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Angiogenesis in the synovium and at the osteochondral junction in osteoarthritis[1]

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Summary

Objectives: We hypothesised that osteochondral and synovial angiogenesis in osteoarthritis (OA) are independent processes. We investigated whether indices of osteochondral and synovial angiogenesis display different relationships with synovitis, disease severity and chondrocalcinosis in patients with OA.

Design: Synovium and medial tibial plateaux were obtained from 62 patients undergoing total knee joint replacement for OA (18 [29%] had chondrocalcinosis) and from 31 recently deceased people with no evidence of joint pathology post-mortem (PM). Vascular endothelium, proliferating endothelial cells (ECs) and macrophages were quantified by immunohistochemistry for CD34, CD31/Ki67 and CD14, respectively. Grades were assigned for radiographic and histological OA disease severity, clinical disease activity and histological synovitis (based on cellular content of the synovium).

Results: Blood vessels breached the tidemark in 60% of patients with OA and 20% of PM controls. Osteochondral vascular density increased with increasing cartilage severity and clinical disease activity scores, but not with synovitis. Synovial EC proliferation, inflammation and macrophage infiltration were higher in OA than in PM controls. Synovial angiogenesis indices increased with increasing histological synovitis, but were not related to osteochondral vascular density or other indices of OA disease severity. OA changes were more severe in patients with concurrent chondrocalcinosis. Chondrocalcinosis was not associated with increased angiogenesis or histological synovitis beyond that seen in OA alone.

Conclusion: Osteochondral and synovial angiogenesis appear to be independent processes. Osteochondral vascularity is associated with the severity of OA cartilage changes and clinical disease activity, whereas synovial angiogenesis is associated with histological synovitis. Modulation of osteochondral and synovial angiogenesis may differentially affect OA disease.

Key words: Synovium, Cartilage, Chondrocalcinosis, Angiogenesis, Inflammation.

Abbreviations: PM post-mortem, OST osteophyte, JSN joint space narrowing, CC chondrocalcinosis, CPPD calcium pyrophosphate dihydrate, OA/CC+ subgroup of patients with OA and chondrocalcinosis, OA/CC− subgroup of patients with OA but with no chondrocalcinosis, ABC avidin–biotin complex, EC endothelial cell.

Introduction

OA is an important cause of pain and disability in the ageing population. Features of osteoarthritis (OA) include cartilage loss, osteophyte (OST) formation and synovial inflammation. Angiogenesis, the formation of new blood vessels from pre-existing ones, may contribute to each of these features of OA[1]. Invasion of blood vessels across the osteochondral junction may contribute to articular cartilage changes in OA by causing cartilage calcification or ossification. Cells present within vascular channels may modulate chondrocyte function, and degrade cartilage matrix[2,3]. Vascular breaching may compromise the normal barrier between articular cartilage and subchondral bone imparted by the layer of calcified cartilage. Additionally, OSTs form by endochondral ossification, a process that is believed to be angiogenesis-dependent. Furthermore, angiogenesis is a key component of chronic inflammation, and inhibition of angiogenesis can inhibit synovitis *in vivo*[4]. Synovitis may contribute not only to symptoms in OA, but through the generation of cytokines and low molecular weight factors, may further impair chondrocyte function and homeostasis of the articular cartilage[5,6].

The synovium plays a key role in maintaining healthy articular cartilage, and cartilage may modulate the function of the adjacent synovium[7]. The synovium and articular cartilage are in close proximity and there is a free flow of solutes between the two, raising the possibility that osteochondral and synovial angiogenesis are driven by common mechanisms. On the other hand, angiogenesis can be driven by a wide variety of factors in different tissues, raising the

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alternative possibility that osteochondral and synovial angiogenesis are parallel, but differentially regulated processes in OA. Synovial angiogenesis has been linked to histological synovitis, in particular macrophage infiltration of the synovium[8]. In addition, changes in articular cartilage may also modulate angiogenic activity through alterations in the matrix composition and expression and release of angiogenic factors[9,10]. Determining the relative contributions of synovitis and cartilage changes to angiogenesis within the joint, therefore requires concurrent assessment of the complex tissues of several joint compartments.

Chondrocalcinosis (CC) results from the deposition of calcium crystals, including calcium pyrophosphate dihydrate (CPPD), in articular and meniscal cartilages, and is often associated with OA. Radiographically apparent CC is associated with higher grades of radiographic OA severity[11]. CC can be associated with episodes of pseudogout, where CPPD crystals stimulate synovial fluid leukocytes, thereby causing severe acute inflammation. It is unknown, however, whether CC is an important cause of subclinical chronic synovitis or angiogenesis in patients with OA.

We have investigated the relationship between indices of angiogenesis in the synovium and the articular cartilage to assess whether a common mechanism may explain both. We also determined whether synovial and osteochondral angiogenesis could be explained by histological, radiographic or clinical evidence of severity, activity or subtype of OA disease.

Patients and methods

PATIENTS

Tissue samples were obtained from a repository of joint tissues and matched clinical data. Informed consent was gained from each donor or next of kin (post-mortem [PM] cases) according to protocols approved by the North Nottinghamshire Research Ethics Committee and Nottingham Research Ethics Committee 1 (Projects NNHA/420, NNHA/544, NNHA/673 and 05/Q2403/24)[12]. Synovium and medial tibial plateaux were obtained from patients undergoing total knee joint replacement surgery for OA, all of whom fulfilled the American College of Rheumatology revised criteria for the diagnosis of knee OA[13]. Patients with RA and other arthritides were excluded from the study. Samples of medial tibial plateaux represented the total number held in the repository from patients undergoing total knee replacement for OA at the time of the study ($n = 45$), excluding samples where no articular cartilage was present ($n = 8$). Five (11%) of the 45 included patients had radiographic CC. Matched synovia from these cases were supplemented from an additional 19 cases, sampled purposively from the repository to create a larger cohort with increased power for subgroup analysis, classified as OA with CC (OA/CC+; $n = 18$, 12 males) or OA without CC (OA/CC−; $n = 44$, 26 females) based on the definite presence or absence of radiographic meniscal calcification in the affected knee. Synovium and medial tibial plateaux from a further 31 patients (one knee each) were collected at post-mortem examination (PM) from people with no history of OA or other rheumatic problems, as a normal control group ($n = 31$, 19 males). Medical records, history from the next of kin and clinical examination of PM patients were used to exclude cases with any clinical evidence of OA. PM cases were excluded if there was evidence of Heberden's/Bouchard's nodes, history of pain, fracture or previous surgery to the knee, or if the articular surfaces displayed OSTs or macroscopic evidence of OA more severe than surface fibrillation. Osteochondral histomorphometry and cartilage scoring was performed on 36 OC/CC− samples, seven OA/CC+ samples and 14 PM samples. Synovial histomorphometry was performed on samples from 25 OA/CC−, 14 OA/CC+ and 16 PM cases.

CLINICAL ASSESSMENT

Clinical disease activity scores were calculated for patients undergoing total knee replacement[14]. This system uses six clinical parameters related to the operated knee (joint line tenderness, morning stiffness, inactivity stiffness, increased temperature, effusion, and soft tissue swelling).

RADIOGRAPHIC CLASSIFICATION AND SCORING

Pre-operative postero-anterior knee radiographs from patients with OA were examined by at least two independent observers including one consultant rheumatologist, all of whom were blinded to patient details and histological findings. Radiographs were not available for PM cases. Misclassification of CC status was minimised by excluding doubtful cases and those where joint space narrowing (JSN) was so severe that no space was visible in the medial tibiofemoral compartment. JSN and OST scores were assigned to each case using a line drawing atlas[15]. Scores for medial and lateral tibiofemoral compartments were summated to give total JSN and total OST scores.

SAMPLE COLLECTION AND PROCESSING

Samples were fixed in neutral-buffered formalin and then wax embedded (tibial plateaux) or mounted unfixed in OCT in melting isopentane and stored at −70°C (synovium). Mid-coronal sections of medial tibial plateaux were decalcified in 10% ethylenediaminetetraacetic acid and 10 mM Tris buffer (pH 6.95) at room temperature prior to wax embedding.

HISTOLOGY AND GRADING

Sections (5 μm) of medial tibial plateau were stained with safranin-O. Following nuclear staining with Weigert's haematoxylin (2 min) and differentiation in acid alcohol (15 s), sections were immersed in 0.02% fast green FCF (Sigma, Poole, UK), then 0.1% safranin-O (Sigma) for 1 min and mounted in DePeX mounting medium. Grades for the severity of OA changes in the articular cartilage (cartilage score) were the sum of four components; *cartilage surface integrity* (grade 0 [normal] to 6 (complete disorganisation)), *chondrocyte appearance* (0 [normal] to 3 [hypocellularity]), *proteoglycan loss* (0 [no loss of safranin-O stain] to 4 [absence of staining]) and *tidemark integrity* (0 [intact] or 1 [crossed by blood vessels])[16].

Synovium sections (5 μm) were stained with haematoxylin and eosin and graded 0–3 (normal to severe inflammation) according to lining cell depth, hypercellularity and lymphocyte infiltration[8].

IMMUNOHISTOCHEMISTRY

Blood vessels at the osteochondral junction of the medial tibial plateau were visualised using monoclonal anti-CD34 antibody and an avidin–biotin complex (ABC)-alkaline phosphatase technique with Fast Red™. Osteochondral

junction, calcified and non-calcified cartilage were discriminated by autofluorescence.

Synovial samples with intimal lining present were used for immunohistochemistry. Double sequential immunohistochemistry was performed as previously described to visualise proliferating endothelial cells (ECs) in the synovium[8]. Briefly, proliferating cell nuclei were visualised using a monoclonal anti-Ki67 antigen antibody and a nickel-enhanced ABC-peroxidase technique; while ECs were visualised with a monoclonal anti-CD31 (PECAM-1) antibody and an ABC-alkaline phosphatase technique with Fast Red™. Sections were counter-stained with 4′−6′-diamidino-2-phenylindole to visualise Ki67-negative nuclei. Macrophages were visualised using a monoclonal anti-CD14 antibody and an ABC-peroxidase technique developed with diaminobenzidine[8]. All antibodies were prepared in buffers containing 0.05% bovine serum albumen and 0.03% horse serum.

IMAGE ANALYSIS

All image analysis was carried out using a Zeiss Axioskop-50 microscope with a ×20 objective lens (Carl Zeiss Ltd, Welwyn Garden City, UK). Osteochondral vascular density was measured as the number of vascular channels with CD34 immunoreactivity within the non-calcified cartilage determined across seven adjacent fields of view (~7 mm) centred on the mid point of the medial tibial plateau. For synovial samples, transmitted light and fluorescence images within 200 μm of the synovial surface were captured for each field using a video camera module and analysed using a KS300 image analysis system (Imaging Associates, Bicester, UK). CD31-immunoreactive blood vessels were thresholded and interactively included based on morphology. Indices for EC fractional areas, EC proliferation and macrophage infiltration were derived as previously described[8].

STATISTICAL ANALYSIS

Data were analysed using SPSS software, version 11 (Chicago, IL, USA). Mann−Whitney U-test was used to compare data from all patients with OA and PM controls, then *post hoc* comparisons between OA/CC+ and OA/CC− subgroups. χ^2 statistic was used to compare frequencies of tidemark breaching between OA and PM. Possible associations between variables are expressed as Spearman's rank correlation coefficients, unless stated as partial correlation coefficients. $P < 0.05$ after correction for multiple comparisons was taken to indicate statistical significance. Data are presented as median and interquartile range (IQR).

MATERIALS

Monoclonal mouse anti- Ki-67 antigen antibody (clone MIB-5) and monoclonal mouse anti-CD34 antibody (clone QBEnd10) were obtained from DakoCytomation, Cambridge, UK. Monoclonal mouse anti-CD31 antibody (clone TLD-3A12) was from Serotec, Oxford, UK. Biotinylated rat-adsorbed horse anti-mouse antibody, Vectastain ABC-peroxidase Elite and ABC-alkaline phosphatase kits were obtained from Vector Laboratories Ltd., Peterborough, UK. Mayer's haematoxylin, eosin and TissueTek™ mounting medium were obtained from Raymond A. Lamb Ltd., Eastbourne, East Sussex, UK. Ammonium chloride, DePeX mounting medium, Superfrost Plus™ microscope slides, phosphate buffered saline (pH 7.5), ethanol and xylene were from WVR International Ltd., Poole, UK. Orthophosphate buffered formalin was from Cell Path PLC, Newtown Powys, Wales, UK. Monoclonal anti-CD14 (clone UCHM-1) and all other chemicals and reagents were from Sigma Chemical Co., Poole, UK.

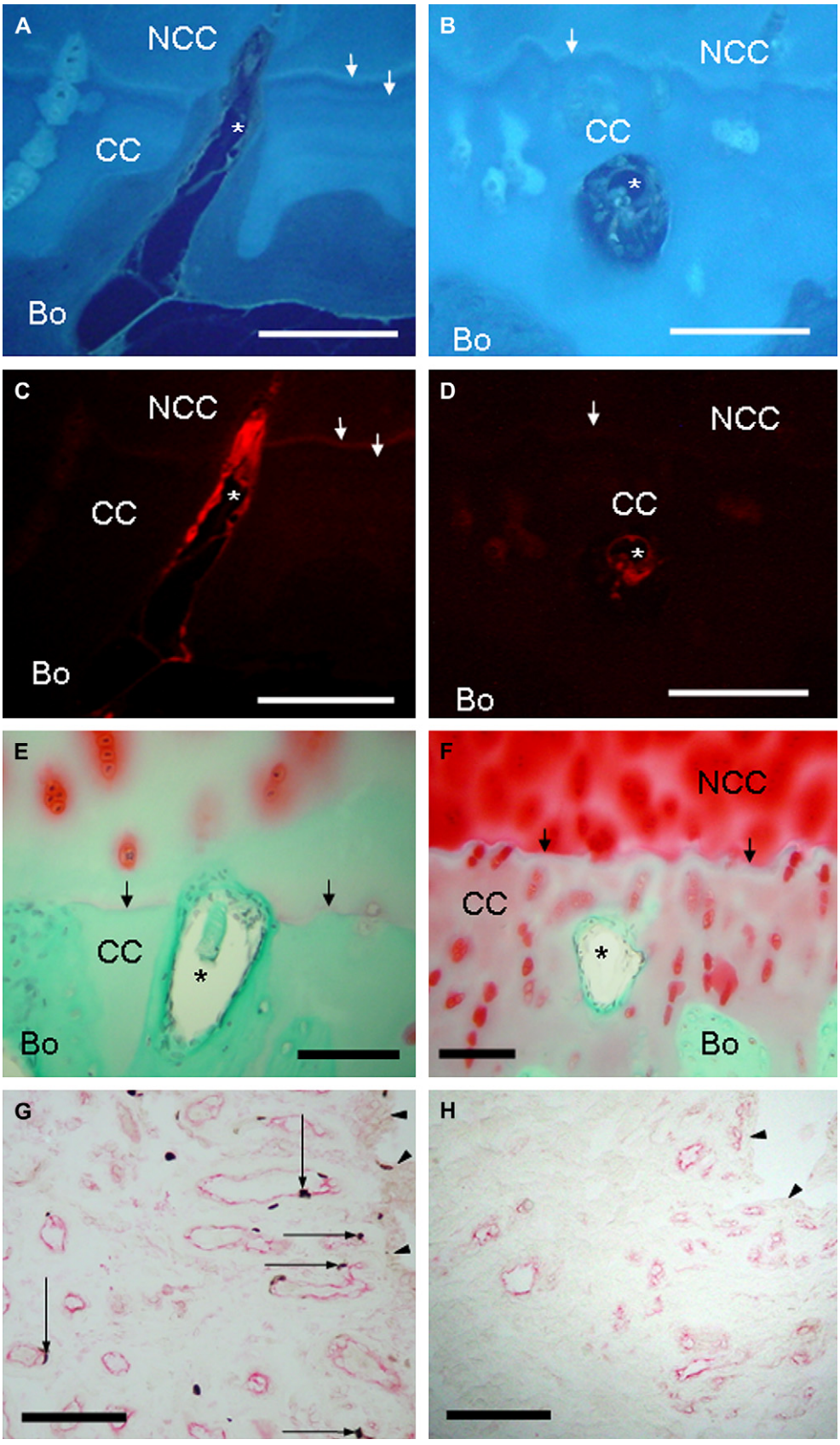
Results

Vascular breaching of the tidemark in the medial tibial plateau was observed mostly in patients with OA [Fig. 1(A−F)]. Fibrovascular tissue, including CD34-immunoreactive vascular endothelium, crossed from the subchondral bone, through the calcified layer of cartilage and breached the deepest tidemark in 27/45 (60%) patients diagnosed with OA and 2/10 (20%) PM controls ($\chi^2 = 5.6$, $P = 0.02$). These osteochondral vessels occurred in regions with low proteoglycan density, as revealed by loss of safranin-O staining [Fig. 1(E−F)]. Vascular density in the non-calcified articular cartilage at the osteochondral junction was higher in patients with OA than in PM controls [Fig. 2(A)].

Vascular density at the osteochondral junction increased with increasing OA disease severity (Fig. 3). Osteochondral vascular density increased with histological cartilage severity score [Fig. 3(A)]. Partial correlation coefficients revealed that vascular density in the non-calcified cartilage increased with increasing total cartilage severity score independent of diagnostic group ($r = 0.38$, $P = 0.005$). Partial correlation coefficients calculated for individual components of the cartilage score indicated that vascular density at the osteochondral junction increased with decreasing cartilage surface integrity ($r = 0.31$, $P = 0.018$), more abnormal chondrocyte appearance ($r = 0.39$, $P = 0.002$) and greater proteoglycan loss ($r = 0.53$, $P < 0.001$), each independent of tidemark integrity score. High osteochondral vascular densities were associated also with high clinical disease activity scores [Fig. 3(D)]. Vascular densities in the articular cartilage were not significantly associated with histological synovitis scores [Fig. 4(A, D)].

The EC proliferation and macrophage infiltration indices and inflammation scores were higher in synovia from patients with OA than in PM [Figs. 1(G, H), 2(B, H, I)]. Synovial EC proliferation increased with increasing synovial EC fractional area ($r = 0.35$, $P = 0.009$), macrophage infiltration [Fig. 4(E)] and inflammation grade [Fig. 4(B)]. Synovial EC fractional area and macrophage infiltration each increased with increasing inflammation grade [Fig. 4(C) and $r = 0.46$, $P = 0.001$, respectively]. Partial correlation coefficients revealed that synovial EC proliferation increased with increasing macrophage infiltration and inflammation grade independent of diagnosis ($r = 0.43$, $P = 0.002$ and $r = 0.37$, $P = 0.01$ respectively). Angiogenesis indices in the synovium were not significantly associated with osteochondral vascular density [Fig. 5(A, B)], cartilage or radiographic severity scores, nor with clinical disease activity scores (Table I). Likewise, inflammatory indices in the synovium were not significantly associated with JSN, OST or clinical disease activity scores (data not shown).

OST scores were slightly higher in OA/CC+ than OA/CC− [Fig. 2(F)]. No qualitative or quantitative differences in osteochondral or synovial angiogenesis, histological synovitis, cartilage severity or clinical disease activity scores were observed between OA/CC+ and OA/CC− cases [Fig. 2(A−E, G−I)].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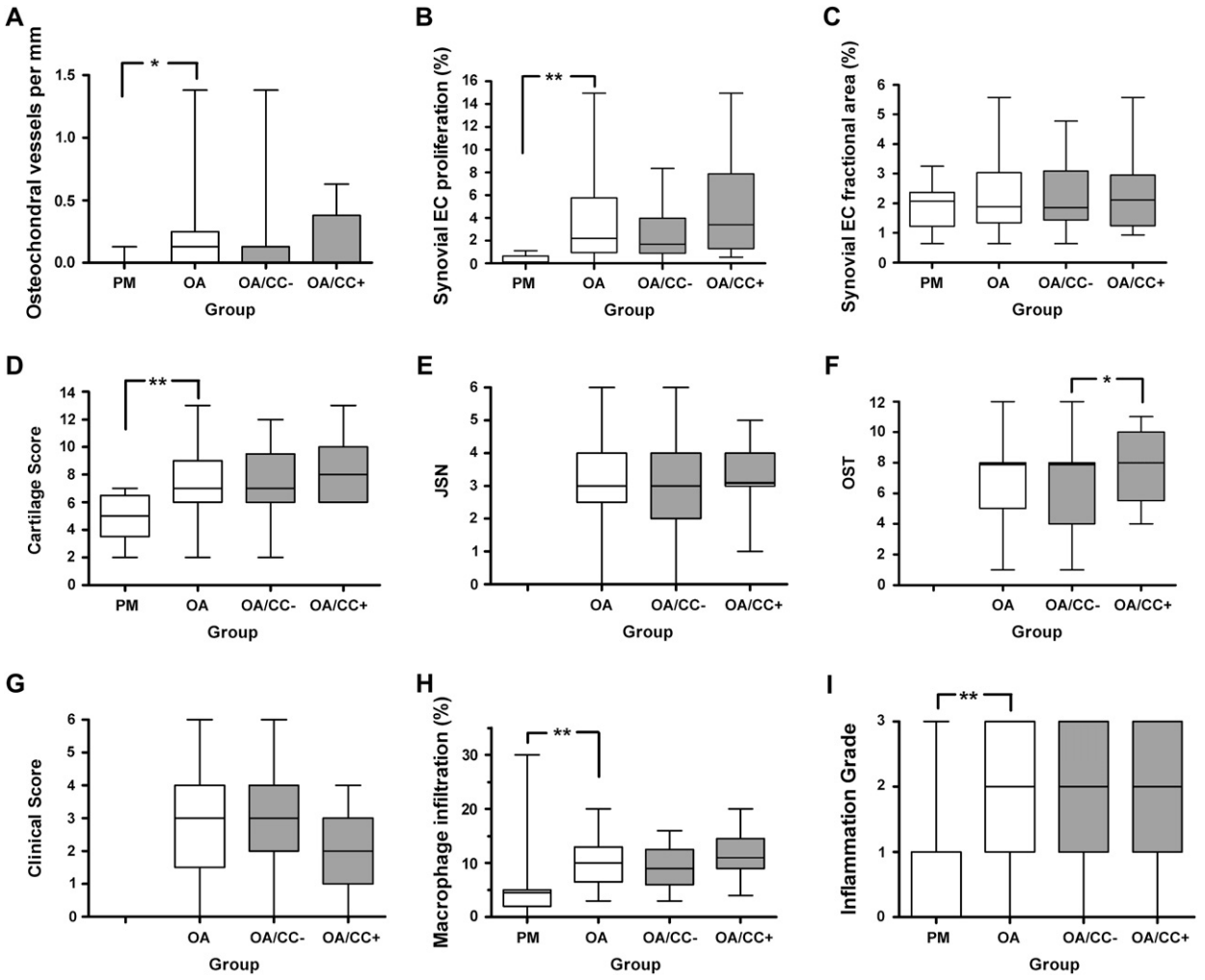

Fig. 2. Angiogenesis, radiological, clinical and inflammation indices compared between patients with OA and PM controls, alongside subgroups of OA with and without CC. (A) Osteochondral vascular density in the non-calcified articular cartilage was higher in patients with OA (median = 0.13, IQR 0.0–0.25) than PM controls (median = 0.0, IQR 0.0–0.03) ($Z = -2.1$, $P = 0.03$). (B) Synovial EC proliferation was higher in patients with OA (median 2.2%, IQR 1.0–5.8%) than in PM (median 0.1%, IQR 0.0–0.7%) ($Z = -4.7$, $P < 0.001$). (C) Synovial vascular densities were similar in all disease groups. (D) Histological cartilage severity score was higher in OA (median 7, IQR 6–9.3) than in PM controls (median = 5.0, IQR 3.5–6.5) ($Z = -3.4$, $P = 0.001$). (E–G) JSN, OST and clinical disease activity scores in surgical samples. OST score was higher in osteoarthritic patients with CC (median = 8, IQR 5.8–10) than in those without CC (median = 8, IQR 4–8) ($Z = -2.0$, $P = 0.045$). (H) Macrophage infiltration was higher in OA (median = 9.7%, IQR 6.3–13.0%) than PM controls (median = 4.3%, IQR 2.3–5.3%) ($Z = -3.7$, $P < 0.001$) (I) Inflammation grade was higher in patients with OA (median = 2, IQR 1–3) than PM controls (median = 0, IQR 0–1) ($Z = -5.1$, $P < 0.001$). Angiogenesis, clinical and inflammation indices in OA did not differ significantly between patients with and those without CC. Box and whisker plots showing median, IQR and range.

Fig. 1. Vascularisation of non-calcified cartilage and synovium in OA compared with PM controls. (A) Vascular channel surrounded by a bone cuff breaching the tidemark and entering the non-calcified cartilage in a patient with OA. Note duplication of tidemark. (B) Vascular channel in the calcified cartilage of a PM control. (C, D) CD34-positive ECs (red fluorescence) within vascular channels of preparations shown in A & B respectively. (E) Vascular channel breaching the tidemark in another patient with OA, showing bone cuff (green) and adjacent depleted proteoglycan (loss of pink stain). (F) Vascular channel within the calcified cartilage of a PM control. (G) CD31-immunoreactive endothelium (red) displaying proliferating nuclei (black) in synovium from a patient with OA. Note the abnormally high vascularity distant from the synovial lining. (H) Synovium from a PM control displaying the normal high density of vessels adjacent to the synovial lining, but few Ki67-positive proliferating nuclei. (A, B) Autofluorescent images under ultraviolet light. (C, D) Red immunofluorescence for CD34. (E, F) Safranin-O and fast green stain. (G, H) Double sequential immunohistochemsitry for CD31 (red) and Ki67 antigen (black). NCC: non-calcified cartilage, CC: calcified cartilage, Bo: bone. Short arrows indicate tidemarks. *Indicates vessel lumen. Long arrows indicate proliferating nuclei within ECs. Arrowheads indicate synovial lining. Scale bars are 100 μm.

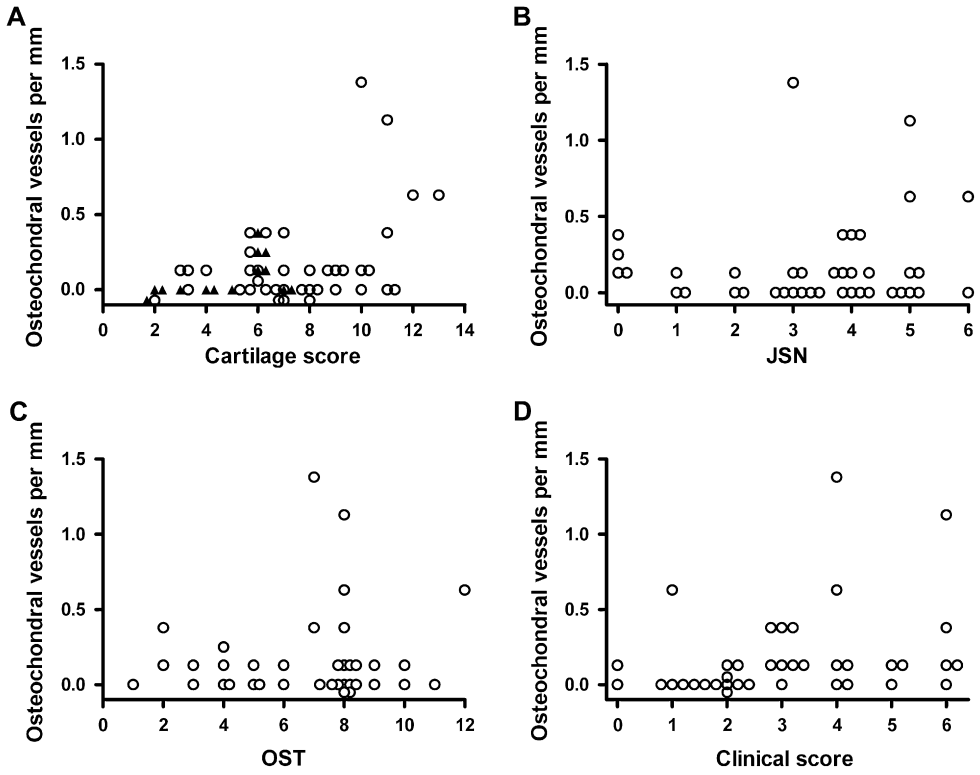

Fig. 3. Osteochondral vascularisation and histological, radiological and clinical OA scores. (A) Osteochondral vascular density increased with increasing histological cartilage score ($r = 0.26$, $P = 0.049$). (B, C) Associations between osteochondral vascular density and radiological OA severity (JSN and OST scores) did not reach statistical significance. (D) Osteochondral vascular density increased with increasing clinical score ($r = 0.41$, $P = 0.009$). Scatter plots of data from patients with OA (○) and PM controls (▲).

Discussion

We found that synovial and osteochondral angiogenesis are both features of OA that occur in parallel. Indices of angiogenesis in the synovium were associated with histological synovitis but not cartilage changes, whereas vascular density at the osteochondral junction was associated with OA changes in the cartilage but not with histological synovitis.

Previous studies of angiogenesis and inflammation in OA have been limited by difficulties in defining disease-free control groups. The PM group in this study was selected to exclude, as far as possible, patients with evidence of OA (or other arthritides). Exclusion criteria for the PM control group included macroscopic appearance of OA, regular mild analgesic use, previous visits to a doctor or hospital about knee pain and previous trauma or surgery to the knee joint. However, mild or preclinical OA still cannot be definitively excluded in the PM group. The observed range of histological cartilage severity scores (2–7) in the PM group may represent mild OA, or may represent appearances of normal adult human articular cartilage[17]. Although they may occur to a lesser degree sometimes in normal knees, increased osteochondral vascularity and synovial EC proliferation, and greater histological synovitis in OA rather than PM cases, indicate that angiogenesis and inflammation are increased in OA.

CC, in the absence of a current episode of pseudogout, did not explain angiogenesis either in the synovium or at the osteochondral junction. Indeed, CC did not appear to be the major determinant of histological synovitis in OA. Despite the well known acute pro-inflammatory actions of CPPD crystals, other factors may be more important determinants of histological synovitis in OA. Our data are consistent with previous findings that CC is associated with more severe radiological change in patients with OA[11]. CC therefore may indicate a discrete subset of the broad spectrum of conditions termed OA[18], however, very large case numbers would be required to discriminate a unique effect of synovitis or vascularisation in this subgroup of OA.

Osteochondral vascularity was associated with higher clinical disease activity scores, indicating that vascularisation of the articular cartilage may contribute to clinical features of OA. Vascular invasion may contribute to symptoms by facilitating sensory nerve growth into the normally aneural articular cartilage[19]. Further work is required to confirm and explain this apparent association.

The distinct associations of angiogenesis at the osteochondral junction and in synovium respectively may indicate that different mechanisms drive vascular growth in these two tissues in OA. It remains possible that synovitis and cartilage changes more weakly contribute to angiogenesis in the adjacent tissue, but local factors within the tissue seem most important. Our findings indicate that future studies of synovial angiogenesis should focus on factors produced by the inflamed synovium, whereas studies of osteochondral angiogenesis should address changes in cartilage matrix composition and chondrocyte function.

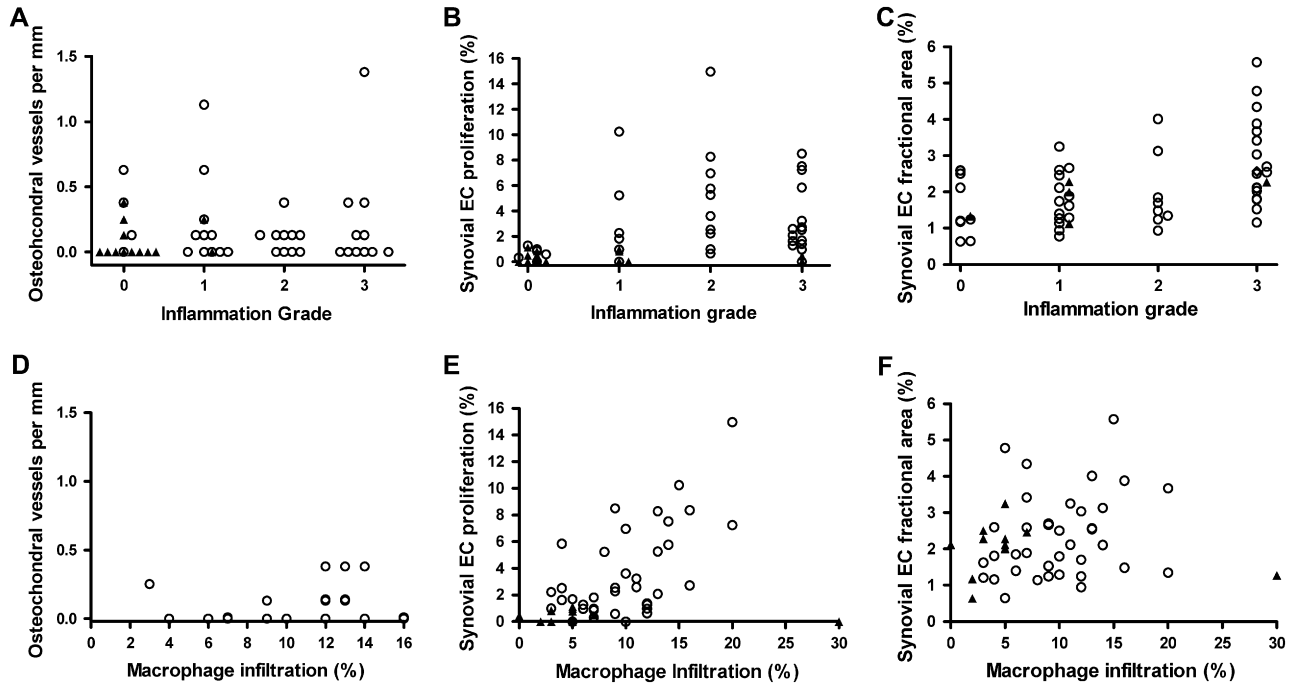

Fig. 4. Osteochondral and synovial vascularisation and synovial inflammation. (A, D) Osteochondral vascular density was not significantly associated with synovial inflammation grade (A) or synovial macrophage infiltration (D). (B, E) Synovial EC proliferation increased with increasing inflammation grade (B, $r = 0.59$, $P < 0.001$), and with increasing synovial macrophage infiltration (E, $r = 0.54$, $P < 0.001$). (C) Synovial EC fractional area increased with increasing inflammation grade (C, $r = 0.42$, $P = 0.002$). (F) Synovial EC fractional area vs macrophage infiltration. Scatter plots of data from patients with OA (○) and PM controls (▲).

Several factors produced by resident and infiltrating cells (such as macrophages[1], lymphocytes[20] and mast cells[21,22]) may contribute to angiogenesis in the inflamed synovium. Of these, vascular endothelial growth factor (VEGF) and its inducer hypoxia-inducible factor-1α have been associated with synovial angiogenesis in OA[8,23]. A large variety of other angiogenic regulators, both positive and negative, may also be present in the synovium[1]. Chondrocytes expressing VEGF also are increased in articular cartilage from patients with OA, although their predominantly superficial localisation, distant from the osteochondral junction may suggest that other factors are more important in its vascular invasion[9].

The association of vascular channels with regions of proteoglycan loss supports the view that changes in cartilage matrix may be important in permitting invasion of the articular cartilage by blood vessels. Loss of resistance to vascular invasion has been associated with proteoglycan loss in explant studies using OA articular cartilage[3,9].

Angiogenesis in OA may contribute to both symptoms and disease progression. Vascular growth at the osteochondral junction may contribute to clinical disease activity, and in the synovium to chronic synovitis. Blood vessel growth into the articular cartilage may contribute to its ossification and to cartilage damage. Novel therapies to manipulate angiogenesis may have potential to relieve the

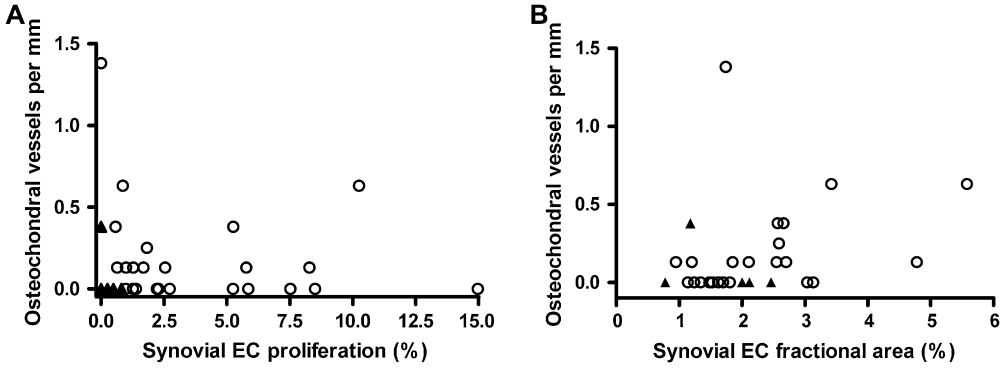

Fig. 5. Osteochondral and synovial vascularisation. Osteochondral vascular density was not significantly associated with synovial EC proliferation (A) and synovial EC fractional area (B). Scatter plots of data from patients with OA (○) and PM controls (▲).

Table I
Synovial and osteochondral angiogenesis indices associated with synovial inflammation and severity of osteoarthritic cartilage changes. Partial correlation coefficients, controlling for OA or PM status are shown

		Synovium			Cartilage	
		EC fractional area	Inflammation grade	Macrophage infiltration	Osteochondral vascular density	Cartilage score
Synovium	EC proliferation	0.21	**0.33***	**0.41****	0.05	0.15
	EC fractional area		**0.42****	0.10	0.07	−0.15
	Inflammation grade			0.21	−0.04	0.17
	Macrophage infiltration				0.19	0.04
Cartilage	Osteochondral vascular density					**0.40****

*$P < 0.05$, **$P < 0.01$.

symptoms and severity of OA by limiting these processes. Such therapies may need to target common mechanisms of angiogenesis, or to separately address the factors that drive vessel growth in the different tissues of the joint. Further work is required to elucidate the diverse mechanisms of angiogenesis that contribute to OA, and the consequences that inhibiting vessel growth may have on the disease.

Acknowledgements

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