Does a pro-angiogenic state exist in the bone–implant interface of aseptically loosened joint prosthesis?

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Neovascularization is indispensable to both bone remodeling and the development of chronic inflammation. A pro-angiogenic state in the periprosthetic tissue may augment the inflammatory response to wear debris. To investigate if a pro-angiogenic state exists in the bone–implant interface of aseptically loosened joint prosthesis, the expression of vascular endothelial growth factor (VEGF) and its receptor Flk-1/KDR were studied by immunohistochemistry. The VEGF-Flk/KDR pathway has been implicated as a key signaling requirement for pathological angiogenesis. The level of vascularization in periprosthetic tissue was semi-quantitatively compared to osteoarthritic (OA) and rheumatoid arthritic (RA) synovium. The level of vascularization in areas of periprosthetic tissue with heavy or low/moderate wear debris were also compared semi-quantitatively by image analysis.

High levels of VEGF expression (16/16 cases) particularly in the implant synovial-like lining layer together with Flk-1/KDR expression by endothelial cells (13/16), suggests that neovascularization is occurring. Morphometric comparison of periprosthetic tissue with RA and OA synovium revealed no significant difference in microvessel density, but did reveal significantly increased microvessel area in RA synovium ($P > 0.05$). Areas of high wear debris infiltrate also contained a significantly smaller microvessel area ($P > 0.01$). Suggesting that wear debris may cause behavioral modification of microvessels. Modifying angiogenesis in the periprosthetic tissue could be a potential therapeutic target in reducing inflammation.

1. Introduction

Poor neovascularization in the bone–implant interface tissue may prevent implant osseointegration due to both a restricted blood supply and lack of endothelial cell associated factors required for bone formation [1]. However, due to the co-dependence between angiogenesis and inflammation [2], a pro-angiogenic state in the periprosthetic tissue may augment the inflammatory response to wear debris.

A pro-angiogenic state has been reported in a number of pathological conditions, including pannus formation in rheumatoid arthritic (RA) synovium, osteoarthritic (OA) synovium, psoriasis, and tumor formation and metastasis. Anti-angiogenic therapy has shown promising results in the treatment of these conditions [3]. Insufficient angiogenesis can also cause pathological conditions and has been implicated in delayed wound healing, heart disease, ulcers, scleroderma and inhibited bone development [3].

Microvessels can actively promote chronic inflammation by the recruitment and trans-endothelial migration of leukocytes via cellular adhesion molecules (CAMs), the production of cytokines, chemokines, and other inflammatory mediators and functioning as antigen presenting cells [4]. The production of cytokines and growth factors by the endothelium induces further angiogenesis and transmigration of leukocytes in an autocrine manner [5].

The importance of angiogenesis in osteogenesis has been recognized for many years. Concurrent microvessel ingrowth occurs with the mineralization in fracture callus, limb lengthening in experimental animals, and is an essential step in endochondral ossification [6–8]. While nutrient, growth factor, and osteoprogenitor supply is vital for osteogenesis, mounting evidence suggests that the role of microvessels extends to that of a vascular conduit. Experimental evidence suggests that the vasculature conveys essential signals required for correct growth plate morphogenesis [8], that some osteoprogenitor cells are derived from endothelial cells (ECs) [9], and that EC culture conditioned medium is mitogenic to osteoblasts [10].

Angiogenesis may therefore exert a direct effect on implant osseointegration, bone remodeling and the

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development of chronic inflammation in the bone–implant interface tissue of prosthetic joints. Certainly several factors produced by the endothelium in the bone–implant interface have a direct effect on bone remodeling or chronic inflammation (e.g. TNFα) [11].

To investigate if a pro-angiogenic state exists in the orthopedic implant interface tissue, the expression of vascular endothelial growth factor (VEGF), and its receptor Flk-1/KDR (VEGF-receptor 2) were assessed in the bone–implant interface tissue obtained during revision surgery of chronically failed joint prosthesis. VEGF and Flk-1/KDR have been implicated as the key endothelial cell-specific signaling pathway required for pathological angiogenesis [12]. VEGF is a potent EC mitogen and is a key regulator of both vasculogenesis and angiogenesis as demonstrated by its vital role in the survival of neonatal mice [13]. VEGF acts directly on cultured ECs to induce endothelium permeability, cell division, cell migration, alter their pattern of gene expression and induce angiogenesis *in vivo*. Anti-VEGF therapy in a RA model prevents pathological osteolysis [14].

VEGF binds to its respective tyrosine kinase receptors Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) both of which are expressed by ECs and are also vital for neonatal mice survival [13]. Experimental evidence links Flk-1/KDR activation to VEGF-induced mitogenesis, angiogenesis and EC survival [15] whilst Flt-1's function is less certain. The expression of VEGF and Flk-1/KDR in the bone–implant interface has not been previously reported.

The levels of vascularization in orthopedic implant interface tissue obtained during revision surgery were compared semi-quantitatively to RA and OA synovium. RA and OA synovium has been previously reported to be vascular and pro-angiogenic [3, 16, 17].

2. Materials and methods

2.1. Patients

A total of 16 patients who had aseptic clinical failure of total hip replacement ($n = 13$) and total knee replacement ($n = 3$) were included in this study. Brief clinical details of each patient were obtained at the time of revision surgery. All patients had degenerated joint condition on revision and there was radiographic indication of osteolysis at various locations around the implant components. The patients included seven males and nine females. The mean age was 69 years (range 41–93 years). The patients had total joint replacement due to primary or secondary osteoarthritis. Two patients had avascular necrosis and one had inflammatory RA. The duration of the implants ranged from 5–21 years. Joint prostheses in these patients comprised a CoCr or titanium metal component and UHMWPE component.

Analysis was carried out on bone–implant interface tissue retrieved during the first, second, or third revision of aseptically loosened joint prostheses in these 16 patients. In addition synovial membranes were obtained from eight patients with active RA and eight patients with OA during primary total joint replacement operations. The specimens were cut into small pieces 1–2 cm in length, frozen in liquid nitrogen cooled isopentane, and stored at $-70\,^{\circ}\mathrm{C}$.

2.2. Immunohistochemistry

Immunostaining of cryostat sections was used to characterize the pattern and the extent of vascular growth and to directly localize the cellular distribution of VEGF and the receptor Flk-1/KDR. Vascular ECs were visualized by labeling with the lectin ULEX Europaeus Agglutinin-I.

Multiple cryostat sections, 5 μm thick, were cut from each specimen of the bone–implant interface or the synovial membranes and immunostained with the biotin streptavidin alkaline phosphatase technique. Briefly sections were fixed for 10–15 min in 50 : 50 mixture of acetone : methanol at $-20\,^{\circ}\mathrm{C}$, washed in phosphate buffered saline (PBS pH 7.4) before adding the primary antibodies. These included mouse monoclonal antibodies to VEGF (1/100, Santa Cruz Biotechnology, Wiltshire, UK), the receptor Flk-1/KDR (1/50, Santa Cruz Biotechnology) or the lectin ULEX (1/200, Vector Laboratories, Peterborough, UK). After one hour incubation the sections were incubated for a further one hour with the secondary antibody (biotinylated horse anti mouse IgG followed by the conjugate streptavidin alkaline phosphatase at a dilution of 1/100 each (both were purchased from Vector Laboratories, UK). Sections immunostained with the lectin were incubated with the biotinylated goat anti-lectin (1/100, Vector Laboratories, UK) followed by the conjugate streptavidin alkaline phosphatase. The substrate reaction was developed with Naphthol AS-BI phosphate and Fast Red TR salt (Sigma, Pools, UK) in 0.1 M Tris-HCl buffer (pH 8.2). Levamisole was added at a final concentration of 10^{-3} M as an inhibitor of endogenous alkaline phosphatase. Negative controls to check for non-specific binding included replacing the primary antibody with tris buffered saline or non-immune immunoglobulin from the same species as the first antibody.

2.3. Semi-quantitative image analysis

The Leica Q500 MC computer based image analysis system was used to semi-quantitatively compare the level of vascularity in bone–implant interface tissues with RA and OA synovium. The five most vascular areas (hot-spots) were manually located in ULEX Europeaus Agglutin I immunolabeled sections with low magnification. A $0.24\,\mathrm{mm}^2$ area of each ''hot spot'' was then quantified with the image analyzer for microvessel density (MVD) and microvessel area. Results were expressed as number of microvessels/mm^2 and total microvessel area/mm^2 respectively.

Heavy particulate wear debris infiltrate was observed in five periprosthetic tissues obtained during revision surgery. Morphometric analysis of the vascularity in these tissues was compared to low or moderate wear debris infiltrate in the remaining 11 cases of periprosthetic tissue.

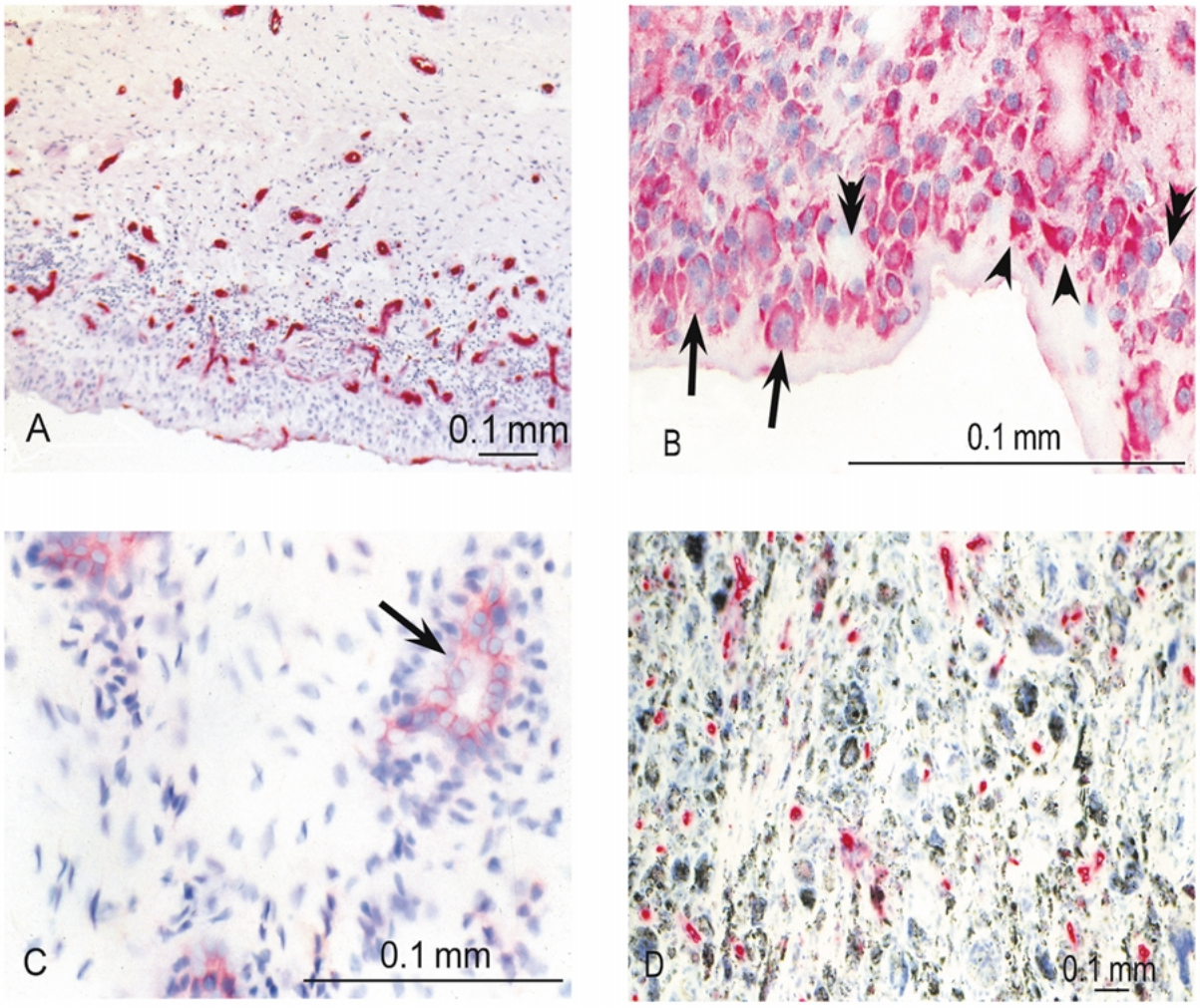

Figure 1 Immuno-streptavidin alkaline phosphatase staining of bone–implant interface tissue. (a) ULEX Europaeus agglutinin staining of implant lining layer. (b) VEGF expression by cells in the implant lining layer, including MNGCs (arrow), synoviocytes (arrow head) and microvessels (double arrowhead). (c) Flk-1/KDR expression by high endothelial venule (arrow), note perivascular inflammatory infiltrate. (d) Endothelial staining in tissue with heavy wear debris infiltrate.

3. Results

The level of vascularization in the bone–implant interface varied considerably both between cases and within each case. Highly cellular areas in the bone–implant interface contained an abundance of blood vessels identified by their immunoreactivity with the lectin ULEX, compared with less vascularized fibrotic areas. A distinct pattern of vascular growth occurred in the implant lining layer (Fig. 1a). The lining layer comprised an acellular, avascular area (5–90 μm thick) followed by a highly cellular synovial-like lining layer, containing a high number of small microvessels without clearly defined lumen (Fig. 1a). In deeper layers the microvessel density often decreased and microvessel size increased. A few cases (3/16) contained, in deeper layers, arterioles or large vessels with ECs surrounded by a clearly defined smooth muscle cell layer.

VEGF was expressed in all periprosthetic tissues studied (16/16). Cells expressing VEGF included macrophages, synoviocytes, spindle shaped cells, endothelial cells, and multinucleated giant cells (MNGC) (Fig. 1b). VEGF expression was concentrated in synoviocytes and macrophages in the implant lining layer (Table I). MNGCs expressed VEGF in 4/16 cases and in most cases appeared to be related to the prescence of wear debris adjacent or in the MNGC.

Flk-1/KDR was expressed by endothelial cells in 80% of patients (13/16). The expression was unevenly distributed throughout the microvasculature of individual sections and between different cases (Table I). Immunolabeling of this receptor revealed the prescence of endothelial swelling and high endothelial venules (HEVs) in several cases (Fig. 1c). Immunolabeling with the lectin ULEX also revealed a visibly reduced size of vessels in cases with high levels of wear debris (Fig. 1d).

Image analysis showed no significant difference between MVD in the bone–implant interface, RA and OA synovium (Fig. 2a). However, microvessels in the RA synovium covered a significantly larger area/mm^2 than those present in the implant-interface tissue ($P < 0.05$) but not OA synovium (Fig. 2b). There was no significant difference in mean MVD or microvessel area between the vasculature in the periprosthetic tissue and OA synovium. Microvessels in the implant interface also contained less vessels with clearly defined lumen than RA synovium. Areas of high MVD also expressed high levels of VEGF.

Comparative morphometric analysis of the vasculature in the bone–implant interface with high wear debris infiltrate to that of low/moderate wear debris infiltrate, demonstrated a significant difference ($P < 0.01$)

TABLE I Summary of the expression of angiogenic factors in the periprosthetic tissue

Cells at the bone-implant interface	Cases in which cells express, VEGF and Flk-1/KDR (%)		Range (mean) of cells expressing VEGF and Flk-1/KDR in positive cases (%)	
	VEGF	Flk-1/KDR	VEGF	Flk-1/KDR
Vessels in implant lining layer	90	70	40–90 (60)	30–90 (40)
Vessels in deeper layers	60	60	10–70 (30)	10–90 (40)
Implant lining layer cells	100	15	80–90 (85)	1–5 (5)
Inflammatory infiltrate	100	0	40–70 (50)	0
MNGC	25	0	10–20 (15)	0

between microvessel area/mm^2, but not microvessel density/mm^2.

4. Discussion

This study demonstrated the prescence of highly vascularized areas in the bone–implant interface retrieved from aseptically failed joint prosthesis. MVD in these tissues was comparable to RA and OA synovial membranes. In addition the identification of potent pro-angiogenic mediators in the bone implant-interface suggests that neovascularization is occurring and that a pro-angiogenic state exists.

While high levels of VEGF were expressed by macrophages, synoviocytes, and microvessels in the periprosthetic tissue implant lining layer, microvessels variably expressed Flk-1/KDR (mean of 35%). Failure of the Flk-1/KDR activation in the prescence of high VEGF expression may indicate an impaired capacity to establish a viable vasculature. High levels of VEGF expression by microvessels in the implant lining layer and to a lesser degree in deeper layers, suggests autocrine regulation of angiogenesis [5].

A number of factors associated with pro-angiogenic activity have been previously reported in the bone–implant interface by several authors investigating the inflammatory response to wear debris, including interleukin-1, fibroblast growth factors, tumor necrosis factor, metalloproteinases, and platelet derived growth factor [18]. However, the co-role of these factors in promoting angiogenesis has not been previously highlighted. An imbalance between angiogenic and angiostatic factors that favor neovascularization in the implant interface would promote inflammation. The periprosthetic tissue exhibits a number of structural and inflammatory similarities to other chronic inflammatory diseases, particularly RA and OA synovium, including synovium lining hyperplasin, fibrotic content, neovascularization, perivascular aggregates of leukocytes, and the development of MNGCs [19]. The similarity in microvessel size between OA synovium and bone–implant interface tentatively suggests greater similarities between these tissues than the vasculature in RA synovium.

Comparison of VEGF and Flk-1/KDR expression in RA and OA synovium to bone–implant interface is difficult due to the contrasting reports [16, 17]. However there is evidence to suggest that the VEGF-Flk-1/KDR pathway is pivotal for pathological angiogenesis in these conditions [12, 16].

Toxicity from wear debris infiltrate may cause morphological and phenotype changes in ECs resulting in significantly smaller microvessels. The physiological consequence of smaller vessels without lumen, in heavy wear debris containing bone–implant interface tissue, could be slower transport of gas and nutrients to the tissues. The surrounding tissue may become hypoxic causing a further inflammatory response by macrophages, ECs, and others cells, which in hypoxic environments produce inflammatory factors [20]. Heavy metal ions have been previously reported to modify the behavior of ECs [21].

A previous study suggested that while highly vascular areas existed in the periprosthetic tissue, non-inflamed synovium was better vascularized than the bone implant-interface and suggested that poor vascularization and reduced blood flow in periprosthetic tissues may prevent implant osseointegration due to a restricted blood supply and abscence of endothelial associated factors needed for

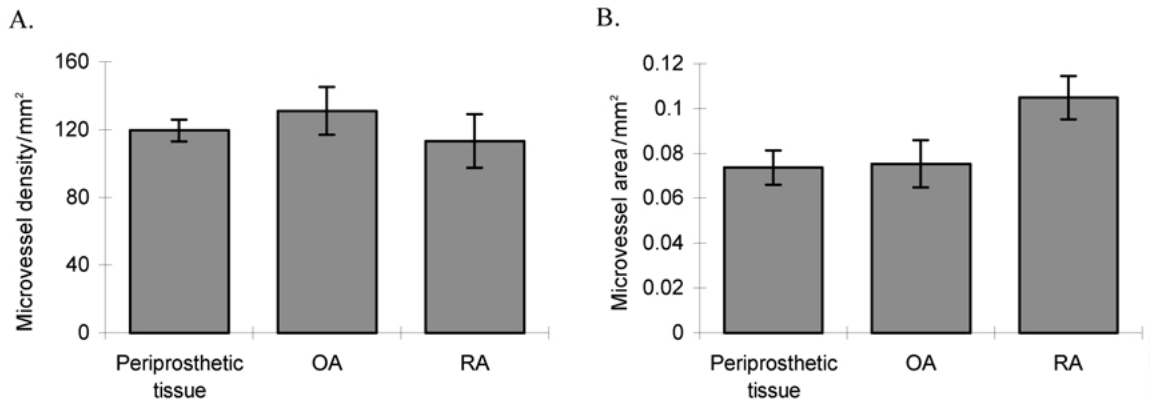

Figure 2 Microvessel density/mm^2 (a) and microvessel area/mm^2 (b) in periprosthetic tissue, osteoarthritic synovium (OA) and rheumatoid arthritic synovium (RA).

bone formation [1]. However, quantifying neovascularization is difficult and often misleading, due to varying analysis techniques and the nature of angiogenic growth. In the angiogenic disease of RA, where neovascularization has been most extensively studied it is still unclear whether the density of vessels in the arthritic synovium/unit area is increased. Some authors suggest that angiogenesis may not keep in pace with the proliferating tissue in RA synovium causing a reduction microvessel density when compared with normal synovium [22], whilst others have reported increased microvessel density in both OA and RA when compared to normal synovium [16, 17].

5. Conclusions

Damaged or impaired microvessel function, due to the toxic effects of wear debris or inflammation may inhibit its vital role in bone formation and thereby inhibit implant osseointegration. However, pathological angiogenesis may play a pivotal role in the inflammatory response to wear debris and the accompanying osteolysis that occurs in the periprosthetic tissue. Anti-angiogenic treatment could therefore be a potential therapeutic target in reducing inflammation and extending orthopedic implant longevity.

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