The innervation of synovium of human osteoarthritic joints in comparison with normal rat and sheep synovium


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SUMMARY
Objective: To study whether osteoarthritis (OA) in the knee is associated with a change of the innervation pattern in the synovial layer.

Design: In synovial tissue from the normal knee joint of rat and sheep we studied the presence of vessels and of nerve fibres using transmission electron microscopy and immunohistochemistry. Synovial material was also obtained from patients who underwent total knee replacement surgery. This material was examined for inflammatory changes, and the presence of vessels and nerve fibres was assessed.

Results: The synovium in the parapatellar region of the normal knee joint of rat and sheep exhibited a dense capillary and neuronal network. It was entered by calcitonin gene-related peptide containing sensory fibres and tyrosine hydroxylase-positive sympathetic nerve fibres. Synovial material from patients with knee OA exhibited different degrees of inflammation. Synovial material without inflammation exhibited a similar vascular and neuronal network as the normal knee joint from rat and sheep. However, in synovium with inflammatory changes we found a significant decrease of nerve fibres in depth ranges close to the synovial lining layer depending on the degree of inflammation whereas deeper regions were less affected.

Conclusions: Inflammationary changes in the synovium of OA joints are associated with a massive destruction of the capillary and neuronal network which is present in normal synovium. Due to the disappearance of the sensory fibres it is unlikely that OA pain is initiated directly in the synovium. The loss of normally innervated vascularisation may have multiple consequences for the physiological functions of the synovium.

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Introduction

Osteoarthritis (OA) is the most frequent cause of joint pain. However, although pain is one of the most significant clinical problems of OA, the pain mechanisms of OA are poorly understood. It is unclear which structures in the joint give rise to OA pain, and the nature of OA pain (nociceptive vs neuropathic) is a matter of discussion.

A hallmark of OA is the progressive destruction of cartilage. Employing near infrared spectroscopy (NIRS) we were able to demonstrate even quantitative tissue alterations in low-grade cartilage defects in OA in sheep as well as in humans. However, in the long-term OA is a disease of the whole joint. The subchondral bone is remodelled, many OA joints show signs of chronic synovitis, and bone marrow lesions may be observed. These multiple changes raise the question which are the most important pathogenic mechanisms of OA. While a major driver of OA progression is aberrant loading or the deterioration of the underlying subchondral bone, many authors stress the significance of inflammation and enzymatic cartilage degradation. OA joints show an aberrant expression of inflammation-related and catabolic genes, nitrogen monoxide, metalloproteinases, cyclooxygenase-2, cathepsins. These mediators are produced by the synovium and chondrocytes which respond to biomechanical perturbation by upregulation of the production of proinflammatory cytokines, stress-induced intracellular

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signals such as reactive oxygen species (ROS), even in the absence of overt inflammation. Also advanced glycation end products (AGEs) seem to play an eminent role in the pathogenesis of OA.

The involvement of the whole joint in the disease process stresses the question which of the pathologies actually cause pain? It is unlikely that the cartilage destruction itself gives rise to pain because cartilage is normally not innervated. Imaging studies showed a greater likelihood of pain in OA knee joints which exhibited magnetic resonance imaging (MRI) abnormalities such as synovitis and bone marrow lesions. However, a major gap is the lack of knowledge whether, and if, how the innervation of the joint is altered in the process of human OA.

Across different species, the joint is innervated by numerous nerve fibres, about 80% of which are unmyelinated. Sensory thick myelinated Aβ fibres with Ruffini-, Golgi- and Pacini-type endings are located in ligaments, fibrous capsule, menisci and adjacent periosteum, but not in synovial tissue and cartilage, and they are thought to function primarily as sensors for pressure and movements. Sensory Aδ and C fibres terminate as free nerve endings in the fibrous capsule, adipose tissue, ligaments, menisci, and the adjacent periosteum. To what extent the synovial layer is innervated by sensory fibres, has been disputed. Several microscopic studies did not find such fibres in the synovial layer, but the staining for the neuropeptides calcitonin gene-related peptide (CGRP) and substance P suggested the presence of sensory fibres at this site.

Most of the sensory Aδ and C fibres are thought to act as nociceptors (see Discussion). In addition to sensory afferent fibres, the joint nerve contains numerous sympathetic postganglionic nerve fibres. Fibres in close approximation to vessels are considered sympathetic fibres. Effector fibres to the knee joint were found to provide vasconstrictor.

In the present study we addressed the innervation of human knee joints with OA by performing light and electron microscopic examinations of material obtained during joint replacement. We focussed on the synovial layer. Because no normal joint tissue can be obtained from humans we studied the innervation of the synovial layer of the normal knee joint of the rat and the sheep. We analysed the pattern of innervation in the material from human OA joints taking into account pathological changes of the synovial tissue.

Methods

Patients and experimental animals

Synovial tissues were obtained from 13 patients with OA who underwent total knee replacement surgery between November 2011 and August 2012. Only patients with presumed idiopathic OA were included, patients with post-traumatic or rheumatoid arthritis (RA) were excluded. The patients were informed about the purpose of tissue sampling and gave written consent after the nature of all examinations was fully explained. The study was approved by the Ethical Committee for Clinical Trials of the Friedrich Schiller University of Jena (1714-01/06 and 2443-12/08). All examinations of material obtained during joint replacement. We examined the innervation of the synovial tissue in the medial and lateral parapatellar region and in the suprapatellar recess was resected. In order to study the innervation of the healthy joint we took synovial tissue from the knee joint of five normal adult Wistar rats (75−90 days, 250−350 g) and of three Merino sheep (3−4 years old, 75−95 kg).

Histochemistry and immunohistochemistry

Human and animal tissue samples were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 24 h at 4°C. After washing with PBS, samples were dehydrated in increasing concentrations of alcohol and embedded in Technovit 9100 methyl methacrylate (Heraeus Kulzer, Wehrheim, Germany) according to manufacturer’s instruction. Using the microtome Polycut S (Reichert–Jung, Heidelberg, Germany), sections of 4 μm were cut from polymerised Technovit 9100 blocks. Before staining, polymer was removed with 2-methoxyethanol, and sections were rehydrated in decreasing concentrations of alcohol.

For histochemistry, sections of materials from patients were stained with Mayer’s Haemalaun solution and Eosin G (Merck, Darmstadt, Germany). Synovitis was scored according to Krenn. It quantifies the enlargement of the lining cell layer (in points; 0: only one cell layer; 1: two to three cell layers; 2: four to five cell layers; 3: more than five cell layers), the cellular density of synovial stroma and pannus formation (0: normal cell density; 1: cell density slightly enhanced; 2: cell density moderately enhanced; 3: high cell density, multinuclear giant cells, pannus tissue), and leukocytic infiltrate (0: no infiltration; 1: single lymphocytes or plasma cells; 2: aggregates of lymphocytes; 3: dense infiltration with lymphocytes or lymph follicles). The total score ranges from 0 to 9 (0−1: no synovitis; 2−3: low-grade, 4−6: medium-grade, 7−9: high-grade synovitis).

For immunohistochemical labelling, heat-induced antigen retrieval was performed. Sections of human and animal tissues were placed in the staining dish containing citrate buffer (10 mM, pH 6.0), transferred to an autoclave and heated to 120°C for 15 min. After cooling down, sections were rinsed with Tris-buffered saline (TBS, 20 mM, pH 7.6) and incubated with blocking solution containing 10% normal goat serum + 1% Triton X-100 in TBS for 30 min at room temperature. Then sections were incubated with primary
antibodies against protein gene product 9.5 (PGP9.5, rabbit polyclonal antibody, 1:500, AbD serotec, Düsseldorf, Germany), tyrosine hydroxylase (TH, rabbit polyclonal antibody, 1:200, Epitomics, Burlingame, CA, USA) or CGRP (mouse monoclonal antibody, 1:100, Acris Antibodies, Herford, Germany) in TBS containing 2% normal goat serum and 1% Triton X-100 at 4°C overnight. After washing with TBS, sections were incubated with goat anti-rabbit immunoglobulin G (IgG) conjugated with Alexa 568 (1:200, Invitrogen, Carlsbad, CA, USA) or goat anti-mouse IgG conjugated with Alexa 488 (1:100, Invitrogen), respectively, for 2 h in darkness. After additional washing, sections were stained with Hoechst 34580 (1:1000, Invitrogen) and embedded in Prolong Gold antifade reagent (Invitrogen). For control of specificity the primary antibodies were left out. Such preparations did not exhibit labelled structures. Immunostaining was analysed using the confocal laser scanning microscope TCS SP5 (Leica, Wetzlar, Germany). Fluorescent dyes were excited with an argon laser, fluorescence signals were detected between 500 and 555 nm using a 63× 1.40 oil objective.

Analysis of nerve fibre bundles

Quantitative analysis of nerve fibre bundles was performed with the microscope Axioplan2 (Zeiss, Oberkochen, Germany). Immunostaining of PGP9.5 from all sections of human samples was photographed in three selected areas of 1 × 0.5 mm using a 20× dry objective. In each area the distance of nerve fibre bundles to synovial surface was measured using the microscopic software Axiovision (Zeiss). All detected nerve fibre bundles were grouped into one of the following categories: 0—50, 50—200, and 200—500 μm from the surface. The total number of nerve fibre bundles was calculated for each category and each patient.

Analysis of the capillary network

For investigation of normal neuronal and capillary network, synovial tissue of rat knee joints was fixed with 4% PFA for 24 h at 4°C. Without embedding, synovial tissues were labelled with PGP9.5 (nerve fibres) and isolectin IB4 (IB4, non-peptidergic nerve fibres) and capillaries. After blocking with 10% normal goat serum and 1% Triton X-100 in TBS for 30 min, synovial tissues were incubated with primary antibody PGP9.5 (1:500, in TBS containing 2% normal goat serum and 1% Triton X-100) at 4°C overnight. Synovial tissues were washed 3× in TBS and incubated with secondary antibody (goat anti-rabbit IgG conjugated with Alexa 568; 1:200, Invitrogen) for 2 h in darkness. Capillaries were stained with IB4 conjugated with fluorescein isothiocyanate (1:20, Sigma–Aldrich, Taufkirchen, Germany) for 45 min in darkness. Finally the synovial tissues were washed 3× in TBS and placed directly on a coverslip with a drop of TBS and were immediately investigated using the inverse confocal laser scanning microscope TCS SP5. Fluorescent dyes were excited with an argon laser (488 nm) or a diode-pumped solid-state laser (561 nm), respectively. The green fluorescence signals were recorded between 500 and 555 nm and the red fluorescence signals were recorded between 570 and 700 nm using a 20× 0.7 dry objective or a 63× 1.40 oil objective. Control preparations without primary antibodies did not show labelled structures.

Transmission electron microscopy

Human and animal tissues were fixed with 4% PFA and 2.5% glutaraldehyde for 24 h at 4°C. After fixation, samples were washed with cacodylate buffer (0.1 M, pH 7.4) and post fixed with 1% osmium tetroxide in cacodylate buffer for 2 h. During the following ascending ethanol series samples were stained with 2% uranyl acetate. The samples were embedded in Araldite resin (Plano, Wetzlar, Germany) according to manufacturer’s instruction. In semithin sections (1 μm) stained with Richardson’s methylene blue the synovial layer was identified. The areas of the samples were then further trimmed down to 500 × 500 μm. Ultra-thin sections of 80 nm thickness were cut using the ultramicrotome Ultracut E (Reichert–Jung, Wien, Austria) and then mounted on Formvar-carbon coated grids (100 mesh). Finally, sections were stained with lead citrate for 4 min and investigated using a transmission electron microscope EM900 (Zeiss).

Statistical analysis

The correlation between the number of nerve fibres at different depth ranges and the synovitis score was calculated using the Spearman Rank correlation (SPSS 19). From each patient one specimen was included. The Bootstrap method was used to calculate the 95% confidence interval. Significance was accepted at P < 0.05.

Results

Localisation of vessels and nerve fibres in the synovial layer of rat, sheep, and human

Sections were made from the synovial layer of the parapatellar region from the knee joint of the rat and the sheep. Figure 1(A) shows an electron microscopic image of the synovial layer of the rat knee joint. Directly below the synovial lining cells there is a quite regular distribution of capillaries (black arrows), and nerve fibre bundles or single nerve fibres (white arrows) which are located either close to the vessels or between the vessels. Figure 1(B–F) shows nerve fibre bundles and blood vessels in higher magnification. The nerve fibre bundles typically consisted of several unmelenated fibres [Fig. 1(C, E)] but occasionally a myelinated fibre was also included [Fig. 1(B)]. A similar pattern of small vessels and nerve fibres was found in the synovial layer of sheep knee joint [Fig. 2(A)].

Synovium from human OA knee joints exhibits regions which appeared quite normal, as well as regions which are considerably altered. In synovial tissue with a “normal” appearance [Figs. 2(B) and 5(A)] the vessels and nerves were similarly arranged as in normal rat and sheep knee joint suggesting similar patterns of vessel distribution and nerve fibres across species.

Distribution and types of nerve fibres in the rat synovial layer

We used PGP9.5 as a general marker of nerve fibres including sensory afferent and sympathetic efferent ones. We aimed to label non-peptidergic sensory neurons with IB4 but found that IB4 clearly labelled the capillaries (as described). Figure 3 displays the vessels in the synovial layer (green) and the nerve fibres (red) of rat knee joint [magnification 20× in Fig. 3(A), 63× in Fig. 3(B)]. The vessels formed a regular network. Judged from the diameters, the vessels were capillaries. Nerve fibre (bundles) were running along the vessels but were also crossing the vessels.

In order to identify the nerve fibres as sensory or postganglionic sympathetic, we used labelling for CGRP or TH. The neuropeptide CGRP is contained in a large proportion of the peptidergic sensory fibres including joint afferents. The enzyme TH is involved in the production of norepinephrine in sympathetic efferent fibres. Figure 4 shows adjacent sections of synovial tissues from a rat, the cell nuclei were stained with Hoechst 34580 for orientation. In Fig. 4(E), nerve fibres were labelled with PGP9.5 (green). Numerous nerve fibres were detected in the most superficial layer as well as in deeper layers (see arrows). The tissue in the white rectangle is also displayed in Fig. 4(E). Figure 4(C) shows CGRP-positive nerve fibres...
(green) in the superficial as well as the deeper layer (see arrows). Figure 4(F) depicts the tissue in the rectangle. Figure 4(D) displays TH-positive nerve fibres (green) around a vessel in the deeper layer [see arrows and Fig. 4(G)]. No labelling was observed when only the secondary antibodies were used [Fig. 4(H and I)]. Thus sensory CGRP-positive nerve fibres were located at both the superficial and the deeper sites whereas TH-positive postganglionic nerve fibres were rather located at deeper layers, in association with small vessels.

**Vessels and nerve fibres in the human OA joint**

We could analyse the synovial material from 13 knee joints which was resected during joint replacement. All patients had OA at K/L stage 3 or 4. Data from these patients are displayed in Table 1. All joints had severe cartilage degeneration, and KOOS indicated symptoms (swelling, stiffness etc.), pain, and a reduction of physical function (ADL). The histology of these preparations showed that such material, concerning inflammatory changes, was very heterogeneous, sometimes even within material from the same joint. We scored synovitis in each tissue specimen according to Krenn et al.24.

The synovial layer in Fig. 5(A) appears quite normal (synovitis score 0–1), the synovial layer in Fig. 5(D) shows a proliferation of synovial lining cells, blood vessels as well as a formation of villi (synovitis score 2). The synovial layer in Fig. 5(G) displays a strong infiltration with inflammatory cells and pannus formation (synovitis score 4). We screened these sections for the presence of capillaries and nerve fibres in the synovial layer. Figure 5(B, E, H) shows in adjacent sections nerve fibres stained with PGP9.5 (white arrows) and nuclei stained with Hoechst. Figure 5(C, F, G) displays the evaluation of these sections for the presence of PGP9.5-stained nerve fibres in an area of 3 mm (length) × 0.5 mm (depth) of synovial tissue. Figure 6 shows for all joints (n = 13) the analysis of
Fig. 3. Vessel network and nerve fibre bundles in the synovial layer. A. Magnification 20×. B. Magnification 63×. Vessels (green) are stained with IB4. Nerve fibre bundles (red) are stained with PGP9.5.

Fig. 4. Nerve fibres in adjacent sections of a preparation of the synovial tissue from rat knee joint. A. Overview (transmission image). In B–I cell nuclei were stained for orientation. B. Nerve fibres labelled with PGP9.5 (green) in the most superficial layer as well as in deeper layers (see white arrows). C. CGRP-positive nerve fibres (green) in the superficial as well as the deeper layer. D. TH-positive nerve fibres (green) around a vessel in the deeper layer. E–G show in higher magnification the areas marked in B–D by rectangles. H. Control section labelled only with goat anti-rabbit IgG conjugated with Alexa 488. I. Control section labelled only with goat anti-mouse IgG conjugated with Alexa 488.
the number of nerve fibre bundles in different depth ranges, in relation to the synovitis score. In samples with no pathological changes numerous nerve fibres were found close to the synovial surface. In contrast, in altered synovial tissues (synovitis score >1) nerve fibres were only observed in deeper layers. The numbers of fibres were negatively correlated to the synovitis score (Spearman Rank correlation) at depth ranges of 0–50 μm (P = 0.022) and 50–200 μm (P = 0.002), and in total (P = 0.035), but at 200–500 μm no significant correlation was found (P = 0.501, for confidence intervals see legends to Fig. 6).

**Discussion**

The present study visualised the dense network of capillaries and nerve fibres in the normal synovial tissue of rat and sheep knee joint. Part of the nerve fibres was identified as unmyelinated CGRP-positive sensory fibres. In deeper layers TH-positive sympathetic fibres were observed in the vicinity of vessels. The synovium of human osteoarthritic knee joints exhibited various degrees of inflammation. While non-inflamed synovium showed a similar vascular and neuronal network as in normal rat and sheep knee joint, regions of inflamed synovium from OA joints exhibited a destruction of the network of capillaries and a dramatic loss of nerve fibres.

The main purpose of the study was to characterise changes of innervation in human OA joints. However, as previous studies we could not obtain tissue from healthy human knee joints. In studies on inflammation such as RA, tissue from OA joints was sometimes used as non-inflamed controls, but we found signs of inflammation in the tissue from all the OA joints. Therefore we used for reference synovium from normal knee joints of rats and sheep and checked whether the rare non-inflamed areas of synovium of human OA joints show similar patterns of the vasculature and nerves. Because the degree of inflammation differed in the samples we assessed the innervation density always in relation to the degree of local synovitis, and in addition we analysed different depths separately. Such an approach was not used before to our knowledge.

The synovium of the normal rat and sheep knee joint and (rare) non-inflamed synovium from OA joints showed a similar capillary and neuronal network. Detailed analysis of rabbit knee joints revealed a very rich capillary bed located very superficially over areolar or adipose tissue, and a rapid decay of capillary density at
Fig. 6. Density of nerve fibre bundles in the synovial layer of 13 OA joints with different grades of synovitis at different depth ranges from the synovial lining layer. A–D. Nerve fibre bundles at a depth range of 0–50, 50–200, 200–500, and 0–500 μm. The data were normalised to 1 mm of synovial surface. For clarity the symbols were slightly shifted. The linear regression line, the Spearman Rank correlation coefficient and its p values are presented. The upper and lower limits (95% confidence intervals) for R are −0.885 to −0.089 (0–50 μm), −0.947 to −0.361 (50–200 μm), −0.805 to −0.476 (200–500 μm) and −0.916 to −0.019 (0–500 μm).

deeper sites29. This vascular bed is thought to be specialised for the supply of fluid to the joint cavity and of nutrients to the distant, avascular cartilage30,31.

Nerve fibres in the synovium were identified by PGP9.5 staining and electron microscopy. The latter showed that almost all nerve fibres were unmyelinated and formed bundles. Thus PGP9.5-stained structures may represent rather nerve fibre bundles than single nerve fibres. About half of the PGP9.5-positive nerve fibres could be identified by staining of adjacent sections with antibodies. CGRP-positive fibres (representing peptidergic sensory afferents) were found below the synovial lining layer and in deeper areas. IB4 staining labelled the capillaries26 (thrombomodulin staining for vessels showed the same pattern, unpublished observation) but we could not clearly identify IB4-positive non-peptidergic sensory nerve fibres. Thus the knee synovium is mainly innervated by peptidergic sensory fibres, similar as the hip joint32 and the bone33 whereas the skin is innervated by both peptidergic and non-peptidergic sensory nerve fibres. The synaptic innervation14,41 we found in the present study that the synovium exhibits a loss of normal and normally innervated vasculature.

Neither PGP9.5 staining nor electron microscopy (performed in some joints) showed the presence of nerve fibres in such inflamed tissue. Previously a strong reduction of the density of the neuropeptides substance P and CGRP was observed in the synovium from patients with RA14,15,17. The reduction of neuropeptides may indicate the depletion of neuropeptides or the loss of innervation. In properly fixed synovial material from rats with polyarthritis a reduced density of innervation was found using PGP and neuropeptide antibodies for labelling28,39. Other authors found mainly a loss of sympathetic nerve fibres in arthritic joints, resulting in the prevalence of sensory substance P-positive fibres40. From the spatial distribution (depth ranges) the present study suggests a reduction of both sensory and sympathetic fibres in OA synovium. Thus, while earlier studies concluded that inflamed synovium from RA patients exhibits a reduction of nerve fibres whereas non-inflamed synovium from OA joints shows an intense peptidergic innervation14,41 we found in the present study that the synovium from OA joints is inflamed at many sites and that the innervation density is strongly reduced, similar as in RA.

The present findings give rise to several conclusions and suggestions. First, in our opinion it is questionable whether OA pain is initiated by stimulation or sensitisation of nociceptive fibres in the inflamed synovial layer itself. On the basis of the analysed synovial samples we did not find a correlation between the pain in the KOOS score and the density of nerve fibres (nor between the pain and the grade of synovitis). This may not be surprising because OA pain similar as other chronic pain states is the result of complex peripheral and central nociceptive mechanisms3,42. However, some
peripheral input seems to contribute because many patients show a significant reduction of pain after joint replacement\(^1\) even if in a substantial number of patients pain relief may not persist\(^4\). Because the innervation density decreases in inflamed synovium we assume that nerve fibres in other structures are important. Many nociceptive fibres of the normal joint respond to strong pressure and noxious movements, and their sensory endpoints are thus presumably located in fibrous structures which are stretched by such stimuli (see Introduction). During inflammation such nociceptors are sensitised and respond even to innocuous mechanical stimuli\(^2\). In awake humans pain sensations in the normal knee joint were particularly elicited by mechanical, thermal and chemical stimulation of fibrous structures (ligaments and fibrous capsule) but rarely by mechanical stimulation of the synovial layer\(^6\). Currently we do not know whether the innervation of the fibrous structures changes during OA. Interestingly, in mice with complete Freund's adjuvant-induced arthritis “hot spots” were identified at the synovial—meniscal interface in which sensory as well as sympathetic fibres showed sprouting resembling neroma formation\(^46\). Whether similar changes occur in human or animal OA joints is unknown.

Studies on the monoiodoacetate (MIA) OA model suggested the presence of a neuropathic component in OA because they found an expression of the “nerve damage marker” activating transcription factor 3 (ATF3) in dorsal root ganglia\(^47,48\). Whether similar changes occur in human or animal OA joints is unknown. The authors thank Dr Harald Schubert and Dr Sabine Bischoff (Institut für Versuchstierkunde, Jena University Hospital) for providing knee joints from sheep, and Mrs Christine Kämmitz for technical assistance. We thank the German Working Compensation (Berufsgenossenschaft für Bauwirtschaft) in Berlin for supporting research work on OA of the knee.

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Conflict of interest

None of the authors has competing interests.

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