Synovial immunopathology in haemochromatosis arthropathy

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*Ann Rheum Dis* published online November 23, 2009
doi: 10.1136/ard.2009.120204

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Synovial immunopathology in haemochromatosis arthropathy


ABSTRACT

Background Hereditary haemochromatosis (HH) is a common autosomal recessive inherited disorder that frequently causes arthritis. The pathophysiology of musculoskeletal involvement is, however, unclear.

Objective To analyse synovial tissue obtained at surgery from patients with HH arthropathy and compare it qualitatively and quantitatively with specimens from patients with rheumatoid arthritis (RA) and osteoarthritis (OA).

Methods Synovial tissue from 15 patients with HH, 20 with RA and 39 with OA was obtained during surgery. A synovitis grading system was used to determine the severity of synovial inflammation. Using immunohistochemistry, synovial neovascularisation and infiltration of macrophages, neutrophils and lymphocytes were quantitatively assessed.

Results Synovitis in HH arthropathy largely resembles OA with mild infiltration of mononuclear cells and lymphocytes, formation of synovial microvessels and a low degree of synovial hyperplasia. While many features of HH arthropathy are reminiscent of OA, macrophage and especially neutrophil invasion is clearly more prominent in HH arthropathy than in primary OA and mimics features of RA. This finding was observed particularly in synovial tissue of HH samples with marked hemosiderin deposition.

Discussion The histological picture of the synovium in HH arthropathy largely resembles a process reminiscent of OA. Neutrophil invasion is, however, markedly increased in HH arthropathy, especially in joints with iron deposition. Accumulation of neutrophils may be crucial for the production of matrix enzymes, which enables cartilage degradation and more rapidly progressive articular damage.

INTRODUCTION

Hereditary haemochromatosis (HH) is the most common autosomal recessive inherited disorder manifesting as progressive iron overload in the liver, pancreas, heart, pituitary and joints.1 In 1964, Schumacher first recognised the relationship between HH and arthritis.2 Although typical clinical findings of HH arthropathy such as involvement of the second and third metacarpophalangeal (MCP) joints are frequently observed, virtually every joint can be affected.3 4 Patients mostly report osteoarthritis-like symptoms with bony swellings and pain during exercise, but intermittent synovitis is also well recognised and sometimes leads to misdiagnosis of seronegative rheumatoid arthritis (RA).5

The pathogenesis of the arthropathy is still uncertain as the relationship between iron overload and the development of arthropathy is unclear and iron removal by phlebotomy does not improve joint pain in a significant proportion of patients. Chondrocalcinosis may be found in some patients and contribute to the degenerative joint processes. Indeed, iron can precipitate calcium pyrophosphate crystal formation in vitro.6 Also, iron can be found in the synovium of patients with HH at the time of joint replacement surgery. However, synovial iron deposition can also occur in other rheumatic joint disorders such as RA.7 Virtually nothing is known about the phenotype and composition of the synovium in HH. This is surprising since HH arthropathy resembles the clinical features of both RA and osteoarthritis (OA) and thus represents an excellent disease model to study the interphase between degenerative and inflammatory joint disease, especially its morphological changes in the synovium. In this study we performed an in-depth analysis of the synovium of patients with HH and compared our findings with synovial tissue samples from patients with OA and RA.

MATERIALS AND METHODS

Tissue samples

Synovial tissue was obtained from 20 patients with RA, 39 with OA and 15 with HH at the time of joint replacement surgery or synovectomy. Surgical sites included 23 hips, 40 knees, 3 ankle joints, 5 wrist joints, 2 interphalangeal joints and 1 MCP joint. Tissues were fixed for 12h in 4% paraformaldehyde. Specimens were processed for paraffin embedding and 1–2 mm serial sections were cut for routine histology and immunohistochemical staining. One section from each sample was stained with haematoxylin and eosin (H&E) for histological assessment. All the patients with RA fulfilled the American College of Rheumatology (ACR) criteria for RA. All the patients with HH were homozygous for the C282Y mutation in the HFE gene and had documented iron overload-related disease.

Antibodies

Paraffin-embedded synovial tissue sections were stained using the following primary antibodies: monoclonal mouse anti-human CD20 (Clone L26; Dako, Glostrup, Denmark) to detect B lymphocytes, monoclonal mouse anti-human CD 31 (Clone JC70A; Dako) to detect endothelial cells, polyclonal rabbit anti human CD3 (Dako) to detect T lymphocytes, monoclonal mouse anti-human CD 68 (Dako) for macrophages, mouse monoclonal anti-human CD16s (Lab Vision Corporation, Fremont, CA) for granulocytes and CD68 for macrophages and, monoclonal anti-human CD16s (Lab Vision Corporation, Fremont, CA).
Fremont, California, USA) to detect activated macrophages and polyclonal rabbit anti-tyrosine hydroxylase (Dako) for polymorphonuclear granulocytes. As isotype control, the following isotype-matched control antibodies were used: mouse IgG1 (Code 0931; Dako), mouse IgG2a (Code 0943; Dako) and rabbit IgG (Vector Laboratories, Burlingame, California, USA).

**Immunohistochemistry**

Immunohistochemistry was performed using an immunoperoxidase technique with diaminobenzidine (DAB) as the chromogen. Expression of CD3, CD20, CD31, CD68, CD163 and myeloperoxidase was examined. Briefly, sections were deparaffinised and rehydrated followed by antigen retrieval in 0.01 M citrate buffer, pH 6.0 at 90°C for 20 min and allowed to cool for at least 30 min. Sections were washed in phosphate buffered saline (PBS), incubated with blocking serum (10% goat serum or 10% rabbit serum diluted in PBS) for 10 min and then incubated with the primary antibody for 60 min. Sections were subsequently incubated with biotinylated goat anti-mouse, goat anti-rabbit or rabbit anti-goat immunoglobulin G (Vector Laboratories) for 30 min followed by biotinylated horseradish peroxidase (HRP)–avidin complex ( Vectastain ABC System, Vector Laboratories) and visualisation with DAB chromogen (Dako Cytomation). The specificity of staining was confirmed by using matched isotype control antibodies. All sections were counterstained with haematoxylin. All incubations were carried out at 25°C and sections were washed between each step with PBS. Slides were examined and photographed using a transmitted light microscope (Nikon Eclipse 80i, Tokyo, Japan) and a Sony DXC 390P digital camera using Improvision imaging software.

**Histological analysis**

Synovial tissues were analysed according to an established synovitis scoring system as previously described. This analytical method, which is applicable to all forms of synovitis irrespective of its aetiology, allows the separate quantitative evaluation of chronic synovitis in three individual joint compartments (synovial lining layer, synovial stroma and inflammatory infiltrates) with a scale ranging from 0 to 9. Parameters were scored as 0 (normal), 1 (mild change), 2 (moderate change) and 3 (severe change). Ulceration of the lining layer accounts for 3 points in the lining score and fibrinoid necrosis accounts for 2 out of 9 points. Inflammatory cells in synovial sections were assessed by direct counting of at least 12 visual fields at ×200 magnification. Positive staining of cellular markers was expressed as the number of positive cells/mm². A total of 370 slides were analysed. The presence of cell aggregates was assessed on anti-CD3 and anti-CD20 stained sections and the percentage of CD20 positive cells was calculated. Aggregate size was measured by Improvision image software. Expression of CD68 and CD163 macrophages was analysed separately in the lining, sublining and stroma.

**Statistical analysis**

Data are presented as mean±SEM. For group comparisons we used one-way factorial analysis of variance with the Bonferroni–Dunn test or the Mann–Whitney U test. A p value <0.05 was considered significant.

**RESULTS**

**Patient characteristics**

The demographic and clinical features of the 74 patients are shown in Table 1. Disease duration was comparable among the three groups: 10 years (range 1–25) in patients with RA, 8.5 years (range 1–13) in patients with OA and 8.5 years (range 1–26) in patients with HH at the time of surgery. Patients with RA and HH were significantly younger than those with OA. Erythrocyte sedimentation rate and C-reactive protein levels were raised in patients with RA but not in those with OA and HH. As expected, the majority of patients with RA (75%) and OA (69%) were women, while the majority of patients with HH were men (87%). Only patients with RA were taking disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; HH, haemochromatosis arthropathy; NSAID, non-steroidal anti-inflammatory drug; OA, osteoarthritis; RA, rheumatoid arthritis.

**Table 1 Clinical data**

<table>
<thead>
<tr>
<th></th>
<th>RA (n=20)</th>
<th>OA (n=39)</th>
<th>HH (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>53.8 (range 30–77)</td>
<td>70.1 (range 38–88)</td>
<td>61.0 (range 36–78)</td>
</tr>
<tr>
<td>M/F (%)</td>
<td>5/15 (25/75%)</td>
<td>12/27 (30.8/69.2%)</td>
<td>13/22 (86.7/13.3%)</td>
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<tr>
<td>Disease duration (years)</td>
<td>10.3 (range 1–23)</td>
<td>8.5 (range 1–18)</td>
<td>8.50 (range 1–26)</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>2.3 (range 0.2–11.7)</td>
<td>0.67 (range 0–2)</td>
<td>0.36 (range 0.1–0.9)</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>23.9 (range 9–41)</td>
<td>8.9 (range 2–24)</td>
<td>5.3 (range 1–10)</td>
</tr>
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<td>Steroids (yes/no)</td>
<td>7 (35%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<td>NSAID (yes/no)</td>
<td>8 (40%)</td>
<td>37 (94.9%)</td>
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<td>DMARD (yes/no)</td>
<td>12 (60%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CD2X2 inhibitors (yes/no)</td>
<td>4 (20%)</td>
<td>0 (0%)</td>
<td>2 (13.3%)</td>
</tr>
<tr>
<td>Opioids (yes/no)</td>
<td>1 (5%)</td>
<td>9 (23.1%)</td>
<td>1 (6.7%)</td>
</tr>
</tbody>
</table>

CDX-2, cyclooxygenase-2 inhibitors; CRP, C-reactive protein; DMARD, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; HH, haemochromatosis arthropathy; NSAID, non-steroidal anti-inflammatory drug; OA, osteoarthritis; RA, rheumatoid arthritis.

**General characteristics of synovial pathology in haemochromatosis**

The degree of synovitis was assessed by an established scoring system. Overall, three key features of synovitis were quantitatively evaluated: (1) proliferation and infiltration of the synovial lining layer, (2) activation of synovial stroma and (3) inflammatory infiltrates. In the present study group, we observed high-grade synovitis in patients with RA (mean score 5.1±1.9, p<0.05 vs OA and HH). As expected, patients with OA displayed low-grade synovitis in the majority of cases (mean score 2.4±0.8). In HH, the histological picture largely resembled primary OA as judged from conventional H&E stainings. These findings were consistent in all three subscales (synovial lining, stroma and inflammatory infiltrates) of the scoring system. Interestingly, we found haemosiderin deposition in 12 of 15 HH synovial tissues but not in RA or OA samples. We therefore suggest that the histological appearance of synovitis in HH arthropathy is reminiscent of OA (figure 1).

**Synovial immune cell infiltration in haemochromatosis arthropathy**

To determine the qualitative alteration of the synovium in HH arthropathy, we analysed immune cell infiltration and neovascularisation (figure 2). We first analysed density of microvessels by staining for the endothelial marker CD31. Compared with patients with OA (mean 8.0±5.8 vessels/mm²), significantly higher numbers of CD31 vessels were found in patients with RA (mean 14.8±12.8 vessels/mm², p<0.05 vs OA) but not in those with HH which were very similar to patients with OA with a low microvessel density (mean 6.6±1.6 vessels/mm², p=NS vs OA). We then assessed immune cell infiltration and
Extended report


infiltration by activated CD163 macrophages in the synovial lining and stroma of patients with RA compared with those with OA and HH. In the sublining, however, activated CD163 macrophages were equally prevalent in all groups of patients.

To define lymphoid infiltration and lymphoid follicle neogenesis we performed stainings for CD3 and CD20. As described previously, patients with RA were characterised by marked infiltration by CD68 macrophages in the synovial lining and stroma of patients with RA compared with those with OA and HH. In the sublining, however, activated CD163 macrophages were equally prevalent in all groups of patients.

Figure 1  Analysis of the severity of synovitis in haemochromatosis arthropathy (HH). Haematoxylin and eosin (H&E) stained sections of synovial tissue from patients with HH, rheumatoid arthritis (RA) and osteoarthritis (OA) were scored using a system assessing three features (enlargement of lining layer, density of resident cells and inflammatory infiltrate) giving a maximum score of 9 points (0–3 points in each category). Data are mean±SEM. *p<0.05.

Figure 2  Quantitative evaluation of immune cells and vessel density. Immunohistochemical analysis of macrophages (CD163), T cells (CD3), B cells (CD45R) and vessels (CD31) in the synovium of patients with haemochromatosis arthropathy (HH), osteoarthritis (OA) and rheumatoid arthritis (RA) was performed. Macrophages were counted in the synovial lining layer, sublining layer and stroma. Microvessel density and lymphocyte infiltration were quantitatively assessed in the synovial stromal tissue. Lymphoid follicle size was quantitatively assessed. Data are mean±SEM. *p<0.05.
lymphocyte infiltration in the synovium. Both T cell counts (mean 151.1±86.4 cells/mm², p<0.05 vs OA and HH) and B cell counts (mean 113.2±56.1 cells/mm², p<0.05 vs OA and HH) were significantly increased in patients with RA compared with those with OA and HH. Also, lymphoid aggregates were found more often and were larger in size in patients with RA (mean aggregate size 38 742±19 849 μm², p<0.05 vs OA and HH) than in those with OA (mean aggregate size 18 946±10 767 μm²) and HH (mean aggregate size 13 706±7656 μm²). Representative stainings are shown in figure 3.

Finally, we assessed the frequency and distribution of neutrophils. Neutrophil infiltration was high in RA synovial tissue (mean 35.8±22.7 cells/mm², p<0.05 vs OA) but very low in OA synovial tissue (mean 5.2±5.1 cells/mm²). Interestingly, we found a similar accumulation of neutrophils in patients with HH as observed in RA (mean 22.5±25.1 cells/mm², p<0.05 vs OA). Notably, neutrophils were especially confined to HH samples with marked haemosiderin deposition as determined from conventional histological stainings. Haemosiderin deposition was found in 12 of 15 HH samples, usually in the lining and sublining layer, and these haemosiderin deposits were usually associated with neutrophilic infiltrates. These findings are shown in figure 4.

**DISCUSSION**

In this study we investigated the synovial pathology of HH arthropathy. The histological picture of the synovium largely resembles a process reminiscent of OA. However, we found that a strong neutrophilic infiltration is a characteristic feature of HH arthropathy.

Arthritis in HH is a well recognised but frequently overlooked organ manifestation. Indeed, about 30–50% of newly diagnosed patients with HH have MCP joint involvement. While any joint can be involved, arthritis of the second and third MCP joints with characteristic hook-like osteophytes on the x-ray is
regarded as a pathognomonic sign.\textsuperscript{10} Importantly, clinical signs and symptoms of MCP joint involvement in HH arthropathy can largely mimic RA with signs of synovial swelling, pain and morning stiffness. Moreover, involvement of other joints in HH such as hip, knee and ankle joints are rather non-specific for HH arthropathy and it is, in fact, difficult to clearly delineate HH arthropathy from idiopathic OA. Chondrocalcinosis, although frequently found, is not specific for HH and can be found in patients with HH without any evidence of joint disease.\textsuperscript{11,12}

The pathophysiological events leading to arthritis in HH are unclear. Clinically, HH arthropathy largely resembles an OA-like disease with an early onset in life. However, intermittent attacks of acute arthritis, most likely in patients with chondrocalcinosis (pseudogout), may occur. Radiologically, osteophytes, cysts and erosions are found in the affected joints.\textsuperscript{10} Histological studies so far have mainly focused on the articular cartilage in patients with late-stage HH undergoing joint replacement surgery.\textsuperscript{13–15} Various pathologies have been described. While cartilage thinning with eburnation of subchondral bone as signs of end-stage damage were uniformly observed, other pathologies were inconsistent. For instance, chondrocalcinosis was found in some studies but was completely absent in other studies. Also, subchondral bone necrosis and cartilage iron deposition were not consistently present in HH arthropathy.\textsuperscript{11,16}

Knowledge of the pathology in the synovium of HH arthropathy is limited. The synovium has been reported to be ‘non-inflammatory’ with iron deposition in some but not all cases.\textsuperscript{17} In this study we performed a more in-depth analysis of the synovium in HH arthropathy. Grading of synovitis using a global synovitis score showed that HH arthropathy largely resembles OA but not RA. We also applied immunohistochemistry to determine the quality and quantity of immune cell infiltration and neovascularisation. Similar to synovitis, the density of microvessels was enhanced in patients with RA and was low in patients with OA and HH arthropathy. Also, we could not detect significant differences between OA and HH with regard to lymphocyte infiltration and lymphoid neogenesis, both of which were significantly less pronounced than in patients with RA. Macrophage infiltration, as assessed by the universal macrophage marker CD68, was more prominent in HH but was significantly less than in RA. Infiltration by activated CD163 macrophages, however, was not significantly different in patients with HH than in those with OA or RA. Most strikingly, however, we found higher numbers of infiltrating neutrophils in patients with HH than in those with OA. Moreover, neutrophil infiltration in HH was confined to samples with significant haemosiderin deposition, suggesting a possible link between iron deposition and neutrophil infiltration. Thus, from the composition of the cellular infiltrate, HH arthropathy can be clearly differentiated from OA by the infiltration of cells of the innate immune response (macrophages, neutrophils), and also from RA by the lack of infiltrating specific immune cells (B cells, T cells).

In spite of neutrophil infiltration being typical in inflammatory arthritis (RA and psoriatic arthritis, in particular), the missing B and T cells suggest a different underlying pathophysiology. Our findings therefore provide further evidence that HH arthropathy should not be treated as premature OA but deserves specific pathophysiology-orientated consideration.

In contrast to RA, iron in patients with HH is mainly deposited in the synovial lining layer and in chondrocytes. Interestingly, synovial iron deposition has been observed in patients with normal iron body stores, indicating a slow iron exchange in joints despite effective venesection therapy.\textsuperscript{11} Indeed, parenteral iron therapy can induce OA-like changes

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**Figure 4** Analysis of neutrophil infiltration in haemochromatosis arthropathy (HH). Neutrophil infiltration was assessed by enumeration of myeloperoxidase (MPO) positive cells in the synovial stroma of patients with HH, osteoarthritis (OA) and rheumatoid arthritis (RA). Haemosiderin deposition was also quantitatively assessed in HH samples. Representative images from patients with HH are shown (from left to right: haematoxylin and eosin (HE) staining, Prussian blue staining and immunohistochemistry for myeloperoxidase; original magnification ×200 and ×400). Data are mean±SEM. *p<0.05.
in immature rabbits, although very high doses are needed. Direct evidence that iron itself is important in the pathogenesis of joint disease comes from a clinical observation. Secondary iron overload due to erythrocyte transfusion or ineffective erythropoiesis can also induce an arthropathy indistinguishable from HH. Moreover, juvenile HH also goes along with typical arthritis of the second and third MCP joints. In vitro, iron complexes can induce lipid peroxidation in phagocytes and chondrocytes, which may contribute to cartilage catabolism and inflammation in HH.

In summary, we show that synovial changes in HH largely resemble those in OA with mild infiltration of mononuclear cells, formation of synovial microvessels and a low degree of synovial hyperplasia. However, it can be clearly differentiated from OA by the presence of neutrophil and macrophage invasion. HH arthropathy is therefore neither typical OA nor RA, as may be suggested by the clinical picture and radiological findings, but histologically it has a different pathophysiology which is possibly related to iron deposition. Indeed, accumulation of neutrophils and macrophages could be a crucial step for production of matrix enzymes, which enable cartilage degradation and rapidly progressing articular damage.

Acknowledgements  The authors thank Isabell Schmidt, Eugenia Scheffler and Cornelia Stoß for their excellent technical assistance.

Funding  This work was supported by the German Society for Rheumatology (to JZ) and the Interdisciplinary Centre for Clinical Research Erlangen (IZKF, project A34, to GS and JZ).

Competing interests  None.

Ethics approval  This study was conducted with the approval of the ethics committee, University of Erlangen-Nuremberg and written informed consent was obtained from patients in accordance with the Declaration of Helsinki.

Provenance and peer review  Not commissioned; externally peer reviewed.

REFERENCES