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 (pO₂). 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 pO₂ levels.

Conclusion
These data demonstrate that decreases in intra-articular pO₂ 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.
VEGF release in hypoxic synovial tissue in CIA / P. J. Etherington et al.

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 micro-electrode 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 DNase (type IV 150 µg/ml; Sigma, UK) in the presence of
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 mm² 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 pO₂ 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 micro-electrodes 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 mm³. The additional current generated at ~1.1V is proportional to the concentration of dissolved nitrous oxide (N₂O) gas within a similar sampling zone (22). By switching between these two voltages, it is possible to assay for both O₂ and N₂O at exactly the same point in the tissue (22-25). A typical N₂O current versus time trace is illustrated in Figure 1.

The pO₂ 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% N₂O, 21% O₂, 5% CO₂ and 44% N₂ 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 N₂O then cleared from the tissue and perfusion was calculated from the exponent of the current-time wash-out curve tissue (23-25).
naive mice or in spleen cells taken from arthritic mice. Subsequently, synovial cells from naive 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 naive 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 naive, immunised but non-arthritic and arthritic (day 10 after disease onset) mice. The joint pO$_2$ level in naive 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. In 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 naive 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 naive control group was not sig-
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 TNF$\alpha$ (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

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.
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 exacerbated 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 PO2 in the joints of naive control mice.

In summary, these observations indicate that intra-arterial PGI2 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


