VEGF release is associated with reduced oxygen tensions in experimental inflammatory arthritis

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Abstract

Objective

The contribution of local VEGF production and subsequent angiogenesis within the synovial membrane to the propagation of arthritis is unclear. The relationship between synovial oxygenation and blood flow in the development of arthritic disease is unknown. We have therefore measured oxygen levels and perfusion rates in the synovial space in a murine model of arthritis.

Methods

Arthritis was induced in DBA/1 mice by immunisation with type II collagen. Oxygen and perfusion levels were measured polarographically using silver needle microelectrodes within the knee joints prior to and 10 days after the onset of arthritis. In addition, synovial cells were isolated from knee joints of naive, pre-arthritic and arthritic mice.

Results

Onset of arthritis was associated with a marked reduction in synovial oxygen tensions (pO_2). The perfusion rates in naive and arthritic animals were not significantly different: in naive mice, the rate was 0.58 ± 0.11 ml/min/g and in arthritic joints, 0.64 ± 0.17 ml/min/g. Furthermore, synovial cells isolated from the knee joints of naive animals did not express mRNA for VEGF, but significant levels were detected in cells from non-arthritic mice immunised with collagen. The onset of arthritis was associated with expression of VEGF mRNA and protein, and correlated negatively with pO_2 levels.

Conclusion

These data demonstrate that decreases in intra-articular pO_2 occur in established arthritic conditions and may be the stimulus for local VEGF production. However, perfusion was not increased in arthritic animals and vascular density was unaltered, suggesting that the neovascularisation associated with inflammatory arthritis, is insufficient to restore oxygen homeostasis in the joint.

Key words

Rheumatoid arthritis, vascular endothelial growth factor, collagen-induced arthritis.

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Introduction

The potential role of the vasculature in perpetuation of inflammatory joint disease has aroused much interest in recent years (for reviews see 1, 2). Normal physiological functioning of synovial membranes is dependent upon the vasculature for adequate delivery of oxygen and nutrients. In human inflammatory joint disease, such as RA, the vasculature plays a key role in recruitment of circulating immune cells to the site of inflammation. When in RA, the metabolic capacity of the synovial tissue increases, the demand for oxygen and nutrients also increases, thus rendering the tissue hypoxic.

Oxygen tensions (pO_2) are reported to be low in aspirated synovial fluid samples taken from human knee joints, with the lowest pO_2 reported in fluids taken from patients with RA (3). Furthermore, several studies have demonstrated that the oxygen consumption in the RA synovium is increased and that glucose is oxidised through the anaerobic, as opposed to the aerobic pathway (4, 5). Oedema is likely to increase intra-articular pressure and occlude blood flow through the existing capillary bed, thus exacerbating the hypoxic state. The homeostatic response is angiogenesis (6) and endothelial cells lining the small blood vessels within RA synovial vasculature have been shown to express cell cycle-associated antigens as well as markers of angiogenesis (7, 8).

A key player in the pathogenesis of angiogenesis-dependent diseases is vascular endothelial growth factor (VEGF) (9-11). VEGF can be detected in synovial fluids from RA patients, and is expressed by RA synovial macrophages and fibroblasts, in addition to vascular endothelial cells in the sublining (12-14). Moreover, both VEGF receptors (Flt-1 and KDR) are expressed on microvascular endothelial cells within the RA synovium (12). Hypoxia is known to be a potent stimulus for VEGF production (15) and its production could be an important adaptation of tissue to hypoxic stress. We and others have reported that in collagen-induced arthritis (CIA) in DBA/1 mice, expression of VEGF correlates with disease (16,17). This observation substantiates the hypothesis that VEGF production is closely associated with the manifestation of arthritis. Although reduced pO_2 has been demonstrated in RA joints, it is unclear whether pO_2 levels correlate with blood flow, vascularity or disease severity.

In the present study, we used a microelectrode technique to measure oxygen within the articular space. The technique is useful tool for measuring both regional perfusion (local blood flow) and tissue pO_2 simultaneously at exactly the same site in the joint, with good spatial resolution and also allows the interrelationship between the 2 variables to be examined. It was therefore possible to investigate the relationship between synovial oxygenation, perfusion, production of VEGF and vascularity. Accordingly, our aim was to determine whether the pro-angiogenic phenotype in arthritis corrects for the hypoperfusion of the synovial tissue and can appropriately restore the oxygen homeostasis.

Materials and methods

Induction and assessment of arthritis

Arthritis was induced in male DBA/1 mice (8-10 weeks old) by id injection of bovine type II collagen (18) in complete Freund's adjuvant (Difco). Joint swelling and/or erythema signified the onset of arthritis and this occurred between day 14 and day 40 after immunisation. Clinical score, a composite of disease severity and the number of limbs affected, was monitored daily from onset of disease and used as an assessment of disease progress. Arthritis increased progressively over 10 days, as reflected by consistent changes in both clinical score and paw swelling.

Synovial membrane cell preparation and culture

Animals were sacrificed and the hind limbs were removed. The synovium was dissected out from the knee joints with the aid of a dissecting microscope, as described previously (19, 20). The synovial tissue was digested with collagenase A (1 mg/ml; Boehringer Mannheim, UK) and DNAase (type IV 150 µg/ml; Sigma, UK) in the presence of

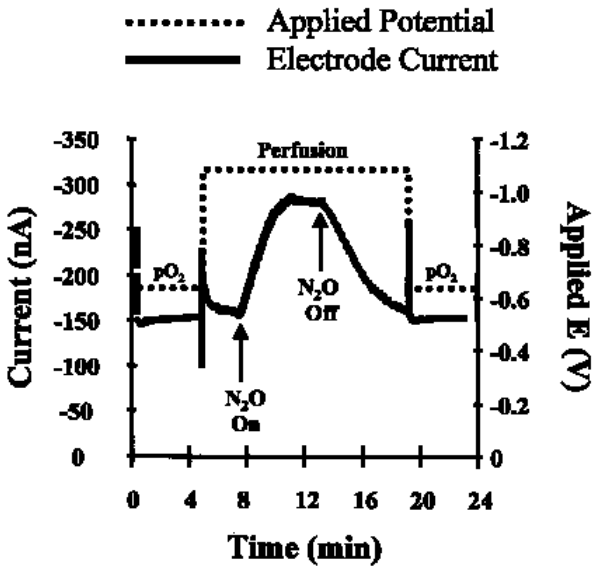

Fig. 1. Representative trace of electrochemical current generated *in vivo*. A microelectrode was inserted into the hind knee joint of an anaesthetised mouse. A current was generated by the reduction of oxygen at an applied voltage of –0.6V and recorded over 5 minutes. The voltage was then changed to –1.1V to reduce N_2O, delivered to the mouse via a nose cup. The N_2O mixture was inhaled until equilibrium was reached, at which point the animal was then switched to breathing air. The current was recorded continuously.

polymixin B sulphate (Sigma, UK) to produce a single cell suspension. Cells were cultured in RPMI (BioWhittaker, UK) containing 10% foetal calf serum (Sigma, UK) at a density of 4 x 10^6 cells in 30 mm^2 wells in flat-bottomed plates at 37°C. Synovial cells isolated from pre-arthritic mice were also transferred to hypoxic chambers and cultured at 37° C. After 24 hours, the supernatants were removed and assayed by ELISA for mouse VEGF (R&D Systems, UK; sensitivity = 5 pg/ml).

VEGF mRNA isolation and polymerase chain reaction (PCR) amplification

Total cytoplasmic RNA was prepared from isolated synovial cells and PCR amplification was used to detect VEGF mRNA as previously described (17).

Measurement of pO_2 and blood flow in vivo

Microelectrodes were constructed in-house as follows: 125 µm insulated silver wire (Goodfellow, Huntingdon, UK) was embedded with epoxy resin in 29G hypodermic needles. Electrical connection to the silver wire was achieved via lightweight and insulated coaxial cable (Radio Spares, UK). The electrochemically active surface was prepared as described previously (21). Mice were anaesthetised (Hypnom 3.3 ml/kg) allowing accurate and reproducible insertion of the needle microelectrodes into the knee joint. Once *in situ*, the electrode was polarised against a calomel reference electrode (Russell pH Ltd, Fife, UK) placed in a saline bath in which the animal's tail was immersed. The voltage was held constant and generated currents displayed using a potentiostat (EMS Ltd, Oxford, UK). The current generated at a potential of –0.6V is a measure of the oxygen concentration in a sampling zone of approximately 1 mm^3. The additional current generated at –1.1V is proportional to the concentration of dissolved nitrous oxide (N_2O) gas within a similar sampling zone (22). By switching between these two voltages, it is possible to assay for both O_2 and N_2O at exactly the same point in the tissue (22-25). A typical N_2O current versus time trace is illustrated in Figure 1.

The pO_2 was calculated from the currents obtained, immediately after the experiment in saline equilibrated with 0%, 10% and 21% oxygen saturation. In order to determine synovial perfusion, animals were allowed to breath a 30% N_2O, 21% O_2, 5% CO_2 and 44% N_2 gas mixture administered via a nose cup with the electrode polarised to –1.1V. When the current was steady, the animal was returned to air. The N_2O then cleared from the tissue and perfusion was calculated from the exponent of the current-time wash-out curve tissue (23-25).

CD31 immunostaining

Synovial tissue was dissected with the aid of a microscope from the hind knee joints of pre-arthritic and arthritic animals and snap frozen. Cryostat sections (7 µm) were cut and processed for immunostaining of CD31, a marker for blood vessels. Briefly, rat anti-mouse IgG2a clone MEC13.3 (Pharmingen) was applied overnight at 4°C and secondary antibody (Amersham Life Science, UK) was applied for 1 hour. The sections were then stained with DAB and counterstained with Mayer's hematoxylin.

Staining was evaluated using an Olympus BH2 microscope (Olympus Optical; Tokyo, Japan), coupled to a JVC KY-F55BE video camera (Victor Company of Japan, Tokyo, Japan) linked to a PC computer as previously described (26). Briefly, digital images were acquired at a magnification of 200x and processed using AnalySIS image analysis software (Soft Imaging Software GmbH, Munster, Germany). Each microscope field was quantified using selected colour detection threshold values for single pixels in the digital image. Multiple fields were analyzed, the total immunopositive area was determined and then expressed as a percentage of the total tissue area.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software package (GraphPad Software, San Diego, CA, USA). Mann-Whitney U test was used to compare the differences between groups of animals with non-Gaussian populations. The parametric Pearson correlation was used to examine the degree of linear association between VEGF production and pO_2 measurements.

Results

Synovial VEGF protein is only produced after arthritis onset

In the current study, we found that the mRNA for VEGF was present in synovial cells of mice not exhibiting clinical arthritis and in those with arthritic symptoms (Fig. 2A). No mRNA was detected in synovial cells taken from

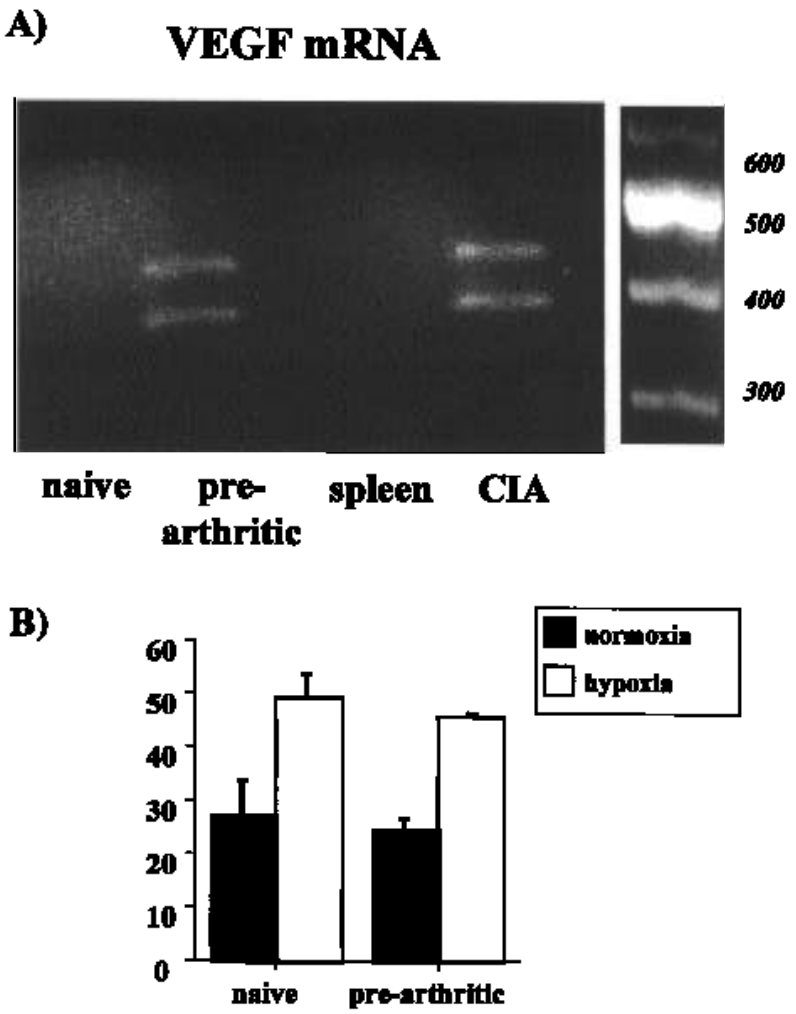

Fig. 2. Synovial cells from mice immunised with collagen type II express VEGF mRNA. (**A**) PCR amplification of VEGF mRNA from synovial cells from either naïve controls or mice immunised with collagen with or without clinical arthritis and from spleen cells from arthritic mice. Standard DNA ladder of 100-660 bp. All samples were probed and standardised for mouse GAPDH mRNA (not shown). (**B**) Synovial cells from naïve (n = 5) or pre-arthritic (n = 5) mice cultured either in normoxia or hypoxia. The supernatants were assayed by ELISA for mouse VEGF.

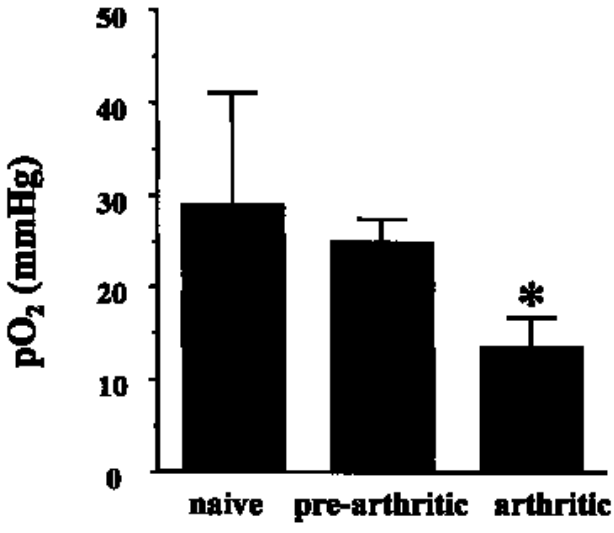

Fig. 3. Oxygen tensions (pO_2) measured in mouse knee joints. Microelectrodes were inserted into the hind knee joints of anaesthetised naïve (n = 16), pre-arthritic (n = 14), and arthritic mice (n = 18). The results are expressed as mean ± SEM.

naïve mice or in spleen cells taken from arthritic mice. Subsequently, synovial cells from naïve and pre-arthritic mice were placed in hypoxic chambers for culture over 24 hours at 37°C. We observed that exposure to hypoxia of the cultured synovial cells had no significant effect on the production of VEGF protein as measured by ELISA from either the naïve or pre-arthritic animals (Fig. 2B).

Oxygen tension correlates with arthritis severity and VEGF production

pO_2 was determined in the hind limb knee joints of naïve, immunised but non-arthritic and arthritic (day 10 after disease onset) mice. The joint pO_2 level in naïve mice was not significantly different from that seen immunised but non-arthritic animals. However joint pO_2 was significantly lower in arthritic mice ($p<0.001$ compared with immunised, non-arthritic by Mann-Whitney U test, Fig. 3).

The effect of mechanical movement/ mobilisation of the joints was then examined ie. when the generated current reached a steady level after insertion, the equilibrium state where the O_2 diffusion field at the electrode tip was established and any transient perturbations resulting from needle insertion had subsided. When the joints of naïve mice were mobilised, the pO_2 increased immediately to a mean of 40 mmHg and then decayed to pre-mobilisation levels after 5 mins (Fig. 4A). In contrast the steady state pO_2 in arthritic mice was initially lower and furthermore upon mobilisation, no change in pO_2 was observed (Fig. 4B).

At the end of the experiments in a portion of each group, isolated synovial cells were cultured and supernatants were assayed for VEGF protein. We found that cells from knees with high pO_2 levels produced markedly lower VEGF levels than those with a low pO_2 (Pearson correlation co-efficient = 0.97, $p < 0.05$; Fig. 5).

Perfusion and vascular density are not altered in arthritic knee

The mean perfusion rate calculated for in the naïve control group was not sig-

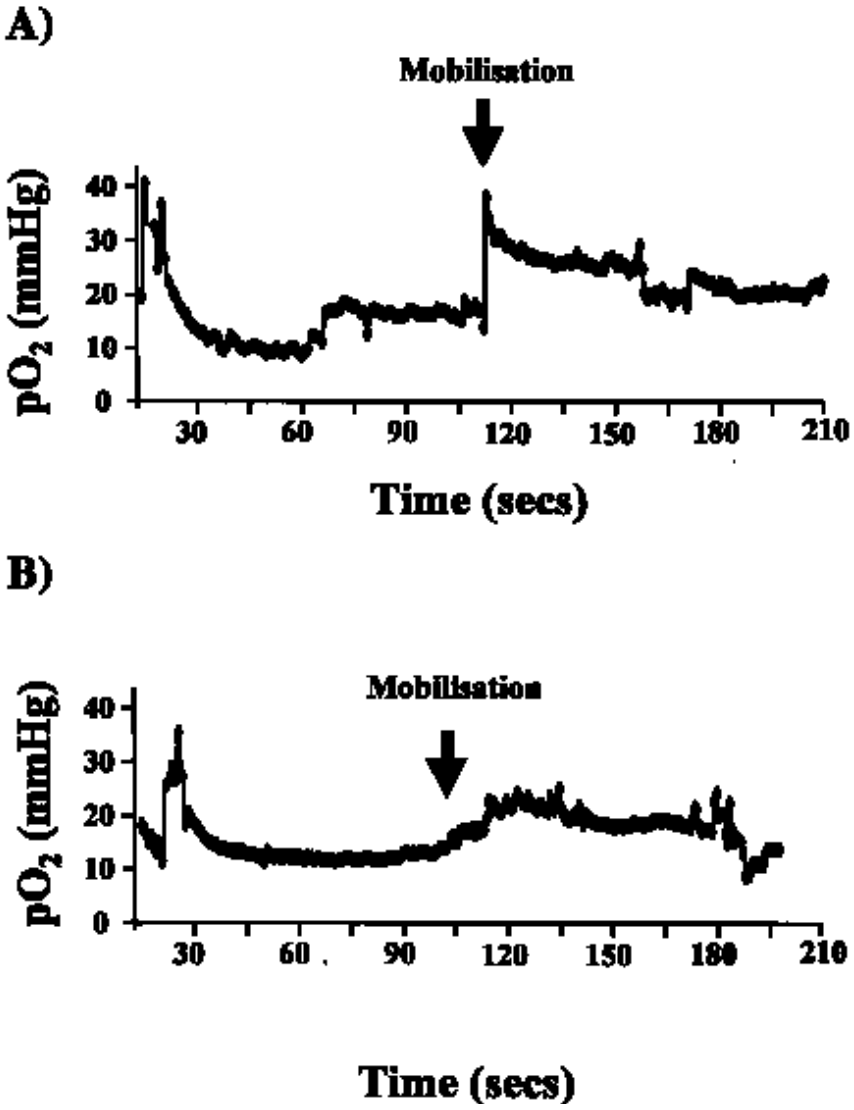

Fig. 4. Effect of mobilisation of the knee joints on synovial tissue oxygen. pO_2 measurements were made in the hind knee joints of (**A**) naïve and (**B**) arthritic mice pre- and post- mobilisation as described in Materials and methods. Results are a mean of 8 animals.

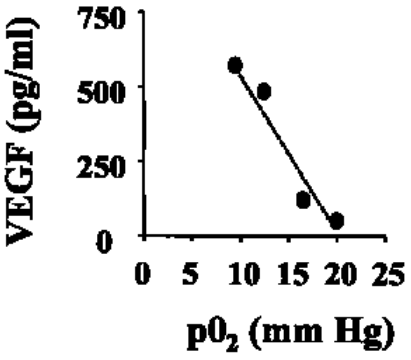

Fig. 5. VEGF production correlates with pO_2. After pO_2 measurements synovial cells were isolated from the knee joints and cultured for 24 hours. VEGF levels in culture supernatants were determined by ELISA.

nificantly different from that measured in arthritic animals as determined by N_2O clearance from synovial tissue. In the naïve mice, perfusion was calculated to be 0.58 ± 0.11 ml/min/g, whereas in the arthritic mice the mean perfusion rate was 0.64 ± 0.17 ml/min/g.

In addition, synovial tissue vascular density was assessed by the level of tissue staining for CD31 and quantified using an image analysis program which measured the fraction of total tissue area expressing CD31. The percentage of synovial tissue which was vascular was not significantly different in immunised, pre-arthritic animals and arthritic animals.

Discussion

In the present study, we have demonstrated that in CIA, intra-articular pO_2 does not decrease until clinically evident arthritis is established. Furthermore, VEGF production was only observed in synovial cell populations derived from mice with arthritis and was noted to vary inversely with intra-articular pO_2. VEGF is a potent pro-angiogenic mitogen and yet we found neither perfusion/blood flow nor synovial vascular density to be increased in the arthritic animals. Although we were able to detect VEGF mRNA in isolated synovial cells from immunised non-arthritic mice, hypoxic culture conditions *ex vivo* were insufficient to promote VEGF gene transcription and release of protein. We have previously reported that VEGF production from synovial cells isolated from mice with established arthritis (day 10) was 445 pg/ml, a 10-fold increase when compared with the levels measured in the present study. Berse *et al.* (27) reported that synovial or dermal fibroblasts cultured in hypoxic conditions secreted significantly lower amounts of VEGF as compared with cells cultured in hypoxia and simultaneously exposed to cytokines. In the CIA model, isolated synovial cells from pre-arthritic animals do not secrete TNFα (unpublished observation). This may explain the absence of VEGF protein upregulation at 24 hours in hypoxic culture conditions despite the presence of VEGF mRNA.

We have previously reported that specific blockade of VEGF activity ameliorates both the clinical and histological features of CIA disease (17). Although VEGF blockade would be predicted to deprive the inflammatory cell mass of essential nutrients and to reduce tissue oedema, it might also be considered potentially detrimental by retarding any restoration of oxygen homeostasis in the joint. Under hypoxic conditions, anaerobic glycolysis begins to take place as does the production of reactive oxygen species which have capability to damage cartilage and bone. In addition, reduced glycosaminoglycan metabolism and disregulation of cartilage homeostasis

can occur. Thus an adequate blood supply is needed to ensure the removal of harmful metabolites as well as delivery of substrates. The observed therapeutic effect of VEGF inhibition in CIA may also be a consequence of inhibiting leukocyte chemotaxis. VEGF has been demonstrated to mediate chemotaxis of monocytes and neutrophils through its action on Flt-1 receptor (29). *In situ* hybridization and immunohistochemical studies indicated that the cells expressing VEGF are macrophage-like synovial lining cells and spindle-shaped cells in the sublining cell layer (12). In addition, neutrophils from RA synovial fluids are reported to contain significant amounts of both VEGF protein and mRNA when compared with peripheral blood neutrophils from either RA patients or healthy controls (30). Both of these cell sources can be considered as important contributors to the pro-angiogenic phenotype observed in RA synovitis. Thus blockade of VEGF, in addition to down-regulating angiogenesis and vascular permeability, may have a significant effect on reducing macrophage recruitment. Thus, the beneficial effects of TNFα blockade in RA (31, 32) may be due in part to downregulation of TNFα-inducible VEGF expression in monocytes found within the synovial tissue and fluid (28).

We have found that the perfusion in the arthritic joint was not increased when compared with controls and this is consistent with our finding that vessel density in the synovial tissue is not significantly increased in arthritic mice. In support of our findings, a previous study also reported that blood flow changes in the joints were almost absent in CIA in the rat and found that any changes in blood flow coincided with an increase in tissue mass (33). The authors used a microsphere method, which gives an average blood flow through the tissue sampled. In contrast, synovial hyperaemia was seen in the knees of carrageenan-induced arthritis in the dog (34). Differences in the species used and/or the nature of the model could explain the discrepancy. So, despite a pro-angiogenic phenotype arising in the arthritic synovium may perpetuate the hypoxic state in RA. This effect would be exarcebated during movement of joint. Indeed, this was found in the present study where mobilisation of the arthritic joints did not enhance oxygen levels, whereas there was an instant but transient increase in pO_2 in the joints of naïve control mice.

In summary, these observations indicate that intra-articular pO_2 is an important stimulus for local VEGF production and yet we found neither perfusion/blood flow nor synovial vascular density to be increased in the arthritic animals. These findings suggest that neovascularisation known to be associated with inflammatory arthritis is probably insufficient to restore oxygen homeostasis in the joint.

References

1. PALEOLOG EM, MIOTLA JM: Angiogenesis in arthritis: Role in disease pathogenesis and as a potential theraeutic target. *Angiogenesis* 1998; 2: 295-307.
2. BALLARA SC, MIOTLA JM, PALEOLOG EM: New vessels, new approaches: angiogenesis as a therapeutic target in musculoskeletal disorders. *Int Journal Exp Pathol* 1999; 80:235-50.
3. LUND-OLESEN K: Oxygen tension in synovial fluids. *Arthritis Rheum* 1970; 13: 769-76.
4. BLAKE DR, MERRY P, UNSWORTH J *et al.*: Hypoxic-reperfusion injury in the inflamed human joint. Lancet 1989;1 (8633):289-293.
5. STEVENS CR, WILLIAMS RB, FARRELL AJ, BLAKE DR: Hypoxia and inflammatory synovitis: observations and speculation. *Ann Rheum Dis* 1991; 50: 124-32.
6. FOLKMAN J: Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Medicine* 1995; 1: 27-31.
7. CEPONIS A, KONTTINEN YT, IMAI S *et al.*: Synovial lining, endothelial and inflammatory mononuclear cell proliferation in synovial membranes in psoriatic and reactive arthritis: a comparative quantitative morphometric study. *Br J Rheumatol* 1998; 37: 170-8.
8. WALSH DA, WADE M, MAPP PI, BLAKE DR: Focally regulated endothelial proliferation and cell death in human synovium. *Am J Pathol* 1998; 152: 691-702.
9. BROWN LF, DETMAR M, CLAFFEY K *et al.*: Vascular permeability factor/vascular endothelial growth factor: A multifunctional angiogenic cytokine. *EXS* 1997; 79: 233-69.
10. ACHEN MG, STACKER SA: The vascular endothelial growth factor family; proteins which guide the development of the vasculature. *Int J Exp Pathol* 1998; 79: 255-65.
11. NEUFELD G, COHEN T, GENGRINOVITCH S, POLTORAK Z: Vascular endothelial growth factor (VEGF) and its receptors. *Faseb J* 1999; 13: 9-22.
12. FAVA RA, OLSEN NJ, SPENCER-GREEN G *et al.*: Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. *J Exp Med* 1994; 180: 341-6.
13. KOCH AE, HARLOW LA, HAINES GK *et al.*: Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. *J Immunol* 1994; 152: 4149-56.
14. NAGASHIMA M, YOSHINO S, ISHIWATA T, ASANO G: Role of vascular endothelial growth factor in angiogenesis of rheumatoid arthritis. *J Rheumatol* 1995; 22: 1624-30.
15. SHWEIKI D, ITIN A, SOFFER D, KESHET E: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992; 359: 843-5.
16. LU J, KASAMA T, KOBAYASHI K *et al.*: Vascular endothelial growth factor expression and regulation of murine collagen-induced arthritis. *J Immunol* 2000; 164: 5922-7.
17. MIOTLA J, MACIEWICZ R, KENDREW J, FELDMANN M, PALEOLOG E: Treatment with soluble VEGF receptor reduces disease severity in murine collagen-induced arthritis. *Lab Invest* 2000; 80:1195-1205.
18. MILLER EJ: Structural studies on cartilage collagen employing limited cleavage and solubilization with pepsin. *Biochemistry* 1972; 11: 4903-9.
19. BUTLER DM, MALFAIT AM, MASON LJ *et al.*: DBA/1 mice expressing the human TNF-alpha transgene develop a severe, erosive arthritis: characterization of the cytokine cascade and cellular composition. *J Immunol* 1997; 159: 2867-76.
20. MALFAIT AM, BUTLER DM, PRESKY DH, MAINI RN, BRENNAN FM, FELDMANN M: Blockade of IL-12 during the induction of collagen-induced arthritis (CIA) markedly attenuates the severity of the arthritis. *Clin Exp Immunol* 1998; 111: 377-83.
21. O'HARE D, WINLOVE CP, PARKER KH: Electrochemical method for direct measurement of oxygen concentration and diffusivity in the intervertebral disc: electrochemical characterization and tissue-sensor interactions. *J Biomed Eng* 1991; 13: 304-12.
22. ALBERY WJ, BROOKS WN, GIBSON SP, HAHN CE: An electrode for PN_2O and PO_2 analysis in blood and gas. *J Appl Physiol* 1978; 45: 637-43.
23. SAIR M, ETHERINGTON PJ, CURZEN NP, WINLOVE CP, EVANS TW: Tissue oxygenation and perfusion in endotoxemia. *Am J Physiol* 1996; 271: H1620-1625.
24. BARRON DJ, ETHERINGTON PJ, WINLOVE CP, JARVIS JC, SALMONS S, PEPPER JR: Muscle transformation in cardiomyoplasty: the effect of conditioning and mobilisation on perfusion, oxygenation and fatigue resistance in the latissimus dorsi muscle. *Eur J Cardiothorac Surg* 1998; 13: 588-98.
25. Al-OBAIDI MK, ETHERINGTON PJ, BARRON DJ, WINLOVE CP, PEPPER JR: Myocardial tissue oxygen supply and utilization during coronary artery bypass surgery: evidence of microvascular no-reflow. *Clin Sci (Colch)* 2000; 98: 321-8.
26. ULFGREN AK, ANDERSSON U, ENGSTROM

M, KLARESKOG L, MAINI RN, TAYLOR PC: Systemic anti-tumor necrosis factor alpha therapy in rheumatoid arthritis down-regulates synovial tumor necrosis factor alpha synthesis. *Arthritis Rheum* 2000; 43: 2391-6.

27. BERSE B, HUNT J, DIEGEL R *et al.*: Hypoxia augments cytokine (transforming growth factor-beta (TGF-beta) and IL-1)-induced vascular endothelial growth factor secretion by human synovial fibroblasts. *Clin Exp Immunol* 1999; 115: 176-82.
28. PALEOLOG EM, YOUNG S, STARK AC, McCLOSKEY RV, FELDMANN M, MAINI RN: Modulation of angiogenic vascular endothelial growth factor (VEGF) by TNFα and IL-1 in rheumatoid arthritis. *Arthritis Rheum* 1998; 41: 1258-65.
29. BARLEON B, SOZZANI S, ZHOU D, WEICH HA,MANTOVANI A, MARME D: Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* 1996; 87: 3336-43.
30. KASAMA T, KOBAYASHI K, YAJIMA N *et al.*: Expression of vascular endothelial growth factor by synovial fluid neutrophils in rheumatoid arthritis (RA). *Clin Exp Immunol* 2000; 121: 533-8.
31. ELLIOTT MJ, MAINI RN, FELDMANN M *et al.*: Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis. *Lancet* 1994; 344: 1105-10.
32. MAINI RN, TAYLOR PC: Anti-cytokine therapy for rheumatoid arthritis. *Annu Rev Med* 2000; 51: 207-29.
33. ANDERSSON SE,EKSTROM GM: Changes in vascular porosity and joint blood flow during development of collagen induced arthritis in the rat. Modulation by indomethacin and L-NAME. *J Rheumatol* 1997; 24: 2188-95.
34. STENDER HANSEN E,HE SZ, HJORTDAL VE, KJOLSETH D, SOBALLE K: Distribution of blood flow in normal and arthritic joints. Role of arteriovenous shunting studied in growing dogs. *Am J Physiol* 1992; 262:H38-46.