et al., 1994) whereas its anti-inflammatory and immunosuppressive action, as demonstrated by the healing of ulcers caused by leprosy, HIV and Behçet's disease, was associated with its reported inhibition of TNFα. Thalidomide was thus considered a very attractive candidate for angiogenic intervention in arthritic models. Thalidomide and its analogues EM-12 and supidimide, were given by oral gavage in rat CIA, and arthritic index, radiological and immunological assessment were measured in an attempt to correlate any changes directly to the expression of both VEGF and TNFα (Oliver et al., 1998). Improvements were observed in arthritis severity and radiological scores of all treated rats, though there was the expected suppression of both collagen-specific delayed type hypersensitivity and antibody responses. In spite of this, both circulating serum VEGF and TNFα levels were elevated in all the treatment and control groups thus eliminating, at least within arthritis, thalidomide having any therapeutic action on VEGF-induced angiogenesis.

The evaluation of changes in vascularity or its markers within these models has contributed in some way to addressing to what extent does angiogenesis play in arthritis but it has not always been linked to VEGF expression. Scola and colleagues evaluated the angiogenic factors produced by synovial tissue taken from patients with juvenile RA (Scola et al., 2001). Though not strictly an RA model, they developed a SCID mouse-human juvenile RA synovium chimera model by transplanting fresh tissue samples subcutaneously in the backs of immunocompromised $Prkdc^{scid}$ mice for up to 10 days. Both mRNA and protein expression of VEGF and its receptors (Flt-1, Flk-1 and Flt-4), together with angiopoietin-1, Tie-1 and -2 receptors, endoglin and CD31, were assessed within the engraftments. An increase in vascularity was clearly observed in the engraftments with juvenile RA and RA tissue, relative to synovium from patients with OA and other non-inflammtory arthropathies. Antibodies against vWF and a murine pan-endothelial cell antigen (anti-MECA-23) allowed for discrimination between human and murine blood vessels, and an increase in newly formed blood vessels density of human origin was found in juvenile RA and RA samples.

Other markers and mediators for neovascularisation include members of the integrin family, αvß3 and αvß5, which are expressed on proliferating and migrating endothelial cells and mediate cell adhesion by recognition of the arg-gly-asp (RGD) sequence (Koch, 1998). Antagonists against the αvß3 have been successfully used to suppress disease activity in both rat AIA (Badger et al., 2001) and rabbit antigen or ovalbumin-induced arthritis (OIA) (Storgard et al., 1999). Immunohistochemical analysis showed expression of vWf and αvß3 in cryosections of rabbit OIA, but not in control, joints, and expression was augmented by intra-articular injection of FGF2. This increased vascularity correlated with inflammatory parameters such as joint swelling, pannus formation and bone and cartilage erosions. These disease indices and increased expression of angiogenic markers was reversed by the use of a αvß3 peptide antagonist, EMD 69601, which contained the RGD sequence (Storgard et al., 1999). A non-peptide αvß3 antagonist, SB273005, confirmed involvement of this integrin when it suppressed paw volume, leukocyte infiltration and increased bone mineral density after oral gavage in the rat AIA model (Badger et al., 2001). This was both after

Table 1. VEGF inhibition reduces joint destruction in mouse collagen-induced arthritis. Mice were immunised with bovine collagen in complete Freund's adjuvant. On the day of arthritis onset, mice were either untreated or treated with sFlt-1 at a dose of 300 μg intra-peritoneally, daily until day 5 of arthritis. An additional group received heat-denatured sFlt-1. Paws were processed for histology, and histology sections were graded for the degree of bone/cartilage erosion and inflammation. Data are percentage of animals exhibiting normal, moderate and severe joint destruction, and were analysed by $\chi 2$ test for trend: sFlt-treated versus untreated: $p<0.001$; sFlt-treated versus Control-treated: $p<0.01$; Untreated versus Control-treated: not significant.

	NORMAL JOINT ARCHITECTURE	SYNOVITIS AND SOME BONE EROSIONS	EXTENSIVE SYNOVITIS AND BONE EROSIONS
Untreated	23%	47%	30%
Control-treated	25%	41%	34%
Soluble Flt-treated	47%	42%	11%

prophylactic and therapeutic regimens.

Recently, with the increased availability of specific antibodies to VEGF and the secreted form of the extracellular domain of VEGF receptor 1 (sFlt-1), a more direct approach could be taken to block this important angiogenic cytokine within arthritic models. Studies from our own group using the murine CIA model firstly showed that single cell suspensions obtained from the synovium of arthritic mice, expressed greater levels of VEGF mRNA and protein than cells from healthy animals (Miotla et al., 2000). Disease severity, as described by either footpad swelling or clinical scores, showed a strong association with magnitude of VEGF release. VEGF blockade using sFlt-1 reduced disease severity (footpad swelling, clinical scores, bone/cartilage destruction and synovitis) when injected intra-peritoneally every day for the first 5 days after disease onset (Table 1). Not only was this study able to link VEGF expression with disease severity and progression, but also showed that VEGF blockade was therapeutic. Other studies were also able to use inhibition of VEGF activity, by using antibodies against this cytokine. A neutralising polyclonal antibody against human VEGF raised in rabbits was shown to both prevent and suppress established disease in mouse CIA when compared to normal rabbit immunoglobulin (Sone et al., 2001). However this was in contrast to a rabbit polyclonal anti-murine VEGF antibody that prevented disease in mouse CIA model but was unable to change disease progression when injected after arthritis onset (Lu et al., 2000). Despite this, increased vascularity was shown by the expression of vWF and localisation of VEGF with synovial fibroblasts and macrophages by immunohistochemical analysis. Furthermore, both vWF and VEGF concentrations, as measured by ELISA, were shown to peak at day 4 after arthritis onset and was considered the height of neovascularisation, suggesting that VEGF-induced neovascularisation was probably crucial in the early inductive phase of arthritis.

These observations demonstrate that anti-angiogenic approaches lead to suppression of arthritis in animal models. In particular, it would appear that blocking the pro-angiogenic and permeability action of VEGF may be beneficial for treatment of RA.

Future perspectives

Angiogenesis is an important process in the development and maintenance of RA. It is likely that clinical trials of VEGF-targeted therapies may be considered for RA, either as stand-alone, or in combination with established therapies such as TNFα inhibitors.

In particular, data from the animal models discussed earlier have contributed to the idea that VEGF-induced angiogenesis potentially plays a vital role in the aetiology of arthritis, and that inhibition of VEGF-mediated effects is desirable. The advantage of this line of therapy is apparent since very few deleterious side affects can be envisaged when targeting neovascularisation because, at least in the adult, it is prevalent in pathological rather than physiological states. The advent of adenoviral gene delivery has allowed the expression of a range of immunosuppressive molecules to modulate arthritis in CIA, including TNF receptor p55 (Quattrocchi et al., 1999), IL-10 (Quattrocchi et al., 2001) and IL-4 (Kim et al., 2000). Such technology would potentially be very intuitive when trying to dissect the role VEGF in the inductive phase of the disease since expression could be controlled more effectively than the repetitive administration required for antibodies or fusion proteins. At present we are using such an adenovirus gene delivery system, expressing the human sFlt-1 transgene, to block VEGF activity in mouse CIA.

However, patients with RA develop cardiovascular problems at an earlier age than the non-arthritic population, and the potential effects of angiogenesis inhibition need to be considered in terms of reducing new vessel formation after an ischemic episode. In theory at least, anti-angiogenic treatment should not be pro-infective, and combined blockade of VEGF and TNFα in RA may be beneficial, without augmenting potential adverse effects.

Acknowledgements. The Kennedy Institute of Rheumatology is a Division of the Faculty of Medicine, Imperial College of Science, Technology and Medicine, and receives a Core Grant from the Arthritis Research Campaign of Great Britain. The authors wish to thank the Nuffield Foundation (YA) and The Sir Jules Thorn Charitable Trust (SK) for financial support. The contributions of Dr. Sundeept Ballara, Dr. Jadwiga Miotla, Dr Peter Taylor and Ms. Sylvia Young, and the support of Professor Marc Feldmann and Professor Ravinder N. Maini, are gratefully acknowledged.

References

Aupperle K.R., Bennett B.L., Boyle D.L., Tak P.P., Manning A.M. and Firestein G.S. (1999). NF-kappa B regulation by I kappa B kinase in primary fibroblast-like synoviocytes. J. Immunol. 163, 427-433.

Aupperle K., Bennett B., Han Z., Boyle D., Manning A. and Firestein G. (2001). NF-kappa B regulation by I kappa B kinase-2 in rheumatoid arthritis synoviocytes. J. Immunol. 166, 2705-2711.

Badger A.M., Blake S., Kapadia R., Sarkar S., Levin J., Swift B.A., Hoffman S.J., Stroup G.B., Miller W.H., Gowen M. and Lark M.W. (2001). Disease-modifying activity of SB 273005, an orally active, nonpeptide alphavbeta3 (vitronectin receptor) antagonist, in rat adjuvant-induced arthritis. Arthritis Rheumatism 44, 128-137.

Ballara S.C., Miotla J.M. and Paleolog E.M. (1999). New vessels, new approaches: angiogenesis as a therapeutic target in musculoskeletal disorders. Int. J. Exp. Pathol. 80, 235-250.

Ballara S.C., Taylor P.C., Reusch P., Marmé D., Feldmann M., Maini R.N. and Paleolog E.M. (2001). Raised serum vascular endothelial growth factor levels are associated with destructive change in inflammatory arthritis. Arthritis Rheumatism 44, 2055-2064.

Bancroft C.C., Chen Z., Dong G., Sunwoo J.B., Yeh N., Park C. and Van Waes C. (2001). Coexpression of proangiogenic factors IL-8 and VEGF by human head and neck squamous cell carcinoma

involves coactivation by MEK-MAPK and IKK-NF-kappaB signal pathways. Clin. Cancer Res. 7, 435-442.

Barrett E.M., Scott D.G., Wiles N.J. and Symmons D.P. (2000). The impact of rheumatoid arthritis on employment status in the early years of disease: a UK community-based study. Rheumatology (Oxford) 39, 1403-1409.

Ben-Av P., Crofford L.J., Wilder R.L. and Hla T. (1995). Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. FEBS Lett. 372, 83-87.

Berra E., Milanini J., Richard D.E., Le Gall M., Vinals F., Gothie E., Roux D., Pages G. and Pouyssegur J. (2000). Signaling angiogenesis via p42/p44 MAP kinase and hypoxia. Biochem. Pharmacol. 60, 1171-1178.

Bottomley M.J., Webb N.J., Watson C.J., Holt L., Bukhari M., Denton J., Freemont A.J. and Brenchley P.E. (2000). Placenta growth factor (PlGF) induces vascular endothelial growth factor (VEGF) secretion from mononuclear cells and is co-expressed with VEGF in synovial fluid. Clin. Exp. Immunol. 119, 182-188.

Brown L.F., Detmar M., Claffey K., Nagy J.A., Feng D., Dvorak A.M. and Dvorak H.F. (1997). Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine. EXS 79, 233-269.

Brown R.A., Weiss J.B., Tomlinson I.W., Phillips P. and Kumar S. (1980). Angiogenic factor from synovial fluid resembling that from tumours. Lancet 1 (8170), 682-685.

Cho C.S., Cho M.L., Min S.Y., Kim W.U., Min D.J., Lee S.S., Park S.H., Choe J. and Kim H.Y. (2000). CD40 engagement on synovial fibroblast up-regulates production of vascular endothelial growth factor. J. Immunol. 164, 5055-5061.

D'Amato R.J., Loughnan M.S., Flynn E. and Folkman J. (1994). Thalidomide is an inhibitor of angiogenesis. Proc. Natl. Acad. Sci. USA 91, 4082-4085.

D'Angelo G., Ladoux A. and Frelin C. (2000). Hypoxia-induced transcriptional activation of vascular endothelial growth factor is inhibited by serum. Biochem. Biophys. Res. Commun. 267, 334-338.

de Bandt M., Grossin M., Weber A.J., Chopin M., Elbim C., Pla M., Gougerot-Pocidalo M.A. and Gaudry M. (2000). Suppression of arthritis and protection from bone destruction by treatment with TNP-470/AGM-1470 in a transgenic mouse model of rheumatoid arthritis. Arthritis Rheumatism 43, 2056-2063.

Fava R.A., Hunt J.A., Yeo K.T., Brown F. and Berse B. (1995). Induction of vascular permeability factor (vascular endothelial growth factor) by TGFß in synovial fibroblasts. Arthritis Rheumatism 38, S343.

Fava R.A., Olsen N.J., Spencer-Green G., Yeo K.T., Yeo T.K., Berse B., Jackman R.W., Senger D.R., Dvorak H.F. and Brown L.F. (1994). Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. J. Exp. Med. 180, 341-346.

Feldkamp M.M., Lau N., Rak J., Kerbel R.S. and Guha A. (1999). Normoxic and hypoxic regulation of vascular endothelial growth factor (VEGF) by astrocytoma cells is mediated by Ras. Int. J. Cancer 81, 118-124.

Feldmann M. and Maini R.N. (2001). Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? Annu. Rev. Immunol. 19, 163-196.

FitzGerald O., Soden M., Yanni G., Robinson R. and Bresnihan B. (1991). Morphometric analysis of blood vessels in synovial membranes obtained from clinically affected and unaffected knee joints of patients with rheumatoid arthritis. Ann. Rheum. Dis. 50, 792-796.

Frater-Schroder M., Risau W., Hallmann R., Gautschi P. and Bohlen P. (1987). Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. Proc. Natl. Acad. Sci. USA 84, 5277-5281.

Fredriksson J.M., Lindquist J.M., Bronnikov G.E. and Nedergaard J. (2000). Norepinephrine induces vascular endothelial growth factor gene expression in brown adipocytes through a beta - Adrenoreceptor/cAMP/Protein kinase A pathway involving src but independently of Erk1/2. J. Biol. Chem. 275, 13802-13811.

Giatromanolaki A., Sivridis E., Athanassou N., Zois E., Thorpe P.E., Brekken R.A., Gatter K.C., Harris A.L., Koukourakis I.M. and Koukourakis M.I. (2001). The angiogenic pathway "vascular endothelial growth factor/flk-1(KDR)-receptor" in rheumatoid arthritis and osteoarthritis. J. Pathol. 194, 101-108.

Harris E.D. Jr. (1986). Pathogenesis of rheumatoid arthritis. Am. J. Med. 80, 4-10.

Hirata S., Matsubara T., Saura R., Tateishi H. and Hirohata K. (1989). Inhibition of in vitro vascular endothelial cell proliferation and in vivo neovascularization by low dose methotrexate. Arthritis Rheumatism 32, 1065-1073.

Huang S., Pettaway C.A., Uehara H., Bucana C.D. and Fidler I.J. (2001). Blockade of NF-kappaB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. Oncogene 20, 4188-4197.

Huang S., Robinson J.B., Deguzman A., Bucana C.D. and Fidler I.J. (2000). Blockade of nuclear factor-kappaB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. Cancer Res. 60, 5334-5339.

Ikeda M., Hosoda Y., Hirose S., Okada Y. and Ikeda E. (2000). Expression of vascular endothelial growth factor isoforms and their receptors Flt-1, KDR, and neuropilin-1 in synovial tissues of rheumatoid arthritis. J. Pathol. 191, 426-433.

Jalkanen S., Steere A.C., Fox R.I. and Butcher E.C. (1986). A distinct endothelial cell recognition system that controls lymphocyte traffic into inflamed synovium. Science 233, 556-558.

Jawed S., Gaffney K. and Blake D.R. (1997). Intra-articular pressure profile of the knee joint in a spectrum of inflammatory arthropathies. Ann. Rheum. Dis. 56, 686-689.

Jiang B.H., Zheng J.Z., Aoki M. and Vogt P.K. (2000). Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. Proc. Natl. Acad. Sci. USA 97, 1749-1753.

Kim K.N., Watanabe S., Ma Y., Thornton S., Giannini E.H. and Hirsch R. (2000). Viral IL-10 and soluble TNF receptor act synergistically to inhibit collagen-induced arthritis following adenovirus-mediated gene transfer. J. Immunol. 164, 1576-1581.

Koch A.E. (1998). Review: angiogenesis: implications for rheumatoid arthritis. Arthritis Rheumatism 41, 951-962.

Koch A.E. (2000). The role of angiogenesis in rheumatoid arthritis: recent developments. Ann. Rheum. Dis. 59 Suppl 1, i65-71.

Koch A.E., Halloran M.M., Hosaka S., Shah M.R., Haskell C.J., Baker S.K., Panos R.J., Haines G.K., Bennett G.L., Pope R.M. and Ferrara N. (1996). Hepatocyte growth factor. A cytokine mediating endothelial migration in inflammatory arthritis. Arthritis Rheumatism 39, 1566-1575.

Koch A.E., Harlow L.A., Haines G.K., Amento E.P., Unemori E.N., Wong

W.L., Pope R.M. and Ferrara N. (1994). Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. J. Immunol. 152, 4149-4156.

Koch A.E., Volin M.V., Woods J.M., Kunkel S.L., Connors M.A., Harlow L.A., Woodruff D.C., Burdick M.D. and Strieter R.M. (2001). Regulation of angiogenesis by the C-X-C chemokines interleukin-8 and epithelial neutrophil activating peptide 78 in the rheumatoid joint. Arthritis Rheumatism 44, 31-40.

Kozawa O., Kawamura H., Hatakeyama D., Matsuno H. and Uematsu T. (2000). Endothelin-1 induces vascular endothelial growth factor synthesis in osteoblasts: involvement of p38 mitogen-activated protein kinase. Cell. Signal. 12, 375-380.

Lee S.S., Joo Y.S., Kim W.U., Min D.J., Min J.K., Park S.H., Cho C.S. and Kim H.Y. (2001). Vascular endothelial growth factor levels in the serum and synovial fluid of patients with rheumatoid arthritis. Clin. Exp. Rheumatol. 19, 321-324.

Levy A.P., Levy N.S., Wegner S. and Goldberg M.A. (1995). Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J. Biol. Chem. 270, 13333-13340.

Levy N.S., Chung S., Furneaux H. and Levy A.P. (1998). Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J. Biol. Chem. 273, 6417-6423.

Lu J., Kasama T., Kobayashi K., Yoda Y., Shiozawa F., Hanyuda M., Negishi M., Ide H. and Adachi M. (2000). Vascular endothelial growth factor expression and regulation of murine collagen-induced arthritis. J. Immunol. 164, 5922-5927.

Lund-Olesen K. (1970). Oxygen tension in synovial fluids. Arthritis Rheumatism 13, 769-776.

Madhok R., Wijelath E., Smith J., Watson J., Sturrock R. and Capell H. (1991). Is the beneficial effect of sulphasalazine due to inhibition of synovial neovascularization? J. Rheumatol. 18, 199-202.

Mangialaio S., Ji H., Korganow A.S., Kouskoff V., Benoist C. and Mathis D. (1999). The arthritogenic T cell receptor and its ligand in a model of spontaneous arthritis. Arthritis Rheumatism 42, 2517-2523.

Matsubara T., Saura R., Hirohata K. and Ziff M. (1989). Inhibition of human endothelial cell proliferation in vitro and neovascularization in vivo by D-penicillamine. J. Clin. Invest. 83, 158-167.

McEntegart A., Capell H.A., Creran D., Rumley A., Woodward M. and Lowe G.D. (2001). Cardiovascular risk factors, including thrombotic variables, in a population with rheumatoid arthritis. Rheumatology (Oxford) 40, 640-644.

Miele C., Rochford J.J., Filippa N., Giorgetti-Peraldi S. and Van Obberghen E. (2000). Insulin and IGF-I induce VEGF mRNA expression via different signaling pathways. J. Biol. Chem. 275, 21695-21702.

Milanini J., Vinals F., Pouyssegur J. and Pages G. (1998). p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. J. Biol. Chem. 273, 18165-18172.

Miotla J., Maciewicz R., Kendrew J., Feldmann M. and Paleolog E. (2000). Treatment with soluble VEGF receptor reduces disease severity in murine collagen-induced arthritis. Lab. Invest. 80, 1195-1205.

Mukhopadhyay D., Tsiokas L., Zhou X.M., Foster D., Brugge J.S. and Sukhatme V.P. (1995). Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. Nature 375, 577-581.

Nagashima M., Yoshino S., Ishiwata T. and Asano G. (1995). Role of vascular endothelial growth factor in angiogenesis of rheumatoid arthritis. J. Rheumatol. 22, 1624-1630.

Nagashima M., Asano G. and Yoshino S. (2000a). Imbalance in production between vascular endothelial growth factor and endostatin in patients with rheumatoid arthritis. J. Rheumatol. 27, 2339-2342.

Nagashima M., Wauke K., Hirano D., Ishigami S., Aono H., Takai M., Sasano M. and Yoshino S. (2000b). Effects of combinations of anti-rheumatic drugs on the production of vascular endothelial growth factor and basic fibroblast growth factor in cultured synoviocytes and patients with rheumatoid arthritis. Rheumatology (Oxford) 39, 1255-1262.

Neufeld G., Cohen T., Gengrinovitch S. and Poltorak Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 13, 9-22.

Okajima E. and Thorgeirsson U.P. (2000). Different regulation of vascular endothelial growth factor expression by the ERK and p38 kinase pathways in v-ras, v-raf, and v-myc transformed cells. Biochem. Biophys. Res. Commun. 270, 108-111.

Oliver S.J. and Brahn E. (1996). Combination therapy in rheumatoid arthritis: the animal model perspective. J. Rheumatol. Suppl 44, 56-60.

Oliver S.J., Banquerigo M.L. and Brahn E. (1994). Suppression of collagen-induced arthritis using an angiogenesis inhibitor, AGM-1470, and a microtubule stabilizer, taxol. Cell. Immunol. 157, 291-299.

Oliver S.J., Cheng T.P., Banquerigo M.L. and Brahn E. (1995). Suppression of collagen-induced arthritis by an angiogenesis inhibitor, AGM-1470, in combination with cyclosporin: reduction of vascular endothelial growth factor (VEGF). Cell. Immunol. 166, 196-206.

Oliver S.J., Cheng T.P., Banquerigo M.L. and Brahn E. (1998). The effect of thalidomide and 2 analogs on collagen induced arthritis. J. Rheumatol. 25, 964-969.

Pal S., Datta K., Khosravi-Far R. and Mukhopadhyay D. (2001). Role of protein kinase Czeta in Ras-mediated transcriptional activation of vascular permeability factor/vascular endothelial growth factor expression. J. Biol. Chem. 276, 2395-2403.

Paleolog E.M. and Fava R.A. (1998). Angiogenesis in rheumatoid arthritis: implications for future therapeutic strategies. Springer Semin. Immunopathol. 20, 73-94.

Paleolog E.M. and Miotla J.M. (1998). Angiogenesis in arthritis: role in disease pathogenesis and as a potential therapeutic target. Angiogenesis 2, 295-307.

Paleolog E.M., Young S., Stark A.C., McCloskey R.V., Feldmann M. and Maini R.N. (1998). Modulation of angiogenic vascular endothelial growth factor (VEGF) by TNFα and IL-1 in rheumatoid arthritis. Arthritis Rheumatism 41, 1258-1265.

Peacock D.J., Banquerigo M.L. and Brahn E. (1992). Angiogenesis inhibition suppresses collagen arthritis. J. Exp. Med. 175, 1135-1138.

Peacock D.J., Banquerigo M.L. and Brahn E. (1995). A novel angiogenesis inhibitor suppresses rat adjuvant arthritis. Cell. Immunol. 160, 178-184.

Pilch H., Schlenger K., Steiner E., Brockerhoff P., Knapstein P. and Vaupel P. (2001). Hypoxia-stimulated expression of angiogenic growth factors in cervical cancer cells and cervical cancer-derived fibroblasts. Int. J. Gynecol. Cancer 11, 137-142.

Pincus T., Sokka T. and Wolfe F. (2001). Premature mortality in patients with rheumatoid arthritis: evolving concepts. Arthritis & Rheumatism

44, 1234-1236.

Pufe T., Petersen W., Tillmann B. and Mentlein R. (2001). Splice variants VEGF121 and VEGF165 of the angiogenic peptide vascular endothelial cell growth factor are expressed in the synovial tissue of patients with rheumatoid arthritis. J. Rheumatol. 28, 1482-1485.

Quattrocchi E., Dallman M.J., Dhillon A.P., Quaglia A., Bagnato G. and Feldmann M. (2001). Murine IL-10 gene transfer inhibits established collagen-induced arthritis and reduces adenovirus-mediated inflammatory responses in mouse liver. J. Immunol. 166, 5970-5978.

Quattrocchi E., Walmsley M., Browne K., Williams R.O., Marinova-Mutafchieva L., Buurman W., Butler D.M. and Feldmann M. (1999). Paradoxical effects of adenovirus-mediated blockade of TNF activity in murine collagen-induced arthritis. J. Immunol. 163, 1000-1009.

Rak J. and Kerbel R.S. (2001). Ras regulation of vascular endothelial growth factor and angiogenesis. Methods Enzymol. 333, 267-283.

Remmers E.F., Sano H., Lafyatis R., Case J.P., Kumkumian G.K., Hla T., Maciag T. and Wilder R.L. (1991). Production of platelet derived growth factor B chain (PDGF-B/c-sis) mRNA and immunoreactive PDGF B-like polypeptide by rheumatoid synovium: coexpression with heparin binding acidic fibroblast growth factor-1. J. Rheumatol. 18, 7-13.

Rooney M., Condell D., Quinlan W., Daly L., Whelan A., Feighery C. and Bresnihan B. (1988). Analysis of the histologic variation of synovitis in rheumatoid arthritis. Arthritis Rheumatism 31, 956-963.

Roshak A., Jackson J.R., Chabot-Fletcher M. and Marshall L.A. (1997). Inhibition of NFkappaB-mediated interleukin-1beta-stimulated prostaglandin E2 formation by the marine natural product hymenialdisine. J. Pharmacol. Exp. Ther. 283, 955-961.

Rothschild B.M. and Masi A.T. (1982). Pathogenesis of rheumatoid arthritis: a vascular hypothesis. Semin. Arthritis Rheumatism 12, 11-31.

Sano H., Engleka K., Mathern P., Hla T., Crofford L.J., Remmers E.F., Jelsema C.L., Goldmuntz E., Maciag T. and Wilder R.L. (1993). Coexpression of phosphotyrosine-containing proteins, platelet-derived growth factor-B, and fibroblast growth factor-1 in situ in synovial tissues of patients with rheumatoid arthritis and Lewis rats with adjuvant or streptococcal cell wall arthritis. J. Clin. Invest. 91, 553-565.

Scola M.P., Imagawa T., Boivin G.P., Giannini E.H., Glass D.N., Hirsch R. and Grom A.A. (2001). Expression of angiogenic factors in juvenile rheumatoid arthritis: correlation with revascularization of human synovium engrafted into SCID mice. Arthritis Rheumatism 44, 794-801.

Semble E.L., Turner R.A. and McCrickard E.L. (1985). Rheumatoid arthritis and osteoarthritis synovial fluid effects on primary human endothelial cell cultures. J. Rheumatol. 12, 237-241.

Shih S.C., Mullen A., Abrams K., Mukhopadhyay D. and Claffey K.P. (1999). Role of protein kinase C isoforms in phorbol ester-induced vascular endothelial growth factor expression in human glioblastoma cells. J. Biol. Chem. 274, 15407-15414.

Shweiki D., Itin A., Soffer D. and Keshet E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359, 843-845.

Sone H., Kawakami Y., Sakauchi M., Nakamura Y., Takahashi A., Shimano H., Okuda Y., Segawa T., Suzuki H. and Yamada N. (2001). Neutralization of vascular endothelial growth factor prevents collagen-induced arthritis and ameliorates established disease in mice. Biochem. Biophys. Res. Commun. 281, 562-568.

Staines N.A. and Wooley P.H. (1994). Collagen arthritis -what can it teach us? Br. J. Rheumatol. 33, 798-807.

Stevens C.R., Williams R.B., Farrell A.J. and Blake D.R. (1991). Hypoxia and inflammatory synovitis: observations and speculation. Ann. Rheum. Dis. 50, 124-132.

Storgard C.M., Stupack D.G., Jonczyk A., Goodman S.L., Fox R.I. and Cheresh D.A. (1999). Decreased angiogenesis and arthritic disease in rabbits treated with an alphavbeta3 antagonist. J. Clin. Invest. 103, 47-54.

Sugishita Y., Shimizu T., Yao A., Kinugawa K., Nojiri T., Harada K., Matsui H., Nagai R. and Takahashi T. (2000). Lipopolysaccharide augments expression and secretion of vascular endothelial growth factor in rat ventricular myocytes. Biochem. Biophys. Res. Commun. 268, 657-662.

Suzuma I., Suzuma K., Ueki K., Hata Y., Feener E.P., King G.L. and Aiello L.P. (2002). Stretch-induced retinal VEGF expression is mediated by PI3 kinase and PKC-{zeta} but not by stretch-induced ERK1/2, Akt, Ras or classical/novel PKC pathways. J. Biol. Chem. 277, 1047-1057.

Tak P.P. and Firestein G.S. (2001). NF-kappaB: a key role in inflammatory diseases. J. Clin. Invest. 107, 7-11.

Taylor P., Patel S., Paleolog E., McCloskey R.V., Feldmann M. and Maini R.N. (1998). Reduced synovial vascularity following TNFα blockade in rheumatoid arthritis. Arthritis Rheumatism 41 Suppl 9, S295 (Abstract 1577).

Trentham D.E., Townes A.S. and Kang A.H. (1977). Autoimmunity to type II collagen an experimental model of arthritis. J. Exp. Med. 146, 857-868.

Tsuji F., Matsuoka H., Aono H., Takai M., Horiuchi M., Nishimura K. and Mita S. (2000). Effects of sulfhydryl compounds on interleukin-1-induced vascular endothelial growth factor production in human synovial stromal cells. Biol. Pharm. Bull. 23, 663-665.

Tsuji F., Oki K., Senda T., Horiuchi M. and Mita S. (1999). Effects of mitogen-activated protein kinase inhibitors or phosphodiesterase inhibitors on interleukin-1-induced cytokines production in synovium-derived cells. Immunol. Lett. 68, 275-279.

Walsh D.A. and Pearson C.I. (2001). Angiogenesis in the pathogenesis of inflammatory joint and lung diseases. Arthritis Res. 3, 147-153.

Walsh D.A., Wade M., Mapp P.I. and Blake D.R. (1998). Focally regulated endothelial proliferation and cell death in human synovium. Am. J. Pathol. 152, 691-702.

Wang D., Huang H.J., Kazlauskas A. and Cavenee W.K. (1999). Induction of vascular endothelial growth factor expression in endothelial cells by platelet-derived growth factor through the activation of phosphatidylinositol 3-kinase. Cancer Res. 59,1464-1472.

Williams R.O., Plater-Zyberk C., Williams D.G. and Maini R.N. (1992). Successful transfer of collagen-induced arthritis to severe combined immunodeficient (SCID) mice. Clin. Exp. Immunol. 88, 455-460.

Yanni G., Whelan A., Feighery C., Fitzgerald O. and Bresnihan B. (1993). Morphometric analysis of synovial membrane blood vessels in rheumatoid arthritis: associations with the immunohistologic features, synovial fluid cytokine levels and the clinical course. J. Rheumatol. 20, 634-638.

Yoshida S., Ono M., Shono T., Izumi H., Ishibashi T., Suzuki H. and Kuwano M. (1997). Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. Mol. Cell. Biol. 17, 4015-4023.

Accepted April 9, 2002